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Primary structure of rat liver alkaline phosphatase deduced from its cDNA

Yoshio MISUMI,* Kosuke TASHIRO,† Masahira HATTORI,‡ Yoshiyuki SAKAKI‡ and Yukio IKEHARA*§
*Department of Biochemistry, Fukuoka University School of Medicine, Jonan-ku, Fukuoka 814-01,
†Department of Biochemistry, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113,
and ‡Research Laboratory for Genetic Information, Kyushu University, Higashi-ku, Fukuoka 812, Japan

Rat liver alkaline phosphatase (ALP) was markedly induced by treatment of rats by bile-duct ligation and colchicine injection. Taking this advantage for enrichment of ALP mRNA, we constructed a λgt11 liver cDNA library using polyadenylated RNA prepared from the treated rat liver, and isolated an ALP cDNA clone. The 2165 bp cDNA contained an open reading frame that encodes a 524-amino-acid-residue polypeptide with a predicted molecular mass of 57737 Da. The precursor protein contained a presumed signal peptide of 17 amino acid residues followed by 28 amino acid residues identical with the *N*-terminal sequence determined from the purified rat liver ALP. It was also confirmed that amino acid sequences of two CNBr-cleavage peptides obtained from liver ALP were contained within the cDNA-encoded protein. Five possible *N*-linked glycosylation sites were found in the molecule and a highly hydrophobic amino acid sequence at the *C*-terminus. The deduced polypeptide of rat liver ALP showed 88% homology to that of the human liver-type enzyme in osteosarcoma cells. RNA blot hybridization analysis identified a single species of ALP mRNA with 2.7 kb in both the control and the treated rat livers. An approx. 20-fold increase of the mRNA was detected in the treated liver at 12 h after the onset of stimulation, compared with that in the control liver.

INTRODUCTION

Alkaline phosphatase (ALP) (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) in mammalian cells is a membrane-bound glycoprotein and a marker enzyme for the plasma membrane (Pekarthy *et al.*, 1972; Fishman, 1974). The enzyme has many interesting aspects to be investigated because of its inducibility, genetic polymorphism, ectopic expression etc. (McComb *et al.*, 1979). At least three types of ALP (isoenzymes) are identified in human tissues: placental, intestinal and tissue-unspecific (referred to below as the liver-type) isoenzymes (Fishman, 1974). Available evidence, however, indicates that only two types of ALP are detectable in other animals, including the rat, where ALP in placenta is the liver-type isoenzyme (Goldstein *et al.*, 1980). Although extensive studies have been done for enzymological and immunological characterization of these isoenzymes, surprisingly little is known about the detailed structure of ALPs. This may be mainly due to the difficulty in complete purification of ALP in a quantity enough for the chemical analysis, a problem commonly observed with membrane-bound enzymes.

Rat liver ALP, though its basal concentration is quite low, is easily inducible in response to various stimuli (Kaplan & Righetti, 1970; Pekarthy *et al.*, 1972). In particular, a much higher concentration of ALP is induced in the liver by treatment of rat by bile-duct ligation in combination with colchicine injection (Ikehara *et al.*, 1978; Oda & Ikehara, 1981). Although the induction mechanism remains to be clarified, we took this advantage for possible enrichment of ALP mRNA in the liver. In the present study we constructed a λgt11 liver cDNA library using poly(A)$^+$ RNA prepared from the treated rat liver, and isolated an ALP cDNA clone. During the progress of this study many efforts have been successfully made for elucidating the molecular detail of human ALPs by cloning and sequencing of cDNAs for the isoenzymes in placenta (Kam *et al.*, 1985; Millán, 1986; Henthorn *et al.*, 1986), intestine (Berger *et al.*, 1987) and osteosarcoma cells (Weiss *et al.*, 1986). All of these tissues and cells are known to possess extremely high ALP activity.

MATERIALS AND METHODS

Materials

Oligo(dT) (12–18-mer), sequencing primers (5′-CAGGAAACAGCTATGAC-3′ and 5′-CCAGTCACGACGACGACGTTGTA-3′) and *Eco*RI linker (pGGAATTC) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden); oligo(dT)–cellulose was from Sigma Chemical Co. (St. Louis, MO, U.S.A.); the λgt11 arms and the Gigapack kit were from Strategene (San Diego, CA, U.S.A.); [α-^{32}P]dCTP (400 or 3000 Ci/mmol) and multiprime DNA-labelling kit were from Amersham–Japan (Tokyo, Japan); [^{35}S]methionine (1120 Ci/mmol) was from New England Nuclear (Boston, MA, U.S.A.); horseradish-peroxidase-conjugated anti-(rabbit IgG) antibody was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Various DNA- and RNA-modifying

Abbreviations used: ALP, alkaline phosphatase; poly(A)$^+$ RNA, polyadenylated RNA.

§ To whom correspondence should be addressed.

These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00714.

enzymes and restriction endonucleases were obtained from Amersham, Takara Shuzo (Kyoto, Japan), Nippon Gene (Toyama, Japan) and Seikagaku Kogyo (Tokyo, Japan). TSK-G 2000SW was from Toyo Soda (Tokyo, Japan) and μBondapak C_{18} from Waters Associates (Milford, MA, U.S.A.).

Animals

Male Wistar rats weighing 350–450 g were used. For the induction of ALP in the liver, rats were treated by bile-duct ligation and colchicine injection (1 mg/kg body wt.), and starved for 12 h before being killed (Ikehara *et al.*, 1978).

Purification of ALP and its CNBr-cleavage peptides

Rat liver ALP was purified by standard biochemical techniques as described previously (Miki *et al.*, 1986). The purified ALP had the specific activity of 1180 units (μmol/min)/mg of protein, and was found to be a single protein with molecular mass 72000 Da when analysed by SDS/polyacrylamide-gel electrophoresis. The purified protein (5 mg) was cleaved with 2.5 M-CNBr in 70% (v/v) formic acid for 24 h at room temperature. The sample was freeze-dried and dissolved in 0.5 ml of 0.1 M-ammonium acetate, pH 7.4. Portions (100 μl each) were subjected to h.p.l.c. on a TSK-G 2000SW column (0.8 cm × 60 cm) and eluted with 0.1 M-ammonium acetate, pH 7.4, resulting in the separation of five major peptide peaks. Fractions of each peak were separately pooled and freeze-dried. Each residue was dissolved in 100 μl of 0.1% (v/v) trifluoroacetic acid and subjected to h.p.l.c. on a μBondapak C_{18} column (0.4 cm × 30 cm), which was eluted with an increasing linear gradient of 0–60% (v/v) acetonitrile in 0.1% trifluoroacetic acid. Two major peptides, one eluted at 30% acetonitrile and the other at 40% acetonitrile, were obtained from the sample that had been eluted as the third peak from the TSK-G 2000SW column.

Protein sequencing

Amino acid sequence analysis of both the purified ALP (20 μg) and its CNBr-cleavage peptides (5 μg each) was performed with an Applied Biosystems model 470A gas-phase sequencer with an on-line model 120A phenylthiohydantoin analyser, with use of the manufacturer's programming.

Antibodies

The purified rat liver ALP (1 mg/ml) was emulsified with an equal volume of Freund's complete adjuvant, and injected intramuscularly into multiple sites on the back of three rabbits (0.5 mg of the antigen/rabbit). At 2-week intervals each rabbit received three injections of the antigen with the same dose in complete adjuvant. At 10 days after the last injection, each rabbit was bled for collection of the antiserum. The antiserum thus obtained was found to be monospecific for the liver-type ALP (Miki *et al.*, 1986). Anti-(liver ALP) IgG was purified from the antiserum by affinity chromatography on Sepharose 4B coupled with the purified rat liver ALP (1 mg of protein/ml of packed gels).

Poly(A)$^+$ RNA extraction and fractionation

Total liver RNA was prepared from the treated rat liver by the method of Chirgwin *et al.* (1979), followed by additional treatment with chloroform/phenol, pH 7.5, and 3.0 M-sodium acetate (Palmiter, 1974). Total poly(A)$^+$ RNA was selected by oligo(dT)–cellulose (Aviv & Leder, 1972). Enrichment of ALP mRNA was carried out by centrifugation of total poly(A)$^+$ RNA on a sucrose density gradient (5–20%, w/v) in 20 mM-Tris/HCl buffer, pH 7.5, containing 2.5 mM-EDTA and 0.02% (w/v) SDS for 18 h at 60000 *g* at 4 °C, followed by fractionation. Identification of a fraction containing ALP mRNA was carried out by translation *in vitro* of the fractionated poly(A)$^+$ RNA in a reticulocyte-lysate system (Pelman & Jackson, 1976). Each fractionated poly(A)$^+$ RNA (2 μg/assay) was incubated in the reaction mixture at 25 °C for 90 min with 50 μCi of [^{35}S]-methionine. ^{35}S-labelled ALP was immunoprecipitated from the translation products and analysed by electrophoresis on SDS/polyacrylamide gels (9.0%, w/v) followed by fluorography, as described previously (Misumi *et al.*, 1983).

cDNA library construction and screening

The poly(A)$^+$ RNA fraction enriched with ALP mRNA (fraction 14 in Fig. 2) was used for construction of the following cDNA library. The first-strand cDNA was synthesized by using reverse transcriptase, oligo(dT) as primer and [α-^{32}P]dCTP as tracer according to the procedure established by Gubler & Hoffman (1983). Second-strand synthesis was carried out by the RNAase H procedure, also with [α-^{32}P]dCTP as tracer (Gubler & Hoffman, 1983). Subsequent processes to construct the λgt11 expression cDNA library followed the method of Young & Davis (1983). A 30 ng portion of double-stranded cDNA was ligated to 1 μg of λgt11 arms, and packaged into bacteriophages by using the Gigapack λ phage packaging system (Maniatis *et al.*, 1982).

The resultant library of 6×10^5 recombinant bacteriophages, without prior amplification, were screened with anti-(liver ALP) IgG in combination with horseradish-peroxidase-conjugated second antibodies, as described previously (Huynh *et al.*, 1985). Plaques of positive clones were isolated and bacteriophage DNAs were digested with *Eco*RI. *Eco*RI-excised cDNA inserts were subcloned into a plasmid vector pUC13 (Vieira & Messing, 1982), and characterized by restriction-endonuclease mapping.

DNA sequencing

After the restriction fragments of the cDNA were subcloned into the plasmid vector pUC13 (see Fig. 3), the nucleotide sequences of both strands were analysed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), in which the denatured plasmid DNA was used as a template (Hattori & Sakaki, 1986). The recombinant plasmid DNA was extracted and purified by rapid alkaline lysis followed by RNAase treatment (Maniatis *et al.*, 1982). The plasmid DNA was then immediately denatured with 0.2 M-NaOH and subjected to a sequencing reaction utilizing synthetic oligonucleotides (5′-CAGGAAACAGCTATGAC-3′ or 5′-CCAGTCACGACGACGACGTTGTA-3′) as primers and [α-^{32}P]dCTP as tracer. The reaction was carried out for 15–20 min at 37 °C, the temperature that was found to be important to avoid the formation of extra bands (Hattori & Sakaki, 1986). The reaction products were analysed by electrophoresis on 7 M-urea/6% (w/v) polyacrylamide wedge gels and 7 M-urea/5% polyacrylamide stretch gels. Under the conditions employed,

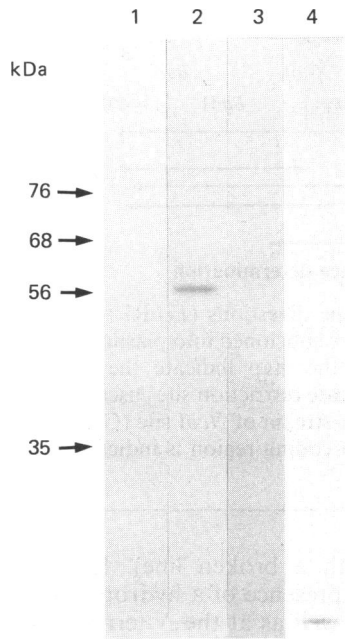

Fig. 1. Immunoprecipitation of translation products directed *in vitro* by rat liver poly(A)$^+$ RNA

Poly(A)$^+$ RNAs were prepared from the control rat liver (lane 1) and from the liver treated for the ALP induction (lanes 2–4) and translated *in vitro* in a reticulocyte-lysate translation system with [^{35}S]methionine, followed by immunoprecipitation with either rabbit anti-(liver ALP) IgG (lanes 1–3) or pre-immune rabbit IgG (lane 4). In lane 3, immunoprecipitation was carried out after the purified ALP (20 μg) was added to the sample. The immunoprecipitates were analysed by SDS/9.0%-polyacrylamide-gel electrophoresis/fluorography. Molecular-mass markers used are rat transferrin (76 kDa), albumin (68 kDa), α_1-proteinase inhibitor (56 kDa) and haptoglobin β-chain (35 kDa).

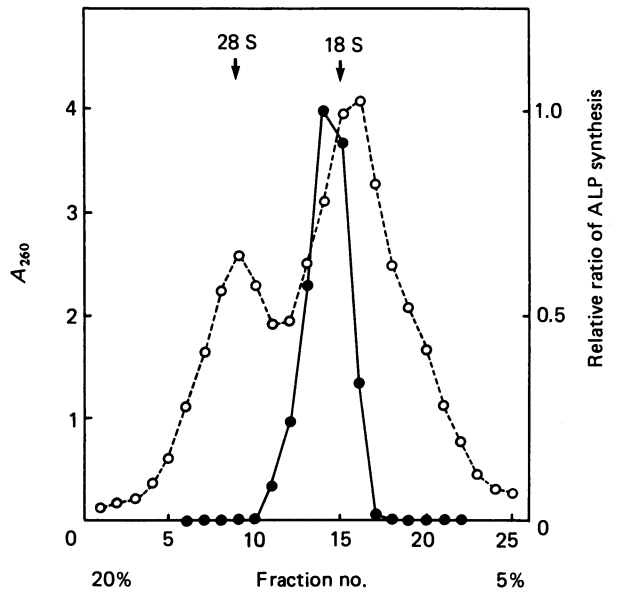

Fig. 2. Sedimentation profile of poly(A)$^+$ RNA of rat liver

Poly(A)$^+$ RNA was isolated from rat liver treated for the ALP induction (12 h). The sample (1.3 mg of RNA) was separated by centrifugation on a linear sucrose density gradient (5–20%, w/v) and fractionated into 25 fractions. RNA in each fraction was precipitated with ethanol and used for translation *in vitro* with [^{35}S]methionine. ALP was immunoprecipitated and analysed by SDS/polyacrylamide-gel electrophoresis/fluorography. The ALP-synthesizing translating activity of each fraction was determined by densitometric tracing of the fluorogram and expressed as a relative ratio taking the maximum densitometric level as 1.0. ○, A_{260}; ●, relative ratio of ALP synthesis. Arrows indicate positions of 28S and 18S rRNA of rat liver.

sequences of more than 500 nucleotide residues could be determined in single experiments (Hattori & Sakaki, 1986).

Northern-blot analysis

A 5 μg portion of poly(A)$^+$ RNA was denatured with 1.0 M-glyoxal and electrophoresed on 1.1% (w/v) agarose gels as described previously (Thomas, 1983). Glyoxalated RNA was transferred to a nylon membrane and hydridized with ^{32}P-labelled ALP cDNA, which had been prepared with a multiprime DNA labelling system (Feinberg & Vogelstein, 1983).

Computer analysis of cDNA and protein

Nucleotide sequence was analysed by using the GENAS System at Kyshu University Computer Center (Kuhara *et al.*, 1984). Hydropathy analysis was carried out in accordance with Kyte & Doolittle (1982).

RESULTS

Enrichment of liver ALP mRNA

Although the basal level of rat liver ALP activity was extremely low, it was increased by double treatment of rats by bile-duct ligation and colchicine injection, reaching a maximum value (about 20-fold) at 24 h after the treatment (Ikehara *et al.*, 1978; Oda & Ikehara, 1981). The elevation of the enzyme activity was found to be due to an increase of ALP mRNA in the treated liver, as shown in Fig. 1. The cell-free translation products with poly(A)$^+$ RNA from the treated rat liver yielded a single component of molecular mass 57 kDa by immunoprecipitation with anti-(liver ALP) IgG (lane 2), but the corresponding component was not detectable in those with poly(A)$^+$ RNA from the control rat liver (lane 1) under the conditions used here. The translation product of molecular mass 57 kDa was identified to be a precursor form of liver ALP (Y. Misumi, K. Oda & Y. Ikehara, unpublished work).

For further enrichment of the ALP mRNA, the poly(A)$^+$ RNA fraction from the treated rat liver was fractionated by centrifugation on a sucrose density gradient (Fig. 2). Cell-free translation of each RNA fraction identified a location of the ALP mRNA (fractions 12–16 in Fig. 2). The fraction 14 most enriched with the ALP mRNA was used for construction of a cDNA library.

Isolation of ALP cDNA from rat liver cDNA library

The rat liver cDNA library was screened with anti-(liver ALP) IgG without prior amplification of recombinant bacteriophages. Screening of 6×10^5

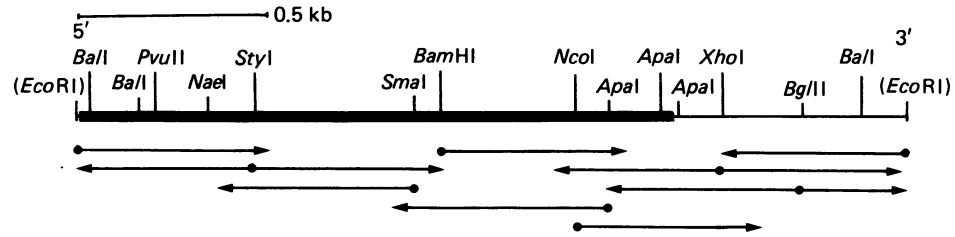

Fig. 3. Restriction map of rat liver ALP cDNA and the strategy for molecular sequence determination

The entire 2.2 kb insert and fragments prepared by appropriate restriction-enzyme digestions (*Eco*RI + *Sty*I, *Eco*RI + *Sma*I, *Eco*RI + *Bam*HI, *Eco*RI + *Apa*I, *Eco*RI + *Nco*I, *Eco*RI + *Xho*I or *Eco*RI + *Bgl*II) were subcloned into plasmid vector pUC13, and sequenced as decribed in the Materials and methods section. Arrows below the map indicate the sequencing strategy representing the extent and direction of each sequence determined. ● symbols indicate restriction sites used for sequencing. The nucleotide sequences on both strands were analysed, and the sequence of the down-stream of *Nco*I site (G + C-rich region) was confirmed by the additional sequence analysis (*Xho*I site subcloning). The protein coding region is indicated by a thick black line.

recombinant bacteriophages yielded six immunoreactive clones. All of these clones continued to be strongly positive through the second and third screenings, and were finally isolated from single plaques. The insert size in the six clones varied from 1.4 up to 2.2 kb. The cDNA inserts of all the positive clones were subcloned into the plasmid vector pUC13, and the relationship among these plasmid inserts was analysed by restriction-endonuclease mapping. The restriction map of the longest insert with 2.2 kb was shown in Fig. 3. Other inserts shorter than 2.2 kb from positive clones were confirmed to be included in this map (results not shown).

Sequencing of rat liver ALP cDNA

The nucleotide sequence of the cDNA insert with 2.2 kb was determined (Fig. 4). The sequence contains an open reading frame, beginning at nucleotide 3, that encodes a 524-amino-acid-residue polypeptide. The predicted molecular mass, 57.7 kDa, of this polypeptide is reasonably consistent with the 57 kDa polypeptide obtained by immunoprecipitation of cell-free translation products directed by poly(A)$^+$ RNA from the liver (Fig. 1, lane 2). The first 17 amino acid residues (-17 to -1 in Fig 4*a*) containing a hydrophobic amino acid cluster may represent a signal peptide. This is supported by the finding that the sequence is followed by 28 amino acid residues identical with the *N*-terminal sequence determined from the purified mature ALP. The chemically determined sequences of two CNBr-cleavage peptides are also identified in the predicted polypeptide sequence, as underlined in Fig. 4(*a*). The putative active site, Asp-Ser, is found at positions 92–93. There exist five possible glycosylation sites in the molecule: Asn^{123}-Thr-Thr, Asn^{123}-Arg-Thr, Asn^{254}-Arg-Thr, Asn^{286}-Leu-Thr and Asn^{413}-Val-Ser. Another characteristic point is that it has a stretch of hydrophobic residues at the *C*-terminus that could participate in membrane localization (Fig. 4*a*, underlined with a broken line). Hydropathy analysis confirmed the presence of a hydrophobic domain at the *C*-terminus as well as at the *N*-terminus (Fig. 4*b*).

Northern-blot hybridization

Fig. 5 shows the Northern-blot hybridization analysis with the ^{32}P-labelled cDNA insert with 2.2 kb as a probe. Since the present cDNA library was constructed from the size-fractionated poly(A)$^+$ RNA, we confirmed that there was no difference in mRNA size of each fractionated sample. A single component with the same size was found in fractions 12 and 15 (lanes 1 and 2 respectively) obtained by sucrose-density-gradient centrifugation (Fig. 2), and estimated to be 2.7 kb. This value is larger than that for the isolated ALP cDNA (2.2 kb), indicating that the latter is not a full-length cDNA for the liver ALP mRNA.

ALP mRNA concentrations of the control and treated rat livers were compared by the same technique. It was found that treatment of rat by bile-duct ligation and colchicine injection caused an about 20-fold increase of the mRNA in the liver at 12 h after the treatment (lane 4) compared with that in the control liver (lane 3), as judged by the radioactivities of the probe hybridized.

DISCUSSION

Our initial attempts with poly(A)$^+$ RNA from the control rat liver failed in isolating the liver ALP cDNA. This might be mainly due to an extreme paucity of ALP mRNA in the RNA preparation used for construction of the cDNA library. The successful cloning has been made in the present study by use of poly(A)$^+$ RNA that was enriched with ALP mRNA by size-fractionation of poly(A)$^+$ RNA from the liver treated for the enzyme induction. Out of 6×10^5 recombinant bacteriophages, six clones reacting with the antibodies were isolated,

Fig. 4. Nucleotide and deduced amino acid sequences of rat liver ALP cDNA

(*a*) Both nucleotides and predicted amino acids are numbered on the left. Amino acid residues that have been determined by protein sequence analysis of purified ALP are underlined. Amino acids -17 to -1 comprise a putative signal peptide. The phosphate-binding serine active site is boxed. Five potential *N*-linked glycosylation signals, Asn-Xaa-Thr/Ser, are indicated by double-underlines. A stretch of hydrophobic amino acids at the *C*-terminus is indicated by a broken line. (*b*) The diagram shows the hydropathicity profile of the entire amino acid sequence of rat liver ALP calculated by the method of Kyte & Doolittle (1982) by using a window of six amino acid residues. Line segments above and below the horizontal axis indicate hydrophobic and hydrophilic portions respectively.

(a)

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  1   CC ATG ATC TTG CCA TTT TTA GTA CTG GCC ATC GGC ACC TGC CTT ACC AAC TCA TTT GTG CCA GAG AAA GAG AAA GAC CCC AGT TAC TGG
-17      Met Ile Leu Pro Phe Leu Val Leu Ala Ile Gly Thr Cys Leu Thr Asn Ser Phe Val Pro Glu Lys Glu Lys Asp Pro Ser Tyr Trp

 90  CGA CAG CAA GCC CAA GAG ACC TTG AAA AAT GCC CTG AAA CTC CAA AAA CTC AAC ACC AAC GTG GCC AAG AAC ATC ATC ATG TTC CTG GGA
 13  Arg Gln Gln Ala Gln Glu Thr Leu Lys Asn Ala Leu Lys Leu Gln Lys Leu Asn Thr Asn Val Ala Lys Asn Ile Ile Met Phe Leu Gly

180  GAT GGT ATG GGC GTC TCC ACA GTG ACA GCT GCC CGC ATC CTT AAG GGC CAG CTA CAC CAC AAC ACG GGC GAG GAG ACC AGG CTG GAG ATG
 43  Asp Gly Met Gly Val Ser Thr Val Thr Ala Ala Arg Ile Leu Lys Gly Gln Leu His His Asn Thr Gly Glu Glu Thr Arg Leu Glu Met

270  GAC AAG TTC CCC TTT GTG GCT CTC TCC AAG ACG TAC AAC ACC AAC GCT CAG GTC CCC GAC AGC GCC GGC ACT GCC ACC TGC TAC CTG TGT
 73  Asp Lys Phe Pro Phe Val Ala Leu Ser Lys Thr Tyr Asn Thr Asn Ala Gln Val Pro Asp Ser Ala Gly Thr Ala Thr Cys Tyr Leu Cys

360  GGC GTG AAG GCC AAC GAG GGC ACC GTG GGA GTG AGC GCG GCC ACT GAG CGC ACG CGA TGC AAC ACC ACT CAG GGG AAT GAG GTC ACG TCC
103  Gly Val Lys Ala Asn Glu Gly Thr Val Gly Val Ser Ala Ala Thr Glu Arg Thr Arg Cys Asn Thr Thr Gln Gly Asn Glu Val Thr Ser

450  ATC CTG CGC TGG GCC AAG GAT GCT GGG AAG TCC GTG GGC ATC GTG ACC ACC ACT CGG GTG AAC CAC GCC ACT CCC AGT GCA GCC TAT GCG
133  Ile Leu Arg Trp Ala Lys Asp Ala Gly Lys Ser Val Gly Ile Val Thr Thr Thr Arg Val Asn His Ala Thr Pro Ser Ala Ala Tyr Ala

540  CAC TCG GCC GAT CGG GAC TGG TAC TCG GAC AAT GAG ATG CCG CCA GAG GCT CTG AGC CAG GGC TGC AAG GAC ATC GCC TAT CAG CTA ATG
163  His Ser Ala Asp Arg Asp Trp Tyr Ser Asp Asn Glu Met Pro Pro Glu Ala Leu Ser Gln Gly Cys Lys Asp Ile Ala Tyr Gln Leu Met

630  CAC AAC ATC AAG GAC ATC GAT GTG ATC ATG GGT GGC GGC CGG AAG TAC ATG TAC CCC AAG AAC AGA ACT GAT GAG GAA TAT GAA CTG GAT
193  His Asn Ile Lys Asp Ile Asp Val Ile Met Gly Gly Gly Arg Lys Tyr Met Tyr Pro Lys Asn Arg Thr Asp Val Glu Tyr Glu Leu Asp

720  GAG AAG GCC AGG GGC ACC AGA CTG GAT GGC CTC GAC CTC ATC ACC ATT TGG AAG AGC TTC AAA CCT AGA CAC AAG CAC TCC CAC TAT GTC
223  Glu Lys Ala Arg Gly Thr Arg Leu Asp Gly Leu Asp Leu Ile Thr Ile Trp Lys Ser Phe Lys Pro Arg His Lys His Ser His Tyr Val

810  TGG AAC CGA ACT GAA CTG CTG GCC CTT GAC CCC TCC AGG GTG GAC TAC CTC TTA GGT CTC TTT GAG CCC GGG GAC ATG CAG TAT GAG TTG
253  Trp Asn Arg Thr Glu Leu Leu Ala Leu Asp Pro Ser Arg Val Asp Tyr Leu Leu Gly Leu Phe Glu Pro Gly Asp Met Gln Tyr Glu Pro

900  AAT CGG AAC AAC CTG ACT GAC CCT TCC CTC TCG GAG ATG GTG GAG GTG GCC CTC AGG ATC CTG ACA AAG AAT CCC AAA GGC TTC TTC TTG
283  Asn Arg Asn Asn Leu Thr Asp Pro Ser Leu Ser Glu Met Val Glu Val Ala Leu Arg Ile Leu Thr Lys Ser Pro Lys Gly Phe Phe Leu

990  CTG GAG GAA GGA GGC AGG ATT GAC CAC GGG CAC CAT GAA GGC AAG GCC AAG CAG GCG CTG CAT GAG GCC GTG GAG ATG GAT GAG GCC ATC
313  Leu Glu Glu Gly Gly Arg Ile Asp His Gly His His Glu Gly Lys Ala Lys Gln Ala Leu His Glu Ala Val Glu Met Asp Glu Ala Ile

1080  GGA AAG GCG GGC ACC ATG ACT TCC CAG AAA GAC ACG TTG ACT CTG GTT ACT GCT GAT CAT CAT CCC ACG TTT TCA CGT TTG GTG GCT ACA
 343  Gly Glu Ala Gly Thr Met Thr Ser Gln Lys Asp Thr Leu Thr Leu Val Thr Ala Asp His His Pro Thr Phe Ser Arg Leu Val Ala Thr

1170  CCC CAG GGC AAC TCC ATT TTT GGT CTG GCA CCC ATG GTG AGT GAC ACG GAC AAG AAG CCC TTC ACA GCC ATC CTC TAT GGC AAC GGG CCT
 373  Pro Gln Gly Asn Ser Ile Phe Gly Leu Ala Pro Met Val Ser Asp Thr Asp Lys Lys Pro Phe Thr Ala Ile Leu Tyr Gly Asn Gly Pro

1260  GGT TAC AAG GTG GTG GAC GGT GAA CGG GAG AAC GTC TCC ATG GTG GAT TAT GCT CAC AAC AAC TAC CAG GCC CAG TCC GCT GTC CCC CTG
 403  Gly Tyr Lys Val Val Asp Gly Glu Arg Glu Asn Val Ser Met Val Asp Tyr Ala His Asn Asn Tyr Gln Ala Gln Ser Ala Val Pro Leu

1350  CGG CAC GAG ACC CAC GGT GGG GAA GAT GTG GCG GTC TTC TGC AAG GGC CCT ATG GCT CAC CTG CTT CAC GGC ATC CAT GAG CAG AAC TAC
 433  Arg His Glu Thr His Gly Gly Glu Asp Val Ala Val Phe Cys Lys Gly Pro Met Ala His Leu Leu His Gly Ile His Glu Gln Asn Tyr

1440  ATC CCC CAC GTC ATG GCG TAT GCC TCC TGC ATT GGA GCC AAC CTT GAC CAC TGT GCC TGG GCC AGC TCT GCG AGC AGC CCC TCC CCA GGG
 463  Ile Pro His Val Met Ala Tyr Ala Ser Cys Ile Gly Ala Asn Leu Asp His Cys Ala Trp Ala Ser Ser Ala Ser Ser Pro Ser Pro Gly

1530  GCC CTG CTG CTT CCA CTG GCT CTG TTC CCC CTA CGC ACC CTG TTC TGA GGGCCCAGGTCCCACAAGAGCCCACAATGGACAGCCGGCTCCCCTCCCTTTGTGG
 493  Ala Leu Leu Leu Pro Leu Ala Leu Phe Pro Leu Arg Thr Leu Phe ***

1633  CCTGCCACCTGGCCGCCCACACTCAACGGGGAGGCCCAGGCAACCTCGAGCAGGAACACAAGTTTGCTACCTGCCTCACTTCCGCCCGGAACCCTCCGTGGGTCGGATTCCTGCTGCCG

1752  TTGTTTCTCTATTCACTGCCTTTTGGCCAGCAGGTGGGTTTCTCTTGGGCCGGCAGGACACAGACTGCGCAGATTCCCAAAGCACCTTATTTTTCTACCAAATATACTCTCCAGACCCT

1871  GCAACCATCATGGAACATTCCAGATCTGACCTTCTCTCCCCTACCCCTTCTCTCTGGAACACTGGGTCCCATAGTCACAGCCAGTCCCTCAACCCAACCCTCCTGGAGAAGACCAGGTC

1990  TGCTCAGGGTGAGACTTCCCAGGAAGCCACCTCCGGGGTTGGTTGTCTACCCAGGGTTGGCCAGGCTGGGAAGAACAACCCAGCGGACAGGACGCACACACACTCCCCACCCAGCTCCA

2109  GAGACTCGCCAACCCTTCACTGAAGCGACTCCCCTGTTTGGAATAGCAAAAAAAAAA
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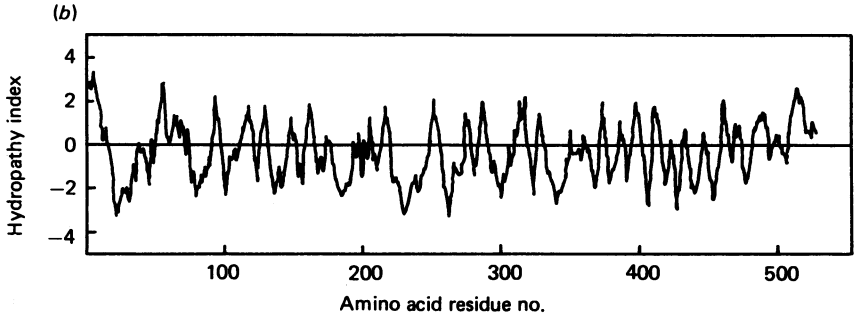
(b)



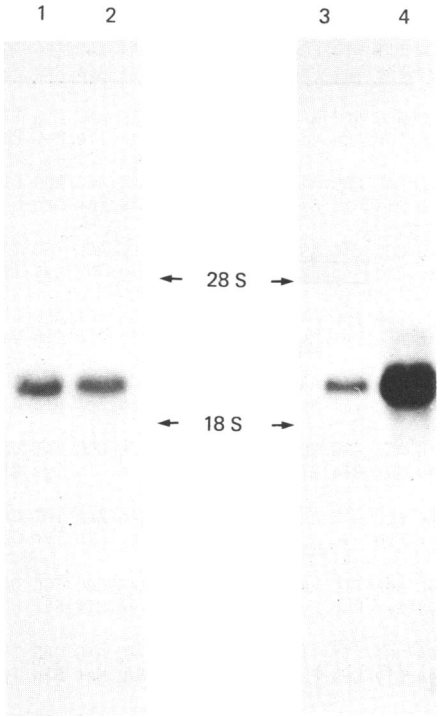


Fig. 5. RNA blot hybridization analysis

Each sample (5 μg of RNA) of poly(A)$^+$ RNA was denatured with 1.0 M-glyoxal and separated by electrophoresis on a 1.1% agarose gel. The separated RNAs were transferred on to a nylon membrane and hybridized with the ^{32}P-labelled 2.2 kb cDNA insert. RNA size markers are 28 S (5.5 kb) and 18 S (2.1 kb) rRNA of rat liver. Lanes 1 and 2, fractions 12 and 15 respectively obtained by sucrose-density-gradient centrifugation of poly(A)$^+$ RNA (Fig. 2); lane 3, poly(A)$^+$ RNA from the control rat liver; lane 4, poly(A)$^+$ RNA from the liver treated for the ALP induction. Exposure was for 12 h (lanes 1 and 2) and for 4 days (lanes 3 and 4).

suggesting that liver ALP mRNA is at most 0.006% of the mRNA selected from rat liver under the present conditions. This value is still extremely low compared with those values obtained for placental ALP mRNA, 0.27% (Kam *et al.*, 1985) and 0.06% (Millán, 1986) of the mRNA from human placenta. The availability of the ALP cDNA probe allowed an approximate estimation of the ALP mRNA concentration in the control rat liver (one-twentieth of that of the treated liver, Fig. 5), and will further enable us to study the regulatory mechanism of ALP in the liver and other tissues under various conditions.

The predicted amino acid sequence of rat liver ALP was found to have 524 amino acid residues, of which the first 17 amino acid residues comprise a signal peptide. The same numbers of residues in the precursor (524) and signal peptide (17) are found in the human liver-type ALP from osteosarcoma cells (Weiss *et al.*, 1986), which shows 88% homology in amino acid sequence to that of the rat liver ALP (Fig. 6). Although two regions that are characterized by less similarity from each other, with about 50% homology, are found in positions 347–374 and 488–507, such variations do not cause a significant change in the similarity of their hydropathic profiles, including these two regions (results not shown). The active site (Asp-Ser^{93}-Ala) and five potential *N*-linked glycosylation sites of rat liver ALP are identified at the same individual positions in the human liver-type enzyme. Such a similarity between the two enzymes is consistent with a preliminary finding that the anti-(rat liver ALP) IgG partially cross-reacts with the human liver ALP (Y. Misumi, K. Tashiro, M. Hattori, Y. Sakaki & Y. Ikehara, unpublished work). It is likely that all or most of the five potential glycosylation sites actually have attached oligosaccharide chains, since the purified rat liver ALP contains a high content of the carbohydrate moiety, amounting to about 18% by weight, although it includes *O*-linked sugar chains in part (Kawahara *et al.*, 1982).

Since no data are available for other rat ALP isoenzymes, we compared the present data with those of human placental and intestinal enzymes. The signal peptide for human intestinal ALP has 19 amino acid residues (Berger *et al.*, 1987), whereas different values have been given for that of placental ALP, 17 (Kam *et al.*, 1985) and 22 amino acid residues (Millán, 1986; Henthorn *et al.*, 1986), though the reason for this discrepancy is not clear. The active site 'Asp-Ser-Ala' is well conserved at similar positions in human intestinal (Ser^{92}), placental (Ser^{92}) and liver-type (Ser^{93}) ALPs as well as in rat liver ALP (Ser^{93}), except that its third residue Ala is replaced by Gly in human placental ALP, probably considered to be one of data supporting the view that human placental ALP is a new evolutionary gene product (Goldstein *et al.*, 1980). There is almost 90% homology in the amino acid sequences of human intestinal and placental enzymes (Berger *et al.*, 1987), the latter of which shows only 52% homology to the human liver-type ALP (Weiss *et al.*, 1986). In addition, the former two isoenzymes contain two potential glycosylation sites at the same positions, Asn^{122} and Asn^{249}, of which only the Asn^{249} is actually glycosylated in the placental enzyme (Millán, 1986), in contrast with a larger number of glycosylation sites in the liver ALP from both species. The absence of immunological cross-reactivity of anti-(rat liver ALP) IgG with both the human intestinal and placental isoenzymes further confirms the structural difference between the intestinal/placental and liver ALP isoenzymes.

Hydropathy analysis demonstrated the presence of a hydrophobic domain at the *C*-terminus (Fig. 5). Such a hydrophobic domain at the *C*-terminus (17–23 amino acid residues) is also commonly observed in primary structures of human ALP isoenzymes predicted by their cDNAs (Kam *et al.*, 1985; Millán, 1986; Henthorn *et al.*, 1986; Weiss *et al.*, 1986; Berger *et al.*, 1987). This domain could be involved in attachment of the ALP molecule to membranes at least during or just after its synthesis. Recent observations, however, suggest another possibility for the membrane-anchoring mechanism of the mature ALP at the cell surface: the hydrophobic domain is cleaved from the *C*-terminus immediately after its synthesis and replaced by a glycophospholipid containing phosphatidylinositol, which in turn functions as a membrane-anchoring component of ALP (Low *et al.*, 1986; Cross, 1987). In fact, rat liver ALP is easily and completely released from the plasma membrane by phosphatidylinositol-specific phospholipase C, resulting in a completely soluble form (Ikezawa *et al.*, 1976; Low & Zilversmit, 1980; Kominami *et al.*, 1985). We also

```
          -17              1        10        20        30        40        50        60        70        80
Rat         MILPFLVLAIGTCLTNSFVPEKEKDPSYWRQQAQETLKNALKLQKLNTNVAKNIIMFLGDGMGVSTVTAARILKGQLHHNTGEETRLEMDKFPFVAL
Human       --S--------------L--------K---D-------Y--E-----------V------------------------P----------------

                 90       100       110       120       130       140       150       160       170       180
Rat       SKTYNTNAQVPDSAGTATCYLCGVKANEGTVGVSAATERTRCNTTQGNEVTSILRWAKDAGKSVGIVTTTRVNHATPSAAYAHSADRDWYSDNEMPPEAL
Human     -----------------A-------------------S-----------------------------------------------------------

                190       200       210       220       230       240       250       260       270       280
Rat       SQGCKDIAYQLNHNIKDIDVIMGGGRKYMYPKNRTDEEYELDEKARGTRLDGLDLITIWKSFKPRHKHSHYVWNRTELLALDPSRVDYLLGLFEPGTNQY
Human     --------------R-----------------K--VG--S--------------VDT------------FI-------T---HN-----------D---

                290       300       310       320       330       340       350       360       370       380
Rat       ELNRNNLTDPSLSEMVEVALRILTKNPKGFFLLEEGGRIDHGHHEGKAKQALHEAVEMDEAIGKAGTMTSQKDTLTLVTADHHPTFSRLVATPQGNSLFG
Human     ------V---------V--IQ--R---------V-----------------------R------SL--SE----V-----SHV-TFGGY--R------

                390       400       410       420       430       440       450       460       470       480
Rat       LAPMVSDTDKKPFTAILYGMGPGYKVVDGERENVSMVDYAHNNYQAQSALPLRHETHGGEDVAVFCKGPMAHLLHGIHEQNYIPHVMAYASCIGANLDHC
Human     ----L----------------------G--------------------V--------------S----------V-----V-------A------G--

                490       500
Rat       AWASSASSPSPGALLLPLALFPLRTLF
Human     -P------LAA-----VA--Y--SV--
```

Fig. 6. Comparison of amino acid sequences of rat liver ALP and human liver-type ALP

The amino acid sequence of rat liver ALP is compared with that of human liver-type ALP from osteosarcoma cells (Weiss *et al.*, 1986). Amino acids are shown in the single-letter code. Amino acid residues of human liver-type ALP that are identical with those of rat liver ALP are indicated by –.

confirmed that there is no significant difference in amino acid composition between the purified membrane and soluble forms of rat liver ALP (Miki *et al.*, 1986), which are characterized by the presence (membrane form) and absence (soluble form) of phosphatidylinositol (S. Ogata, Y. Hayashi, K. Yasutake & Y. Ikehara, unpublished work). Although accumulating findings thus favour the proposed mechanism, the final conclusion must be made by identifying a truncated *C*-terminus that has covalently attached phospholipid in place of the predicted hydrophobic peptide domain.

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REFERENCES

Aviv, H. & Leder, P. (1972) Proc. Natl. Acad. Sci. U.S.A. **69**, 1408–1412

Berger, J., Garattini, E., Hua, J.-C. & Udenfriend, S. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 695–698

Chirgwin, J. M., Prybyla, E., MacDonald, R. J. & Rutter, W. J. (1979) Biochemistry **18**, 5294–5299

Cross, G. A. M. (1987) Cell **48**, 179–181

Feinberg, A. P. & Vogelstein, B. (1983) Anal. Biochem. **132**, 6–13

Fishman, W. H. (1974) Am. J. Med. **56**, 617–650

Goldstein, D. J., Rogers, C. E. & Harris, H. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 2857–2860

Gubler, U. & Hoffman, B. J. (1983) Gene **25**, 263–269

Hattori, M. & Sakaki, Y. (1986) Anal. Biochem. **152**, 232–238

Henthorn, P. S., Knoll, B. J., Raducha, M., Rothblum, K. N., Slaughter, C., Weiss, M., Lafferty, M. A., Fischer, T. & Harris, H. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5597–5601

Huynh, T. V., Young, R. A. & Davis, R. W. (1985) in DNA Cloning: A Practical Approach 1 (Glover, D. M., ed.), pp. 49–78, IRL Press, Oxford and Washington

Ikehara, Y., Mansho, K. & Kato, K. (1978) J. Biochem. (Tokyo) **84**, 1335–1338

Ikezawa, H., Yamanegi, M., Taguchi, R., Miyashita, T. & Ohyabu, Y. (1976) Biochim. Biophys. Acta **450**, 154–164

Kam, W., Clauser, E., Kim, Y. S., Kan, Y. W. & Rutter, W. J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 8715–8719

Kaplan, M. M. & Righetti, A. (1970) J. Clin. Invest. **49**, 508–516

Kawahara, S., Ogata, S. & Ikehara, Y. (1982) J. Biochem. (Tokyo) **91**, 201–210

Kominami, T., Miki, A. & Ikehara, Y. (1985) Biochem. J. **227**, 183–189

Kuhara, S., Matuo, F., Futamura, S., Fujita, A., Shinohara, T., Takagi, T. & Sakaki, Y. (1984) Nucleic Acids Res. **12**, 89–99

Kyte, J. & Doolittle, R. F. (1982) J. Mol. Biol. **157**, 105–132

Low, M. G. & Zilversmit, D. B. (1980) Biochemistry **19**, 3913–3918

Low, M. G., Ferguson, M. A. J., Futerman, A. H. & Gilman, I. (1986) Trends Biochem. Sci. **11**, 212–215

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 368–369, Cold Spring Harbor Laboratory, Cold Spring Harbor

McComb, R. B., Bowers, G. N., Jr. & Posen, S. (1979) Alkaline Phosphatase, pp. 865–902, Plenum Press, New York and London

Miki, A., Tanaka, Y., Ogata, S. & Ikehara, Y. (1986) Eur. J. Biochem. **160**, 41–48

Millán, J. L. (1986) J. Biol. Chem. **261**, 3112–3115

Misumi, Y., Tanaka, Y. & Ikehara, Y. (1983) Biochem. Biophys. Res. Commun. **114**, 729–736

Oda, K. & Ikehara, Y. (1981) Biochim. Biophys. Acta **640**, 398–408

Palmiter, R. D. (1974) Biochemistry **13**, 3606–3614

Pekarthy, J. M., Short, J., Lansing, A. I. & Lieberman, I. (1972) J. Biol. Chem. **247**, 1767–1774

Pelman, R. B. & Jackson, R. J. (1976) Eur. J. Biochem. **67**, 247–256
Sanger, F., Nickleu, S. & Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
Thomas, P. S. (1983) Methods Enzymol. **100**, 255–259
Vieira, J. & Messing, J. (1982) Gene **19**, 259–268
Weiss, M. J., Henthorn, P. S., Lafferty, M. A., Slaughter, C., Raducha, M. & Harris, H. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7182–7186
Young, R. A. & Davis, R. W. (1983) Science **222**, 778–782

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