

Expression and Cloning of the Human X-Linked Hypophosphatemia Gene cDNA

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X-linked hypophosphatemia (XLH), which is a heritable metabolic bone disease characterized biochemically by selective renal phosphate (Pi) wasting, is associated with mutations in the PEX (Phosphate-regulating gene with homologies to Endopeptidases on the X-chromosome) gene. To further explore the physiologic role of PEX and define its effect in XLH we have determined the expression and tissue distribution. Northern analysis found abundant PEX mRNA in a restricted pattern, predominantly in adult ovary and fetal lung. In addition, PEX expression was also found in adult lung and fetal liver. A PEX cDNA of 2550 base-pairs, which contains the full PEX coding region, was isolated from a human ovary cDNA library. The PEX cDNA shows high homology to other membrane-bound zinc metallopeptidases. The presence of PEX in non-osseous tissues strongly suggests features of a systemic role, rather than a unique function in bone development. © 1997 Academic Press

X-linked hypophosphatemia (XLH), which is a heritable metabolic bone disease that features selective renal phosphate (Pi) wasting causing rickets in children and osteomalacia in adults (1), is due to mutations in a gene designated PEX (Phosphate-regulating gene with homologies to Endopeptidases on the X-chromosome) (2,3). Many years of clinical investigation as well as studies using the murine homologue (4), *Hyp*, have established the pathophysiologic importance of the renal Pi wasting (1). Crosstransplantation experiments show that a *Hyp* kidney transplanted into a normal mouse does not transmit the XLH phenotype (5), whereas a normal kidney transplanted into a *Hyp* mouse leads to phosphaturia. This observation substantiates earlier evidence for a circulating phosphaturic factor in XLH (6). Nevertheless, this factor, possibly an important

regulatory hormone in Pi homeostasis, is as yet unidentified. PEX does not appear to encode a hormone; rather, by protein homology, the product appears to be a membrane-bound zinc metallopeptidase. However, the relationship between this metallopeptidase and excessive phosphaturia is still unclear and the substrate for PEX remains unknown.

Though hypophosphatemia appears to have a pivotal role in causing the bone disease of XLH, there is also evidence for an associated osteoblast defect (7,8,9, 10,11). Consistent with a direct role for PEX in bone mineralization Du and coworkers demonstrated the presence of mRNA for the murine homologue, *Pex*, in mouse neonatal calvariae and osteoblasts in culture (12). Though bone matrix mineralization is affected by serum Pi, there is no known connection between the osteoblast and regulation of Pi homeostasis; hence it is unclear how an abnormality in PEX could diminish renal Pi reclamation. The breadth of PEX expression should give clues to the source of Pi regulation.

In addition, when the PEX gene was reported to be involved etiologically in XLH, neither the 5' nor the 3' ends of PEX were identified (2). Mutations in the published coding sequence can be found in up to 30% of the affected patients (3). It is quite likely, however, that at least some XLH patients will have mutations elsewhere in the PEX sequence reflected in the previously unknown portion of the PEX cDNA.

We report the expression of PEX, and the cloning and sequencing of the full coding region of human PEX.

METHODS

(a) *Preparation of cDNA.* Total RNA was isolated from cultured cells derived from Burkitt's Lymphoma [Daudi (ATCC, CRL-7917-NBL) and Raji (ATCC, CRL-7936-NBL)] using guanidium thiocyanate followed by centrifugation in cesium chloride (13). RNA was reverse transcribed using the cDNA Cycle Kit (Invitrogen) with 1 μ g

TABLE 1
Details of Oligonucleotide Primers

name	Strand	Primer sequence	Position
PEX1-Primary	Sense	5'-ACGATCCTCTTCTAGTG-3'	103
PEX1-Primary	Antisense	5'-CTTTCTCTGACCAAAC-3'	552
PEX1-Nested	Sense	5'-CTTTCTAGTGAGTCAAGG-3'	111
PEX1-Nested	Antisense	5'-CTTGGCATCTGCTTTTTC-3'	462
PEX2-Primary	Sense	5'-ATTTGGTGTGGAGAATGG-3'	1094
PEX2-Primary	Antisense	5'-AATACTTGCAGTTTGTAGG-3'	1537
PEX2-Nested	Sense	5'-TTCAGTATAGATGGCTGG-3'	1145
PEX2-Nested	Antisense	5'-TCGGCTTCTGAAAACCTT-3'	1502

The position indicates the position of the 5' end of the primer with respect to the cDNA sequence in Figure 2.

of template and random primers as per the manufacturer's directions.

(b) *Preparation of PEX probes.* Two PEX cDNA fragments, PEX1 and PEX2, were generated by PCR amplification of the Daudi and Raji cell cDNA. Primer pairs were developed that cross PEX exon-intron boundaries and hence amplify from the cDNA but not from the genomic DNA template (Table 1). Polymerase chain reaction (PCR) was performed in PCR buffer (cDNA Cycle Kit, Invitrogen) with the following conditions: 35 cycles of 94°C for 1 min.; 55°C for 2 min.; 72°C for 3 min. PEX1 was amplified using PEX1-Primary primers and 2 μ l of the PCR reaction was reamplified with the nested primers, PEX1-Nested. PEX2 was amplified using PEX2-Primary primers and 2 μ l of the PCR reaction was reamplified with the nested primers, PEX2-Nested. For details on the primers used, see Table 1. Amplification of the expected sized fragments was confirmed by fractionating the PCR products on a 1.2% agarose gel electrophoresis; PEX1 is 352 basepairs (bp) long and PEX2 is 358 bp long (Data not shown). For use in Northern analysis and cDNA library screening, PCR products were subcloned into pCRII using the TA Cloning Kit (Invitrogen). Identity with the published PEX cDNA sequence was confirmed by sequencing using T7 primers and Sequenase (Amersham).

(c) *Northern analysis.* The multiple tissue Northern filters, which are loaded with 2 μ g per lane of poly(A)⁺ RNA derived from a number of human tissues, were purchased from Clontech. Each membrane was hybridized using both PEX probes (PEX1 and PEX2) after *EcoRI* excision from the pCRII vector and gel purification of the insert by fractionation on a 1.2% low-melt agarose gel electrophoresis. ³²P random-primer labelled probes were generated using Rediprime (Amersham). Hybridizations were carried out by standard procedures (13); treatment included a first wash of 2 \times SSC and a final wash with 0.2 \times SSC at 65°C. The filters were exposed to X-ray film at -70°C.

(d) *Isolation of PEX cDNA.* When it was found that PEX was highly expressed in ovary, a human ovary cDNA library made from oligo-dT primed reverse-transcribed RNA, cloned in λ DR2 (Clontech) was screened. The library was plated (8 \times 10⁵ independent clones) and duplicate nitrocellulose filter lifts were made by standard methods (13). Hybridizations with the PEX1 or PEX2 ³²P random-primer labelled probes (Amersham) were done independently with each of the duplicate filters. Hybridizations were carried out by standard procedures (13); treatment included a first wash of 2 \times SSC and a final wash with 0.2 \times SSC at 65°C. The filters were exposed to X-ray film at -70°C. Twelve clones, of 8 \times 10⁵ plated, were found to be positive for both PEX1 and PEX2 suggesting that they were least likely to be partial cDNAs. These clones were isolated and two of them were found to include the 5' most extent of PEX by PCR between an internal λ DR2 primer and an internal PEX primer. These two clones were isolated and converted into a plasmid (pDR2)

using Cre-Lox recombination as per the manufacturer's instructions (Clontech).

(e) *PEX cDNA sequencing and analysis.* The two cDNAs were sequenced on both strands with internal and plasmid-derived oligonucleotide primers using DyeDeoxy Terminators (Applied Biosystems) and an ABI DNA 377 sequencer (Applied Biosystems). Sequence fragments were edited and assembled using Factura and Autoassembler programs (Applied Biosystems). Sequences were analyzed using the Sequence Analysis Software Package Version 8 (Genetics Computer Group).

RESULTS

PEX Expression

In preliminary experiments, nested PCR detected the presence of low levels of PEX mRNA in both Daudi and Raji cells (data not shown). For use in the expression studies and to isolate the full length PEX cDNA, two PEX-specific probes were developed using the PCR products from the Daudi and Raji cells (see Methods).

Multiple tissue Northern analysis, using both probes (PEX1 and PEX2), revealed that PEX has a restricted distribution in human tissues. Definite PEX transcripts, however, were seen in adult ovary and fetal lung (Figure 1A and 1C) on a one day exposure. On a 7 day exposure, expression was also detected in adult lung and fetal liver. The transcript is approximately 6.6 kb making it much longer than the published partial sequence of 1916 bp. Notably (and a possible clue to relative tissue abundance), expression by Northern analysis was undetected in spleen and thymus, which are abundant in B and T lymphocytes. However, the detection of PEX by PCR in Daudi and Raji cells suggests that the PEX message in these cell types is at a very low level (i.e. leaky transcription). Interestingly, though it is present in ovary, it is absent in testes; and though it is present in fetal lung, it is much less abundant in adult lung (Figure 1B).

Isolation and Sequence of PEX cDNA

The human ovary cDNA library was screened using both PEX1 and PEX2 probes. Two clones were isolated

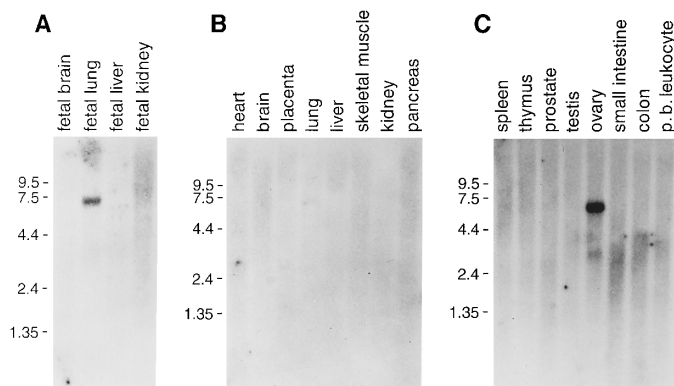


FIG. 1. Northern analysis of PEX expression in adult and fetal human tissues. The labelled probe was hybridized to poly(A)⁺ RNAs (Clontech). Peripheral blood is abbreviated as (p.b.). A 6.6 kb transcript was identified in ovary and fetal lung. A faint band (not visible on the photo) was detected from adult lung and fetal liver.

and converted into plasmids (see Methods). Both strands of the the two clones were completely sequenced using both plasmid-derived and primers obtained from the insert.

The PEX cDNA sequence of 2550 bp (Figure 2) includes an open reading frame of 2247 nucleotides, 159 nucleotides of the 5' untranslated region and 144 bp of the 3' untranslated region. This cDNA contains the 22 PEX exons when comparing with the genomic sequence in Genbank (Y08111-Y08132). Exons all had consensus exon-intron boundary sequences (Table 2).

The human PEX cDNA has a high degree of sequence identity with the murine Pex cDNA (90%). Furthermore, the 749 amino acid human PEX product is 96% identical and 98% similar to the murine Pex product. Based on entries in Genbank the protein is clearly homologous to members of the membrane-bound zinc metalloproteinase family. The human protein product is 60% similar to human neutral endopeptidase 24.11 (NEP), 57% similar to human endothelin-converting enzyme 1, and 53% similar to human Kell antigen. Similarities between human PEX, murine Pex and human NEP include a series of hydrophobic amino acids suggestive of a transmembrane domain (amino acids 21-40) (2,14,15), the conserved cysteine residues (Figure 2) possibly important for secondary structure of the PEX protein, and a zinc-binding motif (Figure 2) (2,11,14,15).

We find three nucleotide differences between the original published partial PEX cDNA sequence (2) and the corresponding region in our sequence, at nucleotides: 1207 (T instead of A, changing amino acid Arg to a Trp) and 1921 and 1922 (GC instead of CG, changing amino acid Ala to a Gly). The sequence given is confirmed by genomic sequencing (Y08121 and Y08129). We also found a polymorphism between the coding sequences of the two isolated cDNAs (G instead of C at

nucleotide 1541; coding for Gly instead of Ala at amino acid 514). The implications of this polymorphism are as yet undetermined.

DISCUSSION

We provide the first evidence of PEX expression in non-osseous tissues, adult ovary, fetal lung, adult lung and fetal liver, and report the sequence of a full-length human cDNA isolated from ovary.

The discovery of the PEX gene and mutations within it in patients with XLH pose an exciting challenge for biologists, because PEX appears to be a key modulator of phosphate homeostasis, yet, as is often the case after identification of a disease gene (16), characterization of PEX has not given satisfactory insight to its physiological role. Hence, detailed expression analyses are a first important step in understanding the function of PEX.

The discovery of PEX RNA in ovary and fetal lung in humans does not seem in keeping with classical Pi regulatory organ systems. However, it indicates that PEX, although expressed in a highly tissue-specific manner, is not expressed only in bone. This suggests that the PEX effect, and possibly the regulation of phosphate transport, does not occur only in bone. In fact, the presence of the defect causing XLH outside of bone is consistent with experimental data demonstrating abnormal vitamin D metabolism at extrarenal sites (17). Identification of the specific cell-types involved in PEX expression is a next step in elucidating the pathophysiology of XLH.

There is strong evidence for a circulating phosphaturic factor, referred to as "phosphatonin", in oncogenic osteomalacia (18,19). At this time it is reasonable to speculate that a PEX substrate, phosphatonin, fails to be degraded when there is loss of PEX function (as in XLH) resulting in phosphaturia.

XLH is inherited in a sex-linked dominant manner but, given that X chromosome inactivation occurs randomly, one might expect a gene dose effect in XLH. Prepubertal patients with XLH, however, do not appear to fit the "Lyon hypothesis"; girls are as severely affected as boys (20,21). Hence, the interaction between PEX and phosphatonin may occur in the same cell or in a local environment where the cells of origin are proximate. If phosphatonin escapes degradation by PEX, then it might not be inactivated elsewhere, because PEX is clearly not widely expressed. The fraction of phosphatonin which escapes degradation must be sufficient for the full expression of the XLH phenotype.

Based on the comparisons with the full length murine Pex, NEP, endothelin converting enzyme 1 and Kell antigen, we are confident that the complete coding region has now been recovered and sequenced. Our data shows that like NEP, PEX produces a large transcript of approximately 6.6 kb. Since the ovary cDNA is 2550 bp, the cDNA sequence does not account for

-159 GAGAAAGGGTGGCGAGGGGAGATTTCCTGACGCGAGTTTCTTAAGCTGTCCATTAGTAGAAGACGAAGAGAGCCTTGGATGTCAACGCCCTCCGCTCTTGAGACACGCCACCAACCACGAA
-39 AAGTGACTTTCTTCTCGTGTCTCTACGCGCCCTTCTGATGGAAGCAGAAACAGGGAGCAGCTGGAGACTGGAAAGAAAGCCAAACAGAGCCACTCGAATGGCCCTGGCTGGTTTGTGTC
M E A E T G S S V E T G K K A N R G T R I A L V V F V 27
82 GGTGGCACCCTAGTTCTGGGCACGATCCTCTTCTAGTGAAGTCTCAAGGTCCTTAAGTCTCAAGCTAAACAGGAGTACTGCCTGAAGCCAGAATGCATCGAAGCGGCTGTGCCATCTTA
G G T L V L V L G T I L F L V S Q G L L S L Q A K Q E Y C L K P E C I E A A A A I L 67
202 AGTAAAGTAAATCTGTCTGGATCCTTGTGATAATTTCTCCGGTTCGCTTGTGATGGCTGGATAAGCAATAATCCAATGCCGAGATATCCAAGCTATGGGGTTTATCCTTGGCTG
S K V N L S V D P C D N F F R F A C D G W I S N N P I P E D M P S Y G V Y P W L 107
322 AGACATAATTTGACCTCAAGTTGAAGGAATTTTGGAGAATCAATCAGTAGAAGCGGGACACCGAAGCCATACAGAAAGCCAAATCCTTTATTTCATCTGCATGAATGAGAAGCG
R H N V D L K L K E L L E K S I S R R R D T E A I Q K A K I L Y S S C M N E K A 147
442 ATTGAAAAGCAGATGCCAAGCCACTGCTACACATCTACGCATTCCACCTTTCCGCTGGCCGCTGCTTGAATCTAATATTGGCCCTGAAGGGTTTTGGTTCAGAGAGAAAGTTCAGCCTT
I E K A D A K P L L H I L R H S P F R W P V L E S N I G P E G V W S E R K F S L 187
562 CTGCAGACCTTCAACGTTTCGTGGTCAATACAGCAATTTGTGTTCATCCGTTTGTATGTGTCCTCCCTGATGACAAAGCATCCAAGTATCTTGAAGCTGGACCAAGCAACACTC
L Q T L A T F R G Q Y S N S V F I R L Y V S P D D K A S N E H I L K L D Q A T L 227
682 TCCCTGGCCGTGAGGGAAGACTACCTTGATAACAGTACAGAAAGCAAGTCTTATCGGGATGCCCTTTACAAGTTCATGGTGGATGCTCCCGTCTTTTAGGAGCTAACAGTCCAGCA
S L A V R E D Y L D N S T E A K S Y R D A L Y K F M V D T A V L L G A N S S R A 267
802 GAGCATGACATGAAGTCAAGTCTGATGATGGAATTAAGTAGCTGAGATAATGATTCACATGAAACCGAAGCCAGCGAGCCATACAAACAAATGAACATTTCTGAAGTGAAGTGTCT
E H D M K S V L R L L E I K I A E I M I P H E N R T S E A M Y N K M N I S E L S A 307
922 ATGATTCCCAGTTCGACTGGCTGGCTACATCAAGAAGGTCATGACACCAGACTTACCCTCCATCGAAAGACATCAGCCCTCCGAGAATGTGGTGGTCCGCTCCCGCAGTACTTT
M I P Q F D T W L G Y I K K V I D T R L Y P H L K D I S P S E N V V R V P Q Y F 347
1042 AAAGATTTGTTAGGATTTAGGCTGAGAGAAAGAACACCTTGGCAACTATTGGTGTGGAGAAATGTTTATTCAGAAATTCCAAACCTTAGCAGCGCTTTCAGTATAGATGGCTG
K D L F R I L G S E R K K T I A N Y L V W R M V Y S R I P N L S R R F Q Y R W L 387
1162 GAATTCCTCAAGGTAATCCAGGGGACCAACACTTTGCTGCCTCAATGGGCAAAATCTGTAACACTTTATGAAAGTCCCTCCCTTATGTTGTTGGAAAGATGTTTGTAGATGTGTACTTC
E F S R V I Q G T T T L L P Q W D K C V N F I E S A L P Y V V G K M F V D V Y F 427
1282 CAGGAAGATAAAGAAATGATGGAGGAATGGTTGAGGGCGTTCGCTGGCCCTTTATGACATGCTAGAGAAAGAAATGAGTGGATGGATGCAGGAACGAAAGGAAAGCAAGAA
Q E D K K E M M E E L V E G V R W A F I D M L E K E N E W M D A G T K R K A K E 467
1402 AAGCGAGAGCTGTTTGGCAAAGTGGCTATCCAGAGTTTATAATGAATGATACTCATGTTAATGAAGCCCTCAAAGCTATCAAGTTTTCAGAAGCCGACTACTTTGGCAACGCTCTA
K A R A V L A K V G Y P E F I M N D T H V N E D L K A I K F S E A D Y F G N V L 507
1522 CAACTCGCAAGTATTAGCACAGTCTGATTTCTTGGCTAAGAAAAGCCGTTCCAAAACAGAGTGGTTTACAACCTCCGACGACTGTCAATGCCTTCTACAGTCCATCCACCAACAG
Q T R K Y L A Q S D F F W L R K A V P K T E W F T N P T T V N A F Y S A S T N Q 547
1642 ATCCGATTTCCAGCAGGAGCTCCAGAAGCCTTTCTTTGGGGAACAGAAATATCTCGATCTCTGAGTTATGGTGTCTATAGGAGTAATTTCTCGGACATGAATTTACACATGGATTTGAT
I R F P A G E L Q K P F F W G T E Y P R S L S Y G A I G V I V G I H E F T H G F D 587
1762 AATAATGGTAGAAAATATGATAAAAATGGAACCTGGATCCTTGGTGTCTACTGAATCAGAAGAAAAGTTTAAAGAAAAACAAAATGCATGATTAACAGTATAGCAACTATTATTGG
N N G R K Y D K N G N L D P W W S T E S E E K F K E K T K C M I N Q Y S N Y Y W 627
1882 AAGAAGCTGGCTAAATGTCAAGGGGAAGAGGACCCCTGGGAGAAAATTTGCTGATAATGGAGCCCTGCGGGAAGCTTTTAGGGCTTACAGGAAATGGATAAATGACAGAAGGCAAGGGA
K K A G L N V K G K R T L G E N I A D N G G L R E A F R A Y R K W I N D R R Q G 667
2002 CTTGAGGAGCTTCTTACAGGCATACATTCACCAACAACAGCTCTTCTCTGAGTATGCTCATGTGAGGTCCAATTTCTACAGACCAGAAGTGCCTGGAGAGAAGTCCAAATT
L E E P L L P G I T F T N N Q L F F L S Y A H V R C N S Y R P E A A R E Q V Q I 707
2122 GGTGCTCACAGTCCCCCTCAGTTTAGGTCATGTTGCAATTTAGTAACCTTTGAAGAATTCAGAAAGCTTTTAACTCTCCACCAATTCACAGATGAACAGAGGATGGACTCTGCCGA
G A H S P P Q F R V N G A I S N F E E F Q K A F N C P P N S T M N R G M D S C R 747
2242 CTCTGGTAGCTGGAGCCTGGTTTATGGCATCTGAGACAGTTGCACAGTCCAGCGGAGGCTGCACNTGACTTCATCGCCCATTCCTTAGCGCTGGAGACTTTTCATTTTAGTGCAT
L W *
2362 TTTTCATTTATTTGGGTAGGTGACCTGCTTGG

FIG. 2. Nucleotide sequence of the full-length PEX human cDNA with the deduced amino acid sequence. Nucleotide numbers are shown on the left and amino acid numbers are on the right. Conserved cysteines between human PEX, murine Pex and neutral endopeptidase are in boxes whereas the zinc-binding motif is underlined. There is a polymorphism between the coding sequences of the two isolated cDNAs (G instead of C at nucleotide 1541; coding for Gly instead of Ala at amino acid 514)(See Results). The sequence has been submitted to GSDB (GSDB: S1154068).

about 4 kb of mRNA detected by Northern analysis (Figure 1C). Since there is only a single band on all Northern analyses (Figure 1)(11) there is no evidence for alternate splicing. There do not appear to be the recovered exons in the 5' region of the transcript, based on exon

predictions of genomic sequence (22). Much of the sequence unaccounted for, thus presumably lies in the 3' untranslated region. This assumption is in accord with the absence of a polyadenylation signal in the recovered cDNA. Again by analogy to NEP, a long 3' untranslated

TABLE 2
Positions of Exon-Intron Borders in cDNA

Exon	Start	End	Size (bp)	Exon	Start	End	Size (bp)
1		118		12	1303	1404	102
2	119	187	69	13	1405	1482	78
3	188	349	162	14	1483	1586	104
4	350	436	87	15	1587	1645	59
5	437	663	227	16	1646	1700	55
6	664	732	69	17	1701	1768	68
7	733	849	117	18	1769	1899	131
8	850	933	84	19	1900	1965	66
9	934	1079	146	20	1966	2070	105
10	1080	1173	94	21	2071	2147	77
11	1174	1302	129	22	2148		

region may confer message stability and enable post-transcriptional regulation (23).

The hypothesis that PEX processes a hormone is also consistent with the function of its homologous gene, NEP (15,24,25). The varied tissue distribution of PEX may also suggest that PEX, like NEP, has multiple substrates (15). Thus PEX may have multiple roles, and its presence in the ovary and fetal lung maybe related to a role in addition to Pi homeostasis.

Given that mutations in the published coding sequence of XLH account for less than half of patients with XLH (12), it is likely that other mutations arise in parts of the PEX transcript not yet analyzed. The additional mutations will be necessary for making accurate genotype-phenotype correlations and ultimately understanding the role of PEX in XLH and human metabolism.

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