Cell Cycle-dependent Expression of Nek2, a Novel Human Protein Kinase Related to the NIMA Mitotic Regulator of Aspergillus nidulans

Sharon J. Schultz, Andrew M. Fry, Christine Sütterlin, Thomas Ried, and Erich A. Nigg
Swiss Institute for Experimental Cancer Research, Chemin des Boveresses 155, CH-1066 Epalinges, Switzerland; and Department of Human Genetics, University of Heidelberg, Im Neuenheimer Feld 328, D-6900 Heidelberg, Germany [T. R.]

Abstract
The serine/threonine protein kinase NIMA of Aspergillus nidulans is required for entry into mitosis and may function in parallel to the universal mitotic inducer p34cdc2. Here, we report the isolation of complementary DNAs encoding Nek2 and Nek3, two novel human protein kinases structurally related to NIMA. Sequence comparisons revealed several unique features which may define a family of NIMA-related protein kinases. Nek2 was chosen for further study since it represents the closest known mammalian relative of NIMA. Chromosomal mapping of the nek2 gene identified two independent loci on chromosomes 1 and 14, and Northern blot analyses revealed the expression of two distinct mRNAs of approximately 2.4 and 4.7 kilobases in all human cell lines examined. In HeLa cells synchronized by both drug arrest and elutriation, a strikingly cell-cycle-dependent pattern of Nek2 expression could be observed; Nek2 protein was almost undetectable during G1, but accumulated progressively throughout S, reaching maximal levels in late G2. These observations demonstrate that Nek2 resembles Aspergillus NIMA, not only in its catalytic domain, but also in its cell-cycle-dependent expression. Hence, the human Nek2 protein kinase may also function at the onset of mitosis.

Introduction
Reversible protein phosphorylation plays a key role in controlling progression through the cell cycle in all eukaryotes. Prominent among the serine/threonine protein kinases involved in cell cycle regulation are p34cdc2 and structurally related cdk's. These kinases require cyclin binding for activity, and they are regulated by reversible phosphorylation (1-4). Different cdk/cyclin complexes control distinct transitions in the cell cycle, particularly entry into mitosis and the initiation of DNA synthesis (5-7). Although the cardinal importance of cdk/cyclin complexes in cell cycle progression is well established, these protein kinases are unlikely to function as the sole regulators of the cell cycle. In particular, several lines of evidence suggest that activation of p34cdc2 protein kinase may not be sufficient to initiate all the events necessary for mitosis and that other regulatory pathways may cooperate with cdk/cyclin complexes in controlling cell cycle progression (8-11).

In the filamentous fungus Aspergillus nidulans, a serine/threonine-specific protein kinase termed NIMA has been implicated in controlling entry into mitosis, although it is structurally unrelated to p34cdc2 (11-14). A temperature-sensitive nimA allele arrests cells in G2 at the nonpermissive temperature (15, 16), whereas overexpression of the NIMA protein kinase drives cells into mitosis from any stage of the cell cycle (13). Most interestingly, cells expressing inactive NIMA arrest in G2, despite the fact that they exhibit active p34cdc2; conversely, cells expressing inactive p34cdc2 arrest in G2 with active NIMA (11). These results suggest that p34cdc2 and NIMA may be activated independently and that the activation of both kinases is required for entry into mitosis.

Many of the basic features of cell cycle control have been conserved in all eukaryotes from yeast to man (6). Thus, it would seem likely that protein kinases functionally analogous to Aspergillus NIMA might be expressed in vertebrates as well. In support of this possibility, a NIMA-related protein kinase has recently been identified in the mouse (17, 18). This kinase, Nek1, was isolated by screening an expression library with antiphosphotyrosine antibodies and displays dual specificity for serine/threonine and tyrosine residues. While its function remains unknown, the high levels of Nek1-specific transcripts detected in male and female germ cells suggest a role for Nek1 in meiosis (18).

Using a polymerase chain reaction approach with degenerate oligonucleotides corresponding to sequences conserved within the catalytic domain of NIMA, we have recently isolated three different partial cDNAs, all representing human NIMA-related protein kinases (19). While one of these cDNAs (HsPK 20) was closely related to the murine Nek1, the other two (HsPK 21 and HsPK 36) represented novel NIMA-related protein kinases and accordingly were named Nek2 and Nek3, respectively. Here we report the isolation and sequencing of near full-length cDNAs coding for Nek2 and Nek3. The nek2 cDNA encodes the entire Nek2 protein, but the nek3 cDNA lacks some coding information at its 5' end. A comparison of the structural organization and catalytic domain sequences of Nek2 and Nek3 with those of NIMA and Nek1 reveals that all four kinases share unique features and appear to be members of a related family. Because Nek2 is most closely related to NIMA, it was analyzed in further detail. Specifically, we have studied the chromosomal localization of the nek2 gene and its expression in several human cell lines.
Furthermore, we show that Nek2 displays a strikingly cell cycle-dependent pattern of expression with maximal levels of protein being detectable prior to the onset of mitosis. These data are consistent with the notion that human Nek2, like Aspergillus NIMA, may function in cell cycle control, particularly at the G2-M transition.

Results

Isolation of the Human nck2 and nck3 cDNAs. Two short (340–360 bp) cDNA fragments encoding parts of the catalytic domains of Nek2 and Nek3, respectively, were originally isolated using a polymerase chain reaction-based strategy aimed at identifying human protein kinases related to the cell cycle regulator NIMA of A. nidulans (19). Here, these cDNA fragments were used to screen several human cDNA libraries by DNA hybridization. Six cDNAs were isolated for nck2 (Fig. 1A) and seven cDNAs for nck3 (Fig. 1B). They were subcloned into pGEM vectors and partially or entirely sequenced as described in detail in "Materials and Methods.”

Fig. 2 shows the 2051-bp sequence of the largest nck2 insert (cDNA clone 21–29) and the translation product corresponding to the largest open reading frame (nucleotides 83–1417). The predicted Nek2 polypeptide has 445 residues with a calculated molecular mass of 51.7 kDa; it displays all of the hallmarks of a protein kinase (20). The first in-frame ATG codon (beginning at nucleotide 83) is within a suitable sequence context for an initiator methionine (21), suggesting that this cDNA contains the entire coding information for Nek2. This interpretation is supported by the results shown below (see Fig. 8).

The longest insert obtained for nck3 (cDNA 36-8) was 1787 bp in length. However, cDNA clones 36-11 and 36-10 were found to contain additional 5' and 3' sequences not present in clone 36-8 (Fig. 1B). The combined sequence information from these cDNAs is shown in Fig. 3. The nck3 sequence begins with an open reading frame (nucleotides 1–1377) but lacks the initiator methionine and hence does not represent the entire coding region for the Nek3 protein. The available protein sequence codes for 459 amino acids, indicating that Nek3 has a molecular mass of at least 51 kDa. The sequence displays the characteristic features of a protein kinase but lacks the most NH2-terminal GXGXXG motif of the catalytic domain. So far, we have been unable to isolate a cDNA spanning the entire coding region of Nek3. However, considering that the catalytic domains of NIMA, Nek1 and Nek2 are all located at the NH2 terminus (Figs. 4A and 5), it is attractive to postulate that Nek3 may have a similar overall structure, in which case, sequence information encoding approximately 50 amino acids would be missing from the nck3 cDNA described here.

The open reading frames of the nck2 and nck3 cDNAs were confirmed by in vitro translation (data not shown); in the case of the nck3 cDNA, an initiator methionine was provided by the NH2-terminal addition of a myc epitope tag (for a detailed description, see “Materials and Methods”).

Nek2 and Nek3 Are Related to NIMA and Nek1. When the sequences of nck2 and nck3 were compared against databases, both were found to be unique, with Aspergillus NIMA (13) and murine Nek1 (18) being their closest relatives. Like NIMA and Nek1, Nek2 has its catalytic domain located at the NH2 terminus, and its COOH terminus also displays a very basic isoelectric point, although it is substantially shorter (Fig. 4A). In contrast, the COOH terminus of Nek3 is acidic (Fig. 4A). The extent of similarity between the three proteins was determined using the BestFit program (Fig. 4B). Of all presently known mammalian kinases, Nek2 displays the highest degree of sequence similarity with NIMA. Over the catalytic domain, Nek2 shares, at the amino acid level, 47% sequence identity with Aspergillus NIMA but only 43% with murine Nek1. In contrast, the catalytic domain of Nek3 appears to be more closely related to that of Nek1 (58%) than to that of NIMA (43%), at least as judged from the incomplete sequence presently available. The COOH termini of the three kinases show little sequence similarity, but those of Nek1, Nek2, and Nek3 appear more closely related to each other (identities ranging from 24 to 26%) than to that of NIMA (14 to 19%).

Several features in the catalytic domains of NIMA and the Nek protein kinases are unique and may thus define a family of NIMA-related protein kinases (Fig. 5). One shared motif is the sequence LX(I/L)X(M/E)DXCXXGD found in subdomain V; this motif is clearly distinct from similar motifs found in members of the cAMP-dependent protein kinase family (20). Another potentially diagnostic motif is the sequence CX(L/K)MYXLC located just COOH-terminal to the conserved DXWXXG sequence found in subdomain IX. Finally, it is noteworthy that within the GXGXXGXV motif of
and tentative cDNA signal. Fig. 2, Chromosomal localization plays a role in kinases. Chromosomes 1, 2, 3, 4, 5, and 6 are indicated by numbers in parentheses. Amino acid residues that are highly conserved among protein serine/threonine kinases (20) are underlined. A potential cyclin-like mitotic destruction box (spanning residues 361–369) and adjacent lysine residues (381–383) in the COOH-terminal end domain are marked by arrowheads and Q, respectively.

Fig. 2. Nucleotide and deduced amino acid sequence of nek2. Amino acids are given in single letter code. Amino acid positions are numbered, and nucleotide positions from the beginning of the cDNA are indicated by numbers in parentheses. Amino acid residues that are highly conserved among protein serine/threonine kinases (20) are underlined. A potential cyclin-like mitotic destruction box (spanning residues 361–369) and adjacent lysine residues (381–383) in the COOH-terminal end domain are marked by arrowheads and Q, respectively.

Chromosomal Localization of nek2 Identifies Two Loci on Chromosomes 1 and 14. To identify the chromosomal localization of the nek2 gene, 18 randomly selected metaphase plates were analyzed by fluorescence in situ hybridization using a biotinylated nek2 cDNA as a probe (22). In 60% of the metaphases, two distinct chromosomal mapping positions could be observed; these were on the long arm of chromosome 1 at position 1q32.2–1q41 and on the long arm of chromosome 14 at position 14q12. The signals observed on chromosome 14 were consistently more intense than those on chromosome 1, and no additional signals were detected. The ideogram presented in Fig. 6 shows the chromosomal localizations of nek2 in a schematic form. The hybridization of two distinct loci with the nek2 probe might indicate that the human genome harbors either a pseudogene or a second, closely related functional gene.
Human Cell Lines Express Two Distinct nek2 RNAs. To examine the expression of the nek2 gene, RNA was isolated from several human cell lines and analyzed by Northern blot hybridization using a nek2-specific probe (Fig. 7). Using stringent hybridization conditions, two different RNAs of approximately 2.4 and 4.7 kb were detected. The expression levels of both RNAs, as well as the ratio between the two RNAs, differed between the cell lines examined (Fig. 7). Furthermore, the expression of nek2 transcripts appears to be developmentally regulated (Fig. 7), as indicated by the different levels observed in promyelocytic HL-60 leukemia cells that were induced to differentiate along either a macrophage/monocytic lineage or a granulocytic lineage (in response to treatment with TPA or dimethyl sulfoxide, respectively). At present, we do not know which of the two RNA transcripts gave rise to the 2-kb cDNA described in Fig. 2.

Identification of the Nek2 Protein. Polyclonal antibodies were generated against a bacterially expressed recombinant fusion protein containing the carboxyl terminal 343 amino acids of Nek2. Fig. 8 illustrates the specificity of affinity-purified (R31) anti-Nek2 antibodies as determined by immunoblotting. In total lysates of HeLa (Fig. 8, Lane 2) or promyelocytic HL-60 leukemia cells (Fig. 8, Lane 3), R31 antibodies detected one major protein migrating at approximately 46 kDa (Fig. 8, arrow). However, a minor immunoreactive protein migrating at about 70 kDa (Fig. 8, arrowhead) was also visible in both HeLa and HL-60 cells.

Fig. 3. Nucleotide and deduced amino acid sequence of nek3. Amino acids are given in single letter code. Amino acid positions are numbered, and nucleotide positions from the beginning of the cDNA are indicated by numbers in parentheses. Note that this Nek3 protein sequence is partial and lacks the NH2 terminus. Amino acid residues that are highly conserved among protein serine/threonine kinases (20) are underlined. The translation stop codon and the polyadenylation signal at the 3' end of the nek3 cDNA are also underlined.
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Fig. 4. Protein domain structures and similarities of the NIMA-related protein kinases. A, the domain structures of the NIMA, Nek2, Nek3, and Nek1 protein kinases are illustrated with boxes, and the NH2-terminal catalytic domains are indicated in black. The isoelectric points of the COOH termini were calculated using the ISOELECTRIC program from the University of Wisconsin GCG sequence alignment software package. ND indicates that the total number of amino acids for Nek3 remains unknown. B, summary of the amino acid identities shared between NIMA, Nek2, Nek3, and Nek1.

Discussion

Identification of Human NIMA-related Genes. In this study, we report the identification of two human protein kinases, termed Nek2 and Nek3, both of which are structurally related to the mitotic regulator NIMA of *A. nidulans*. Together with Nek1, a dual-specificity kinase identified recently in mouse (Refs. 17 and 18; see also Ref. 19), Nek2 and Nek3 appear to belong to a novel family of NIMA-related mammalian protein kinases. Within the catalytic domains of all of these kinases, there are two motifs that might tentatively be considered as diagnostic features for this family. The first motif spans the 13 amino acids LY(I/L)XM(E/D)YCXGGDL in subdomain V; the second comprises a somewhat shorter sequence, CX(L/M)YELC, in subdomain IX. The COOH termini of the presently known NIMA-related kinases show little relatedness to each other in either size or amino acid sequence, but it is noteworthy that those of NIMA, Nek1, and Nek2 are all highly basic.

At present, the relationship between *Aspergillus* NIMA and mammalian Nek1, Nek2, and Nek3 is based primarily on structural criteria. As yet, none of the mammalian Nek kinases has been shown to functionally complement a mutation in the *nima* gene of *A. nidulans*, and it remains uncertain to what extent these mammalian kinases are functionally related to NIMA. In particular, all our attempts to complement a *nima* ts mutation by the human *nek2* cDNA have so far been unsuccessful.

Characterization of Nek2. Since several protein kinases have been implicated in the etiology of human diseases (27, 28), we considered it of interest to determine the chromosomal localization of Nek2. Surprisingly, we found that a nek2-specific probe hybridized to two distinct loci, one on the long arm of chromosome 1 (at position 1q32.2–1q41) and the other on the long arm of chromosome 14 (at position 14q12). This result might be explained if one of the two loci were to harbor a pseudogene; alternatively, these two

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loci may code for two functional, closely related \textit{nek2} genes. In the latter context, it is intriguing that both of the loci identified in this study have previously been genetically linked to two different forms of Usher syndrome (29–31), the most commonly recognized cause of combined visual and hearing loss in developed countries (32). Our present results thus indicate that \textit{nek2} and its hypothetical relative might be candidate genes for Usher syndrome. Clearly, more detailed studies will be required to substantiate or refute this possibility.

Consistent with the possible existence of two functional \textit{nek2} loci in the genome, we have also detected two distinct RNAs hybridizing with \textit{nek2}-specific probes in all human cell lines analyzed. It is possible that the two RNAs might represent transcripts originating from two distinct genes on chromosomes 1 and 14. Alternatively, a single active \textit{nek2} gene might give rise to two RNAs by differential splicing. At present, we do not know whether the two observed RNAs of 2.4 and 4.7 kb differ in coding information or merely in nontranslated flanking sequences.

When affinity-purified anti-Nek2 antibodies were used to identify Nek2 protein in human cell lines, they reacted not only with a 46-kDa protein but also with a 70-kDa protein. Whereas the 46-kDa protein has been positively identified as the product of the \textit{nek2} cDNA described here, the molecular identity of the 70-kDa protein remains to be determined. In light of the findings discussed above, it is tempting to speculate that the 70-kDa protein might represent a Nek2-related protein kinase. However, alternative explanations, such as the presence of cross-reacting epitopes on an otherwise unrelated protein, cannot be excluded. In any event, we consider it highly unlikely that the 70-kDa protein could be either Nek1 or \textit{nek3}. The mouse Nek1 is in fact considerably larger (88 kDa) than the cross-reactive protein detected by our anti-Nek2 antibodies, and Northern analyses using \textit{nek3}-specific probes failed to reveal a band comigrating with the Nek2 transcripts.\footnote{C. Sütterlin and S. J. Schultz, unpublished results.}

One important question concerns the substrate specificity of NIMA-related kinases. \textit{Aspergillus} NIMA was reported to phosphorylate both itself and \textit{in vitro} substrates, such as casein, exclusively on threonine and serine residues (33). In contrast, murine Nek1 was shown to be a dual-specificity kinase with the capacity to phosphorylate tyrosine as well as serine and threonine (18). Based on the examination of

\begin{figure}
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\caption{Alignment of the amino acids comprising the catalytic domains of the NIMA-related protein kinases. The catalytic domain sequences of NIMA (residues 1–295), Nek2 (residues 1–271), Nek3 (residues 1–210 of the partial sequence), and Nek1 (residues 1–258) are shown in single letter code. The approximate positions of the subdomains as defined by Hanks and Quinn (20) are indicated above in bold Roman numerals. The heights of the black bars below each residue indicate whether 1 (line), 3 (low bar) or 4 (high bar) residues are conserved among all four kinases. Dots, gaps that were introduced to optimize the alignments.}
\end{figure}
Fig. 6. Chromosomal localization of nek2. A schematic diagram depicting the chromosomal localizations of nek2 is shown. Hybridization of the \(^{32P}\) labeled nek2 cDNA to human metaphase chromosome spreads identified two distinct sites. These sites are indicated with vertical bars on the left sides of chromosome (chr) 1 at position 1q32.2-1q41 and chromosome 14 at position 14q12. The arms of the chromosomes are also indicated (p and q).

Fig. 7. Northern blot analysis of nek2 RNA expression. RNA was prepared from different human cell lines (indicated above each lane) or from HL-60 cells differentiated into granulocytes with 1% dimethyl sulfoxide or macrophages with 30 \(\mu\)M TPA (indicated above first and second lane). Total RNA (10 \(\mu\)g) was electrophoresed in a 2 \(\times\) formaldehyde-1% agarose gel, transferred to nitrocellulose, and probed with a \(^{32P}\)-labeled nek2-specific probe. Size markers are indicated in kilobases (kb).

sequence motifs that may be characteristic for either serine/threonine- or tyrosine-kinases (34, 35), both Nek2 and Nek3 appear to be protein-serine/threonine kinases (Figs. 2 and 3, respectively). However, the location of an alanine at position 145 of Nek2 is reminiscent of small, nonpolar amino acids found at the corresponding position in catalytic subdomain VI in some dual-specificity protein kinases (35). Thus far, we have not been able to determine the substrate specificity of Nek2 experimentally since no specific kinase activity could be detected in Nek2 immunoprecipitates. While these negative results may in part reflect the low abundance of Nek2 protein in all cultured cell lines examined, no activity was detected even in immunoprecipitates from HeLa cells overexpressing Nek2.\(^6\) Thus, it is possible that we have not yet found the right substrate(s) or assay conditions for Nek2 kinase or that our antibodies are inhibitory. In an attempt to overcome these problems, studies are in progress to overexpress Nek2 in insect cells using a baculovirus expression system.

Do the Mammalian NIMA-related Kinases Have a Role in Cell Cycle Regulation?\(^7\). Genetic evidence strongly suggests that the activity of the Aspergillus NIMA kinase is required for the onset of mitosis (11-13, 15, 16), but the precise function of this fungal kinase remains to be determined at a biochemical level. Likewise, no definitive information is presently available on the functions of the mammalian members of the purported NIMA kinase family, except that circumstantial evidence implicates Nek1 in meiosis (18).

In the present study, we have elucidated one important property of Nek2 which strongly suggests that this kinase may have a role at the G2/M transition. Cell synchronization studies based on nocodazole arrest-release experiments as well as on centrifugal elutriation demonstrated

\(^6\) S. J. Schultz, A. M. Fry, and E. A. Nigg, unpublished results.
Human nimA-related Protein Kinases

that the expression level of Nek2 protein is markedly regulated during the cell cycle. In nocodazole-arrested pseudomitotic cells, Nek2 protein was undetectable, but levels increased upon release into interphase. Likewise, in cells that were synchronized by centrifugal elutriation, levels of Nek2 were extremely low in G1 cells but increased progressively during S and reached maximal levels at the G2-M transition. This expression pattern is highly reminiscent of that of A- and B-type cyclins, the regulatory subunits of the mitotic inducer p34cdc2 (36). A- and B-type cyclins reach maximal levels at the G2-M transition, before they are proteolytically destroyed at the onset of anaphase, in a process that involves polyubiquitination (37). We do not presently know how the levels of the Nek2 protein kinase are regulated. However, it is intriguing that the COOH terminus of Nek2 contains a motif (RXLXXXXX; residues 361−369) which constitutes a perfect match to a cyclin destruction box (36); furthermore, this potential destruction box is followed by a block of three lysine residues (381−384) which could potentially function as acceptors for polyubiquitination. Studies are currently in progress to determine whether this potential destruction box is functionally implicated in the mitotic degradation of Nek2.

Regardless of the molecular mechanisms underlying the cell cycle-dependent expression of Nek2, it is remarkable that both nimA mRNA levels and NIMA activity also peak at the G2-M transition (38−40). Based on this parallel, we conclude that mammalian Nek2 and fungal NIMA may not only be structurally related to each other but may also share similar functions at the onset of mitosis.

Materials and Methods

Cells. HL-60, Raji, M14, and M43 cells were kindly provided by S. Carr, 3229 cells by P. Cerutti, TEK-4 cells by R. Tyrrell, and HaCat cells by P. Beard, all of the Swiss Institute for Experimental Cancer Research. SIMA, HT29, and A431 cells were provided by J. Bartek, Danish Cancer Institute, Copenhagen, Denmark. Cell culture was carried out as described previously (10, 19). To induce differentiation of HL-60 cells along the macrophage or granulocyte lineages, they were grown in culture medium supplemented with 30 nm TPA or 1% dimethyl sulfoxide, respectively (41).

cDNA Library Screening. Plaque-forming units (5−6 × 10^6) from several human λ phage cDNA libraries were screened by DNA hybridization. For nek2, a Agt1 HUT-78 T-cell library, a Agt1 Jurkat CD4+ T-cell cDNA library, a Agt1 hepatoma library, and a Agt1 placenta library (all from Clontech Laboratories), a Agt1 nasopharyngeal carcinoma library (42), and a AAZAPI HL-60 library (Stratagene) were screened. For nek3, the above HL-60 and placenta and nasopharyngeal carcinoma libraries were screened.

For screenings, plaques were transferred to nitrocellulose filters (Schleicher and Schuell) which were treated with 1.5 M NaCl, 0.5 M NaOH for 2 min, then with 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) for 5 min, and finally with 0.2 M Tris-HCl (pH 7.5), 2X SSC (1X SSC = 0.15 M NaCl and 15 mm sodium citrate) for 30 s. Filters were baked in a vacuum oven at 80°C for 2 h and prehybridized at 42°C for at least 4 h in 0.1 M PIPES (pH 7.0), 0.8 M NaCl, 0.1% N-lauroyl sarcosine, 5X Denhardt’s solution, 200 μg/ml salmon sperm DNA and 50% formamide. (1X Denhardt’s solution is 0.02% bovine serum albumin, 0.02% ficoll, and 0.02% polyvinylpyrrolidone). Filters were hybridized with 32P-labeled nek2 or nek3-specific probes (described below) at 42°C overnight in 0.1 M 1.4-piperazinediethanesulfonic acid (pH 7.0), 0.8 M NaCl, 0.1% N-lauroyl sarcosine, 2X Denhardt’s solution, 100 μg/ml salmon sperm DNA, 50% formamide, and 10% dextran sulfate. Filters were washed two times for 15 min at room temperature in 2X SSC and 0.1% SDS, followed by four washes for 15 min each at 50°C in 0.2X SSC and 0.1% SDS. Filters were dried and exposed to Kodak X-OMAT autoradiography film. Recombinant phages from positive plaques were plaque-purified and subjected to two additional rounds of purification. Phage DNA was prepared using Lambdaorb (Promega Corp.) and characterized by restriction enzyme analysis.

To prepare nek2- or nek3-specific 32P-labeled probes for hybridization, the 363-bp and 342-bp inserts of the partial nek2 (originally designated as HsPK21) and nek3 (originally designated as HsPK36) cDNAs (19) were excised by digestion with PstI and KpnI, gel-isolated, and labeled with...
α-[32P]dATP (Amersham) using a Random Primed DNA labeling kit (Boehringer Mannheim). For nek3, the original 342-bp probe was used for all library screenings. For nek2, the original 363-bp probe was used to screen the HL-60, hepatoma, Jurkat CD4± T-cell, and Hut-78 T-cell libraries. The 790-bp 21-1 clone obtained from the Hut-78 library was then used for subsequent library screenings and for Northern blot analysis (see below).

**Sequence Analysis**. Inserts were excised from recombinant λ phages by EcoRI digestion and subcloned into the EcoRI site of pGEM-32Zf(−) (Promega Corp.). Plasmid DNAs were prepared using a Magic Miniprep kit (Promega Corp.). Nested deletion templates for sequence analysis were generated using the Erase-A-Base kit (Promega Corp.). Double-stranded DNA sequencing was carried out by the dideoxynucleotide method using Sequenase 2.0 (United States Biochemical Corp.). DNA sequences were determined for both strands using combinations of nested deletion templates and synthetic oligonucleotide primers.

Sequences were analyzed on a VAX system using the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package. The sequences of the nek2 and nek3 cDNAs were compared (February 1994) in library searches against the GenBank, EMBL, and SwissProt databases using the FASTA program and the BLAST network service of the National Center for Biotechnology information.

**In Vitro Transcription and Translation**. Nek2 protein was produced in vitro using pGEM-nek2 or pCMV-nek2 (see below), T7 RNA polymerase, and the TNT Coupled Reticulocyte Lysate System (Promega Corp.) in the presence of [35S]methionine (Amersham). To similarly express Nek3 protein and confirm the open reading frame of the partial nek3 cDNA, a start codon was introduced by an in-frame fusion between nek3 and a 104-bp DNA encoding a 14-amino acid myc epitope tag. To this end, the 1787-bp nek3 EcoRI fragment was inserted downstream of the myc tag in a pBluescript plasmid (pBTmyc; Ref. 43).

**RNA Isolation and Northern Blotting**. RNA was prepared by the acid guanidinium-thiocyanate-phenol-chloroform method (44) as described previously (19). Total RNA (10 μg) from various cell lines was electrophoresed in a denaturing 1% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose membrane, and hybridized with the [32P]-labeled 790-bp fragment obtained from the 21-1 cDNA as described for library screenings, except that the hybridizations were carried out at 50°C. Ethidium bromide staining of the 18S and 28S RNAs was used to determine equal loading for each sample.

**Bacterial Expression of Nek2 Proteins**. Poly histidine-tagged Nek2 fusion proteins were generated using the QIAxpert bacterial expression system (QIAGEN, Inc.). The plasmid pQE-Ne2 was constructed by subcloning the PstI-EcorI insert of the original nek2 partial cDNA (HspK 21; 19) into the pQE9 vector; this yielded a 144-amino acid recombinant protein comprising an NH2-terminal poly-His tag fused to a 121-amino acid product of the Nek2 catalytic domain. The plasmid pQE-Ne2pNe2K was created by subcloning the 1.7-kb Styl-PstI fragment from pGEM-nek2 (missing the first 102 amino acids of Ne2) into the pQE9 vector, resulting in the expression of a 355-amino acid recombinant protein comprising an NH2-terminal poly-His tag fused to the 343-amino acid COOH terminus of Ne2. Overexpression using pQE-Ne2 was found to be optimal in Escherichia coli strain M15[pREP4] using LB medium (45), while overexpression using pQE-Ne2pNe2K was optimal in E. coli strain SG13009[pREP4] using Super medium (25 g bacto-tryptone, 15 g bacto-yeast extract, and 5 g NaCl per liter). Recombinant proteins were expressed and purified under denaturing conditions essentially as described by the manufacturer (QIAGEN), except that all chromatographic solutions were supplemented with 10 mM 2-mercaptoethanol. Recombinant proteins were eluted in buffer E (8 M urea, 0.1 M NaH2PO4, 10 mM 2-mercaptoethanol, and 10 mM Tris-HCl, pH 4.5) and stored without further purification at −20°C.

**Production of Polyclonal Antibodies against Bacterially Expressed Nek2 Protein**. For rabbit immunizations, 250 to 300 μg of the purified 355-amino acid recombinant Nek2 protein in buffer E was emulsified with an equal volume of Freund’s complete or incomplete adjuvant for the initial or subsequent injections, respectively. Intramuscular injections were carried out at 2- to 3-week intervals, and immunoreactivity for Nek2 protein was monitored by immunoblotting analysis.

For affinity purification, 700 μg of the purified 355-amino acid recombinant Nek2 protein was covalently coupled to CNBr-activated Sepharose 4B (Pharmacia) as recommended by the manufacturer. This affinity matrix was used to purify antibodies from immune serum (R31) essentially as described by Harlow and Lane (46).

**DNA Transfections**. For transient expression in HeLa cells, the 1.7-kb Nael-Xbal fragment containing the entire nek2 coding sequence was excised from pGEM-nek2 and introduced into the pRcCMV vector (Invitrogen Corp.) at the HindIII and Xbal sites to generate pCMV-nek2. For this construction, the HindIII site of pRCMV was filled in using the Klenow fragment of DNA polymerase I (45). HeLa cells (2.5 × 106) seeded on 60-mm plates were transfected with 12.5 μg of pCMV-nek2 as described in Krek and Nigg (10) using the method of Chen and Okuyama (47).

**Immunoblotting Analyses**. Products of in vitro translation reactions were mixed with an equal volume of 2X gel sample buffer [100 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, and 0.02% bromphenol blue] and boiled for 10 min. Cell lysates were prepared by addition of 2X sample buffer, boiling for 10 min and centrifuging at 10,000 × g for 10 min (46). Proteins were resolved on SDS-10% polyacrylamide gels and transferred to nitrocellulose. Immunoblotting analysis was performed using affinity-purified R31 anti-Nek2 antibodies at 0.72 μg/ml, followed by goat anti-rabbit secondary antibodies coupled to either horseradish peroxidase or alkaline phosphatase. Immunoreactive proteins were visualized by ECL using an ECL kit (Amersham) or by using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Promega Corp.), respectively.

**Cell Cycle Synchronization**. Cells were synchronized in M by the addition of 500 ng/ml nocodazole for 15 h and released as described previously (23). Relative levels of DNA synthesis were determined at 3-h intervals after release by labeling cell cultures with 2.5 μCi/ml of [3H]thymidine (New England Nuclear/Du Pont) for 30 min at 37°C. Cells were lysed by the addition of 0.5 M NaOH-10 mM EDTA, and the lysates were transferred to an Eppendorf tube. After vortexing, lysates were incubated for 15 min at 60°C and 50–100 μl aliquots were spotted onto GF/C glass fiber filters (Whatman) previously impregnated with 100 μl of 50 mM sodium pyrophosphate-50 mM EDTA. DNA was precipitated on the filters with 5% trichloroacetic acid and
10 mM sodium pyrophosphate, and filters were washed and counted as described previously (40).

For synchronization by centrifugal elutriation, HeLa suspension cells (1 × 10⁷) were cultured in S-Minimal Essential Medium adapted for spinner culture (GIBCO) supplemented with 5% fetal calf serum and penicillin-streptomycin (100 international units/ml and 100 µg/ml, respectively). Centrifugal elutriation, to sort cells by size, was carried out as described by Draetta and Beach (25). Briefly, cells were transferred to icecold phosphate-buffered saline containing 0.3 mM EDTA, 1% (v/v) calf serum, and 0.1% (w/v) glucose, loaded onto a J5.0 rotor (Beckman) and collected by stepwise increases in flow rates from 22 to 60 ml/min at 2900 rpm. Portions of each fraction were prepared for flow cytometric (FACS) analysis on a FACS II instrument (Becton Dickinson) as described by Krek and Nigg (23). The remainder of each fraction was washed in phosphate-buffered saline, resuspended in gel sample buffer, and boiled prior to analysis by SDS-polyacrylamide gel electrophoresis and immunoblotting.

Chromosomal Localization. Chromosomal mapping was carried out by fluorescence in situ hybridization as described previously (22). In brief, metaphase spreads were prepared from peripheral blood lymphocytes following standard procedures (49). The 2051-bp nek2 cDNA was labeled via nick translation by substituting dTTP with biotin-11-dUTP (Sigma). Labeled probe (50 ng) was precipitated with 10 µg of salmon sperm DNA and resuspended in 10 µl of hybridization solution (50% formamide, 2X SSC, and 10% dextran sulfate). The probe was heat-denatured for 5 min at 76°C, chilled on ice, applied to denatured chromosome preparations, covered with an 18 x 18 mm² coverslip, and sealed with rubber cement. Chromosomes were denatured in a solution containing 70% formamide-2X SSC (pH 7.0) for 2 min at 80°C and dehydrated through an ethanol series. After overnight incubation at 37°C, slides were washed three times in 50% formamide-2X SSC at 45°C (5 min each), followed by three washes in 0.1X SSC at 60°C (5 min each). After blocking in 4X SSC-3% bovine serum albumin for 30 min at 37°C, the probe was visualized with avidin conjugated to fluorescein (Vector laboratories). Chromosomes were counterstained with 4,6-diamidino-2-phenylindole and embedded in an antifading solution (DABCO) to reduce photobleaching. Gray scale images were recorded with a cooled CCD camera (Photometrics) mounted on a Zeiss Axiopt microscope equipped for epifluorescence, converted to tint scale, and merged using the computer software “Gene Join” (50).

Accession Numbers. The nucleotide sequence data reported in this paper will appear in the EMBL Data Library under the following accession numbers: ZZ39066, nek2-2; and ZZ29067, nek3.

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References