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Kid-1, a Putative Renal Transcription Factor: Regulation during Ontogeny and in Response to Ischemia and Toxic Injury

RALPH WITZGALL,[1,2] EILEEN O'LEARY,[1] REINHARD GESSNER,[1,2] ANDRE J. OUELLETTE,[3,4,5] AND JOSEPH V. BONVENTRE[1,2]*

Medical[1] and Surgical[3] Services, Massachusetts General Hospital, Departments of Medicine[2] and Surgery,[4] Harvard Medical School, and Shriners Burns Institute,[5] Boston, Massachusetts 02114*

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We have identified a new putative transcription factor from the rat kidney, termed *Kid-1* (for kidney, ischemia and developmentally regulated gene 1). *Kid-1* belongs to the C_2H_2 class of zinc finger genes. Its mRNA accumulates with age in postnatal renal development and is detected predominantly in the kidney. *Kid-1* mRNA levels decline after renal injury secondary to ischemia or folic acid administration, two insults which result in epithelial cell dedifferentiation, followed by regenerative hyperplasia and differentiation. The low expression of *Kid-1* early in postnatal development, and when renal tissue is recovering after injury, suggests that the gene product is involved in establishment of a differentiated phenotype and/or regulation of the proliferative response. The deduced protein contains 13 C_2H_2 zinc fingers at the COOH end in groups of 4 and 9 separated by a 32-amino-acid spacer. There are consensus sites for phosphorylation in the NH_2 terminus non-zinc finger region as well as in the spacer region between zinc fingers 4 and 5. A region of the deduced protein shares extensive homology with a catalytic region of Raf kinases, a feature shared only with TFIIE among transcription factors. To determine whether Kid-1 can modulate transcription, a chimeric construct encoding the Kid-1 non-zinc finger region (sense or antisense) and the DNA-binding region of GAL4 was transfected into COS and LLC-PK_1 cells together with a chloramphenicol acetyltransferase (CAT) reporter plasmid containing GAL4 binding sites, driven by either a minimal promoter or a simian virus 40 enhancer. CAT activity was markedly inhibited in cells transfected with the sense construct compared with the activity in cells transfected with the antisense construct. To our knowledge, this pattern of developmental regulation, kidney expression, and regulation of transcription is unique among the C_2H_2 class of zinc finger-containing DNA-binding proteins.

The kidney is a very heterogeneous organ with various different types of cells expressing different phenotypic characteristics. Kidney development and cellular differentiation are likely regulated at the level of transcription and involve tissue-specific gene expression (16, 32, 34). One of the ways to achieve kidney-specific gene expression is to selectively express *trans*-acting factors, kidney-specific transcription factors, which may play important roles in determination of the differentiated phenotypes of kidney cells and renal development. These proteins may be important not only for renal development and differentiation but also for the processes involved in repair of the kidney after an injury. The process of kidney injury and repair recapitulates many aspects of development since it involves dedifferentiation and regeneration of epithelial cells followed by differentiation (4, 35, 56). The ability to restore differentiated function and regenerate epithelial structure after an ischemic or toxic insult is an important property of the kidney, an ability not possessed by the heart and the brain, in which myocytes and neurons are not replaced.

One goal of the present study was to identify genes of potential importance in kidney development and repair which are preferentially expressed in the kidney. We report the cloning of a novel cDNA which encodes a protein containing 13 zinc fingers in groups of 4 and 9. The gene, which we call *Kid-1* (for kidney, ischemia and developmentally regulated gene 1), is expressed primarily in the kidney and is a single-copy gene. *Kid-1* mRNA levels accumulate with increasing postnatal age. mRNA levels are reduced after ischemia and reperfusion or folic acid administration. There are protein sequence consensus motifs in the non-zinc finger region and in the region separating the fourth and fifth zinc fingers of the deduced protein, which suggests that the protein may be regulated by phosphorylation and may have kinase activity.

To determine whether Kid-1 can modulate transcription, a chimeric construct encoding the Kid-1 non-zinc finger region (sense or antisense) and the DNA-binding region of GAL4 was transfected into COS cells, a large-T-antigen-transformed green monkey kidney cell line, and LLC-PK_1 cells, a highly differentiated porcine epithelial kidney cell line, together with a chloramphenicol acetyltransferase (CAT) reporter plasmid containing GAL4 binding sites, driven by either a minimal promoter or a simian virus 40 (SV40) enhancer. CAT activity was markedly inhibited in cells transfected with the sense construct compared with that in cells transfected with the antisense construct.

MATERIALS AND METHODS

Construction and screening of cDNA libraries. cDNA libraries were constructed from normal and postischemic rat kidneys. Male Sprague-Dawley rats (Charles River Breeding Labs, Wilmington, Mass.) weighing 250 to 300 g were anesthetized with an intraperitoneal injection of pentobarbital (6.5 mg/100 g of body weight). The blood supply to both

* Corresponding author.

kidneys was interrupted by placement of a microaneurysm clamp (Roboz Surgical Instrument Co., Washington, D.C.) on the renal pedicles for 30 min. The clamps were then removed, and blood flow was reestablished for 1 h. Total RNA was prepared from these kidneys and normal kidneys from other rats by previously reported techniques (39). Poly(A) RNA was selected by oligo(dT)-cellulose chromatography. First-strand cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase. cDNA was ligated into λgt10 with *Eco*RI linkers. Enzymes and components for library construction were obtained from GIBCO BRL, Gaithersburg, Md. In addition, kidney λgt10 and λgt11 libraries were purchased from Clontech Laboratories, Palo Alto, Calif. For initial screening of the libraries we constructed, approximately 125,000 phage were plated on Luria-Bertani agar plates. Subsequent screenings of libraries involved plating of 10^6 phage. Two replicas of each plate were made with GeneScreen (New England Nuclear [NEN], Boston, Mass.) or Hybond (Amersham, Arlington Heights, Ill.) nylon filters. Replica filters were then hybridized with oligonucleotides or cDNA by standard protocols (48).

DNA sequencing. Inserts from purified phage were subcloned into pBluescript (Stratagene, La Jolla, Calif.). Double-stranded DNA was sequenced by the chain termination method (49). Each clone was sequenced on both strands. T3 and T7 Bluescript sequencing primers and oligonucleotide primers corresponding to the cDNA were synthesized by Oligos etc., Inc., Wilsonville, Oreg. Products of sequencing reactions were electrophoresed on Hydrolink Long Ranger (AT Biochem, Malvern, Pa.) modified acrylamide gels. Computer analysis was carried out with the sequence analysis software package of the Genetics Computer Group at the University of Wisconsin, Madison (17). The predicted amino acid sequence of the Kid-1 protein was compared with sequences in the SwissProt, National Biomedical Research Foundation Protein Identification Resource, and GenBank data bases, using the BLAST and FASTA algorithms.

Animal protocols. In experiments designed to examine *Kid-1* mRNA levels with ontogeny, the kidneys of young Sprague-Dawley rats which were 0.5, 10, 15, 20, or 40 to 45 days old were rapidly frozen in liquid nitrogen and then homogenized in 6 M guanidium isothiocyanate for preparation of total RNA (48). Kidneys of three or four animals ≤20 days old were pooled. For older animals, RNA was prepared from each kidney separately.

For the ischemia-reperfusion experiments, a left-flank incision was made in 150- to 250-g male rats and the renal pedicle was clamped with a microaneurysm clamp. The contralateral, nonischemic kidney served as a control. The incision was closed temporarily until 30 min later, when the clamp was removed. The incision was then sutured closed, and, after the stated time of reperfusion, both kidneys were removed for extraction of RNA without freezing.

In other adult male rats, kidney injury was produced by intraperitoneal administration of 250 or 350 mg of folic acid per kg of body weight. Kidneys were harvested at 3, 8 (350-mg-treated animals), and 24 (250-mg-treated animals) h after the injection. Controls were injected with the carrier only (0.3 mmol of $NaHCO_3$). The surgical wounds were sutured, and the animals were returned to their cages until removal of both kidneys under pentobarbital anesthesia.

Northern (RNA) blot analysis. Total RNA was isolated by standard protocols (48), with the exception that 6 M guanidinium isothiocyanate was used. Twenty-five micrograms (unless otherwise noted) of total RNA was lyophilized and dissolved subsequently in 16 μl of buffer A [20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 8 mM Na acetate, 1 mM EDTA (pH 8.0), 6.2% formaldehyde, 50% formamide]–4 μl of buffer B (10 mM EDTA [pH 8,0], 0.25% bromophenol blue, 50% glycerol, 0.1 mg of ethidium bromide per ml) (31). Samples were heated 10 min at 65°C prior to being loaded onto a formaldehyde-agarose gel. After electrophoresis, the gel was treated for 30 min in 50 mM NaOH, 15 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 15 min in 10× SSC before being blotted onto GeneScreen Plus (NEN). Blots were hybridized with a 356-bp *Eco*RI-*Xmn*I non-zinc finger fragment of Z5.9 (Fig. 1), Z5.9zf−, labelled by random priming (19, 20). Hybridization was performed at 55°C with 2.5×10^5 cpm/ml in 2× SDE (2× SDE is 100 mM NaCl, 50 mM sodium phosphate [pH 7.0], and 5 mM EDTA)–5% sodium dodecyl sulfate (SDS)–100 μg each of yeast tRNA and denatured salmon sperm DNA per ml (55). The sulfated glycoprotein 2 (SGP-2) rat cDNA (1,364-bp) probe was obtained from R. Buttyan, Columbia, University. A full-length human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was provided by M. Alexander-Bridges, Massachusetts General Hospital. High-stringency washes were done twice for 10 min at 65°C with 1× SSC (Z5.9zf−), twice for 5 min at 65°C with 0.1× SSC (SGP-2), or twice for 10 min at 65°C with 2× SSC (GAPDH). Membranes were exposed at −70°C to Kodak X-OMAT film with intensifying screens for 3 to 14 days.

Southern blot analysis. Genomic DNA was prepared from human leukocytes by the protocol of John et al. (25) and from rat and mouse liver according to standard protocols (3). Ten micrograms of genomic DNA was digested with the indicated restriction enzymes (rat DNA) or with *Eco*RI only (mouse and human DNA). Samples were separated by agarose gel electrophoresis and transferred onto GeneScreen Plus membranes (NEN). Hybridization with Z5.9zf− (2.5×10^5 cpm/ml) was performed at 55°C in 2× SDE–5% SDS–100 μg each of yeast tRNA and denatured salmon sperm DNA per ml (55). High-stringency washes were done twice for 10 min at 65°C with 1× SSC (rat DNA) or 4× SSC (human and mouse DNAs). Membranes were exposed at −70°C to Kodak X-OMAT film with intensifying screens.

Amplification of RNA by PCR. A 515-bp fragment of *Kid-1* (nucleotides 441 to 955 in the cDNA) was amplified from 1 μg of total RNA from various organs by using primers 5′-AATTTCTCTCCACTATGG-3′ (antisense) and 5′-CTGG AGAATTACAGCAACC-3′ (sense) and a GeneAmp kit (Perkin-Elmer, Norwalk, Conn.). The initial denaturation step involved incubation for 5 min at 95°C. This was followed by 40 cycles of 1 min at 95°C, 1 min at 45°C, and 1 min at 72°C, with final extension for 10 min at 72°C. No RNA was added to the negative control. Equal aliquots of the polymerase chain reaction (PCR) products were separated on an agarose gel, blotted, and hybridized as described under "Southern blot analysis." The blot was hybridized with the random-primed 515-bp fragment prepared by PCR from the Z5.9/16 *Kid-1* cDNA clone. After a 1-h exposure, the areas on the membrane corresponding to the signal on the film were cut out and the radioactivity was quantitated in a scintillation counter. To ensure the quality of the RNA, amplification was also carried out with primers for GAPDH.

Transfection protocols. LLC-PK_1 and COS cells, growing in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, were plated at a density of approximately 2.5×10^5 cells per 100-mm-diameter dish 2 days prior to transfection. For transfection, cells were exposed to a

total of 20 μg of DNA (including carrier DNA) in 5 ml of Dulbecco's minimal essential medium–10% NuSerum (Collaborative Biomedical Products, Bedford, Mass.)–400-μg/ml DEAE-dextran (Sigma, St. Louis, Mo.)–0.1 mM chloroquine (Sigma). Four hours after the addition of DNA, the medium was removed and cells were shocked for 2 min at room temperature with 10% dimethyl sulfoxide in 1× phosphate-buffered saline (PBS). After the shock treatment, the cells were washed once with PBS and new medium was added. Cells were harvested 48 h after transfection for CAT and luciferase assays.

CAT and luciferase assays. Transfected cells were washed twice with PBS, scraped with a rubber policeman into a microcentrifuge tube, and spun down. The cell pellet was resuspended in 200 μl of 0.25 M Tris Cl, pH 7.8, and subsequently broken up by freeze-thawing three times by alternating a dry ice-ethanol bath and a 37°C water bath. The supernatant was assayed for CAT activity by standard protocols (48). Thin-layer chromatography with silica gel plates (Silica gel IB; J. T. Baker, Phillipsburg, N.J.) was used to separate acetylated from nonacetylated forms of chloramphenicol. Spots corresponding to monoacetylated and nonacetylated [^{14}C]chloramphenicol were scraped from the thin-layer chromatography plate and quantitated in a liquid scintillation counter. CAT activity is expressed as the ratio of monoacetylated [^{14}C]chloramphenicol to total [^{14}C]chloramphenicol and normalized to luciferase activity derived from a cotransfected luciferase reporter plasmid. One microgram of a luciferase-expressing plasmid, poLucSV/T1 (53), was included in all cotransfection experiments to enable normalization for transfection efficiencies. Cell extracts were assayed for luciferase activity with the luciferase assay system from Promega (Madison, Wis.) according to the manufacturer's instructions using a model 20e Luminometer (Turner Designs, Mountain View, Calif.).

Nucleotide sequence accession number. The GenBank accession number for the *Kid-1* sequence is M96548.

RESULTS

Isolation of cDNA clones. cDNA libraries constructed from normal and postischemic kidneys were screened with a degenerate oligonucleotide [(A/G)TANGG(C/T)TT(C/T)TC NCCNGT(A/G)TG] encoding the conserved peptide, HT GEKPY, which lies between the individual zinc fingers of the C_2H_2 class of transcription factors (52). More than 150 hybridizing plaques were found in each library. Nineteen were further purified, and five of them were subcloned into pBluescript. Partial sequencing of these clones revealed the presence of C_2H_2 zinc finger motifs, confirming the success of the pursued strategy.

One of these cDNAs, which we call Z5.9, derived from the postischemic library, contains 1,381 bp and hybridizes to a 2.8-kb transcript which is expressed primarily in the kidney and accumulates with age in the course of postnatal development of the kidney. Furthermore, the levels of the transcript encoded by this gene, which we call *Kid-1* (kidney, ischemia and developmentally regulated), decrease after ischemia-reperfusion and folic acid administration. This reduction in mRNA levels requires more than 1 h of reperfusion to be observed, accounting for our ability to isolate the cDNA from the 1-h postischemia kidney library.

To obtain cDNA clones which overlap Z5.9 and extend 5′ and 3′, λgt10 and λgt11 rat kidney cDNA libraries from Clontech were screened with Z5.9. Positive plaques were picked and subjected to PCR with internal Z5.9-specific primers and external lambda-specific primers. Clones extending further 5′ or 3′ were plaque purified, subcloned, and sequenced. Two overlapping cDNA clones were isolated, corresponding to a total of 2,563 bp (Fig. 1).

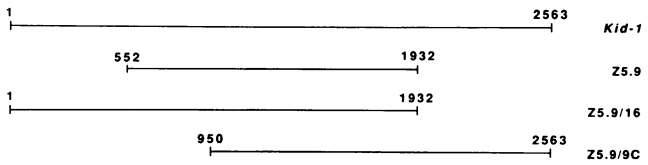

FIG. 1. Relationship between clones analyzed. Z5.9 was the initial clone isolated from the λgt10 kidney cDNA library. Clones Z5.9/16 and Z5.9/9C were selected by using PCR with 5′ and 3′ primers from Z5.9 and lambda primers. The relationship of these clones to the full-length *Kid-1* cDNA is presented. Base pair numbers are indicated.

Sequence analysis of *Kid-1*. The nucleotide sequence of *Kid-1* contains a long open reading frame in the cDNA, from which a predicted protein product of approximately 66 kDa was deduced (Fig. 2). This size estimate is based on the usage of the 5′-proximal ATG codon at position 312. An in-frame ATG at position 468 could be an alternative start codon since, except for the presence of a pyrimidine rather than a purine at position −3, it lies in an almost perfect consensus context to optimize translation initiation (30). At the carboxy end of the predicted protein are 13 zinc fingers, which are clustered in groups of 4 and 9. Zinc fingers 4 and 5 are separated by a 32-amino-acid spacer. Upon alignment of the zinc fingers (Fig. 3), the invariant cysteines and histidines are clearly distinguished. In addition, there is significant conservation of many amino acid residues within the fingers themselves as well as in the H-C link. There is more identity among the amino acids of the individual fingers in the group of nine zinc fingers than in the group of four.

The amino-terminal non-zinc finger region of Kid-1 contains a Krüppel-associated box (KRAB) domain (5) which is predicted to form an α-helix and may have a role in protein-protein interactions. If the alternative methionine codon at position 468 is used, the KRAB-A box would not be expressed, possibly resulting in altered interaction of the Kid-1 protein with other proteins. Kid-1 also contains a nuclear translocation signal, as previously described for nucleoplasmin (44).

Comprehensive sequence data base searches for genes encoding similar protein sequences revealed regions of identity in the mouse genes *Zfp-35* (55%) (14) and *Kr2* (58%) and the human gene *Hf.10* (51%). In each case, however, the identity was found in the zinc finger region of the protein sequence and there was no similarity seen in the non-zinc finger region. Thus, *Kid-1* encodes a novel zinc finger protein. Even though there is considerable homology with other genes in the zinc finger region, Northern analysis of total kidney RNA, using as a probe Z5.9, which includes 11 of the 13 zinc fingers, revealed only one identifiable band under conditions of high-stringency washes (data not shown). Therefore, while we cannot exclude the possibility of more than one transcript of similar size, these data further suggest that there is no significant cross-hybridization of *Kid-1* clones with transcripts of other genes.

Kid-1 mRNA has two possible polyadenylation signals (57), one located 33 nucleotides from the poly(A) tail and another within 69 bases of the stop codon. There are structural features of the *Kid-1* mRNA and deduced protein sequence which suggest that both may be short-lived (Fig.

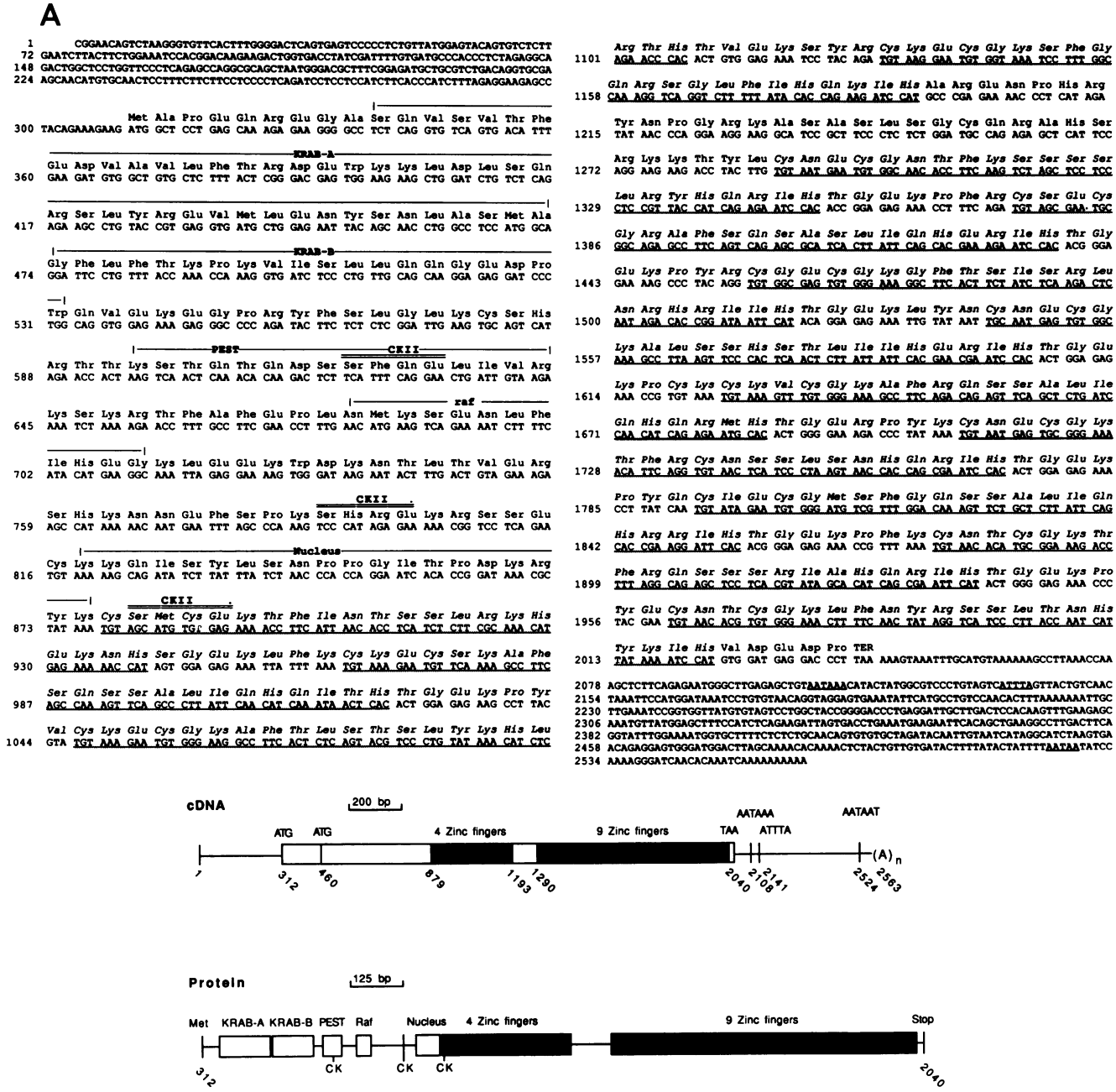

FIG. 2. Structure of *Kid-1*. (A) Sequence of the *Kid-1* cDNA and the predicted protein product. Nucleotide sequence is written in the 5′-to-3′ direction. The deduced amino acid sequence is shown from the putative start codon at position 312. The zinc fingers are underlined, and the protein sequence is in italics. KRAB-A and -B, the PEST sequence, the *raf* homology domain, the nuclear translocation signal (Nucleus), and casein kinase II consensus sites (CKII) are indicated. Putative polyadenylation sequences (AATAAA and AATAAT) and an instability motif (ATTTA) in the 3′ untranslated region are underlined. (B) Relationships of the *Kid-1* cDNA and structural domains of the Kid-1 protein. CK, casein kinase.

2). An AUUUA sequence at residues 2137 to 2141 may contribute to a short half-life of the message, since this pentanucleotide sequence confers mRNA instability and has been found in the 3′ untranslated regions of several rapidly turning over mRNAs, including those of cytokines, lymphokines, and oncogenes (7). One could also speculate that there exists another form of mRNA in which the polyadenylation signal further 5′ was used and which, because it does not carry the AUUUA sequence, would be predicted to have a longer half-life. At present, we do not know whether this mRNA species exists and, if it does, whether it exerts a distinct biological function. There also is a consensus region (PEST sequence) in the NH_2 terminus of the deduced Kid-1 protein which is present in proteins with short half-lives (45). This peptide sequence, rich in proline, acidic, serine, and threonine residues, has been proposed to be the correlate of the AUUUA sequence on the protein level.

Another important feature of the predicted Kid-1 polypep-

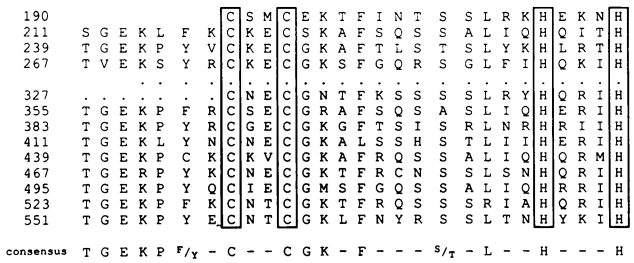

FIG. 3. Zinc finger domains of Kid-1. Finger repeats are aligned with consensus amino acids indicated at the bottom. Invariant cysteines and histidines are boxed. There is a 32-amino-acid spacer between fingers 4 and 9. Amino acid numbers are indicated to the left of the repeats.

tide is the presence of a 12-amino-acid motif shared by all members of the Raf family of serine/threonine kinases (22) (Fig. 4). This motif lies in the catalytic subdomain VI of protein kinases (22, 28, 29), suggesting that Kid-1 may have kinase activity. The glutamic acid residue in the catalytic domain, together with the aspartic acid residue 7 amino acids downstream, may support ATP binding (9). A region somewhat homologous to kinase subdomain VI has been identified in the deduced protein sequence of one of the subunits of TFIIE, a general transcription factor (40).

There are structural aspects of the deduced Kid-1 protein which suggest that it may be a substrate for phosphorylation. There are three potential casein kinase II phosphorylation sites (41), two in the non-zinc finger region and one in the first zinc finger. In addition, the 32-amino-acid spacer between the fourth and fifth zinc fingers contains four serines and one threonine. Two of the serines and the threonine are preceded by an arginine at position −3, a consensus motif for cyclic AMP (cAMP)-dependent protein kinase and protein kinase C (27).

Southern analysis. Southern analysis of rat genomic DNA cut with *Bam*HI, *Eco*RI, *Hin*dIII, or *Pst*I and hybridized with Z5.9zf− yielded only one band in each case (Fig. 5A). Thus, *Kid-1* is a single-copy gene. When mouse and human genomic DNAs were cut with *Eco*RI and rat Z5.9zf− cDNA was used as a probe, there was a single band seen in each lane, indicating that *Kid-1* is conserved among rodents and primates (Fig. 5B).

Developmental and tissue expression of *Kid-1*. *Kid-1* mRNA levels change with kidney ontogeny. *Kid-1* mRNA is only marginally detectable by Northern analysis in kidneys taken

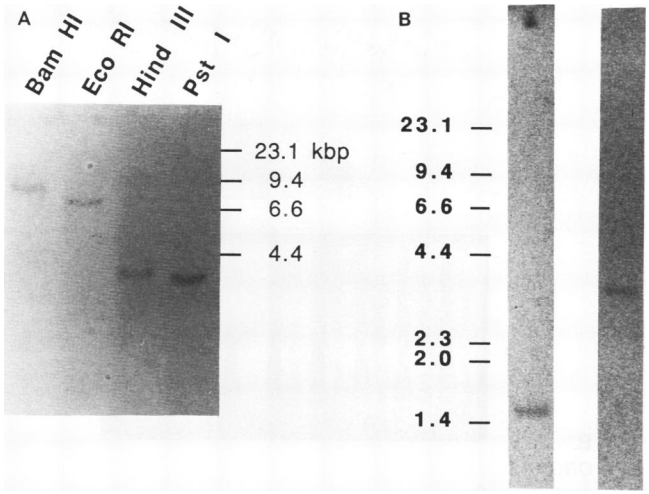

FIG. 5. Southern analysis of *Kid-1*. (A) Rat genomic DNA hybridized with *Kid-1* cDNA. When 10 μg of rat genomic DNA cut with each of four different restriction enzymes was probed with Z5.9zf−, the non-zinc finger region of Z5.9, only one band could be detected in each lane. (B) Mouse and human genomic DNA cut with *Eco*RI and hybridized with rat Z5.9zf−. A single band can be seen in mouse (left lane) and human (right lane) genomic DNAs.

at the time of birth but becomes easily seen at 15 days and accumulates to greater levels in the adult rat (Fig. 6).

Kid-1 is expressed predominantly in the kidney. When 50 μg of total RNA from a number of different organs was hybridized with Z5.9zf−, a 2.8-kb band could be detected only in the kidney (Fig. 7A). To further increase the sensitivity of detection of *Kid-1* mRNA in different organs, we also used the PCR to amplify *Kid-1* mRNA sequences, using reverse transcriptase and primers selected to amplify a 515-bp fragment of the *Kid-1* cDNA sequence (see Materials and Methods). A probe was made from Z5.9/16, amplified with the same primers, and labelled by random priming. The probe was hybridized to products of the PCR in a Southern blot. Hybridization was quantitated by cutting out the bands. There was a very strong signal for the kidney and signals near background level for other organs (Fig. 7B). On the Southern blot itself, very faint but clearly present bands

Kid-1	NMKSENLFIHEG
Human c-raf	D---N-I-L---
Rat c-raf	D---N-I-L---
Xenopus c-raf	D---N-I-L---
Human A-raf	DL--N-I-L---
Rat A-raf	DL--N-I-L---
Mouse A-raf	DL--N-I-L---
Human putative ser/thr kinase	DL--N-I-L---
Human B-raf	DL--N-I-L--D
Mouse B-raf	DL--N-I-L--D
Drosophila raf	DL--N-I-L--D

FIG. 4. Homology of Kid-1 with members of the *raf* family of serine/threonine kinases. A catalytic region of the Raf protein family is highly conserved in Kid-1. Hyphens denote identity with Kid-1. The single-letter amino acid code is employed.

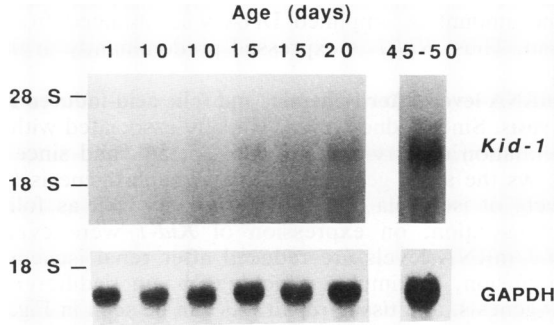

FIG. 6. Northern analysis of total RNA collected from rats at various stages of postnatal development. Blots were hybridized with Z5.9zf− and GAPDH cDNA probes.

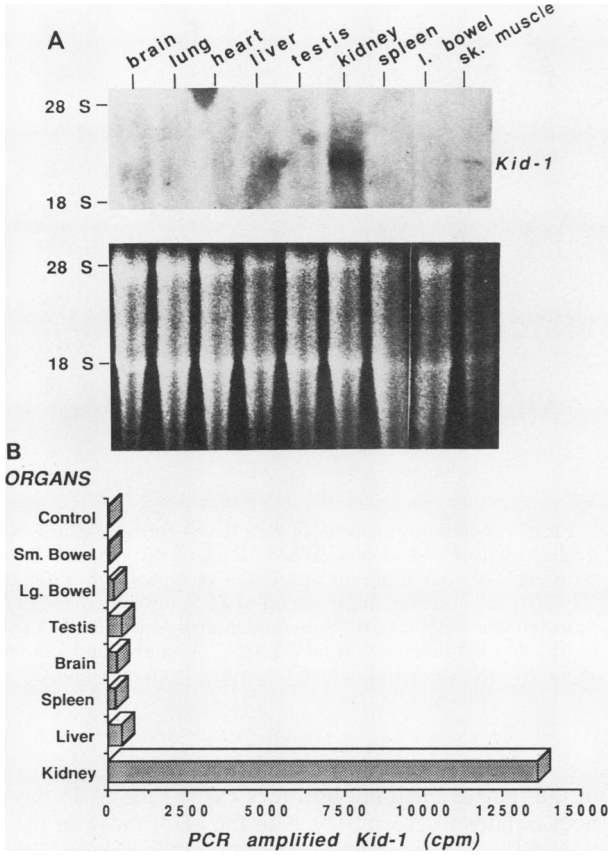

FIG. 7. Expression of *Kid-1* in various tissues. (A) Northern blot analysis of 50 μg of total RNA isolated from each of the indicated rat organs. Among the large number of rat organs tested, *Kid-1* is expressed only in the kidney. Neither brain, lung, heart, liver, testis, spleen, large bowel, nor skeletal muscle tissue shows any detectable expression of *Kid-1* mRNA. The lower part of panel A shows the ethidium bromide staining of the gel, indicating approximately the same amounts of RNA in each lane, as reflected by the relative intensities of the 28S and 18S bands. (B) Analysis of expression of *Kid-1* in various tissues by PCR. cDNA was prepared from different tissue RNAs with reverse transcriptase. By using specific primers for *Kid-1*, a fragment of the *Kid-1* cDNA was amplified by PCR and samples of the amplified product were then subjected to Southern blot analysis with Z5.9zf− labelled with ^{32}P by random priming. Bands were cut out and quantitated by liquid scintillation counting.

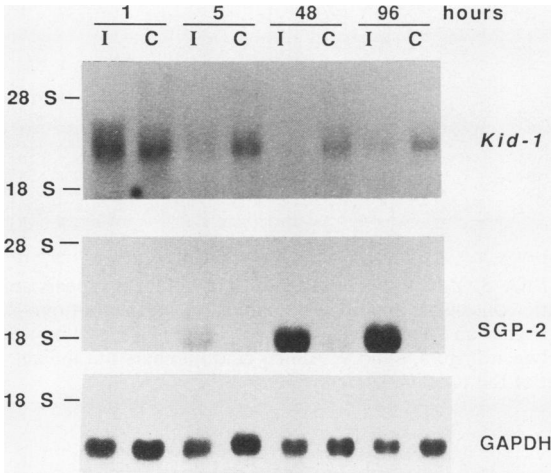

FIG. 8. Northern analysis of kidney RNA obtained after ischemia and various periods of reperfusion. Northern blots of total RNA taken from kidneys which underwent 30 min of ischemia and various times of reperfusion (lanes I) or from the contralateral control kidneys (lanes C) were hybridized with either Z5.9zf−, SGP-2, or GAPDH cDNA probes.

were seen in samples from the spleen, brain, liver, and testis (data not shown). When PCR was performed with oligonucleotides complementary to GAPDH, approximately the same amount of amplified DNA was obtained from each organ. Thus, *Kid-1* is expressed predominantly in the kidney.

mRNA levels after ischemia- and folic acid-induced tubular necrosis. Since kidney injury is likely associated with dedifferentiation of surviving cells (6, 35, 56), and since repair follows the same general pattern as nephrogenesis (4), the effects of ischemia and reperfusion, as well as folic acid administration, on expression of *Kid-1* were evaluated. *Kid-1* mRNA levels are reduced after renal ischemia and reperfusion, a stimulus which results in dedifferentation, mitogenesis, and tissue repair. As can be seen in Fig. 8, the levels of *Kid-1* mRNA decline after 30 min of unilateral ischemia and 5 h of reperfusion. There is a marked decrease in the steady-state levels of *Kid-1* mRNA up to 96 h after reperfusion. Normal mRNA levels are restored after 7 days of reperfusion (data not shown). To prove that the decline in mRNA levels was not a nonspecific response to tissue damage or energy depletion, which might have a general effect on transcription, we hybridized the same blot with the cDNA for sulfated glycoprotein 2 (SGP-2), a gene developmentally regulated in the kidney whose expression is downregulated in epithelia that are terminally differentiated (23). This gene has also been implicated in prostate cell death (10) and has previously been shown to be induced with renal ischemia and reperfusion (46). There was a marked increase in SGP-2 mRNA levels in the postischemic kidney at 48 and 96 h of reperfusion, at a time when *Kid-1* mRNA levels were reduced, compared with levels in the contralateral kidney.

To evaluate whether a decrease in *Kid-1* mRNA levels could be reproduced by another stimulus for kidney epithelial cell proliferation, we measured *Kid-1* mRNA levels after rats were administered folic acid. High concentrations of folic acid induce acute tubular necrosis with subsequent regeneration of epithelial cells derived from the surviving cells (2, 21). *Kid-1* mRNA levels were suppressed 3 h after folic acid administration and remained suppressed for at least 24 h (Fig. 9). The same blots were hybridized with a cDNA for SGP-2. SGP-2 mRNA accumulated to high levels at 8 and 24 h after folic acid administration, at the same times that *Kid-1* mRNA levels were reduced.

Effect of the N-terminal, non-zinc finger region of Kid-1 on transcriptional activity. The *Kid-1* cDNA was modified in the following way. A 5′ fragment was amplified by PCR with primers spanning position 312 (5′-CCAACATTTAAGCT TCTAGACTGCAGCTCGAGGCCACC**ATG**GCTCCT GAGCAAAG-3′ [the ATG starting at position 312 is underlined and in boldface type]) to position 541 (5′-TCCACC TGCCAGGGATCCTCT-3′). The upstream primer contained a perfect Kozak box for ATG-312 and recognition sites for *Hin*dIII, *Xba*I, *Pst*I, and *Xho*I. The downstream primer included the internal *Bam*HI site at position 524, so that the resulting PCR fragment could be cut with *Bam*HI and ligated to the *Bam*HI-*Bsa*HI 1.6-kbp fragment which

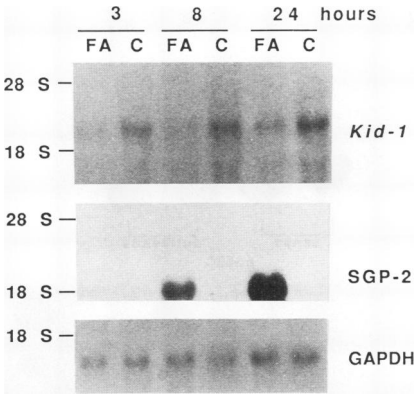

FIG. 9. Northern analysis of kidney RNA collected 3, 8, or 24 h after folic acid administration. Northern blots were hybridized with Z5.9zf−, SGP-2, and GAPDH cDNAs. Lanes FA, RNA from a kidney from an animal treated with folic acid; lanes C, RNA from a kidney from a vehicle-treated control animal.

was isolated from a phage clone containing the full-length *Kid-1* cDNA. Sequencing confirmed the absence of any mutations. The non-zinc finger domain of *Kid-1* was subsequently excised and ligated to the DNA-binding region of *GAL4* (encoding amino acids 1 to 147) in the vector pBXG1, both in sense and antisense directions, yielding pBXG1/Kid-1N sense or pBXG1/Kid-1N antisense (Fig. 10A). The reporter plasmid contained five GAL4 binding sites and a CAT gene downstream from a minimal promoter (pG5EC) or SV40 enhancer (pG5SV-BCAT). pBXG1, pG5EC, and pG5SV-BCAT were generous gifts of M. Ptashne (26, 47).

Ten micrograms of either pBXG1/Kid-1N sense or pBXG1/Kid-1N antisense was cotransfected with 3 μg of the pG5EC reporter plasmid to evaluate whether the N-terminal, zinc finger-free domain of Kid-1 was able to modulate transcriptional activity when coupled to the DNA-binding region of GAL4. We chose COS cells, a large-T-antigen-transformed green monkey kidney cell line, and LLC-PK_1 cells, a highly differentiated porcine epithelial kidney cell line, to assay for transcriptional regulation in two different eukaryotic environments. There was low-level constitutive expression from the pG5EC reporter plasmid, which contains only a weak promoter (47). In neither cell line could we detect any positive influence of the GAL4–Kid-1N chimeric protein on transcription. In fact, the fusion protein exerted a negative effect on transcription in both cell types (Fig. 10B and C).

To further evaluate the possibility that the GAL4–Kid-1N chimeric protein might alter transcription, we performed experiments with pG5SV-BCAT, a reporter plasmid in which the CAT gene is driven by the strong SV40 enhancer (33). This construct showed strong CAT activity when cotransfected with pBXG1/Kid-1N antisense; however, CAT activities dropped markedly when pBXG1/Kid-1N sense was the expression plasmid (Fig. 10B and C). In a subsequent experiment, we evaluated how the suppression of transcription varied with the amount of expression plasmid transfected. A 50% reduction in CAT activity was seen when 1 μg of expression plasmid was cotransfected with 3 μg of reporter plasmid, with greater reductions observed as larger amounts of the expression plasmid were cotransfected (Fig. 10D).

DISCUSSION

The kidney is a complex organ, consisting of cells with highly varied differentiated phenotypes. This phenotypic complexity is likely determined in part by differential expression of various genes, some of which may be expressed primarily in the kidney. Kidney-specific expression may be modulated in part by kidney-specific transcription factors. Our studies reveal a novel *Kid-1* cDNA which hybridizes to a 2.8-kb mRNA transcript which is expressed primarily in the kidney and accumulates in the course of postnatal development of the kidney. It is possible that *Kid-1* plays a role in renal development. Whereas in humans renal development is complete at the time of birth, in rats a substantial amount of development takes place after birth (50). S-shaped bodies can still be found 4 to 5 days after birth (12), and thymidine incorporation does not decrease to background levels until 15 to 35 days after birth (12, 54). In the newborn rat kidney, *Kid-1* is barely detectable. *Kid-1* is much more strongly expressed in the adult. Its mRNA levels increase between days 15 and 20 after birth, at a time when the thymidine incorporation begins to decrease to baseline (12, 54). Thus *Kid-1* mRNA levels are correlated with epithelial cell differentiation in postnatal development.

The potential importance of *Kid-1* is also underscored by the fact that its expression is predominantly kidney specific, a feature comparable to that of the helix-loop-helix protein MyoD in muscle (15). Some of the other known zinc finger genes are preferentially expressed in particular cells, and some are developmentally regulated. *MZF-1*, a human zinc finger gene, is preferentially expressed in myeloid cells (24). *ZFX* and *ZFY* are zinc finger genes on the Y chromosome which may have a role in testis development (51). *ZFX* and *ZFY* are transcribed, however, in many human tissues. The mouse gene *Zfp-35*, which shares homology with *Kid-1* in the zinc finger region only, contains 18 zinc fingers and is expressed predominantly in the testis (14). The Wilms' tumor gene has four C_2H_2 zinc fingers and is expressed primarily in the kidney and the spleen (11). To our knowledge, *Kid-1* is the first example of a developmentally regulated putative zinc finger transcription factor expressed primarily in the kidney.

Kid-1 encodes the same number of zinc fingers (13) as *MZF-1*, *ZFX*, and *ZFY*. MZF-1 has a 24-amino-acid spacer between the fourth and fifth zinc fingers, a pattern similar to that of Kid-1, in which the spacer consists of 32 amino acids. The amino acids of the spacer regions of the two proteins are different, however. In MZF-1, the spacer is rich in glycines and prolines. In the deduced Kid-1 protein, the spacer region contains four serines and one threonine. Two of the serines and the threonine are preceded by an arginine at position −3, a consensus motif for cAMP-dependent protein kinase and protein kinase C (27). The zinc fingers in ZFX and ZFY are not divided by a spacer.

Decreased *Kid-1* mRNA levels are seen when epithelial cells are dedifferentiated and proliferate: early in postnatal development, during ischemia and reperfusion, and after folic acid administration. It is possible that the gene product may exert a positive effect on kidney cell differentiation and/or a negative effect on growth. We have shown that the non-zinc finger NH_2 terminus of Kid-1 (Kid-1N) can serve as a strong transcriptional suppressor when fused to the DNA-binding domain of GAL4. This suppressor effect is not species dependent, since the GAL4–Kid-1 construct inhibits CAT activity from a reporter plasmid containing GAL4 binding sites in both COS cells, derived from the green

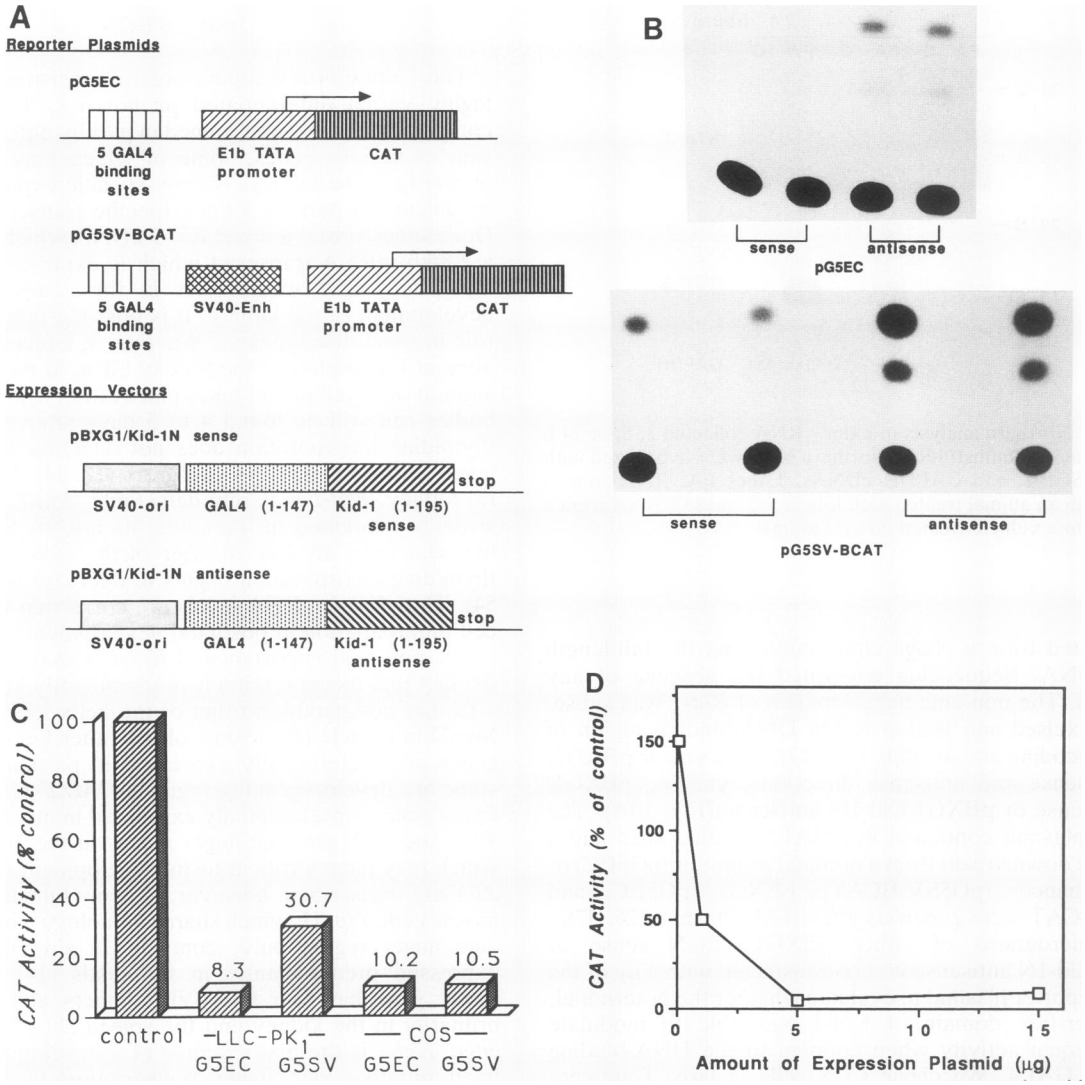

FIG. 10. Effects of the non-zinc-finger region of Kid-1 on transcription. (A) Plasmid constructs used for transfection experiments. Two reporter plasmids were used. The CAT gene was driven by either a minimal promoter only, containing a TATA box of the E1b gene (pG5EC), or the strong SV40 early enhancer (pG5SV-BCAT). In both plasmids, five GAL4 binding sites were located upstream of the promoter and enhancer regions. The expression vectors contained sequences encoding the NH_2-terminal 147 amino acids of GAL4 and the NH_2-terminal 195 amino acids of Kid-1, with the latter in either the sense (pBXG1/Kid-1N sense) or the antisense (pBXG1/Kid-1N antisense) orientation. (B) Effect of expression of the chimeric Kid-1N–GAL4 protein on CAT activity in COS cells cotransfected with either pG5EC or pG5SV-BCAT. CAT activity was suppressed when the chimeric protein included the Kid-1N sequence in the sense orientation compared with the activity with the antisense orientation, whether the reporter plasmid was driven by a weak or a strong promoter. The upper spots on these thin layer chromatography plates reflect the acetylated forms of chloramphenicol. (C) Effect of expression of the Kid-1N–GAL4 protein on CAT activity in pG5EC and pG5SV-BCAT in LLC-PK_1 and COS cells. CAT activities observed with the sense construct are expressed as percentages of those measured when equal amounts of the antisense construct were cotransfected, with the latter activity arbitrarily set at 100%. Values were normalized to luciferase activity derived from a cotransfected luciferase reporter plasmid (poLucSV/T1). Means of two or more experiments are presented. (D) Effect of transfection of various amounts of expression plasmid on CAT activity. Different amounts (0.1, 1, 5, or 15 μg) of pBXG1/Kid-1N sense or antisense were cotransfected with 3 μg of pG5SV-BCAT. CAT activities observed with the sense construct are expressed as percentages of those measured when equal amounts of the antisense construct were cotransfected. Values were normalized to luciferase activity derived from a cotransfected luciferase reporter plasmid.

monkey, and LLC-PK_1 cells, derived from the porcine kidney. Nor does transcriptional suppression depend on the differentiation state of the cell line (COS cells are fibroblastoid, whereas LLC-PK_1 cells are highly differentiated epithelial cells). Transcriptional repression in eukaryotes is poorly understood (43), and hence statements regarding the mechanism of Kid-1 repression must be considered highly hypothetical at this time. Kid-1 may cause a local distortion of the DNA structure around the promoter site and thus impede binding of other transcription factors to the SV40 enhancer in the reporter plasmid containing the SV40 enhancer (pG5SV-BCAT). The fact that the non-zinc finger region of Kid-1 also inhibits CAT activity from the reporter plasmid containing a minimal promoter (pG5EC) suggests,

however, that Kid-1 may interact with proteins of the basal transcription factor machinery or may interfere with the proper assembly of basal transcription factors. At this point, we cannot discriminate between these or other possibilities.

Proteins implicated in renal differentiation include cytoskeletal proteins (e.g., vimentin and cytokeratin) (36), enzymes (e.g., gamma glutaryl transferase and alkaline phosphatase) (12), other transcription factors (e.g., Wilms' tumor gene and *myc*) (37, 38, 42), extracellular matrix genes (e.g., laminin and collagen IV genes), and cell surface proteins (e.g., N-CAM and uvomorulin) (4, 8, 18, 50). Genes encoding these proteins are potential target genes for regulation by Kid-1.

Features of the putative Kid-1 protein suggest potential mechanisms of regulation. The KRAB domains in the amino terminus of the putative protein have been proposed to form helical structures which may promote protein-protein interactions (5). Many transcription factors interact with other proteins in the regulation of gene expression (for examples, see reference 13). The presence of casein kinase II, cAMP-dependent protein kinase, and protein kinase C consensus regions in the Kid-1 protein suggests that the protein may be modulated by phosphorylation. Thus, in addition to the involvement of zinc fingers in DNA binding, interactions of the Kid-1 protein with other proteins and/or DNA may be modulated by the KRAB domains and/or by phosphorylation of the protein. Furthermore, the presence of a region of high-level homology to a catalytic domain of each member of the Raf family of transforming proteins suggests the possibility of kinase activity for Kid-1, an activity which may also be important for protein-protein and protein-DNA interactions. There is evidence that phosphorylation is necessary to optimize the ability of some proteins to initiate transcription. This is the case, for example, with RNA polymerase II (1). The largest subunit of this protein has a serine- and threonine-rich C-terminal domain which is phosphorylated on many sites, and this phosphorylation is believed to be necessary for transcriptional initiation (1).

In conclusion, we report the cloning and partial characterization of a putative transcription factor whose expression is modulated in kidney ontogeny and whose mRNA accumulates primarily in the kidney. *Kid-1* mRNA levels are also influenced by other stimuli associated with change in the differentiated phenotype and the rate of cell proliferation. The *Kid-1* gene product may play an important role in kidney cell differentiation and mitogenesis and possibly in renal repair mechanisms.

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