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NYD-SP6, a Novel Gene Potentially Involved in Regulating Testicular Development/Spermatogenesis

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Using cDNA microarray hybridization from a human testicular cDNA library, a novel gene exhibiting 30-fold difference in expression level between adult and embryo human testes was cloned and named NYD-SP6, which was 1858 bp in length with 87% nucleotide identity with the mouse homologue sequence. The deduced protein structure of NYD-SP6 was found to contain two plant homeodomain (PHD) finger domains, believed to be involved in activating transcriptional regulation. Tissue distribution analysis using Northern blot indicated that the NYD-SP6 gene was expressed in a wide range of tissues, with a high expression level in the testis. Its expression in human and mouse testes by *in situ* hybridization was confined to Sertoli cells and the expression was developmentally regulated as demonstrated by cDNA microarray, *in situ* hybridization, and semiquantitative PCR in mouse testes. GFP/NYD-SP6 protein was predominantly localized in the nucleus of the transfected CHO cells, indicating its role in transcriptional regulation. In contrast, the N-terminal truncated NYD-SP6 (tNYD-SP6) localized in the nuclear envelope, indicating this region function as a nuclear localization signal. Further Northern blot analysis of gene expression in patients with spermatogenesis arrest revealed that NYD-SP6 was absent in one patient whose spermatogenesis was blocked at the stage of spermatocytes. Taken together, these results suggest that the putative protein of NYD-SP6 may play an important role in stimulating transcription involved in testicular development and/or spermatogenesis.

Key Words: **gene; NYD-SP6; PHD domain; Sertoli cell; testis; spermatogenesis.**

Spermatogenesis is the progress of cell division and cell differentiation, undergoing spermatogenia, spermatocytes, spermatids, and leading to the production of spermatozoa in the testis. Spermatogenesis is a highly regulated complex process involving both intrinsic and extrinsic regulators, as well as interaction between germ cells and Sertoli cells (1). The intrinsic regulation of gene expression in spermatogenesis occurs at three levels: transcription, translation, and posttranslation. Spermatogenesis is further subjected to secondary regulation by endocrine, paracrine, and autocrine signals transmitted indirectly through surrounding somatic cells including Sertoli cells (2). Growing evidence indicates that the interaction between germ cells and Sertoli cells in seminiferous tubules may play a pivotal role in regulation of the spermatogenic cycle (1). Sertoli cells are also recognized to play important roles in the initiation of testicular differentiation and establishment of the framework for subsequent sperm production (3). However, the molecular mechanisms regulating germ cell behavior and testicular development remain largely unknown.

Defective spermatogenesis may result from systemic disease, malnutrition, endocrinological disorders, genetic defects, anatomical obstruction, infections and environmental toxins (4). It is now recognized that a significant number of cases of sever oligozoospermia or azoospermia have underlying genetic etiologies, including chromosomal abnormalities, microdeletions of the Y chromosome, meiotic abnormalities and mutations associated with the cystic fibrosis gene leading to congenital absence of the vas deferens (5, 6). Spermatogenic arrest is an interruption of germ cell differentiation that may result in either oligozoospermia or azoospermia. There are different levels of specific cell types of spermatogenic arrest, and the diagnosis can be made by testicular biopsy (7). Mutations or absent of genes expressed at different development levels of

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spermatogenesis may lead to spermatogenic arrest and infertility (8).

Many of the genes involved in the development of the testis and normal spermatogenesis are transcription factors, such as hormone receptors and growth factors. Intriguingly, two PHD (plant homeodomain) zinc finger domains are identified in putative NYD-SP6 protein localized in nucleus. Many nuclear transcription factors, which are important for germ cell development, have been reported to contain PHD finger domain, which is localized in nucleus and involved in transcriptional regulation (9, 10). This domain is characterized by a highly conserved Cys_4-His-Cys_3 motif identified in a number of proteins in modulating transcription including the trxG protein TRX, Absent, small or homeotic discs 1 and 2 (ASH1, ASH2) (9, 12). The PHD finger has been found in many regulatory proteins from plants to animals, which are frequently associated with transcriptional activation (13). Furthermore, PHD is suggested to mediate protein–DNA interactions. Conservation of this region from yeast to human suggested that it is important in NYD-SP6 function.

We report here the identification and characterization of the complete sequence of a novel gene, NYD-SP6, which is highly expressed in human testis and contains two PHD finger domains. The development-dependent and functional expression of NYF-SP6, as well as its subcellular localization are consistent with its deduced protein structure characteristic and indicate its potential role in regulating transcription during testicular development and/or spermatogenesis.

MATERIALS AND METHODS

Preparation of Human Testis cDNA Microarray

9,216 positive phage clones were picked up randomly from Human Testis Large Insert λ phage cDNA Library (Clontech, HL5503U) and then converted into plasmid clones (according to Clontech's Manual PT3003-1). The inserts were amplified by PCR using 5′-CCATTGTGTTGGTACCCGGGAATTCG-3′ as a forward primer and 5′-ATAAGCTTGCTCGAGTCTAGAGTCGAC-3′ as a reverse primer. PCR products were used to make human testis cDNA microarray. Protocol for cDNA microarray preparation and hybridization signal analysis has been recently described (14).

Sequence and Analysis of Interest Clones

Clones of interest were selected and extracted by DNA extraction system (Qiagen, Hilden, Germany), inserted into PT3003-1 vector and sequenced by ABI 377 in HuaDa Gene Center in China. NYD-SP6 was one of those interest clones whose complete nucleotide sequences have been accepted by GenBank. The Accession No. AY014283 has been offered by GenBank. To determine sequence homology with other components, all sequences were blasted in Data Bank of National Center for Biotechnology information (NCBI) (http://www.ncbi.nlm.nih.gov/blast). High homologous proteins were compared by CLUSTER and phylogenetic relations were calculated with neighbor joining methods of Mega 1.02 (Molecular Evolution Genetics Analysis). Meanwhile, nucleotide and putative protein was analyzed by Generunner.

Multiple Tissue Northern Blots Analysis

Total RNA of 16 adult human tissues was purchased from Clontech Co. NYD-SP6 cDNA probe (nucleotides 1048–1760) was produced by PCR labeling method. Poly(A)$^+$ RNA was purified by Oligotex dT-30 (Roche Molecular Biomedicals) absorption according to the manufacturer's introduction. Poly(A)$^+$ RAN (2 μg each) or total RAN (10 μg each) was denatured with formaldehyde, electrophoresed on 1% agarose gels, transferred onto Hybond-N$^+$ nylon membranes (Amersham Pharmacia Biotech). The blots were hybridized with the probe of digoxigenin-labeled NYD-SP6 cDNA at 60°C in a solution containing 10% dextran sulfate, 1× Denhardt's solution, 0.1% SDS, 6× SSC and single-stranded salmon sperm DNA for 16 h. For integrity of RNA, the membrane was rehybridized with a human β-actin cDNA probe, which was labeled as a positive probe through PCR. Its upstream primer is 5′-CGGTTGGCCTTGGGGTTCAGGGGG-3′ and downstream primer is 5′-ATCGTGGGGCGCCCCAGGCACCA-3′.

NYD-SP6 Expression in the Testes of Azoospermia

Testicular tissues were obtained from a total of 22 azoospermic patients undergoing testicular biopsy for clinical diagnosis. Total RNA (about 3.5 μg/μl) of each sample was extracted using TRIzol Reagent (GIBCO). All the following experimental procedures including poly(A)$^+$ RNA preparation, tranferation and hybridization were performed as mentioned above. The same NYD-SP6 and β-actin cDNA probe were used.

Semiquantitative Analysis of NYD-SP6 mRNA in Mouse testes of Different Stage

After analyzing NYD-SP6 in GenBank database, high homology of NYD-SP6 was found between human and mouse. To evaluate the quantity of NYD-SP6 expression in mouse testes of different stage, semiquantitative PCR was performed. Total RNA of week 1, week 4, and week 7 mouse testes were isolated using TRIzol Reagent (GIBCO Co.) respectively. Different cDNA was obtained by means of reverse transcription according to the manufacturer's manual (M-MLV Reverse Transcriptase, Promega). Primers were desired according to the mouse sequence (GenBank Accession No. AK005258) homology with human (NYD-SP6): P1, 5′-TCT GTA TGA GCA AGG CAG AGA C-3′; P2, 5′-GGA TTC TCT TCC AGG CTG GTA G-3′. PCR was carried out using PE-5700 (PE Co.). To obtain relatively accurate result, each template of different mouse testes was all conducted in three columns in the same PCR mixture and profile. β-Actin mRNA was run in parallel with the same template as positive control under the same procedure. Statistically significant differences among groups were determined by Stata 7.0 software. $P < 0.05$ was considered statistically significant.

Tissue in Situ Hybridization

Tissue processing. Detailed practical protocols for fixation, paraffin, embedding, mounting, and sectioning of adult human and week 1, 4, 7 mouse testis have been described recently (15).

Human RNA probes by PCR labeling. Dig-labeled probes were made by *in vitro* transcription. The purified probe templates were generated using PCR amplification method followed by *in vitro* RNA transcription. Two primers contain T7 and SP6 promoter sequence, respectively. And desired fragment span two introns in genome. Purified The PCR products using PT3003-1-NYD-SP6 as template were labeled respectively through SP6/T7 RNA polymerase using DIG-RNA labeling Mix (Roche) according to the manufacturer's manual.

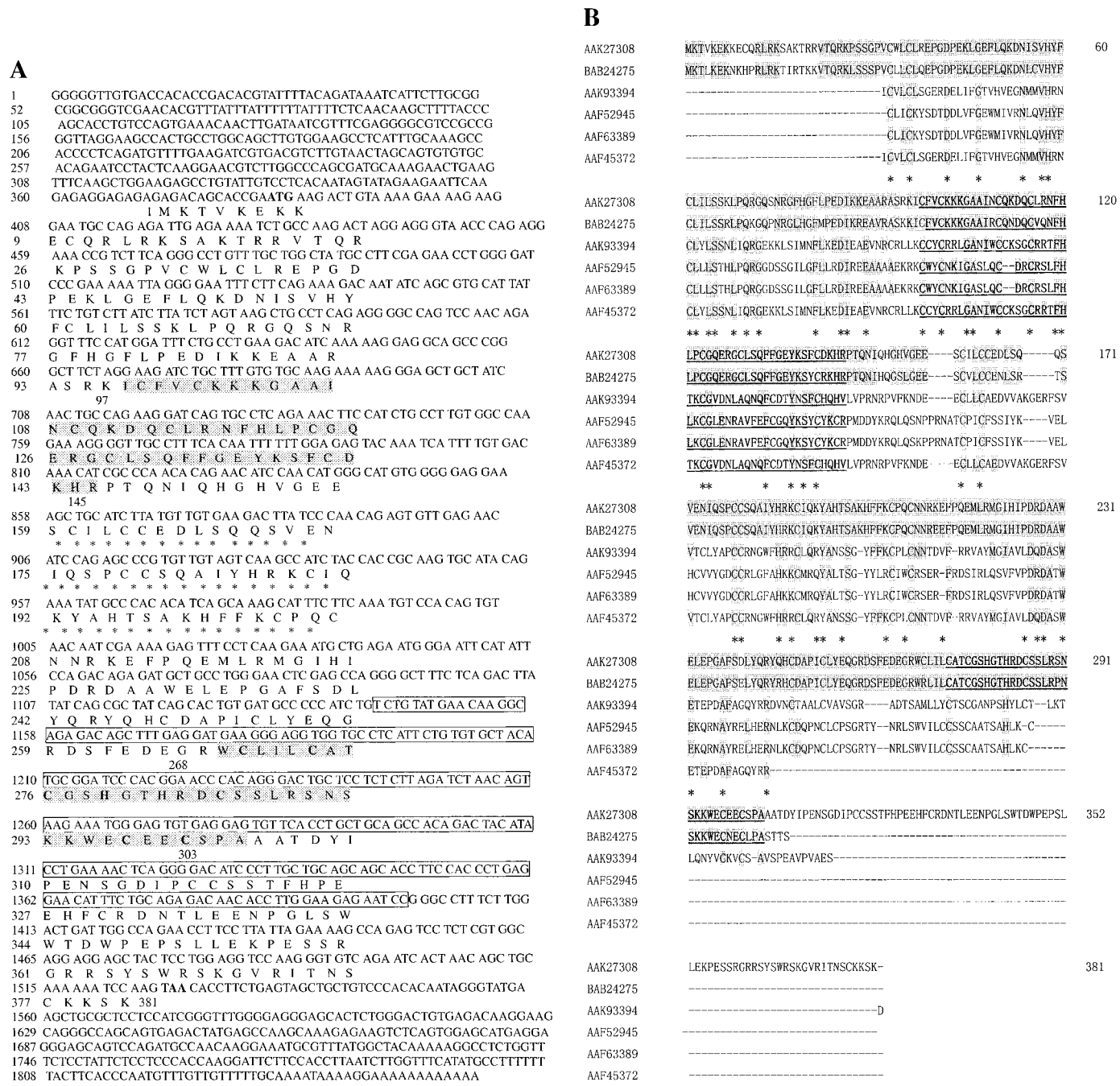

FIG. 1. (A) Nucleotide and deduced amino acid sequences of human NYD-SP6. The sequence of PHD domains is shaded, and the polyadenylation signal is underlined. The ring finger sequence is marked with *. Numbers of nucleotide and amino acid are shown on the each line. Complete sequence of NYD-SP6 has been accepted in GenBank under Accession No. AY014283. Amplification sequence of semiquantitative PCR is shown in boxes. (B) Amino acid sequence comparison of putative NYD-SP6 protein (Accession No. AAK27308) with mouse (Accession No. BAB24275), *Drosophila melanogaster* (Accession Nos. AAK93394, AAF52945, AAF63389, AAK454372). Identical residues are shaded gray. Highly conserved amino acids are marked with *. Homologous PHD finger domain is underlined.

Plasmid-derived mouse RNA probes. Dig-labeled probes were made by *in vitro* transcription by constructing Pinpoint Xa-1 vector (3.331kb) containing Sp6 promoter (from Promega). Mouse cDNA was amplified by PCR. Primers were as follows: P1, 5′-TCT GTA TGA GCA AGG CAG AGA C-3′; P2, 5′-GGA TTC TCT TCC AGG CTG GTA G-3′. The purified insert templates were generated using PCR amplification method using PT3003-1-NYD-SP6 as templates (Clontech) followed by *in vitro* RNA transcription. Antisense and sense probe templates were selected through PCR using Pinpoint primer, Sp6 primer and P1, P2, respectively. The positive combination plasmid was digested by restriction enzyme *Hin*dIII followed by precipitation with 70% ethanol in −20°C overnight and resuspended in 10 μl DEPC water. Plasmid was labeled with DIG-RNA labeling Mix (Roche) according to the manufacturer's manual.

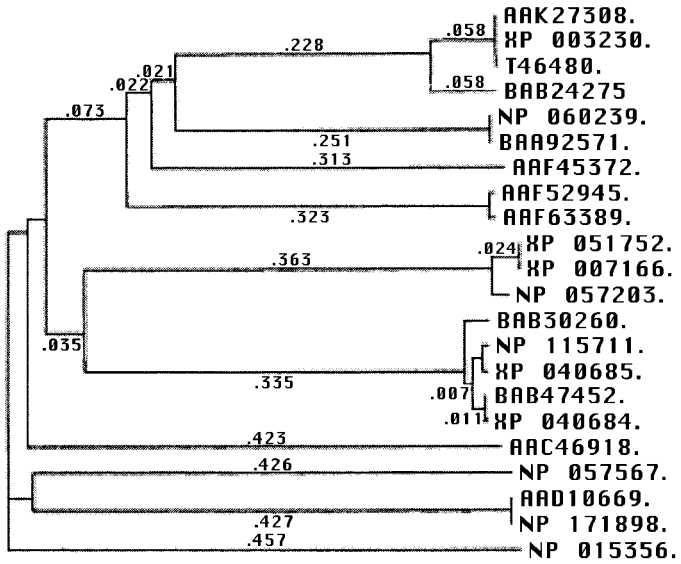

FIG. 2. Unrooted dendrogram showing the phylogenetic relations between deduced NYD-SP6 protein (AAK27308) and homologous proteins by neighbor-joining methods of Mega 1.02. Numbers indicate the phylogenetic branch distance.

In situ hybridization (ISH) After removal of paraffin, sections were rinsed in DEPC water, 0.01 M PBS, and 4% formaldehyde polymerase for 2, 5, and 10 min, respectively. The pretreatment of the sections consisted only of proteolytic digestion for 20 min at 37°C with 10 μg/ml proteinase K dissolved in 50 mM EDTA and 0.1 M Tris–HCl, followed by a 5-min rinse in DEPC water and two rinses of 5 min in 0.01 M PBS. Sections were prehybridized in hybridization mix without human or mouse probe for 1 h at 60°C and then hybridized overnight at 60°C, which was followed by stringy wash. Digoxigenin-labeled probe was detected with alkaline phosphatase-conjugated antidigoxigenin and visualized with NBT/BCIP chromogen.

Subcellular Localization Based on Green Fluorescent Protein Fusion Product

Plasmid construction of the pEGFP-c3 fusion protein. pEGFP-C3-NYD-SP6 was generated by inserting the PCR-derived ORF (nucleotides 384–1529) of NYD-SP6 into the *Sal*I/*Sac*I site of pEGFP-c3 vector (Clontech). The truncated NYD-SP6 (tNYD-SP6, Fig.7 (top)) was cloned into the *Hin*dIII/*Sal*II site of pEFGP-C3. The identity of the insert OFR of 1146bp and truncated fragment was confirmed by DNA sequencing.

Lipofection of recombination vector to CHO cell line. CHO cell (Chinese hamster ovary cell) was maintained in DMEM/F12 medium (v/v, 50/50, GIBCO BRL) supplemented with 10% fetal calf serum (from SI JI QING, Hangzhou, People's Republic of China). The trans-

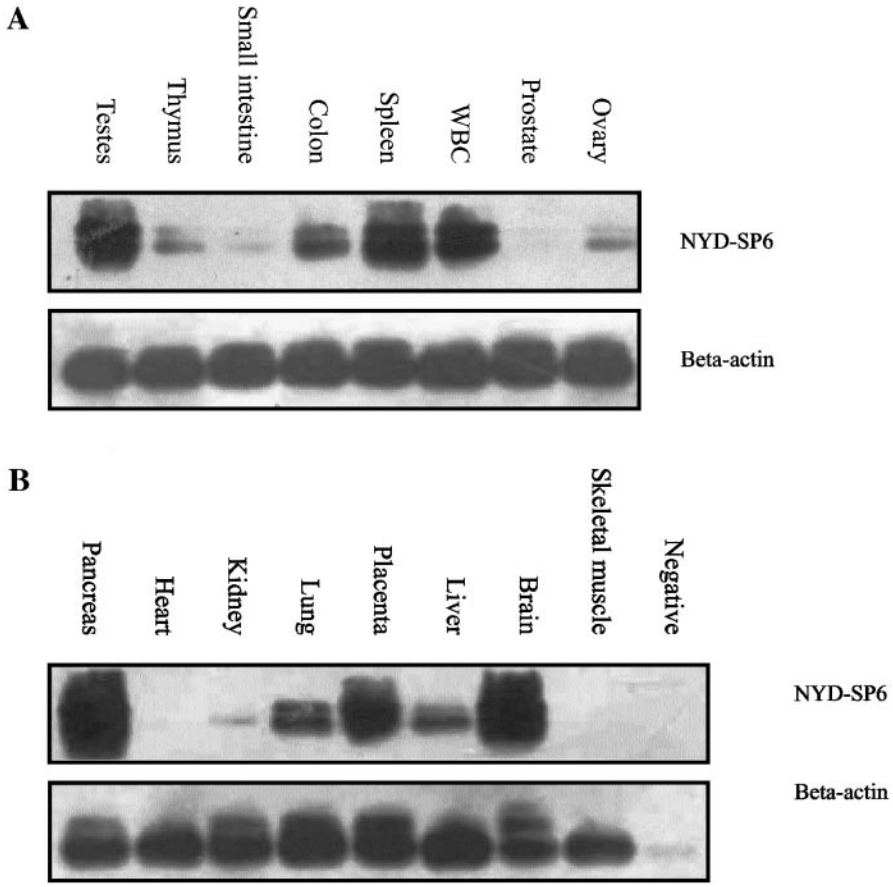

FIG. 3. Tissue distribution of NYD-SP6 mRNA expression in human tissue. Northern blot of 16 human tissues showed that high expression of NYD-SP6 (A) could be found in testes, pancreas, brain, placenta, spleen, and white blood cells. As a control, human β-actin, was coexpressed (B).

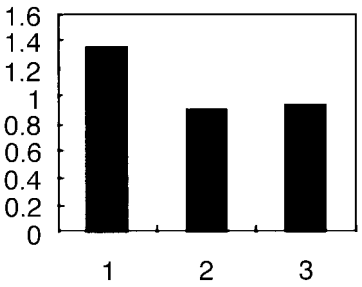

FIG. 4. Result of semiquantitative PCR in week 1, 4, and 7 mouse. The cycles of initial exponential phase was produced by PE-5700. Ratios between NYD-SP6 and β-actin of each stage were 1.35 ± 0.2256, 0.90 ± 0.3216, and 0.92 ± 0.1957. These ratios were analyzed by Stata 7.0 statistic software. $P < 0.05$ was considered statistically significant. Histograms 1, 2, and 3 represent the ratio of week 1, 4, and 7 mouse, respectively.

fection of the plasmid into CHO cells was performed as described by Matsusushita *et al.* (16). As a control, pEGFP-C3 vector without insert was transfected into CHO cells at the same time under the same condition.. The fusion protein expression was examined using a confocal laser microscope (Zeiss). Transfection ratio has been confirmed using a flow cytometer. About 15% of the cells were transfected consistently under these conditions.

RESULTS

Positive Clone with High Expression in Adult Testis

Among the hybridization clones which exhibited differential expression in embryo and adult testis, NYD-SP6, one of the 58 genes accepted by GenBank, was identified having hybridization signal intensities of 2126.37 and 58.74 for adult and embryo testis probes, respectively, with an expression level in the adult about 36.20-fold higher than that in the embryo. The human cDNA microarray was used to hybridize with mouse probes of postnatal 1- and 4-week testes and the hybridized signals were 12.14 and 145.28, respectively, exhibiting a 12-fold higher expression in 4-week testis than that in 1-week testis, as observed in human testis. Additionally, homology of this clone between human and mouse was implicated in this data.

High Conservation during Evolution

Multiple sequence comparison among different species by program CLUSTER and BLAST showed that putative NYD-SP6 protein shared approximately 35% amino acid sequence identity with that of *Drosophilas*, especially the 84% with that of mouse at protein level (Fig. 1B) and 87% at nucleotide level (data not shown). 34% highly conserved residues was found. The potential function of this mouse mRNA, however, has not been elucidated. Unrooted phylogenetic trees (Fig. 2) further suggested that putative NYD-SP6 protein (AAK27308) is more close to three *Drosophila melanogaster* proteins (AAF45372, AAF52945, and AAF63389), especially mouse protein BAB24275 (nucleotide in GenBank AK005852), than other selected proteins. Data presented above indicate that NYD-SP6 gene is highly conserved during evolution and is one human-mouse homologous gene.

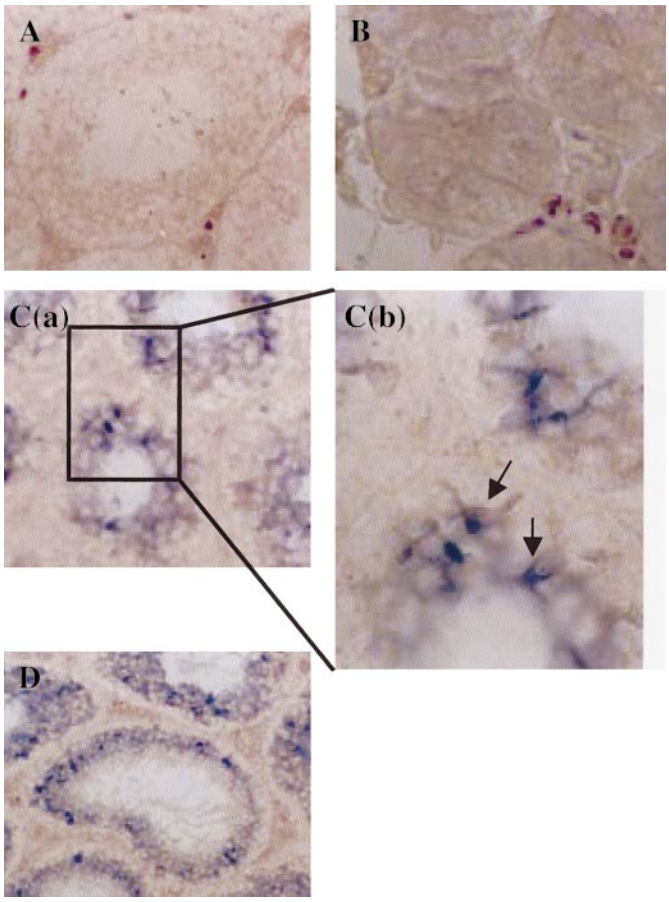

FIG. 5. Analysis of NYD-SP6 expression during postnatal testicular development using *in situ* hybridization. Testes were obtained from postnatal week 1 (B), 4 (C, E), and 7 (D) mouse and *in situ* hybridization was performed using antisense and sense riboprobes, respectively. Specific signal of NYD-SP6 transcripts was solely observed with NYD-SP6 antisense probe, whereas no signal was seen with sense probe. Magnifications are 400× for A, B, and C(b); 200× for C(a); 100× for D. Picture C(b) showed the same position in C(a) (black box). Arrows indicate Sertoli cells. NYD-SP6 is shown located in Sertoli cells.

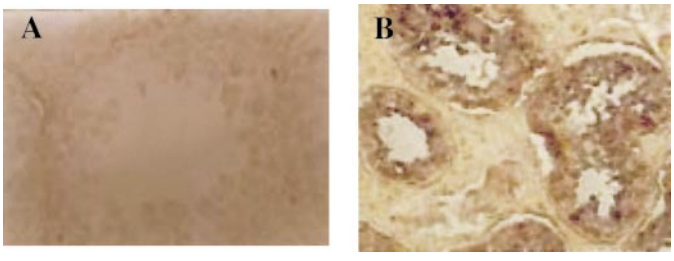

FIG. 6. Analysis of NYD-SP6 expression in adult human using *in situ* hybridization. Specific signal of NYD-SP6 transcripts was solely observed with NYD-SP6 antisense probe (B), whereas no signal was seen with sense probe (A). Magnification: 200× for A; 100× for B. NYD-SP6 was confined to Sertoli cells as shown.

Deduced Protein Containing Two PHD Finger Domains

NYD-SP6 was 1858 bp in length spanning 11 exons in genome, while the deduced protein had 381 amino acids (Fig. 1A). Blast search of the GenBank database (http://www.ncbi.nlm.nih.gov/) revealed that this gene is located on chromosome 3p24.3. Smart analysis showed that it contained two PHD finger domains, which rang from 97 to 145 and 268 to 303 amino acid, respectively (http://smart.embl-heidelberg.de) (Fig. 1A). Homologous sequence of Ring finger domain has also been identified spanning 160 to 207 amino acid. Deduced protein analysis through Generunner indicated a cluster of phosphorylation sites, including protein kinase C, casein kinase II, cAMP and cGMP-dependent kinase phosphorylation sites, which may allow posttranslational modification by respective kinases.

Wide Distribution in Human Tissue

Expression profile of NYD-SP6 in human tissues was studied using Northern blot hybridization. Of the 17 organs/tissues examined, NYD-SP6 was highly transcribed in human testes, pancreas, placenta, spleen, white blood cells, and brain, but was absent in the heart, skeletal muscle, and prostate (Fig. 3). The amount and integrity of the RNAs used for this Northern blot were verified by rehybridizing the membrane with digoxigenin-labeled human β-actin cDNA probe.

Development-Dependent Expression in Mouse Testis

To overcome the limitation on availability of human samples, development-dependent expression of NYD-SP6 mRNA was conducted using its mouse homologues and semiquantitative PCR. Amplified homologous sequence between AY014283 and AK005258 was showed in Fig. 2B. Cycles entering exponential phase reflected the expressed amount of mRNA. The cycles of initial exponential phase produced by GeneAmp5700 Sequence Detection System (Applied Biosystems) was about 16.5 ± 0.4358 and 16.95 ± 0.3905 in week 4 and week 7 mouse, respectively, but 25.19 ± 0.2594 in week 1 mouse. The cycle numbers for β-actin of the three samples, as a control, were 18.62 ± 0.3568, 18.30 ± 0.2865, 18.28 ± 0.3270, respectively. Ratios between NYD-SP6 and β-actin of weeks 1, 4, and 7 were 1.35 ± 0.2256, 0.90 ± 0.3216, and 0.92 ± 0.1957, respectively. These ratios were analyzed by Stata 7.0 statistic software. $P < 0.05$ was considered statistically significant. While no statistically significant difference between week 4 and 7 mouse testes, the expression level at week 1, compared to that of weeks 4 and 7, was significantly lower (Fig. 4), suggesting that NYD-SP6 might be involved in testicular development.

Sertoli Cell Localization in Testis

Based on the high homology of NYD-SP6 between human and mouse, *in situ* hybridization was conducted to identify the type of testicular cells in which NYD-SP6 gene was expressed, using paraffin sections of adult human testis and mouse testes of three different stages. ISH results obtained from mice tissue sections showed that NYD-SP6 mRNA expression was confined to Sertoli cells but not in germ cells. As shown in Figs. 5 and 6, positive signal indicating the expression of NYD-SP6 gene appeared as a dark purple precipitate, which could only be found in the Sertoli cell of week 4 and 7 mouse testes and adult human testes. No signal was observed in week 1 mouse testis. Negative control using sense probe confirmed the specific expression of NYD-SP6 mRNA (Figs. 5A and 6A). In additional, its strong expression in week 4 and 7 mouse testis section consist with the result of semiquantitative PCR suggest this gene is development-dependent.

Expression and Localization of NYD-SP6 Fusion Protein

The deduced structure of NYD-SP6 protein product appeared to be a regulatory protein containing a PHD finger domain. We examined the subcellular localization of the fusion protein by transient transfection in CHO cells. Fusion protein was exclusively expressed in the nucleus (Fig. 7 (top), C and D) while the single EGFP protein, as a control, was evenly distributed throughout the whole cell without any compartmentalization (Fig. 7 (top), A and B). To test whether the N-terminal amino acid residues was functional, the truncated NYD-SP6 (tNYD-SP6) construct (Fig. 7, bottom) which lack the N terminal 118 amino acids was transfected into CHO cells. It located around the nucleus (Fig. 7 (top), E and F) suggesting the N-terminal region may act as a nuclear localization signal.

mRNA Analysis in Spermatogenesis Arrest Testicular Samples

To probe the physiological function NYD-SP6, its expression in testicular samples obtained from patients with spermatogenesis arrest was examined by Northern blot analysis. Tissue samples from patients whose spermatogenesis were arrested either in spermatocyte and spermatid, or with Sertoli cell-only syndrome. Of 22 patients, no signal was seen in one patient whose spermatogenesis was blocked in spermatocyte (Fig. 8). The tissue section showed no spermatid and sperm in the seminiferous tubule (Fig. 9).

DISCUSSION

Using cDNA microarray constructed from a human testicular cDNA library, the present study has identified a novel gene, NYD-SP6, which consists of 1858bp with an open reading frame of 1146 bp.

Hybridization of cDNA microarray with probes from adult and embryo human/mouse testes has indicated NYD-SP6 was strongly expressed in adult testis, which was then in agreement with sequence comparison and phylogenetic tree analysis. Based on this homology between human and mouse, limitation of obtaining human sample used in examination could be overcome, which is the reason we test NYD-SP6 expression in mouse testis of different stage and carried out mouse ISH. Phylogenetic trees analysis of selected proteins ranging from vertebrate and invertebrate animals and sequence alignment showed NYD-SP6 protein genesis from the similar gene with three *Drosophila melanogaster* proteins. This high conservation suggests there exists important roles of NYD-SP6 during evolution.

The primers used in PCR for tissue distribution analysis guaranteed the impossible amplification in genomic DNA. The PCR product of 713 bp detected by Northern blot showed a wide distribution of NYD-SP6 in human tissues among which the testis has a high level of expression. The observed wide tissue distribution is in good agreement with the expression information produced by Unigene database (http://www.ncbi.nlm.nih.gov/UniGene/). cDNAs belonging to the same Unigene cluster (Hs.30127), such as NYD-SP6, are expressed in various human tissues, indicating that NYD-SP6 is a gene likely to be expressed in somatic cells. Indeed, *in situ* hybridization analysis of the testicular tissues of both human and mouse has demonstrated its expression confined to Sertoli cells but not germ cells.

Hybridization of cDNA microarray has indicated a 30-fold higher expression of NYD-SP6 in adult than that in embryo testis, indicating its development-dependent expression. Further examination on development-dependent expression using semiquantitative PCR on cDNA isolated from postnatal mouse testes of different development stages was performed. The results clearly showed that the amount of mRNA expressed in mouse testis was significantly increased from weeks 1 to 4; however, no further increase was observed from weeks 4 to 7. Previous study has revealed that spermatocytes in rats and mice begin to undergo meiosis and to generate haploid round spermatids on postnatal days 25–30, and round spermatids are first apparent on day 25 in seminiferous tubules (17). Results from *in situ* hybridization have also revealed positive signals found in week 4 and 7 mouse testes, but not in week 1 mouse. These results imply that the expression of NYD-SP6 is developmentally regulated, suggesting that NYD-SP6 may play a role in testicular development and/or spermatogenesis.

Program Smart analysis predicts that NYD-SP6 contains two PHD domains. PHD domain, also called leukemia associated protein domain (LAP domain), was first identified in HAT3.1 (histone acetyl-transferase) (18), a protein involved in plant root development. The function of human members of the LAP family sharing a high degree of LAP domain is not known, although they appear to be widely expressed (19), which is in accordance with the expression pattern of NYD-SP6. PHD finger domain has an average length of 51 residues, which is characterized by a Cys_4-His-Cys_3 motif. It folds into an interleaved type of zinc-finger chelating 2 Zn ions in a similar manner to that of Ring and FYVE domain (13). Initially, this zinc finger was implicated in DNA recognition (18). However, this motif often occurs in various regulatory genes, such as members of the trithorax (TRX-G) or polycomb (PC-G) groups (11) and leukemia-associated proteins (LAP) (20), which led to the suggestion that the PHD finger is involved in chromatin-mediated transcription control (11). PHD has been found in more than 300 eukaryotic proteins and most of them are localized in the nucleus and are involved in transcription. The result of subcellular localization of NYD-SP6 fusion protein, pEGFP-C3-NYD-SP6, is in good agreement with its structural analysis. Furthermore, PHD fingers found in regulatory eukaryotic genes (ZMHOX1a/b, 2a/b, HAT3.1, PRHA, PRHP) all activate transcription in yeast cells and mutation of this motif caused severely decreased transcription activation (21). It has been suggested that the PHD finger activates transcription in yeast, plant and animal cells (13). As a result, PHD-containing proteins are believed to function in activating transcription. Data presented here suggests that PHD-containing NYD-SP6 is likely to be involved in transcriptional regulation, possibly for activating gene transcription. A recent examination of the mammalian spermatogenic pathway supports that cell fate is medicated by global changes in chromatin conformation (22). Conservation of this domain from yeast to human implies its conservation of function during evolution and importance in NYD-SP6 function. Therefore, it is quite meaningful for us to investigate function of PHD-containing proteins.

Selected PHD finger-domain proteins belong to LAP protein was grouped (20). All of them have been found one or two even more PHD finger domains, which seem to be organized in different patterns. Function of these proteins was related to transcription (23) and protein-protein interaction (18). As PHD domain was also named LAP (leukemia-associated protein) domain, deduced NYD-SP6 protein might be a novel member of LAP protein family. NYD-SP6 may activate transcription through PHD zinc finger, regulating potential protein likely responsible for cell proliferation including germ cell development. Therefore, investigation of Sertoli cell-localized and PHD domain-containing NYD-SP6 may provide new insight into chromatin-mediated transcription regulation in spermatogenesis.

It has been suggested that zinc finger domain not only function in DNA recognition (24), but also in determining subcellular localization (26). tNYD-SP6

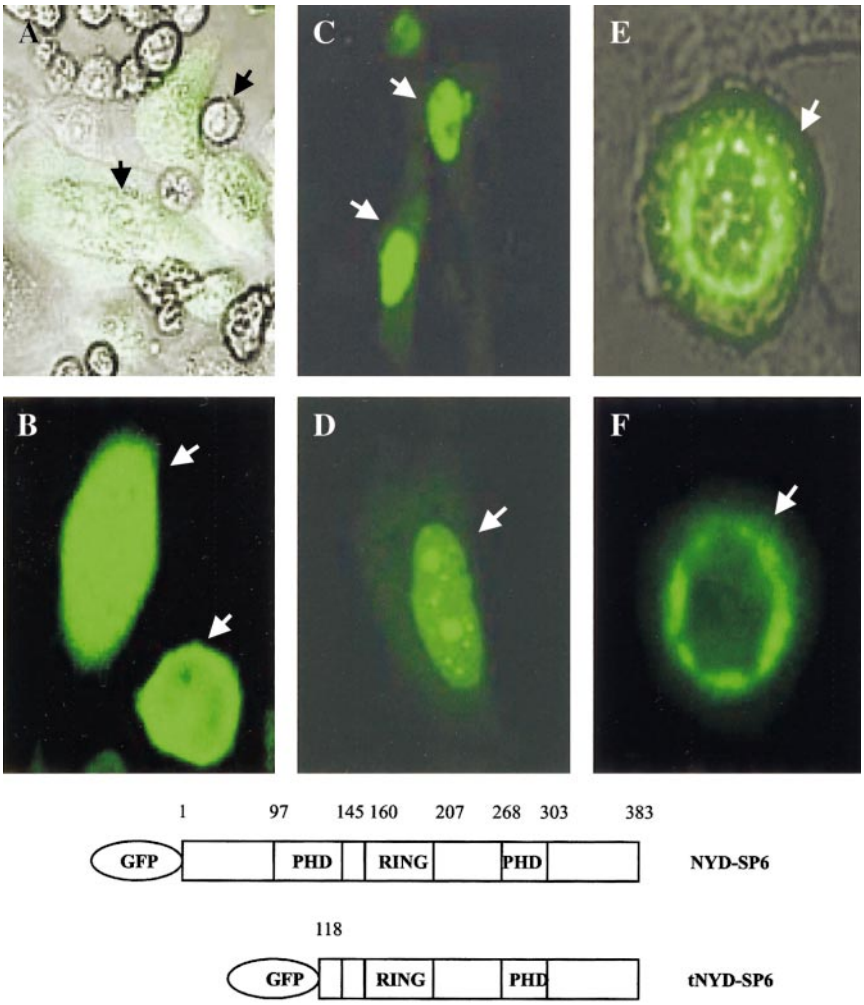

FIG. 7. (Top) Confocal laser microscope detection of pEGFPC3-NYD-SP6 fusion protein transiently expressed in CHO cells. NYD-SP6 fusion protein located in the nucleus is shown. Plasmid: A and B, pEGFP-C3 vector (control); C and D, pEGFP-C3-NYD-SP6 recombination vector; E and F, pEGFP-C3-tNYD-SP6 vector. Image was analyzed under 488 nm fluorescence. Arrows indicate fluorescent cells. Magnifications are 200× for C, 400× for A, B, D, E, and F. A and E were examined under DIC. (Bottom) Schematic representation of NYD-SP6 and N-terminally truncated NYD-SP6 (tNYD-SP6). GFP tag was fused to the N-terminus of two constructs so that they could be detected with confocal laser microscope. PHD finger and ring finger domains were labeled in boxes.

(119–383) localized around the nucleus by comparison with the nuclear localization of NYD-SP6. Taken together, our results suggest that one signal, which lies within N-terminus amino acids 1–118 and interrupts the N terminal PHD zinc finger domain, is necessary to harbor NYD-SP6 to the nucleus, implying the NYD-SP6 nuclear localization may result from a signal-dependent nuclear translocation. Further study is needed to investigate the localization function of truncated two PHD domains and their mutation. It should also be noted that NYD-SP6 contains another amino acid sequence homologous to Ring finger, which also functions in nuclear regulation (11).

Differentiation of male germ cells in adult mammals involves a cross-talk between Sertoli cell, the somatic component which supports and controls germinal differentiation, and germ cells at their successive maturation stages (26). The finding of significant *in situ* hybridization signal in Sertoli cells but not germ cells suggests that NYD-SP6 may exert its effect on spermatogenesis by influencing Sertoli cells function. Evidence indicates that germ cell development is dependent on the production of substances by Sertoli cells (3). A carefully defined Sertoli cell-monocyte co-culture system indicates that adjacent Sertoli cells are crucial to neonatal testicular development in the rat (27). In the meantime, several transcription factors which have been demonstrated to play important roles in testicular development, including Sry gene encoding SRY protein, are produced by Sertoli cells (28). Taken together, Sertoli cell influence germ cells through various ways.

On the other hand, physiological function of NYD-SP6 might be embroiled with PHD finer domain. Point

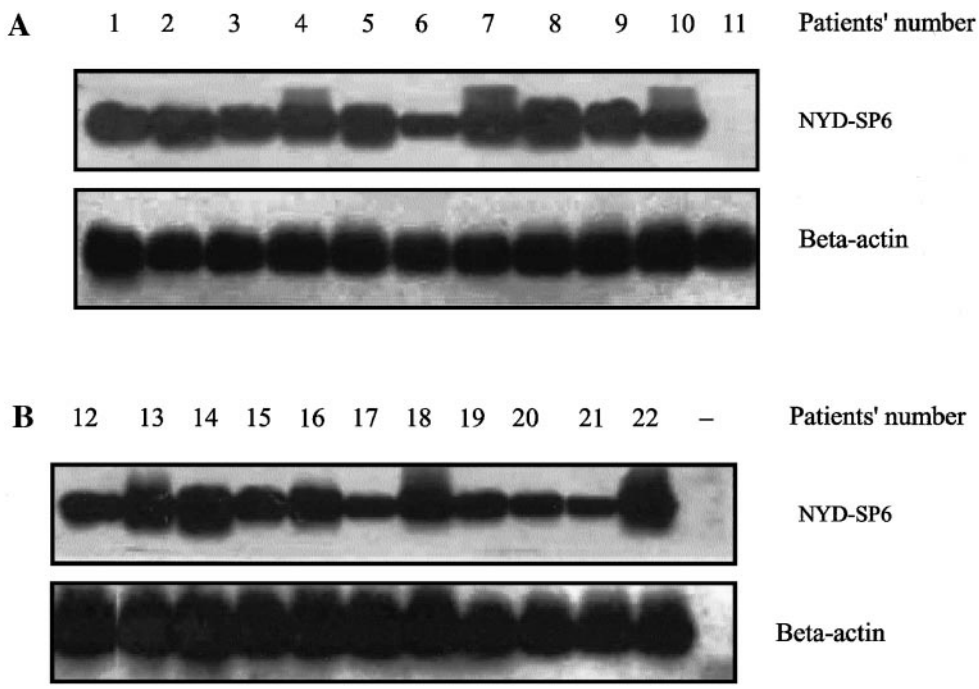

FIG. 8. mRNA expression of NYD-SP6 in 22 patients of spermatogenic arrest. Poly(A)$^+$ RNA from testes of various patients were subjected to Northern blot analysis using the NYD-SP6 cDNA as a probe. Nearly no expression of NYD-SP6 was detected in the 11th patient testes. In contrast, expression was detected in all patients with Sertoli cell-only syndrome (1, 6, 16, 18, 20). The lower panel showed the expression of human β-actin mRNA, used as a control.

mutations in the PHD motifs of human proteins have been reported to be associated with development of diseases (10). The presence of PHD fingers in genes up-regulated in leukemia, associated with autoimmune disease APECED (rare autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) or participating in euchromatin to heterochromatin modulation, like the TPX-G or PC-G genes, indicates that this motif may involved in a variety of pathways (29). The complexity of the action pathways and operators of PHD domains has raised our interest to further detect the regulation of PHD containing proteins involved in spermatogenesis. In testis, this Sertoli cell expressed gene may regulate gene transcription of Sertoli cell, protein of which might work on Sertoli cell itself, germ cell or Leidig cell, resulting in functioning spermatogenesis through extrinsic mechanism. Further investigation of the functional role of NYD-SP6 using such a co-culture system may prove to be advantageous.

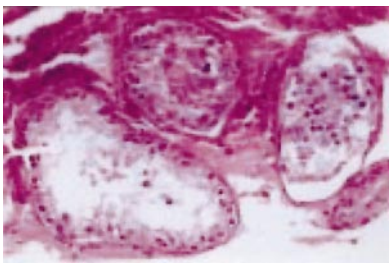

FIG. 9. Testicular biopsy section of the 11th patient. This tissue section showed that no spermatid and sperm in semiferous tube was found.

The present study has provided the first evidence implicating the possible involvement of NYD-SP6 in spermatogenesis. The expression of NYD-SP6 was not detectable in a patient whose spermatogenesis was arrested at the stage of spermatocytes, as diagnosed by testicular biopsy. Although further studies with larger sample size may be required, it is still tempting at this point, considering its structural features, development-dependent expression and cellular localization, to speculate a possible role of NYD-SP6 in the process of spermatogenesis, absence or mutation of which may lead to pathological conditions of the testis or infertility.

In summary, together with the structural characteristics of NYD-SP6 having numerous important functional domains, the present findings have revealed a novel gene, NYD-SP6, which may be involved in regulation of spermatogenesis, testicular development or pathophysiology of the testis. These results warrant further investigation of the new gene and related regulatory mechanisms underlying interaction between germ cells and Sertoli cells, testicular development and spermatogenesis.

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