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Cloning, sequencing, and chromosomal localization of human term placental alkaline phosphatase cDNA

(DNA sequence analysis/synthetic oligodeoxynucleotide probe/amino acid sequence identity/isozymes/serine hydrolase)

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ABSTRACT **A human term (third trimester) placental alkaline phosphatase (PLAP; EC 3.1.3.1) cDNA was isolated from a human placental λgt11 cDNA library. The expression library was screened by using rabbit antibodies against PLAP and oligonucleotide probes. DNA sequence analysis of a positive clone with an insert of 2.7 kilobase pairs allowed us to predict the complete amino acid sequence of PLAP (530 residues), which coincided with the reported 42 N-terminal amino acid sequence of PLAP except at position 3. Contrary to the previous supposition that there was no amino acid sequence homology between PLAP and *Escherichia coli* alkaline phosphatase (471 residues), we found 30% overall homology, with regions of strong homology including the putative active site and the metal-binding sites. The 44-residue C-terminal extension of PLAP has a stretch of 17 hydrophobic amino acids, which presumably anchors the protein to the plasma membrane, a change perhaps necessary for the transition from a bacterial periplasmic enzyme to a mammalian membrane-associated enzyme. We have also localized PLAP-related DNA sequences mainly on chromosome 2 and to a lesser degree on chromosome 17. It seems likely therefore that the *PLAP* gene resides on chromosome 2 and other member(s) of the alkaline phosphatase family may exist (on this chromosome and) on chromosome 17.**

Alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] is a metalloenzyme that hydrolyzes phosphate esters with a peculiarly high pH optimum (pH 10–10.5). Its presence in nearly all living organisms, with the exception of some higher plants, argues for its physiologic importance, but its exact physiological role is not known (1). Human alkaline phosphatase may consist of five tissue-specific forms or isozymes: term placental (PLAP; Regan isozyme); intestinal (IAP), tissue unspecific (found in liver, bone, and kidney), PLAP-like (Nagao isozyme), and IAP-like (Kasahara isozyme) (2). Of these, PLAP is of interest because of its genetic polymorphism, reported in 1961 (3), and its use as a tumor marker owing to its ectopic expression in some human cancer tissue and cell lines (2, 4, 5).

PLAP is a homodimer, membrane-associated glycoprotein enzyme with a 58- to 70-kDa subunit (2, 6, 7). It has immunological cross-reactivity to IAP and PLAP-like but not to tissue-unspecific alkaline phosphatase, and it differs from IAP in heat stability, enzyme inhibition, and monoclonal antibody reactivity (2). Surprisingly little is known about the structure of human alkaline phosphatases, except for a recent determination of the N-terminal 42 amino acids of PLAP (7). A better understanding of the structural differences in the various forms of this human enzyme and their expression may improve their clinical diagnostic values. Here we report the cloning, complete nucleotide sequencing, and chromosomal localization of related sequences of a PLAP cDNA. Perhaps because the prokaryotic alkaline phosphatase is soluble and the PLAP is membrane bound, it has been assumed that the molecules were quite different. However, the predicted amino acid sequence of PLAP shows homology with the recently reported sequence of *Escherichia coli* alkaline phosphatase (8, 9), especially in the regions of the putative active site and the metal-binding sites.

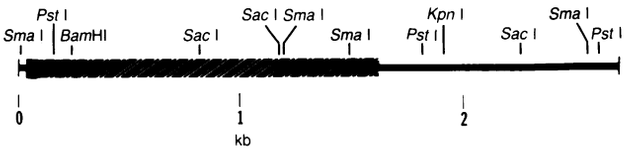

FIG. 1. Schematic representation of human PLAP cDNA clone λAP27. Untranslated sequences are represented by a thick line. Coding sequences are represented by a rectangle: the black portion represents the putative signal peptide; the hatched region encodes the protein.

MATERIALS AND METHODS

Screening of Human Placental cDNA Expression Library with Antibody and Mixed Oligonucleotide Probes. An oligo(dT)-primed human placental λgt11 cDNA library, kindly provided by Ebina *et al.* (10), was screened with a polyclonal antibody to PLAP (Dako, Santa Barbara, CA), using an immunoscreening kit, CLIK (Clontech, Palo Alto, CA) (11). Further screening was performed by hybridizing positive clones from the antibody screening with two sets of ^{32}P-labeled, 26-base oligonucleotide probes (12) (5′-TTCC-A$^{A}_{G}$AA$^{A}_{G}$TC$^{A}_{G}$GG$^{A}_{G}$TT$^{C}_{T}$TC$^{C}_{T}$TC$^{C}_{T}$TC-3′ and 5′-TTCCA$^{A}_{G}$AA-A$^{A}_{G}$TC$^{C}_{T}$GG$^{A}_{G}$TT$^{C}_{T}$TC$^{C}_{T}$TC$^{C}_{T}$TC - 3′), which were synthesized with a Beckman System 1 automated DNA synthesizer (13) on the basis of the amino acid sequences of the N terminus (residues 5 to 13, see Fig. 1). These probes were labeled at the 5′ end with [γ-^{32}P]ATP (7000 Ci/mmol; 1 Ci = 37 GBq) and T4 polynucleotide kinase by the standard method (ref. 14, pp. 122–123).

Subcloning of λgt11 Inserts and Preparation of Cloned cDNA. Insert cDNA from *Eco*RI digestion of the positive clones was ligated to the *Eco*RI-digested pMT21 vector (15). Plasmid DNA was prepared from the transformed *E. coli* HB101 cells and was purified by gradient centrifugation in cesium chloride (ref. 14, pp. 88–94).

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Abbreviations: PLAP, placental alkaline phosphatase; IAP, intestinal alkaline phosphatase; kb, kilobase(s).

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FIG. 2. Nucleotide and predicted amino acid sequences of the term human PLAP. Nucleotides are numbered at the right; amino acids are numbered throughout. The amino acid sequence used to design the synthetic DNA probe is underlined with a broken line. Sites of potential asparagine-linked glycosylation are indicated by asterisks. Boxes indicate the putative signal peptide (box A, as indicated on the left), phosphate-binding serine active site (box B), and transmembrane region (box C). The poly(A) addition sequences (AATAAA) are underlined.

Restriction Endonuclease Mapping. Restriction mapping of the cDNA insert of λgt11 was performed by single or double endonuclease digestions of the recombinant phage, according to the suppliers' recommendations (Bethesda Research Laboratories, New England Biolabs, and Boehringer-Mannheim).

DNA Sequence Analysis. The entire insert and the restriction fragments were subcloned in phage M13mp18 and M13mp19. Single-stranded phage DNA was prepared as described (16). The entire DNA sequence of one of these inserts, λAP27, was determined by sequencing of both strands by the dideoxynucleotide chain termination technique (17) using deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate (650 Ci/mmol; Amersham) or [α-^{32}P]dCTP (3000 Ci/mmol; Amersham), and the 17-mer universal primer and, in selected areas, specific oligonucleotide primers.

RNA Blot Hybridization Analysis. Poly(A)$^+$ RNA from human placenta, human PLC/PRF/5 hepatoma cells, human liver, and mouse testis were electrophoresed on a 1% agarose/2.2 M formaldehyde gel, blotted to nitrocellulose filter according to Thomas (18), and hybridized with nick-translated ^{32}P-labeled 1.9-kilobase (kb) *Kpn* I-digested fragment from λAP27 (under *high* stringency conditions) (ref. 14, pp. 387–389). Autoradiography was performed overnight and for 10 days at −70°C.

Genomic Mapping and Chromosome Localization of the PLAP. Various restriction endonuclease-treated human

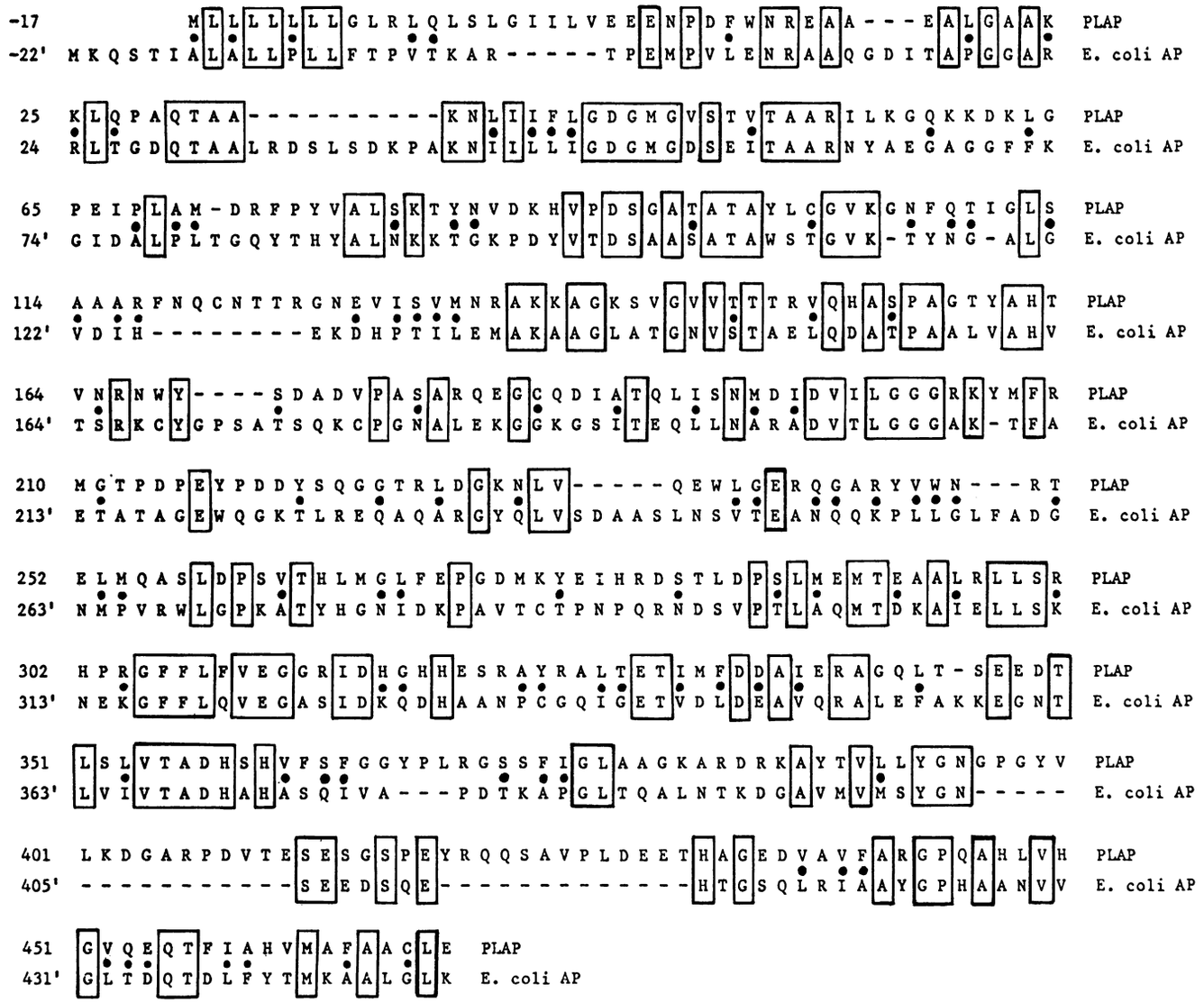

FIG. 3. Comparison of homologous regions of human PLAP and *E. coli* alkaline phosphatase (AP). Unprimed and primed residue numbers are shown on left for PLAP and *E. coli* alkaline phosphatase, respectively. Identical residues are boxed. A ● between the two amino acids represents conservative replacements; a - represents insertion to produce alignment. The sequences are shown in single-letter code, which corresponds to the three-letter code as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

DNAs were electrophoresed in a 1% agarose gel. Southern transfer to a nitrocellulose filter (19) was followed by hybridization under high stringency conditions with the nick-translated ^{32}P-labeled 1.9-kb *Kpn* I restriction fragment derived from λAP27 (ref. 14, pp. 387–389). Autoradiography was performed overnight and for 10 days. The chromosomal location of the gene was analyzed by direct hybridization of the same probe as used for the Southern blot to the DNA of chromosomes resolved by dual laser chromosome sorting as previously described (20).

RESULTS AND DISCUSSION

Isolation of Human PLAP cDNA and Comparison with *E. coli* Alkaline Phosphatase. The human placental λgt11 cDNA library was screened at high cell density with the polyclonal antibody to PLAP. Out of 150,000 recombinant phages, 68 clones reacting with the antibody were isolated, suggesting that PLAP mRNA is at most 0.27% of the mRNA from human placenta. After rescreening at lower cell density, *Eco*RI digestion of DNA from 29 of the cells exhibiting the strongest signals during antibody screening yielded 23 cDNA inserts ranging from 1.9 to 2.7 kb.

To confirm the identity of these positives (since the antibody used was not affinity purified) and to identify potentially full-length PLAP cDNA clones, we used ^{32}P-labeled oligonucleotide probes corresponding to the N-terminal peptide sequence to screen the positives obtained by immunoscreening. Nine out of 23 clones hybridized to both sets of probes and all had inserts of approximately 2.7 kb. The restriction map of λAP27 is shown in Fig. 1. Cleavage sites for *Xba* I, *Hin*dIII, and *Sal* I were not found in the insert. Both strands of λAP27 (2698 base pairs) were sequenced (Fig. 2). The longest open reading frame, which starts with a methionine codon preceded by 25 nucleotides of 5′ untranslated sequence and ends with a TGA codon, encodes a protein of 530 amino acids. This coding sequence indicates a M_r of 57,504, which is in good agreement with the 60,000 M_r estimate for the unglycosylated polypeptide from *in vitro* translation (6). The predicted amino acid composition and tryptic peptide maps also agree with those already determined (1, 7, 21). Although the initiating methionine codon does not have the typical initiation consensus sequences

around it [CC$^{A}_{G}$CCAUGG (22)], we assume it is the initiation site for translation because it is followed by a predicted signal peptide just prior to the known N-terminal amino acids. The predicted M_r of 1872 of this signal peptide agrees with the previously estimated value of 2000 determined in expression experiments (6). This leucine-rich signal peptide is similar to the signal peptides of mouse epidermal growth factor precursor and human pancreatic polypeptide hormone precursor (23, 24). Except for the Pro/Leu difference at position 3, representing a single-base difference, the predicted sequence precisely matches the published N-terminal sequence of 42 amino acids (7). Although the possibility of errors in the amino acid sequence determination cannot be ruled out, the discrepancy could be due to a substitution (C→T) that occurred as a result of reverse transcriptase errors during cDNA synthesis or of the well-studied genetic polymorphism at the PLAP locus (2). The first four N-terminal amino acids are hydrophobic; it is common for hydrophobic amino acids to be found next to the signal peptide (2).

The amino acid sequences of PLAP and *E. coli* alkaline phosphatase bear a remarkable homology throughout the molecule, including the signal peptide. PLAP has 30% identity with the 471 residues of *E. coli* alkaline phosphatase (Fig. 3). The PLAP residues corresponding to residues in the proposed α-helix and the A to J strands within the β-sheet of *E. coli* alkaline phosphatase are within conserved areas (25, 26). We therefore presume that the general structures of the bacterial alkaline phosphatase and PLAP are similar. All but one of the residues in the putative catalytic active site and metal-binding sites are identical. Thus, serine residue 92 (PLAP) and residue 102′ (*E. coli* alkaline phosphatase) (the ′ designates *E. coli* alkaline phosphatase residues) which bind to a phosphate group (27) and the other important amino acids believed to be involved in the metal-binding sites (25, 26) [Arg (residue 166, 166′), His (residues 320, 331′; 358, 370′; 360, 372′; and 432, 412′), Asp (residues 42, 51′; 357, 369′)] are all identical and within highly conserved areas. The exception is residue Thr-155′, which is replaced by Ser-155. The residues immediately adjacent to serine (residue 92) correspond to the characteristic sequence Asp/Glu-Ser-Ala/Gly found in most of the serine hydrolases with the exception of subtilisin (1). More significantly, both *E. coli* alkaline phosphatase and PLAP have eight carboxyl anions and two amino cations in the putative active site region (assuming the histidines are neutral). The resulting net charge of −6 equals the combined charge of three divalent metal ions (25).

Two longer insertions of 16 residues (396–411), and 13 residues (419–431) shift one of the histidines at a putative metal-binding site (residues, 432, 412′) of PLAP further toward the C terminus relative to the equivalent position in *E. coli* alkaline phosphatase. Since the corresponding residues (412–418, 405′–411′) between these two insertions are not involved in the proposed α-helix–β-sheet structure or in the metal-binding or active sites in the *E. coli* alkaline phosphatase, they may form a loop-out structure and the overall structure of PLAP could resemble that of the *E. coli* enzyme. There are two N-linked glycosylation sites (residues 122, 249), in agreement with previous evidence that PLAP is only slightly glycosylated (28); both occur at insertion/deletion sites. The positions for cysteine, however, are not conserved in the PLAP.

PLAP is 59 residues longer than *E. coli* alkaline phosphatase, in part because of a net gain from insertion/deletion in the body of the protein but also because of a 44-residue extension (487–530) at the C terminus with a stretch of 17 hydrophobic amino acids (491–507). These C-terminal amino acids presumably anchor the protein to the plasma membrane (29), and they explain this fundamental difference between the bacterial periplasmic enzyme and the mammalian membrane-associated enzyme. This assignment of a C-terminal anchor agrees with previous experimental evidence suggesting that IAP and PLAP are inserted at their C termini (2).

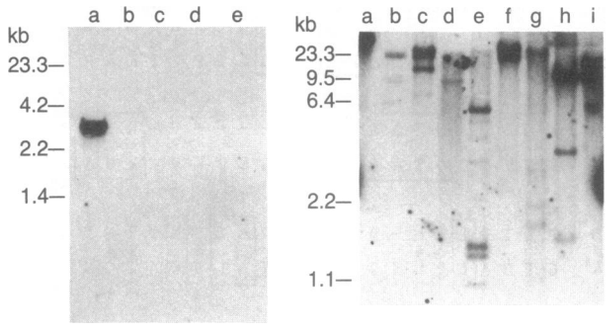

FIG. 4. (*Left*) Size of PLAP mRNA by gel analysis of poly(A)$^+$ RNA from human and mouse. Samples are 4 μg each of total poly(A)$^+$ RNA from human placenta (lane a), human PLC/PRF/5 hepatoma cell (Alexander cell) (lane b), human liver (lane c), 1 μg of mouse testis total poly(A)$^+$ RNA (kindly provided by R. Cann) (lane e), and DNA markers (*Hin*dIII-digested λ phage DNA and *Hin*cII-digested φX174 phage DNA) (lane d). (*Right*) Southern gel analysis of undigested and various restriction-enzyme-digested human genomic DNAs. Eighteen micrograms was loaded in each lane of a 1% agarose gel. Lanes: a, undigested human DNA; b, size markers of *Hin*dIII-digested λ DNA and *Hin*cII-digested φX174 DNA; c–i, human DNA digested by *Kpn* I (c), *Xba* I (d), *Sst* I (e), *Sal* I (f), *Pst* I (g), *Hin*dIII (h), and *Eco*RI (i).

Until recently, it was generally assumed that membrane-spanning domains are α-helical and that at least 20 amino acids are required to span the lipophilic core of the membrane (30). The putative transmembrane domain in the β subunit of the T-cell receptor, like PLAP, also contains an uncharged segment of only 17 amino acids (31, 32). In fact, recent reports using deletion mutations (33) and oligonucleotide-directed mutagenesis (34) generating deletions in the membrane anchor suggest that as few as 12 to 14 amino acids may allow attachment to the membrane.

The strong homology of PLAP with the *E. coli* enzyme through most of the molecule and the sharp divergence at the C terminus containing the anchor suggests that the mammalian enzyme may have been formed by transposition of an exon containing the C-terminal domain onto the 3′ terminus of the primitive bacterial alkaline phosphatase type gene. Thus, the structure of the PLAP gene is of interest. An intron might be found in the region between the two domains at approximately residue 470.

There are 1083 nucleotides of 3′ untranslated sequence following the coding sequence. Many other membrane protein cDNAs have 1–3 kb of 3′ untranslated sequence (10). The 3′ sequence contains six polyadenylylation signals (AATAAA). Multiple polyadenylylation sites have been described in other gene systems (35, 36), but it is unusual to find five (two overlap) within a short stretch of 38 nucleotides (2654–2691) at the 3′ end.

RNA Blot Hybridization Analysis. By this analysis, probably only one species of PLAP mRNA, 2.8 kb, was detected (Fig. 4 *Left*). Since the mRNA is only 0.1 kb larger than our cDNA, the poly(A)$^+$ stretch at the 3′ end of the sequence may be part of the poly(A)$^+$ tail. The analysis also indicates the absence or low abundance of PLAP mRNA or other closely related mRNAs in human liver, Alexander cells, and mouse testis. Thus, liver alkaline phosphatase probably does not have close sequence homology with PLAP, as is reflected by the lack of immunological cross-reactivity between these isozymes (2).

Genomic Mapping and Chromosome Localization. At least three loci have been postulated for the various forms of

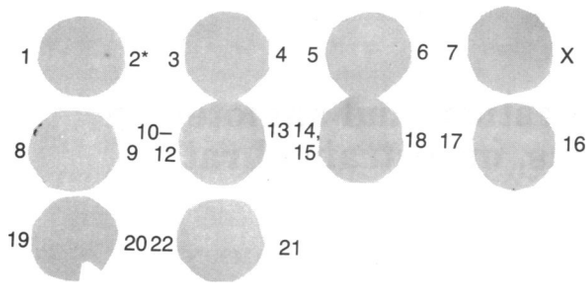

FIG. 5. Autoradiogram of dot-blot filters of sorted chromosomes.

human alkaline phosphatase on the human chromosome(s) (37). The chromosomal location of the *PLAP* gene was analyzed by direct hybridization to the DNA of chromosomes resolved by dual laser chromosome sorting (20). A strong signal is found on chromosome 2 (Fig. 5), which, interestingly, is also the location of the acid phosphatase gene (38). However, with longer exposure a weaker signal also appeared on chromosome 17, and we speculate that this may be the location of a related gene(s). Although we cannot assign the chromosomal location of the *PLAP* gene with certainty because the number of closely related genes and the degree of their cross-hybridization are not known, it is likely that the *PLAP* gene is located on chromosome 2 and that one or more of the other closely related genes (IAP or PLAP-like genes) is located on chromosome 17 and perhaps on chromosome 2 also. Southern blot analysis of human DNA treated with several hexanucleotide specific restriction endonucleases revealed several bands hybridizing to a probe containing the entire coding sequence of the cDNA (Fig. 4 *Right*). The interpretation of the data is also limited by our lack of knowledge of the number of cross-reacting genes. Clarification of both sets of data must await isolation and sequence information from the other alkaline phosphatase genes.

PLAP has no known causal relationship with any human disease, but presumably is essential for normal fetal development, especially in the last trimester, when the level of the enzyme is high. Recently, a monoclonal antibody specific for IAP has been used for prenatal diagnosis of cystic fibrosis (39). The availability of PLAP cDNA and knowledge of its chromosomal location will allow further study of this gene family and of its possible relationship with human diseases.

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