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A mammalian dual specificity protein kinase, Nek1, is related to the NIMA cell cycle regulator and highly expressed in meiotic germ cells

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Screening of mouse cDNA expression libraries with antibodies to phosphotyrosine resulted in repeated isolation of cDNAs that encode a novel mammalian protein kinase of 774 amino acids, termed Nek1. Nek1 contains an N-terminal protein kinase domain which is most similar (42% identity) to the catalytic domain of NIMA, a protein kinase which controls initiation of mitosis in *Aspergillus nidulans*. In addition, both Nek1 and NIMA have a long, basic C-terminal extension, and are therefore similar in overall structure. Despite its identification with anti-phosphotyrosine antibodies, Nek1 contains sequence motifs characteristic of protein serine/threonine kinases. The Nek1 kinase domain, when expressed in bacteria, phosphorylated exogenous substrates primarily on serine/threonine, but also on tyrosine, indicating that Nek1 is a dual specificity kinase with the capacity to phosphorylate all three hydroxyamino acids. Like NIMA, Nek1 preferentially phosphorylated β-casein *in vitro*. *In situ* RNA analysis of *nek1* expression in mouse gonads revealed a high level of expression in both male and female germ cells, with a distribution consistent with a role in meiosis. These results suggest that Nek1 is a mammalian relative of the fungal NIMA cell cycle regulator.

Key words: cell cycle/NIMA-related kinase

Introduction

The majority of known protein kinases fall into two mutually exclusive subgroups, based on their primary structures and substrate specificities. All protein kinases contain highly conserved residues that are important for nucleotide binding and phosphotransfer (Hanks *et al.*, 1988; Moran *et al.*, 1988; Knighton *et al.*, 1991a,b). In addition, protein kinases that specifically phosphorylate either serine/threonine or tyrosine contain residues that are diagnostic of their substrate specificity, and are probably instrumental in discriminating between different hydroxyamino acid phosphoacceptors (Hanks *et al.*, 1988). Recently, however, a small number of protein kinases have been identified that do not conform to these conventions. These kinases have sequence motifs that would initially suggest that they are serine/threonine-specific, but are able to autophosphorylate on both serine/threonine and tyrosine, and in some instances can also phosphorylate exogenous substrates on tyrosine (reviewed in Lindberg *et al.*, 1992). Examples of such dual specificity protein kinases include the *MCK1* gene product of *Saccharomyces cerevisiae*, which is involved in meiotic recombination (Dailey *et al.*, 1990), the *S.cerevisiae SPK1* product (Stern *et al.*, 1991), the mammalian mitogen-activated protein (MAP or ERK) kinases, which are implicated in mitotic and meiotic signaling, (Crews *et al.*, 1991; Seger *et al.*, 1991; Wu *et al.*, 1991) and the murine Clk/Sty protein kinase (Ben-David *et al.*, 1991; Howell *et al.*, 1991).

Although no conventional tyrosine kinases have been identified in yeast, tyrosine phosphorylation is important in control of the yeast cell cycle. Phosphorylation of the cell cycle regulator $p34^{cdc2}$ on Tyr15 inhibits its activity in the G_2 phase of the cell cycle (Gould and Nurse, 1989). Recent evidence suggests that the protein kinase encoded by the *wee1* gene of fission yeast, which has sequence elements generally considered characteristic of protein serine/threonine kinases, can phosphorylate $p34^{cdc2}$ on Tyr15 (Featherstone and Russell, 1991; Parker *et al.*, 1991) and thereby repress its activity. In doubly null mutants of *wee1* and a second related protein kinase gene, *mik1*, $p34^{cdc2}$ is not tyrosine phosphorylated, and the cells are propelled prematurely into mitosis (Lundgren *et al.*, 1991). Dual specificity protein kinases therefore appear to share a demonstrated or implicit involvement in signal transduction, and in the control of mitosis and meiosis.

Although the *cdc2* gene has been generally considered as the universal regulator of the cell cycle, recent data from both *S.cerevisiae* and *Aspergillus nidulans* have suggested the existence of an additional pathway involved in controlling entry into mitosis (A.H.Osmani *et al.*, 1991a; Amon *et al.*, 1992; Sorger and Murray, 1992). In particular, the *nimA* gene of *A.nidulans* is required, independently of *cdc2*, for the initiation of mitosis, for spindle formation and for nuclear envelope breakdown (A.H.Osmani *et al.*, 1991a,b; S.A.Osmani *et al.*, 1988a). The extraordinary functional and structural conservation of *cdc2* among eukaryotes (Lewin, 1990) raises the possibility that such *cdc2*-independent mitotic regulators might also be conserved during evolution. Here we describe the identification and expression patterns of a novel mouse dual specificity kinase that is structurally and biochemically related to the *nimA* gene product.

Results

Isolation of the nek1 mammalian cDNA

The mouse *nek*1 cDNA was originally isolated in a functional screen designed to identify protein tyrosine kinases

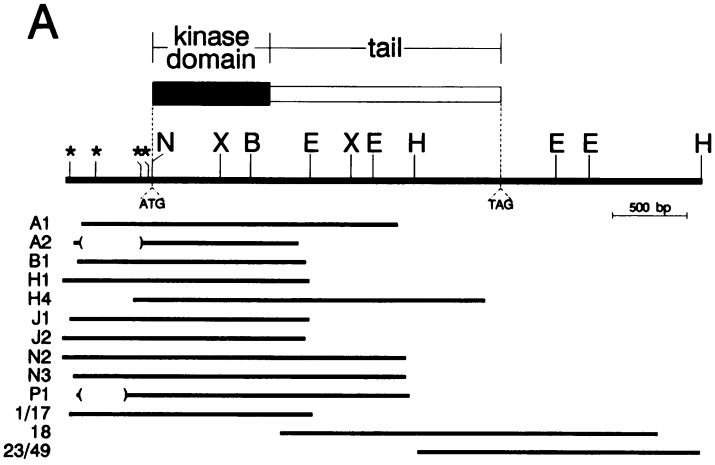


Fig. 1. Sequence and structure of the *nek1* cDNAs and the predicted translation product. (**A**) The structure of the 4.2 kb composite *nek1* cDNA is shown above the individual cDNA isolates, with the initiation and termination codons indicated. In-frame stop codons situated in the 5′-nontranslated region are represented by asterisks. Restriction endonuclease sites are also shown (B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; X, *Xho*I). The expected protein product is depicted above the composite cDNA, with the catalytic domain and C-terminal tail indicated. Deletions in the 5′-nontranslated regions of the A2 and P1 clones are indicated. (**B**) The sequence of the composite *nek1* cDNA is shown with the sequence of the expected translation product. The single letter amino acid designation is used. Conserved residues diagnostic of a protein kinase catalytic domain are highlighted. In-frame stop codons situated in the 5′-nontranslated region are underlined.

(Ben-David *et al.*, 1991). Mouse erythroleukemia cell cDNA expression libraries were probed with antibodies to phosphotyrosine, which identify recombinant bacteriophage with cDNA inserts encoding enzymatically active tyrosine kinases. The mouse cDNAs isolated in this screen all encoded polypeptides with the hallmarks of protein kinases, of which several were identical or closely related to known protein tyrosine kinases. Three cDNAs encoded the dual specificity Clk kinase. The most frequently isolated cDNA sequence, designated *nek1*, encoded a novel protein kinase.

The ten *nek1* cDNAs isolated in this anti-phosphotyrosine antibody screen are illustrated in Figure 1. Since the library was unamplified, each isolate represents a unique cDNA. Restriction endonuclease mapping and partial sequence analysis revealed that all but two of the *nek1* cDNA isolates are colinear, and are identical in the regions where they overlap, suggesting that they are derived from the same mRNA species. Relative to other clones, both the A2 and P1 cDNAs contain small internal deletions in the untranslated region near their 5′-ends. Nucleotides 138–544 are lacking in A2, and residues 138–422 are missing from P1 (see Figure 1). As the regions missing from both the A2 and P1 clones are flanked by consensus splice donor and splice acceptor sites, they presumably represent differentially spliced mRNAs.

A composite sequence of *nek1* cDNA was constructed from two clones, N2 and H4 (see Figure 1A). This sequence is 2828 nt long and contains a single long ORF, beginning at nt 576 (see Figure 1B). There are several in-frame stop codons located upstream of this residue, the closest being only a few residues distant. The regions missing in A2 and P1 are therefore derived from 5′-nontranslated sequence, and might be involved in translational control. No termination codon was found for the long ORF, suggesting that additional 3′-coding sequences were missing.

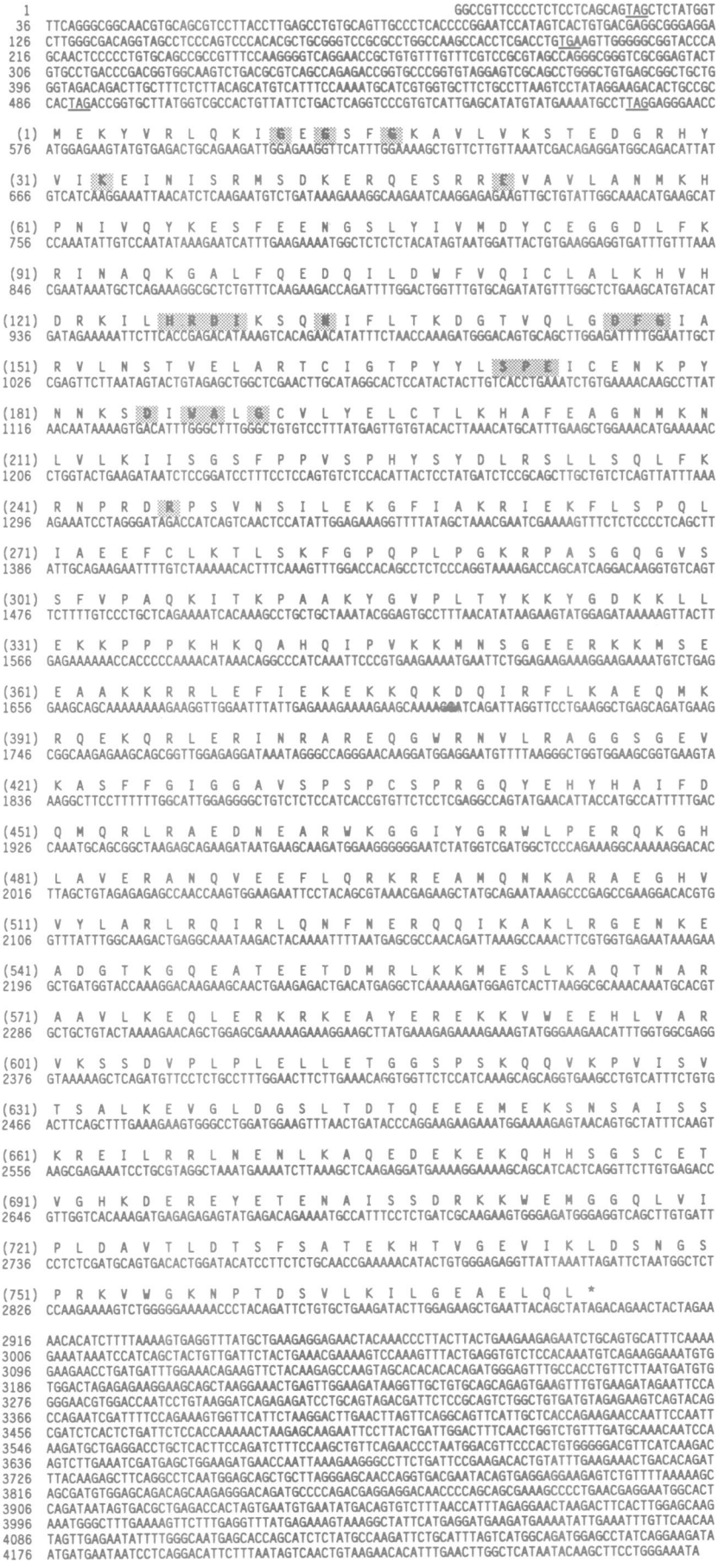
In order to clone the remaining 3′-coding region, we rescreened the original λgt11 cDNA expression libraries using H4 DNA as a probe. Six positive clones were isolated and sequenced (see Figure 1A). Sequence data obtained from clones 23 and 49 extended the composite *nek1* sequence to 4263 nt. A termination codon beginning at residue 2898 was found for the ORF initiating at residue 576. The resulting translation product would be 774 amino acids in length, with an expected molecular weight of 88.4 kDa.

Nek1 encodes a protein kinase related to NIMA

Residues 1–258 of the *nek1* cDNA product (Nek1) have sequence motifs characteristic of a protein kinase catalytic domain (highlighted in Figure 1B). In particular, highly conserved amino acids which are involved in ATP-binding and phosphotransfer are present in Nek1. Because the original screen was designed to isolate enzymatically active protein tyrosine kinases, it was not surprising that each of the ten individual clones identified in the original anti-phosphotyrosine antibody screen encodes an uninterrupted and complete catalytic domain. In the sequence elements that normally distinguish protein tyrosine kinases from protein serine/threonine kinases, Nek1 is most similar to serine/threonine-specific protein kinases. Within subdomain VIB (Hanks *et al.*, 1988; Lindberg *et al.*, 1992), tyrosine kinases generally have the sequence Leu-Ala-Ala-Arg-Asn, whereas serine/threonine kinases have Leu-Lys-Pro-Glu-Asn, with the lysine being highly conserved. Nek1 retains the conserved lysine, diagnostic of serine/threonine kinases, which is followed by Ser-Gln (i.e. Leu-Lys-Ser-Gln-Asn). It is of interest that the *Dictyostelium discoideum* tyrosine kinase, DPYK2 (Tan and Spudich, 1990), also has Ser-Gln at these latter two positions. Nek1 also contains residues characteristic of serine/threonine-specific kinases in subdomain VIII. Residues 259–774 of Nek1 form a long C-terminal extension, containing a high proportion of charged residues, with a preponderance of basic amino acids.

A search of the EMBL and GenBank databases with the Nek1 sequence confirmed that it is a novel protein. This search also revealed that Nek1 is most closely related to NIMA, a protein kinase that controls the initiation of mitosis in the fungus *A.nidulans*, and is required for the G_2 to M transition (Morris, 1976; Oakley and Morris, 1983; S.A.Osmani *et al.*, 1988b; A.H.Osmani *et al.*, 1991a). The Nek1 and NIMA proteins are 42% identical in their catalytic

B



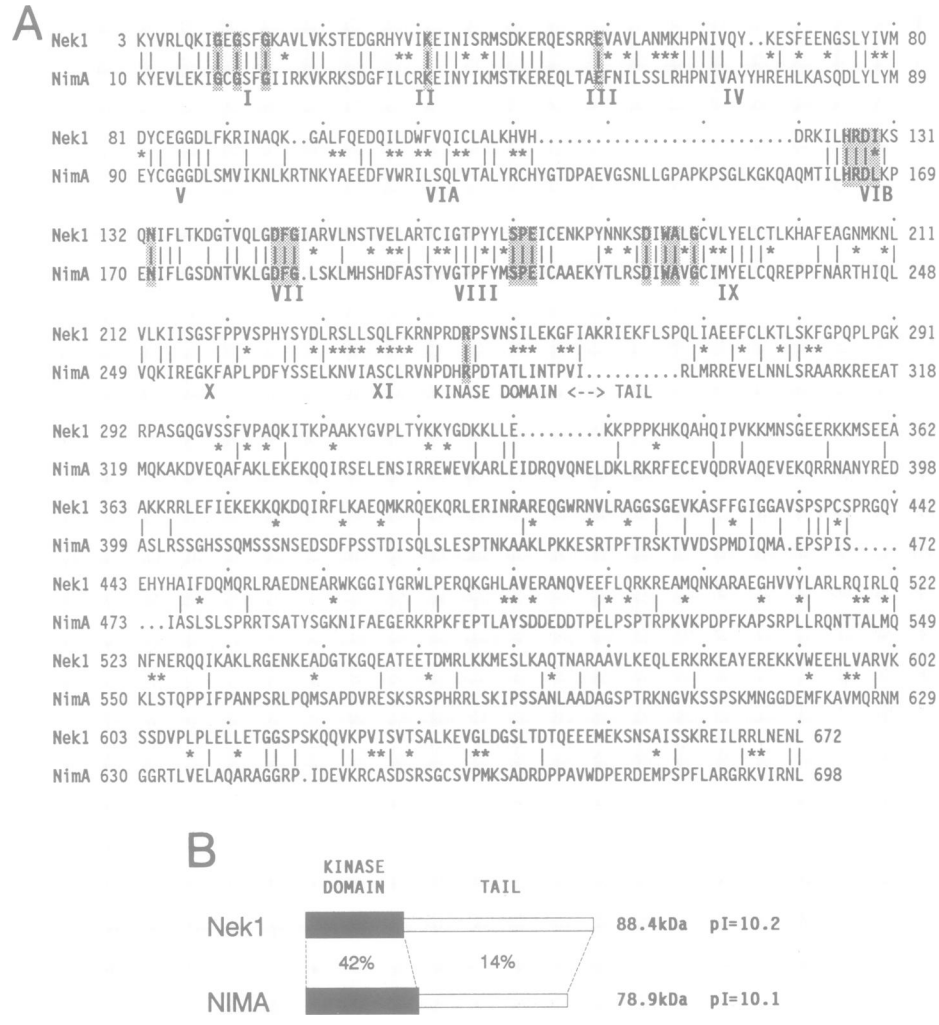


Fig. 2. Comparison of the Nek1 and NIMA proteins. (**A**) Sequence comparison of the Nek1 and NIMA proteins. The single letter amino acid designation is used. The alignment was generated using the BESTFIT program from the University of Wisconsin GCG sequence analysis software package. Identical residues are indicated by vertical lines, similar residues by asterisks. Similar residues were grouped as follows: hydrophobic/aromatic (LVAIMCYFW), polar (STQN), acidic (ED), basic (HKR) and helix breakers (GP). Residues that are highly conserved in the catalytic domains of all protein kinases are highlighted. The kinase domains and C-terminal tail regions of both proteins, and protein kinase subdomains I–XI (Hanks *et al.*, 1988) are indicated. (**B**) Structural comparison of the Nek1 and NIMA proteins. The structures of both the Nek1 and NIMA proteins are shown with the percent identity between the N-terminal kinase domains and the C-terminal tails indicated. The predicted molecular weights and the calculated isoelectric points are shown for both proteins.

domains (Figure 2A), excluding a 27 amino acid sequence within the NIMA kinase domain which is absent from Nek1, and 24% identical across their entire length. In addition to this primary sequence similarity, the two proteins are related in other ways. Nek1 and NIMA are similar in their overall structural arrangement, with their kinase domains at the extreme N-teminus, followed by a long C-terminal extension (Figure 2B). Both proteins are exceptionally basic, having calculated isoelectric points >10. Within the Nek1 C-terminal extension there are four clusters of basic amino acids (residues 322–339, 347–357, 364–378 and 580–591), each of which conforms to the consensus for a bipartite nuclear targeting motif (Dingwall and Laskey, 1991). Due to these similarities in sequence and structural organization, we have named this novel kinase nek1, for *n*imA-r*e*lated *k*inase. The nek1 kinase also shows significant homology (~38%) with the kinase domain of an *S.cerevisiae* protein kinase, KIN3 (Jones and Rosamond, 1990), but has much lower levels of sequence identity to other known protein kinases.

Nek1 phosphorylates bacterial proteins on serine, threonine and tyrosine

The sequence of the *nek1* cDNA suggested that it encodes a serine/threonine-specific protein kinase, while its isolation with anti-phosphotyrosine antibodies raised the possibility that its product has protein tyrosine kinase activity. To investigate Nek1 kinase activity, the presumptive Nek1 kinase domain (residues 1–263) was expressed in *E.coli* as a fusion protein with the T7 gene 10 leader peptide (Tag). Induction of Tag–Nek1 expression correlated with the synthesis of a series of proteins ranging from 30–37 kDa, which were detected by anti-Tag antibodies in immunoblots (Figure 3A) or in anti-Tag immunoprecipitates of cells labeled with $^{32}P_i$ (Figure 3B) or [^{35}S]methionine/cysteine (Figure 3C). This is consistent with the expected size of the Tag–Nek1 fusion protein (30 kDa). The apparent heterogeneity of the polypeptide could be due to differential phosphorylation (see below).

To test for tyrosine-specific kinase activity, bacteria harboring the Tag–Nek1 expression plasmid were analyzed

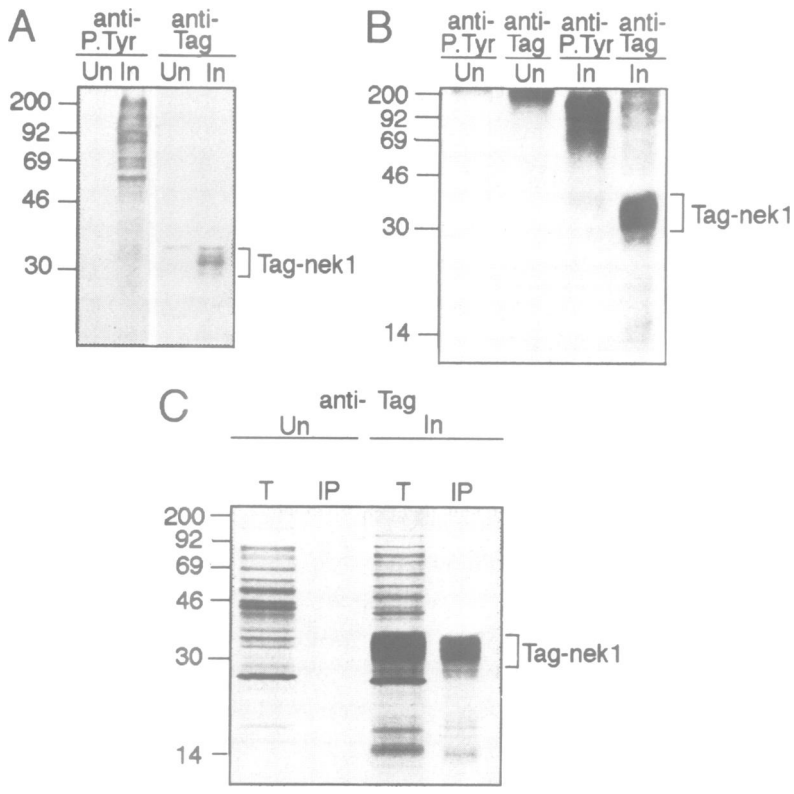


Fig. 3. Induction of tyrosine kinase activity by Tag−Nek1 and immunoprecipitation of *in vivo* labeled Tag−Nek1. (**A**) Immunoblot of lysates from uninduced and induced Tag−Nek1 expressing bacteria probed with anti-phosphotyrosine antibody (anti-P.tyr) or anti-T7 gene 10 leader peptide antiserum (anti-Tag). The band detected with anti-Tag antibody in the uninduced lane is non-specific. (**B**) Anti-phosphotyrosine and anti-Tag immunoprecipitates from uninduced and induced ^{32}P-labeled Tag−Nek1 bacteria. (**C**) Anti-Tag immunoprecipitates prepared from uninduced or induced ^{35}S-methionine/cysteine-labeled bacteria expressing Tag−Nek1. The position of the Tag−Nek1 fusion protein is indicated.

by immunoblotting with antibodies to phosphotyrosine. Induction of Tag−Nek1 expression correlated with the appearance of numerous bacterial polypeptides that were recognized by anti-phosphotyrosine antibodies (Figure 3A). Similarly, several phosphoproteins were immunoprecipitated from lysates of ^{32}P-labeled bacteria expressing the Tag−Nek1 polypeptide by anti-phosphotyrosine antibodies (Figure 3B). The same results were obtained with an expression plasmid, pGEMEX-N2, encoding Nek1 residues 1−576 (data not shown). Total bacterial proteins, or three prominent phosphoproteins immunoprecipitated with anti-phosphotyrosine antibodies, were isolated from ^{32}P-labeled cells expressing pGEMEX-N2, and subjected to phosphoamino acid analysis (Figure 4). Lysates of cells expressing the Nek1 kinase contained phosphoserine and phosphothreonine and low amounts of phosphotyrosine (Figure 4A). Each of the immunoprecipitated phosphoproteins contained similar ratios of phosphoserine, phosphothreonine and phosphotyrosine (Figure 4B). Taken together these results indicate that expression of the Nek1 kinase domain in *E.coli* induces the phosphorylation of bacterial proteins on serine/threonine and tyrosine residues.

We next performed *in vitro* Nek1 kinase assays, using Tag−Nek1 immunoprecipitated from induced bacterial lysates with anti-Tag antibodies (Figure 5). The ^{32}P-labeled products generated in a Tag−Nek1 immune complex kinase assay are shown in Figure 5A. In the absence of exogenous substrate, Tag−Nek1 primarily phosphorylated the Ig heavy chain (Figure 5A, lane 1). Additional minor phosphoproteins were detected following prolonged autoradiographic exposure, some of which were of the size expected for Tag−Nek1. These latter proteins could be re-immunoprecipitated with anti-Tag sera, indicating that they represent autophosphorylated Tag−Nek1 (Figure 5A, lane 2). *In vitro* Nek1 kinase activity, as measured by Ig heavy chain phosphorylation, was optimal at pH 7.5, much preferred Mn^{2+} to Mg^{2+} and Ca^{2+} [while Zn^{2+} was ineffective (all at 5 mM)] and was stimulated in the presence of the reducing agent dithiothreitol at 10 mM (data not shown). Phosphoamino acid analysis of labeled Ig heavy chain and Tag−Nek1 indicated that both proteins contained a similar ratio of all three phosphoamino acids, with phosphothreonine and phosphoserine predominating over phosphotyrosine (Figure 5B). Indeed, the phosphotyrosine signal was relatively weak, suggesting that *in vitro* Nek1 tyrosine kinase activity is modest.

To examine exogenous substrate phosphorylation by immunoprecipitated Tag−Nek1, *in vitro* kinase assays were performed in the presence of increasing concentrations of added poly(Glu:Tyr), β-casein and H1 histone (Figure 6). Tag−Nek1 displayed *in vitro* kinase activity against all three substrates in a concentration-dependent manner, with preference for β-casein ≥ H1 histone > > poly(Glu:Tyr) (Figure 6A). No substrate phosphorylation was observed in the absence of immunoprecipitated Tag−Nek1 (data not shown). Phosphoamino acid analysis of labeled exogenous substrates (Figure 6B) indicated that phosphorylation of β-casein and H1 histone was on serine and threonine residues only, while poly(Glu:Tyr) phosphorylation, as expected, was on tyrosine residues. A bacterial Tag−Nek1 fusion protein

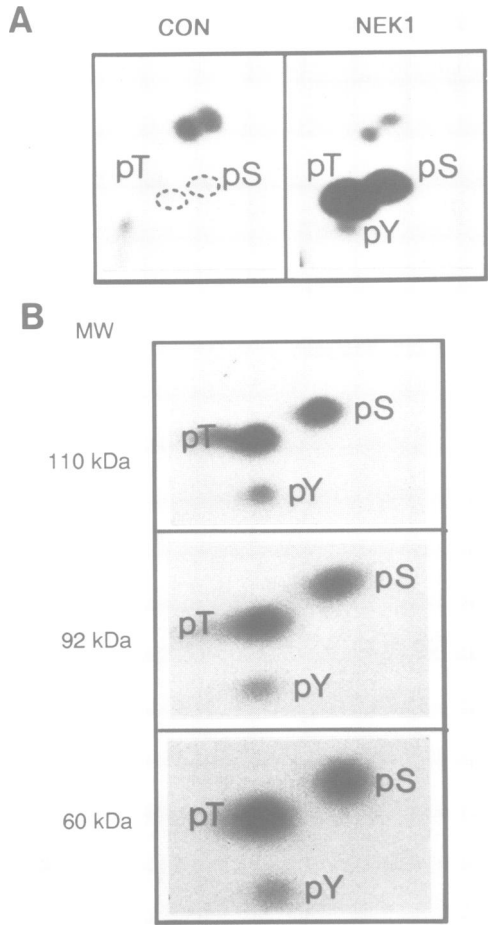


Fig. 4. Detection of proteins containing phosphoserine, phosphothreonine and phosphotyrosine in bacteria expressing the Nek1 kinase. (**A**) Phosphoamino acid analysis of total ^{32}P-labeled protein from induced bacteria expressing the parental plasmid pGEMEX-1 (CON) or pGEMEX-N2, which encodes Nek1 residues 1–576 (NEK1). (**B**) Phosphoamino acid analysis of selected ^{32}P-labeled proteins obtained by anti-phosphotyrosine immunoprecipitation of induced pGEMEX-N2 lysates. Molecular weights ($\times 10^{-3}$) of the three bacterial proteins are indicated. pS = phosphoserine; pT = phosphothreonine; pY = phosphotyrosine.

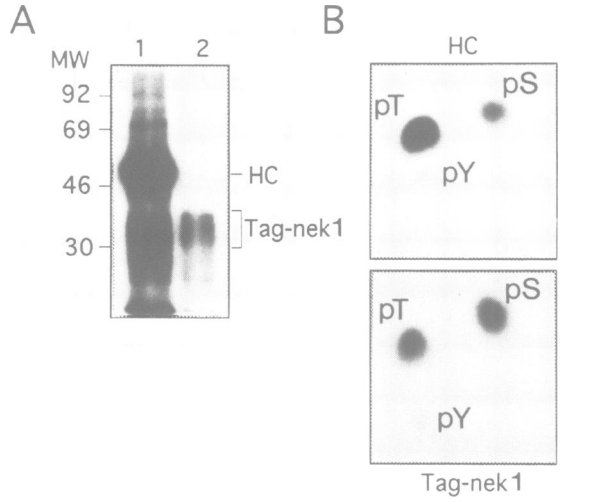


Fig. 5. *In vitro* kinase activity and autophosphorylation of bacterially expressed Tag–Nek1. A lysate was prepared from bacteria expressing Tag–Nek1, and immunoprecipitated with anti-Tag antibodies. Immune complex kinase assays were performed in the presence of [γ-^{32}P]ATP, as described in Materials and methods. (**A**) Total ^{32}P-labeled products of the anti-Tag immune complex kinase assay (lane 1); proteins in the anti-Tag immune complex kinase reaction were boiled in 2% SDS, and the solubilized proteins were diluted to 0.1% SDS and re-immunoprecipitated with anti-Tag antibody (lane 2). The positions of radiolabeled Ig heavy chain (HC) and Tag–Nek1 fusion protein(s) are indicated. (**B**) Phosphoaminoacid analysis of ^{32}P-labeled proteins present in the Tag–Nek1 *in vitro* kinase reaction. Ig heavy chain and Tag–Nek1 were isolated from the samples shown in lanes 1 and 2, respectively, of panel A.

containing the entire Nek1 polypeptide gave very similar results in these kinase assays to the C-terminally truncated Nek1 protein (data not shown). The substrate specificity of Nek1 is therefore related to that of NIMA, which preferentially phosphorylates β-casein *in vitro* (A.H.Osmani *et al.*, 1991b).

In situ localization of nek1 transcription in the gonads

The expression of the murine *nek1* gene was analyzed initially by Northern blot analysis using RNA from adult mouse tissues. Under stringent hybridization conditions, two major *nek1* RNA transcripts of 6.5 and 4.4 kb were observed. The levels of *nek1* RNA were ~10-fold higher in testis than in other tissues examined, though transcripts were also detected in ovary, thymus and lung (Figure 7). After longer exposure *nek1* RNA was detected in all tissues examined (data not shown).

To identify the cell types expressing *nek1* we localized *nek1* transcripts in both male and female gonads by *in situ* RNA hybridization analysis. Tissue sections of gonads from embryos at 15.5 days post-coitum (dpc) and from adult gonads were hybridized to *nek1* antisense probes and to a sense control probe. Three different antisense probes, complementary to a section of the coding region or to 3′-untranslated regions, were used for the *in situ* analysis (see Materials and methods). These probes gave essentially identical results, confirming specificity of the signal for *nek1*. The sense control probe did not give a specific signal in any of the tissues examined (data not shown).

In the ovaries of female mouse embryos at 15.5 dpc, high levels of *nek1* expression were restricted to the germinal tubules (Figure 8A and B). At this stage, oocytes are in the zygotene or pachytene stages of meiosis (Monk and McLaren, 1981). At higher magnification it was evident that *nek1* transcripts were present both in oocytes and in cells that immediately surround the oocytes, presumably granulosa cells (data not shown). In the adult ovary the expression of *nek1* RNA was also restricted to oocytes and granulosa cells, but varied with the developmental stage of the follicles (Figure 8C and D) (staging of follicle maturation according to Ross, 1985). *nek1* RNA was undetectable in primordial and primary follicles. However, as follicles entered the secondary stage, characterized by the acquisition of a distinct theca layer, high levels of *nek1* RNA were expressed in the oocytes, while much lower levels were present in the surrounding, proliferating granulosa cells. Oocytes continued to express high levels of *nek1* in successive stages until ovulation, whereas *nek1* expression in the antral granulosa cells declined slightly, concomitant with the cessation of granulosa cell proliferation. Another site of *nek1* expression was the early corpus luteum. No signal was observed in proliferating theca or endothelial cells at any stage of follicular growth, nor in interstitial cells.

The patterns of *nek1* expression in the male reproductive

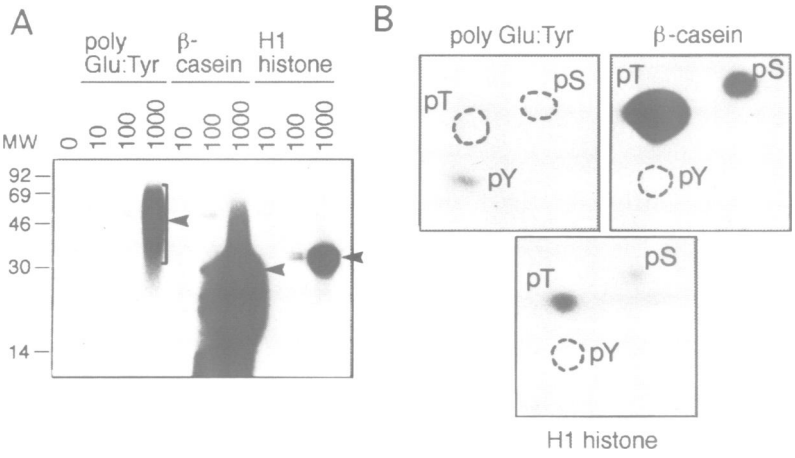


Fig. 6. Bacterially expressed Tag−Nek1 phosphorylates the exogenous substrates poly(Glu:Tyr), β-casein and H1 histone. (**A**) Tag−Nek1 immune complex kinase assays performed in the presence of increasing concentrations (0−1000 μg/ml) of the three exogenous substrates. The mobilities of the three substrates detected by Coomassie blue staining of the same gel are shown by arrows. Note that the β-casein preparation contained a minor proportion of smaller polypeptides (<30 kDa) which were also phosphorylated in this assay. (**B**) Phosphoamino acid analysis of ^{32}P-labeled poly(Glu:Tyr), β-casein and H1 histone obtained following the Tag−Nek1 *in vitro* kinase assay.

system were essentially homologous to those found in the female. In the 15.5 dpc testis, *nek1* transcripts were largely confined to the germinal tubules and, as in the female embryo, both germ cells (prospermatogonia) and the surrounding Sertoli cells expressed *nek1* (data not shown). However, the levels of *nek1* expression were lower than those observed in corresponding female cells. In the mature testis, the highest levels of *nek1* expression were observed in germ cells (Figure 9). Interestingly, *nek1* expression was clearly stage specific. Low levels of *nek1* RNA were observed in peripheral spermatogonia, while much higher levels were found in spermatocytes as they proceeded through the prophases of meiosis, and in the immediate post-meiotic round haploid spermatids. As round spermatids developed into elongated spermatids and spermatozoa (at around stage 10 of spermiogenesis: Oakberg, 1956), the *nek1* signal disappeared and *nek1* transcripts were not observed in spermatozoa stored in the epididymis (Figure 9). Low levels of *nek1* RNA were also observed in Sertoli cells, but not in interstitial Leydig cells, paralleling the patterns of *nek1* expression in the ovary, in which granulosa cells were positive while theca and interstitial cells were negative.

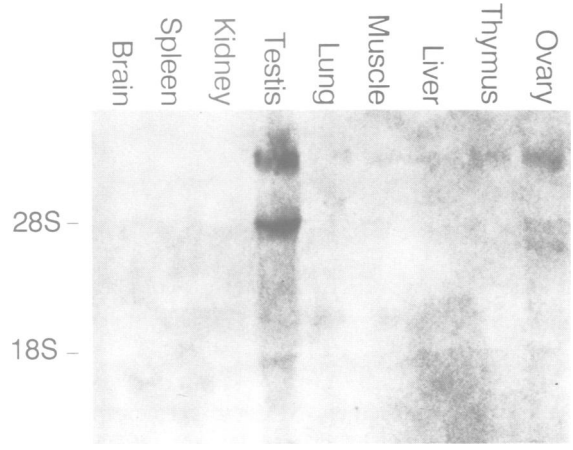


Fig. 7. Northern blot analysis of *nek-1* expression in adult mouse tissues. 10 μg of total RNA extracted from the indicated tissues was resolved by formaldehyde denaturing gel electrophoresis and transferred onto a nitrocellulose filter. The filter was hybridized to a ^{32}P-labeled probe randomly primed from the H4 cDNA (Figure 1A) and washed at high stringency prior to autoradiography.

Discussion

A mammalian gene product related to NIMA

Screening of mouse cDNA expression libraries with anti-phosphotyrosine antibodies has netted several protein kinases that are related to the products of genes involved in signal transduction and cell cycle control. One of these, *nek1*, is most similar to the *nimA* cell cycle gene of *A.nidulans*. In *A.nidulans*, the cell cycle is apparently controlled by two independently acting genes, *cdc2* and *nimA* (A.H.Osmani *et al.*, 1991a). The p34^{cdc2} protein kinase of eukaryotic cells is a pleiotropic and central regulator of the cell cycle, whose activity is required both for transit through G_1 into S phase, and for passage from G_2 into M phase (Lewin, 1990; Nurse, 1990).

Mutations in the *nimA* gene of *A.nidulans* arrest cells in G_2 and prevent their entry into mitosis (Oakley and Morris, 1983). Genetic evidence suggests that the *nimA* gene product represses the action of *bimE*, which normally inhibits entry into mitosis (A.H.Osmani *et al.*, 1991b). Conversely, overexpression of *nimA* advances entry into mitosis (S.A.Osmani *et al.*, 1988b). *nimA* encodes a protein kinase (NIMA) whose activity is regulated during the cell cycle, and peaks at mitosis (A.H.Osmani *et al.*, 1991b). In addition to controlling the initiation of mitosis, *nimA* is also important for the organization of the mitotic spindle and the nuclear envelope (S.A.Osmani *et al.*, 1988a; A.H.Osmani *et al.*, 1991b). The activities of p34^{cdc2} and NIMA in *A.nidulans* are independently regulated during the cell cycle, suggesting that they lie on separate pathways, and that both are necessary for entry into mitosis (A.H.Osmani *et al.*, 1991a).

The mouse Nek1 and *A.nidulans* NIMA proteins are 42% identical in their kinase domains, suggesting that they may have similar biochemical functions and substrate specificities. Indeed, both the NIMA and Nek1 kinases are active in the phosphorylation of β-casein *in vitro*. Although the close sequence relationship between Nek1 and NIMA is confined to the kinase domain, both proteins have a long, exceptionally basic C-terminal extension. Hence NIMA and Nek1 have

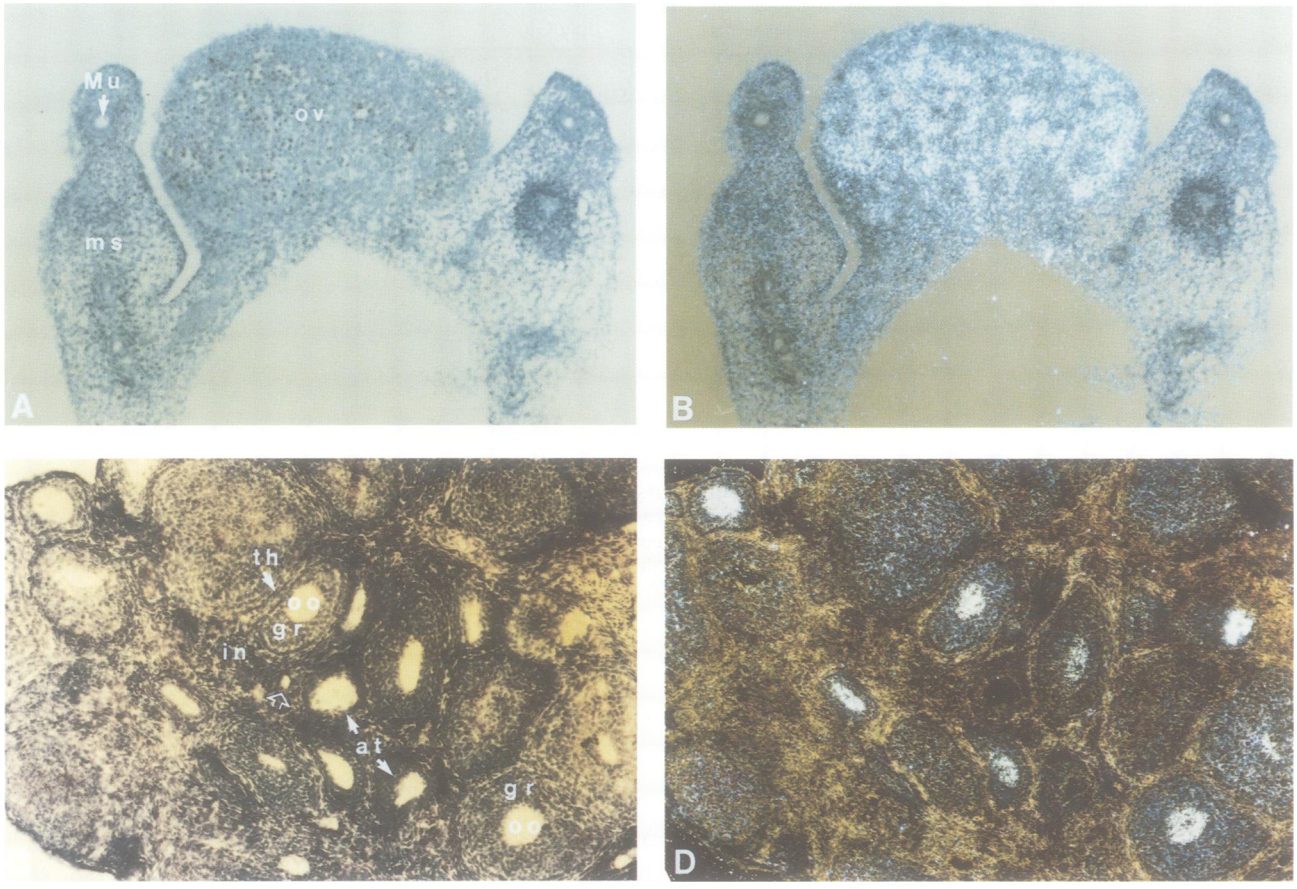


Fig. 8. *In situ* analysis of mouse *nek1* RNA in the ovary. (**A**) Bright-field and (**B**) combined bright- and dark-field micrographs of a transverse section through a 15.5 dpc embryonic ovary hybridized to a *nek1* antisense probe. ms, mesonephric tubules; Mu, Muellerian duct; ov, ovarian follicle. (**C**) Bright-field and (**D**) dark-field micrographs of an ovary from a 25 day-old mouse hybridized to a *nek1* antisense probe. at, atretic follicle; gr, granulosa (follicular) layer; in, interstitial cells; oo, oocyte; th, theca layer; open arrow, primary follicle (no hybridization).

the same overall structure. NIMA may therefore be the prototype for a family of protein kinases, of which Nek1 is the first mammalian member. It remains to be established whether Nek1 is the precise mammalian homologue of NIMA from *A.nidulans*, or whether there are mammalian family members more closely related in sequence or function to NIMA. The identification of *nek1* indicates that a relative of *nimA* exists in the mouse, consistent with the notion that *nimA*-related genes, like *cdc2* and CDKs (Lee and Nurse, 1987; Tsai *et al.*, 1991) have been conserved during eukaryotic evolution.

Nek1 is a dual specificity kinase

nek1 cDNAs were the most frequently isolated clones in a functional screen designed to identify mammalian sequences encoding protein tyrosine kinases. The Nek1 gene product, however, has sequence motifs that are generally considered diagnostic of protein serine/threonine kinases. This suggested that Nek1 might belong to a novel group of protein kinases that can phosphorylate both serine/threonine and tyrosine residues. Consistent with this possibility, Nek1 induced the tyrosine phosphorylation of exogenous proteins in *E.coli*, although its principal effect was to increase markedly the phosphorylation of serine and threonine residues. Thus, under the conditions of this assay, Nek1 is primarily a protein serine/threonine kinase, with a more modest tyrosine kinase activity. Similarly, in *in vitro* kinase assays, Nek1 was most active in the phosphorylation of β-casein and H1 histone, which were phosphorylated exclusively on serine and threonine residues. Whether its potential to phosphorylate both serine/threonine and tyrosine is manifested in mammalian cells remains to be established. There is, as yet, no evidence that NIMA itself can phosphorylate tyrosine residues, although it is an active protein kinase which, like Nek1, can utilize β-casein as substrate (A.H.Osmani *et al.*, 1991b).

It is intriguing that protein kinases linked directly or circumstantially to control of the mitotic and meiotic cell cycle, such as Wee1, Nek1, Clk and the MAP kinase family, have the potential for tyrosine phosphorylation. These results imply that, in addition to the conventional tyrosine kinases acting at the plasma membrane to initiate and regulate cytoplasmic signaling pathways, there is a second group of dual specificity protein serine/threonine and tyrosine kinases acting in the immediate control of the cell cycle. Phosphorylation of Tyr15 of $p34^{cdc2}$ is known to be important in regulating G_2-M phase transition. It seems possible that other tyrosine phosphorylation events are also involved in cell cycle control.

The structural features of dual specificity protein kinases that permit the phosphorylation of all three hydroxyamino acids are not immediately obvious (Lindberg *et al.*, 1992). Two motifs have been suggested as possible hallmarks of dual specificity kinases. One involves the sequence Arg-X-Tyr-Arg in subdomain VIII (Wu *et al.*, 1991); this is absent from Nek1, as well as Wee1. The second motif is in

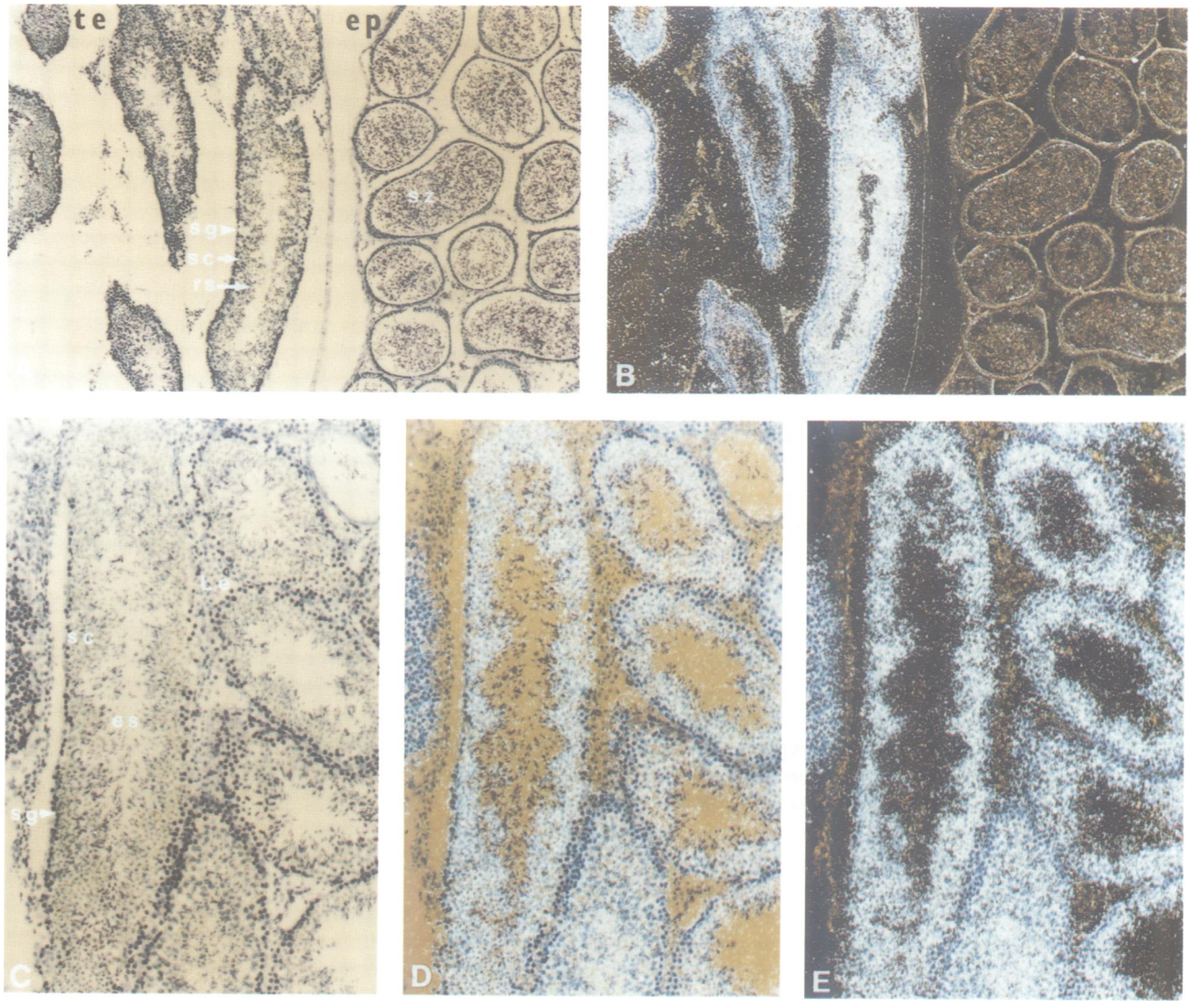


Fig. 9. (**A**) Bright-field and (**B**) dark-field micrographs of sections through the testis (te) and epididymis (ep) of a 2 month-old mouse, hybridized to a *nek1* antisense probe. Note the very high levels of expression in spermatocytes (sc) and round spermatids (rs) and the lack of signal over spermatozoa (sz) stored in the epididymis. (**C**) Bright-field, (**D**) combined bright- and dark-field and (**E**) dark-field high power micrographs of seminiferous tubules of adult testis hybridized to a *nek1* antisense probe. Note the low levels of expression in the peripheral spermatogonia (sg) and the lack of expression in elongated spermatids (es) and in the interstitial Leydig (Le) cells.

subdomain XI, where several dual specificity kinases have related sequences (Seger *et al.*, 1991). However, the possible influence of this region in substrate specificity is unclear, since it is some distance from the active site observed in the crystal structure of cAMP-dependent protein kinase (Knighton *et al.*, 1991a,b).

Possible involvement of nek1 in meiosis

As noted above, the NIMA kinase in *A.nidulans* is known to be involved in cell cycle control. By determining the sites of *nek1* expression, we sought to determine whether this kinase could serve a similar role in mammals. The Northern and *in situ* RNA analyses do not support an absolute requirement for *nek1* in mitosis. In some cases there was a correlation between cell proliferation and the levels of *nek1* expression. For example, in the granulosa cell layer, the highest levels of *nek1* expression correlated with rapid mitotic activity, and the decline in granulosa cell proliferation coincided with a decrease in *nek1* RNA levels. However, there was no general correlation between *nek1* expression and the mitotic activity of the tissues we examined. For example, only low levels of *nek1* transcripts were observed in the spleen, even following mitogenic activation with lipopolysaccharide for 24 h (data not shown). In addition, *in situ* RNA analysis showed that *nek1* expression was not detectable in proliferating theca and endothelial cells of growing ovarian follicles. However, as discussed above, it is possible that *nek1* is the founding member of a family of mammalian NIMA-related kinases that have similar or overlapping roles during mitosis of cells within different lineages. It is also possible that constitutively low levels of *nek1* RNA are expressed by all proliferating cells, and that *nek1* activity is governed by post-transcriptional and/or post-translational modifications of this protein kinase.

Although our results do not support a general role for *nek1* in mitosis, the patterns of *nek1* expression are consistent with the possible involvement of this gene in meiosis. Specifically, much higher levels of *nek1* RNA were found in meiotic 15.5 dpc oocytes than in mitotically arrested prospermatogonia of the same age. In the postnatal ovary, *nek1* expression was not detected in resting primordial and primary oocytes; however, *nek1* transcripts accumulated at the secondary

follicular stage and persisted at high levels until ovulation. Similar patterns of expression in oocytes have been reported for the c-*mos* serine/threonine kinase (Keshet *et al.*, 1988; Mutter *et al.*, 1988) and the c-*kit* receptor tyrosine kinase (Manova *et al.*, 1990; Orr-Urtreger *et al.*, 1990). c-*mos* activates maturation promotion factor thus enabling oocytes to mature (Sagata *et al.*, 1988), while c-*kit* is involved in germ cell survival (Dolci *et al.*, 1991; Godin *et al.*, 1991). However, as most of the mRNA transcribed by a growing oocyte is translated only later during meiotic maturation, reductive divisions and very early embryogenesis (for review see Bachvarova, 1985), the Nek1 protein kinase could function in any of these processes.

The patterns of *nek1* expression in the testis are also associated with meiosis. During spermatogenesis *nek1 is* highly expressed in germ cells actively involved in meiosis, and a sharp decline in *nek1* mRNA levels was observed around the transition from round to elongated spermatids. Stage-specific transcription during sperm development has been reported previously for c-*mos*, c-*abl*, *fer*T, *mak* and c-*kit* protein kinases (Goldman *et al.*, 1987; Meijer *et al.*, 1987; Propst *et al.*, 1988; Keshet *et al.*, 1990; Manova *et al.*, 1990; Matsushime *et al.*, 1990). Our results suggest that Nek1 may be one of several protein kinases required for the survival and development of germ cells. In summary, we have described the isolation of a novel mammalian dual specificity protein kinase that is related both structurally and biochemically to the NIMA mitotic kinase of *A.nidulans*, and is implicated in the control of meiosis. *nimA*-related kinases have therefore apparently been conserved in eukaryotic evolution.

Materials and methods

Isolation of cDNA clones

Construction of the λgt11 cDNA expression libraries and expression screening using anti-phosphotyrosine antibodies has been described previously (Letwin *et al.*, 1988; Ben-David *et al.*, 1991). The libraries were rescreened using H4 DNA in a nucleic acid hybridization screen to isolate additional *nek*1 sequences.

Subcloning and nucleotide sequence determination

Phage DNA was digested with *Not*I and the cDNA insert was gel purified prior to subcloning into pGEM-5Zf(+). For complete sequence determinations, nested deletions spanning the cDNA insert of desired clones were generated using the method of Hennikoff (1987). These constructs were sequenced by the dideoxynucleotide chain termination method using reagents and protocols supplied by United States Biochemicals Corporation. Sequences were obtained for the ends of all clones, but only clones N2, H4, 23 and 49 were fully sequenced. Reported sequence was confirmed on both strands.

Sequence comparisons

All sequence manipulations were done on a micro VAX using the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package. A library search against the entire GenBank and EMBL databases was conducted with the *nek*1 sequence using the FASTA program.

Construction of Nek1 bacterial expression vectors

The plasmid pN_2Not12 was constructed by subcloning the 2.3 kb *Not*I cDNA insert of λN2 into the *Not*I site of pGEM 5Zf(+). The expression vector pGEMEX-N2 was constructed by subcloning the 1.7 kb *Nco*I (blunted with Klenow)−*Sal*I fragment of pN_2Not12 into the *Nhe*I (blunted with Klenow) and *Sal*I sites of pGEMEX-1. The *nek*1 specific insertion covers the N-terminal 576 amino acids of the protein (including the entire catalytic domain plus 318 amino acids of the tail region) fused in-frame with the first three amino acids of the T7 gene 10 leader sequence located in the pGEMEX-1 polylinker region.

For routine plasmid growth, pGEMEX-N2 was transformed into JM101 and grown in Luria broth (LB) supplemented with 100 μg/ml ampicillin (amp). Initial Nek1 protein induction experiments indicated that the expressed product was toxic to the JM101 host. Therefore, to study Nek1 protein expression, pGEMEX-N2 was transformed into *E.coli* BL21 (λDE3plysS)(Novagen) to suppress basal transcription of the toxic *nek1* gene product maximally.

The expression vector pTag−Nek1 was constructed by subcloning the 798 bp *Nco*I(blunted with Klenow)−*Xmn*I(blunted with Klenow) cDNA fragment from pN_2Not12 (see above) into the *Bam*HI (blunted with Klenow) site of pET11a (Novagen). The resultant insert contains the N-terminal 263 amino acids of Nek1 (including the entire catalytic domain plus five amino acids of the tail region) fused in-frame with the first 13 amino acids of the upstream T7 gene 10 leader peptide (Tag) of pET11a. To study pTag−Nek1 protein expression, the expression vector was transformed into *E.coli* BL21 (λDE3plysS) (Novagen).

Preparation of bacterial lysates

E.coli BL21 (λDE3plysS) harboring the Nek1 expression plasmids pGEMEX-N2, pTag−Nek1, or appropriate control plasmids were grown to saturation in LB-amp supplemented with 30 μg/ml chloramphenicol (chlor) at 37°C. For Nek1 protein induction, overnight cultures were diluted 1/100 into 1 ml LB-amp-chlor, grown at 37°C and induced at OD_{600} = 0.5 with 1 mM isopropyl-β-D-thio-galactopyranoside (IPTG). Typically, cultures were harvested between 2 and 4 h after IPTG induction and growth at 37°C (or as indicated in the relevant Figure legends) and stored as pellets at −70°C.

Immunoblotting of bacterial lysates

Frozen bacterial pellets were lysed in 200 μl of 100°C Laemmli sample buffer (2.3% SDS, 5% 2-mercaptoethanol, 62.5 mM Tris−HCl pH 6.8, 10% glycerol) and maintained at 100°C for 5 min. Equivalent amounts of lysate (as judged by Coomassie blue staining) were resolved by SDS−PAGE and transferred to nitrocellulose. Blotted proteins were probed with 1 μg/ml affinity-purified rabbit anti-phosphotyrosine antibodies as described (Letwin *et al.*, 1988) or with rabbit anti-T7 gene 10 leader peptide sera (anti-Tag) as recommended by the manufacturer (Novagen). Visualization of bound antibodies was mediated by a goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) and the chromogenic substrates NBT and BCIP.

Immunoprecipitation of radiolabeled bacterial lysates

Following a 2−4 h induction period, 1 ml cultures of *E.coli* BL21 (λDE3plysS) harboring the control plasmid pGEMEX-1 or the Nek1 expression plasmids pGEMEX-N2 or pTag−Nek1 were labeled directly in LB-amp-chlor (plus IPTG where indicated) with either 1 mCi/ml $^{32}P_i$ (ICN:800 mCi/ml) or 500 μCi/ml [^{35}S]methionine/cysteine (ICN Translabel:15 mCi/ml) for 1 h at 37°C. The radiolabeled cells were stored at −70°C. Bacterial pellets were either extracted for total ^{32}P-labeled protein as described (Cortay *et al.*, 1991) or lysed by sonication in PLC lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM $NaPP_i$, 100 mM NaF, 1 mM PMSF) plus 1 mM DTT. The lysate was clarified at 20 000 *g* for 15 min and the supernatant was immunoprecipitated, where indicated, with either 5 μg/ml anti-phosphotyrosine serum or anti-Tag serum as recommended by the manufacturer (Novagen) using protein A−Sepharose beads (Pharmacia). Immunoprecipitates were washed in PLC lysis buffer plus DTT, resuspended in Laemmli sample buffer and heated at 100°C for 5 min. Radiolabeled proteins were resolved by SDS−PAGE and visualized by autoradiography.

In vitro kinase assay of bacterially expressed Tag−Nek1

Anti-Tag immunoprecipitates from lysates of induced bacteria expressing pTag−Nek1 were prepared as described above. Following extensive washing in PLC lysis buffer plus DTT, immunoprecipitates were washed in optimized kinase reaction buffer (100 mM Tris−HCl, pH 7.5, 5 mM $MnCl_2$, 2 mM DTT, 1 μM ATP) and finally resuspended in kinase reaction buffer containing [γ-^{32}P]ATP (Amersham) (3 μCi per reaction) and where indicated exogenous polypeptide substrate (total reaction volume = 50 μl). The kinase reactions were incubated for 30 min at 22°C then terminated by addition of an equal volume of 2× Laemmli sample buffer and heating at 100°C for 5 min. The *in vitro* kinase reactions were resolved by SDS−PAGE, and the ^{32}P-labeled proteins visualized by autoradiography.

Phosphoamino acid analysis

^{32}P-labeled proteins resolved by SDS−PAGE were transferred to PVDF membranes (Immobilon; Millipore Corp.), visualized by autoradiography, and pertinent regions of the PVDF membrane were excised. Isolated ^{32}P-labeled proteins were subjected to phosphoamino acid analysis (Kamps and Sefton, 1988) as previously described (Ben-David *et al.*, 1991).

In situ RNA hybridization

C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). *In situ* RNA hybridization analysis was performed on 10 μm cryostat sections as described previously (Motro *et al.*, 1991). Adjacent sections were hybridized with *nek1* antisense or sense control probes. To assess the specificity of the signal observed, three different fragments were used as antisense probes. Fragment 1 was derived from the N2 subclone (Figure 1) linearized at the *Eco*RI site. The RNA transcribed by SP6 was 259 bases long and spans nucleotides 2046–2304. This region encodes part of the 'tail' domain. Fragment 2 was derived from the 23 subclone (Figure 1) linearized at the *Bgl*II site. The RNA transcribed by SP6 was 691 bases long and spans nucleotides 3571–4261 in the 3′-noncoding region. Fragment 3 was derived from the entire 23 subclone [linearized at the *ApaI* site in the pGEM-5Zf (Promega) vector]. The RNA transcribed by SP6 was 2278 bases long and spans nucleotides 2384–4261. This region includes the last 61 coding bases of the 'tail' and the 3′-noncoding sequences. Fragment 3, synthesized in the opposite direction by T7 polymerase, was used as a sense control. Post-hybridization washings included treatment with 50 μg/ml RNase A (Sigma) at 37°C for 30 min and two stringent washes of 20 min each at 60°C in 0.1×SSC. Following dehydration, the slides were dipped into NTB-2 emulsion (Kodak), exposed at 4°C for 5–8 days, developed and stained with toluidine blue. All three antisense probes gave similar results, confirming the specificity of the signal observed. The sense control probe did not give a specific signal over any of the slides examined. All experiments were done on multiple sections from different animals. Ovaries were examined from both naturally cycling and superovulated females (Hogan *et al.*, 1986) at different points in their cycle.

RNA extraction and Northern blotting

Total cellular RNA from adult mouse tissues was isolated and analyzed by Northern blotting as previously described (Ben-David *et al.*, 1991). The filter was hybridized with a random primed *nek1* cDNA probe corresponding to the H4 clone.

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