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Decreased Expression of Cold-Inducible RNA-Binding Protein (CIRP) in Male Germ Cells at Elevated Temperature

Hiroyuki Nishiyama,*† Shouzou Danno,*‡ Yoshiyuki Kaneko,* Katsuhiko Itoh,* Hiromichi Yokoi,* Manabu Fukumoto,§ Hiroshi Okuno,† José Luis Millán,¶ Tadashi Matsuda,‡ Osamu Yoshida,† and Jun Fujita*

From the Departments of Clinical Molecular Biology, Urology,† and Pathology,§ Faculty of Medicine, Kyoto University, Shogoin Kawahara-cho, Sakyo-ku, Kyoto, Japan; the Department of Urology,‡, Faculty of Medicine, Kansai Medical University, Moriguchi, Japan; the Burnham Institute,¶ La Jolla, California; and the Department of Medical Genetics,¶ Umeå University, Umeå, Sweden*

Physiological scrotal hypothermia is necessary for normal spermatogenesis and fertility in mammals. Cirp is a recently identified cold-inducible RNA-binding protein that is inducible at 32°C in mouse somatic cells *in vitro*. Cirp is constitutively expressed in the testis of mouse and structurally highly similar to RBM1, a candidate for the human azoospermia factor. To elucidate the role played by Cirp in spermatogenesis, we investigated its expression levels during spermatogenesis and after heat stress. In the mouse testis, *cirp* mRNA was detected in the germ cells, and the level varied depending on the stage of differentiation. Also, a high level of Cirp protein was detected immunohistochemically in the nucleus of primary spermatocytes. Expression of Cirp was decreased in the GC-2spd(ts) mouse germ cell line when culture temperature was raised from 32°C to 37°C. When mouse testis was exposed to heat stress by experimental cryptorchidism or immersion of the lower abdomen in warm (42°C) water, the expression of Cirp was decreased in the testis within 6 hours after either treatment. In human testis with varicocele analyzed immunohistochemically, germ cells expressed less Cirp protein than those in the testis without varicocele. These results demonstrated that CIRP expression is down-regulated at elevated temperature in male germ cells of mice and humans. Analysis of Cirp expression in the testes will help elucidate the molecular mechanisms leading to male infertility. *(Am J Pathol 1998, 152:289–296)*

Most mammals have testes within a scrotum, the temperature of which is maintained at 30 to 33°C, 5 to 7°C lower than the body cavity temperature.[1,2] This temperature difference is believed to be important for normal testicular function.[3,4] In experimental animals, surgical induction of cryptorchidism or exposure to heat stress causes disruption of spermatogenesis, leading to infertility.[3,5] Detailed investigations of experimentally cryptorchid testes have shown that the earliest cellular changes noticed are in primary spermatocytes and early spermatids.[6–8] Especially in primary spermatocytes, apoptotic cell death is induced within 2 to 4 days.[8] Various exogenous thermal factors, including those observed in welders and paraplegic patients in wheelchairs, are proposed to be risk factors for human male infertility.[9–11] Cryptorchidism and varicocele of the spermatic veins are associated with male infertility, and their pathogenesis is attributed to thermal factors.[2,12–15] The molecular mechanisms of the thermal effect on spermatogenesis are, however, just beginning to be explored.[16–18]

Organisms have developed sophisticated strategies to adapt themselves to varying environmental temperature. In mammalian cells, gene expression is regulated in response to cold as well as heat. Recently, we have identified a novel cold-inducible protein in mouse cells and designated it as Cirp.[19] Lowering the culture temperature of mouse fibroblasts from 37°C to 32°C induces the expression of Cirp, and Cirp suppresses their proliferation. Cirp is structurally composed of an amino-terminal consensus-sequence RNA-binding domain (CS-RBD) and a carboxyl-terminal glycine-rich region.[19] CS-RBD, also referred to as RNP motif, RNP consensus sequence, or RNA recognition motif, is one of the major RNA-binding motifs.[20,21] Although proteins with CS-RBD are involved in the post-transcriptional regulation of gene expression,[21] it remains to be elucidated how Cirp suppresses cell growth.

Some proteins with CS-RBD are known to play important roles in spermatogenesis. For example, chromosomal deletion of the *Rb97D* gene encoding a potential

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Address reprint requests to Dr. Jun Fujita, Department of Clinical Molecular Biology, Faculty of Medicine, Kyoto University, Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606, Japan. E-mail: jfujita@virus.kyoto-u.ac.jp.

RNA-binding protein results in azoospermia in *Drosophila*.[22] The Y chromosome genes *RBM1* (RNA-binding motif gene) and *DAZ* (deleted in azoospermia) are candidate genes for azoospermia factor, which controls human spermatogenesis.[23,24] Interestingly, Cirp shares with RBM1 a sequence similarity in the CS-RBD (59%) and is constitutively expressed in the testis. In the present study, to elucidate the function of Cirp in spermatogenesis, we identified the cells expressing Cirp in mouse testis and assessed the effect of heat stress on its expression.

Materials and Methods

Tissue Samples and Cells

Sterile WBB6F1-*W/W^v* mutant mice were obtained through the mating between congenic C57BL/6-*W^v*/+ and WB-*W*/+ mice. Sexually mature (4-month-old) C57BL/6 wild-type (+/+) mice and WBB6F1-*W/W^v* mice were purchased from Japan SLC Co. (Hamamatsu, Japan).

Biopsy specimens of histologically normal testis were obtained from five patients, from 19 to 41 years old, at the time of surgical removal of the contralateral testicular tumor. Seven biopsy specimens of testis were obtained from four patients, 30 to 38 years old, with varicocele at the male infertility clinic. One biopsy specimen of testis lacking germ cells was obtained from a 32-year-old patient with Sertoli-cell-only syndrome. Informed consent was obtained from all patients.

The GC-2spd(ts) mouse male germ cell line[25] was cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Dainippon Pharmaceutical Co., Osaka, Japan), 1% nonessential amino acids solution (Gibco), and 100 IU/ml penicillin at 32°C in a humidified atmosphere of 5% CO_2 in air.

Purified germ cell preparations were obtained from the testis of +/+ mice using centrifugal elutriation and Percoll density gradient separation methods as described previously.[26] We routinely obtained a purified pachytene spermatocyte population (95% pure) and a purified early spermatid population (95% pure).

Heat Stress

For temperature-shift experiments, GC-2spd(ts) cells were grown for 24 hours at 32°C and then shifted to 37°C in a humidified atmosphere of 5% CO_2 in air. After 12 hours, the cells were scraped, frozen in liquid nitrogen, and stored at −80°C until analysis.

To induce unilateral cryptorchidism, 4-month-old +/+ male mice were anesthetized by intraperitoneal injection of 25 mg/kg pentobarbital sodium, and a small incision was made in the abdomen. On one side, the testis was fixed in the abdominal cavity by suturing its capsule to peritoneum. On the other side of the same animal, the testis was not fixed and used as a control. The animals were sacrificed at different intervals after surgery. The testes were removed, frozen in liquid nitrogen, and stored at −80°C until analysis.

Lower abdominal heat treatment was performed as described previously.[27] Briefly, 4-month-old male +/+ mice were anesthetized by intraperitoneal injection of 25 mg/kg pentobarbital sodium, and the scrotal regions of the animals were submerged in a water bath maintained at 30°C or 42°C. After 30 minutes, they were returned to room temperature and allowed to recover. After a predetermined interval of recovery, the animals were sacrificed and the testes were processed as described above.

Northern Blot Analysis

Samples were dissolved in TRIzol reagent (Life Technologies, Grand Island, NY) and RNA was extracted by following the manufacturer's instructions. A 20-μg aliquot of total RNA of each sample was separated in 1.0% agarose/formaldehyde gels by electrophoresis and was blotted onto nylon filters (Hybond-N$^+$, Amersham, Little Chalfont, UK). The filters were hybridized with [α-^{32}P]dCTP-labeled random-primed cDNA fragments, washed under stringent conditions (65°C for 30 minutes in a washing buffer composed of 0.1X SSC and 0.1% SDS), and detected by autoradiography. A mouse *cirp* cDNA (nucleotide positions 82 to 597 in Ref. 19) was used as a probe. The filters were stripped and rehybridized with a cDNA probe for the S26 ribosomal protein VASA or PGK2.[19,28,29]

In Situ *Hybridization Histochemistry*

In situ hybridization histochemistry was performed using sections from the adult +/+ or *W/W^v* mutant mouse testes fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in wax. A 650-bp *Sac*I-*Eco*RI fragment of *cirp* cDNA was cloned into the *Sac*I-*Eco*RI site of vector pBluescript SK(−) (Stratagene, La Jolla, CA). The plasmids were linearized with the appropriate enzymes and then transcribed and labeled with digoxigenin using a DIG RNA labeling kit (Boehringer Mannheim Biochemica, Mannheim, Germany) following the manufacturer's instructions. Hybridization was carried out as described previously.[26] The hybridized digoxigenin-labeled probe was detected with a nucleic acid detection kit (Boehringer Mannheim) following the manufacturer's instructions. The control was hybridized with the sense probe.

Western Blot Analysis

Protein extraction and Western blot analysis using a rabbit polyclonal antibody recognizing the carboxyl-terminal oligopeptide of Cirp were performed as described previously.[19] A 10-μg aliquot of protein extracted from cultured cells or tissues was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, blotted onto polyvinylidene difluoride membrane (Millipore, Tokyo, Japan), and treated with the anti-Cirp antibody (1:20,000). Protein concentrations were determined us-

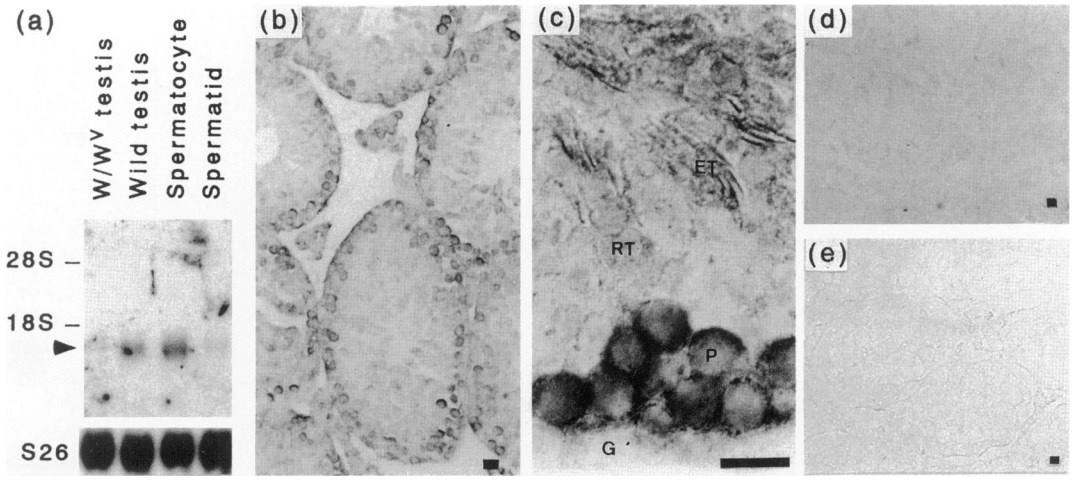

Figure 1. Expression of *cirp* mRNA in mouse testis. **a:** Northern blot analysis of *cirp* mRNA. **Lane 1**, testes of 4-month-old *W/W^v* mice; **lane 2**, testes of 4-month-old +/+ mice; **lane 3**, pachytene spermatocytes; **lane 4**, early spermatids. Each lane contained 20 μg of total RNA. Using the coding sequence of *cirp* cDNA as a probe, a 1.3-kb band was detected (**arrowhead**). The filter was rehybridized with a probe for the S26 ribosomal protein to correct for the amount of RNA loaded. **b** to **e:** *In situ* hybridization histochemistry of *cirp* mRNA in mouse testis. Sections were obtained from the testis of 4-month-old +/+ mice (**b** to **d**) and the testis of *W/W^v* mice (**e**). **b, c,** and **e:** Hybridization with the antisense RNA probe. **d:** Hybridization with the sense RNA probe as a control. G, spermatogonium; P, primary spermatocyte; RT, round spermatid; ET, elongated spermatid. Bar, 20 μm.

ing the Bradford reagent (BioRad Laboratories, Hercules, CA). Bound antibody was detected using a goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad) and an enhanced chemiluminescence system (Amersham).

Immunohistochemistry

Immunohistochemistry was performed using sections from the human and mouse testes fixed in 4% paraformaldehyde in PBS and embedded in wax. The anti-Cirp antibody was used at a dilution of 1:10,000. Detection was performed with Envision-labeled polymer reagent (DAKO, Kyoto, Japan), which is a mixture of goat anti-rabbit and goat anti-mouse immunoglobulins conjugated to the peroxidase-labeled reagent, following the manufacturer's instructions. As a control, the preimmune antiserum was used at the same dilution.

Results

By Northern blot analysis using the *cirp* cDNA as a probe, a single band of 1.3 kb was detected in the testis (Figure 1a). To determine whether the *cirp* transcript was expressed in germ cells and/or somatic cells in the testis, testis from dominant spotting (*W*) mutant mice were examined. The *W/W^v* mutant mice lack germ cells in the adult, although the somatic cell elements are apparently normal.[30] The expression of *cirp* was not detected in the testes of *W/W^v* mice (Figure 1a), suggesting that the *cirp* transcripts were predominantly expressed in the germ cells. To define the stages at which germ cells express *cirp*, enriched populations of pachytene spermatocytes and round spermatids were analyzed. The *cirp* transcripts were detected in the former but barely in the latter (Figure 1a).

In situ hybridization histochemistry with the antisense *cirp* RNA probe revealed that signals were observed in primary spermatocytes but not in spermatogonia, round spermatids, Sertoli cells, or Leydig cells (Figure 1, b and c). No significant signal was observed when a sense strand was used as a probe (Figure 1d) nor when the testis of *W/W^v* mice was hybridized with an antisense strand (Figure 1e). These observations demonstrated that *cirp* transcripts were expressed in the germ cells, and its level of expression was modulated during germ cell development.

The expression of Cirp protein in the adult +/+ mouse testis was examined by immunohistochemistry using the anti-Cirp polyclonal antibody that specifically recognizes an 18-kd protein.[19] As shown in Figure 2a, strong signals were detected within the seminiferous tubules. No significant signals were observed in the seminiferous tubules when the preimmune serum was used as a control, although a strong nonspecific staining was observed in the interstitial tissues (Figure 2b). Spermatogenesis in the mouse has been divided into 12 stages (I to XII) on the basis of the germ cell types present in the seminiferous epithelium.[31] Examination of several tubules at various stages (Figure 2, c to f, and data not shown) revealed that strong signals were present in the nucleus of primary spermatocytes, especially preleptotene, leptotene, zygotene, and early pachytene spermatocytes. Faint signals were detected in late pachytene spermatocytes, meiotic phase, and round spermatids. In round spermatids at stages I to III, signals were present in the cytoplasm, but not in the nucleus (Figure 2c). No significant signals were detected in spermatogonia, elongated spermatids, or Sertoli cells.

To examine the effect of heat stress on Cirp expression in the testis *in vivo*, we made use of experimental crypt-

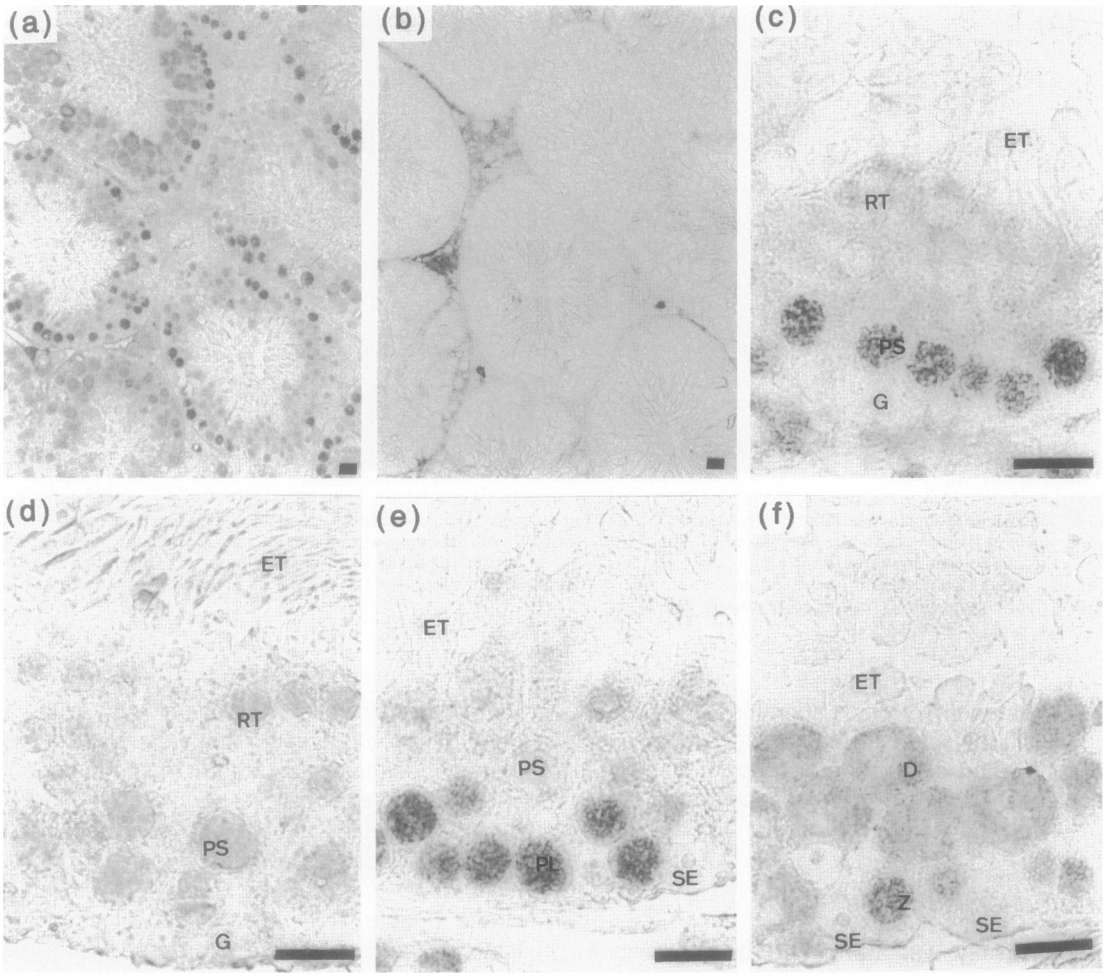

Figure 2. Localization of Cirp protein in mouse testis. Immunohistochemical staining was performed using the anti-Cirp antibody (**a** and **c** to **f**) or preimmune antiserum (**b**). **c** to **f**: High-magnification views of testis sections representing stages I to III (**c**), stages VI and VII (**d**), stages IX and X (**e**), and stage XI (**f**). Note strong signals in the nucleus of preleptotene, zygotene, and pachytene spermatocytes at stages I to III. G, spermatogonium; PL, preleptotene spermatocyte; Z, zygotene spermatocyte; PS, pachytene spermatocyte; D, diplotene spermatocyte; RT, round spermatid; ET, elongated spermatid; SE, Sertoli cell. Bar, 20 μm.

orchidism. As shown in Figure 3a, Northern blot analysis revealed that *cirp* transcripts were decreased in the cryptorchid testes within 3 hours after the surgery as compared with the contralateral sham-operated testes in the same animals. In contrast to the *cirp* expression, experimental cryptorchidism did not affect the expression of other genes, such as *vasa*[28] and *pgk-2*,[29] that are predominantly expressed in primary spermatocytes and/or round spermatids.

To further assess the effect of temperature shift on *cirp* expression, we exposed the mouse lower abdomen to heat or cold stress. The expression of *cirp* transcripts was decreased in the testis within 6 hours at 42°C as compared with that at 30°C. Although in Figure 3b, it is difficult to evaluate the status of expression at 6 hours after the 42°C treatment, given the apparently aberrant high level of control, it was confirmed to be elevated in repeated experiments. Next, we examined the expression of Cirp at the protein level. In accord with the results of Northern blot analysis, Cirp protein expression was decreased in both the experimentally cryptorchid testis and the testis exposed to lower abdominal heat stress compared with those in the controls (Figure 3c). In GC2-spd(ts) male germ cells, expression of *cirp* mRNA was decreased when culture temperature was raised from 32°C to 37°C (Figure 3d).

We have recently isolated a cDNA clone encoding the human cold inducible RNA-binding protein (CIRP),[32] a human homolog of mouse Cirp. Its amino acid sequence was 95.3% identical to that of mouse, and the carboxyl-terminal 11 peptides used for raising antibody was completely identical. As expected, the human CIRP was recognized by the anti-mouse-Cirp antibody (Figure 4a, lane 1). When we examined normal human testis by immunohistochemistry using the anti-Cirp antibody, signals were detected in the nucleus of almost all cell types except for elongated spermatids, and the strongest signal was in the spermatocytes (Figure 4, b to d). When preimmune serum was used as a control, no significant signals were detected (data not shown). Western blot analysis and immunohistochemical analysis on the testis of a Sertoli-cell-only syndrome patient lacking germ cells confirmed that human CIRP was expressed in somatic cells of human testis (Figure 4a, lane 2, and Figure 4e).

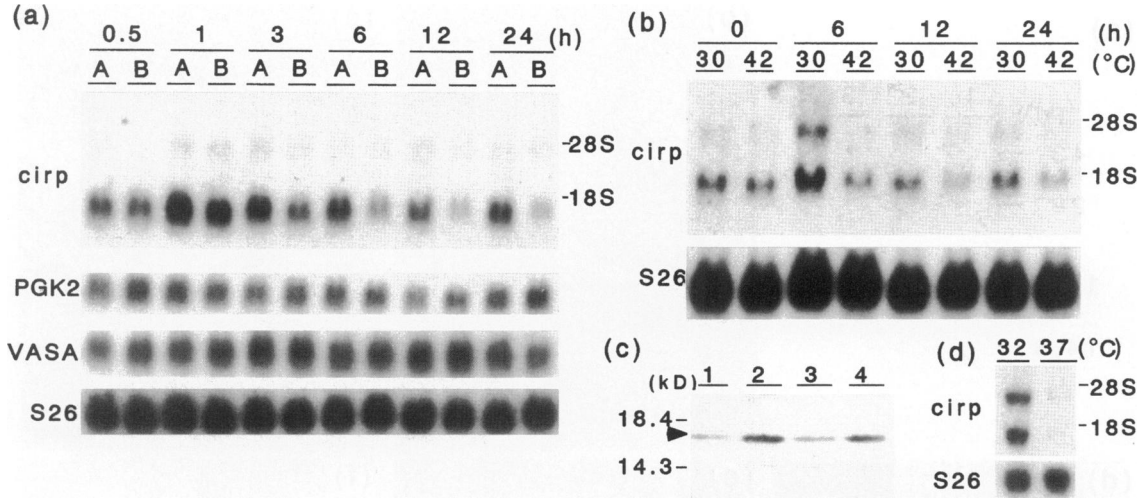

Figure 3. Effect of heat stresses on expression of *cirp* mRNA in mouse testis. **a:** Northern blot analysis of total RNAs from cryptorchid testis (**B**) or sham-operated testis (**A**) removed at the indicated time after the operation. **b:** Northern blot analysis of total RNAs extracted from testes exposed to heat stress. Mice were immersed in a water bath at 30°C or 42°C for 30 minutes and sacrificed at the indicated times after return to room temperature. The coding sequence of *cirp* cDNA was used as a probe. The positions of 18 S and 28 S ribosomal RNAs are indicated on the right. The filter was rehybridized with mouse *pgk-2*, *vasa*, or S26 ribosomal protein cDNA probe. **c:** Effect of heat stress on expression of Cirp protein in mouse testis. Western blot analysis was carried out using the anti-Cirp polyclonal antibody. Cell lysates were prepared from cryptorchid testes (**lane 1**), sham-operated testes (**lane 2**), or testes exposed to 42°C (**lane 3**) or 30°C (**lane 4**). Samples (10 μg) were separated by 14% SDS-PAGE. Mobilities of co-electrophoresed molecular size markers and Cirp are indicated on the left (**numbers** and **arrowhead**, respectively). **d:** Northern blot analysis of total RNA from GC-2spd(ts) germ cells. GC-2spd(ts) cells were cultured at 32°C and then harvested at 12 hours after the indicated temperature shift. The coding sequence of *cirp* cDNA was used as a probe. The positions of 18 S and 28 S ribosomal RNAs are indicated on the right. The filter was rehybridized with mouse S26 ribosomal protein cDNA probe.

To examine whether the presence of varicocele in spermatic veins affected the expression of CIRP in human testis, we immunohistochemically analyzed the biopsy samples from varicocele patients. As shown in Table 1, almost all seminiferous tubules in normal testis were filled with germ cells positively stained with the anti-CIRP antibody, and CIRP-negative germ cells were barely observed. In contrast, the percentage of tubules filled with CIRP-positive germ cells was significantly decreased in the testis of varicocele patients compared with control (66.8 ± 21.7 *versus* 99.7 ± 0.7; $P < 0.01$; Figure 4f and Table 1). These results demonstrated that CIRP expression was decreased in the human testis with varicocele.

Discussion

In the present study, we demonstrated that the expression of Cirp was restricted to germ cells in mouse testis and that its level was regulated during spermatogenesis. Spermatogonia develop into primary spermatocytes after several mitotic divisions.[33] In primary spermatocytes, mitotic cell division is suppressed, and DNA replication, homolog pairing, and recombination occur.[33] And then spermatocytes give rise to four haploid cells by two meiotic divisions. Mouse Cirp was strongly expressed in primary spermatocytes but not in spermatogonia. These results suggest a specific role played by Cirp in primary spermatocytes. Interestingly, overproduction of Cirp suppressed the mitotic proliferation of GC-2spd(ts) germ cells as well as fibroblasts[19] *in vitro* (H. Nishiyama and J. Fujita, unpublished data). These findings suggest a possibility that Cirp may be involved in suppression of the mitotic cell cycle after differentiation of spermatogonia to spermatocytes. GC-2spd(ts) cells were originally established and characterized as a germ cell line capable of undergoing meiotic cell division *in vitro*,[26] although a contradictory report exists.[34] Unfortunately, the GC-2spd(ts) cells did not produce haploid cells under the present conditions, and the effects of Cirp on the meiotic cell cycle is presently unknown.

The temperature of the testis is maintained at a specific temperature between 30°C and 33°C depending on the species.[1,2] The present study demonstrated that the level of *cirp* expression was regulated by temperature in germ cells. The *cirp* mRNA expression was decreased at the elevated temperature within 6 hours. The finding that the expression of other genes known to be expressed in the *cirp*-expressing cells was not affected suggests that the decrease is not due to a decrease in the number of cells. In the absence of information concerning the half-life and stability of these mRNAs, however, it is not known whether the *cirp* expression is selectively down-regulated transcriptionally compared with other mRNA. At the body cavity temperature, male germ cells are easily damaged.[6–8] The earliest cellular changes are noticed in primary spermatocytes and early spermatids,[6–8] in which Cirp was found to be strongly expressed at scrotal temperature. At the body cavity temperature, apoptotic cell death will be induced in primary spermatocytes within 2 to 4 days.[8] As the timing of mitosis and meiosis in spermatogenesis is strictly controlled,[31] these findings, coupled with the effect of Cirp on mitosis, suggest that decreased expression of Cirp adversely affects the coordinated regulation of mitosis and meiosis, leading to disruption of spermatogenesis.

Proteins with CS-RBD are involved in post-transcriptional regulation of gene expression.[20,21] In eukaryotic

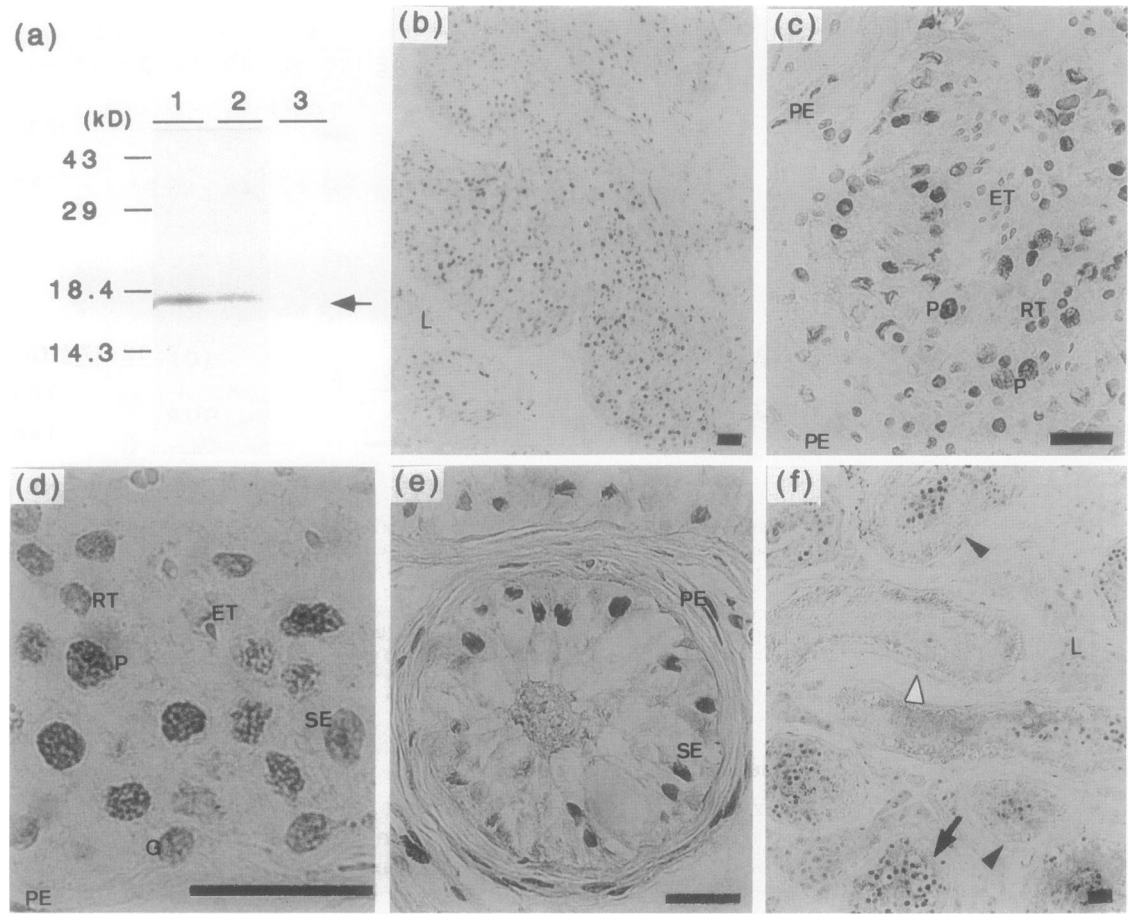

Figure 4. Expression of CIRP protein in human testis. **a**: Using the anti-Cirp antibody (**lanes 1** and **2**) and preimmune serum (**lane 3**), Western blot analysis was performed on the normal human testis (**lanes 1** and **3**) and the testis from a patient with Sertoli-cell-only syndrome (**lane 2**). Note that the anti-Cirp antibody specifically recognizes an 18-kd protein in the testes with and without germ cells. **b** to **f**: Using the anti-Cirp antibody, immunohistochemical analysis was performed on the testes from a patient without varicocele (**b** to **d**), a patient with Sertoli-cell-only syndrome (**e**), and a patient with varicocele (**f**). Note the presence in **f** of three staining patterns in the tubules: homogeneous staining (**arrow**), heterogeneous staining (**arrowheads**), and no staining (**white arrowhead**). No tubule was stained positively with preimmune antiserum (data not shown). G, spermatogonium; P, primary spermatocyte; RT, round spermatid; ET, elongated spermatid; SE, Sertoli cell; L, Leydig cell; PE, peritubular myoid cell. Bar, 50 μm.

Table 1. Decreased CIRP Expression in Human Testis with Varicocele

Patient number	Age (years)	Side	Number of tubules assessed	% of tubules belonging to class		
				1	2	3
Testis with varicocele						
1	36	Left	32	56	9	34
2	35	Left	85	94	6	0
		Right	66	27	12	60
3	30	Left	22	77	23	0
		Right	24	58	42	0
4	38	Left	29	79	21	0
		Right	4	75	25	0
Testis without varicocele						
101	26	Left	84	100	0	0
102	36	Left	24	100	0	0
103	36	Right	21	100	0	0
104	19	Left	64	98	2	0
105	41	Right	76	100	0	0

CIRP expression was analyzed immunohistochemically using the anti-mouse Cirp antibody, which cross-reacts with human CIRP. Each seminiferous tubule was classified according to the content of CIRP-positive germ cells as follows: class 1, more than 95%; class 2, between 15 and 95%; class 3, less than 15% of germ cells positively stained. The percentages of tubules in a testis belonging to each class are indicated.

cells, mRNAs are produced in the nucleus from the primary transcripts of protein-coding genes, pre-mRNAs, by a series of processing reactions including mRNA splicing and polyadenylation.[20,21,35] In the cytoplasm, the translation and stability of mRNAs are also subject to regulation.[21,35] Immunohistochemical analysis of testes revealed that the strong signals for Cirp were present in the nucleus of spermatocytes in both mice and humans, indicating that Cirp may play an important role(s) in RNA biogenesis in the nucleus. In round spermatids at stages I to III of mice, however, signals were detected in cytoplasm but not in the nucleus, suggesting an additional function(s) of Cirp in the cytoplasm of haploid cells.

Immunohistochemical analysis revealed that the expression of CIRP was decreased in the testes of varicocele patients compared with that in nonvaricocele patients. The prevalence of varicocele is estimated to be 38% in infertile males, and varicocele has been considered to cause male infertility, although the mechanisms of pathogenesis are still poorly understood.[13,36–38] The scrotal temperature in patients with varicocele is increased compared with that in normozoospermic men,[12,15,39] indicating that the elevation of testicular temperature is one of the possible causes. Our results in human testis, combined with the results in experimental animals, suggest that the decrease in CIRP expression may be related to the pathogenesis of infertility in patients with varicocele. Analysis of CIRP expression in the testes of patients with cryptorchidism and those exposed to various exogenous thermal factors[9–11] will help elucidate the molecular mechanisms leading to male infertility.

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