

Materials and methods

Amplification of mMAN2B2 mRNA by the reverse transcriptase-polymerase chain reaction.

The RNA fractions purified from the porcine or mouse caput, corpus and cauda epididymis, and testis were incubated at 65°C for 10 min. The first strand cDNAs were synthesized using the "First-Strand cDNA Synthesis Kit" (Pharmacia Biotech). The oligonucleotides were synthesized according to the porcine MAN2B2 cDNA (5'-GCA TCC ATG TTC ACA CGC TAC ATG T-3' and 5'-GGT ACT GCT GCT CGA GCC GTT GGC AGA-3'), and were used as primers for the amplification of cDNAs by Taq polymerase (Boehringer-Mannheim).

Isolation of a cDNA clone encoding mMAN2B2 from the mouse testis cDNA library.

The mouse testis cDNA library was constructed using the "cDNA Cloning System λ gt11" (Amersham) as described previously. The cDNA library was screened with the DIG-labeled RT-PCR product as a probe

using the "DIG Nucleic Acid Detection Kit" (Boehringer-Mannheim). The positive inserts were excised from the vector by digestion with EcoRI, and were subcloned into the vector pUC118. Dideoxynucleotide sequencing was performed using the "Dye Primer Cycle Sequencing Kit" (Applied Biosystems). In order to obtain the sequence of the 5'-region of mMAN2B2 mRNA, the 5'-RACE method was employed using the "5'-RACE System for Rapid Amplification of cDNA Ends" (Life Technologies). The PCR product was ligated into the pCR vector of "TA Cloning System" (INVITROGEN) and then sequenced as described above.

Isolation of gene clone encoding mMAN2B2 from the mouse genomic library.

The 129SVJ mouse genomic library was screened with the digoxigenin (DIG) labeled cDNA and oligonucleotide synthesized according to the sequence of the mMAN2B2 homologue cDNA using the plaque hybridization technique. Three positive phage clones were selected from approximately 6×10^5 plaques and subcloned into the pUC118 plasmid vector or into the pCR11 plasmid vector of "TA cloning system" (INVITROGEN). Dideoxynucleotide sequencing was performed using the "Dye Primer Cycle Sequencing Kit" (Applied Biosystems).

Chromosome mapping of the mMAN2B2 gene.

The direct R-banding fluorescence *in situ* hybridization (FISH) method was used for chromosomal assignment of the mouse MAN2B2 homologue gene (Abe *et al.*, 1999). A plasmid containing the gene (7kbp) of the mouse MAN2B2 homologue gene was labeled with biotin by nick translation for *in situ* hybridization on mouse chromosome spreads. The hybridized probe DNA on chromosomes was detected by the FITC-labeled streptavidin/biotinylated anti-streptavidin antibody system, and the R-band of the chromosomes was induced simultaneously by staining with propidium iodide (PI). FITC and PI signals were collected separately with a cooled CCD camera, and electronically overlaid.

In situ hybridization.

Fresh mouse testis was frozen quickly in liquid nitrogen and embedded in O.C.T. compound (Miles). The sections were fixed with 4% paraformaldehyde and then treated with 5 μ g/ml proteinase K. After post-fixation with 4% paraformaldehyde, the sections were treated with 0.2M HCl, with 0.1M triethanolamine-HCl, pH8.0 and 0.25% acetic anhydride, 0.1M triethanolamine-HCl, pH8.0, successively. After the sections were dehydrated and air-dried, hybridization was carried out

using 1.5 μ g/ml DIG-oligo-DNA probe dissolved in 50% formamide containing 200 μ g/ml tRNA, 1 \times Denhardt's solution, 10% dextran sulfate, 0.6M NaCl, 0.25% SDS, 1mM EDTA and 10mM Tris-HCl, pH7.6 at 37°C for 16h in a moist chamber equilibrated with 50% formamide in 10mM Tris-HCl, pH7.6. The sections were then washed with 5 \times SSC at 37°C for 5min, followed by 2 \times SSC, 50% formamide at 40°C for 30 min and then washed again with 0.5M NaCl containing 1mM EDTA and 10mM Tris-HCl, pH7.6 (TNE) at 37°C for 10 min. The sections were further treated with 1 μ g/ml RNase A at 37°C for 30 min, then washed with TNE at 37°C for 10min, followed by 2 \times SSC at 40°C for 20 min and then twice with 0.2 \times SSC at 40°C for 20 min, successively. The colorization was performed using the "DIG Nucleic Acid Detection Kit" as described above.

Oligonucleotide probes.

Probe used for Northern blot analysis consisted of a mixture of the following three oligonucleotides : 5'-CAG GAG CTG GAC CTG CTG CTG TTT-3', 5'-CAG GGT AGG GGT CGT TGC TGA TG-3', 5'-GGG TCT TCA TGG TCC AGG AAA GCT-3'. The antisense oligonucleotide probe used for *in situ* hybridization was complementary to mRNA encoding amino acids 8-22 of the mMAN2B2 : 5'-CTG AGC TGC CCG CGG CCA CAA CAG CTG GCC GAG TAG CGG-3'. The sequence of the sense oligonucleotide probe

was 5'-CCG CTA CTC GGC CAG CTG TTG TGG CCG CGG GCA GCT CAG-3'. Probe used for isolation of gene clone from the mouse genomic library consisted of a mixture of the following three oligonucleotides : 5'-CAG GAG CTG GCG GAC CTG CTG CTG TTT-3', 5'-CAG GGT AGG GGT CGT TGC TGA TG-3', 5'- GGG TCT TCA CGG TCC AGG AAA GCA-3'.

Construction of bacterial expression vector pGEX-2T and expression of recombinant GST-mMAN2B2 fusion protein.

The clone 41-3 whose insert contained about 70% protein of the mMAN2B2 cDNA (868-3054) was digested with EcoR1. The fragment (2186bp) was ligated into Eco R I site of the bacterial expression vector pGEX-2T, which coded to produce GST-mMAN2B2 fusion protein. The plasmid construct was transformed into E.coli JM109. The transformed single colony cells were cultured in LB broth medium containing ampicillin at 37°C for overnight. Then, nine volumes of LB broth medium containing ampicillin were added and cells were further incubated at 37°C until the absorbance of the culture at 600nm reached 0.6~0.8. IPTG (beta-isopropylthiogalactopyranoside) was added to the medium and incubated for further 3h at 37 °C . The cells were collected by centrifugation, resuspended in PBS and lysed by sonication on ice. As the

fusion protein formed inclusion body, the cell debris were precipitated by centrifugation and solubilized in 1% SDS. The GST-mMAN2B2 fusion protein was purified by the preparative SDS-PAGE.

Preparation of antiserum against the GST-mMAN2B2 fusion protein.

Rabbit antiserum against the GST-mMAN2B2 fusion protein was produced by subcutaneous injection of 300 μ g purified antigen with Freund's adjuvant-complete (SIGMA). Three additional booster injections of 200 μ g purified antigen with Freund's adjuvant-complete were followed at intervals of two weeks.

Preparation of monospecific antibody.

As the rabbit antiserum recognized not only mMAN2B2 but also GST, antibody specific for mMAN2B2 was prepared from the antiserum. The clone 41-3 was digested by BamH I and EcoR I and subcloned into BamH I / EcoR I sites of bacterial expression vector pET-30c which coded to produce His.Tag-mMAN2B2 fusion protein. The plasmid construct was transformed into E.coli BL21 (DE3). His.Tag-mMAN2B2 fusion protein was obtained by similar method as used for bacterial

expression vector pGEX-2T. The cell lysate was mixed with SDS-PAGE denaturation buffer and boiled for 5min and separated by 10% SDS-polyacrylamide gel electrophoresis. The proteins were transferred from the gel to a nitrocellulose membrane by semi-dry system. The protein bands were visualized by staining 0.5% ponceau S in 2% TCA . The band of His.Tag-mMAN2B2 fusion protein was cut out from the membrane, which was blocked with 3% skim milk in PBS. The membrane was then exposed to the antiserum for 5 h at room temperature and washed with 0.15M NaCl and PBS. In order to extract specific for mMAN2B2 from the membrane, the membrane was soaked in elution buffer for 10 min at room temperature. The eluted monospecific antibody was neutralized to about pH7.0 as soon as possible by 1M Tris-base and stored at 4°C.

Immunohistochemical analysis of mouse testis.

Immunohistochemical analysis was carried out by avidine-biotin complex (ABC) method (Noguchi *et al.*, 1997). Adult and infant (20 days of age) mouse testes were fixed by soaking in Bouin solution (saturated picric acid : chloroform : acetic acid = 15 : 5 :1) at room temperature for 24 h. Fixed testis was then consecutively dehydrated in 90% and 100% ethanol for 24 h at room temperature each two times and in xylene at room temperature for 1 h three times. After embedded in paraffin, the

block was sectioned in a thickness of $0.4\ \mu\text{m}$ and mounted on slides coated with silane. After deparaffinization, sections were soaked in 0.1M sodium citrate buffer at $37\ ^\circ\text{C}$ and washed with DW. Sections were then exposed to 0.5% peroxidic acid for inactivation of the intracellular peroxidase activity. Sections were washed with Tris-saline (0.15 M NaCl in 50mM Tris, pH7.6) and blocked with 0.5% casein, 0.1% sodium azide in Tris-saline (blocking buffer A) at $37\ ^\circ\text{C}$ for 1 h. Sections were exposed to the monospecific anti mMAN2B2 antibody dissolved in the blocking buffer A and incubated at $37\ ^\circ\text{C}$ for 1 h in a moist chamber. Specificity control for the immunohistochemical reaction was carried out on adjacent sections, which were incubated with the preimmune serum instead of the monospecific anti mMAN2B2 antibody. Sections were then washed with Tris-saline and exposed to the anti rabbit biotinized goat IgG dissolved in the blocking buffer A at $37\ ^\circ\text{C}$ for 1 h in a moist chamber. After washed with Tris-saline three times, sections were exposed to the avidin-biotin-peroxidase complex solution (VECTASTAIN ; VECTOR Laboratories, Inc) for 30 min and washed progressively with Tris-saline and DW. Counter staining of sections had been done by hematoxylin and eosin. After washed with DW and Tris-saline , the peroxidase activity was revealed by 0.025% diaminobenzidine tetrahydrochloride (DAB), 0.003% H_2O_2 , 0.02% $(\text{NH}_4)\text{NiSO}_4$, 0.025% CoCl_2 in Tris-saline. Sections were washed with DW, dehydrated, mounted and observed under microscope.

Immunocytochemical analysis of mouse sperm.

A dense sperm mass was squeezed out from the cauda epididymis of mature mouse into 400 μ l HTF medium (Quinn *et al.*, 1985). The sperm were diluted to a final concentration of 4×10^6 cells/ml in HTF medium and used for capacitation and acrosome reaction. Capacitation was achieved by incubating sperm in HTF medium at 37°C for 1 h under 5% CO₂ in air. Acrosome reaction was induced by incubating the capacitated sperm in HTF medium containing 5 μ g/ml calcium ionophore A23187 at 37°C for 1 h under 5% CO₂ in air. Immunocytochemical analysis of mouse sperm had been done by avidine-biotin complex (ABC) method using a "VECTASTAIN Elite Kit" (VECTOR Laboratories, Inc). Acrosome reacted or acrosome intact sperm suspension was placed on the slide coated with VECTABOND (VECTOR Laboratories, Inc). After dryness on hot plate at 50°C, sperm were fixed by the treatment with 4% paraformaldehyde in PBS on ice for 30 min. The fixed sperm were washed with PBS three times, and blocked with the normal goat serum in PBS (blocking buffer B) and with "Avidine-Biotin Blocking Kit" (VECTOR Laboratories, Inc) at room temperature each for 30 min. Sperm were exposed to the monospecific anti mMAN2B2 antibody dissolved in the blocking buffer B at 37 °C for 1 h in a moist chamber. Specificity control

for the immunohistochemical reaction were carried out with the preimmune serum instead of the monospecific anti mMAN2B2 antibody. Sperm were washed with PBS three times and incubated with the anti rabbit biotinized IgG dissolved in blocking buffer B at room temperature for 30 min. After washed with PBS three times, sperm were incubated with the *Elite* ABC solution at room temperature for 30 min. After washed with PBS three times, the peroxidase activity was revealed by "VECTASTAIN DAB Substrate Kit" (VECTOR Laboratories, Inc) and observed under microscope.

in vitro fertilization.

Immature female mice at four weeks of age were ovulated by consecutive i.p. injections of 5 IU Pregnant Mare Serum Gonadotropin : PMS (Teikokuzouki, Ltd.) followed 48 h later by 5 IU Human Chorionic Gonadotropin : hCG (Teikokuzouki, Ltd.). Oocytes were collected 15 h after the injection of hCG from the oviductal ampulla region and were transferred to 200 μ l drops of HTF medium under mineral oil equilibrated with 5% CO₂ in air at 37°C. The oocytes were preincubated in 200 μ l drops of HTF medium containing 0 μ g/ml (control) or 100 μ g/ml of monospecific anti mMAN2B2 antibody for 1 h. The capacitated sperm were added to the preincubated oocytes in HTF medium at final

concentration of 4×10^4 cells/ml followed by incubation under 5% CO₂ in air at 37°C for 24 h. On the other control experiment, the oocytes were preincubated in 200 μ l drops of HTF medium with the normal rabbit IgG (100 μ g/ml). The successful fertilization was assessed by the formation of the two cell embryos at 24 h after insemination.