Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 5597–5601, August 1986
Genetics

Products of two common alleles at the locus for human placental alkaline phosphatase differ by seven amino acids

(cDNA expression libraries/DNA sequence analysis/allelic variation/enzyme polymorphism)

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Contributed by Harry Harris, April 7, 1986

ABSTRACT **Amino-terminal amino acid sequences (42 residues) were determined for the products of the three common alleles at the human placental alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] gene locus. The sequences differ at position 3, which is proline in types 1 and 2 but is leucine in type 3. cDNA libraries were constructed in phage λgt11 and used to isolate clones covering the coding regions of types 1 and 3 cDNAs. Comparison of the deduced amino acid sequences of the types 1 and 3 proteins showed 7 differences out of 513 amino acids, each due to a single base substitution. cDNA sequence comparisons showed three silent substitutions in the coding regions and three base differences in the greater than 1 kilobase pairs of 3′ untranslated sequences.**

Studies by enzyme electrophoresis have shown that human placental alkaline phosphatase [ALP, orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] is highly polymorphic. Three common alleles, ALP^1_p, ALP^2_p, and ALP^3_p, give rise to six common phenotypes; types 1, 2, and 3 representing homozygotes and types 2-1, 3-1, and 3-2 representing heterozygotes (1, 2). In addition, many different so-called rare alleles occur (3, 4). These produce rare variant electrophoretic phenotypes, most of which represent one of the rare alleles in heterozygous combination with one or another of the three common alleles. Although individually uncommon, such rare variants occur in 3–5% of placentas (4). Here we report the cloning and sequencing of the cDNA products of two of the common placental ALP alleles, ALP^1_p and ALP^3_p, and consider their differences.

MATERIALS AND METHODS

Placental ALP Typing. Extracts from term placentas were prepared, assayed, and typed electrophoretically as described (5, 6).

Purification of Placental ALP. ALP was isolated from single placentas by standard biochemical techniques as described (7) or by immunoaffinity chromatography as described (8).

Antiserum. Antiserum to human placental ALP was raised in a rabbit, using purified type 1 placental ALP as antigen.

Protein Sequencing. Amino acid sequence analysis of both peptides and proteins was performed with an Applied Biosystems model 470A gas-phase sequencer, using the manufacturer's programming. The phenylthiohydantoin amino acid derivatives were identified by reverse-phase high-performance liquid chromatography using a Waters Associates automated high performance liquid chromatography system with a Waters Nova-Pak C_{18} steel column. Chromatographic conditions were as previously described (9).

Construction of a cDNA Library from a Type 1 Placenta. Total RNA was extracted from 40 g of a placenta, from a Caesarian section delivery, as described by Seeburg *et al.* (10), except that frozen placenta was homogenized in the guanidine thiocyanate solution of Chirgwin *et al.* (11). Polyadenylylated RNA was isolated by two cycles of oligo(dT) affinity chromatography (12). cDNA was synthesized as described (13), using oligo(dT) to prime the first strand and S1 nuclease to digest the hairpin loop. The double-stranded cDNA was made blunt-ended by treatment with DNA polymerase (Klenow fragment) and then methylated, ligated to *Eco*RI linkers, and cloned as described below. From 300 ng of cDNA, 2×10^7 independent clones were generated, of which >99% were recombinant.

Construction of a cDNA Library from a Type 3-1 Placenta. RNA was isolated from 120 g of a term placenta from a Caesarian section delivery. Frozen tissue was pulverized in a Waring blender and polyadenylylated RNA was isolated as described (14).

Three micrograms of polyadenylylated RNA was converted to cDNA by the procedure of Friedman and Rosbash (15). The second strand synthesis was carried out according to Gubler and Hoffman (16). Blunt ends were generated in the presence of 33.3 mM Tris acetate at pH 7.8, 66.6 mM potassium acetate, 10 mM magnesium acetate, 500 μM dithiothreitol, 50 μM β-nicotinamide adenine dinucleotide, each of the four deoxynucleoside triphosphates at 300 μM, ribonuclease A at 15 μg/ml, 1.5 units of ribonuclease H, 5 units of *Escherichia coli* DNA ligase, and 4.5 units of T4 DNA polymerase in a reaction volume of 40 μl for 60 min at 37°C. Ten micrograms of yeast transfer RNA was added prior to extractions with organic solvents and precipitations with ethanol. Methylation of *Eco*RI sites was carried out as recommended by the supplier (New England Biolabs). Phosphorylated *Eco*RI linkers (New England Biolabs) were ligated to the double-stranded cDNA and excess linkers were removed by *Eco*RI digestion in 100-fold enzyme excess. Unligated linkers were separated by Sepharose CL-4B chromatography (5-ml bed volume; Pharmacia). The remaining 16 ng of double-stranded cDNA was ligated to 0.6 μg of λgt11 arms (Promega Biotec, Madison, WI) and packaged into phage heads as described (13). The resulting library of 7×10^5 recombinant phages was amplified on *E. coli* strain Y1088 prior to screening.

Screening cDNA Libraries with Antibody Probes and Oligonucleotides. The type 3-1 placental cDNA library was screened with antiserum to human placental ALP and horseradish peroxidase-conjugated second antibody [rabbit IgG

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Abbreviations: ALP, alkaline phosphatase; kb, kilobase pairs.

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$F(ab')_2$, Jackson ImmunoResearch, Avondale, PA], as described by Young and Davis (17). DNA from phage candidates was digested with *Eco*RI, electrophoresed in agarose gels, and transferred to GeneScreen*Plus* membranes (New England Nuclear) according to the manufacturer's recommendations. DNA on the membranes was hybridized overnight to two separate mixtures of oligonucleotides (Biosearch, San Rafael, CA) in the presence of 0.9 M NaCl/0.09 M sodium citrate/1% $NaDodSO_4$ at 30°C. Membranes were washed in 0.3 M NaCl/0.03 M sodium citrate/1% $NaDodSO_4$ at 42°C before autoradiography.

The oligonucleotide mixture of sequence 5′-GA$\frac{A}{G}$GA$\frac{A}{G}$-GA$\frac{A}{G}$AA$\frac{T}{C}$CC-3′ (Biosearch) corresponds to amino acid positions 5 through 9, while the oligonucleotide mixture of sequence 5′-TT$\frac{T}{C}$TGGAA$\frac{TC}{CA}$GNGA-3′ is derived from amino acid positions 11 through 15 of the placental ALP protein sequence.

The type 1 placental ALP cDNA library was screened exactly as described by Young and Davis (17), using the anti-human ALP antiserum and ^{125}I-labeled staphylococcal protein A. Clones of interest were identified by comparison of DNA sequence to amino-terminal placental ALP peptide sequence (ref. 18; C.S., unpublished data).

DNA Sequencing. DNA sequences were determined by enzymatic (19) and chemical methods (20). Various M13 phage subclones were generated by forced cloning and by the T4 polymerase deletion method of Dale *et al.* (21). All of the type 1 and 3 placental ALP cDNA clones were sequenced at least once on both DNA strands. The identities of bases at nucleotide positions 611, 818, 867, and 1180 in the type 3 cDNA sequence were confirmed by the finding that the type 3 placental ALP cDNA clone was cleaved by restriction enzymes as predicted. The order of the bases G and C at positions 800 and 801 in the type 3 cDNA sequence was ambiguous as determined by both enzymatic and chemical methods. The base sequence at these two positions was demonstrated by the hybridization of the oligonucleotide TGGCGAA*GC*ACCAGGG and the lack of hybridization of the oligonucleotide TGGCGAA*CG*ACCAGGG under stringent conditions. These oligonucleotides were kindly provided by Eric Rappaport.

RESULTS

Amino-Terminal Amino Acid Sequence of Types 1, 2, and 3 Human Placental ALP. The amino-terminal amino acid sequences of the products of the common alleles ALP^1_p, ALP^2_p, and ALP^3_p were determined from placentas of phenotypes 1, 2, and 3. Over their first 42 amino acids, the ALP^1_p and ALP^2_p polypeptide products are identical to the previously published placental ALP amino-terminal sequence (18). The ALP^3_p product shows a proline-to-leucine substitution at amino acid position 3. In addition, polypeptides were generated by cyanogen bromide cleavage of ALP isolated from a type 1 placenta. The amino acid sequences of these peptides are underlined in Fig. 1.

Isolation of cDNA Clones for the ALP^1_p and ALP^3_p Alleles. Two fresh placentas obtained by Caesarian section were used as mRNA sources for the construction of placental cDNA libraries. Starch gel electrophoresis of ALP isolated from the placentas indicated they were phenotypes 1 and 3-1 (data not shown). A cDNA library was constructed from each placenta and screened by using polyclonal antiserum raised against purified placental ALP, as described in *Materials and Methods*.

Four immunopositive clones were isolated from the type 1 placental cDNA library. These clones fell into three categories of overlapping DNA fragments, as determined by hybridization and restriction enzyme mapping studies (data not shown). The sequence of the 2.7 kilobase pairs (kb) of DNA spanned by these clones is shown in Fig. 1. The reading frame beginning at nucleotide positon 81 encodes a polypeptide that contains the first 42 amino acids of the type 1 placental ALP protein. From amino acid +1 (isoleucine), this polypeptide is 513 amino acids in length. The deduced amino acid sequence agrees completely with the sequences of additional cyanogen bromide polypeptides from type 1 placental ALP. The locations of these peptide sequences are shown by lines under the deduced amino acid sequence in Fig. 1.

A cDNA clone coding for the ALP^3_p product was isolated from the cDNA library constructed from the type 3-1 placental mRNA. Of 10 phage candidates that bound anti-placental ALP antiserum, 1 phage contained a 2.7-kb DNA insert that hybridized to a mixture of oligonucleotides specific for amino acid residues 5 through 9 of the amino-terminal placental ALP sequence (see *Materials and Methods*). The DNA sequence of the amino-terminal portion of this cDNA clone encodes a placental ALP polypeptide that has a leucine residue at position 3. Therefore this 2.7-kb insert encodes the ALP^3_p protein product. The complete sequence of this 2.7-kb fragment was determined.

Comparison of ALP^1_p and ALP^3_p cDNA Sequences. The comparison of the sequences of the ALP^1_p and ALP^3_p cDNAs is shown in Fig. 1. Only those bases that differ between the two sequences are shown for the ALP^3_p cDNA. Also shown are the presumed amino acid differences between the resulting polypeptide chains. There are 12 base substitutions and a single base length difference between the two DNA sequences. In addition, there are differences between the sequences at their 5′ and 3′ ends, which probably do not reflect actual differences in mRNA and gene sequences, respectively (discussed below). Seven of the 12 base substitutions cause amino acid differences between the two polypeptides. These substitutions occur at amino acid positions 3, 44, 241, 255, 263, 367, and 372. Each of the amino acid substitutions is attributable to a single base change. Three of the base substitutions, at nucleotide positions 611, 686, and 818, are silent substitutions (they do not change the amino acid sequence). The remaining two substitutions and the single base length difference occur within the 1 kb of 3′ untranslated DNA. These differences are summarized in Table 1.

The differences between the 5′ ends of the ALP^1_p and ALP^3_p cDNA sequences are probably a result of the method used to clone the ALP^1_p cDNA. Specifically, nucleotides 66 through 76 differ between the two sequences. However, these bases in the ALP^1_p sequence are the exact reverse complement of bases 8 through 18 in the ALP^3_p cDNA sequence. We suspect that these bases, GCATGTCTGGA, in the ALP^1_p cDNA sequence were introduced at the 5′ end of the single-stranded cDNA during cloning and do not represent sequences actually present in the ALP^1_p mRNA. Similar artifacts due to S1 nuclease digestion of hairpin loops and misrepair have been described (22).

Apparent base differences between the allelic placental ALP sequences are present at the immediate 3′ end. Since these base differences occur within the poly(A) sequence of the type 1 sequence, we cannot assume that they are differences in the gene sequences. Rather, they may result from differential use of poly(A) addition signals, as discussed below. The ALP^3_p cDNA sequence contains six poly(A) addition signals (AATAAA), beginning at nucleotide positions 2416, 2658, 2666, 2679, 2683, and 2690. The signals beginning at positions 2416 and 2960 are located within a pair of 19-base-pair direct repeats of the base sequence AAT*AATAAA*AGGAAGTGTT.

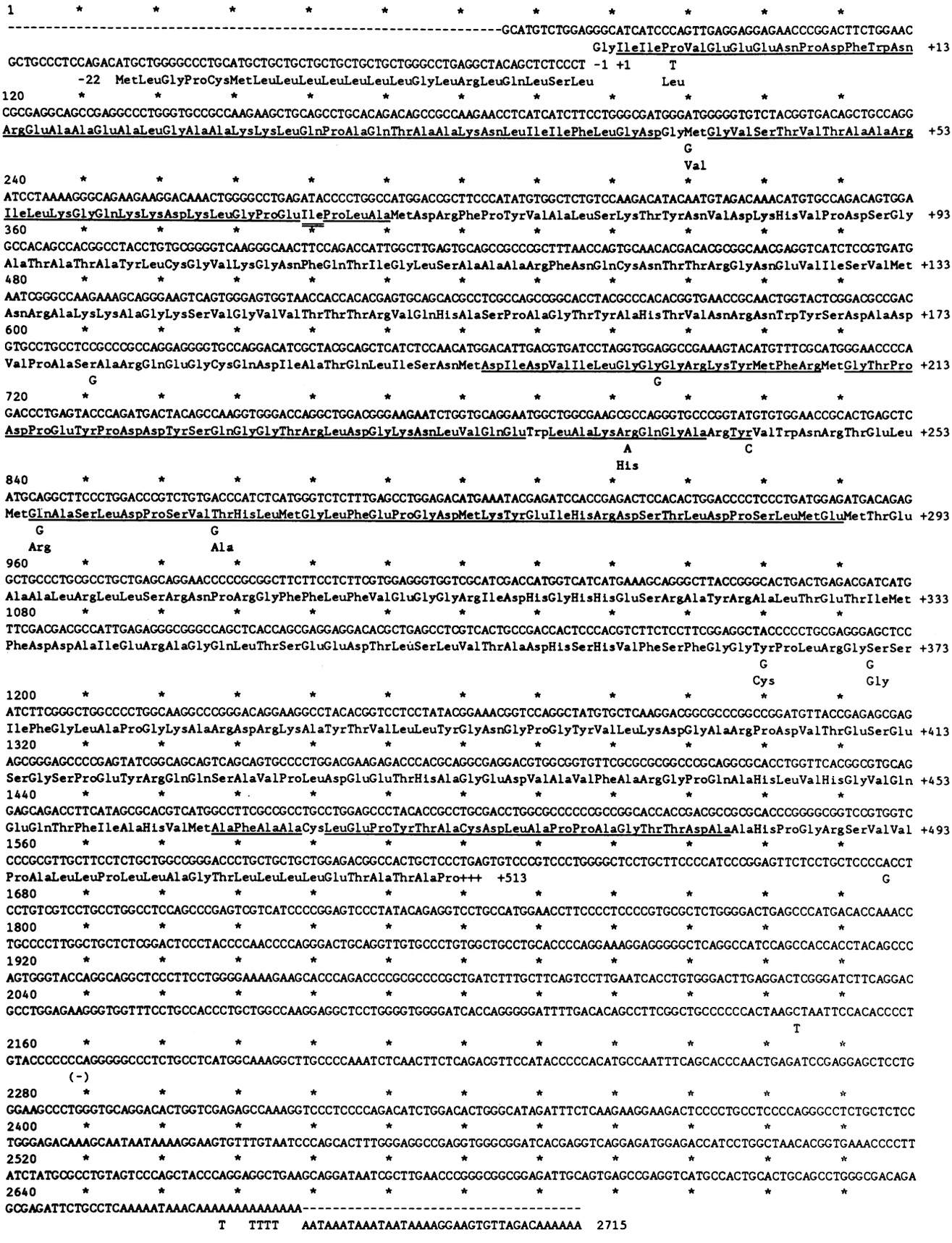

FIG. 1. DNA sequences of cDNA clones of types 1 and 3 placental ALP. The entire sequence of the type 1 placental ALP cDNA is shown. The deduced amino acid sequence of the ALP_p^1 protein product is also shown. Amino acid sequences determined by protein sequencing experiments are underlined. At amino acid position 67 (indicated with a double underline), both leucine and isoleucine were seen. The type 3 placental ALP cDNA sequence is identical to the type 1 sequence except as indicated. Bases that differ are shown below the type 1 sequence. Resulting changes in amino acids are also shown. The single length difference is indicated as (-). Nucleotide position numbers are with respect to the longer type 3 sequence and are shown on the left. Amino acid position numbers, shown at the right and within the sequence, are preceded by + or −. Asterisks occur at 10-base intervals. The termination codon is marked as +++.

Table 1. Base differences in the cDNA sequences of types 1 and 3 placental ALP alleles

Nucleotide position	Nucleotide Type 1	Nucleotide Type 3	Amino acid position	Amino acid Type 1	Amino acid Type 3	Silent or 3'UT
88	C	T	3	Pro	Leu	
210	A	G	44	Met	Val	
611	C	G				Silent
686	A	G				Silent
802	G	A	241	Arg	His	
818	T	C				Silent
844	A	G	255	Gln	Arg	
867	A	G	263	Thr	Ala	
1180	A	G	367	Tyr	Cys	
1194	A	G	372	Ser	Gly	
1676	A	G				3'UT
2144	C	T				3'UT
2169	C	—				3'UT

Nucleotide positions refer to Fig. 1. 3'UT, 3' untranslated.

DISCUSSION

It is unexpected and surprising that the polypeptide products of two common alleles at the placental ALP locus differ by as many as seven amino acid substitutions. It seems probable that the finding is related to another unusual feature of placental ALP that was detected by enzyme electrophoresis. In addition to the six common electrophoretic phenotypes, there are an unusually large number of different rare variants (1–3). They mainly represent heterozygotes for one or another of an extensive series of rare alleles (frequencies <0.01) with one of the three common alleles (1–3). Such rare alleles also occur, of course, at other loci. However, in electrophoretic surveys of enzymes determined by 43 different loci it was found that the heterozygosity for rare alleles at the placental ALP locus, estimated as 35–49 per 1000 (4) was much greater than at the other loci. The average heterozygosity for rare alleles at these 42 other loci was estimated to be about 1.14 per 1000 (3). The reasons for this high degree of allelic diversity at the ALP locus are not known. They may involve an unusual degree of intragenic crossing-over, gene conversion, single base change mutations, or other causes. Whatever the phenomenon involved, it seems likely that it also accounts for the remarkable number of amino acid substitutions between the two common alleles described here. It is noteworthy that the protein products of two common alleles at the locus coding for the vitamin D binding protein of human serum (*GC*) have been reported to differ by four amino acid substitutions (23, 24) and that in this case also an extensive series of rare alleles has been identified in population surveys using electrophoresis or isoelectric focusing (25, 26).

A near-full-length cDNA clone for the ALP type 1 (also referred to as type S) polypeptide chain has recently been isolated and sequenced by Millan (27). This sequence differs from the type 1 cDNA sequence reported here at two nucleotide positions within the protein coding regions. One of these differences (a G at position 635 as reported by Millan) is the same silent substitution at nucleotide position 611 as is seen in the type 3 cDNA sequence. In the other difference, a C at nucleotide position 730 of Millan's sequence replaces a G at position 706 in Fig. 1. This results in a proline for an arginine substitution in the polypeptide chain at amino acid position 209 (Fig. 1). Thus the type 1 sequence reported by Millan differs by eight amino acid substitutions from the type 3 polypeptide reported here. The difference between the two type 1 sequences may, if not due to cloning or sequencing artifacts, reflect allelic differences within the type 1 phenotype. We have previously raised this possibility of type 1 heterogeneity in the analysis of monoclonal antibody studies (28).

Kam *et al.* (29) have recently reported the sequence of a human placental ALP cDNA clone of unknown type that is different from both the ALP^1_P and ALP^3_P cDNA sequences. It differs from the type 1 polypeptide at 7 amino acid positions (3, 239, 240, 302, 374, 375, and 379) and from the type 3 polypeptide at 12 amino acid positions (44, 239, 240, 241, 255, 263, 302, 367, 372, 374, 375, and 379). At the positions of the six silent and 3' untranslated region sequence differences between types 1 and 3, the sequence of Kam *et al.* is identical to that of type 1 at two positions (686 and 1676) and is like that of the type 3 cDNA sequence at the remaining four positions (611, 818, 2144, and 2169). This untyped cDNA sequence is unique at two silent site nucleotide positions, 671 and 983, and at one 3' untranslated position, 2629, where it has four G residues instead of three as found in the type 1 and 3 cDNA sequences. This cDNA sequence is obviously not either type 1 or type 3 because of the multiple unique substitutions it shows. We also assume it is not type 2 because it has a leucine at amino acid position 3 instead of proline. We presume that it represents one of the many rare variant alleles at this locus.

Several aspects of the distribution of base differences between the ALP^1_P and ALP^3_P cDNA sequences are of interest, although we cannot yet place any statistical significance on these observations. The substitutions in the protein coding portion of the cDNA sequence are approximately equally distributed between amino acid replacement sites (7 out of 1107) and silent sites (3 out of 432). However, the frequency of substitutions in the protein coding region (10/1539 = 0.0065) is twice that in the 3' untranslated region (3/1045 = 0.0029). Also, upon inspection there appears to be some clustering of base substitutions, especially between base positions 802 and 867. Two of these features, clustered mutations and highly conserved 3' untranslated sequences, have been observed among the alleles of class I histocompatibility genes (30, 31).

The 5' ends of the type 3 cDNA clone described here and of the type 1 cDNA clone of Millan (27) both contain two in-phase initiation codons at amino acid positions −22 and −17 (base positions 15–17 and 30–32, respectively). The nucleotide sequence surrounding the initiation codon at amino acid position −22 shows the best agreement with a consensus sequence derived from sequence comparisons of initiation codons and from *in vitro* studies (32, 33). However, the sequence of the placental ALP allele determined by Kam *et al.* (29) shows an initiation codon only at amino acid position −17, and the base sequence 5' to this differs markedly from the type 3 sequence reported here and that of the type 1 sequence reported by Millan (27).

The immediate 3' ends of the four placental ALP cDNA clones differ in length and in number of poly(A) addition sites. The two longest cDNA clones, the type 3 clone reported here and the clone of Kam *et al.* (29), both have five poly(A) addition sites at their immediate 3' ends but differ in length. The two type 1 placental ALP cDNA clones, the one reported here and that of Millan (27), have one and three poly(A) addition sites, respectively, and differ in length from each other and from the other two placental ALP cDNA clones. Although cloning artifacts cannot be eliminated as a possible explanation for these differences, it is possible that the 3' ends of these cDNA clones may reflect heterogeneity in placental ALP mRNA that is caused by differential use of poly(A) addition sites.

The unusual degree of allelic variation present in placental ALP combined with the finding of multiple amino acid substitutions between two common alleles may make this system particularly useful in the study of mechanisms by which allelic diversity is generated and maintained.

Note Added in Proof. Further analysis of sequences has revealed an *Alu* family repetitive element at the extreme 3′ end of placental ALP cDNAs. Homology with the consensus *Alu* sequence (34) begins at nucleotide 2432 and terminates at the nucleotide 2686 (254 base pairs). The *Alu* sequence in the cDNA lacks 23 nucleotides from the 5′ end and 36 nucleotides from the center when compared to the consensus *Alu* sequence. The direct repeats 5′-AATAATAAAAG-GAAGTGTT-3′, discussed in the text, flank the *Alu* sequence. Consequently, of the six poly(A) addition signals in the type 3 cDNA, two are in the duplicated sequences that flank the *Alu* sequence and four are within the *Alu* sequence itself. This suggests that one original poly(A) addition signal (at position 2416) was duplicated during insertion of the *Alu* sequence.

We thank Ms. Joanne L. Borthwell for her assistance in the preparation of this manuscript and Drs. S. Woo, A. Dillela, and V. Kidd (Baylor College of Medicine) for supplying packaging extracts and *Eco*RI-cut dephosphorylated λgt11 DNA used in the construction of the type 1 placental cDNA library. This work was supported by National Institutes of Health Grant GM 27018 and March of Dimes Grant 858, both to H.H., and by the American Cancer Society and the University of Texas Medical School Biomedical Research Support Grant for B.J.K. Also, P.S.H. is supported by National Institutes of Health Training Grant HD 07107 to the Division of Biochemical Development and Molecular Diseases, Children's Hospital of Philadelphia. M.W. is a trainee of the Medical Scientist Training Program and is supported by National Institutes of Health Grant GM 07170.

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