Results

Cloning of cDNA encoding the mMAN2B2.

Porcine MAN2B2 is mainly secreted from the proximal corpus epididymis and binds to the maturating sperm. In order to determine whether mRNA of the MAN2B2 homologue is also present in the mouse male reproductive system, RT-PCR was performed using oligonucleotides derived from the porcine MAN2B2 cDNA as primers. As shown in Fig.1, in boar, MAN2B2 mRNA was most abundant in the corpus epididymis whereas only trace levels of mRNA were observed in the other parts of the epididymis and testis in consistence with the previous results. On the other hand, in mice, testis RNA produced higher amounts of the RT-PCR product than the epididymis as shown in Fig.1. There was no difference in the amounts of the RT-PCR product among the different regions of mouse epididymis. The RT-PCR products of both testis and the epididymis were found to have exactly the same nucleotide sequences, which were 61% homologous to the corresponding part of the porcine MAN2B2 cDNA (data not shown). All these data suggest that the MAN2B2 homologue is also expressed in mouse reproductive tissues.

cDNA clones encoding mMAN2B2 were screened from the mouse testis cDNA library with the RT-PCR product as a probe for plaque hybridization. Six positive clones were selected from 2×10^5 clones. Clone L8-2-1 whose insert contained the inserts of five other clones was analysed as shown in Fig.2. The 5'-region of the cDNA was extended by the 5'-RACE method as described in the Materials and Methods. Fig.3 shows the nucleotide sequence of the mMAN2B2 cDNA. The first potential translation initiation codon, ATG, is preceded by 52 base pairs containing Kozak's conserved sequence (Kozak, 1984). An open reading frame is formed by 3054 base pairs that code 1018 amino acids, followed by 198 base pairs of 3'-untranslated region. The polyadenylation signal AATAAA is located 20 base pairs upstream of the polyadenylation site. From the deduced amino acid sequence, the molecular weight was estimated to be 115622. As suggested for porcine MAN2B2, mMAN2B2 also contained 9 possible N-glycosylation sites, although there was no similarity in the distribution of the N-glycosylation sites between the porcine and mouse proteins.

As shown in Fig.4 the predicted amino acid sequence of mMAN2B2 was significantly homologous (62% identity in the overall amino acid sequence) to that of porcine MAN2B2. It was found that the region between the 29th and 217th amino acid of mMAN2B2 was 80% identical to the corresponding region of porcine MAN2B2.

Cloning and characterization of the mMAN2B2 gene.

Genomic clones encoding mMAN2B2 were screened from the 129SVJ mouse genomic library with the oligonucleotides synthesized according to the sequence of the mMAN2B2 cDNA as a probe for plaque hybridization. Three positive clones were isolated and characterized. Those clones overlapped each other and contained 25475bp, which covered all of the mMAN2B2 cDNA. As shown in Fig.5, the mMAN2B2 gene was composed of 19 exons and 18 introns. Several possible consensus sequences for the binding of the transcription factors were found within 1.7kbp length of 5'-franking region, whereas TATA box was not identified, as shown in Fig.6.

In Fig.7 (a), the chromosomal localization of the mMAN2B2 gene was determined by the R-banding FISH method as described in Materials and Methods. In Fig.7 (b), mMAN2B2 was localized to 5ChrB, where the synteny with human 4Chr has been demonstrated.

Distribution of mRNA encoding the mMAN2B2.

Although porcine MAN2B2 is specifically expressed in the regions between the distal caput and the proximal corpus epididymis, mMAN2B2

was found to be expressed to a greater extent in the testis compared to the epididymis, as shown in Fig.1. Fig.8 shows the distribution of the mMAN2B2 mRNA among various mouse tissues. By Northern blot hybridization, mRNA was detected only in the testis among the organs tested.

In order to determine the cell type in the testis where mMAN2B2 is expressed, we employed the *in situ* hybridization method. As shown in Fig.9, mMAN2B2 mRNA was found only in the germ cells bordering on the basement membrane of the seminiferous tubule, which were mainly composed of spermatogonia, and not either in spermatocytes at late pachytene stage, spermatid, Sertoli cells nor in the interstitial cells. Furthermore, spermatogonia in all cross sections of the seminiferous tubules were not stained, suggesting that the mMAN2B2 RNA was expressed at the specific stages of spermatogenesis.

As shown in Fig.10, the sections used for *in situ* hybridization were counter stained with methyl green, and the stage of the germ cell was identified on the basis of the nuclear morphology and topographical relationships in the seminiferous tubule according to the Monesi's, Russell's and Takahashi's definition of the stages of the mouse spermatogenesis (Monesi, 1964 ; Russell *et al.*, 1990 ; Takahashi, 1994), as shown in Fig.11. There was no mRNA signal in testis at stages Ⅶ and Ⅷ. mRNA of the mMAN2B2 was stained intensely in spermatogonia at stages

Ⅸ~Ⅺ and rather weakly at stages Ⅰ~Ⅵ and Ⅻ.

Localization of mMAN2B2 during spermatogenesis.

In order to determine the distribution of the mMAN2B2 in testis, we carried out immunohistochemical analysis using the monospecific anti mMAN2B2 antibody purified as described in Materials and Methods. The signal of mMAN2B2 was not observed in the testis from the mouse of 20 days of age in which spermatid had not been developed yet, as shown in Fig.12.

As shown in Fig.11 and.13, the process of development of spermatid is divided into 16 steps. The signals of mMAN2B2 were observed in the acrosome whose shape gradually changed along with the progress of spermiogenesis. Namely, mMAN2B2 signal was first observed in the proacrosomal granule and round acrosomal vesicle in the spermatids at Steps 2 and 3. mMAN2B2 was then observed in acrosomal vesicle which flatted over the surface of the nucleus in the spermatids at Steps 4~7. The subtend angle of the area containing the mMAN2B2 signal became approximately 150° at Step 7. During Steps 8~9 spermatids, mMAN2B2 was observed in the slightly elongate shaped acrosome. In the Steps 10~16 spermatid, mMAN2B2 was clearly observed in the sickle shaped acrosome between the ventral and dorsal fin of the head.

The localization of mMAN2B2 on the mature sperm prepared from the cauda epididymis was also studied. The signals of mMAN2B2 were still observed in the acrosomal region of the mature sperm. But it was found that the mMAN2B2 signal in the acrosome was not detected after the acrosome reaction, as shown in Fig.14.

Effect of the anti mMAN2B2 antibody on the fertilization rate.

mMAN2B2 was located in the acrosomal region of mature sperm, where was an important region for the sperm-egg interaction. In order to determine whether mMAN2B2 have any effects on the fertilization, the effects of the anti mMAN2B2 antibody was studied in *in vitro* fertilization system. As shown in Table 1, the fertilization rate was determined by the ability to form two cells embryo. The fertilization rate in the absence of the antibody was 86.9±7.4% under the present condition described in Materials and Methods. It was found that the antibody caused 21.0% reduction of the fertilization rate to be 65.9±6.0%. The normal rabbit IgG had no effect on the fertilization rate (data not shown).