ABSTRACT **Extracellular-signal regulated kinases/microtubule-associated protein kinases (Erk/MAPKs) and cyclin-directed kinases (Cdks) are key regulators of many aspects of cell growth and division, as well as apoptosis. We have cloned a kinase, *Nlk*, that is a murine homolog of the *Drosophila nemo* (*nmo*) gene. The *Nlk* amino acid sequence is 54.5% similar and 41.7% identical to murine Erk-2, and 49.6% similar and 38.4% identical to human Cdc2. It possesses an extended amino-terminal domain that is very rich in glutamine, alanine, proline, and histidine. This region bears similarity to repetitive regions found in many transcription factors. *Nlk* is expressed as a 4.0-kb transcript at high levels in adult mouse brain tissue, with low levels in other tissues examined, including lung, where two smaller transcripts of 1.0 and 1.5 kb are expressed as well. A 4.0-kb *Nlk* message is also present during embryogenesis, detectable at day $E_{10.5}$, reaching maximal steady state levels at day $E_{12.5}$, and then decreasing. Nlk transiently expressed in COS7 cells is a 60-kDa kinase detectable by its ability to autophosphorylate. Mutation of the ATP-binding Lys-155 to methionine abolishes its ability to autophosphorylate, as does mutation of a putative activating threonine in kinase domain VIII, to valine, aspartic, or glutamic acid. Subcellular fractionation indicates that 60–70% of Nlk is localized to the nucleus, whereas 30–40% of Nlk is cytoplasmic. Immunofluorescence microscopy confirms that Nlk resides predominantly in the nucleus. Nlk and Nmo may be the first members of a family of kinases with homology to both Erk/MAPKs and Cdks.**

Extracellular-signal regulated kinases/microtubule-associated protein kinases (Erk/MAPKs) and cyclin-directed kinases (Cdks) are two large families of serine/threonine kinases that are loosely related by sequence homology and by their substrate specificity as proline-directed kinases (1–3). Both families of kinases are of critical importance in directing many aspects of cellular growth, division, and differentiation, in response to external stimuli. The Erk/MAPK family members are key components of intracellular signaling cascades, and consist of at least 10 members (4–6). They and their upstream activators are phosphorylated and activated almost immediately after stimulation of cells with growth factors and hormones, or after various cellular stresses such as heat, UV, and inflammatory cytokines. Although Erk/MAPKs have several cytoplasmic targets, they have also been shown to be important as transporters of messages that have been relayed from the plasma membrane to the cytoplasm by upstream kinases, into the nucleus where they phosphorylate transcription factors and thereby alter gene transcription patterns (7, 8). Erk/MAPK family member substrates include c-fos, c-jun, ATF2, and the ETS family members Elk-1, Sap1a, and c-Ets-1 (9–13).

Cdks are crucial regulators that control transitions between the successive stages of the cell cycle. Activity of Cdks is tightly controlled by various phosphorylation events and by the association of cyclins, whose expression fluctuates throughout the cell cycle. At least eight members of the Cdk family have been identified, with even more cyclins reported. The activity of Cdks is also negatively regulated by the association of small inhibitory molecules (14, 15). Targets of Cdks include various transcriptional coactivators such as p110Rb and p107, and transcription factors such as p53, E2F, and RNA polymerase II, as well as many cytoskeletal proteins and cytoplasmic signaling proteins (14, 16–21).

In 1994 Choi and Benzer (22) reported the characterization of a *Drosophila melanogaster* mutant, *nemo* (*nmo*). A null mutation (nmo^{P1}) causes greatly reduced viability in *Drosophila*, and results in a phenotype of incomplete rotation of photoreceptor cells in the eye. The predicted amino acid sequence of Nmo has ≈37–41% homology to Erk/MAPKs and Cdks, and is thus more closely related to these kinases than to other families of kinases (22). Nmo differs from the Erk/MAPKs and Cdks in its larger size, as its two predicted isoforms possess carboxy-terminal regions ≈50–90 amino acids longer than those of Erk/MAPKs and Cdks. Nmo also differs from Erk/MAPKs in that the Nmo amino acid sequence in the phosphorylation lip in conserved kinase domain VIII is unlike that of either the Erk/MAPKs or Cdks (22). Based on its sequence, Nmo therefore appears to be a member of an extended family of Erk/MAPK-like and Cdk-like kinases.

We report the cloning and characterization of a mammalian homolog of *nmo*, called *Nlk*. *Nlk*, although highly identical to *nmo*, has a longer amino-terminal region that is rich in proline, alanine, glutamine, and histidine. This 124 amino-acid stretch bears similarity to regions in many transcription factors. Nlk protein overexpressed in COS7 and HEK293 cells is a 60-kDa protein kinase that autophosphorylates, and localizes to a large extent in the nucleus. Nlk and Nmo may therefore function to phosphorylate and regulate transcription factors.

MATERIALS AND METHODS

Synthesis of $E_{14.5}$ Mouse Brain cDNA and PCR Screen for Nlk. $E_{14.5}$ mouse brain polyadenylated RNA used as template in PCR was isolated as described below, and cDNA was synthesized utilizing the StrataScriptTM RT-PCR kit (Stratagene). Degenerate oligonucleotide primers were designed and synthesized. The primers utilized in the initial PCR screen were: domain VI, CCGGAATTCGG(GATC)CT(GATC)AA(GA)TA(TC)-AT(GATC)CA(TC)TC(GATC)G; domain IX, CGCGGATC-

C(GA)CA(GATC)CC(GATC)AC(GATC)GACCA(GAT)A-(GT)(GA)TC. The primers used in the second PCR were: domain I, CCGGAATTCAT(ATC)GG(GATC)TA(TC)GG-(GATC)GC(GATC)TT(TC)GA(GATC)G; domain VII, CGCGGATCCAACACAATTGCTGTTCACAAG. PCRs were carried out at 94°C for 1 min, 46°C for 2 min, and 72°C for 2 min, for 33 thermal cycles. PCR products were extracted with chloroform, ethanol precipitated, digested with *Eco*RI and *Bam*HI, and cloned into Bluescript SK$^-$.

Isolation and Sequencing of the Nlk cDNA Clone. Approximately 1×10^6 plaques of a BALB/c mouse neonatal brain cDNA library (Stratagene) were screened by using as probe the subcloned PCR products described above, labeled by random oligonucleotide priming. Filters for screens were hybridized in 7% SDS, 0.5 M sodium phosphate (pH 7.2), 1% BSA, and 1 mM EDTA, at 65°C for 24 hr, and then washed several times in 1% SDS, 20 mM sodium phosphate (pH 7.2), and 1 mM EDTA, at 65°C for 1 hr. Positive clones were purified and rescued with ExAssist helper phage (Stratagene) into Bluescript SK$^-$. Clones obtained were analyzed by restriction analysis and sequenced by the dideoxynucleotide chain termination method by using Sequenase, version 2.0 (United States Biochemical).

RNA Preparation and Northern Blot Analysis. Tissues were isolated from adult or embryonic mice and quick frozen in liquid nitrogen. RNA was prepared by using the Ultraspec RNA Isolation Reagent (Biotecx Laboratories, Houston). Briefly, tissues were Dounce homogenized in Ultraspec reagent, extracted with chloroform, and centrifuged at 12,000 × *g* for 15 min. The aqueous phase was precipitated with isopropanol, and centrifuged again for 10 min. Precipitates were washed with 70% ethanol, and resuspended in .1% diethyl pyrocarbonate/10 mM Tris·HCl, pH 8.0/1 mM EDTA. Polyadenylated RNA was purified utilizing the PolyATtract mRNA Isolation System (Promega). RNAs were electrophoresed as described (23) and transferred to Hybond-N (Amersham) filters, UV crosslinked, and probed with the entire *Nlk* cDNA, in 0.5 M sodium phosphate (pH 7.2), 7% SDS, 1% BSA, and 1 mM EDTA, at 65°C for 18 hr. The blot was then washed twice in 1% SDS, 20 mM sodium phosphate (pH 7.2), and 1 mM EDTA, at 65°C for 30 min. Northern blots were exposed to X-Omat AR film (Kodak).

Cell Culture and Transient Transfections. COS7 and HEK293 cells were cultured in DMEM (GIBCO/BRL) supplemented with 5% fetal calf serum (HyClone) and 0.1% penicillin/streptomycin (GIBCO/BRL). Transient transfections were performed by using the calcium phosphate technique. Briefly, cells were seeded 20 hr before transfection at 8×10^5 cells/ml on 10-cm tissue culture plates. Hemagglutinin (HA)-tagged wild-type and mutant *Nlk* cDNAs (5.0 μg), cloned into the mammalian expression vector *pJ3ΩHA* (24), were added to 450 μl sterile H_2O and 50 μl of 2.5M $CaCl_2$. This mixture was added dropwise to 500 μl of 2×HBS, pH 7.05 (280 mM NaCl/50 mM Hepes/1.5 mM sodium phosphate) and incubated at room temperature for 20 min. It was then added dropwise onto seeded cells. After 18 hr the transfection mixture was replaced with fresh medium. Cells were harvested at 48 hr.

Tissue Lysis, Immunoprecipitations, Kinase Assays, and Western Blot Analysis. 48 hr after transient transfection, cells were lysed in modified RIPA buffer with protease inhibitors [150 mM NaCl/50 mM Tris·HCl, pH 7.5/1.0% Nonidet P-40/0.25% deoxycholic acid/2 mM EGTA/1 mM EDTA/1 mM sodium orthovanadate/1 mM 4-(2-aminoethyl) benzenesulfonyl-fluoride (PefablocSC, Boehringer Mannheim)/10 μg/ml leupeptin/10 μg/ml pepstatin A]. Lysates were centrifuged at 16,000 × *g* for 10 min, and the supernatants used for immunoprecipitations. Immunoprecipitations were performed by adding 3 μg anti-HA antibody to lysates, incubating on ice for 1 hr, and then adding 30 μl of protein A-Sepharose beads (Zymed) with rocking, for 30 min. Immunoprecipitates were washed three times with modified RIPA buffer, and once with ST buffer (100 mM NaCl/10 mM Tris·HCl, pH 8.0). Immunoprecipitates were then incubated with 25 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 100 μM ATP, and 5 μCi [γ^{32}P]-ATP (1 Ci = 37 GBq), in a total volume of 30 μl, for 20 min at 30°C. Samples were boiled in 1× sample buffer (500 mM Tris·HCl, pH 6.8/10% SDS/20% glycerol/0.05% bromophenol blue/1% 2-mercaptoethanol) for 5 min, and electrophoresed on an SDS/PAGE gel. Proteins were transferred to polyvinylidene fluoride Immobilon membrane (Millipore). For anti-HA Western blots, membranes were blocked in 3% BSA in Western buffer (150 mM NaCl/10 mM Tris·HCl, pH 8.0/0.1% Triton X-100) at room temperature for 1 hr, incubated with 1 μg/ml anti-HA antibody for 1 hr, then washed with Western buffer four times for 10 min, incubated with protein A-horseradish peroxidase (Amersham) at a dilution of 1:2,000, in Western buffer with 3% BSA at room temperature for 25 min, and washed again, four times in Western buffer for 10 min each. Membranes were then subjected to enhanced chemiluminescence (Amersham) and exposed to X-Omat AR film (Kodak).

Biochemical Fractionation for Subcellular Localization and Immunofluorescence. For subcellular fractionation, 48 hr after transient transfection, cells were harvested and resuspended in 1 ml hypotonic lysis buffer (1 mM EGTA/1 mM EDTA/2 mM $MgCl_2$/10 mM KCl/1 mM DTT/10 mM β-glycerophosphate/1 mM sodium orthovanadate). Cells were Dounce homogenized with 25 strokes, the lysate was loaded onto 1 ml of 1 M sucrose in hypotonic lysis buffer (sucrose-hypotonic buffer), and centrifuged at 1,600 × *g* for 10 min. Nuclei in the pellet were washed once by resuspending in 1 ml sucrose-hypotonic buffer, and centrifuging at 1,600 × *g* for 5 min. Nuclei were then resuspended in 1× sample buffer and boiled for 5 min. The supernatant from the original lysate was centrifuged at 150,000 × *g* for 30 min, and the pellets, containing membranes, were resuspended in 1× sample buffer and boiled for 5 min. Sample buffer was added to the supernatant, containing cytosol, to 1× concentration, and boiled. Equivalent fractions of each sample were loaded on a SDS/PAGE gel, electrophoresed, transferred to membranes, and Western blotted with anti-HA antibody, as described above.

Immunofluorescence staining of transiently transfected HEK293 cells plated 24 hr posttransfection onto collagen coated coverslips (Vitrogen-100, Celltrix) was as follows: at 48 hr posttransfection, coverslips were washed 2× with PBS, fixed by incubation in 4% paraformaldehyde for 10 min, washed twice with PBS, and permeabilized by incubation with methanol for 2 min. They were washed four times with PBS, and incubated with anti-HA antibodies at 10 μg/ml, in PBS with 3% BSA for 45 min. Coverslips were washed with PBS three times, and incubated with anti-mouse-fluorescein isothiocyanate (GIBCO/BRL) at a dilution of 1:200, for 30 min, washed three times in PBS with 0.1% Triton X, and once in PBS. Coverslips were mounted on slides, sealed, and examined under a fluorescence microscope.

RESULTS

PCR Screening for Erk/MAPK Family Members. cDNA derived from polyadenylated RNA from $E_{14.5}$ mouse brain was used as a template for PCR, using degenerate primers based on Erk/MAP family members Erk-1, Erk-2, and Erk-5, to conserved kinase domains VI and IX (3). The resulting 350-bp PCR products were cloned into Bluescript SK$^-$ and sequenced. One clone possessed a sequence that was highly identical to the *Drosophila melanogaster* gene *nemo* (*nmo*), which displays similarity to both Erk/MAPKs and Cdks (22). Degenerate primers were then made based on the *nmo* sequences in conserved kinase domains I and VII, and PCR utilizing the

$E_{14.5}$ mouse brain cDNA as template, was performed. The resulting 400-bp product was cloned into Bluescript SK$^-$ and sequenced, and was also found to possess high levels of identity to *nmo*. The overlapping regions of the first and second PCR products (between kinase domains VI and VII) were identical, suggesting that both PCR products were fragments of the same gene, possibly a murine homolog of the *Drosophila nmo* gene.

Isolation and Characterization of the Nlk cDNA. A murine neonatal brain cDNA library was screened for cDNA corresponding to the PCR fragments, by using both PCR products as probe. Numerous cDNA clones were obtained, all of them different size fragments of the same gene. Upon sequencing the largest cDNA, which was ≈2.6 kb in length, an ORF was observed to begin at nucleotide 323 and end at 1,867 (Fig. 1*A*). Several in frame stop codons upstream of the initiating ATG indicate that the entire ORF has been isolated. This sequence encodes a polypeptide with an overall similarity of 79% and identity of 73% to one of the two isoforms of the *nmo* gene, *nmo* II (Fig. 1). This high degree of identity throughout the protein suggests that we have isolated the murine homolog of the *Drosophila nmo* II gene. We therefore suggest the murine gene to be denoted *nemo-l*ike *k*inase (*Nlk*). However, unlike *nmo*, the ORF of *Nlk* begins roughly 250 bp further upstream than in the *Drosophila* cDNA, to encode a protein of predicted molecular weight of 57 kDa, as opposed to the predicted Nmo II molecular weight of 48 kDa (Fig. 1*B*). Another methionine is encoded in the murine cDNA near the site of the analogous methionine in Nmo. However, *in vitro* transcription/translation of the *Nlk* cDNA clones yields a protein of at least 60 kDa, corresponding more closely to the predicted size of the translational product if the first upstream methionine were utilized. In addition, the sequence surrounding this upstream methionine appears optimal for translation initiation, as predicted by the Kozak rule (25).

Within its kinase domain, the Nlk peptide sequence predicts a protein kinase, that, like the predicted Nmo amino acid sequence, has distinct homology to Erk/MAPK and Cdk family members. Nlk is 54.5% similar and 41.7% identical to murine Erk-2, and 49.6% similar and 38.4% identical to human Cdc2. Although overall it possesses a higher level of similarity to Erk/MAPKs, Nlk does not possess the characteristic MAPK activating phosphorylation sequence TXY in the so-called phosphorylation lip just upstream of conserved kinase domain VIII. Instead it possesses the sequence TQE at the analogous site, more similar to the sequence THE at the analogous site in Cdc2 (Fig. 1*A*). These high levels of identity to both Erk/MAPKs and Cdks suggest that Nlk and Nmo may also be proline-directed kinases. The 129 amino-terminal residues of Nlk upstream of the beginning of the kinase domain, encode a region extremely rich in histidine, proline, glutamine, and alanine (Fig. 1*A*). In fact, these four amino acids account for >70% of the amino acid composition of Nlk in this region. This repetitive region does not appear to be an artifact of the cDNA library, as several independently isolated cDNAs of varying lengths all bear this same sequence. Moreover, probing a tissue Northern blot with the region encompassing only the first 75 amino acids, detects the same size messages as a blot probed with the *Nlk* kinase domain alone, or the entire *Nlk* cDNA (data not shown).

Northern Blot Analysis of Nlk. Probing of polyadenylated RNA from various adult mouse tissues with the entire 2.6-kb *Nlk* cDNA reveals a 4.0-kb message expressed at relatively high steady state levels in brain tissue, and less strongly in heart, kidney, lung, and liver (Fig. 2*A*, *Upper*). Although low amounts of RNA are present in the lung tissue sample, two smaller messages of ≈1.0 and 1.5 kb are also observed to be strongly expressed (Fig. 2*A, Upper*). These 1.0- and 1.5-kb messages are also observed in many cultured lung carcinoma cell lines (data not shown), and may be alternate splice products of the *Nlk* mRNA. Polyadenylated RNA was also probed with rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to indicate amounts of RNA in each lane (Fig. 2*A, Lower*). Although the GAPDH signal is less intense in lung RNA, the band detected shows that the RNA is not degraded. *Nlk* expression was also investigated in polyadenylated RNA isolated from whole

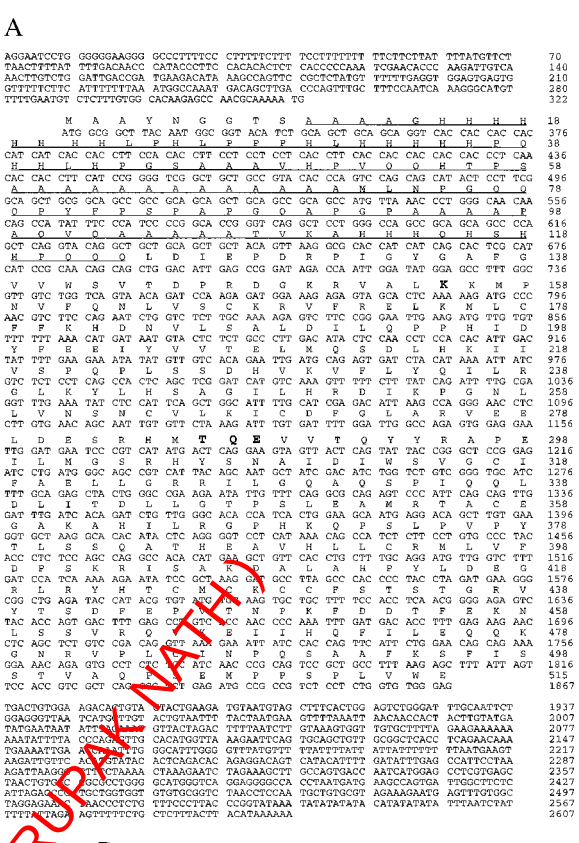

FIG. 1. Nlk sequence and comparison to *nmo*. (*A*) Complete nucleotide and amino acid sequence of *Nlk*. Highlighted are Lys-155, the putative ATP-binding site; and Thr-286, Gln-287, and Glu-288, a putative phosphorylation/regulatory site. A glutamine, alanine, histidine, and proline-rich N-terminal region is underlined. (*B*) Comparison of the murine *Nlk* and the *Drosophila nmo* amino acid sequences. Lines between the two sequences indicate sequence identity. Double dots indicate conserved amino-acid residues, single dots indicate semiconserved residues.

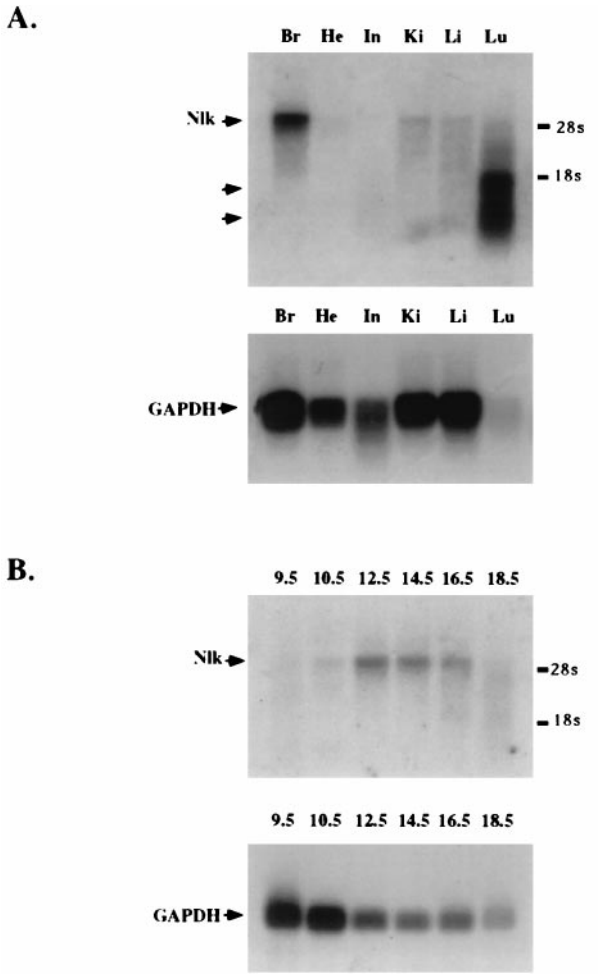

FIG. 2. Nlk expression in adult and embryonic tissue. (*A*) Polyadenylated RNA isolated from adult mouse brain (Br), heart (He), intestine (In), kidney (Ki), liver (Li), and lung (Lu), was probed with the entire *Nlk* cDNA (*Upper*), or rat GAPDH (*Lower*). 28S and 18S ribosomal RNAs were used as markers. (*B*) Polyadenylated RNA isolated from entire mouse embryos, from days $E_{9.5}$–$E_{18.5}$, was electrophoresed, transferred to membranes, and probed with the entire *Nlk* cDNA (*Upper*), or rat *GAPDH* (*Lower*). 28S and 18S ribosomal RNAs were used as markers.

mouse embryos from days $E_{9.5}$ to $E_{18.5}$ (Fig. 2*B, Upper*). The 4.0-kb *Nlk* message is undetectable at day $E_{9.5}$, and is present at low steady state levels at day $E_{10.5}$. However, by $E_{12.5}$, high levels of *Nlk* are observed (Fig. 2*B, Upper*); levels then diminish progressively at $E_{14.5}$, $E_{16.5}$, and $E_{18.5}$.

Nlk Protein Expression and Kinase Activity. The *Nlk* cDNA was cloned into the mammalian expression vector *pJ3ΩHA*, and a HA-tagged Nlk protein (HA-Nlk) was transiently expressed in COS7 cells. After immunoprecipitation and an *in vitro* kinase assay under autophosphorylating conditions, an autophosphorylating protein of 60–63 kDa was readily detected by autoradiography, which was also recognized by anti-HA antibodies (Fig. 3, lanes 2 and 8). Kinase assays utilizing myelin basic protein, casein, histone, glutathione *S*-transferase-retinoblastoma protein, and glutathione *S*-transferase-Erk-1, reveal that none of these proteins is phosphorylated by the transiently expressed HA-tagged Nlk (data not shown). Various point mutations were introduced into the *Nlk* cDNA, encoding changes of the putative ATP-binding Lys-155 to methionine (HA-Nlk(KM)), and encoding for mutation of Thr-286, which is located in the putative phosphorylation lip of Nlk, to valine, aspartic acid, or glutamic acid [HA-Nlk(TV), HA-Nlk(TD), and HA-Nlk(TE), respectively].

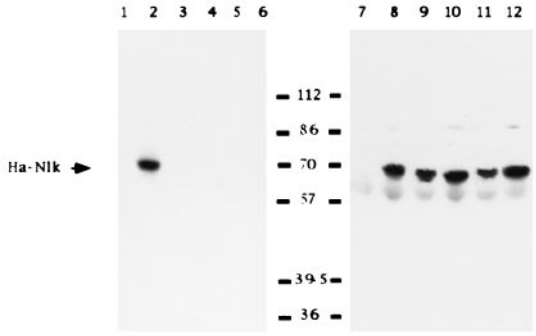

FIG. 3. Nlk is a 60 kDa kinase that autophosphorylates strongly. HA-tagged wild-type Nlk and Nlk mutants KM, TV, TE, and TD, were transiently expressed in COS7 cells and harvested after 48 hr, lysed and immunoprecipitated, and an *in vitro* kinase autophosphorylation assay performed. After electrophoresis, samples were subjected to autoradiography (lanes 1–6), and then analyzed by Western blot with anti-HA antibody (lanes 7–12). Lanes 1 and 7, mock transfection; lanes 2 and 8, wild-type HA-Nlk; lanes 3 and 9, HA-Nlk(KM); lanes 4 and 10, HA-Nlk(TV); lanes 5 and 11, HA-Nlk(TE); and lanes 6 and 12, HA-Nlk(TD). Molecular mass standards (in kDa) are indicated.

After transient transfection into COS7 cells, all four mutants were expressed at high levels, but were no longer able to detectably autophosphorylate (Fig. 3, lanes 3–6, and 9–12).

Subcellular Localization of Nlk. The subcellular localization of Nlk was examined first by biochemical fractionation of COS7 cell lysates from COS7 cells transiently transfected with HA-tagged *Nlk* cDNA. After electrophoresis of samples and Western blot analysis with anti-HA antibody, it was observed that ≈60–70% of overexpressed Nlk is present in the nuclear fractions, whereas 30–40% is observed in the cytoplasmic fractions (Fig. 4*A*, lanes 3 and 4). No Nlk was detectable in membrane fractions (a light background band that co-migrates with Nlk is also observed in membrane fractions from untransfected COS7 cell lysates) (Fig. 4*A*, lane 2, and data not shown). The subcellular localization of HA-tagged Nlk was also determined by immunofluorescence microscopy of HA-

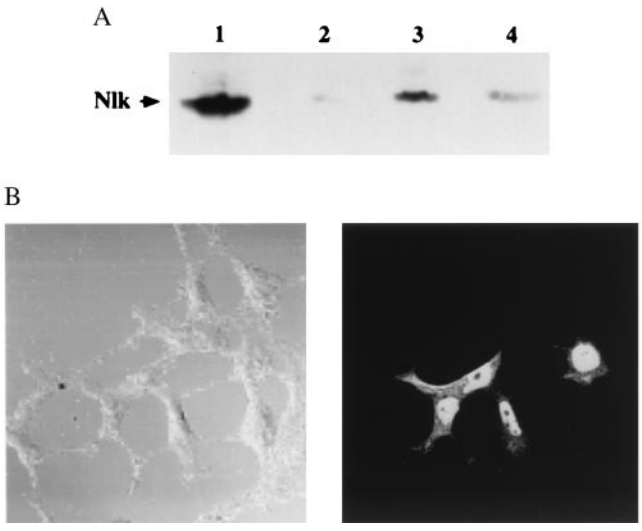

FIG. 4. Nlk localizes primarily to the nucleus. (*A*) COS7 cells transiently expressing wild-type HA-Nlk were harvested after 48 hr and fractionated into membrane (lane 2), nuclear (lane 3), and cytosolic (lane 4) fractions, and electrophoresed. Whole cell lysate was also electrophoresed (lane 1). After transfer to membranes, fractions were Western blotted with anti-HA antibody. (*B*) HEK293 cells transiently expressing wild-type HA-Nlk were permeabilized and then probed with anti-HA antibody, followed by anti-mouse-fluorescein isothiocyanate. Cells on coverslips were visualized by Nomarski optics (*Left*), or by fluorescence microscopy (*Right*).

Nlk transiently expressed in HEK293 cells. After probing with anti-HA antibodies, it was apparent that once again, ≈60–70% of Nlk was present in the nucleus, whereas 30–40% appeared to stain diffusely throughout the cytoplasm (Fig. 4*B*). A truncated form of Nlk, lacking 72 of the 129 amino acids from the region upstream of the kinase domain, was also observed to localize to the nucleus to the same extent as wild-type Nlk (data not shown).

DISCUSSION

We report the cloning and initial characterization of a mammalian kinase that bears sequence identity to both Erk/MAPKs and to Cdks. We have named this kinase *nemo-l*ike *k*inase, or *Nlk*, after its homolog, *nmo*, in *Drosophila* (22). Two isoforms of *Drosophila nmo* have been cloned, *nmo* I and *nmo* II, that predict differing carboxy-terminal regions outside their kinase domain. The *Nlk* clone described here exhibits marked sequence similarity to the smaller of the two clones, as it is 79% similar and 73% identical to *nmo* II. Perhaps a homolog of the *nmo* I isoform may be expressed in some murine tissues. However, as all 20 of the *Nlk* clones isolated from the neonatal brain cDNA library are identical and are similar to *nmo*II, if other isoforms exist they will most likely be present in other tissues besides neonatal brain. Probing of RNA isolated from various embryonic and adult tissues indicates that in many of the tissues examined, a *Nlk* message of 4.0 kb is observed. This is substantially larger than the 2.6-kb cDNA that we have isolated as our largest clone, indicating that the entire *Nlk* cDNA has not been isolated. However, the entire ORF of *Nlk* appears to be present in this 2.6-kb cDNA. In adult tissues, *Nlk* is expressed at a relatively high level in brain, suggesting that it plays a role in neural tissue function. Lower levels are present in other adult tissues examined, as low steady state amounts of a 4.0-kb *Nlk* message can be detected in heart, kidney, liver, and lung. The 4.0-kb *Nlk* message is also present in embryonic RNA beginning at day $E_{10.5}$ and peaking at day $E_{12.5}$. The level of expression appears to decrease after day $E_{16.5}$, however. This may reflect a decrease in *Nlk* expression in certain tissues as organogenesis and differentiation progress. Other tissues, such as neural tissues, may continue to express high levels of *Nlk* mRNA.

The *Nlk* protein product bears significant similarity to Erk/MAPK members in its kinase domain. In particular, the Nlk amino acid sequence exhibits sequence similarity to the kinases Erk-1, Erk-2, and Erk-5, of various species. Its closest murine relative is Erk-2, with 54.5% similarity and 41.7% identity. It has lower levels of similarity to Jnk/stress-activated protein kinases, p38, and various Cdks. However, unlike the Erk/MAPK members, it does not possess the characteristic MAPK phosphorylation motif, TXY, in conserved kinase domain VIII. Instead, at the analogous region it exhibits the sequence TQE, which resembles the THE sequence found in some Cdks (15). Nlk is thus probably not a substrate for a Erk/MAPK activator such as MEK1. In an effort to constitutively activate the Nlk kinase activity, Thr-286 was mutated to aspartic acid or glutamic acid. However, as autophosphorylation was no longer detected, these mutations appear to inactivate Nlk kinase activity. Alternatively, Thr-286 may be a major autophosphorylation site of Nlk.

Based on its close relationship to Erk/MAPKs and Cdks, Nlk may also exhibit a proline-directed substrate specificity (1, 2). HA-Nlk transiently expressed in COS7 cells does not phosphorylate myelin basic protein, casein, histone, glutathione *S*-transferase-retinoblastoma, or glutathione *S*-transferase-Erk-1. This suggests that Nlk is not a promiscuous kinase, but has specificity for a limited number of substrates. However, optimal activation of Nlk may require stimulation of cells in which it is naturally expressed, such as neural cells, resulting in modifications such as phosphorylation by an upstream kinase, or association with an activating subunit.

Both subcellular fractionation and immunofluorescence microscopy indicate that transiently expressed Nlk localizes primarily in the nucleus. While conclusive studies of Nlk subcellular localization await analysis of endogenous protein by anti-Nlk antibodies, these results indicate that Nlk may localize to the nucleus under appropriate conditions. Nlk does not appear to possess a basic sequence that might clearly be identified as a nuclear localization signal. However, no apparent nuclear localization signals have yet been identified in Erk/MAPKs and Cdks, many of which have been shown to reside in the nucleus, or to migrate into it after cellular stimulation. Evidence suggests that some Erk/MAPKs and Cdks complex with other proteins that carry them into the nucleus (26, 27); this may also be the case for Nlk.

A feature that distinguishes Nlk from Erk/MAPKs, Cdks, and also Nmo, is its extended amino-terminal domain. This region is extremely glutamine, alanine, histidine, and proline rich, and bears similarity to repetitive regions found in many transcription factors, in regions distinct from their DNA-binding domains. Interestingly, a long run of alanines flanked by glutamines, prolines, and sometimes histidines, is a characteristic of defined repression domains in the gap gene Kruppel, a zinc-finger type transcription factor (28), and in some homeotic transcription factors such as Engrailed, and Even-skipped (28, 29). Nlk may utilize this region to interact with transcription factors or coactivators, and then modify them by phosphorylation. As many Erk/MAPKs and Cdks have been shown to associate with and phosphorylate transcription factors or transcriptional coactivators, this unique domain may reflect the substrate specificity of Nlk.

Nlk and Nmo may constitute the first members of a new family of enzymes that are related to the proline-directed kinases, Erk/MAPKs and Cdks, but like these important regulators of cell division, differentiation, and apoptosis, possess unique domains that enable them to interact with their specific regulators and substrates. Further work will determine the role of these kinases during development and in adult neural tissue.

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