Development 124, 2167-2177 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV4784

The NIMA-related kinase 2, Nek2, is expressed in specific stages of the meiotic cell cycle and associates with meiotic chromosomes

Kunsoo Rhee[1,3] and Debra J. Wolgemuth[1-4]

Departments of [1]Genetics and Development and [2]Obstetrics and Gynecology, and [3]The Center for Reproductive Sciences and [4]The Columbia Cancer Center, Columbia University College of Physicians and Surgeons, New York, NY 10032, USA

*Author for correspondence at address 1 (e-mail: djw3@columbia.edu)

SUMMARY

The *Aspergillus nimA* gene encodes a Ser/Thr protein kinase which is required for mitosis, in addition to Cdc2, and which has been suggested to have a role in chromosomal condensation. In this study, we isolated a potential murine homologue of *nimA*, *Nek2*, which was shown to be expressed most abundantly in the testis of the adult tissues examined. Its expression in the testis was restricted to the germ cells, with highest levels detected in spermatocytes at pachytene and diplotene stages. Immunohistochemical analysis revealed that Nek2 localized to nuclei, exhibiting a non-uniform distribution within the nucleus. Nek2 appeared to be associated with meiotic chromosomes, an association that was better defined by immunolocalization to hypotonically dispersed meiotic chromosomes. This localization was more apparent in regions of dense chromatin, including the sex vesicle, and was also obvious at some of the chromosome ends. The presence of Nek2 protein was not unique to male germ cells, as it was found in meiotic pachytene stage oocytes as well. Furthermore, in an in vitro experimental setting in which meiotic chromosome condensation was induced with okadaic acid, a concomitant induction of Nek2 kinase activity was observed. The expression of *Nek2* in meiotic prophase is consistent with the hypothesis that in vivo, Nek2 is involved in the G_2/M phase transition of the cell cycle. Our results further provide evidence that in vivo, mouse Nek2 is involved in events of meiosis, including but not limited to chromosomal condensation.

Key words: cell cycle, chromosome condensation, NIMA-related kinases, spermatogenesis, oogenesis

INTRODUCTION

A key regulator of the cell cycle in mitotic cells is the Ser/Thr protein kinase Cdc2, which functions, along with its regulatory partner cyclin B, in the G_2/M progression. However, it has recently been shown that induction of Cdc2 activity alone may not be sufficient to drive cells into and through mitosis in certain organisms, such as the yeasts and *Aspergillus nidulans* (reviewed by Gallant et al., 1995). A gene that appears to be critical to this progression in *Aspergillus nidulans* has been identified as *nimA* (Osmani et al., 1988, 1991). NIMA is a Ser/Thr protein kinase, but is distinct from Cdc2, structurally as well as biochemically (reviewed by Fry and Nigg, 1995). While the activity of NIMA is regulated by its state of phosphorylation, as is Cdc2, much less is known about the kinases responsible for this activation. Recent studies have actually implicated the Cdc2 kinase in NIMA activation (Ye et al., 1995). NIMA and Cdk kinases also have different consensus sequences as substrates for phosphorylation, with histone H1 serving as a good in vitro substrate for most Cdks whereas NIMA prefers β-casein (Lu et al., 1993, 1994).

A role for a NIMA-like activity in vertebrate cells has been documented. Ectopically expressed NIMA has been shown to induce germinal vesicle breakdown in *Xenopus* oocytes (Lu and Hunter, 1995) and premature mitotic events in HeLa cells (Lu and Hunter, 1995; O'Connell et al., 1994), independent of Cdc2 activity. Dominant negative mutant versions of the NIMA protein, in contrast, arrest HeLa cells in G_2 phase (Lu and Hunter, 1995). Three putative human homologues of the *Aspergillus nimA* gene, designated *NEK1*, *NEK2*, and *NEK3*, have been identified by virtue of structural similarity (Schultz and Nigg, 1993; Schultz et al., 1994; Levedakou et al., 1994). Although these *Nek* genes have not been extensively studied, it is known that they exhibit in vitro kinase activity with peptide substrate specificity similar to that of NIMA (Fry et al., 1995). One of the putative mammalian *nimA* homologues, human *NEK2*, exhibits cell cycle-specific expression (Schultz et al., 1994; Fry et al., 1995) and another, murine *Nek1*, is highly expressed in germ cells at particular stages of their differentiation (Letwin et al., 1992). However, the *Nek* genes have not successfully rescued *nimA* deficiency in *Aspergillus* nor has the misexpression of *Nek* genes in cultured cells resulted in a phenotype (reviewed by Fry and Nigg, 1995). Thus, the function of the vertebrate *nimA* homologues remains a mystery, despite the clear critical function of this gene in various organisms.

Our laboratory has been interested in understanding the control of the mitotic and meiotic cell cycles of the germ line cells. Our observations on cell cycle gene expression in both male and female germ cells in vivo in the genetically and

experimentally amenable mouse system have provided important new insights into our understanding of the control of the cell cycle in higher eukaryotic cells in general and in the control of meiosis in particular (reviewed by Wolgemuth et al., 1995). For example, we recently discovered that there are two distinct *cyclin A* genes in the mammalian genome, one of which is expressed only in the germ line cells (Ravnik and Wolgemuth, 1996; Sweeney et al., 1996). We have also detected patterns of expression of *Cdk*s at the level of both RNA and protein that correlate with differentiation rather than proliferation (Rhee and Wolgemuth, 1995). One of the Cdks, Cdk5, actually appears to be involved with apoptotic cell death rather than cell division, at least in the developmental paradigms examined (Zhang et al., 1997). These observations suggest that control of the cell cycle and cellular differentiation is complex in higher organisms and underscore the importance of studying the function of putative cell cycle regulating genes in vivo.

Recently, we have extended our studies of cell cycle regulating genes in the germ line to include the *nimA* homologues. In this paper, we describe the isolation and characterization of the murine *Nek2* gene, showing that it is expressed most abundantly in male germ cells. We further present data which suggest that in vivo, Nek2 is involved in the organization and/or condensation of meiotic prophase chromosomes during male and female germ cell development.

MATERIALS AND METHODS

Sources of tissues and probes

Normal tissues were obtained from Swiss Webster mice (Charles River, Wilmington, DE). Fetal ovaries were obtained from d16.5 embryos (day of vaginal plug = d0.5). Neonatal testes were obtained from mice at postnatal days 7, 17 and 19 and adult tissues from mice at least 35 days old. The mouse mutant strains *atrichosis* (*at;* ATEB/Le *a/a d/d* + *at/eb* +) and *white spotting* (*W/W^v*; WBB6F$_1$/J-*W/W^v*) were obtained from The Jackson Laboratory (Bar Harbor, ME). Dissected tissue specimens were frozen in liquid nitrogen prior to RNA isolation. Dissected tissues for in situ hybridization and immunohistochemistry analyses were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. Testicular cells for immunoblot analysis were separated on a 2-4% BSA gradient at unit gravity as described by Wolgemuth et al. (1985).

The cDNA probes for *Cdc2*, *Cdk2*, *Cdk4*, *Pctaire-1*, and *Pctaire-3* have been described previously (Rhee and Wolgemuth, 1995). The murine *Cdk5* cDNA was obtained from Dr L.-H. Tsai (Tsai et al., 1994). All radioactive nucleotides used in this study were obtained from New England Nuclear (Wilmington, DE). DNA probes for the cDNA library screening were labeled with [^{32}P]dCTP using the multiprime DNA labeling kit (Amersham, Arlington Heights, IL). Riboprobes were prepared using T7 or T3 RNA polymerases in the presence of [^{32}P]UTP for northern blot hybridization analysis and [^{35}S]UTP for in situ hybridization analysis. For the northern blot hybridization analysis of *Nek2* expression, the full length *Nek2* cDNA clone was used as a template for the probe. For the in situ hybridization analysis, the 1.3 kb *Eco*RI fragment, which contains most of the *Nek2* coding sequences, was used to generate both sense and antisense riboprobes.

Isolation and sequence analysis of murine Nek2

A cDNA library was constructed with RNA from testis of adult mice, using the Uni-Zap XR cDNA synthesis kit (Stratagene, La Jolla, CA). The cDNA library was screened with reduced stringency following the protocols outlined in Sambrook et al. (1989). As probes, we used a mixture of mouse *Cdc2*, *Cdk2*, *Cdk4*, *Cdk5*, *Pctaire-1*, and *Pctaire-3* cDNAs. Filters were washed at a final stringency of 2× SSC, 0.1% SDS at room temperature for 2 hours. Positive clones were plaque purified twice and inserts from the tertiary screen were in vivo-excised following Stratagene's protocol. The phagemids contained the cDNA at the *Eco*RI and *Xho*I site of pBluescript SK–. Clones isolated from this screen were sequenced using an Applied Biosystems Model 373A DNA sequencer (Applied Biosystems, Foster City, CA). Sequences were analyzed using the GCG sequence analysis program for the VAX (Devereux et al., 1984).

Northern blot hybridization analysis

RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) as described previously (Rhee and Wolgemuth, 1995). Total RNA was electrophoresed in a denaturing 0.85% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose membrane, and hybridized with a *Nek2* riboprobe. Ethidium bromide staining of the 18S and 28S RNAs was used to determine equal loading for each sample.

In situ hybridization analysis

Paraffin embedded tissues were cut into 5 μm sections and analyzed by in situ hybridization as described previously (Rhee and Wolgemuth, 1995). ^{35}S-labeled riboprobes of *Nek2* were synthesized in the antisense orientation using T3 RNA polymerase or sense (control) orientation using T7 RNA polymerase. Slides were exposed to emulsion (Kodak type NTB-2) for 10 days, developed, stained with hematoxylin and eosin, mounted and viewed on a Leitz photomicroscope under bright-field and epiluminescence optics.

Antibodies

For generating an antibody specific to mouse Nek2, a nearly full length coding sequence (aa 4-442) of *Nek2* was cloned into the bacterial expression vector pET21 which contains a histidine tag. The fusion protein was purified by immobilized metal affinity chromatography (Novagen, Madison, WI), and injected into a rabbit (Pocono Farm & Laboratory, Canadensis, PA). The antisera were affinity-purified by incubation with a strip of nitrocellulose membrane blotted with the Nek2 fusion protein and eluting with 100 mM glycine, pH 3.0. Both anti-Cdc2 antibody and p13^{suc1} conjugated-agarose were obtained from UBI (Lake Placid, NY).

Immunoblot analysis

Samples from total testis or testicular cells were solubilized in 1× Laemmli sample buffer (Laemmli, 1970), boiled for 5 minutes, and kept frozen until use. The protein concentrations of the tissue lysates were determined with the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA). Protein samples were resolved by 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membrane was blocked by soaking in Blotto (1× TBS, 0.05% Tween 20, 5% non-fat dried milk) for 1 hour, incubated with the primary antibody diluted with Blotto for 3 hours, washed three times with Blotto, incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG; Boehringer-Mannheim, Indianapolis, IN) for 1 hour, and washed with TBST (1× TBS, 0.05% Tween 20) three times. Affinity purified anti-Nek2 antibody was diluted to 1:100, anti-Cdc2 antibody was diluted to 0.5 μg/ml, and the secondary antibody was diluted to 1:5,000. The ECL western blotting detection reagents (Amersham, Arlington Heights, IL) were used according to the manufacturer's recommendations.

Immunohistochemical localization

Immunohistochemical localization was carried out with sections of paraffin embedded tissue samples or air-dried cytogenetic preparations treated with a hypotonic solution. After deparaffinization, slides were boiled in 0.01 M citrate buffer, pH 6.0, in a microwave (Shi et

al., 1991) for 10 minutes and washed extensively with H_2O. The slides were treated with 0.03% H_2O_2 in methanol for 20 minutes, washed with PBST (1× PBS, 0.1% Triton X-100), and blocked for over 1 hour with 2.5% goat serum in PBST. The slides were incubated with the primary antibodies in a humidified chamber overnight at 4°C, washed three times with PBST and stained with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). For testis sections, DAB-stained slides were counterstained with hematoxylin. Cytogenetic preparations were not counter-stained. Alternatively, an indirect immunofluorescent detection method was applied using FITC-conjugated goat anti-rabbit IgG antibody (Boehringer-Mannheim, Indianapolis, IN) as a secondary antibody. Chromosomes were counterstained with propidium iodide (0.3 μg/ml). Affinity purified anti-Nek2 antibody was diluted 1:20, anti-Cdc2 antibody was diluted to 0.2 μg/ml, preimmune serum or anti-Nek2 antisera was diluted 1:100, and the FITC-conjugated secondary antibody was diluted 1:100.

Cytogenetic methods

Slides for cytogenetic analysis were prepared by modifying the procedures described by Meredith (1969). Testes or fetal ovaries were dissected, teased apart into small pieces in 10 ml of 0.5% KCl solution and left for 1 hour at room temperature. About 10 drops of a fixative (1 part acetic acid and 3 parts methanol) were added to the solution. A few pieces of seminiferous tubules or fetal ovaries were smeared on a clean slide and air-dried. For cytogenetic analysis of cultured testicular cells, 0.5 ml of 0.5% KCl solution was used to disperse cell pellets. Twenty-five minutes later, the hypotonically treated cells were fixed with a few drops of the fixative, pelleted at 800 ***g***, suspended with 0.5 ml fixative, left for 1 hour at room temperature, pelleted at 800 ***g***, and resuspended with 50 μl of the fixative. Two or three drops of the cell suspension were placed on a clean slide. Meiotic substages were determined by staining slides with Giemsa in a phosphate buffer, pH 7.0. Stages in the progression of cells from the pachytene stage through metaphase of meiosis I were scored by light microscopy at 400× using classical cytogenetic criteria.

Telomere in situ hybridization

Detection of telomeric sequences in meiotic chromosomes was performed following directions recommended by Oncor (Gaithersburg, MD). In brief, chromosomal DNA was denatured by incubating the slides in 60% formamide/2× SSC at 72°C for 2 minutes and quickly transferring them into ice-cold ethanol. Digoxigenin-labeled telomere probe (Oncor) was applied to the slides and incubated overnight at 37°C. After hybridization, the slides were washed with 50% formamide/2× SSC at 43°C for 15 minutes followed by 2× SSC at 37°C for 8 minutes. FITC-conjugated anti-digoxigenin antibody was used to detect the signal. Chromosomes were counterstained with propidium iodide (0.3 μg/ml). Photomicrographs were taken using a Zeiss LSM 410 confocal microscope with 1,000-fold magnification. The same slides were used for immunostaining with the Nek2 antibody.

Short term culture of testicular cells

The short term culture of testicular cells was carried out using modifications of the procedures of Wiltshire et al. (1995). Approximately 12 testes from 19-day-old mice were decapsulated and placed into 10 ml of DMEM medium containing 0.5 mg/ml collagenase. The cells were dissociated by repeated pipetting for 5 minutes, passed through an 85 μm nylon mesh, and washed twice with DMEM medium containing 5% fetal calf serum. The concentration of the cells was adjusted to 2×10^6 cells/ml. The testicular cells were incubated overnight in DMEM medium with 5% fetal calf serum at 32°C. The following morning, the cells were treated with okadaic acid (ICN, Costa Mesa, CA) at a final concentration of 2.5 μM. Cells were harvested at 0, 2, 4, and 6 hour intervals. At each time point, an aliquot (~10%) of the cells was used for assessing chromosome condensation and the rest of the cells were used to prepare cell lysates. A portion of the lysate was used for immunoblot analysis and the remaining portion to assay kinase activity.

In vitro Nek2 kinase assay

Immunoprecipitation followed by kinase assay for Nek2 was carried out following procedures described by Fry et al. (1995). Testicular cells from the short term culture were suspended in lysis buffer containing 50 mM Hepes, pH 7.5, 5 mM $MnCl_2$, 10 mM $MgCl_2$, 5 mM EGTA, 2 mM EDTA, 100 mM NaCl, 5 mM KCl, 0.1% Nonidet P-40, 3 U/ml of DNase I (Boehringer-Mannheim, Indianapolis, IN), 30 μg/ml RNase A, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of leupeptin and pepstatin A, 0.1 μg/ml aprotinin, 1 μM okadaic acid, and 1 μg/ml heparin. After 30 minutes on ice, cells were lysed with a Dounce homogenizer and the lysates were spun down at 15,000×*g* for 10 minutes. The protein concentration of the supernatant was determined and used for immunoblot analysis and for immunoprecipitation. For immunoprecipitation of Nek2, aliquots of lysate containing 100 μg protein were incubated with affinity-purified Nek2 antibody (1:20 dilution), anti-Nek2 antisera (1:200) or preimmune sera (1:200) in the lysis buffer for 2 hours at 4°C, followed by incubation with 3 μg of protein A-sepharose for 1 hour. Immune complexes were collected by centrifugation and washed four times with the lysis buffer and once with the Nek2 kinase buffer, which consists of 50 mM Hepes, pH 7.5, 5 mM $MnCl_2$, 5 mM NaF, 5 mM β-glycerophosphate, 1 μM okadaic acid, and 1 μg/ml heparin.

The assay of in vitro Nek2 kinase activity was carried out for 20 minutes at 30°C in the Nek2 kinase buffer supplemented with 4 μM ATP, 1 mM dithiothreitol, 0.5 mg/ml dephosphorylated casein (Sigma, C4032), and 10 μCi of [γ-^{32}P]ATP in a total volume of 30 μl. The reactions were stopped by the addition of 30 μl of 2× Laemmli sample buffer (Laemmli, 1970) and boiled for 2 minutes. Reaction products were visualized by SDS-PAGE and autoradiography. The relative amounts of the kinase activities were quantitated by densitometric analysis of the autoradiographs and counting the radioactivity in bands of substrate excised from the gels.

MPF activity assay

The procedures for preparation of testicular cell lysates for MPF precipitation were identical to those for Nek2 immunoprecipitation. MPF complexes were precipitated from 50 μg of cellular lysate protein by incubation with 10 μg of p13^{suc1} agarose for 2 hours at 4°C. The agarose beads were washed three times with the lysis buffer and once with the MPF kinase buffer, which consists of 80 mM β-glycerophosphate, 10 mM $MgCl_2$, 1 mM DTT, and 20 mM EGTA. MPF activity assay was carried out as described previously (Chapman and Wolgemuth, 1994). Histone H1 kinase reactions were performed in the MPF kinase buffer supplemented with 10 μM ATP, 5 μM cAMP-dependent protein kinase inhibitor, 50 μg/ml calf thymus histone H1 (Boehringer-Mannheim, Indianapolis, IN), and 4 μCi of [γ-^{32}P]ATP in 30 μl for 20 minutes at 30°C. The reactions were stopped by the addition of 30 μl of 2× Laemmli sample buffer (Laemmli, 1970) and boiled for 2 minutes. Reaction products were visualized by SDS-PAGE and autoradiography.

RESULTS

Cloning of murine *Nek2* cDNA

We have isolated a cDNA corresponding to the murine *Nek2* gene in a search for novel Ser/Thr protein kinases expressed during male germ cell development. A mouse testis library was screened with reduced stringency using several *Cdk* cDNA probes. Sequence analysis of a clone of 3.1 kb, encoding 443 a.a. (Fig. 1), was shown to have strong structural homology with NIMA-related kinases (Schultz et al., 1994). We designated this clone as the mouse *Nek2*, based on its highest

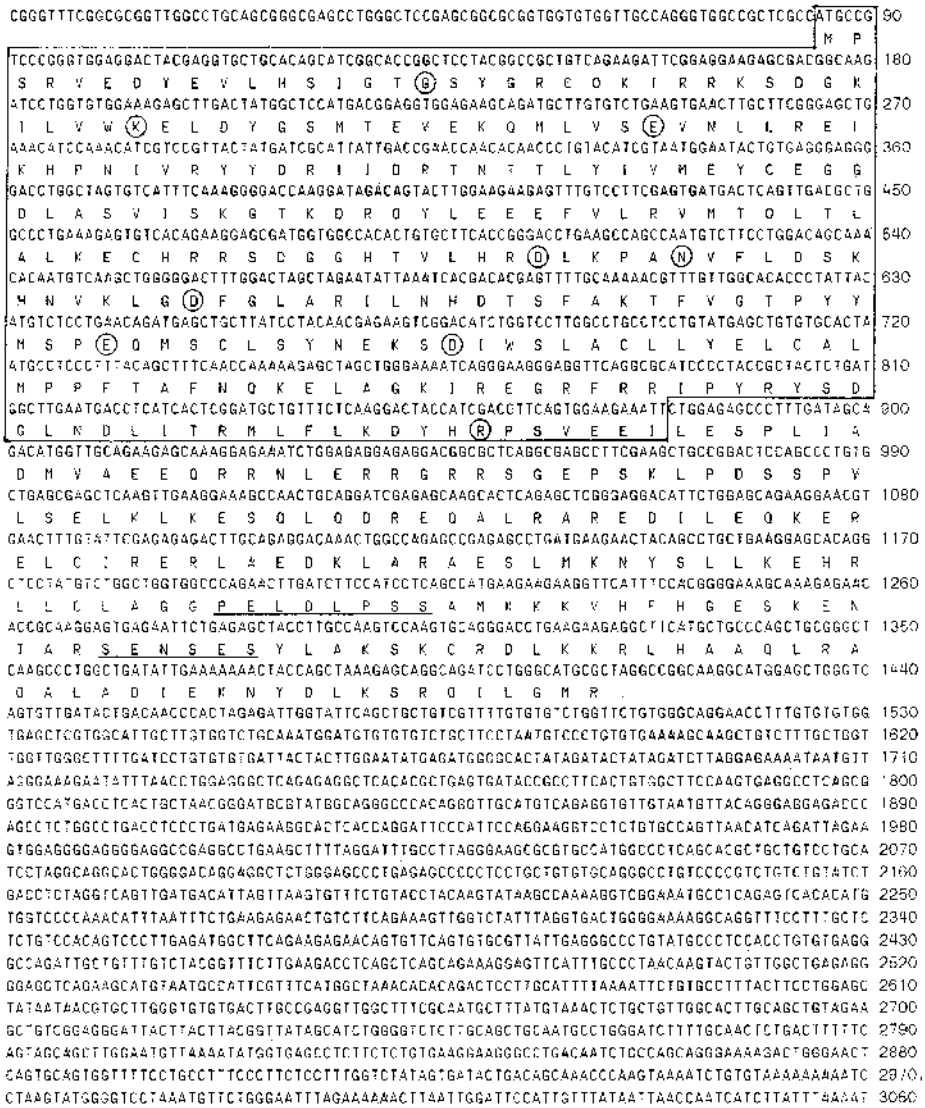

Fig. 1. Nucleotide and deduced amino acid sequences of murine *Nek2* cDNA. The boxed region outlines the N-terminal catalytic domain. Amino acid residues that are highly conserved among Ser/Thr kinases are circled. Two putative PEST sequences are underlined (aa 368-377, 396-401). The sequences have been deposited into the GenBank database under accession number U95610.

identity at the amino acid level with human *NEK2* (86%; Schultz et al. 1994). Mouse *Nek2* is 32% identical to *Aspergillus nimA* (Osmani et al., 1988), 28% to mouse *Nek1* (Letwin et al., 1992), and 24% to human *NEK3* (Schultz et al., 1994).

The NIMA and Nek2 polypeptides can be divided into two domains, the catalytic domain at the N terminus and the regulatory domain at the C terminus (Schultz et al., 1994). The catalytic domain includes amino acid residues conserved among Ser/Thr protein kinases (marked as circles in Fig. 1; Hanks and Hunter, 1995). The regulatory domain of NIMA was reported to include sequences for destruction, nuclear localization, protein-protein interaction, and phosphorylation sites for Cdc2 (Lu and Means, 1994; O'Connell et al., 1995; reviewed by Fry and Nigg, 1995). In mouse Nek2, two putative PEST sequences (Rechsteiner and Rogers, 1996) were found at aa 368-377 and 396-401, as underlined in Fig. 1. We believe that these PEST motifs are functional in mouse Nek2, as preliminary experiments have indicated that deletion of these sequences results in an approx. 10-fold increase of the truncated protein as compared with the full length Nek2 in transfected tissue culture cells (data not shown).

Expression of mouse *Nek2*

Northern blot hybridization analysis was carried out to determine the pattern of expression of the murine *Nek2* gene in various tissues. Low levels of *Nek2* transcripts of 3.4 kb were detected in samples of mid-gestation embryo, ovary and placenta. By far the highest levels of *Nek2* expression were detected in the testis (Fig. 2A). Lineage- and developmental stage-specificity of *Nek2* expression in the testis was determined by examining RNA samples from germ cell-deficient and immature testes (Fig. 2B). Mice homozygous at the *atrichosis* (*at*) or *white-spotting* (*W*) loci are devoid of germ cells, but have the normal complement of somatic cell types, including Leydig, Sertoli, and peritubular myoid cells (Mintz and Russell, 1957; Hummel, 1964). The heterozygous littermates have the normal somatic and germ cell complements and are fertile. Little, if any,

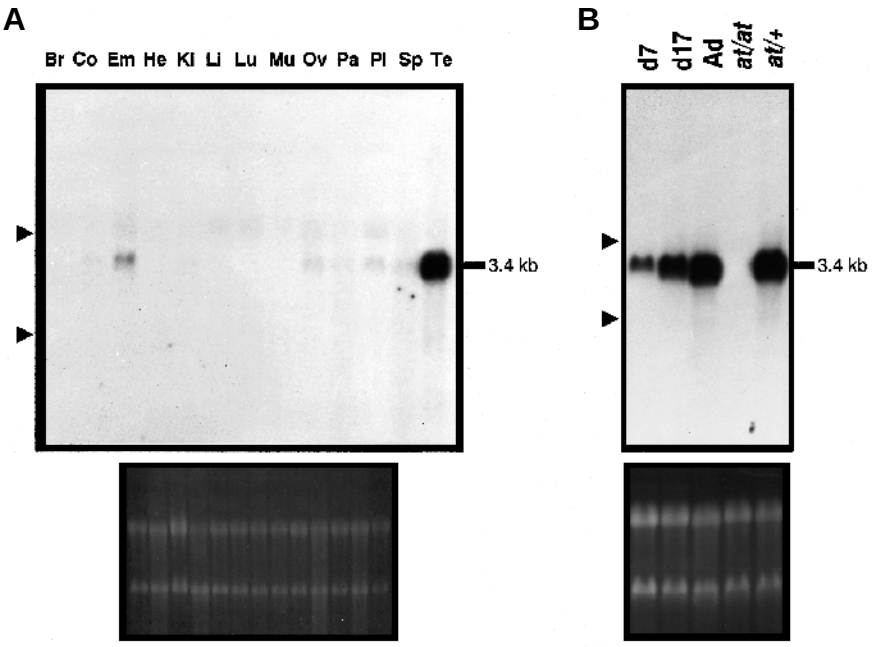

Fig. 2. Northern blot analyses of *Nek2* expression. Total RNA (10 μg) was isolated from various mouse tissues (A) or from testes (B) of immature (d7, d17), germ cell-deficient (*at/at*), or adult (Ad) mice. The size of the specific *Nek2* transcript is indicated on the right side of the figures. Arrowheads mark position of the 28S and 18S ribosomal RNA bands. The lower figures show the ethidium bromide-stained ribosomal RNA bands. Br, brain; Co, colon; Em, 12.5-day embryo; He, heart; Ki, kidney; Li, liver; Lu, lung; Mu, muscle; Ov, ovary; Pa, pancreas; Pl, placenta; Sp, spleen; Te, testis. Exposure for 24 hours.

Nek2 transcripts were detected in testes from *atrichosis* homozygotes (*at/at*) whereas comparable amounts of *Nek2* transcripts were detected in testes both from the heterozygotes (*at/+*) and wild-type adult mice (Ad). This result indicated that *Nek2* is expressed mostly in the germ cell compartment in the testis.

The first wave of male germ cell development initiates after birth and yields fully developed spermatozoa in 35 days (Bellve et al., 1977). At postnatal day 7, the mouse testis contains only spermatogonia, and at day 17 contains both spermatogonia and spermatocytes. As shown in Fig. 2B, *Nek2* transcripts were more abundant in adult testis than in immature testes, suggesting higher levels of *Nek2* expression in meiotic and/or postmeiotic as opposed to mitotic stages of germ cell development.

In situ hybridization analysis was performed on histological sections of testes from adult (Fig. 3A,B), immature (Fig. 3C,D), and germ cell-deficient mutant (W/W^v; Fig. 3E) mice to determine more precisely the testicular cell types in which *Nek2* is expressed. Low levels of *Nek2* signals were detected in spermatogonia, as shown in immature testes (Fig. 3C,D). However, *Nek2* transcripts were clearly most abundant in spermatocytes (Fig. 3A,B,D). *Nek2* transcripts were barely

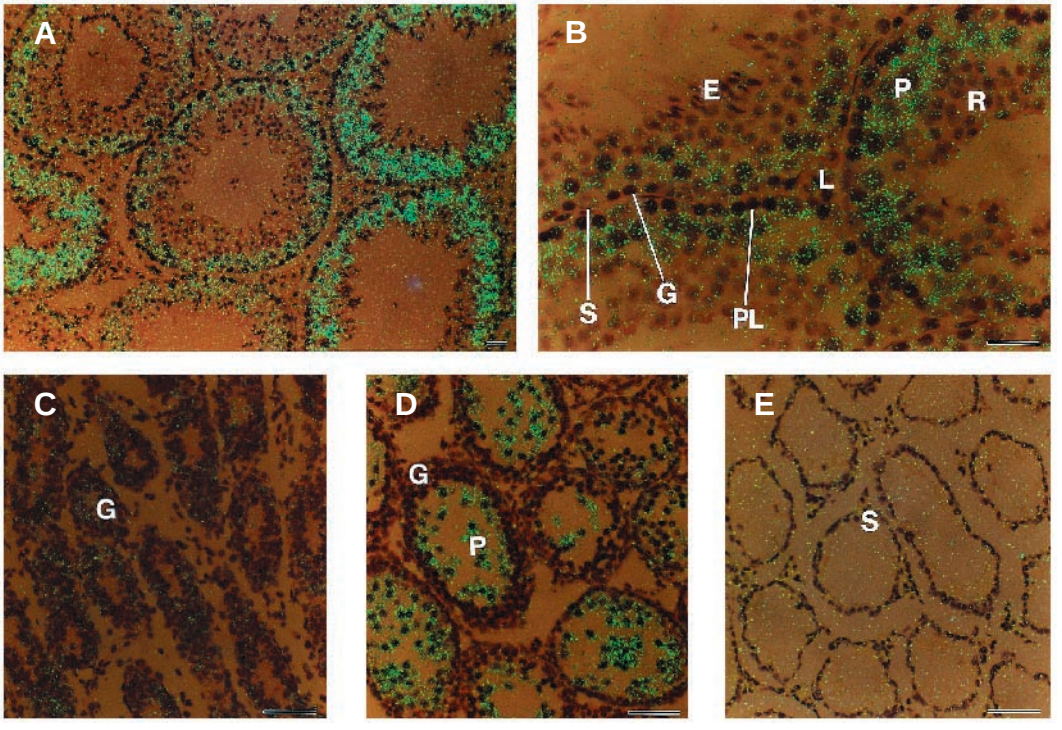

Fig. 3. In situ hybridization analyses of *Nek2* expression in testis. An ^{35}S-labeled antisense probe specific to *Nek2* was hybridized with sections of paraffin-embedded testis samples from adult (A,B), 7-day-old (C), 17-day-old (D), and germ cell-deficient (W/W^v) (E) mice. Exposure time was 10 days. The photomicrographs in this figure were taken using epiluminescence optics with a low bright-field background, so that signals were visualized with a bright green color. G, spermatogonia; PL, preleptotene spermatocytes; P, pachytene spermatocytes; R, round spermatids; E, elongating spermatids; S, Sertoli cells; L, Leydig cells. Bar = 50 μm.

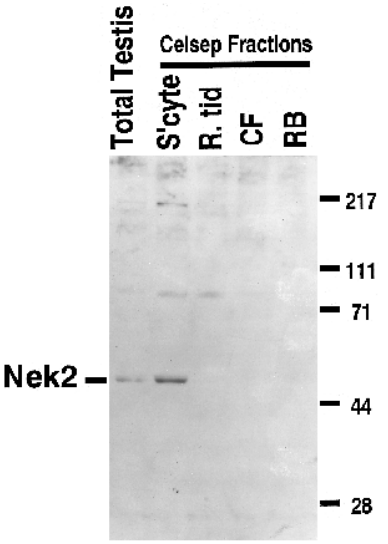

Fig. 4. Immunoblot analysis of Nek2 in male germ cells. Lysates were prepared from total testis or from testicular cell fractions enriched in spermatocytes (S'cyte), round spermatids (R. tid), cytoplasmic fragments (CF), and residual bodies (RB). Each lane contained 50 μg protein. The blot was incubated with an affinity-purified rabbit polyclonal antibody against a Nek2 fusion protein. The Nek2 specific band and the sizes of the protein markers ($M_r \times 10^{-3}$) are indicated on the right and left sides of the figure, respectively.

detected in spermatids after meiosis (Fig. 3A,B) or in somatic testicular cells (Fig. 3E). Probes synthesized in the sense orientation were used as controls and produced no signal above background (data not shown).

Immunodetection of Nek2 protein in the testis

A polyclonal antibody was raised against a bacterially expressed Nek2 fusion protein and affinity-purified. Immunoblot analysis was performed with lysates from adult testis and from enriched populations of spermatocytes and spermatids, separated according to our standard procedures (Wolgemuth et al., 1985). A specific band of 46×10^3 M_r in size was detected in the total testis (Fig. 4). This size is similar to that reported for human NEK2 (Schultz et al., 1994). This 46×10^3 M_r band was readily detected in the sample from enriched spermatocytes but not in samples from round spermatids and elongating spermatids (CF and RB). This result is consistent with the RNA expression data which indicated that *Nek2* was expressed predominantly in spermatocytes and

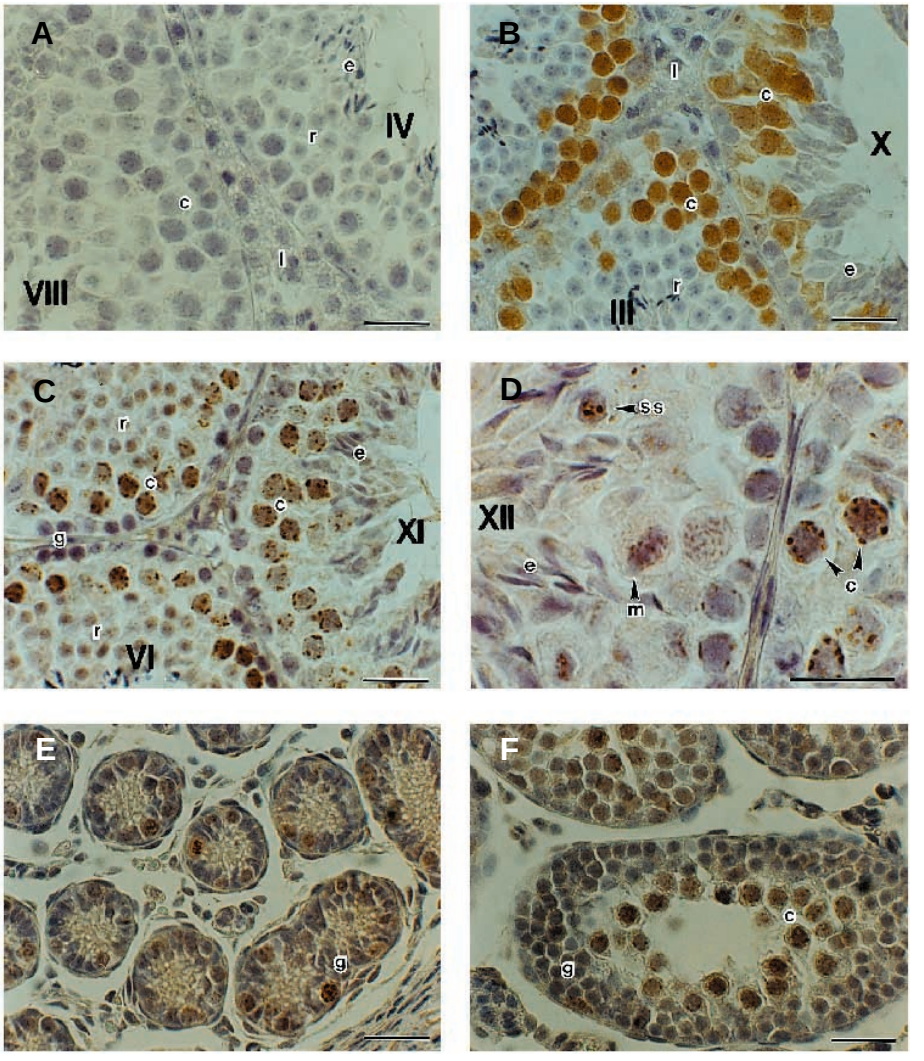

Fig. 5. Immunostaining of Cdc2 and Nek2 in the testis. Paraffin-embedded sections from the adult testis were immunostained with preimmune serum (A), a Cdc2 antibody (B), and Nek2 antibody (C,D). D was photographed using higher magnification. Testis sections from 7-day-old (E) and 17-day-old (F) immature mice were also immunostained with the Nek2 antibody. Roman numerals indicate the stage of the seminiferous tubule (Russell et al., 1990). g, spermatogonia; c, spermatocytes; m, spermatocyte undergoing meiosis; ss, secondary spermatocytes; r, round spermatids; e, elongating spermatids; l, Leydig cells. Bar, 50 μm.

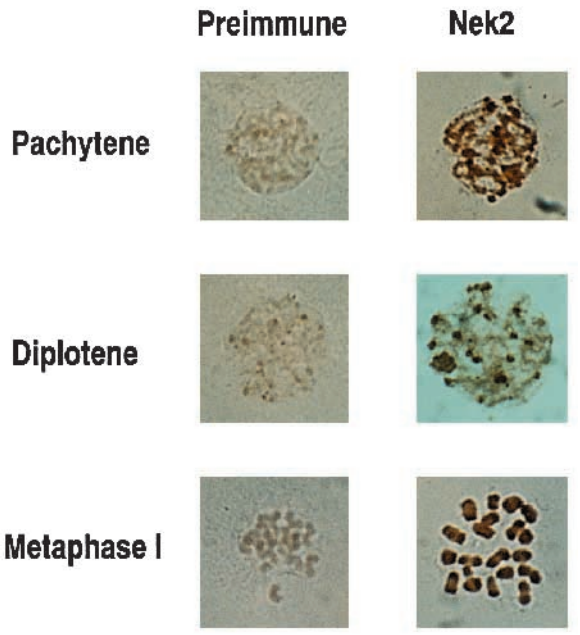

Fig. 6. Immunostaining of Nek2 in cytogentic preparations of spermatocytes. Mouse testicular cells were subjected to hypotonic treatment, spread for cytogenetic analysis, and immunostained with preimmune serum or anti-Nek2 anti-serum, as indicated.

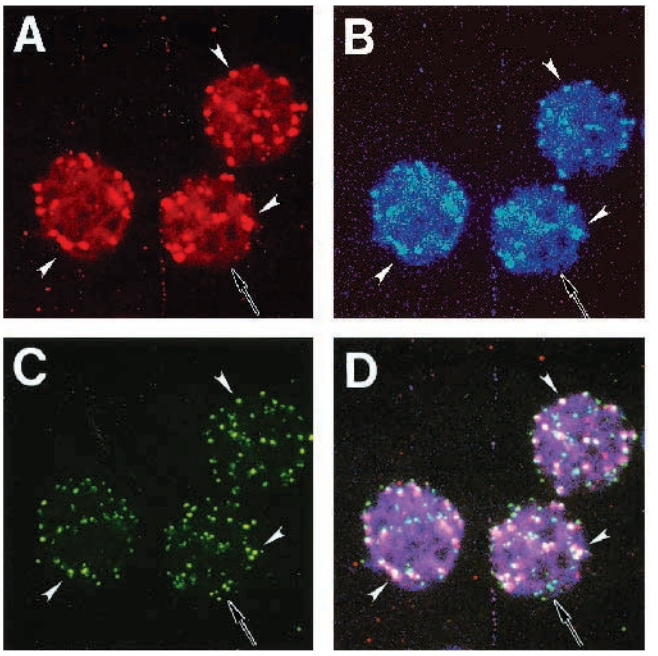

Fig. 7. Confocal microscopic analysis of localization of Nek2, telomere sequences, and chromatin in cytogenetic preparation of spermatocytes. Chromatin was stained with propidium iodide (red, A). Nek2 immunostaining (blue, B) and telomere in situ analysis (green, C) were carried out as described in Materials and Methods. The three images were superimposed in D. Since Nek2 was colocalized with chromatin, the staining in D became purple. The white arrowheads indicate representative positions where telomere sequences are colocalized with strong staining of Nek2 and chromatin. However, not all telomere signals colocalize with strong Nek2 staining (black arrow).

further suggests that obvious translational regulation is not key in *Nek2* expression.

To determine the subcellular distribution of Nek2 within the spermatocytes, paraffin-embedded sections of adult and immature testes were immunostained with the Nek2 antibody. The localization of Cdc2 within spermatocytes was determined in parallel, as a control and for comparison, since we have shown that Cdc2 is present in these cells (Chapman and Wolgemuth, 1994; S. E. Ravnik and D. J. Wolgemuth, unpublished observations). Both Nek2 and Cdc2 antibodies specifically stained spermatocytes of all tubules in the adult testis (Fig. 5B,C). The Nek2 antibody also stained spermatocytes in the 17-day-old testis (Fig. 5F). There was little staining in haploid germ cells (Fig. 5C,D), except the nucleolus of early round spermatids in stage I to III seminiferous tubules (staged according to Oakberg, 1956; Russell et al., 1990; data not shown). These staining patterns of Cdc2 and Nek2 are consistent with previous results at both the RNA and protein levels (Chapman and Wolgemuth, 1994; Rhee and Wolgemuth, 1995; Figs 2-4). However, the intracellular distributions of Cdc2 and Nek2 were quite distinct. Cdc2 antibody uniformly stained nuclei of pachytene spermatocytes and both nuclei and cytoplasm of late pachytene/diplotene spermatocytes which are present in stage X to XII seminiferous tubules (Fig. 5B). In contrast, the Nek2 antibody stained nuclei of spermatocytes, beginning at the zygotene stages, with a uniquely punctate pattern (Fig. 5C,D,F). Such a punctate staining pattern was observed in spermatocytes undergoing meiosis as well as in secondary spermatocytes (Fig. 5D). The Nek2 antibody also stained nuclei of a subset of what appear to be spermatogonia in the 7-day-old testis with a punctate pattern (Fig. 5E). This non-uniform distribution of Nek2 suggested its association with distinct nuclear compartments, possibly with chromosomes, in specific developmental stages.

An association of Nek2 with chromosomes was confirmed by localization of Nek2 in spermatocytes treated with a hypotonic solution to disrupt the cytoplasmic and nuclear membranes and disperse the chromosomes. Under these conditions, the Nek2 antibody clearly localized to the meiotic chromosomes, but with a non-uniform distribution. More intense staining was observed at the ends of the some of the chromosomes, several of which were positioned together at the periphery of the nucleus, as well as in regions of dense chromatin (Fig. 6).

To determine whether the Nek2 antibody localized at both ends of the chromosomes, i.e. in the region of telomeres, we carried out in situ hybridization with telomere-specific sequences (Allshire et al., 1988; Moyzis et al., 1988). Hypotonically dispersed nuclei were hybridized with telomere sequences, then exposed to the Nek2 antibody, and finally stained with propidium iodide to visualize DNA. The chromatin staining pattern visualized with propidium iodide revealed a significant overlap with that observed for the Nek2 protein (Fig. 7A,B). The localization of Nek2 protein coincided with some but not all of the regions of telomere sequences (Fig. 7B-D). These results indicate that abundance of Nek2 correlates with chromatin density which is generally higher at the ends of chromosomes, but not with telomeres per se.

To extend the possible role of Nek2 in meiotic chromosome function, we asked whether Nek2 was also associated with meiotic chromosomes in oocytes. To examine comparable

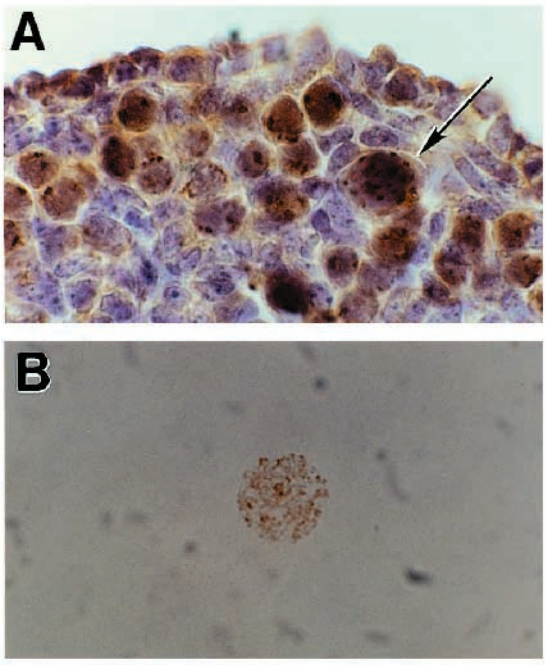

Fig. 8. Immunostaining of Nek2 in pachytene oocytes. (A) Fetal ovary from day 16.5 embryo was immunostained with the Nek2 antibody . An arrow indicates a representative pachytene oocyte. (B) The fetal ovary cells were subjected to hypotonic treatment, spread for cytogenetic analysis, and immunostained with the Nek2 antibody.

stages of meiosis in the female, we performed immunohistochemistry analysis on ovaries obtained from 16.5-day-old embryos. Nek2 antibody localized in nuclei of pachytene oocytes in a punctate pattern, similar to that observed in spermatocytes (Fig. 8A). Hypotonically dispersed meiotic chromosomes from pachytene oocytes were also stained specifically with the Nek2 antibody (Fig. 8B). These results indicated that during meiosis, Nek2 is associated with prophase chromosomes of both male and female germ cells. To date, we have not detected Nek2 protein in histological sections of oocytes from adult mice (data not shown), suggesting that Nek2 may not be involved in the chromosomal condensation that occurs in the meiotically maturing oocyte immediately preceding meiosis I.

Induction of Nek2 activity during testicular cell culture

To begin to test the hypothesis that during meiosis, Nek2 is involved in chromosomal organization and/or condensation, we turned to an in vitro system in which meiotic chromosome condensation is induced. Recently, Wiltshire et al. (1995) reported that treatment of cultured pachytene spermatocytes with okadaic acid, an inhibitor of subtype 1 and 2A phosphatases, induced a rapid onset of events leading to M phase, visualized cytologically by nuclear envelope breakdown and chromosome condensation. While the target(s) of okadaic acid in this experimental model has not been established, the induction is rapid (within 6 hours) and occurs in the absence of new protein synthesis. If Nek2 is involved in meiotic chromosome condensation, we predicted that its activity would be induced concomitantly.

Testicular cell suspensions were prepared from 19-day-old mice, in which about 59% of the cells are meiotic prophase spermatocytes (Bellve et al., 1977). At indicated time intervals after the addition of 2.5 μM okadaic acid, cells were harvested and an aliquot was used for cytogenetic analysis to determine the stage of meiotic progression of the spermatocytes. The remainder of the sample was used to prepare lysates for immunoblot analysis and kinase assays. Lysates from the cultured cells were immunoprecipitated using the Nek2 antibody and assayed for kinase activity using casein as substrate (Fry et al., 1995). The assay for Nek2 activity in vitro was optimized using tissue culture cells transfected with Nek2 constructs (K. Rhee and D. J. Wolgemuth, unpublished observations). For comparison, MPF activity was determined in precipitates using $p13^{suc1}$-conjugated agarose and histone H1 as a substrate.

In agreement with the report of Wiltshire et al. (1995), cytogenetic analysis revealed that okadaic acid induced a rapid onset of meiotic chromosome condensation, so that over 75% of the spermatocytes reached metaphase I within 6 hours after addition of okadaic acid (Fig. 9A). Few if any mitotic nuclei

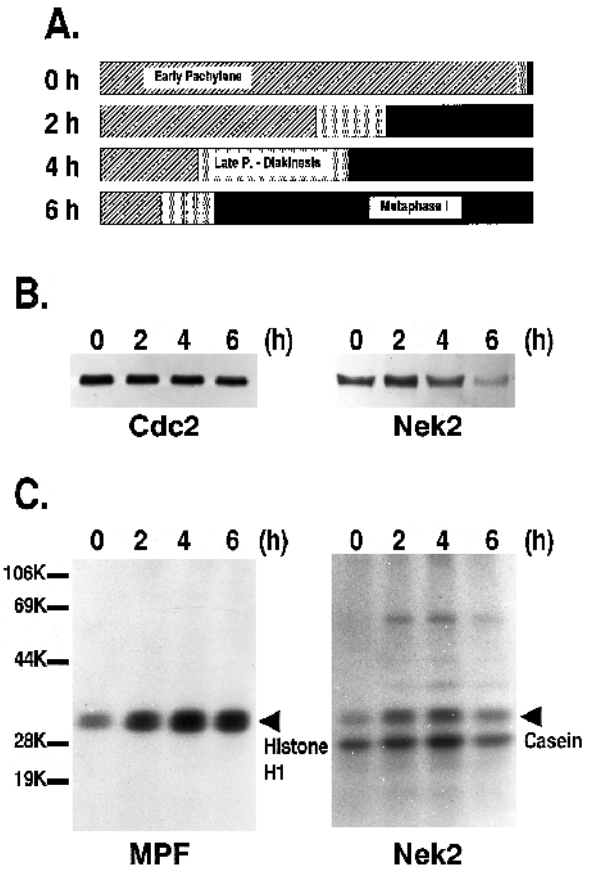

Fig. 9. Effects of okadaic acid on MPF and Nek2 activities of cultured testicular cells. Testicular cells from 19-day-old mice were cultured overnight and treated with 2.5 μM okadaic acid (Wiltshire et al., 1995). At 0, 2, 4, and 6 hours after addition of okadaic acid, cells were harvested and analyzed for meiotic progression of chromosomes (A), for protein levels of Cdc2 and Nek2 by immunoblot analysis (B), and for in vitro kinase activities of MPF and Nek2 using precipitates with $p13^{suc1}$ and anti-Nek2 antibodies, respectively (C). Histone H1 and casein were used as in vitro substrates for MPF and Nek2 kinase activity assays, respectively.

that might have originated from spermatogonia or Sertoli cells were observed. When the cytogenetic preparation was exposed to the Nek2 antibody, only meiotic prophase chromosomes were stained. Therefore, we believe that most of Nek2 and Cdc2 activities (see below) were derived from spermatocytes rather than other cell types.

We then determined the protein levels of Nek2 and Cdc2 in the cultures by immunoblot analysis (Fig. 9B). The levels of Cdc2 or Nek2 did not appear to increase during this period, consistent with the previous report that protein synthesis is not required for chromosomal condensation (Wiltshire et al., 1995). While the levels of Cdc2 appear to remain constant, the levels of Nek2 decreased 6 hours after treatment with okadaic acid.

The activities of MPF and Nek2, however, were induced by the treatment (Fig. 9C). The levels of induction of kinase activity of Nek2 and MPF were quantified and analyzed statistically using data from three independent experiments (Fig. 10). Nek2 activity increased ~3.5 fold within 2 hours and declined at 6 hours. Such a decrease may have been due, at least in part, to the concomitant decrease in the levels of Nek2 protein. MPF activity increased over 4 fold at 6 hours after addition of okadaic acid. These results are consistent with the hypothesis that Nek2 activity, along with MPF activity, is involved in the events of meiotic progression, including chromosomal condensation.

DISCUSSION

Of the three putative mammalian *nimA* homolgues identified to date, *Nek2* appears to be most similar to the *Aspergillus* gene at several levels. Structurally, it bears the highest homology to NIMA at the amino acid level (Schultz et al., 1994). Perhaps even more striking is the observation that both *NEK2* and *nimA* exhibit cell cycle-specific regulation of their expression and activity. NIMA has been shown to accumulate when

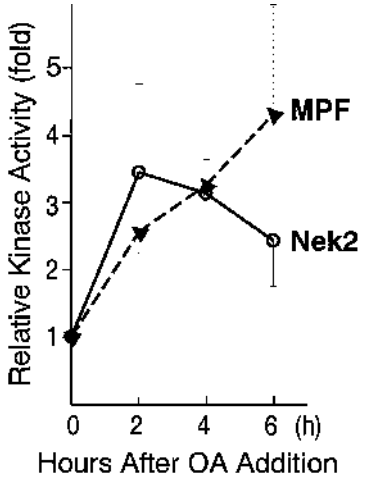

Fig. 10. Quantification of induction of MPF and Nek2 kinase activities by okadaic acid. The experiments described in Fig. 9 were repeated three times. The in vitro kinase activities were quantitated relative to the 0 hour groups, as described in Materials and Methods (mean ± s.e.). MPF, filled triangles with broken lines; Nek2, open circles with solid lines.

Aspergillus cells are arrested in G_2 phase and to be degraded in order for the cells to exit mitosis (Pu and Osmani, 1995). Similarly, in synchronized HeLa cells, human *NEK2* begins to be expressed during S phase, is most abundant at G_2, and is reduced to basal levels after mitosis (Schultz et al., 1994; Fry et al., 1995). The data presented in the present study provide several lines of additional evidence for these similarities, importantly, in a normal, in vivo system. The expression of *Nek2* was limited to what appeared to be mitotic M phase cells of 7-day-old testis, suggesting cell cycle-stage specificity. More strikingly, such putative cell cycle stage-specificity was clearly evidenced in the germ line cells once they had committed to meiosis. *Nek2* was most abundant in meiotic prophase spermatocytes, a stage after DNA synthesis is completed but before M phase. The levels of *Nek2* mRNA and protein dropped dramatically in spermatogenic cells once the chromosome divisions at meiosis I and meiosis II were completed.

Previous studies on the function of the *Aspergillus* gene *nimA* in yeast and vertebrate cells had suggested a role in the process of chromosomal condensation. The expression of dominant negative mutants of *nimA* in fission yeast, *Xenopus* oocytes or mammalian tissue culture cells resulted in an arrest at the G_2 phase (O'Connell et al., 1994; Lu and Hunter, 1995), a phenotype similar to that seen for *nimA* mutants in *Aspergillus* (Lu and Means, 1994). Ectopic expression of the normal NIMA protein resulted in an arrest at M phase, with condensed chromatin (O'Connell et al., 1994; Lu and Hunter, 1995). Interestingly, NIMA localized to the prematurely condensed chromatin in HeLa cells (Lu and Hunter, 1995).

The hypothesis that the putative *nimA* homologue *Nek2* functions in chromosomal organization and/or condensation was supported at several levels by our observations. Nek2 protein was shown to be associated with meiotic chromosomes of both sexes. Its presence at some of the ends of pachytene/diplotene stage chromosomes was particularly intriguing as it is known that telomeres are attached to the nuclear membrane during meiotic prophase spermatocytes (Scherthan et al., 1996), a configuration that may be important for efficient pairing of homologous chromosomes at the zygotene stage (reviewed by Dernburg et al., 1995). Nek2 was also readily detected in the sex vesicle, a structure consisting of the sex chromosomes in a unique heterochromatic conformation which persists until the end of diplotene (reviewed by Handel and Hunt, 1992).

Additional support for a role for Nek2 in chromosome organization and/or condensation resulted from our studies on the induction of premature chromosome condensation in spermatocytes which were cultured in a short-term culture system and treated with okadaic acid. We observed an induction of Nek2 and MPF kinase activities, as assayed in vitro, coincident with the condensation of the meiotic prophase chromosomes. In the female germ line, okadaic acid treatment of oocytes has also been shown to induce chromosome condensation, even in meiotically incompetent oocytes (Gavin et al., 1992; de Vantery et al., 1996). The effect of okadaic acid is not unique to meiotic cells, as it has also been shown to induce mitotic chromosome condensation in F210 cells (Guo et al., 1995). The cell cycle of F210 cells, which bear a temperature-sensitive lesion in the *Cdc2* gene, is blocked at G_2 phase at the restrictive temperature. Treatment of these G_2-arrested F210

cells with okadaic acid resulted in full chromosome condensation in the absence of Cdc2 activity or histone H1 hyperphosphorylation.

The localization of Nek2 to meiotic chromosomes also provides insight as to its site of action and substrates. The potential substrates of NIMA itself are only beginning to be elucidated. Recently, Lu et al. (1996) isolated a human cDNA in a yeast two-hybrid screen for proteins interacting with NIMA in HeLa cells. This cDNA encodes a peptidyl-prolyl isomerase (PIN1) which appeared to be essential for G_2/M transition of mitosis. That is, overexpression of PIN1 resulted in arrest at G_2 while antisense oligonucleotide-mediated reduction of PIN1 resulted in an accumulation of cells in M phase with condensed chromatin (Lu et al., 1996). Whether any of the NEK proteins, as opposed to NIMA, are associated with PIN1 remains to be determined. There are other isomerases that have been shown to be associated with meiotic chromosomes as phosphoproteins, such as topoisomerase II (Moens, 1990; Rose et al., 1990; Kimura et al., 1994), which could be tested for association with Nek2. The SMC (structural maintenance of chromosomes) family proteins, which function in mitotic chromosome condensation (Hirano and Michison, 1994; reviewed by Gasser, 1995) may also be candidates, although it is not known whether SMC proteins must be phosphorylated for activation.

An enduring enigma in the role of the NIMA homologues in vertebrate cells concerns the inability to demonstrate an effect on cell cycle progression by either suppression of the normal Nek activity by dominant-negative mutants or ectopic expression of the *Nek* genes, analogous to that elicited by NIMA. One possible explanation is that there are additional, as yet unidentified vertebrate *nimA* homologues which will function in a manner more similar to *nimA*. To this end, the comment by Hunter and colleagues (Lu and Hunter, 1995) of the possibility of as many as six *Nek* family genes should be noted. Alternatively or in addition, it may be that the vertebrate *Nek* genes function in very specific physiological contexts in vivo. If, as we speculate, Nek2 functions in meiotic prophase in the course of the highly specialized structuring and condensation of meiotic chromosomes, it is possible that its function will not be revealed by assays in generic tissue culture cell systems. Indeed, it is likely that function will only be elucidated by direct manipulation of the endogenous gene and modulation of its products in vivo, a first step toward which has been presented here.

We thank Dr Weng Kong Sung for helping with cytogenetic methods and Ms Theresa Swayne with the confocal microscopy. We are grateful to Dr Xiang Yuan Wang and Mr Chris Marshall for technical assistance. We also thank Drs Tej K. Pandita, Erich A. Nigg, Alan I. Packer and Dorothy Warburton for helpful discussion. This work was supported in part by grants from the NIH, P50 HD05077 (D. J. W.) and F32 HD 07968 (K. R.).

REFERENCES

Allshire, R. C., Gosden, J. R., Cross, S. H., Cranston, G., Rout, D., Sugawara, N., Szostak, J. W., Fantes, P. A. and Hastie, N. D. (1988). Telomeric repeat from *T. thermophila* cross hybridizes with human telomeres. *Nature* **332**, 656-659.

Bellve, A. R., Cavicchia, J. C., Millette, C. F., O'Brien, D. A., Bhatnager, Y. M. and Dym, M. (1977). Spermatogenic cells of the prepubertal mouse: Isolation and morphological characterization. *J. Cell Biol.* **74**, 68-85.

Chapman, D. L. and Wolgemuth, D. J. (1994). Regulation of M-phase promoting factor activity during development of mouse male germ cells. *Dev. Biol.* **165**, 500-506.

Chomczynski, P. and Sacchi, N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Dernburg, A. F., Sedat, J. W., Cande, W. Z. and Bass H. W. (1995). Cytology of telomeres. In *Telomeres*(ed. Blackburn, E. H. and Greider), pp. 295-338. C. W. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

de Vantery, C., Gavin, A. C., Vassalli, J. D. and Schorderet-Slatkine, S. (1996). An accumulation of $p34^{cdc2}$ at the end of mouse oocyte growth correlates with the acquisition of meiotic competence. *Dev. Biol.* **174**, 335-344.

Devereux, J., Haeberli, P. and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids Res.* **12**, 387-395.

Fry, A. M. and Nigg, E. A. (1995). The NIMA kinase joins forces with Cdc2. *Curr. Biol.* **5**, 1122-1125.

Fry, A. M., Schultz, S. J., Bartek, J. and Nigg, E. A. (1995). Substrate specificity and cell cycle regulation of the Nek2 protein kinase, a potential human homolog of the mitotic regulator NIMA of *Aspergillus nidulans*. *J. Biol. Chem.* **270**, 12899-12905.

Gallant, P., Fry, A. M., and Nigg, E. A. (1995). Protein kinases in the control of mitosis: focus on nucleocytoplasmic trafficking. *J. Cell Sci. Supp.* **19**, 21-28.

Gasser, S. M. (1995). Coiling up chromosomes. *Curr. Biol.* **5**, 357-360.

Gavin, A. C., Vassalli, J. D., Cavadore, J. C. and Schorderet-Slatkine, S. (1992). Okadaic acid and $p13^{suc1}$ modulate the reinitiation of meiosis in mouse oocytes. *Mol. Reprod. Dev.* **33**, 287-296.

Guo, X. W., Th'ng, J. P. H., Swank, R. A., Anderson, H. J., Tudan, C., Bradbury, E. M. and Roberge, M. (1995). Chromosome condensation induced by fostriecin does not require $p34^{cdc2}$ kinase activity and histone H1 phosphorylation, but is associated with enhanced histone H2A and H3 phosphorylation. *EMBO J.* **14**, 976-985.

Handel, M. A. and Hunt, P. A. (1992). Sex-chromosome pairing and activity during mammalian meiosis. *BioEssays* **14**, 817-822.

Hanks, S. K. and Hunter, T. (1995). The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.* **9**, 576-596.

Hirano, T. and Mitchison, T. M. (1994). A heterodimeric coiled-coil protein required for mitotic chromosome condensation in vitro. *Cell* **79**, 449-458.

Hummel, K. P. (1964). *Mouse News Lett.* **34**, 31-32.

Kimura, K., Nozaki, N., Saijo, M., Kikuchi, A., Ui, M. and Enomoto, T. (1994). Identification of the nature of modification that causes the shift of DNA topoisomerase IIβ to apparent higher molecular weight forms in the M phase. *J. Biol. Chem.* **269**, 24523-24526.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **335**, 251-254.

Letwin K., Mizzen, L., Motro, B., Ben-David, Y., Bernstein, A. and Pawson, T. (1992). A mammalian dual specificity protein kinase, Nek1, is related to the NIMA cell cycle regulator and highly expressed in meiotic germ cells. *EMBO J.* **11**, 3521-3531.

Levedakou, E. N., He, M., Baptist, E. W., Craven, R. J., Cance, W. G., Welcsh, P. L., Simmons, A., Naylor, S. L., Leach, R. J., Lewis, T. B., Bowcock, A. and Liu, E. T. (1994). Two novel human serine/threonine kinases with homologies to the cell cycle regulating *Xenopus* MO15, and NIMA kinases: cloning and characterization of their expression pattern. *Oncogene* **9**, 1977-1988.

Lu, K. P., Hanes, S. D. and Hunter, T. (1996). A human peptidyl-prolyl isomerase essential for regulation of mitosis. *Nature* **380**, 544-547.

Lu, K. P., Kemp, B. E. and Means, A. R. (1994). Identification of substrate specificity determinants for the cell cycle-regulated NIMA protein kinase. *J. Biol. Chem.* **269**, 6603-6607.

Lu, K. P. and Means, A. R. (1994). Expression of the catalytic domain of the NIMA kinase causes a G2 arrest in *Aspergillus nidulans*. *EMBO J.* **13**, 2103-2113.

Lu, K. P. and Hunter, T. (1995). Evidence for a NIMA-like mitotic pathway in vertebrate cells. *Cell* **81**, 413-424.

Lu, K. P. and Means, A. R. (1994). Expression of the noncatalytic domain of the NIMA kinase causes a G2 arrest in *Aspergillus nidulans*. *EMBO J.* **13**, 2103-2113.

Lu, K. P., Osmani, S. A. and Means, A. R. (1993). Properties and regulation of

the cell cycle-specific NIMA protein kinase of *Aspergillus nidulans*. *J. Biol. Chem.* **268**, 8769-8776.

Meredith, R. (1969). A simple method for preparing meiotic chromosomes from mammalian testes. *Chromosoma* **26**, 254-258.

Mintz, B. and Russell, E. S. (1957). Gene-induced embryological modifications of primordial germ cell in the mouse. *J. Exp. Zool.* **134**, 207-230.

Moens, P. B. (1990). Unravelling meiotic chromosomes: topoisomerase II and other proteins. *J. Cell Sci.* **97**, 1-3.

Moyzis, R. K., Buckingham, J. M., Cram, L. S., Dani, M., Deaven,, L. L., Jones, M. D., Meyne, J., Ratliff, R. L. and Wu, J.-R. (1988). A highly conserved repetitive DNA sequence, $(TTAGGG)_n$, present at the telomeres of human chromosomes. *Proc. Natl. Acad. Sci. USA* **85**, 6622-6626.

Oakberg, E. F. (1956). A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. *Am. J. Anat.* **99**, 391-409.

O'Connell, M. J., Norbury, C. and Nurse, P. (1994). Premature chromatic condensation upon accumulation of NIMA. *EMBO J.* **13**, 4926-4937.

Osmani, A. H., McGuire, S. L. and Osmani, S. A. (1991). Parallel activation of the NIMA and $p34^{cdc2}$ cell cycle-regulated protein kinases is required to initiate mitosis in A. nidulans. *Cell* **67**, 283-291.

Osmani, S. A., Pu, R. T. and Morris, N. R. (1988). Mitotic induction and maintenance by overexpression of a G2-specific gene that encodes a potential protein kinase. *Cell* **53**, 237-244.

Pu, R. T. and Osmani, S. A. (1995). Mitotic destruction of the cell cycle regulated NIMA protein kinase of *Aspergillus nidulans* is required for mitotic exit. *EMBO J.* **14**, 995-1003.

Ravnik, S. E. and Wolgemuth, D. J. (1996). The developmentally restricted pattern of expression in the male germ line of a murine *cyclin A, cyclin A2*, suggests roles in both mitotic and meiotic cell cycles. *Dev. Biol.* **173**, 69-78.

Rechsteiner, M. and Rogers, S. W. (1996). PEST sequences and regulation by proteolysis. *Trends Biochem. Sci.* **21**, 267-271.

Rhee, K. and Wolgemuth, D. J. (1995). *Cdk* family genes are expressed not only in dividing but also terminally differentiated mouse germ cells, suggesting their possible function during both cell division and differentiation. *Dev. Dynam.* **204**, 406-420.

Rose, D., Thomas, W. and Holm, C. (1990). Segregation of recombined chromosomes in meiosis I requires DNA topoisomerase II. *Cell* **60**, 1009-1017.

Russell, L. D., Ettlin, R. A., Sinha Hikim, A. P. and Clegg, E. D. (1990). *Histological and Histopathological Evaluation of the Testis* Cache River Press, Clearwater, FL.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scherthan, H., Weich, S., Schwegler, H., Heyting, C., Harle, M. and Cremer, T. (1996). Centromere and telomere movements during early meiotic prophase of mouse and man are associated with the onset of chromosome pairing. *J. Cell Biol.* **134**, 1109-1125.

Schultz, S. J., Fry, A. M., Sutterlin, C., Ried, T. and Nigg, E. A. (1994). Cell cycle-dependent expression of Nek2, a novel human protein kinase related to the NIMA mitotic regulator of *Aspergillus nidulans*. *Cell Growth Diff.* **5**, 625-635.

Schultz, S. J. and Nigg, E. A. (1993). Identification of 21 novel human protein kinases, including 3 members of a family related to the cell cycle regulator *nimA* of *Aspergillus nidulans*. *Cell Growth Diff.* **4**, 821-830.

Shi, S.-R., Key, M. E. and Karla, K. L. (1991). Antigen retrieval in formalin-fixed paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* **39**, 741-748.

Sweeney, C., Murphy, M., Kubelka, M., Ravnik, S. E., Hawkins, C. F., Wolgemuth, D. J. and Carrington, M. (1996). A different mammalian cyclin A, murine cyclin A1, is expressed exclusively in germ cells and during early embryogenesis. *Development* **122**, 53-64.

Tsai, L.-H., Delalle, I., Caviness, V. S., Chae, T. and Harlow, E. (1994). p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. *Nature* **371**, 419-423.

Wiltshire, T., Park, C., Caldwell, K. A. and Handel, M. A. (1995). Induced premature G2/M-phase transition in pachytene spermatocytes includes events unique to meiosis. *Dev. Biol.* **169**, 557-567.

Wolgemuth, D. J., Gizang-Ginsberg, E., Engelmyer, E., Gavin, B. J. and Ponzetto, C. (1985). Separation of mouse testis cells on a Celsep apparatus and their usefulness as a source of high molecular weight DNA or RNA. *Gamete Res.* **12**, 1-10.

Wolgemuth, D. J., Rhee, K., Wu, S. and Ravnik, S. E. (1995). Genetic control of mitosis, meiosis and cellular differentiation during mammalian spermatogenesis. *Reprod. Fertil. Dev.* **7**, 669-683.

Ye, X. S., Xu, G., Pu, R. T., Fincher, R. R., McGuire, S. L., Osmani, A. H. and Osmani, S. A. (1995). The NIMA protein kinase is hyperphosphorylated and activated downstream of $p34^{cdc2}$/cyclin B: coordination of two mitosis promoting kinases. *EMBO J.* **14**, 986-994.

Zhang, Q., Ahuja, H. S., Zakeri, Z. F. and Wolgemuth, D. J. (1997). Cyclin-dependent kinase 5 is associated with apoptotic cell death during development and tissue remodeling. *Dev. Biol*. (in press).

(Accepted 2 April 1997)