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Expression of a Novel RAD23B mRNA Splice Variant in the Human Testis

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ABSTRACT: A gene coding a novel human RAD23B protein named RAD23-like protein B, presumably involved in spermatogenesis, was identified and characterized using a complementary DNA (cDNA) microarray. In the human testis, its expression was 2.33 times higher than it was in the embryo testis, with a particularly high expression in ejaculated human spermatozoa. The full length of this gene is 1548 bp, and the putative protein is 338 amino acids long. This protein is homologous to RAD23B, which is one of two human homologs of *Saccharomyces cerevisiae* RAD23, and is involved in both nucleotide excision repair (NER) and ubiquitin (Ub)-dependent proteolysis. However, RAD23-like protein B lacks the Ub-like (UbL) domain that functions as a proteasome localization signal. Multiple-tissue expression profile of the messenger RNA (mRNA) that encodes the RAD23-like protein B also showed that it is highly expressed in the human testis and in ejaculated spermatozoa. Our present study indicates that this novel alternative splicing form of RAD23B is correlated with human spermatogenesis.

Key words: RAD23, alternative splice, ubiquitin, spermatogenesis, proteolysis, self-balance.

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RAD23B is one of two human homologs of the *Saccharomyces cerevisiae* nucleotide excision repair (NER) gene product RAD23 and a component of a protein complex that specifically complements the NER defect of xeroderma pigmentosum group C (XP-C) cell extracts in vitro. Most NER gene difficulties cause a marked decrease in survival (De Laat et al, 1999). In contrast, the deletion of the yeast RAD23 gene results in intermediate sensitivity to UV light (Watkins et al, 1993), suggesting that its product plays a regulatory role. An amino acid (aa) sequence comparison of the yeast RAD23 and its mammalian homologs showed the existence of at least 4 distinct domains that are well conserved among these proteins (van der Spek et al, 1996). First, this class of proteins is predominantly characterized by a ubiquitin-like (UbL) sequence at the amino terminus. This UbL domain can bind to the proteasome, and the removal of this domain from the yeast RAD23 prevented interaction with the proteasome and was associated with increased sensitivity to UV light (Watkins et al, 1993; Schauber et al, 1998). The second and fourth domains from the amino terminus are Ub-associated (UBA) domains, suggesting the involvement of RAD23 in certain pathways of Ub metabolism (Hofmann and Bucher, 1996). By deletion and truncation analysis of the recombinant RAD23 protein, the third domain, the stress-induced phosphoprotein (STI1), has recently been found to be responsible for binding the XP-C protein (Masutani et al, 1997).

Ubiquitination is likely one of the most versatile cellular regulatory mechanisms controlling physiologic and pathologic events. A variety of elements involved in the Ub-dependent proteolysis system have been detected in the testis, epididymis, and seminal plasma (Baarends et al, 1999; Bebington et al, 2001). The activity of the Ub system is relatively high during spermatogenesis. Thus, it can be implicated in protein degradation/recycling or the elimination of defective cells as well as apoptosis, not only during spermatogenesis but also during later epididymal maturation (Sutovsky et al, 2003).

Using a high-throughput adult testis complementary DNA (cDNA) microarray that was prepared by our laboratory, a comparison of the genes expressed in the embryo, adult testis, and human ejaculated spermatozoa was made. We report a novel isoform of RAD23B, named RAD23-like protein B, which is expressed both in the adult and embryo testis although much more strongly in the adult than in the embryo. Compared to RAD23B, the RAD23-like protein B lacks a UbL domain. Furthermore, the expression profile of the messenger RNA (mRNA) that encodes for RAD23-like protein B indicates that it is highly expressed in the testis and in ejaculated spermatozoa. The present results also suggest that the encoded

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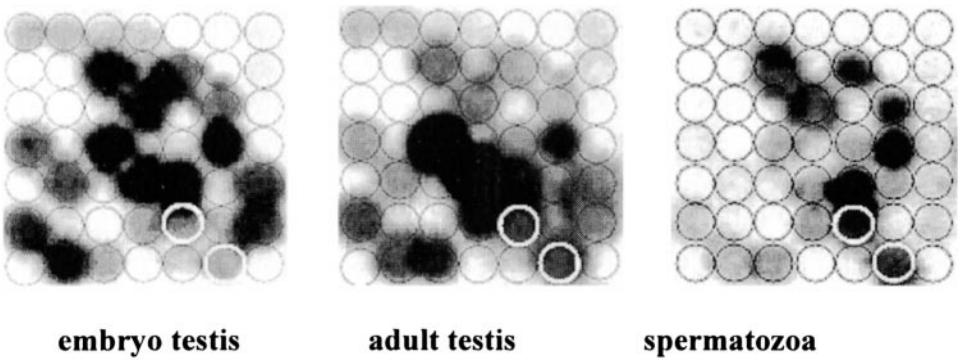


Figure 1. Partial complementary DNA (cDNA) hybridization images showing the differential expression of the gene for the RAD23-like protein B product in the **(left)** embryo testis, **(center)** adult testis, and **(right)** spermatozoa. White rings indicate RAD23-like protein B cDNA. The intensity in embryo testis, adult testis, and spermatozoa was 77.46, 180.26, and 190.44, respectively.

protein of the gene for RAD23-like protein B influences spermatogenesis by a Ub-dependent proteolytic pathway.

Materials and Methods

Samples

Consent forms were signed by all participants, and approval to conduct this research was granted by the ethics committee of Nanjing Medical University before sample collection was begun. Human adult testes were obtained from deceased people. Embryo testes were obtained from accidentally aborted 6-month fetuses. Human ejaculates were obtained from healthy volunteers of proven fertility and of normal semen quality as assessed by World Health Organization criteria (1999) (ie, $>20 \times 10^6$ spermatozoa/mL with more than 50% active sperm, more than 25% sperm moving forcefully in 1 direction [rapid and linearly progressive], and less than 1 lymphocyte per high-power field [40× magnification]).

Preparation of the Human Testis cDNA Microarray

A total of 9216 positive phage clones were chosen randomly from the Human Testis Insert phage λ cDNA library (clontech, Hl5503U) and were amplified by polymerase chain reaction (PCR). Then, the PCR products were spotted on the membrane to create a human testis cDNA microarray. The detailed methods were identical to those previously described (Cheng et al, 2002; Sha et al, 2002).

Screening of Genes Differentially Expressed in Embryo Testis, Adult Testis, and Human Ejaculated Spermatozoa

The adult testis cDNA microarray was hybridized with ^{33}P-labeled cDNA probes from the embryo testis, adult testis, and human ejaculated spermatozoa. Then, sequence identification and data analysis were performed (Cheng et al, 2002; Zhou et al, 2002).

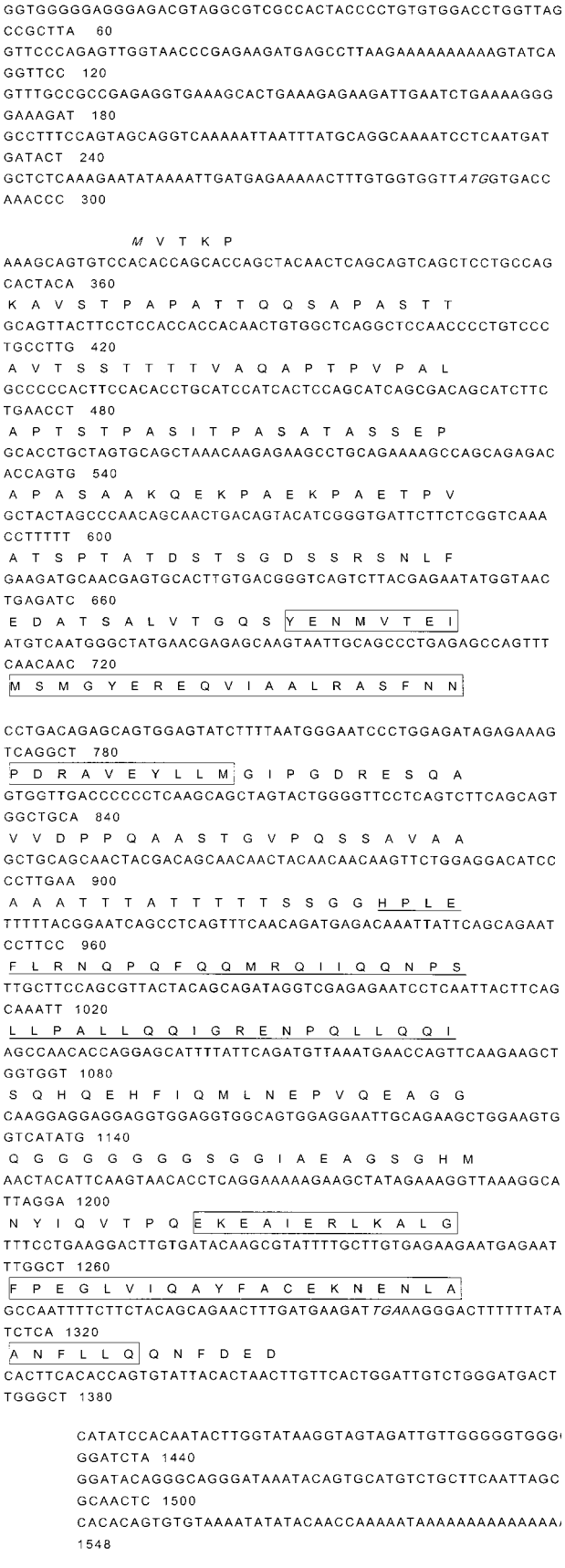
Tissue Distribution of the mRNA That Encodes for RAD23-Like Protein B

The screening experiments identified a novel gene named RAD23-like protein B in the human testis. The tissue distribution of the mRNA that encodes for RAD23-like protein B was determined using PCR. Human testis and ejaculated spermatozoa total RNAs were isolated using Trizol reagent (GIBCO), and cDNAs were amplified using reverse transcriptase (RT)-PCR in our laboratory. Multiple-tissue cDNA panels, including 16 kinds of human tissue (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and blood leukocytes), were purchased from Clontech (K1420-1 and K1421-1). The primers of the gene for the RAD23-like protein B were as follows: F 5′TTAGCCGCTTAGTTCCCAG3′ and R 5′GGTCACCATAACCACCAC3′. The PCR product size was 240 bp. G3PDH (glyceraldehyde 3-phosphate dehydrogenase) and β-actin were used as positive controls. PCR was performed according to the manufacturer's instructions, and conditions were as follows: denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 30 seconds. The first cycle had a denaturation period of 5 minutes. The last cycle had an extension period of 7 minutes. Thirty-five cycles were performed, and the PCR products were analyzed after electrophoresis.

Results

cDNA Microarray Hybridization

After hybridization and data analysis, the genes that were differentially expressed in human adult and embryo testes were considered related to testis development and spermatogenesis. Altogether, 386 Unigene clusters were identified. Of these, 68 Unigene clusters were identified as having novel alternative splicing forms in the microarray (see http://www.reprod-lab.net/). RAD23-like protein B is just the novel alternative splicing form of RAD23B. The hybridization intensity of the mRNA that encodes for RAD23-like protein B in the adult testis, embryo testis, and ejaculated spermatozoa was 180.26, 77.46, and 190.44, respectively (Figure 1). Obviously, the mRNA that encodes for RAD23-like protein B was expressed in both the adult and embryo testis, but the expression level in the adult testis was about 2.33-fold higher than that in



embryo testis. Furthermore, the mRNA that encodes for RAD23-like protein B was highly expressed in ejaculated spermatozoa. On the basis of what has already been described, we predicted that the protein encoded by the alternatively spliced mRNA would be related to spermatogenesis.

Features of cDNA and Its Deduced Protein

The nucleotide acid length of the gene for RAD23-like protein B was 1548 bp. The putative protein was 338 aa (Figure 2). A blast search in the human genome database localized the RAD23-like protein B gene to 9q31–q32. This gene possessed 10 exons (Figure 3). Analysis of the aa sequence using SMART software (http://smart.embl-heidelberg.de/) revealed that the encoded protein had 2 UBA domains and 1 STI1 domain (Figure 2). The first UBA domain was located at 118–155 aa, and the second was located at 294–331 aa. The gene for the RAD23-like protein B product has GenBank accession number AY313777.

Homologous Comparison Between RAD23-Like Protein B and RAD23B

Blast searches showed that the gene for RAD23-like protein B was highly homologous to the gene for RAD23B (GenBank accession number NM_002874). The cDNA for RAD23-like protein B is shorter than that for RAD23B, indicating that the 2 cDNAs were transcripted from the same DNA and that they then underwent alternative splicing. A splicing comparison of the 2 cDNAs showed that both have 8 identical exons in the middle of their cDNAs. The differences were at the first and last exons (Figure 3). Because the nucleic acids differed, the sequences of proteins differed. Like RAD23-like protein B, RAD23B also contains 2 UBA domains and 1 STI1 domain. But RAD23B has a UbL domain added at its amino-terminal end (Figure 4).

Tissue Distribution of the mRNA That Encodes for RAD23-Like Protein B

PCR and electrophoresis showed that the mRNA that encodes for RAD23-like protein B was widely expressed in human tissues, with a high expression level occurring in the human testis and ejaculated spermatozoa (Figures 5 and 6). Almost no expression signal was detected in the human ovary, indicating that this alternative splice product is involved only in male reproduction.

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Figure 2. Nucleic acid and deduced amino acid sequences of the complementary DNA (cDNA) for the RAD23-like protein B gene. The initiation and stop codons are in italic type. The 2 ubiquitin-associated (UBA) domains are boxed, and the stress-induced phosphoprotein (STI1) domain is underlined.

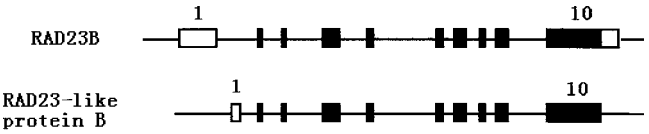

Figure 3. A transcript and splicing comparison between the genes for RAD23-like protein B and RAD23B. The 8 identical exons are shown with black rectangles. Differences are shown with empty rectangles.

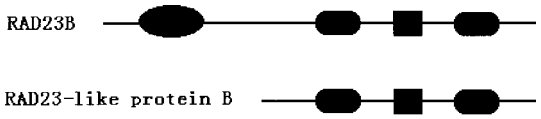

Figure 4. A comparison of the protein domains of RAD23-like protein B and RAD23B. A black oval indicates the ubiquitin-like (UbL) domain. A larger black ellipse indicates ubiquitin-associated (UBA) domain. A black rectangle indicates the stress-induced phosphoprotein (STI1) domain.

Discussion

Recently, a testis cDNA microarray was used to identify the genes related to testis development and spermatogenesis. A RAD23B-like gene, named the RAD23-like protein B gene, was identified. Our present data showed that cDNA microarray analysis was an efficient method for identifying testis gene expression profiles (Sha et al, 2002); additionally, RAD23-like protein B was shown to be a UbL protein that may influence spermatogenesis by a Ub-dependent proteolytic pathway.

The RAD23 protein was originally isolated during a screening for UV-sensitive mutants (Haynes and Kunz, 1981) and was the first UbL protein identified in yeast (Watkins et al, 1993). Previous studies have shown that the RAD23 protein inhibits multi-Ub chain formation and the degradation of proteolytic substrates (Ortolan et al, 2000). Multi-Ub chains are required for the degradation of most proteolytic substrates and are recognized by specific subunits in the proteasome. Further research has been conducted to investigate which domain is responsible for this action. Chen et al (2001) generated RAD23 mutants that were expressed in yeast as fusions to glutathione *S*-transferase (GST), and they thereafter carried out ubiquitination assays in vitro. Both GST-RAD23 and GST-$^{\Delta ubL}$Rad23 (a mutant lacking UbL) could bind Ub and showed potent inhibitory activity, whereas GST-UbL (which contained only the UbL domain) had no effect. Then, they investigated the interaction between Ub and GST-UBA^1 and GST-UBA^2 fusion proteins. A significant interaction was detected with GST-UBA^1 in one study, and a weaker binding was noted with GST-UBA^2 in another. All of the above results demonstrated that both UBA domains contributed to the functioning of RAD23, and they interacted with ubiquitinated substrates to inhibit the expansion of a nascent multi-Ub chain. The subsequent translocation of the tethered substrates to the proteasome, next to the UbL domain, could facilitate degradation by proteasome-associated E2 and E3 factors (Xie and Varshavsky, 2000). The UbL domain was not required for RAD23/Ub binding or for inhibiting multi-Ub chain formation, and so it may function primarily as a proteasome localization signal.

Besides the above research, Ng et al (2002) constructed the mouse homolog of RAD23B-deficient mice. They discovered that a disruption of the mouse RAD23B gene resulted in severely compromised phenotypes, such as mice with impaired embryonic development and intrauterine death, retarded growth, facial dysmorphology, and especially male sterility. A knockout of the mouse RAD23B gene caused defective spermatogenesis, which resulted in the absence of developing germ cells and a phenotype similar to that of the Sertoli cell–only syndrome. Mouse RAD23B genes are expressed in all mouse tissues and organs, but they show enhanced mRNA levels in the testis (van der Spek et al, 1996); thus, this gene could be crucial to germ cell development, and the loss of the encoded proteins might have specific gonadal consequences.

RAD23-like protein B is homologous to RAD23B, but it lacks the UbL domain at its amino terminus. Based on the previous research by Chen et al (2001), we can speculate that the RAD23-like protein B has a similar function to that of RAD23B, because they both have 2 UBA domains that contribute to the functioning of the entire protein, indicating that the RAD23-like protein B interacts with a ubiquitinated substrate to inhibit the expansion of a nascent multi-UbL chain and ultimately inhibits the degradation of proteolytic substrates. On the other hand, the mRNA of the RAD23-like protein B was highly expressed in the human adult testis but not in the human ovary. This is good evidence that the RAD23-like protein B is as involved in male germ cell development as is RAD23B. To further illustrate its function, we explore its location in the human testis. Although ejaculated spermatozoa are terminally differentiated cells, some studies have suggested that the mRNAs seen in mature spermatozoa are remnants of untranslated stored mRNA and that they provide a historic record of spermatogenesis (Miller et al, 1994). The mRNA of RAD23-like protein B is highly expressed in normal ejaculated spermatozoa, and so we think this is a historic record of the function of RAD23-like protein B in spermatogenesis (ie, the gene for RAD23-like protein B may be translated at some time during spermatogenesis and may play an important role at that time).

Recent results from the human genome project indicate that fewer genes than originally thought are necessary to express the complexity seen in human cells, such as germ cells. The limited numbers of gene products available in a cell must therefore perform diversified functions (Bal-

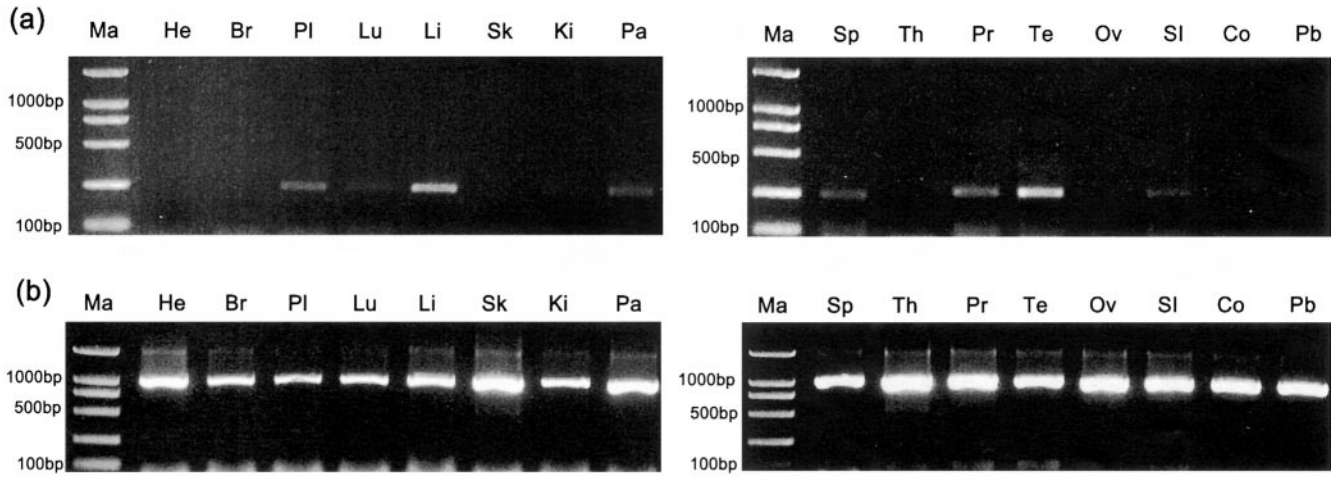

Figure 5. Tissue distribution of **(a)** RAD23-like protein B with **(b)** glyceraldehyde 3-phosphate dehydrogenase (G3PDH) as the control as shown by electrophoresis. RAD23-like protein B was widely expressed in human tissues, with a high expression level in the human testis. G3PDH was expressed in all tissues. Ma indicates marker; He, heart; Br, brain; Pl, placenta; Lu, lung; Li, liver; Sk, skeletal muscle; Ki, kidney; Pa, pancreas; Sp, spleen; Th, thymus; Pr, prostate; Te, testis; Ov, ovary; Si, small intestine; Co, colon; and Pb, P. blood leukocyte.

timore, 2001). Ub and UbL proteins are prime examples of such strategies. They control the degradation of substrates that are as diverse as cyclins, viral envelope proteins, and plasma membrane receptors. Ub-dependent proteolysis plays a proven role in germ cell differentiation inside the testicular seminiferous tubules and in cell cycle control throughout spermatogenesis, such as the dramatic reduction of cytoplasmic volume and the replacement of the spermatid's nuclear histones by transition proteins, followed by permanent substitution with protamines dur-

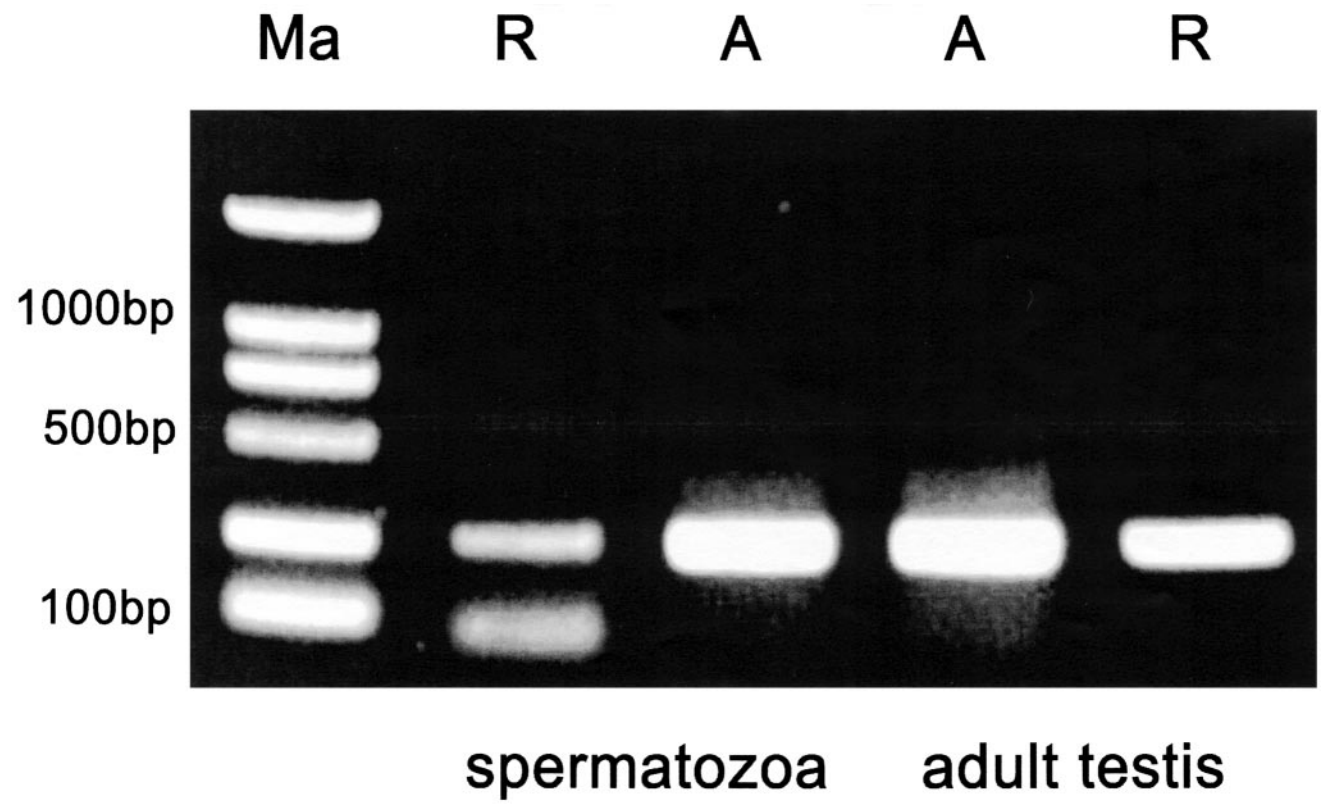

Figure 6. The expression of RAB23-like protein B in the human adult testis and spermatozoa with β-actin as the control as shown by electrophoresis. RAD23-like protein B was highly expressed in both tissues as the result of hybridization. Ma indicates marker; R, RAD23-like protein B; and A, β-actin.

ing spermatid elongation (Chen et al, 1998; Oko and Clermont, 1998). It is well known that our bodies' mechanisms are balanced by homeostasis. So, although ubiquitination is relatively high in spermatogenesis, proteins must also exist that inhibit ubiquitination to maintain a healthy balance (Sakks et al, 1999). The mRNA that encodes for RAD23-like protein B is highly expressed in human adult testis and spermatozoa; hence, perhaps its purpose is that of a protein inhibitor that competes for the binding of Ub in order to prevent Ub-dependent proteolysis.

Spermatozoa must be normal and must function normally to ensure successful fertilization and embryonic development; thus, during mammalian spermatozoa maturation, defective sperm must be eliminated (Rajapurohitam et al, 2002; Toshimori, 2003). A series of experiments showed that both normal and defective sperm carry intrinsic, constitutively ubiquitinated substrates; however, only the defective sperm become surface ubiquitinated during epididymal passage and subsequently phagocytosed by the epididymal epithelial cells (Sutovsky et al, 2001). Because RAD23-like protein B is expressed in ejaculated spermatozoa and because normal sperm escape ubiquitination and maintain their functional structure during their maturation in the epididymis, we speculate that many proteins play a functional role in this process. The RAD23-like protein B may be only one of these proteins that maintain normal sperm structure and function by inhibiting Ub-dependent proteolysis.

In summary, the encoded protein of RAD23-like protein B, with the high level of expression of its mRNA in the human adult testis and spermatozoa, may play an important role in spermatogenesis and later sperm maturation by inhibiting Ub-dependent proteolysis. Further study is required for a better understanding about the exact role that the encoded protein of RAD23-like protein B plays in the mechanism and regulation of spermatogenesis.

References

Baarends WM, Roest HP, Grootegoed JA. The ubiquitin system in gametogenesis. *Mol Cell Endocrinol.* 1999;151:5–16.

Baltimore D. Our genome unveiled. *Nature.* 2001;409:814–816.

Bebington C, Doherty FJ, Fleming SD. The possible biological and reproductive functions of ubiquitin. *Hum Reprod Update.* 2001;7:102–111.

Chen HY, Sun JM, Zhang Y, Davie JR, Meistrich ML. Ubiquitination of histone H3 in elongating spermatids of rat testes. *J Biol Chem.* 1998; 273:13165–13269.

Chen L, Shinde U, Ortolan TG, Madura K. Ubiquitin-associated (UBA) domains in RAD23 bind ubiquitin and promote inhibition of multiubiquitin chain assembly. *EMBO Rep.* 2001;2:933–938.

Cheng LJ, Zhou ZM, Li JM, et al. Expression of a novel HsMCAK mRNA splice variant, tsMCAK gene, in human testis. *Life Sci.* 2002; 71:2741–2757.

De Laat WL, Jaspers NGJ, Hoeijmakers JHJ. Molecular mechanism of nucleotide excision repair. *Genes Dev.* 1999;13:768–785.

Haynes RH, Kunz BA. DNA repair and mutagenesis in yeast. In: Strathern JN, Jones EW, Breach JR, eds. *Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1981:371–414.

Hofmann K, Bucher P. The UBA domain: a sequence motif present in multiple enzyme classes of the ubiquitination pathway. *Trends Biochem Sci.* 1996;21:172–173.

Masutani C, Araki M, Sugasawa K, et al. Identification and characterization of XPC-binding domain of hHR23B. *Mol Cell Biol.* 1997;17: 6915–6923.

Miller D, Tang PZ, Skinner C, Lilford RJ. Differential RNA fingerprinting as a tool in the analysis of spermatozoal gene expression. *Hum Reprod.* 1994;9:864–869.

Ng JMY, Vrieling H, Sugasawa K, et al. Developmental defects and male sterility in mice lacking the ubiquitin-like DNA repair gene mHR23B. *Mol Cell Biol.* 2002;22:1233–1245.

Oko R, Clermont Y. Spermiogenesis. In: Knobil E, Neil JD, eds. *Encyclopedia of Reproduction.* Vol IV. San Diego, Calif: Academic Press; 1998:602–609.

Ortolan TG, Tongaonkar P, Lambertson D, Chen L, Schauber C, Madura K. The DNA repair protein RAD23 is a negative regulator of multiubiquitin chain assembly. *Nat Cell Biol.* 2000;2:601–608.

Rajapurohitam V, Bédard N, Wing SS. Control of ubiquitination of proteins in rat tissues by ubiquitin-conjugating enzymes and isopeptidases. *Am J Physiol Endocrinol Metab.* 2002;282:E739–E745.

Sakks D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG, Bianchi U. Origin of DNA damage in ejaculated human spermatozoa. *Rev Reprod.* 1999;4:31–37.

Schauber C, Chen L, Tongaonkar P, Vega I, Lambertson D, Potts W, Madura K. RAD23 links DNA repair to the ubiquitin/proteasome pathway. *Nature.* 1998;391:715–718.

Sha JH, Zhou ZM, Li JM, et al. Identification of testis development and spermatogenesis-related genes in human and mouse testis using cDNA microarray. *Mol Hum Reprod.* 2002;8:511–517.

Sutovsky P. Ubiquitin-dependent proteolysis in mammalian spermatogenesis, fertilization, and sperm quality control: killing three birds with one stone. *Microsc Res Tech.* 2003;61:88–102.

Sutovsky P, Moreno R, Ramalho-Santos J, Dominko T, Thompson WE, Schatten G. A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. *J Cell Sci.* 2001;114:1665–1675.

Toshimori K. Biology of spermatozoa maturation: an overview with an introduction to this issue. *Microsc Res Tech.* 2003;61:1–6.

van der Spek PJ, Visser CE, Hanaoka F, Smit B, Hagemeijer A, Bootsma D, Hoeijmakers JH. Cloning, comparative mapping, and RNA expression of the mouse homologous of the *Saccharomyces cerevisiae* nucleotide excision repair gene RAD23. *Genomics.* 1996;31:20–27.

Watkins JF, Sung P, Prakash L, Prakash S. The *Saccharomyces cerevisiae* DNA repair gene RAD23 encodes a nuclear protein containing a ubiquitin-like domain required for biological function. *Mol Cell Biol.* 1993;13:7757–7765.

Xie Y, Varshavsky A. Physical association of ubiquitin ligases and the 26 S proteasome. *Proc Natl Acad Sci U S A.* 2000;97:2497–2502.

Zhou ZM, Sha JH, Li JM, et al. Expression of a novel reticulon-like gene in human testis. *Reproduction.* 2002;123:227–234.