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MOLECULAR CLONING OF HUMAN CLASS IV ALCOHOL DEHYDROGENASE cDNA

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SUMMARY A cDNA encoding a new type of alcohol dehydrogenase was cloned from a human stomach cDNA library. PCR amplification of 5'-stretch human stomach λgt11 library, using degenerate inosine-containing oligonucleotide probes compatible with peptide sequences of human σ-ADH, resulted in a single product. Subsequently, internal non-degenerate primers were constructed according to the sequences occurring in the product. By PCR with combinations of these new primers and λgt11 forward and reverse primers, fragments of the cDNA containing its 5' and 3' ends were amplified. The full length cDNA sequence has 1125 nucleotides with a 72% similarity to those of human class I ADH. The polypeptide sequence, predicted from the cDNA, corresponds to 373 amino acids with a high degree of similarity (96%) to fragments of σ-ADH previously reported. Northern hybridization analysis with the specific probe for the mRNA of this protein showed that it is expressed in the human stomach but not in the liver. These data indicate that the cDNA we cloned is that of human class IV ADH.

Human alcohol dehydrogenase (ADH, EC 1.1.1.1.) comprises a family of enzymes that participate in oxido-reductions between various alcohols and the corresponding aldehydes. The cDNA structures of five subunits (α, ß, γ, π, χ) have been determined and the corresponding genes (*ADH1*, *ADH2*, *ADH3*, *ADH4*, and *ADH5*,) have been mapped (1, review). Moreover, a gene encoding an additional class of isozyme (*ADH6*) has been characterized (2).

Recent studies have identified a new type of ADH in the human stomach (3-6), which has structural similarities to that present in rat glandular stomach (5). Observations by starch gel electrophoresis indicate that this ADH isozyme is absent in the liver. It has been considered to constitute a new class (class IV) of ADH. The subunit of this enzyme is named σ (or μ) and its partial amino acid sequence has been determined (5,7). The aim of this study was to resolve the entire structure of human class IV ADH by molecular cloning of the enzyme's cDNA.

MATERIALS AND METHODS

Amplification of DNA fragments of a new alcohol dehydrogenase from human stomach library. A 5'-stretch human stomach λgt11 phage library was purchased from Clontech Laboratories,

0006-291X/94 $5.00

reaction (PCR) is illustrated in Figure 1. PCR was carried out with 2.5-10 units of Taq polymerase (Promega; Madison, WI) in PCR buffer (50 mM KCL, 10 mM Tris-HCl, pH=9.0 at 25°C, 0.1% Triton X-100) with 0.2 mM each dNTPs (Pharmacia; Piscataway, NJ) and 5mM $MgCl_2$. All oligonucleotides for PCR primers were synthesized by Geneset (La Jolla, CA).

The library was diluted to 5 x 10^7 phages/ml and 2 µl of the solution was subjected to PCR amplification, (30 cycles: 94°C, 30 sec; 42°C, 2 min; 72°C, 2 min), using degenerate inosine (i) containing oligonucleotide probes (8) according to the reported partial amino acid sequence of σ-ADH (7). The primer sequences are expressed left to right as 5' to 3' of nucleotides and parentheses indicate degenerated portions. The sense primer (sense-1; GCi GCi CTi (TC)Ti TGG GA(AG) CA) was compatible with the 11-17 peptides of the isolated σ-ADH and that of the antisense primer (antisense-1; GT(GA) AAi A(AG)i A(AG)C ATi GG(AG) TC(GA) TAi GTi A(AG)C) with the 309-299 peptide.

The PCR fragment was subcloned into plasmid (pGEM3Zf(+); Promega) and its nucleotide sequence was analyzed by the fluorescent dye labelled deoxynucleotide-termination method with an ABI 373A automatic DNA sequencer (Applied Biosystems; Foster City, CA).

To obtain the 5' and 3' end fragments of the ADH cDNA, internal non-degenerate primers (sense-2; GTT CCT CCA TCA GCC AAG ATG and antisense-2; ACT TGG ACA CCA TTG TTC CTT) were synthesized according to the sequences occurring in the medial portion of the first PCR product. To obtain 5' end fragments of the ADH cDNA, PCR amplification (30 cycles: 94°C, 30 sec; 50°C, 1 min; 72°C, 1 min) with the antisense-2 and the λgt11 forward primers (GGT GGC GAC GAC TCC TGG, (9)) was carried out from the λgt11 phage solution. To obtain 3'end fragments, PCR amplification was done under the same conditions with the sense-2 and the λgt11 reverse primers (TTG ACA CCA GAC CAA CTG GT (9)). The PCR products were subcloned and their nucleotide sequences were determined as described above.

The homology between the full cDNA sequence with those of other ADHs was estimated at the nucleotide and amino acid levels by MacVector (version 4.0.1; Kodak, Rochester NY) with a Macintosh IICi (Apple Computer Japan, Inc. Tokyo, Japan).

Northern hybridization analysis. For this analysis, a DNA probe of 497 bp was prepared by PCR using sense-3 (ACi TT(TC) ACi GA(AG) TA(CT) ACi GTi GTi GA(TC) GA) and antisense-1 primers and then labeled with [α-P^{32}] dCTP (3000 Ci/mmol; DuPont NEN, Boston, MA), using a multiprime DNA labeling kit (Amersham, Arlington Heights, IL). Human stomach and liver specimens were obtained at surgery from obese patients undergoing gastroplasty and from liver transplantation donors. Total RNAs were extracted by the guanidinium thiocyanate method (10) and their concentrations were measured by optical absorbance at 260 nm. Twenty µg of each sample was applied to agarose/formaldehyde gel electrophoresis and transferred to Nylon membrane (Hybond N; Amersham) (11). After pre-hybridization for 3 hours, hybridization with the heat denatured probe was carried out in 50% (v/v) formamide, 5 X SSPE, 1 X Denhardt's solution, 0.005% (W/V) sodium pyrophosphate, 0.2 mg/ml salmon sperm DNA (Sigma) and 0.1% sodium dodecylsulfate (SDS; Sigma) for 72 hours at 42°C (11). The blots were washed twice with 2 X SSC / 0.1% SDS at room temperature for 5 minutes and 0.2 X SSC / 0.1% SDS for 5 minutes at room temperature and 0.2 X SSC / 0.1% SDS for 15 minutes at 42°C (11), and exposed to X-Omat AR film (Kodak) for 72 hours at -70°C with two intensifying screens.

RESULTS AND DISCUSSION

The full length cDNA sequence encoding a human class IV ADH was obtained by molecular cloning from a human stomach library following the PCR strategy illustrated in figure 1.

The first PCR amplification of the stomach cDNA library with the degenerate primers (sense-1 and antisense-1) yielded a single product with 896 bp. The polypeptide sequence predicted from the nucleotide sequence of this product showed 94% similarity with the reported polypeptide sequences of σ-ADH fragments (7) (79/84: 79 matched in 84 amino acids).

PCR primed by combinations of antisense-2 and λgt11 forward primers or sense-2 and λgt11 reverse primers amplified several products, respectively. These results may reflect the presence of

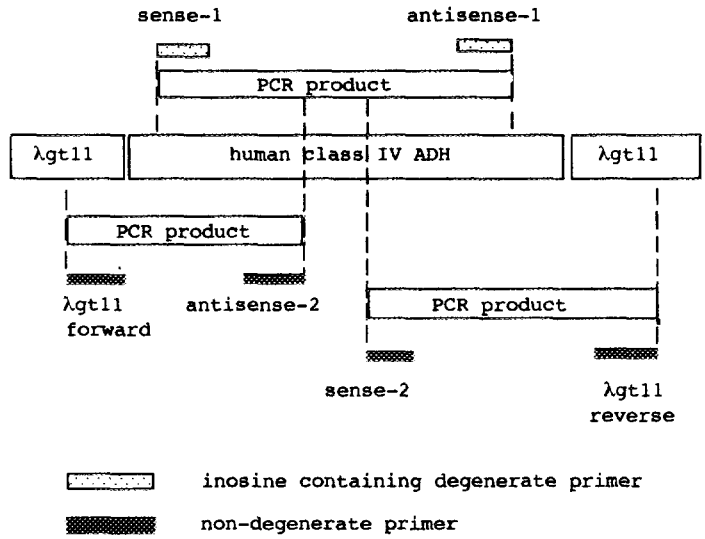

Figure 1. Strategy of cDNA cloning from λgt11 by PCR.

phages containing different size fragments of the targeted cDNA in the library. Since products of 283 bp and 721 bp were expected to contain the 5' and 3' ends of the ADH cDNA, because of their molecular sizes, these clones were subcloned and sequenced. The peptide sequences deduced from their nucleotide sequence showed high degrees of similarities with the reported peptide fragments of σ-ADH (7) (100% ; 18/18, 98% ; 43/44, respectively). As expected, start (ATG) and stop (TGA) codons were found in the predicted positions.

The nucleotide sequence of the cDNA derived from these 3 PCR fragments is shown in Figure 2 (Gene Bank accession number; L-33179). The full-length cDNA predicts a 373 amino acid protein. This sequence shows similarities with those of human class I, II, and III ADH isozymes. The homology with class I (α-ADH), II, and III ADH were 72%, 62%, and 64% at the nucleotide level and 70%, 59%, and 60% at the amino acid level, respectively. The amino acid sequence predicted from the cDNA sequence indicates the presence of cysteine in position 46 and 173, and of histidine in position 67, which are compatible with characteristics of ADH which have been implicated in the binding of zinc and the formation of the active site (12). Moreover, the protein contains several domains which are considered to be interaction sites for NAD+, coenzyme of ADH (such as histidine 51, asparagine 222, 270, lysine, 227, valine, 202, arginine 367, etc). These findings support the conclusion that the cDNA we cloned is that of a member of the ADH family.

The amino acid sequence corresponding to this cDNA was 93% (67/72) compatible with the fragments reported as σ-ADH by Paré's et al. (5) and 96% (130/136) with those by Stone et al. (7). This may reflect an incorrect assignment by previous studies, or the presence of subclasses

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1/1                                          31/11
GGC ACT GCT GGA AAA GTT ATT AAG TGC AAA GCA GCT GTG CTT TGG GAG CAG AAG CAA CCC
gly thr ala gly lys val ile lys cys lys ala ala val leu trp glu gln lys gln pro
61/21                                        91/31
TTC TCC ATT GAG GAA ATA GAA GTT GCC CCA CCA AAG ACT AAA GAA GTT CGC ATT AAG ATT
phe ser ile glu glu ile glu val ala pro pro lys thr lys glu val arg ile lys ile
121/41                                       151/51
TTG GCC ACA GGA ATC TGT CGC ACA GAT GAC CAT GTG ATA AAA GGA ACA ATG GTG TCC AAG
leu ala thr gly ile cys arg thr asp asp his val ile lys gly thr met val ser lys
181/61                                       211/71
TTT CCA GTG ATT GTG GGA CAT GAG GCA ACT GGG ATT GTA GAG AGC ATT GGA GAA GGA GTG
phe pro val ile val gly his glu ala thr gly ile val glu ser ile gly glu gly val
241/81                                       271/91
ACT ACA GTG AAA CCA GGT GAC AAA GTC ATC CCT CTC TTT CTG CCA CAA TGT AGA GAA TGC
thr thr val lys pro gly asp lys val ile pro leu phe leu pro gln cys arg glu cys
301/101                                      331/111
AAT GCT TGT CGC AAC CCA GAT GGC AAC CTT TGC ATT AGG AGC GAT ATT ACT GGT CGT GGA
asn ala cys arg asn pro asp gly asn leu cys ile arg ser asp ile thr gly arg gly
361/121                                      391/131
GTA CTG GCT GAT GGC ACC ACC AGA TTT ACA TGC AAG GGC AAA CCA GTC CAC CAC TTC ATG
val leu ala asp gly thr thr arg phe thr cys lys gly lys pro val his his phe met
421/141                                      451/151
AAC ACC AGT ACA TTT ACC GAG TAC ACA GTG GTG GAT GAA TCT TCT GTT GCT AAG ATT GAT
asn thr ser thr phe thr glu tyr thr val val asp glu ser ser val ala lys ile asp
481/161                                      511/171
GAT GCA GCT CCT CCT GAG AAA GTC TGT TTA ATT GGC TGT GGG TTT TCC ACT GGA TAT GGC
asp ala ala pro pro glu lys val cys leu ile gly cys gly phe ser thr gly tyr gly
541/181                                      571/191
GCT GCT GTT AAA ACT GGC AAG GTC AAA CCT GGT TCC ACT TGC GTC GTC TTT GGC CTG GGA
ala ala val lys thr gly lys val lys pro gly ser thr cys val val phe gly leu gly
601/201                                      631/211
GGA GTT GGC CTG TCA GTC ATC ATG GGC TGT AAG TCA GCT GGT GCA TCT AGG ATC ATT GGG
gly val gly leu ser val ile met gly cys lys ser ala gly ala ser arg ile ile gly
661/221                                      691/231
ATT GAC CTC AAC AAA GAC AAA TTT GAG AAG GCC ATG GCT GTA GGT GCC ACT GAG TGT ATC
ile asp leu asn lys asp lys phe glu lys ala met ala val gly ala thr glu cys ile
721/241                                      751/251
AGT CCC AAG GAC TCT ACC AAA CCC ATC AGT GAG GTG CTG TCA GAA ATG ACA GGC AAC AAC
ser pro lys asp ser thr lys pro ile ser glu val leu ser glu met thr gly asn asn
781/261                                      811/271
GTG GGA TAC ACC TTT GAA GTT ATT GGG CAT CTT GAA ACC ATG ATT GAT GCC CTG GCA TCC
val gly tyr thr phe glu val ile gly his leu glu thr met ile asp ala leu ala ser
841/281                                      871/291
TGC CAC ATG AAC TAT GGG ACC AGC GTG GTT GTA GGA GTT CCT CCA TCA GCC AAG ATG CTC
cys his met asn tyr gly thr ser val val val gly val pro pro ser ala lys met leu
901/301                                      931/311
ACC TAT GAC CCG ATG TTG CTC TTC ACT GGA CGC ACA TGG AAG GGA TGT GTC TTT GGA GGT
thr tyr asp pro met leu leu phe thr gly arg thr trp lys gly cys val phe gly gly
961/321                                      991/331
TTG AAA AGC AGA GAT GAT GTC CCA AAA CTA GTG ACT GAG TTC CTG GCA AAG AAA TTT GAC
leu lys ser arg asp asp val pro lys leu val thr glu phe leu ala lys lys phe asp
1021/341                                     1051/351
CTG GAC CAG TTG ATA ACT CAT GTT TTA CCA TTT AAA AAA ATC AGT GAA GGA TTT GAG CTG
leu asp gln leu ile thr his val leu pro phe lys lys ile ser glu gly phe glu leu
1081/361                                     1111/371
CTC AAT TCA GGA CAA AGC ATT CGA ACG GTC CTG ACG TTT TGA
leu asn ser gly gln ser ile arg thr val leu thr phe OPA
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Figure 2. Nucleic acid sequence and deduced amino acid sequence of human class IV ADH. The amino acid counting begins after the start codon. OPA: stop codon.

within class IV ADH. The later can not be excluded at this time, since subclasses and polymorphism are well established in class I ADH (1). Thus, the cDNA we cloned encodes σ-ADH itself or an ADH isozyme which is closely related to σ-ADH.

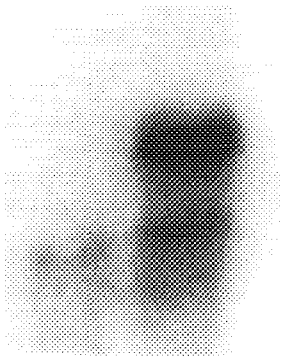

Figure 3. Detection of the class IV ADH-specific mRNA in the human stomach and liver. Total RNAs prepared from the stomach and liver tissues were electrophored and blotted onto nylon membranes. The RNA was hybridized with the human class IV ADH-specific probe. Specific signals were observed in the stomach but not in the liver.
Lane S: total RNA from stomach. Lane L: total RNA from liver.

By northern hybridization analysis, labelled mRNAs of approximately 2 kb and 3 kb were observed in the stomach samples, whereas these signals were not detected in those of the liver (Figure 3), indicating that this protein is expressed in the stomach but not in the liver. This organ expression is in keeping with that of human class IV ADH (3, 7), further supporting the fact that this cDNA encodes a human class IV ADH. The probe used in this study seems to be specific for the mRNA of this new ADH, since it did not hybridized with mRNAs of class I, II or III ADHs or that corresponding to *ADH6* all of which have been shown to be expressed in the human liver (2,13). The finding that mRNAs with two different sizes correspond to this ADH is not surprising, since similar observations have been made for the other ADHs (13).

In conclusion, the complete structure of a new type of ADH was determined by molecular cloning from a human stomach library using PCR. The amino acid sequence predicted from the full length cDNA showed a high degree of similarity with that of σ-ADH. The mRNA corresponding to this protein was expressed in the stomach but not in the liver. Taken together, these data indicate that the cloned cDNA encodes human class IV ADH.

REFERENCES

1) Yoshida, A., Hsu, L.C., and Yasunami, M. (1991) Prog. Nucleic Acid Res. Mol. Biol. 40, 225-287.
2) Yasumami, M., Hsu, L.C., and Yoshida, A. (1991) Proc. Natl. Acad. Sci. USA. 88, 7610-7614.
3) Moreno, A., and Pares, X.(1991) J.Biol.Chem.266, 1128-1133.
4) Hernandez-Munoz, R., Caballeria, J., Baraona, E., Uppal, R. Greenstein, R. and Lieber, C.S. (1990) Alcoholism:Clin.Exp.Res 14, 946-950.
5) Pares, X., Moreno, A., Cederlund, E., Hoog, J.-O. and Jornvall, J. (1990) FEBS Lett. 277, 151-118.
6) Yin, S.J., Wang, M.F., Liao, C.S., Chen, C.M., and Wu, C.W. (1990) Biochem. Int. 22, 829-835.

7) Stone, C.L., Thomasson, H.R, Bosron, W., F. and Li, T.K.
(1993) Alcoholism:Clin.Exp.Res 17, 911-918.
8) Knoth, K., Roberds, S., Poteet, C and Tamkun, M. (1989) Nucleic Acid res. 16, 10932.
9) Young, R. and Davis, R. (1983) Proc. Natl. Acad. Sci. USA 80, 1194-1197.
10) Chomczynski,P. and Sacchi, N. (1987) Anal Biochem. 162, 156-159.
11) Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) In Molecular Cloning: A Laboratory Manual, 2 nd ed. Cold spring Harbor Laboratory, Cold Spring harbor, NY.
12) Eklund, H. (1989) Biochem.Soc.Trans, 17, 293-305.
13) Engeland, K. and Maret, W. Biochem. Biophys. Res. Commun. (1993) 193, 47-53.