Expression of a Y-box protein, YB2/RYB-a, precedes protamine 2 expression during spermatogenesis in rodents

Yoshihito Iuchi1, Takashi Kobayashi1, Tomoko Kaneko1, Masatoshi Takahara2, Toshihiko Ogino2 and Junichi Fujii1,3

1Department of Biochemistry and 2Department of Orthopedics, Yamagata University School of Medicine, 2-2-2 Iidanishi, Yamagata 990-9585, Japan
3To whom all correspondence should be addressed. E-mail: jfujii@med.id.yamagata-u.ac.jp

Y-box binding proteins, a large family of proteins, are involved in a variety of functions. The present study describes the expression of YB2, a rat Y-box binding protein, and/or RYB-a, an alternatively spliced product of the YB2 gene during spermatogenesis. YB2/RYB-a is thought to be the rat orthologue of mouse Y-box protein 3 (MSY3). An antibody which recognizes YB2/RYB-a was developed and applied in an immunochemical study of rat and mouse testes. We also carried out an in-situ hybridization study and Northern blot analysis of YB2/RYB-a mRNA expression. Both YB2/RYB-a mRNA and the proteins appeared in prepubertal mouse testes, prior to the expression of the mouse protamine 2 mRNA. The mRNA and protein were present at high levels in spermatocytes, decreased in round to elongated spermatids, and were absent in spermatozoa. Since the protamine 2 mRNA was present at high levels in round and elongating spermatids, the proposed function of the YB2/RYB-a protein as a translational repressor of the mRNA was supported in mouse. The level and localization of YB2/RYB-a mRNA and protein expression in the rat testis was comparable to that in mouse testis, although rat testis is known to express a very low level of protamine 2, but is also likely to affect the expression of other proteins (including protamine 1) during spermatogenesis.

Key words: in-situ hybridization/protamine/spermatogenesis/translational repression/Y-box proteins

Introduction

Information which has accumulated to date in genetic databases allows the identification of genes that encode proteins with similar primary structures or domains across different species. The Y-box protein family, identified by a homology search, is recognized as one of the most evolutionarily conserved nucleic acid binding protein families defined to date and members are found in bacteria, plants, and animals. The Y-box proteins contain a cold-shock domain (CSD), the sequences of which are conserved with >40% homology in small bacterial cold shock proteins (Wistow, 1990; Wolffe et al., 1992) and bind single-stranded nucleic acids (Schnuchel et al., 1993). In addition to the CSD, all vertebrate Y-box proteins contain basic/aromatic islands that bind RNA (Ladomery and Sommerville, 1994; Murray, 1994) in their C-terminal region. The bacterial cold shock proteins act as transcription factors (Jones et al., 1992). A property common to the Y-box proteins is the binding of the reversed CCAAT box element, ATTTG, and the ability either to promote or to repress transcription of a variety of genes (Lloberas et al., 1995; Nikolajczyk et al., 1995; Bargou et al., 1997; Shibao et al., 1999).

Genes for many Y-box proteins, including Xenopus (frog) FRGY1 and FRGY2 genes and mouse homologous genes, MSY1 and MSY2 (Kwon et al., 1993; Tafuri et al., 1993; Gu et al., 1998), have been cloned from vertebrates. Some Y-box proteins are thought to serve as widespread translational repressors in both male and female germ cells (Matsumoto and Wolfe, 1998; Evdokimova and Ovchinnikov, 1999). In Xenopus oocytes, FRGY2 is abundant and is bound to cytoplasmic maternal mRNA in which translation is repressed (Murray et al., 1991). In mouse testis, MSY-2 has been detected in both the nuclei and cytoplasm of cells using an immunological approach (Kwon et al., 1993; Nikolajczyk et al., 1995; Oko et al., 1996; Yiu et al., 1997).

Protamine mRNA is one of the promising candidates as a target of the Y-box proteins (Fajardo et al., 1994; Davies et al., 2000). Protamines are small, arginine- and cysteine-rich proteins which are expressed only during the post-meiotic stages of spermatogenesis. Two protamines, protamine 1 and protamine 2, which differ in size and amino acid sequences have been found in the mouse. The nuclear compaction of the sperm nucleus during spermatogenesis involves the sequential replacement of histones by transition proteins and, finally, by protamines (Hecht, 1990). Protamine mRNA represent well-
known members of a large group of mRNA that are translationally repressed in round spermatids and are actively translated in elongated spermatids after the cessation of transcription (Kleene, 1996). It has actually been reported that MSY4, another mouse Y-box protein, is able to bind the 3’ untranslated region of the mouse protamine 1 mRNA (Davies et al., 2000). Mastrangelo and Kleene (2000) have reported developmental expression of the MSY3 gene which is actually the same gene as MSY4. Since MSY3 was originally deposited in the database as a pseudogene of MSY1, MSY3 would be the appropriate name for this gene.

Rat Y-box binding protein-a (RYB-a) was isolated from a rat liver cDNA library by affinity for a sequence from the aldolase B gene promoter (Ito et al., 1994). RYB-a is an alternatively spliced product of the gene for rat Y-box binding protein, YB2 (Sapru et al., 1996). The RYB-a protein, which consists of 292 amino acids, differs in the carboxyl terminus in that it is 69 amino acids smaller than the YB2 protein. Rat YB2 exhibits 98.6% amino acid identity to MSY3 and, hence, is thought to be a rat orthologue of MSY3 (Mastrangelo and Kleene, 2000). The expression of RYB-a mRNA in adult liver is very low, but is induced during liver regeneration and by serum stimulation of quiescent cells as well as in immunoblot analyses. We also employed in-situ hybridization chemical studies for localization of the protein as well as in immunohistochemistry for rat YB2/RYB-a and used it in some immunohistochemical staining procedures.

The mouse protamine 1 mRNA (Davies et al., 2000) was originally deposited in the database as a pseudogene of MSY1, MSY3. It is 69 amino acids smaller than the YB2 protein. The expression of RYB-a mRNA in adult liver is very low, but is induced during liver regeneration and by serum stimulation of quiescent fibroblasts, suggesting that RYB-a is responsible for cell proliferation (Ito et al., 1994).

In this study, we raised an antibody against the recombinant protein for rat YB2/RYB-a and used it in some immunohistochemical studies for localization of the protein as well as in immunoblot analyses. We also employed in-situ hybridization to investigate the distribution of YB2/RYB-a and protamine 2 mRNA in the developing testes of mice and rats. The results showed that YB2/RYB-a mRNA and protein appeared in prepubertal mouse testes and preceded the expression of the protamine 2 mRNA, supporting its potential role as a translational repressor of mRNA during spermatogenesis. Although the expression of YB2/RYB-a in the rat testis was similar to that in the mouse, the expression level of protamine 2 in the rat is known to be very low, suggesting the influence of YB2/RYB-a on other mRNA species.

Materials and methods

Animals
Mice (Std/dvy) and rats (Wistar), purchased from Japan SLC (Shizuoka, Japan), were maintained under conventional conditions at the Laboratory Animal Center, Yamagata University School of Medicine, at least 2 weeks before use. This study was conducted in accordance with the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH, 80-23). Experiments using animals were performed in accordance with the Declaration of Helsinki under the protocol approved by the Animal Research Committee in our institution. Mice and rats were anesthetized with diethyl ether and killed either by perfusion with Bouin’s fixative through the heart or by cutting the vein.

Construction of a phylogenetic tree
Multiple alignment was done using Clustal W program (Thompson et al., 1994). The neighbour-joining method (Saitou and Nei, 1987) was used for constructing a phylogenetic tree. We also performed a bootstrap analysis to gain a statistical measure of confidence in the phylogenetic tree. By bootstrap analysis, a total of 1000 trees were generated from the initial data set, and the percentage of trees containing a particular clade was measured.

cDNA cloning of mouse protamine 2 by reverse transcription–polymerase chain reaction (RT–PCR)

The mouse protamine 2 (mP2) probe was constructed by RT–PCR cloning. Total RNA was extracted from mouse testicular tissue with Isogen (Wako, Osaka, Japan), according to the manufacturer’s protocol. RT–PCR was performed with the BcaBEST RNA PCR kit (Takara, Kyoto, Japan) using a pair of primers, mP2-F (5’-CTCGAGTTATGATGTTGCGGCCATCGG-3’) and mP2-R (5’-AACTGGATTGATGTTGCGGCCATCGG-3’), which flank the entire mP2 coding region and have EcoRI and Xhol sites in their 5’ ends, respectively. RT–PCR conditions were: 1 min at 95°C, then 25 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 1.5 min; then the products were kept at 4°C. The result of 312 bp mP2 cDNA was digested with EcoRI/Xhol, subcloned into pBluescript KS+ (Stratagene, La Jolla, CA, USA) at EcoRI/Xhol site, and subjected to DNA sequencing for confirmation.

Digoxigenin (DIG)-labelling of cRNA probes for YB2/RYB-a and mP2 mRNA detection
A 396 bp DNA fragment (nucleotide numbers 757–1152) was excised by digestion with HaeIII and Smal from the full length RYB-a cDNA in pBluescript KS+ (Ito et al., 1994) and was ligated into the EcoRV site of the vector. The vector containing the RYB-a cDNA was linearized by HindIII and EcoRI digestion for the production of sense and antisense cRNA respectively. The pBluescript KS+ vector containing the mP2 insert was linearized by Xhol and EcoRI for the production of the sense and antisense cRNA respectively. In-vitro transcription to produce sense and antisense digoxigenin (DIG)-labelled cRNA was performed using the RNA–DIG labelling mix (Boehringer Mannheim, Mannheim, Germany) and T3 and T7 RNA polymerases.

Northern blot analysis
RNA samples were electrophoresed in 1% agarose–formaldehyde gels, transferred to nylon membranes (MSI, MA, USA), and hybridized at 60°C overnight in hybridization buffer [50% formamide, 0.5% sodium dodecyl sulphate (SDS), 5% Irish Cream Liqueur, 0.75 mol/l NaCl, 43 mmol/l Na2PO4, and 6.25 mmol/l EDTA] containing DIG-labeled cRNA probes. The nylon membranes were washed sequentially, finally with 0.1×SSC containing 0.1% SDS at 65°C. The membranes were then incubated for 30 min at room temperature with an anti-DIG Fab-antibody conjugated with alkaline phosphatase (Boehringer Mannheim). The membranes were reacted with diosodium 3-(4-methoxyxyspiro[1,2-dioxetane-3,2-yl]phenyl phosphate (CSPD) (Boehringer Mannheim) and exposed to X-ray film to detect the signals from the enzymatic reaction.

Raising an anti-YB2/RYB-a antibody
A 327 bp fragment from the 3’ region of the RYB-a cDNA was amplified from the RYB-a clone (Ito et al., 1994) by PCR using a pair of primers: YB2-F (5’-AAGAATTCATGGTTCGACTCCAATGTCCTACAG-3’) and YB2-R (5’-AACTGGATTGATGTTGCGGCCATCGG-3’), which flank the entire RYB-a coding region and have EcoRI and Xhol sites in their 5’ ends, respectively. The PCR product which is common to YB2 and RYB-a was digested with EcoRI/Xhol, subcloned into pBluescript KS+ (Stratagene, La Jolla, CA, USA) at EcoRI/Xhol site, and subjected to DNA sequencing for confirmation.
gene expression was induced in the bacteria using a final concentration of 10 mmol/l isopropyl-β-D-thiogalactopyranoside overnight. Cells were harvested and subjected to purification for the GST–YB2/RYP–a fusion protein by the Bulk GST Purification Module (Amersham Pharmacia Biotech) according to the manufacturer’s protocol. Anti-YB2/RYP–a antiserum was raised by immunizing a female rabbit with the purified GST–YB2/RYP–a protein.

Western blot analysis

Tissues dissected from mice and rats were broken into pieces under liquid nitrogen and suspended in a buffer containing 25 mmol/l Tris–HCl, 50 mmol/l NaCl, 0.5% Na-deoxycholate, 100 mmol/l NaF, 2% NP-40, 0.2% SDS, and 200 μmol/l NaVO₄ supplemented with protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.57 mmol/l phenylmethylsulphonyl fluoride, and 10 μg/ml pepstatin). The lysate was centrifuged at 10 000 g for 5 min in a microcentrifuge. Protein concentrations of the supernatant were determined using a BCA kit (Pierce, Rockford, Illinois, USA). Total proteins (10 μg) were separated on 10% SDS–polyacrylamide gels and electroblotted onto PVDF membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). The blots were blocked with 10% non-fat dry milk in phosphate-buffered saline (PBS) and then incubated with the anti-YB2/RYP–a antisera diluted in PBS overnight at 4°C. After washing twice in PBS containing 0.1% Tween 20 and twice in PBS for 30 min, the blots were incubated with goat anti-rabbit IgG antibody conjugated with horseradish peroxidase. After washing as above, the presence of the enzyme was revealed by chemiluminescence with an ECL plus detection reagent (Amersham Pharmacia Biotech) and exposure to X-ray films.

Preparation for tissue sections

After perfusion with the Bouin’s fixative, the testes were removed from mice and rats, cut into pieces, and immersed in the Bouin’s fixative for a period ranging from 3 h to overnight. They were embedded in paraffin, sectioned at 4 μm thickness, and used for both in-situ hybridization and immunohistochemical detection.

In-situ hybridization

In-situ hybridization was performed as previously reported (Steger et al., 1998). Briefly, sections on the slides were deparaffinized, digested with proteinase K (20 μg/ml) for 5 min at room temperature, post-fixed in 4% paraformaldehyde, 0.2% glutaraldehyde for 20 min, followed by incubation in the prehybridization buffer containing 50% formamide, 0.5% SDS, 5% Irish Cream Liquor, 0.75 mol/l NaCl, 43 mmol/l Na₂PO₄, and 6.25 mmol/l EDTA. The sections were then hybridized with the DIG-labelled antisense YB2/RYP–a or mP2 RNA (2 μg/ml) in hybridization buffer containing 50% formamide, 1% SDS, 0.75 mol/l NaCl, 10 mmol/l PIPES, 0.05% heparin, and 100 μg/ml yeast tRNA (Sigma) at 50°C overnight. After washing with PBS, the tissue samples were incubated for 30 min at room temperature with an anti-DIG Fab-antibody conjugated with alkaline phosphatase (Boehringer Mannheim). Positive signals were visualized by the reaction of the alkaline phosphatase with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Boehringer Mannheim) under protection from light in a humid chamber. After dehydration by passing the slides through a series of graded ethanol solutions, they were mounted. For each test, control experiments using the DIG-labelled sense cRNA probe were performed.

Immunohistochemical staining

The paraffin sections were deparaffinized in xylene and hydrated in a series of graded ethanol solutions. After hydration, endogenous peroxidase was inactivated in 3% hydrogen peroxide. Prior to immuno-

staining, the non-specific binding of the antibody was blocked with 2% swine serum in Tris-buffered saline for 10 min. The slide glasses were immered in 50 μl of solution containing the anti-YB2/RYP–a antisera at a dilution of 1:250 in PBS, with the tissue face down, then incubated at room temperature in a humid chamber overnight. Following three consecutive washes in PBS for 5 min each, the sections were incubated at room temperature for 30 min with horseradish peroxidase-conjugated goat anti-rabbit IgG polymer (Dako, Carpinteria, CA, USA). To visualize the signals, the reaction was completed by incubating the sections in dianamobenidine tetrahydrochloride (DAB) reaction reagent (Dako) for several seconds. The resulting slides were then washed in water, dehydrated by passing through a series of graded ethanolos, and mounted. Photographs were taken using a digital camera under BX50 light microscope (Olympus, Tokyo, Japan).

Results

A phylogenetic tree of Y-box proteins

A phylogenetic tree was constructed from amino acid sequences of human DNA binding protein A (DBPA) and B (DBPB), rat YB2, mouse MSY1, MSY2 and MSY3, and frog FRGY1 and FRGY2 as shown in Figure 1A. Multiple alignment was done using Clustal W program (Thompson et al., 1994). The neighbour-joining method (Saitou and Nei, 1987) was used for constructing the phylogenetic tree. Vertebrate Y-box proteins could be classified into three large groups, although many Y-box proteins play multi-functional roles (Wolffe, 1994; Kleene, 1996; Matsumoto and Wolffe, 1998). YB2 and RYP–a are rat counterparts of mouse MSY3-L and MSY3-S respectively.

Expression of YB2/RYP–a mRNA

We first examined the levels of rat YB2/RYP–a mRNA in several tissues of the adult rat by Northern blot analysis of the total cellular RNA and identified strong signals at ~1.6–1.8 kb in testis, heart, and skeletal muscle (data not shown). This pattern was similar to that of the mouse (Mastrangelo and Kleene, 2000). We focused on testes because, of the tissues examined, the expression was the highest and a putative role of Y-box proteins has been proposed in spermatogenesis (Wolffe et al., 1992; Kleene, 1996). Since YB2 and RYP–a mRNA derive from the same gene by alternative splicing (Sapru et al., 1996; Mastrangelo and Kleene, 2000), a pair of primers that flank the alternative exon was used for RT–PCR to distinguish the two closely spaced transcripts. Two bands, corresponding to YB2 and RYP–a, were detected at similar levels in rat in testes, consistent with data reported for mouse testes by Mastrangelo and Kleene (2000), and indicating that both YB2 and RYP–a mRNA are simultaneously expressed (data not shown).

Specificity of the antibody to YB2/RYP–a

To analyse YB2/RYP–a protein, we raised a polyclonal antibody against the recombinant protein. The resultant antiserum reacted with GST–YB2/RYP–a in E.coli extracts as well as with the purified protein, but not with untransformed E.coli extract (Figure 2). The antibody recognized proteins of various sizes in rat and mouse testes. The proteins detected by this
Y. Iuchi et al.

**Figure 1.** (A) Phylogeny of Y box proteins from human (DBPB and DBPA), mouse (MSY1, MSY2 and MSY3), rat (YB2/RYB-a) and frog (FRGY1 and FRGY2). The tree was estimated from amino acid sequences using a neighbour-joining method. Thus, the branch lengths are meaningful. (B) Schematic representation of RYB-a and YB2 cDNA. A 396 bp DNA fragment, nucleotide numbers from 757 to 1152 of RYB-a cDNA, was digoxigenin-labelled and used as the cRNA probe for Northern blot analysis and in-situ hybridization. A 327 bp fragment of the 3' coding region of the RYB-a cDNA was amplified from the RYB-a clone by polymerase chain reaction (PCR) using a pair of primers, YB2-F and YB2-R. The PCR product was ligated to the GST fusion vector to produce a GST–YB2 fusion protein which was then used for antibody production. Boxes indicate the open reading frame. Shaded boxes indicate the region encoding the highly conserved cold-shock domain (CSD). Exon 6, represented as a black box, is an alternatively spliced exon found only in YB2.

**Figure 2.** Specificity of the anti-YB2/RYB-a antibody and immunoblot analysis of mouse and rat testes. Lane 1, 10 ng of control E.coli extract; lane 2, 10 ng of extract of E.coli expressing the GST–YB2/RYB-a fusion protein; lane 3, 10 ng of affinity-purified GST–YB2/RYB-a fusion protein; lane 4, 10 µg of total protein extract of mouse testis; lane 5, 10 µg of total protein extract of rat testis.

**Figure 3.** Expression of YB2/RYB-a and mP2 during development of mouse testes. (A) Northern blot analysis was performed using total RNA (10 µg) isolated from various stages of prepubertal and adult (>3 months) mouse testes. Two closely spaced transcripts for YB2/RYB-a were detected. (B) Western blot analysis for YB2/RYB-a in protein extracts (10 µg) from testes at the same developmental stages as in (A) and in spermatozoa with the antisera specific to rat YB2/RYB-a. (C) The same blot as in (A) was deprobbed and hybridized with the mP2 cRNA probe. (D) Ribosomal RNA stained with ethidium bromide are shown in the agarose gel used for Northern analysis.

antibody, however, appeared to be products of the same YB2/RYB-a gene because the proteins were detected only in tissues with high mRNA expression. Bands of 48 and 55 kDa were observed in mouse and rat testes respectively, although the predicted size from the cDNA sequences is 38 kDa. Such a divergence in size from the theoretical value is peculiar, but is commonly seen for the Y-box proteins (Davies et al., 2000). Differences in the molecular sizes of YB2/RYB-a among tissues is probably due to the differences in post-translational modifications, although the actual mechanisms responsible for this are still unknown. This antibody was used in the following experiments.

**Changes in YB2/RYB-a expression during mouse development**

Since an involvement of Y-box proteins in spermatogenesis is proposed, we examined the levels of the YB2/RYB-a mRNA and the corresponding proteins at different developmental stages of mice and compared them with the mRNA levels for mP2 (Figure 3). The levels of both the YB2/RYB-a mRNA and protein were low in the testes of prepubertal mice before 17 days of age, and increased in the testis after the animals reached the age of 20 days. The YB2/RYB-a protein was not detected in spermatozoa. On the other hand, the mP2 mRNA was initially detected in the testis of 26 day old mice, when spermiogenesis occurs. Thus, the presence of YB2/RYB-a protein preceded mP2 transcription, and was not carried over into spermatozoa.
Expression of a Y-box protein during spermatogenesis

**Figure 4.** Localization of the YB2/RYB-a protein and the mP2 mRNA in adult mouse testis. Immunohistochemistry of YB2/RYB-a (a, c and e) and in-situ hybridization of mP2 (b, d and f) were carried out for serial sections of the adult testis. Different magnifications are shown; (e) and (f) are enlargements of the boxed area in (c) and (d) respectively. Roman numerals indicate the stage of the seminiferous epithelial cycle (Clermont, 1972). Bar = 100 µm.

**Localization of YB2/RYB-a in developing and adult mice testes**

We investigated which cells express the YB2/RYB-a protein and the mP2 mRNA by immunohistochemistry and in-situ hybridization respectively. A control incubation with DIG-labelled YB2/RYB-a cRNA sense probe was completely negative (data not shown). In adult mouse testis, the mP2 mRNA was strongly detected in round spermatids and elongating spermatids, and to a lower extent in early elongated spermatids in the seminiferous epithelial cycle (Figure 4). These observations are almost consistent with previous works (Mali et al., 1989; Morales et al., 1991). Although Mali et al. (1989) first detected transition protein 1, protamine 1 and protamine 2 mRNA in step 7 round spermatids, Morales et al. (1991) detected protamine 1 and transition protein 1 mRNA as early as step 1. The discrepancy could be due to sensitivity of the detection methods used by them as supposed by Morales et al. (1991). The YB2/RYB-a protein was observed in all stages of the adult seminiferous tubules and was present at high levels in spermatocytes, decreased in round to elongated spermatids and was absent in spermatozoa. The intracellular localization of YB2/RYB-a was predominantly cytoplasmic. Signals for YB2/RYB-a and mP2 coexisted in stages I–III and VII–XII.

We then investigated when the expression of YB2/RYB-a mRNA and protein, and of mP2 mRNA, occurred during mouse development. In-situ hybridization carried out for serial sections of mouse testes around sexual maturation indicated that the YB2/RYB-a mRNA, hybridizable to the antisense YB2/RYB-a cRNA probe, began to appear in mice at the age of 14 days (Figure 5b). The number of cells expressing the mRNA increased during sexual maturation of the testes (Figure 5b,f,j,n). Consistent with the results in Figure 4, pachytene spermatocytes were found to be positive, but no expression was observed in spermatogonia that localize in the peripheral region of the seminiferous tubules. Round to elongated spermatids also expressed YB2/RYB-a to a lesser extent. The cells
Expression of a Y-box protein during spermatogenesis

which produced the YB2/RYB-a protein precisely matched those which expressed the mRNA (Figure 5c,g,k,o), suggesting that the translation of YB2/RYB-a occurred concomitantly with transcription. The mP2 mRNA became detectable at the age of 28 days and, consistent with that observed in Figure 4, was strongly detected in round and elongating spermatids, and to a lesser extent, in elongated spermatids in adult testes (Figure 5d,h,l,p).

**YB2/RYB-a expression in rat testis**

We also investigated the expression of YB2/RYB-a in rat testis in which the relative abundance of the protamine 2 mRNA is ~50-fold lower than that in mouse testes (Bower *et al*., 1987). Northern blot analysis was carried out for total RNA, obtained from sexually maturing rat testes. In-situ hybridization and immunohistochemical staining were also performed, in order to specify cells which express YB2/RYB-a (Figure 6B). The rat YB2/RYB-a mRNA was detectable in pachytene spermatocytes (Figure 6B: b) and increased in testes of rats from 21 to 25 days of age (Figure 6A). In the adult testis, the YB2/RYB-a protein, which was detectable by immunohistochemistry, appeared in pachytene spermatocytes and round spermatids (Figure 6B: d), consistent with findings in mouse testis (Figure 4).

**Discussion**

The expression of one of the mammalian Y-box proteins, YB2/RYB-a, during spermatogenesis and the changes accompanying testicular maturation in rodents has been investigated. The Y-box protein family is assumed to function both as a transcriptional activator in the nucleus and as a translational repressor in the cytoplasm (Wolffe, 1994). In testis, a number of mRNA are known to be regulated at the translational stage via the formation of complexes with mRNA binding proteins. Protamine mRNA is a well-known transcript that is transcribed

---

**Figure 5.** YB2/RYB-a mRNA and protein and mP2 mRNA in prepubertal and adult mouse testes at different developmental stages. Serial sections from mouse testes at 14 days (a–d), 21 days (e–h), 28 days (i–l), and adult (m–p) were stained with haematoxylin and eosin (HE) (a, e, i and m). In-situ hybridization was performed using a digoxigenin-labelled YB2/RYB-a cRNA probe (b, f, j and n) and a digoxigenin-labelled mP2 cRNA probe (d, h, l and p). Immunohistochemical staining was performed with the anti-YB2/RYB-a antisera (c, g, k and o). Bar = 100 μm.

**Figure 6.** Expression of YB2/RYB-a in rat testes. (A) Total RNA (10 μg) were isolated from rat testes at 14, 21, 25 and 30 days and 6 and 18 months and were used for Northern blot hybridization. Two closely spaced transcripts for YB2/RYB-a were detected. (B) Localization of YB2/RYB-a mRNA and proteins in the adult rat testis. In-situ hybridization was performed for sections of 21 day (a), 25 day (b) and adult (c) rat testes. Immunohistochemical staining of YB2/RYB-a protein was performed in adult rat testis (d). Bar = 100 μm.
in round spermatids, stored in a translationally inactive form for several days, and is then actively translated in elongated spermatids (Kleene, 1996). Previous investigations have revealed the cell type-specific distribution of human protamine 1 (PRM-1) and protamine 2 (PRM-2) mRNA in adult human testis (Steger et al., 2000). During normal spermiogenesis, both PRM-1 and PRM-2 mRNA were found in round spermatids and elongating spermatids, but are completely absent in elongated spermatids. Thus the timing of mRNA and protein expression suggests that tightly bound protein repressors must prevent the PRM-1 and PRM-2 transcripts from being translated in round elongating spermatids.

The YB2/RYB-a protein initially appeared in pachytene spermatocytes of the prepubertal testis and persisted until the early elongated spermatid stage in adult rodent testes. A strong signal for YB2/RYB-a was detected in the cytoplasm of the pachytene spermatocytes and in all round spermatids, and weak signal was detected in elongating spermatids, while the mP2 mRNA appeared in round spermatids, elongating spermatids and in early elongated spermatids. Hence, the YB2/RYB-a protein and mP2 mRNA coexisted in round spermatids and in elongating spermatids, but not at other stages. The level of the YB2/RYB-a protein decreased in the late elongated spermatids and finally became undetectable in spermatozoa. These observations are generally consistent with previous studies of MSY3 (Davies et al., 2000) and protamine mRNA (Mali et al., 1989) and, hence, support the proposal that the YB2/RYB-a protein masks protamine 2 mRNA in round spermatids to elongating spermatids and prevents them from being translated (Davies et al., 2000; Steger et al., 2000). Accordingly, a substantial fraction of the free mRNA in pachytene spermatocytes to round spermatids in mice could be expected to be translationally inhibited (Tafuri et al., 1993) as these spermatogenic cells contain high levels of the Y-box proteins. Two proteins in the Y-box family, FRGY1 and FRGY2, are believed to function as non-specific translational repressors in Xenopus oocytes as judged from the abundance of free mRNA, the low sequence specificity of binding to mRNA, and the inhibition of translation both in vitro and in vivo (Tafuri and Wolff, 1990; Wolff, 1994). The mouse MSY3 protein, a mouse counterpart of the YB2/RYB-a protein, and MSY2 have been shown to bind protamine 1 mRNA in a sequence-specific manner (Davies et al., 2000). Our observations about the expression of the YB2/RYB-a protein and the mP2 mRNA suggest that the Y-box protein might also interact with protamine 2 mRNA in the mouse testis. However, the YB2/RYB-a protein could also bind mRNA other than protamine mRNA in the testis due to its abundance in these mouse and rat tissues. In meiotic and early haploid cells, large amounts of mRNA, which are transcribed from various genes, are masked by RNA binding proteins. MSY2, a mouse counterpart of Xenopus FRGY2, exhibits a similar distribution to that of YB2/RYB-a in the mouse testis and binds to RNA in a sequence- and structure-independent manner (Oko et al., 1996; Gu et al., 1998). Thus, YB2/RYB-a may also function as both a sequence-specific and a non-specific mRNA binding protein. The involvement of YB2/RYB-a with other mRNA was suggested by the rat spermatocytes which expressed levels of the YB2/RYB-a protein similar to those of the mouse cells even though they contain only a very low level of proteamine 2 mRNA (Bower et al., 1987). When masked, the mRNA would only become translatable in elongated spermatids, at which time the masking proteins, such as the YB2/RYB-a protein, would be replaced by a translational activator (Lee et al., 1996; Zhong et al., 1999).

DBPA, a human counterpart of the rat YB2/RYB-a, plays a functional role in the transcriptional regulation of the major histocompatibility complex I–αβ gene (Lloberas et al., 1995). To investigate the possibility that YB2/RYB-a functions not only as a translational regulator but also as a transcriptional regulator, we performed a gel mobility shift assay using an oligonucleotide from mP2 promoter sequences and nuclear extracts of mouse testis. However, we failed to detect a specific signal which was shifted by the anti-YB2/RYB-a antibody or to show the presence of the YB2/RYB-a protein in the nuclear extract of testis by immunoblot analysis (data not shown). This is consistent with the immunohistochemical data in which the nuclei were negative for antibody binding. Since only a few molecules are required for transcriptional activity, further detailed analysis will be required to clarify this point.

In addition to its testicular expression, YB2/RYB-a is also found in other tissues, including skeletal muscle, heart, and regenerating liver (Ito et al., 1994). Its function in these tissues remains unknown, but it may play a role in cell cycle regulation. Moorhamer et al. (1999) have reported that human DBPA binds cyclin-dependent kinase 5 (Cdk5) and Cdk4 and inhibits their activity. Although Cdk5 is expressed in both proliferative and differentiated cells, its expression is relatively abundant in nerve and muscle cells. Essential roles of Cdk5 and Cdk4 kinase activity for the differentiation of these cells have actually been reported (Nikolic et al., 1996; Chae et al., 1997; Lazaro et al., 1997; Philpott et al., 1997). Thus, it is possible that YB2/RYB-a may also play a role in the cell cycle through interaction with Cdk5 or Cdk4 or both, resulting in an alteration in gene expression during differentiation.

In conclusion, we have observed that expression of YB2/RYB-a precedes that of protamine 2 mRNA in mouse, suggesting a role for YB2/RYB-a as a translational repressor of protamine 2. However, an additional function is implied as the expression of YB2/RYB-a was also high in rat testis which expresses a very low level of proteamine 2 mRNA.

Acknowledgements
We thank the staff of the Laboratory Animal Center, Yamagata University School of Medicine, for housing and caring for the rats. We are grateful to Dr. Takuki Naruse, Department of Orthopedics, Yamagata University School of Medicine, for valuable advice on in-situ hybridization. This work was supported, in part, by a Grant-in-Aid for Scientific Research (C) (No. 13670111) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan and by Pfizer Pharmaceuticals Inc.

References
Expression of a Y-box protein during spermatogenesis


Received on April 2, 2001; accepted on August 22, 2001