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Identification and Characterization of Human *PKNOX2,* a Novel Homeobox-Containing Gene

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The three-amino-acid loop extension (TALE) homeodomain proteins are highly conserved transcription regulators. Since cooperative function among members of this growing family is critical for regulating transcription, we have tried to explore novel members to understand their regulatory mechanisms in cellular proliferation and differentiation. Here we report identification of PKNOX2, a novel TALE homeodomain protein that shows distinct homology with PKNOX1, a stable partner of PBX proteins. PKNOX2 is composed of 460 amino acids and contains HR1, HR2, and homeodomain, which are highly similar to PKNOX1, suggesting that PKNOX2 may also interact with PBX proteins as well as the same DNA sequence as PKNOX1. Genomic organization of *PKNOX2* also showed high similarity to *PKNOX1,* though *PKNOX2* lies on a different chromosomal region, 11q24. Unlike PKNOX1, which was broadly expressed in many tissues, PKNOX2 showed a more restricted pattern of mRNA expression. Nuclear localization of PKNOX2 was confirmed by transfection of epitope-tagged cDNA. Taken together, these data indicate that PKNOX2 is a novel PKNOX-related protein and may interact with PBX proteins and play a tissue-specific regulation of transcription.

Key Words: **PKNOX2; homeodomain; PKNOX1; TALE family; 11q24.**

Homeodomain protein superfamily comprises a large number of sequence specific transcription factors sharing a highly conserved DNA-binding homeodomain, and play fundamental roles in cellular proliferation, differentiation, and death (1). The three-amino-acid loop extension (TALE) superclass of homeodomain proteins is characterized by an extension of three amino acids between α-helices 1 and 2 within the homeodomain (2, 3). Members of this superclass, such as MEIS-, TGIF-, and PBX-related proteins and PKNOX1, are highly conserved, and are present in the common ancestor of plants, fungi, and animals (3). Interestingly, cooperative function among TALE family members is critical for regulating transcription (4–6), and several members have been shown to function as essential contributors to the HOX-mediated developmental program (7, 8). These features had led us to search for novel members of this growing and interesting superfamily, in an effort to better understand regulatory mechanisms involving homeodomain proteins. In previous search using computer-assisted method, actually, we have successfully identified one of the TGIF-related proteins, TGIF2 (9).

PKNOX1, a member of TALE superfamily homeodomain protein, has been identified as one of the components of human transcription factor complex urokinase enhancer factor 3 (UEF3) (10). The PKNOX1 homeodomain is most closely related to those of the MEIS and TGIF proteins and, like these, recognizes a TGACAG motif. Interestingly, this protein stably heterodimerizes with PBX1, another subunit of UEF3, independent of DNA binding, resulting in a strong DNA binding affinity of the heterodimerized complex towards the TGACAG target site. The PKNOX1-PBX1 complex binds DNA cooperatively with other transcription factors, including HOX family proteins, and PKNOX1 is able to act as a co-activator in the transcription of PBX-HOX activated promoters (11, 12). In some genes including glucagons (13), PKNOX1-PBX heterodimers also can repress the transcription. Thus, PKNOX1 may be a stable intracellular partner of PBX proteins to regulate transcription of specific genes positively and negatively.

PKNOX1 is ubiquitously expressed in almost all tissues examined, though the PBX isoforms vary in different tissues. In addition, PKNOX1-related proteins have never been identified except PKNOX1, whereas other members of the TALE superfamily form a superfamily (3). In order to explore other members of this subfamily, which may show unique tissue expression

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pattern, we applied a combination of computerized database searches, analysis of expressed sequence tag (EST) clones, and reverse transcription-polymerase chain reaction (RT-PCR), as in a previous study (9). Here we report the identification and characterization of the second member of this subfamily, designated as PKNOX2.

MATERIALS AND METHODS

Cloning and sequencing of human PKNOX2. We searched for PKNOX-related proteins by comparing human PKNOX1 sequence (GenBank accession number: NM_004571) against the databases of ESTs and genomic sequences, using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Of several EST clones identified by this search, we purchased three clones from Incyte Genomics Inc. (St. Louis, MO): IMAGE clones 347065, 265097, and 1583683. Since all these EST clones contain part of the entire coding sequence, we used RT-PCR to obtain cDNA fragments containing the entire coding sequence. cDNA was synthesized using mRNA derived from human brain, and then PCR amplifications were performed using primers generated on the basis of EST and genomic sequence (GenBank accession number AC009807) and Platinum *Pfx* DNA polymerase (Gibco BRL, Gaithersburg, MD) according to the manufacturer's directions. PCR products were sub-cloned for sequencing with a 377 ABI autosequencer (PE Biosystems, Foster City, CA). Analyses of sequences and comparisons of data were performed using BLAST, MOTIF (http://motif.genome.ad.jp/) and PSORT II (http://cookie.imcb.osaka-u.ac.jp/nakai/psort.html) programs.

Northern blot analysis. Northern blots of RNA from different human tissues (Human 12-lane MTN blot) were obtained from Clontech, Inc. (Palo Alto, CA). A cDNA probe containing full coding sequence of *PKNOX2* was labeled with [α^{32}P]dCTP by random priming (Megaprime, Amersham Pharmacia Biotech), and hybridized to the prehybridized blots. Blots were washed in a solution of 0.1× SSC/0.1% SDS, and then exposed for 72 h.

Fluorescence in situ hybridization (FISH). FISH analyses were performed as described previously (9, 14), using a bacterial artificial chromosome (BAC) containing *PKNOX2* (RP11-417F7) as the probe. Digoxigenin-11-dUTP-labeled probe was hybridized to metaphase chromosomes prepared from normal male lymphocytes, and specific signals were detected with rhodamine anti-digoxigenin antibody (Roche Diagnostics, Tokyo, Japan). Precise localization of PKNOX2 was determined on elongated prometaphase chromosomes.

Expression construct and transfection. A plasmid construct encoding an epitope-tagged form of PKNOX2 was assembled by cloning the coding sequence of this gene in-frame with Xpress epitope into the pcDNA3.1/HisC vector (Invitrogen, Carlsbad, CA). After confirming the sequence, we transfected pcDNA3.1-His-PKNOX2 into COS-7 cells using FUGEGE6 (Roche Diagnostics) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were washed with phosphate-buffered saline and fixed with acetone/methanol (1:1 v/v). Epitope-tagged PKNOX2 protein was localized within cells using monoclonal anti-Xpress antibody (Invitrogen) and FITC-conjugated anti-mouse secondary antibody (MBL, Nagoya, Japan) according to the manufacturer's suggestions.

RESULTS AND DISCUSSION

PKNOX2 Is a Novel Member of the TALE-Superfamily of Homeodomain Proteins

PKNOX1 encodes a DNA-binding homeodomain protein that can interact with PBX (10). Evidence for the existence of a novel *PKNOX1*-related gene was gathered during the process of comparing PKNOX1 amino acid and nucleotide sequences against public databases using the BLAST program. With this approach, we identified genomic sequence present in BAC RP11-417F7 (GenBank accession number AC009807), which contains a gene predicted to encode a protein similar to PKNOX1 and several ESTs including three clones analyzed in this study. We used RT-PCR to connect gaps between the partial PKNOX1-related sequences of the ESTs and to confirm the exonic sequences predicted from the sequence of ESTs and BAC. In this manner we isolated a cDNA that provided the entire coding sequence for a novel 460-amino-acid (50.8 kDa) protein containing an atypical homeodomain referred to as TALE (Fig. 1).

The homeodomain of PKNOX2 resembles those of the divergent TALE class of homeodomain proteins (Fig. 1B) and is characterized by a conserved insertion of three amino acids between helices 1 and 2 (3). The homeodomain of PKNOX2 is 94% identical to the corresponding domain within PKNOX1 (Fig. 1). In addition, PKNOX2 homeodomain sequence has 45/63 (71%) residues identical with those of human MEIS-related proteins, 32/63 (51%) with human TGIF-related proteins, 24/63 (38%) with human PBX1, 3 and 4, 23/63 (37%) with human PBX2, and 14/60 (23%) with the homeodomain consensus (15). PKNOX1 displays a specific, albeit low, affinity for the TGACAG motif, which is identical to those bound by human MEIS and TGIF proteins (3). PKNOX1, TGIF and MEIS are almost identical in the third helix of their homeodomains, i.e. the DNA-recognition helix, and in particular they have an isoleucine at the third position of the highly conserved homeodomain motif, WF-N-N, of the third helix. Since isoleucine is rarely found at this position in homeodomains (3, 15), these proteins represent a novel emerging subclass of homeodomains that shows binding preference for a TGACAG-like motif. Based on the high similarity of homeodomains, especially the third helix, between these proteins and PKNOX2, the PKNOX2 homeodomain may recognize the same TGACAG motif as well, suggesting this protein is the novel member of this subclass.

Outside of the homeodomain, two regions of 22 and 40 amino acids, respectively HR1 and HR2 (10), display strong homology to similar regions in PKNOX1 and MEIS proteins (Fig. 1). These regions are not found in any other members in human TALE class homeodomain proteins. Their position relative to the homeodomain is also conserved among these proteins. HR1 and HR2 domains have been shown to mediate interactions of PKNOX1 proteins with N-terminal region of PBX proteins, and are essential for PBX-PKNOX1 heterodimerization (10). This association with PBX increases the affinity of PKNOX1 for DNA binding (10, 13). In addition, the PBX-PKNOX1 com-

A

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PKNOX2 MMATQNVPPPPYQDSPQMTATAQPPSKAQAVHISAPSAAASTPVPSAPIDPQAQLEADKR  60
PKNOX  MMATQTLSIDSYQDGQQMQVVTELKTEQDP----NCSEPDAEGVSPPPVESQTPMDVDKQ  56

PKNOX2 AVYRHPLFPLLTLLFEKCEQATQGSECITSASFDVDIENFVHQQEQEHKPFFSDDPELDN  120
PKNOX  AIYRHPLFPLLALLFEKCEQSTQGSEGTTSASFDVDIENFVRKQEKEGKPFFCEDPETDN  116
       HR1

PKNOX2 LMVKAIQVLRIHLLELEKVNELCKDFCNRYITCFKTKMHSDNLLRNDLGGPYSPNQPSIN  180
PKNOX  LMVKAIQVLRIHLLELEKVNELCKDFCSRYIACLKTKMNSETLLSGEPGSPYSPVQS---  173
       HR2

PKNOX2 LHSQDLLQNSPNSMSGVSNNPQGIVVPASALQQGNIAMTTVNSQVVSGGALYQPVTMVTS  240
PKNOX  -------QQIQSAITG-TISPQGIVVPASALQQGNVAMAT-----VAGGTVYQPVTVVTP  220

PKNOX2 QGQVVTQAIPQGAIQI---QNTQVNLDLTSLLDNEDKKSKNKRGVLPKHATNIMRSWLFQ  297
PKNOX  QGQVVTQTLSPGTIRIQNSQLQLQLNQDLSILHQDDGSSKNKRGVLPKHATNVMRSWLFQ  280

PKNOX2 HLMHPYPTEDEKRQIAAQTNLTLLQVNNWFINARRRILQPMLDASNPDPAPKAKKIKSQH  357
PKNOX  HIGHPYPTEDEKKQIAAQTNLTLLQVNNWFINARRRILQPMLDSSCSE-TPKTKKKTAQN  339
       HD                                              ●●●●●●● NLS

PKNOX2 RPTQRFWPNSIAAGVLQQQGGAPGTNPDGSI--------NLDNLQSLSSDSATMAMQQAM  409
PKNOX  RPVQRFNPDSIASGVAQPPPSELTMSEGAVVTITTPVNMNVDSLQSLSSDGATLAVQQVM  399

PKNOX2 MAA--HDDSLDGTEEEDEDEMEEEEEEELEEEVDELQTTNVSDLGLEHSDSLE        460
PKNOX  MAGQSEDESVDSTEEDAGALAPAHISGLVLENSDSLQ                        436
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B

Name	Amino acid sequence	Identity
	Helix 1 *Helix 2* *Helix 3*	
Human PKNOX2	SKNKRGVLPKHATNIMRSWLFQHLMHPYPTEDEKRQIAAQTNLTLLQVNNWFINARRRILQPM	
Human PKNOX1	----------V--------IG---------K----------	**59/63** (94%)
Human MEIS1	KRH---IF--V------A------T----S-EQ-K-L-QD-G--I----------V---	**45/63** (71%)
Human MEIS2	RQK---IF--V------A------T----S-EQ-K-L-QD-G--I----------V---	**45/63** (71%)
Human MEIS3	RNK---IF--V------A------S----S-EQ-K-L-QD-G--I----------V---	**45/63** (71%)
Human TGIF	KRRR--N---ESVQ-L-D--YE-RYNA--S-Q--ALLSQ--H-ST---C---------L-PD-	**32/63** (51%)
Human TGIF2	KRKR--N---ESVK-L-D--YL-RYNA--S-Q--LSLSG----SV--IC---------L-PD-	**32/63** (51%)
Human PBX1	ARR---NFN-Q--E-LNEYFYS--SN---S-EA-EEL-KKCGI-VS--S---G-K-I-YKKNI	**24/63** (38%)
Human PBX2	ARR---NFS-Q--EVLNEYFYS--SN---S-EA-EEL-KKCGI-VS--S---G-K-I-YKKNI	**23/63** (37%)
Human PBX3	ARR---NFS-Q--E-LNEYFYS--SN---S-EA-EEL-KKCSI-VS--S---G-K-I-YKKNI	**24/63** (38%)
Human PBX4	ARR---NFS-Q--EVLNEYFYS--NN---S-EA-EEL-RKGG--IS--S---G-K-I-YKKNI	**24/63** (38%)
HOX consensus	RRR--TAYTRYQLLELEKEFHFN R-L-RRRRIEL-HSL---ER--KI--Q-R-MKWKKEN	**14/60** (23%)

FIG. 1. (A) Alignment of human PKNOX1 and PKNOX2 protein sequences (GenBank accession numbers NM_004571 and DDBJ entry ID: 20010706081615.75447, respectively). Numbers at right refer to amino acid residues. Identical amino acids are highlighted by shading. The HR1 and HR2 domains are underlined, and homeodomain (HD) sequences are boxed. A putative nuclear localization signal is indicated by a series of solid circles. (B) Amino acid homologies with PKNOX2 homeodomain. Amino acid identity with respect to PKNOX1 is indicated by (–). Residue identities and similarities of the different homeodomain with respect to PKNOX2 are indicated as a absolute number (in bold) and in percentage (in parentheses).

plexes are very stable and form independently of DNA (10, 16). Taken together, these data indicate that PKNOX1 is the predominant partner of PBX proteins, suggesting potentially widespread functions for PBX-PKNOX1 that are likely to play key roles in the regulation of coordinated gene transcription. Since HR1

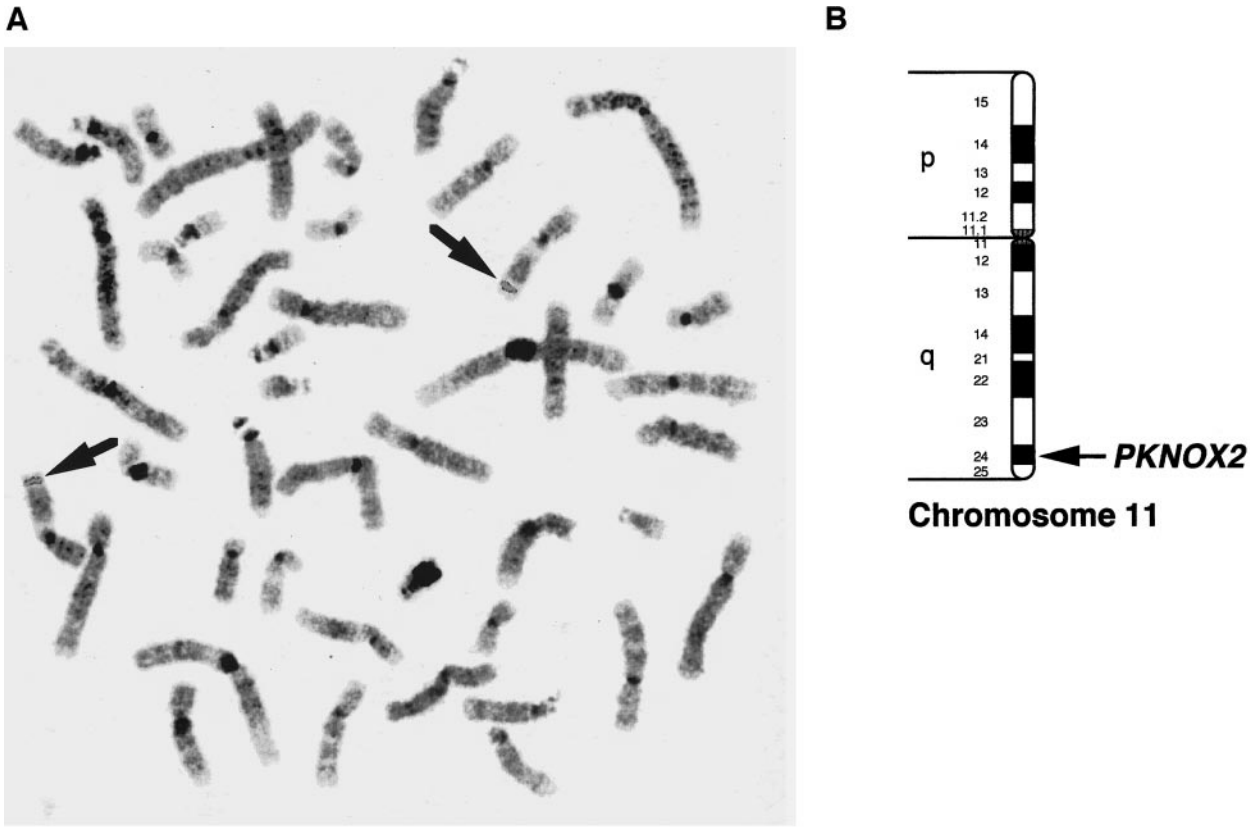

FIG. 2. (A) Mapping of the *PKNOX2* gene by FISH. Metaphase chromosomes from human diploid cells were hybridized with labeled genomic DNA from BAC RP11-417F7. Typical hybridization signals appear on both chromosomes 11 at band q24–q25 (arrows). (B) *PKNOX2*-specific signals were sublocalized at 11q24 in elongated prometaphase chromosomes.

and HR2 of PKNOX2 have high similarity with those of PKNOX1, it is conceivable that PKNOX2 may interact with PBX proteins as well, and modulate PBX-HOX functions.

Apart from the homeodomain and the HR domains, the PKNOX2 protein share relatively high sequence similarity with the PKNOX1 (Fig. 1), but not with the MEIS proteins (10). Like PKNOX1 (17), therefore, PKNOX2 may function differently from the MEIS proteins, although PKNOX1 can substitute for MEIS proteins in several cases (18, 19). Further study identifying biochemical and biological difference between PKNOX2 and PKNOX1 will contribute to understand the role of PKNOX2 protein.

Chromosomal Localization and Genomic Organization of PKNOX2

FISH using BAC clone RP11-417F7 as a *PKNOX2*-specific probe exhibited clear signals on chromosomes 11q24–q25 (Fig. 2A), and further on sub-band 11q24 in elongated prometaphase chromosomes (Fig. 2B). Previous study demonstrated that the *PKNOX1* gene is localized on human chromosome 21q22.3 (20). Thus, the *PKNOX* genes were not clustered within the genome. Previous studies have demonstrated that human chromosome 11q24–q25, to which *PKNOX2* is localized, is deleted in various malignancies, such as breast cancer and ovarian cancer (21, 22). In addition a restricted chromosomal breakpoint site around this region has also been demonstrated in various hematological malignancies (23). Complex genetic and biological interactions between members of the TALE family and HOX proteins appear to be important for leukemic transformation (17, 24, 25). Thus, the aberrant expression of this gene might be involved in the development of solid tumors and/or a subset of leukemia. Further studies will be required to address this possibility.

The exon-intron structure for *PKNOX2* gene was determined by comparing the sequence from the 3178-bp cDNA (DDBJ entry ID 20010706081615.75447) to the corresponding genomic sequence (Table 1). The *PKNOX2* gene contains at least 10 exons. Comparison of the genomic sequences demonstrates the high degree of similarity between the exon-intron structure of *PKNOX2* and *PKNOX1* (Table 1). Exons 2 and 3, exons 3 and 4, and exons 7 to 9 of *PKNOX2* encode the same domains as their *PKNOX1* counterparts. Moreover, all exons, including two exons with the exact same basepair length in

TABLE 1

Delineation of *PKNOX2* Exon–Intron Boundaries and the Exon Size and Domain Comparison between the *PKNOX2* and *PKNOX1* Genes

PKNOX2					*PKNOX1*[b]	
Exon number	Exon size (bp)	Splice acceptor[a]	Splice donor[a]	Domains	Exon number	Exon size (bp)
1	79		CAGCCCACAG **gt**gagtgcgc		1	111
2	140	ctctctgc**ag** ATGACGGCAA	CTGTATACAG **gt**aggagaca	**HR1**	2	128
3	172	gtccctgc**ag** GCACCCTCTT	GGACAATCTG **gt**aaagaccc	**HR1, 2**	3	172
4	189	ctctccac**ag** ATGGTGAAGG	TCACTCACAG **gt**aacaccca	**HR2**	4	171
5	130	tcactgcc**ag** GACCTCCTGC	GTTGTGTCAG **gt**cggtgcaa		5	100
6	98	gtctcctc**ag** GTGGAGCCTT	GAACACACAG **gt**gagtgtgt		6	98
7	120	tgccctga**ag** GTTAACCTTG	GCATCTCATG **gt**gagtgtgt	**HD**[c]	7	129
8	76	ctcgtttc**ag** CACCCCTACC	GTAAACAACT **gt**gagtttgc	**HD**	8	77
9	180	ctctgggc**ag** GGTTCATCAA	AACCCCGATG **gt**aagaactg	**HD**	9	173
10	1994	tctctcgc**ag** GTTCCATCAA			10	2035

[a] Exon sequences are in uppercase letters and intron sequences are in lowercase letters.
[b] PKNOX1 exons are numbered according to Gen Bank accession number NM_004571 (cDNA) and NT_011514 (genomic DNA).
[c] HD; homeodomain.

both proteins, have similar size. This shared exon-intron pattern parallels the high level of homology between the shared protein domains of both *PKNOX2* and *PKNOX1*. These data clearly suggest that *PKNOX2* and *PKNOX1* are paralogous genes.

Tissue Distribution of PKNOX2 mRNA

Northern-blot analysis of RNA derived from various human tissues was performed to gain insight into the spatial distribution of *PKNOX2* mRNA. As shown in Fig. 3, a single 4.6-kb *PKNOX2* transcript, which is different from *PKNOX1* transcript in size (20), was expressed in several tissues. Abundant expression was revealed in brain and skeletal muscle, and the transcript was almost undetectable in colon, liver, and peripheral leukocytes. This expression pattern is completely different from that of *PKNOX1;* for example, the latter is ubiquitously expressed and two mRNA species with different sizes were detected in all tissues (20). Tissue specific expression of *PKNOX2* gene indicates that PKNOX1 and PKNOX2 may have distinct roles through forming heterodimers with the different PBX proteins and/or PKNOX2 may modulate the regulatory function of PKNOX1 in a tissue-specific manner.

The transcript is significantly longer than the isolated and assembled cDNA sequence. The difference in the size is likely due to additional 5′-untranslated region (UTR) and poly(A) tail sequence of *PKNOX2*.

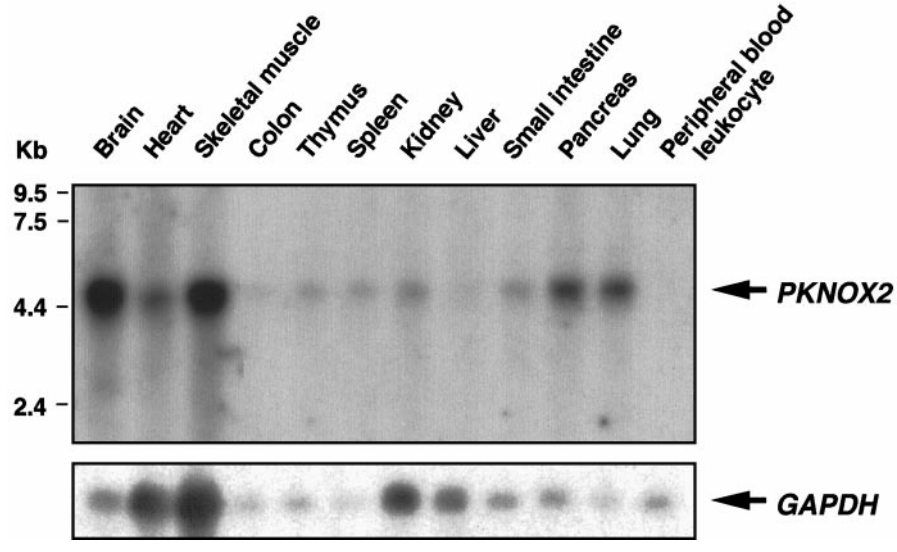

FIG. 3. Expression of *PKNOX2* mRNA in normal human tissues. A multitissue Northern blot (Clontech) was probed with *PKNOX2* cDNA. The same blot was rehybridized with a *GAPDH* probe, as a control for RNA loading and transfer.

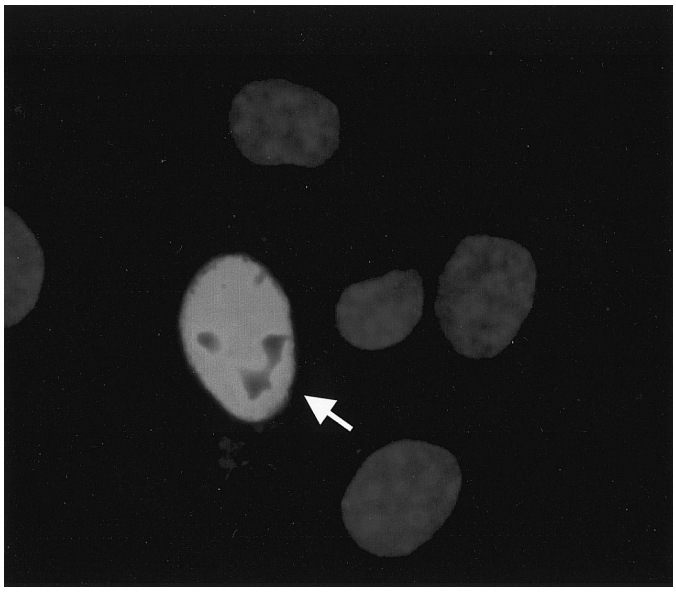

FIG. 4. Nuclear localization of PKNOX2. COS-7 cells were transiently transfected with a vector containing Xpress epitope-tagged *PKNOX2* cDNA. The presence of tagged protein was detected microscopically after being stained with an anti-Xpress antibody and a FITC-conjugated anti-mouse secondary antibody. The preparations were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Arrow indicates nuclear staining of the *PKNOX2* construct.

Nuclear Localization of PKNOX2

Since a computer-based analysis using the PSORT II program predicted nuclear localization (Fig. 1), we investigated the sub-cellular localization of this protein by adding an epitope-tag that would be detected by immunofluorescence. Using this approach we confirmed that PKNOX2 is present in the nucleus but is excluded from nucleoli (Fig. 4). No detectable staining was observed in cells transfected with the parental plasmid as a control (data not shown). As it contains a DNA-binding motif, PKNOX2 may function as a nuclear transcription factor. Interestingly, it has been reported that mouse homolog of PKNOX1 is localized mainly in the cytoplasm, and its interaction with PBX1 is necessary for its nuclear localization as a PBX1-PKNOX1 complex (18). It will be interesting to determine whether the constitutive nuclear localization of human PKNOX2 is regulated by similar mechanism.

In summary, we have identified *PKNOX2* as a novel TALE homeodomain-encoding gene, located at 11q24. The structure and sub-cellular localization of PKNOX2 indicate that this protein functions as a nuclear transcription factor.

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