Identification and Characterization of a Haploid Germ Cell-specific Nuclear Protein Kinase (Haspin) in Spermatid Nuclei and Its Effects on Somatic Cells*

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We have cloned the entire coding region of a mouse germ cell-specific cDNA encoding a unique protein kinase whose catalytic domain contains only three consensusesubdomains (I–III) instead of the normal 12. The protein possesses intrinsic Ser/Thr kinase activity and is exclusively expressed in haploid germ cells, localizing only in their nuclei, and was thus named Haspin (for haploid germ cell-specific nuclear protein kinase). Western blot analysis showed that specific antibodies recognized a protein of Mr 83,000 in the testis. Ectopically expressed Haspin was detected exclusively in the nuclei of cultured somatic cells. Even in the absence of kinase activity, however, Haspin caused cell cycle arrest at G1, resulting in growth arrest of the transfected somatic cells. In a DNA binding experiment, approximately one-half of wild-type Haspin was able to bind to a DNA-cellulose column, whereas the other half was not. In contrast, all of the deletion mutant Haspin that lacked autophosphorylation bound to the DNA column. Thus, the DNA-binding activity of Haspin may, in some way, be associated with its kinase activity. These observations suggest that Haspin has some critical roles in cell cycle cessation and differentiation of haploid germ cells.

Maturation of male germ cells in mammals involves numerous structural and functional changes that are precisely timed. These complex processes, known collectively as spermatogenesis, may be represented by the following three major events: proliferation and differentiation of spermatogonia, meiotic events at prophase of spermatocytes, and drastic morphological change during differentiation from the haploid round spermatids to sperm (1). To uncover the mechanism of spermatogenesis, many germ cell-specific molecules have been studied using various strategies (2–5).

During haploid germ cell differentiation, or spermiogenesis, the round spermatid undergoes marked morphological change to become a sperm without cell division; and the nucleus is shaped, mitochondria are rearranged, the flagellum is developed, and the acrosome is generated (6). Over this long period of time, ~2 weeks for the mouse, no division of haploid germ cells occurs. Some of the regulatory proteins localized in the nucleus must participate in this precise regulation.

It appears that there are at least two types of mechanisms at work in haploid germ cell-specific gene regulation: one involves the CRE, and the other, as yet uncharacterized, does not. Several proteins are specifically expressed in the nuclei of haploid germ cells: transition protein 7 (7), protamine (8), histone H1t (9), zinc finger proteins (10), testis-specific HMG (11), lamin B3 (12), and CREM* (13). Through knockout of the Crem gene in mice, CREM* capable of binding to the sequence of CRE has been shown to play important roles in the regulation of spermiogenesis (14, 15). In contrast, some of the proteins specifically expressed in haploid germ cells do not have any CRE motifs in the promoter region (16), implying that some other regulatory mechanism exists.

We have isolated many cDNA clones specifically expressed in germ cells using a subtracted cDNA library prepared from supporting cells of mutant testes and the wild-type testis (4). Using a partial cDNA clone previously obtained, we have cloned the entire coding region and characterized a novel gene encoding a protein with various well known motifs. The protein, which we named Haspin (haploid germ cell-specific nuclear protein kinase), is specifically expressed in haploid germ cells, localizes in nuclei of round spermatids, binds to DNA, and has Ser/Thr protein kinase activity. Since transfection of haspin cDNA into cultured somatic cells caused cessation of cell proliferation, Haspin could be involved in regulation of proliferative activity as well as specific gene expression in haploid germ cells.

EXPERIMENTAL PROCEDURES

Construction of the Subtracted Library and Screening—Total RNAs were extracted by the guanidine thiocyanate-CsTFA method (17) from the testes of adult wild-type C57BL/6 mice and 4-month-old WW mutant mice lacking germ cells (4). The corresponding cDNA libraries were prepared as described by Gubler and Hoffman (18), with some modifications. Prepared cDNA fragments were directionally inserted between NotI (dephosphorylated) and BglII sites of the pAP3neo vector (4). The ligated DNAs were electroporated into MC1061A cells as described previously (19). The complexities of the cDNA libraries obtained were ~6 × 107 colony-forming units in both cases. A germ cell-specific cDNA library was generated by subtracting cDNAs of mutant (WW) testis that contains no germ cells from wild-type testis cDNAs (4).

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† The abbreviations used are: CRE, cAMP response element; RACE, rapid amplification of cDNA ends; PMASP, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; EGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorter.

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Plasmid DNA of each clone randomly picked from the subtracted cDNA library was subjected to cDNA dot-blot analysis. As a probe to select testicular germ cell-specific cDNA clones, RNAs of testis cDNA libraries of wild-type and mutant mice were generated with T7 RNA polymerase and labeled with the hapten digoxigenin. To clone the complete cDNA of gsg2, a testis-specific partial cDNA obtained previously by screening the subtracted library (4), a library of Escherichia coli MC1061A cells carrying the adult wild-type testis cDNAs was diluted to seed at 1 x 10^5 colony-forming units on a nitrocellulose filter placed on an LB plate. After incubation at 37 °C, grown colonies were transferred to two nylon replica filters and lysed by sequential soakings in the following solutions at room temperature: 5 min in 0.5M NaOH and 1.5 M NaCl, 5 min in 0.5 M Tris-HCl (pH 7.4) and 1.5 M NaCl, and 5 min in 2 x SSC. After baking at 80 °C for 2 h, the filters were washed, and the bacterial debris was removed. A 32P-labeled probe was prepared with a BCaBest random primer kit (Takara, Osaka, Japan) using a 1.3-kilobase EcoRI-NcoI fragment of the partial gsg2 cDNA fragment (4). The filters were then hybridized with the partial clone gsg2 probe at 65 °C for 20 h (4 x SSC, 10 x Denhardt's solution, 0.1% SDS, and 100 μg/ml denatured sonicated salmon sperm DNA). Several positive clones were isolated by screening 2 x 10^6 colonies.

5'-RACE—To isolate the 5'-end of the gsg2 cDNA, we performed 5'-RACE (20, 21) using a 5'-AmpliFINDER RACE kit (CLONTECH). Synthesis of the single-stranded cDNA was carried out using 10 pmol of a first antisense oligonucleotide primer (20-mer) designed from the sequence data of the gsg2 clone, which corresponded to nucleotides 913–932 of the gsg2 cDNA (see Fig. 1), and 2 μg of mouse testis poly(A)+ RNA. After hydrolysis of RNA with NaOH, single-stranded DNAs were purified by GENO-BIND (CLONTECH) and ligated to AmpliFINDER Anchor (5'-phosphorylated 3'-aminated oligonucleotide, 5'-CAGGAT-
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TCATCTATGATCTGGAACCTTCAGG-NH₂-3' with T4 ligase. Polymerase chain reaction amplification of the 5'-end region was performed using a second antisense linker oligonucleotide primer (19-mer corresponding to nucleotides 317–335 and a BamHI recognition sequence) and an anchor primer (5'-CTGTTCCGCCACCTCTGAGG-GTCCCAAGATCTCCGTAGAT-3'). Amplification products were then digested with EcoRI and BamHI and ligated to EcoRI-BamHI-cut pBluescript II SK̂ plasmid DNAs. Three independent clones were selected and sequenced.

Northern Blot Analysis and DNA Sequencing—Freshly removed organs of adult mice (C57BL/6 strain) were homogenized in RNAzol® B (Tel-Test, Inc., Tokyo, Japan). Germ and other somatic cells of the testes were prepared as described in our previous report (22). Cryptorchid testes and those of various mutants defective in germ cell differentiation such as jsd/jsd, Sl17H/Sl17H, and W/Wv were also used (5). Total RNAs were extracted according to the manufacturer's recommendations (Tel-Test Inc., Tokyo, Japan) and quantified by optical density measurements. RNA samples containing 2.2 μg formaldehyde were subjected to electrophoresis on a 1.1% agarose gel containing 0.66 M formaldehyde (23). RNAs were transferred to a nitrocellulose filter in 20× SSC. Hybridization was performed with 32P-labeled cDNA prepared from total RNAs extracted according to the manufacturer's recommendation in a sodium chloride-SDS-denaturing solution. Filters were washed twice in 1× SSC and 0.1% SDS at 60 °C. Signals of the bands were detected with a Fuji image analyzer.

Deoxy chain termination sequencing reactions (24) were performed with fluorescent dye-labeled primers and thermal cycle sequencing kits containing 4× dye mix and 50% formamide. Dideoxy chain termination sequencing reactions (24) were performed with fluorescent dye-labeled primers and thermal cycle sequencing kits purchased from Li-Cor. The reaction products were analyzed by a Li-COR model 4000 DNA Sequencer. GenBank™, EBI, DDBJ, SWISS-PROT, and PIR data banks were searched for homology to the haspin cDNA or amino acid sequence. The deduced amino acid sequence of the haspin cDNA was analyzed by the Fujitsu Biosearch/PR system.

Antiserum Preparation—Two kinds of synthetic peptides (KKK-1, TT PreH=H4S5K6K7K8K9K10K11K12K13K14K15K16K17K18K19K20K21) and (25). Glutathione-S-transferase fusion proteins were produced in Escherichia coli and purified with glutathione-agarose beads. Polyclonal antisera were obtained by injecting the above antigens followed by booster injections at 3-week intervals, seven times in total, into New Zealand White rabbits.

Each anti-Haspin rabbit antiserum (KKK-1, DDP-2, and HAS-3) reacted with the same molecule when examined by Western blot analysis. The most effective anti-Haspin antiserum was used for each biochemical assay.

Western Blot Analysis—Freshly prepared organs or cell fractions of adult C57BL/6 mice or cultured cells were homogenized on ice with a lysis buffer containing 10 mM Na₂HPO₄ (pH 7.2), 160 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, 0.3% SDS, and 2 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma). After centrifugation, the protein concentration of each supernatant was estimated by the Bradford protein assay (Bio-Rad). Each extract containing ~100 μg of protein was subjected to SDS-PAGE as reported by Laemmli (26), followed by electroblotting onto polyvinylidene difluoride membrane filters (Millipore Corp., Bedford, MA). The filters were blocked with 5% nonfat dry milk and washed for 15 min with TBS-T (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20). The filters were then reacted with anti-Haspin rabbit antiserum (KKK-1, 1,300 dilution) in TBS at 25 °C for 1 h, washed with TBS-T for 3 min, washed again three times for 5 min each time, and incubated with peroxidase-conjugated anti-rabbit Ig (1:500, Dako A/S, Glostrup, Denmark) at 25 °C for 1 h. After further washing, reactive bands were visualized by development with diamino benzidine in 50 mM Tris-HCl (pH 7.5) plus 0.3% H₂O₂.

Immunohistochemistry—To prepare frozen tissue specimens, testes were immersed in O.T.C. compound embedding medium (Tissue-Tek, Sakura, Tokyo, Japan) and frozen at −20 °C. Sections of 10 μm in thickness were prepared by using a cryomicrotome (Microm HM 5000M) and were fixed with 80% methanol at −20 °C for 5 min. Each section was treated with 0.3% hydrogen peroxide, incubated with 20% normal goat serum in phosphate-buffered saline, washed twice in 0.1% Triton X-100, 0.1% SDS, and 0.05% Tween 20, and then reacted with anti-Haspin rabbit antiserum (HAS-3) diluted at a ratio of 1:100 after blocking with a blocking kit (Vector Labs, Burlingame, CA). Sections were reacted with biotinylated sheep anti-rabbit Ig (Amersham-Pharmacia Biotech, Tokyo, Japan) diluted at 1:500 and subjected to a peroxidase/avidin system (Vectastain ABC kit, Vector Labs), followed by incubation with 0.05% diaminobenzidine in 50 mM Tris-HCl buffer (pH 7.2). Some of the sections were counterstained with hematoxylin.

Construction of Expression Vectors of Haspin (pEGFP-HASP) and a Deletion Mutant of the ATP-binding Site (pEGFP-dHASP)—Polymerase chain reaction amplification of the haspin cDNA coding region was performed using a primersense primer for the 5′-region of the haspin cDNA (5′-GGCTTTGTTGAAAACCCCGGG-3′, nucleotides 1–21) and a primer (BanHI) oligonucleotide primer of the 3′-region (5′-CGCAATTC-CTGGCTTACCTAAATAGAC-3′, nucleotides 2283–2304). Amplification products were then digested with KpnI, which corresponded to nucleotides 58–63 of the haspin cDNA (see Fig. 1), and with BamHI, whose recognition site had been included in the linker oligonucleotide primer, and ligated at the KpnI-BamHI site of the mammalian expression vector pEGFP-C1 (CLONTECH). The resultant clone was capable of expressing the EGFP-Haspin fusion protein.

The expression vector with a deletion mutant of haspin was also constructed by polymerase chain reaction amplification using the haspin cDNA as a template. For this, the following strategy was used. First, the haspin cDNA was cut into two pieces in the region of the ATP-binding site; then, using the 5′- and 3′-fragments, a deletion was introduced by polymerase chain reaction; and the two DNA fragments were joined later to obtain a complete deletion mutant. The linker (HindIII)-containing oligonucleotide primer sequence corresponding to

FIG. 2. Schematic presentation of major motifs in the Haspin protein and comparisons of Haspin with the ATP-binding site of CDC2 kinase and with transcription factor MEF2B. A, schematic presentation of locations of major motifs present in the Haspin amino acid sequence, B, comparisons of Haspin and the deletion mutant of Haspin with the mouse CDC2 kinase catalytic domain. Roman numerals in A and B indicate subdomains of the kinase catalytic domain. Underlining also indicate subdomains of the kinase catalytic domain. C, comparison of Haspin and mouse MEF2B. Numbers at both ends indicate amino acids of each protein. A.A., amino acids.
was done with anti-Haspin antiserum (KKK-1) as described under “Experimental Procedures.” 28S and 18S RNA was hybridized with the haspin cDNA probe. After exposure, the same filter was rehybridized with the β-actin and protamine cDNA probes. Arrows indicate ribosomal RNA positions. B, ~100 μg of protein of each tissue prepared from an adult mouse was electrophoresed and electrotransferred to a membrane filter. Western blot analysis was done with anti-Haspin antisera (KKK-1) as described under “Experimental Procedures.” Arrows indicate molecular weights (×10^5) of marker proteins. The asterisk indicates the Haspin protein.

nucleotides 1354–1368 (5′-CCAAGCTTCTCACCAGGCCTC-3′) and the antisense primer of the 5′-region as described above were used for the 5′-region DNA fragment of the deletion mutant haspin. The linker (HindIII)-containing oligonucleotide primer sequence corresponding to nucleotides 1406–1421 (5′-AGAAGCTTAACTCAAGCCTCC-3′) and the linker (BamHI) oligonucleotide primer of the 3′-region as described above were used for the 3′-region DNA fragment of the deletion mutant haspin. The two DNA fragments were subcloned into polycloning sites of Bluescript II to obtain the deletion mutant haspin cDNA (see Fig. 2A). The cDNA fragment obtained, i.e. the deletion mutant of haspin, was ligated to the Kpn1-BamHI site of the mammalian expression vector pEGFP-C1.

Transfection of Cultured Cells with Expression Vectors—COS-7 (27) and HEK-293 (28) cells were transfected with expression vectors pEGFP-HASP and pEGFP-dHASP using calcium phosphate (29) and LipofectAMINE Plus reagent (Life Technologies, Inc.), respectively. Sixteen hours after transfection, COS-7 cells were washed sequentially with 20% MeSO, 10% MeSO, and phosphate-buffered saline and then incubated in Dulbecco’s modified Eagles medium. However, in the case of HEK-293 cells, transfected cells were washed with the medium and incubated. After 2 days, for in vitro protein kinase assay, the cells were washed with phosphate-buffered saline and scraped with a plastic policeman under a microscope. The expression vector pEGFP-C1 alone without the haspin cDNA was used as a negative control.

In Vitro Kinase Assay—Mouse testes and transfected cells were lysed with a lysis buffer consisting of 10 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.03% SDS, 0.15 mM NaCl, and 0.01 mM PMSF, and the lysates were centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was treated with protein A-Sepharose at 4 °C for 1 h to eliminate nonspecific binding materials. Preimmune normal rabbit serum or specific antisera (DDP-2) was then added at a 1:500 dilution and shaken at 4 °C for 2 h. The samples were incubated with protein A-Sepharose beads at 4 °C for 1 h and centrifuged. The precipitated protein A-Sepharose beads were washed three times with the lysis buffer and then twice with kinase assay buffer (40 mM HEPES (pH 7.4), 10 mM MgCl_2, 3 mM MnCl_2, 5 mM CaCl_2, and 150 mM NaCl) and incubated at 37 °C for 10 min in 40 μl of kinase assay buffer with 10 μCi of γ-[32P]ATP (3000 Ci/mmol; Amersham Pharmacia Biotech). Phosphoamino acid analysis was performed as described (30) in the protein kinase resource of the World Wide Web. 2

FACS Analysis—After transfection with expression vector DNAs, on days 2, 4, and 6, HEK-293 cells were harvested, suspended in tubes, and fixed with 70% methanol. After being treated with 0.1 μg/ml RNase, cells were stained with propidium iodide (10 μg/ml). The cell suspensions were analyzed with a fluorescence-activated cell sorter (FACS-Calibur, Becton Dickinson Co., Ltd.).

DNA Binding Assay of Haspin—The affinity between the Haspin protein and DNA was examined by monitoring an elution profile of the Haspin protein by DNA affinity column chromatography. Two days after being transfected with pEGFP-HASP or pEGFP-dHASP, HEK-293 cells were sonicated in 0.5× TBS-T (12.5 mM Tris-HCl (pH 7.5), 75 mM NaCl, 25 mM KCl, and 0.05% Tween 20) containing 1 mM PMSF. Cell lysates were centrifuged at 10,000 rpm for 10 min at 4 °C and filtered through a 0.45-μm microfilter (Kurabo, Tokyo). Supernatants were applied to chromatography columns (0.5-μl bed volume; Bio-Rad, Tokyo) with calf thymus native DNA-cellulose (P-L Biochemicals). Materials were eluted stepwise sequentially with 1-ml volumes of 0.1, 0.2, 0.3, 0.4, 0.6, and 1.0 mM NaCl in 0.5× TBS-T containing 1 mM PMSF after washing two times with 5-ml volumes of buffer consisting of 0.5× TBS-T containing 1 mM PMSF. The eluates were immunoblotted after SDS-PAGE and subjected to Western blot analysis with anti-Haspin rabbit antisera.

2 Available at http://www.sdsc.edu/.
RESULTS

Structural Characterization of haspin cDNA and Its Deduced Protein—Twenty independent cDNA clones have been isolated by screening $2 \times 10^6$ clones from a pAP3 neo mouse testis cDNA library with the $^{32}$P-labeled gsg2 probe that had been previously cloned as a germ cell-specific cDNA (4). All nucleotide sequences of the five independent isolates with inserts of $2.6$ kilobase pairs revealed a single long open reading frame. As no stop codon was found anywhere upstream of the 5'-coding region, we performed 5'-RACE using a gsg2-specific antisense primer prepared from the sequence data and isolated five independent clones. Since a stop codon was located 24 bases upstream of the ATG sequence in all five clones, we assumed the ATG to be the translation initiation codon of the cDNA (Fig. 1). The complete nucleotide sequence and its deduced amino acid sequence are shown in Fig. 1. The protein consisted of 754 amino acids beginning from the first methionine at nucleotide 34. A computer-assisted homology search for the nucleotide sequence revealed that no homologous sequence has ever been reported. The cDNA sequence was without a poly(A) tail, but contained a 3'-untranslated region of 532 nucleotides, including two possible consensus AATAAA polyadenylation signals at nucleotides 2512 and 2788.

The deduced amino acid sequence of the cDNA showed the following well known motifs (Figs. 1 and 2A): a nuclear localization signal (KKKKR) at amino acids 320–324 (31); a leucine zipper at residues 585–613 (32); and parts of protein kinase consensus sequences over the region of amino acids 440–498 (33, 34), which was in particular homologous to CDC2 kinase (Fig. 2B). It also contained many potential target sites for protein kinases: phosphorylation sites for protein kinase C (consensus (S/T)X(R/K)) (35) located at residues 24–26, 338–340, 437–439, 480–482, and 705–707; a cAMP- and cGMP-dependent protein kinase phosphorylation site (consensus RKX(ST)) (36) at residues 323–326; and 11 target sites for casein kinase II (consensus (S/T)XX(D/E)) (37) in Haspin (Fig. 1). It had also a region homologous to murine MEF2B (myocyte-specific enhancer factor 2B) (38) at residues 144–162 (Figs. 1 and 2). We named this protein Haspin.

Expression of Haspin mRNA and Protein—Northern blot analysis showed that haspin mRNA was specifically detected in the testis as a major transcript of 2.8 kilobases, expressed exclusively in germ cells within the testis, but was not detectable in somatic tissues such as the brain, heart, intestine, kidney, liver, lung, muscle, and spleen (Fig. 3A). No transcript was observed in the ovary, either. In both the cryptorchid and

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**Fig. 5. Immunohistochemical staining with anti-Haspin rabbit antiserum.** Frozen sections of an adult mouse testis were stained with anti-Haspin antiserum (HAS-3) using the streptavidin method as described under “Experimental Procedures.” Testicular sections were treated as follows: control (preimmune rabbit serum; A) and stained with anti-Haspin antiserum (B–F). Testicular sections of C and D include round and elongated spermatids in seminiferous tubules, respectively. E indicates a blow-up of early spermatids. F was stained with hematoxylin after being immunostained with anti-Haspin antiserum. Spermatids in this section can be classified into two types: round spermatids at steps 2 and 3 and elongated spermatids at steps 14 and 15. Numbers at the top of F indicate developmental steps of spermatids. Bars = 100 μm (A–D) and 20 μm (E and F).
mutant mouse testes, in which there are no differentiated germ cells (5), no transcript was observed. Western blot analysis with anti-Haspin rabbit antiserum detected one positive signal with $M_r$ 83,000 specifically in mouse testis extracts (Fig. 3B), consistent with the results of Northern blot analysis. A positive signal with a low molecular weight was also detected in the muscle with this antiserum. However, the protein was believed to be a cross-reacting molecule since no haspin mRNA was detected in the muscle by Northern blot analysis. During male germ cell development, the transcript was not detected in neonatal mouse testis before 24 days of age. Then, the signal strengthened up to adulthood (Fig. 4A). The chronological change in the level of the haspin transcript was very similar to the pattern for protamine mRNA, indicating that its expression in the testis is haploid germ-cell specific. In the case of Western blot analysis, Haspin was first detected in the 24-day-old testis, and the signal gradually increased with age until adulthood. This result was positively correlated with the mRNA expression (Fig. 4B). Thus, these results indicate that both transcription and translation of the haspin gene occur exclusively in haploid male germ cells and that the timing of gene expression is precisely regulated during the development of male germ cells.

Immunohistochemical Identification of the Haspin Protein in Male Germ Cells—Immunohistochemical examination of Haspin in adult mouse testis showed that some germ cells stained positively, but somatic cells such as Leydig and Sertoli cells were negative (Fig. 5, B–D). The expression of the Haspin protein was first detected in haploid round spermatids at step 1 (Fig. 5E). However, the accumulating rate gradually decreased as differentiation proceeded further. In late spermatids at steps 15 and 16, in which nuclear condensation was almost completed, no signal was detected. No germ cells present in the first layer of the seminiferous epithelium (spermatogonia and leptotene and zygotene spermatocytes) were stained (Fig. 5, B–D). These observations were in good agreement with the results of Western blot analysis, indicating that Haspin is a novel differentiation-associated protein whose expression level peaked at the appearance of haploid spermatids. Subcellular localization of Haspin was limited to the nuclei of round (Fig. 5E) and elongated (Fig. 5F) spermatids. However, upon development of spermatids undergo remodeling of the nucleus and acrosome formation, localization of Haspin appeared to change from the whole nucleus to a limited area of the nucleus, in the vicinity of the acrosome (Fig. 5F).

In Vitro Kinase Assay—Haspin has a core kinase catalytic domain that includes consensus kinase subdomains I–III, but lacks the C-terminal consensus subdomains IV–XII. To examine the biochemical function of Haspin, we investigated its kinase activity. Adult testis lysates were immunoprecipitated with DDP-2 antiserum. An aliquot of the immunocomplexes was subjected to SDS-PAGE followed by Western blot analysis with KKK-1 antiserum. By using the same polyvinylidene difluoride filter, phosphorylated proteins labeled with $^{32}$P were detected by autoradiography, and Haspin proteins were detected by immunostaining. Two prominent bands of $M_r$ 83,000 and 50,000 labeled with $^{32}$P were detected (Fig. 6A). These two bands corresponded to phosphorylated Haspin and the heavy chain of immunoglobulin, respectively. In addition, one weaker band was also detected at $M_r$, −75,000. Because the band was detected with all three different anti-Haspin rabbit antisera (see “Experimental Procedures”), it might be a specific substrate associated with Haspin kinase. Alternatively, it could be a degradation product of Haspin. To demonstrate that the kinase activity was intrinsic to Haspin, a deletion of 10 amino acids was introduced into kinase subdomain I, where ATP is supposed to bind (Fig. 2, A and B). The expression vectors were constructed to produce the wild-type and mutant Haspin proteins, each fused with green fluorescence protein (pEGFP-C1 vector) and cloned into the pEGFP-C1 vector; D, the deletion mutant haspin cDNA in the pEGFP-C1 vector; M, mock transfection with the pEGFP-C1 vector DNA alone without an insert; IgG, IgG heavy chain. Arrows on the right indicate molecular weights (×10$^3$) of marker proteins. The results from the phosphoamino acid analysis of Haspin are shown (C). In vitro phosphorylation was done as described under “Experimental Procedures.” After hydrolysis of Haspin, phosphorylated amino acids were analyzed by thin-layer chromatography. The positions of the three phosphoamino acids are represented schematically in panel a. The profiles of amino acids reacted with ninhydrin solution in panel b and of $^{32}$P-labeled phosphoamino acids in panel c are shown.
both serine and threonine were specifically phosphorylated. Taken together, these results indicate that Haspin has intrinsic serine/threonine kinase activity undergoing autophosphorylation.

**Ectopic Expression of Haspin Inhibits Proliferation of HEK-293 and COS-7 Cells**—To understand the function of Haspin, we have examined its localization and the effects of its ectopic expression on cultured cells. When HEK-293 and COS-7 cells were transfected with the cDNA of wild-type haspin (pEGFP-HASP) or deletion mutant haspin (pEGFP-dHASP), both proteins were localized exclusively in the nuclei of the cultured cells (Fig. 7), similar to the physiological localization of Haspin in haploid spermatids (Fig. 5). In the cultured cells, however, localization in Haspin was restricted to some subnuclear foci, discretely present in a punctate form within the nucleus (Fig. 7, J and H). Because transfected cells did not proliferate, we have examined the effect of Haspin on cell cycle progression. Cultured HEK-293 cells were transfected with wild-type haspin or deletion mutant haspin cDNA. After an appropriate period of culture, cells were harvested and separated into two groups, i.e. fluorescence-positive cells (Fig. 8, peak a) and fluorescence-negative cells (peak b). The latter negative cells were supposedly the non-transfected cells; thereby, these cells could be considered as the negative control for the former positive cells capable of expressing exogenous Haspin. The control cells in both mutant and wild-type haspin-transfected cultures showed the normal log phase growth pattern (Fig. 8, A, peak b; and B, peak b). In contrast, the fluorescence-negative cells showed cell cycle arrest at G1 phase (Fig. 8, A, peak a; and B, peak a). Thus, the transfected cells capable of expressing exogenous Haspin were not able to proliferate, and furthermore, almost all the cells had detached from the culture dishes 7 days after transfection. The expression level of deletion mutant Haspin lacking the kinase activity was similar to that of wild-type Haspin in transfected HEK-293 cells judging from the fluorescence intensity of EGFP-fused Haspin (data not shown). Nevertheless, the deletion mutant induced a quicker response than did the wild type: 2 days after transfection, all of the HEK-293 cells expressing mutant Haspin had undergone cell cycle arrest at G1 phase, whereas those with wild-type Haspin showed cell cycle arrest 6 days after transfection. It should be pointed out that the negative control EGFP protein spread over the whole cell (Fig. 7, E, F, K, and L) and did not affect the cell cycle or growth of the transfected cells (data not shown).

**DNA-binding Ability of Haspin**—Since both the physiological localization and ectopic expression of Haspin were restricted to nuclei, we examined the association of Haspin with nuclear DNA. The binding affinities of Haspin and mutant Haspin for DNA were compared by monitoring their elution profiles by DNA affinity column chromatography. Extracts from the cells transfected with the wild-type or mutant haspin cDNA were applied to calf thymus native DNA-cellulose mini-columns, and proteins were eluted stepwise with increasing concentrations of NaCl. After SDS-PAGE, the EGFP-Haspin proteins were detected by Western blot analysis with anti-Haspin antiserum. As shown in Fig. 9, more than half the amount of the Haspin protein was recovered from the flow-through fraction, and the rest was retained by the DNA column. With 0.1 M NaCl, almost all the bound Haspin was eluted. In contrast, all of the mutant Haspin defective in kinase activity was able to bind to the DNA column and was eluted with 0.1 M NaCl. Thus, these results indicate that the mutant Haspin lacking the autophosphorylation activity has higher affinity for DNA than the wild type and suggest that Haspin is present in at least two forms in terms of DNA-binding ability.

**DISCUSSION**

Recently, we have isolated various germ cell-specific cDNAs from a subtracted cDNA library of the mouse testis (4) and investigated the roles of genes in spermatogenesis. The present study demonstrated that Haspin is a haploid germ cell-specific nuclear protein expressed exclusively in spermatids from steps 1 to 14 and decreasing thereafter as the spermatids mature. Western blot analysis using three anti-Haspin rabbit antisera (KKK-1, DDP-2, and HAS-3) capable of recognizing three different epitopes in the Haspin molecule showed one positive band of Mr 83,000 exclusively in the testis. In addition, another positive band having a smaller size was detected in the muscle with KKK-1 antiserum. However, the protein must be a cross-reactive molecule other than Haspin since no positive band was detected with the other two antisera (data not shown) and also no haspin transcript was observed in the muscle by Northern blot analysis.

Haspin contains several known motifs in the deduced amino acid sequence. First, a core part of the protein kinase consensus sequence was found. Eukaryotic protein kinases belong to a very extensive family of proteins sharing a catalytic core consisting of 12 conserved motifs common to both the serine/
Haspin and deletion mutant Haspin in the HEK-293 cells transfected with pEGFP-C1 vector. To examine whether Haspin really has kinase activity, thus indicating that Haspin has intrinsic protein kinase activity.

Second, Haspin contains many potential target sites for protein kinases, i.e., potential phosphorylation sites for protein kinase C (consensus (S/T)X(R/K)) (35), casein kinase II (consensus (S/T)XX(D/E)) (37), and cAMP- and cGMP-dependent protein kinases (consensus RKXX(S/T)) (36). It is possible that the biological activity of the phosphorylated form of Haspin is different from that of the non-phosphorylated form. We have shown that Haspin association with DNA was different between wild-type and kinase-negative mutant Haspin proteins, of which the former was capable of autophosphorylation and the latter was not. The interaction with DNA may cause some biological activity, and such phosphorylation might be involved in the interaction.

Third, a leucine zipper pattern could also be constructed within the region of amino acids 585–613 using the consensus sequence LX_6L, suggesting that Haspin associates with some other proteins to form protein complexes. Recently, many leucine zipper proteins have been reported and revealed to play key roles in cell growth and differentiation (39). In haploid germ cells, a specific cAMP response element modulatory protein (CREM) is believed to control some gene expression (40, 41). Haspin may associate with such a factor to play a regulatory role in haploid-specific gene expression. Haspin has a nuclear localization signal (KKKRK) (31) at residues 320–324. However, when the sequence was deleted, ectopically expressed Haspin was still observed exclusively in nuclei of COS-7 or HEK-293 cells (data not shown). Thus, the nuclear localization signal (KKKRK) is unlikely to play an important role in specific localization of Haspin in spermatid nuclei. It is possible that the basic amino acid sequence in the N-terminal region of Haspin functions for the specific localization in the nucleus. Within the nucleus, Haspin seems to localize in a punctate form, implying its involvement with some subnuclear foci such as nucleoli.

Ectopic expression of Haspin caused cell cycle arrest at G1 phase in transfected HEK-293 and COS-7 cells (Fig. 8) and ES cells (data not shown). The deleterious effect of Haspin, which was not due to the toxicity of the EGFP tag (42) in these cultured cell lines, was not due to the kinase activity either since the same or a rather severer effect was observed with the kinase-negative deletion mutant Haspin protein. However, when we examined the DNA-binding ability of Haspin using the DNA-cellulose minicolumn, there was a clear difference observed between the wild-type and mutant Haspin proteins. Approximately one-half of EGFP-fused wild-type Haspin was bound to DNA, but the rest was not and was recovered from the flow-through fraction. In contrast, EGFP-fused kinase-negative mutant Haspin bound to the DNA column completely. This indicates that wild-type Haspin has heterogeneity for DNA affinity, possibly by the modification of phosphorylation. The N-terminal basic region of Haspin might serve as a DNA-binding domain, and its binding ability might be negatively controlled by phosphorylation. In this way, autophosphorylation may partic-

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**Fig. 8. FACS analysis of HEK-293 cells transfected with the haspin and deletion mutant haspin cDNAs in expression vector pEGFP-C1.** The HEK-293 cells in the culture dish transfected with cDNA were separated into transfected and non-transfected cells according to intensities of EGFP fluorescence on days 2, 4, and 6 after transfection. A, the cells were subjected to FACS analysis. A gate was set with EGFP fluorescence (488 nm) to separate transfected HEK-293 cells (peak a) from non-transfected HEK-293 cells (peak b) in the same culture dish. B, shown are changes in the DNA content profiles of transfected (peak a) and non-transfected (peak b) HEK-293 cells after transfection with the haspin and deletion mutant haspin (d-Haspin) cDNAs in the pEGFP-C1 vector.

**Fig. 9. Elution of the EGFP-Haspin fusion protein from a DNA-cellulose column.** Supernatants from the HEK-293 cells transfected with the pEGFP-HASP or pEGFP-dHASP expression vector were applied to native DNA-cellulose minicolumns and then eluted stepwise sequentially with 0.1, 0.2, 0.3, 0.4, 0.6, and 1.0 M NaCl. T, F, and W indicate the applied sample and the flow-through and washing fractions, respectively. Haspin in each fraction was visualized by Western blot analysis with KKK-1 antiserum. d-Haspin; deletion mutant Haspin.
ipate in the regulation of cell cycle control. Since Haspin caused cell cycle arrest in somatic cells, it may exert a somewhat similar function in the testis. It should be noted, however, that Haspin expression was observed only in the haploid germ cells that were no longer able to replicate. In conclusion, Haspin may participate in the negative control of the cell cycle progression of spermatids.

Haspin has a region homologous to MEF2B at residues 144–162 (38) and similarly has a basic amino acid region at its N terminus. The mef2B gene encodes a MADS box transcription factor, which regulates the expression of many muscle-specific genes (43). As Haspin does not have the MADS box and MEF2B does not have the protein kinase domain, Haspin should differ in function from MEF2B. However, Haspin has the basic domain at its N terminus and can bind to DNA. Thus, it is possible that Haspin is another type of testis-specific transcription factor or a cell cycle regulatory factor of haploid germ cells.

The haspin gene has been mapped to mouse chromosome 11 (44). A known mouse mutant locus close to haspin is the ovum mutant (om), which shows a notable phenotype related to fertilization (45). Its corresponding gene acting in spermatozoa is the S element. Haspin might be a kind of germ cell factor. Further studies are now in progress to elucidate the molecular function of Haspin in testicular germ cell proliferation and differentiation.

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