

# Cloning and expression of the bovine intestinal alkaline phosphatase gene: biochemical characterization of the recombinant enzyme

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A complete genomic clone and a full-length cDNA coding for bovine intestinal alkaline phosphatase have been isolated and sequenced. The gene (5.4 kb) contains 11 exons separated by ten small introns at positions identical to those of other members of the eukaryotic tissue-specific alkaline phosphatase family. In addition, 1.5 kb of upstream sequences contain putative regulatory elements showing sequence similarity to human and mouse intestinal alkaline phosphatase promoter sequences. To achieve recombinant bovine intestinal alkaline phosphatase expression, the coding region of the gene was subcloned into the pcDNA I eukaryotic expression vector and transfected into Chinese ham-

ster ovary cells. Recombinant bovine intestinal alkaline phosphatase displays enzymatic properties comparable with those of purified native bovine intestinal alkaline phosphatase, a slightly increased thermal stability and, upon desialylation, it shows a homogeneous behaviour in agarose gel electrophoresis and isoelectric focusing. The availability of the recombinant bovine intestinal alkaline phosphatase and the elucidation of its primary sequence will help to accelerate our efforts to obtain the first crystallographic model of a eukaryotic alkaline phosphatase molecule.

## INTRODUCTION

Recent progress in understanding the complexity of the human and mouse alkaline phosphatase (AP; EC 3.1.3.1.) gene family and the tissue-specific expression of the individual isoenzyme genes has excited a renewed interest in understanding the biological function of these molecules. APs are membrane-bound homodimeric glycoenzymes, characteristically dependent on the metal ions zinc and magnesium, that exist in all species from bacteria to man (McComb et al., 1979). Sequence comparisons (Millán, 1988; Kim and Wyckoff, 1989) between the mammalian APs and the *Escherichia coli* AP, for which the three-dimensional structure is known (Sowadski et al., 1985), indicate that they conform to the basic  $\alpha/\beta$  architecture of the *E. coli* enzyme with a highly-conserved central  $\beta$ -sheet. However, the eukaryotic enzymes possess highly variable loops and major insertions with no equivalents in the *E. coli* structure (Millán, 1992). These variable regions are in some instances associated with unique properties of the mammalian APs, such as uncompetitive inhibition (Hummer and Millán, 1991; Hoylaerts and Millán, 1991; Hoylaerts et al., 1992) and binding to extracellular proteins (Tsonic et al., 1988; Makiya and Stigbrand, 1992). Because these regions may be responsible for isoenzyme-specific functions, structural-functional relationships based on the *E. coli* AP structure are not informative in understanding the biological role of mammalian APs, and, consequently, elucidation of the three-dimensional crystallographic structure of at least one mammalian AP is required.

We have chosen the bovine intestinal AP (bIAP) as the model enzyme for the following reasons: (a) purified bIAP is available from a number of manufacturers in large quantities as a reagent with applications in biotechnology, (b) bIAP crystallizes readily and this property can be used as one of the steps in the large-scale

purification of the enzyme, (c) bIAP displays the highest specific activity of any AP known and its structure should provide a reference with which the conformation of specific mutants and catalytically less efficient isoenzymes could be compared. Whilst bIAP was the first mammalian AP for which the sequence surrounding the active site serine was determined (Milstein, 1964), only very limited protein sequence information (Culp et al., 1985; Besman and Coleman, 1985) is available for bIAP. In this paper we present the cloning of a full-length cDNA and genomic clones for bIAP and we report the entire primary sequence of the enzyme. These data will greatly facilitate our efforts to unravel the three-dimensional structure of bIAP.

## MATERIALS AND METHODS

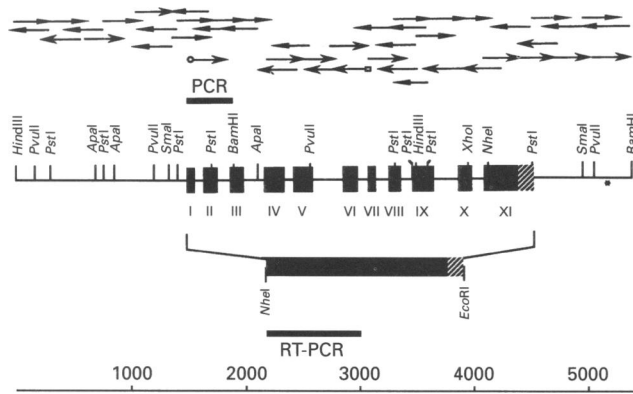
### Library screening, sequencing and genomic analysis

A  $\lambda$ gt11 cDNA library prepared from adult cow intestine (Clontech Laboratories, Palo Alto, CA, U.S.A.) was screened using a 2.2 kb *Hind*III fragment containing the 5' end of the mouse IAP gene (Manes et al., 1990) and a 700 bp *Eco*RI cDNA fragment coding for the C-terminal half of rabbit IAP (unpublished results). The 2.1 kb R201 cDNA clone and the 1.1 kb BB204 cDNA clone isolated from the cow intestinal cDNA library were used to screen an EMBL3 SP6/T7 genomic library prepared from adult cow liver (Clontech Laboratories). Probes were radiolabelled and positive clones were plaque-purified and expanded as described (Manes et al., 1990). Large-scale phage DNA preparation was performed as described (Sambrook et al., 1989). Genomic clones were characterized by Southern blot analysis and sequenced following procedures already described (Manes et al., 1990). Nucleic acid and protein sequences were assembled and analysed using the MacVector sequence analysis program (International Biotechnologies, Inc. New Haven, CT,

Abbreviations used: bIAP, bovine intestinal alkaline phosphatase; AP, alkaline phosphatase; TSAP, tissue-specific alkaline phosphatase; RT-PCR, reverse transcriptase-polymerase chain reaction; CHO, Chinese hamster ovary; EAP, embryonic alkaline phosphatase; PLAP, placental alkaline phosphatase.

The nucleotide sequence data in this paper have been submitted to the GenBank Nucleotide Sequence database under the accession number L07733.

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**Figure 1** Sequencing strategy, genomic organization and restriction map of the bIAP gene and the constructed cDNA

The sequencing strategy is indicated above the genomic structure. The arrows represent the universal SK, KS, T7 and T3 Bluescript primers (←) and those primers synthesized at our facility (→). The primers beginning with an open circle and square were used as 5' and 3' PCR primers for the RT-PCR completion of clone BB204. The crosshatched region of exon XI represents that part of the 3' untranslated region which was identified by comparison with the isolated cDNA fragment BB204. An asterisk marks the approximate position of the putative polyadenylation signal.

U.S.A.). Genomic DNA was isolated from adult cow liver and Southern blot analysis was performed using standard protocols (Sambrook et al., 1989). Restriction enzymes were obtained from Gibco BRL, Boehringer Mannheim and New England Biolabs.

### Expression of recombinant bIAP

The bIAP gene was subcloned into the expression vector pcDNA I as follows: a 23-mer primer with sequence 5'-GCTAGCCATGCAGGGGCCTGCG-3' (arrow with open circle, Figure 1), designed to introduce a new *NheI* site, was used to amplify basepairs 1497–1913 of the 5'-end of the bIAP gene which had been subcloned as a 1.9 kb *HindIII/BamHI* fragment into Bluescript KS<sup>+</sup> (Stratagene, San Diego, CA, U.S.A.). The universal SK primer was used as a complementary reverse primer in the PCR amplification of this region. The amplified fragment was directly subcloned into the T-modified *EcoRV* site of Bluescript (Marchuk et al., 1990) in the orientation for  $\beta$ -galactosidase transcription. This fragment was used together with a 3.2 kb *BamHI/SmaI* fragment of the bIAP gene for directional subcloning into a *HindIII/EcoRV*-cut pcDNA I vector (Invitrogen, San Diego, CA, U.S.A.). The bIAP-pcDNA I construct was transfected into Chinese hamster ovary (CHO) cells by means of calcium phosphate precipitation (Gorman et al., 1982). A pSV2-dhfr plasmid (Schimke, 1982) was cotransfected to allow selection of transfectants in nucleoside-free medium and possible amplification of the transfected gene upon methotrexate treatment. Stable transfectants were selected as described by Hummer and Millán (1991). In order to construct a full-length cDNA, reverse transcriptase-PCR (RT-PCR) was performed as follows: total RNA from a bIAP-pcDNA I stably-transfected CHO/cell clone was isolated by acid guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski and Sacchi, 1987). The RT-PCR reaction was performed according to manufacturer's instructions (Promega, WI, U.S.A.) using the 5' primer described above and the sequence 5'-TCGGCCGCTGAAGGAGC-3' as complementary reverse primer for PCR.

### Characterization of the recombinant enzyme

Recombinant bIAP was extracted from the CHO-bIAP cells essentially as previously described for site-directed placental AP (PLAP) mutants (Hoylaerts et al., 1992).  $K_m$  and  $K_i$  values for L-Phe inhibition were derived from Lineweaver-Burk plots upon incubating recombinant or native bIAP in the presence of increasing concentrations of the substrate *p*-nitrophenyl phosphate (final concentration 0.1–10 mM) and inhibitor (0–20 mM) in 1 M diethanolamine buffer (pH 9.8) containing 20  $\mu$ M ZnCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>, essentially as described by Hoylaerts et al. (1992). To determine  $k_{cat}$  ( $V_{max}/[E^0]$ ) values, the enzyme concentrations were measured with the BCA protein assay reagent (Pierce Laboratory) in the case of the purified Calzyme Laboratories and Boehringer-Mannheim preparations. For recombinant bIAP, the enzyme concentration was measured by enzyme-antigen immunoassay upon progressive saturation of 100 000-fold-diluted polyclonal mouse anti-bIAP IgG, using the Calzyme Laboratories preparations as a standard.

Prior to heat-inactivation kinetics, bIAP samples were diluted in 10 mM phosphate buffered saline (pH 7.5) containing 10% saturated casein, 20  $\mu$ M ZnCl<sub>2</sub> and 1 mM MgCl<sub>2</sub> and incubated in a water bath at 65 °C. After fixed time intervals 50  $\mu$ l samples were removed and pipetted into each well of a microtitre plate kept on ice. Residual activities were then measured in duplicates upon simultaneous addition to the wells of 200  $\mu$ l of substrate solution. Isoelectric focusing was performed using the Resolve-ALP system (Isolab, Akron, OH, U.S.A.) according to Griffiths and Black (1987) and agarose gel electrophoresis on Paragon gels (Van Hoof et al., 1988). Samples of recombinant and purified enzyme were treated with neuraminidase (Sigma, St. Louis, MO, U.S.A.), phosphatidylinositol-specific phospholipase C (Sigma) and serum phospholipase D either singly or in combination, before electrophoretic separation.

## RESULTS AND DISCUSSION

### Characterization of the bIAP gene

Initially, one positive clone was obtained upon duplicate screening of the  $\lambda$ gt11 cDNA library with the mouse IAP gene fragment and a partial rabbit IAP cDNA clone. The sequence of this 2.1 kb fragment (R201) revealed an incomplete cDNA encoding exons VI to XI of an IAP-like gene as identified by sequence comparison to all known AP genes (data not shown). This cDNA fragment also contained all corresponding unspliced introns as well as several stop codons and two frameshift mutations in the coding exons, suggesting that it might represent a transcribed pseudogene. R201 was used as a probe for further screening of the  $\lambda$ gt11 library. Two additional cDNA clones were subsequently isolated and identified as transcripts of a different AP gene; a 0.8 kb fragment (BB203) was reverse-transcribed from an incompletely processed RNA (data not shown), whereas a 1.1 kb fragment (BB204) was derived from a fully processed mRNA and extended from exon V to exon XI. Since further screening of the intestinal cDNA library did not yield full-length clones, both R201 and BB204 were used to screen an EMBL3 SP6/T7 bovine genomic DNA library. Two positive clones were obtained and analysed by Southern blotting. One clone contained sequences identical (except for a few bp changes) to R201. The other proved to contain the entire coding region for the bIAP gene, as identified by comparison of the deduced amino acid sequence with partial peptide sequences available for bIAP (Culp et al., 1985; Besman and Coleman, 1985) and similarity with the human, rat and mouse IAP sequences (Berger et al., 1987; Henthorn et al., 1988; Manes et al., 1990; Lowe et al., 1990). The

AAGCTTTACCTTCTCTGAAACAGAGAGACAGTCTCAGCCCCAGTCTCACCCTTCTACCTCCCTGCCTGATGCCAGGCAATCATCTGGTGGCGTGACCTCCCTCTGTCCCATG 120  
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 AGGAAATGCTACCACCTCCAGTATTCTTGGCTGGATAATCCCATGGACAGAGAGTGTGGCAGGCTGCAGACCAATAGGTAGAAAGAGTACAGCATGACTGAGCACAATAGCACAATAT 360  
 TCCACTGGATATATCATCTTTGTTCATCCATTTGTCTGTGGATGGTGTGAGTGGCTGTGCTTGGCTTCTGGCTTCTGGATATGCTACTAAATGTGAGTGTGCNAATACCTTTATA 480  
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 GCCTGGCCCTGAGACCCGGCTCCCAAGTCCATCTGACCTCTGCCATCACACAGCC ATG CAG GGC TGC GTG CTG CTG CTG CAT CTA CAG 1545  
 Met Gln Gly Ala Cys Val Leu Leu Leu Leu Gly Leu His Leu Gln

CTC TCC CTA GGC CTC GTC CCA G GTAATCAGGGGGCTCCAGCAGCCCTACTCACAGGGGGCGCTCTAGGCTGACCTGACCAACACTCTCCCTTGGGCG TT GAG GAG 1654  
 Leu Ser Leu Gly Leu Val Pro V al Glu Glu

GAA GAC CCC GGC TTC TGG AAC CGC CAG GCA GCC CAG GGC CTC GAT GTG GCT AAG AAG CTG CAG CCC ATC CAG ACA GCC GCC AAG AAT GTC 1744  
 Glu Asp Pro Ala Phe Trp Asn Arg Gln Ala Ala Gln Ala Leu Asp Val Ala Lys Lys Leu Gln Pro Ile Gln Thr Ala Ala Lys Asn Val

ATC CTC TTC TTG GGG GAT G GTAGTACATGAGCCAGCCACCCCTGTCCCTGACAGCCCTGGAACCTGTGATGCCGGTGCACCGAGTGGCCCGAAGAACTCGGACCT 1857  
 Ile Leu Phe Leu Gly Asp G

GAGACACTGTACTCTCAG GG ATG GGG GTG CCT ACG GTG ACA GCC ACT CGG ATC CTA AAG GGG CAG ATG AAT GGC AAA CTG GGA CCT GAG ACA 1951  
 ly Met Gly Val Pro Thr Val Thr Ala Thr Arg Ile Leu Lys Gly Gln Met Asn Gly Lys Leu Gly Pro Glu Thr

CCC CTG GCC ATG GAC CAG TTC CCA TAC GTG GCT CTG TCC AAG GTAAGGCCAAGTGGCTCAGGGTGTCTACACCAGAGGGGTGGTGTGGCCCTAGGGAGCAGGG 2057  
 Pro Leu Ala Met Asp Gln Phe Pro Tyr Val Ala Leu Ser Lys

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G ACA TAC AAC GTG GAC AGA CAG GTG CCA GAC AGC GCA GGC ACT GCC ACT GCC TAC CTG TGT GGG GTC AAG GGC AAC TAC AGA ACC ATT 2265  
 Thr Tyr Asn Val Leu Pro Asp Gln Val Thr Ala Thr Ala Tyr Leu Cys Gly Val Lys Thr Lys Gly Asn Tyr Arg Thr Ile

GGT GTA AGT GCA GCC GGC CGC TAC AAC CAG TGC AAA ACG ACA CGT GGG AAT GAG GTC ACG TCT GTG ATG AAC CGG GCC AAG AAA GCA G GT 2355  
 Gly Val Ser Ala Ala Ala Arg Tyr Asn Gln Cys Lys Thr Thr Arg Gly Asn Glu Val Thr Ser Val Met Asn Arg Ala Lys Lys Ala G

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 ly Lys Ser Val Gly Val Val Thr Thr Arg

GTG CAG CAT GCC TCC CCA GCC GGG GGC TAC GCG CAC ACG GTG AAC CGA AAC TGG TAC TCA GAC GCC GAC CTG CCT GCT GAT GCA CAG ATG 2554  
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AAT GGC TGC CAG GAC ATC GCC GCA CAG CTG GTC AAC AAC ATG GAT ATT GAC GTCCGACATGTTGGGCACAGGGCGGGCTGGCCACAGGTGGTGGGCACACT 2657  
 Asn Gly Cys Gln Asp Ile Ala Ala Gln Leu Val Asn Asn Met Asp Ile Asp

CGCAACACAGTGTAGGTAACCTCCAGCCTCGGCTGTTTCAGGGTTTTCATGGTFTTGTGTGTGTGTATGTGTGGTGGGCTGGCCACTGTAGGAGTGGGGACAGCCCTTCCCA 2777  
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 Val Ile Leu Gly Gly Arg Lys

TAC ATG TTT CCT GTG GGG ACC CCA GAC CCT GAA TAC CCA GAT GAT GCC AGT GTG AAT GGA GTC CGG AAG CGA AAG CAG AAC CTG GTG CAG 2977  
 Tyr Met Phe Pro Val Gly Thr Pro Asp Pro Glu Tyr Pro Asp Asp Ala Ser Val Asn Gly Val Arg Lys Arg Lys Gln Asn Leu Val Gln

GCA TGG CAG GCC AAG CAC CAG GTAATGGGGGCTCAGGATGTGGGGTACAGTGGGGCTGGCCCTGGGGTGTGGCTATGGCTAGGCGCTGTTCTCCCTCCAG GGA 3086  
 Ala Trp Gln Ala Lys His Gln Gly

GCC CAG TAT GTG TGG AAC CGC ACT GCG CTC CTT CAG GCG GCC GAT GAC TCC AGT GTA ACA CAC CTC ATG G GTAACGACTCCACCCACCTCACTGT 3182  
 Ala Gln Tyr Val Trp Asn Arg Thr Ala Leu Leu Gln Ala Ala Asp Asp Ser Ser Val Thr His Leu Met G

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 ly Leu Phe Glu Pro Ala Asp Met Lys Tyr Asn

GTT CAG CAA GAC CAC ACC AAG GAC CCG ACC CTG CAG GAA ATG ACA GAG GTG GCC CTG CGA GTC GTA AGC AGG AAC CCC AGG GGC TTC TAC 3378  
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 Leu Phe Val Glu G ly Gly Arg Ile Asp

CAC GGT CAC CAT GAT GAC AAA GCT TAT ATG GCA CTG ACC GAG GCG GGT ATG TTT GAC AAT GCC ATC GCC AAG GCT AAT GAG CTC ACT AGC 3578  
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GAA CTG GAC ACG CTG ATC CTT GTC ACT GCA GAC CAC TCT CAT GTC TTC TCT TTT GGT GGC TAT ACA CTG CGT GGG ACC TCC ATT TTT G GT 3668  
 Glu Leu Asp Thr Leu Ile Leu Val Thr Ala Asp His Ser His Val Phe Ser Phe Gly Gly Tyr Thr Leu Arg Gly Thr Ser Ile Phe G

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TTGCTACTGACAGCAGACTGGCCATGGCTCTGCACACAGGGAGCACAAGCTAGGTGATGATCAGGGGTCCCTCTCCCTGAAG GT CTG GCC CCC AGC AAG GCC 3898  
 ly Leu Ala Pro Ser Lys Ala

TTA GAC AGC AAG TCC TAC ACC TCC ATC CTC TAT GGC AAT GGC CCA GGC TAT GCG CTT GGC GGG GGC TCG AGG CCC GAT GTT AAT GAC AGC 3988  
 Leu Asp Ser Lys Ser Tyr Thr Ser Ile Leu Tyr Gly Asn Gly Pro Gly Tyr Ala Leu Gly Gly Gly Ser Arg Pro Asp Val Asn Asp Ser

ACA AGC G GTAAGTGTAGTGGGGGCTGGGAGGTGGGACCCCTGGCCAGAAATGTGGGAGGGGAGGCTGCCTCCCTTGTACATTAACCTCCCTTCTTCTGGCCAG AG 4102  
 Thr Ser G lu

GAC CCC TCG TAC CAG CAG GCG GCC GTG CCC GAG GCT AGC GAG ACC CAC GGG GGC GAG GAC GTG GCG GTG TTC GCG GCG GCC CCG CAG 4192  
 Asp Pro Ser Tyr Gln Gln Gln Ala Val Pro Gln Ala Ser Glu Thr His Gly Gly Glu Asp Val Ala Val Phe Ala Arg Gly Pro Gln

GCG CAC CTG GTG CAC GGC GTC GAG GAG GAG ACC TTC GTG GCG CAC ATC ATG GCC TTT GCG GGC TGC GTG GAG CCC TAC ACC GAC TGC AAT 4282  
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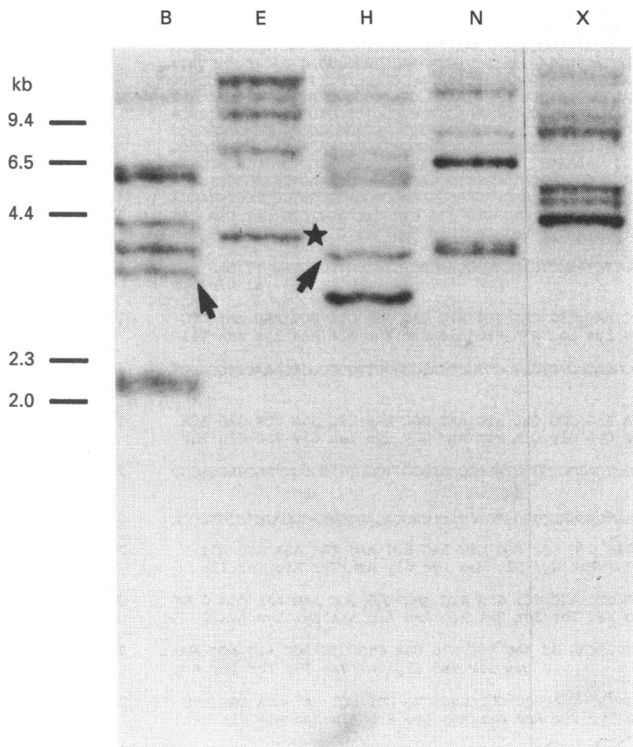
CTG CCA GCC ACC ACC GGC ACC GGC ATC CCC GAC GCG CAG CTG GCG GCC AHC CCG CCT CCA CTG GCG CTG CTG GCT GCG GCG ATG 4372  
 Leu Pro Ala Pro Thr Thr Ala Thr Ser Ile Pro Asp Ala Ala His Leu Ala Ala Ser Pro Pro Pro Leu Ala Leu Leu Ala Gly Ala Met

CTG CTG CTG CTG GCG CCC ACC TTG TAC TAA CCCCCACAGTTCAGGCTCTGGGATTTCCCGCTCTCTGCCAAAACCTCCAGCTCAGGCCCTACGGAGCTACCA 4482  
 Leu Leu Leu Ala Pro Thr Leu Tyr \*\*\*

TCAGATCCCCACCCGAGTGTCTTCTAGCTGOCATCTCTCAGACCCGACCCGGGCCACCACAGAGTTCACCTCCAGCAGTATTCACATTCAGCATTGAAGGAGCCTCAGC 4602  
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 GCAAGCCAAAGCAACTCAGAGAGACAGTGTGTCAGACTGGTGTGTCTCAGCAGGGGACTGTGGGGATCC 5399

Figure 2 Nucleotide sequence of the bIAP gene

The deduced amino acid sequence of the bIAP protein is indicated. Consensus sequences for a TATA box and a polyadenylation signal are underlined. The deduced amino acid sequence of the bIAP protein is written under the codons.



**Figure 3** Southern blot analysis of bovine genomic DNA digested with different enzymes

The blot was probed with a 1.6 kb *Bam*HI/*Hind*III fragment of the bIAP gene (see Figure 2). Bands marked with arrows were identified as corresponding to restriction fragments of the bIAP gene. An asterisk marks a band corresponding to the isolated pseudogene. Abbreviations for the restriction enzymes used are: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; N, *Nhe*I; X, *Xba*I.

sequencing strategy as well as the restriction map and genomic structure of the bIAP gene are shown in Figure 1. A full-length bIAP cDNA was constructed by ligation of clone BB204 with an 830 bp 5' bIAP RT-PCR fragment amplified from RNA extracted from CHO cells stably transfected with the bIAP-pcDNA I construct.

Figure 2 shows the complete sequence of a 5.4 kb genomic clone containing the bIAP gene. The bIAP gene contains 11 exons and ten introns of very compact nature. Exon and intron borders were determined by comparison with BB204 and other known AP genes (Henthorn et al., 1988; Knoll et al., 1988; Millán and Manes, 1988; Manes et al., 1990). The exact length of exon XI could not be determined since no cDNA with a poly(A) tail had been isolated. The estimate given is based on the identification of a putative polyadenylation site AATAAA (bp 5183–5188) in the 3' non-coding region of the gene (underlined in Figure 2). Interestingly, the entire coding region of exon XI shows a high G/C content of over 60–80% compared with an approximately equal ratio of G/C to A/T throughout the whole structural gene. Other regions of biased nucleotide content were found at bp 270 to bp 490, which shows a high A/T content and in a region preceding the polyadenylation site, which shows a high G/C content. The 1.5 kb sequence preceding the first exon contains the same variant TATA box sequence ATTTAA and the GGGAGGG sequence reported previously in the promoter region of the mouse tissue-specific AP (TSAP) genes (Manes et al., 1990) and two human TSAP genes (Millán, 1987; Millán and Manes, 1988). A stretch of 36 alternating pyridines and purines is found at position 732 and is mainly composed of cytosine and adenine nucleotides. Identical structures are reported for the human germ-cell AP (GCAP) gene (Millán and Manes, 1988)

and are thought to form Z-DNA structures of regulatory significance (Nordheim and Rich, 1983).

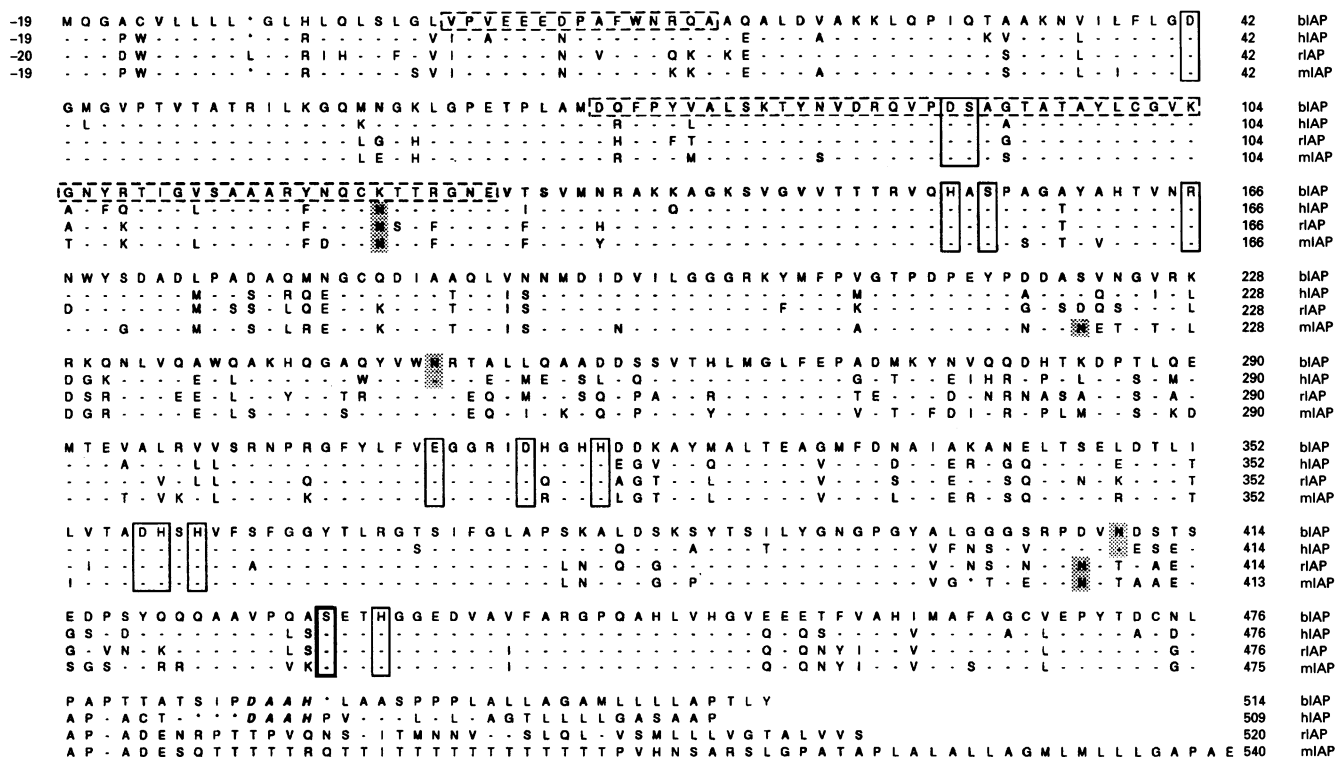
The complexity of the bIAP and related genes in the bovine genome is demonstrated in Figure 3. Two bands in the genomic Southern blot could be identified as fragments derived from the bIAP gene (marked with arrows in Figure 3) while one 4.0 kb *Eco*RI band hybridizes with, and probably corresponds to, the R201 pseudogene. The only other non-human mammalian genome investigated extensively for TSAP genes is the mouse (Manes et al., 1990) where three loci, embryonic AP (EAP), IAP and a pseudogene have been cloned. There is biochemical evidence of only three AP isoenzymes expressed in cows, a tissue non-specific AP (Garattini et al., 1987) and two tissue-specific APs with differential expression in calf and adult intestine (Besman and Coleman, 1985). The bIAP gene characterized in this paper codes for the adult bovine intestinal isoenzyme (see below). The identity of the fetal isoenzyme remains to be determined. The expression of two AP isoenzymes in the cow intestine is analogous to the mouse system, where both EAP and IAP mRNA are found in the adult intestine (Hahnel et al., 1990). Expression of AP in rat intestine seems to be even more complex (Eliakim et al., 1990) with alternatively spliced mRNA being present. Since R201 was cloned from a cDNA library it may represent a transcribed pseudogene or an unusual AP isoenzyme being co-expressed in the adult intestine. Determining whether this gene is equivalent to the EAP isoenzyme in the mouse or is identical to the fetal intestinal AP form described by Besman and Coleman (1985) will require its full characterization.

#### Deduced amino acid sequence

The deduced amino acid sequence (Figure 4) of the bIAP protein is highly homologous to other known IAPs (Berger et al., 1987; Henthorn et al., 1988; Manes et al., 1990; Eliakim et al., 1990) and contains two stretches of amino acids corresponding to the partial peptide sequences previously determined for adult bovine IAP (Culp et al., 1985; Besman and Coleman, 1985) (Figure 4; hatched boxes). Conserved residues and/or conservative amino acid substitutions are found within structurally important regions (Figure 4; boxed residues), such as the Zn and Mg binding sites and the active site residues Asp-91, Ser-102 and Arg-166. Residue 429, found to play a major role in determining the uncompetitive inhibition properties of human placental and germ cell AP (Hummer and Millán, 1991; Hoylaerts and Millán, 1991; Hoylaerts et al., 1992), is occupied by Ser in bIAP as in the other known IAPs (Figure 4, boxed in bold lines). Structural variability is almost exclusively found at the C-terminal end and in the highly variable surface loops (Millán, 1988, 1992). Asp-487 of bIAP resides within a sequence of 4 amino acids (Figure 4; bold italics) conserved between human IAP and human PLAP. The equivalent Asp-484 was shown to be the attachment site of a phosphatidylinositol membrane anchor in PLAP (Micanovic et al., 1988). Recent evidence (Hoffman-Blume et al., 1991) and results presented below indicate that bIAP is also anchored to the plasma membrane by this mechanism and Asp-487 may serve as the attachment site. Two putative glycosylation sites are conserved between human IAP and bIAP. Three other possible sites found in the other IAP sequences were not found in bIAP (Figure 4; shaded residues).

#### Characterization of the recombinant bIAP

The cloned bIAP gene was stably transfected and expressed in CHO cells and the extracted recombinant enzyme was compared



**Figure 4** Comparison of deduced amino acid sequences of bIAP, human IAP (hIAP; Henthorn et al., 1988), mouse IAP (mIAP; Manes et al., 1990) and rat IAP (rIAP; Lowe et al., 1990)

Residues of bIAP that are outlined in hatched lines are identical to sequences obtained by direct protein sequencing (Culp et al., 1985; Besman and Coleman, 1985). Shaded asparagines represent putative *N*-linked glycosylation sites. Residues outlined with solid lines are involved in co-ordinating the essential metal ions Zn and Mg and are active site residues (Sowadski et al., 1985). Residue 429, outlined with bold lines, influences the uncompetitive inhibition properties of APs (Hummer and Millán, 1991; Hoylaerts and Millán, 1991; Hoylaerts et al., 1992). Asp-487 and the following three amino acids are shown in bold italics. Asterisks mark gaps which were introduced to optimize the sequence alignment.

kinetically and biochemically with two commercially-available purified bIAP preparations (Boehringer-Mannheim and Calzyme Laboratories, Inc.). As can be observed in Table 1, recombinant bIAP displays  $K_m$ ,  $K_i$  (for the uncompetitive inhibition by L-Phe) and  $k_{cat}$  values comparable to those of commercially-available bIAP sources. The recombinant bIAP preparation is slightly more heat stable and desialylation does not influence stability with any of the three preparations. However, it is very probable that the CHO-expressed recombinant bIAP differs in other aspects of its glycosylation compared with the natural sources of bIAP and these additional differences may be responsible for the increased heat stability. The recombinant bIAP is clearly sialylated since treatment with neuraminidase reduces the electrophoretic mobility of the enzyme (Figure 5). Similarly the recombinant bIAP contains a hydrophobic phosphatidylinositol

**Table 1** Biochemical comparison of different bIAP preparations

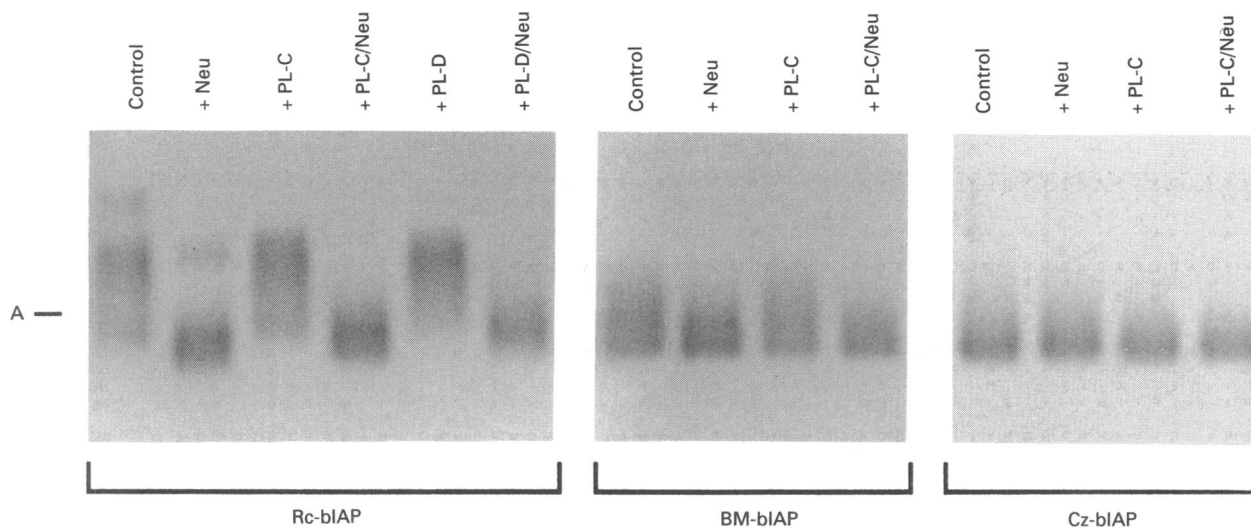
Rc-bIAP, recombinant bIAP; BM-bIAP, Boehringer-Mannheim bIAP; Cz-bIAP, Calzyme Laboratories, Inc. bIAP.

	$K_m$ (mM $\pm$ S.D.)	$K_i$ (mM $\pm$ S.D.) (L-Phe)	$k_{cat}$ ( $s^{-1}$ )	$T_{50}$ (65 °C) (min)	
				- Neu	+ Neu
Rc-bIAP	0.50 $\pm$ 0.024	18.1 $\pm$ 0.6	3485 $\pm$ 140	144	130
BM-bIAP	0.54 $\pm$ 0.026	15.0 $\pm$ 1.0	3630 $\pm$ 120	111	108
Cz-bIAP	0.66 $\pm$ 0.023	16.4 $\pm$ 0.5	3435 $\pm$ 84	120	120

anchor that is mainly cleaved during extraction at pH 5.5 by endogenous CHO cell-derived phospholipase C. The remaining anchor can be cleaved by adding phosphatidylinositol-specific phospholipase C and phospholipase D (Figure 5). The desialylated, anchorless recombinant bIAP shows a discrete isoelectric focusing pattern compared with the untreated enzyme (data not shown). Industrial bIAP preparations consist mainly of anchorless enzyme containing, however, various degrees of residual sialic acid. This is illustrated in Figure 5 where the Boehringer-Mannheim preparation is slightly neuraminidase-sensitive in contrast to the Calzyme Laboratories preparation that appears fully desialylated. Both industrial preparations cover a wider range of pI values in isoelectric focusing compared with recombinant bIAP, a behaviour not related to sialic acid content. The heterogeneity observed in different industrial preparations is probably due to the varying degree of autolytic degradative processes occurring in the bovine intestine after slaughtering. This heterogeneity may explain the variability observed in the performance of different batches of commercially available bIAP during different labelling procedures for immunoassays.

## Conclusion

The bIAP sequence presented in this paper and the availability of fully-active recombinant bIAP enzyme with help to solve the three-dimensional crystal structure of the most efficient AP isoenzyme known. This information will be critical in understanding the structural-functional relationship of several AP domains that may be involved in protein-protein interaction and substrate recognition *in vivo*. In addition, the recombinant bIAP



**Figure 5** Isopal electrophoresis of recombinant (Rc-bIAP), Boehringer-Mannheim (BM-bIAP) and Calzyme Laboratories, Inc. (Cz-bIAP) bIAP, native and after single or combined treatment with neuraminidase (Neu), phosphatidylinositol-specific phospholipase C (PL-C) and phospholipase D (PL-D)

A, application point.

and mutants produced by site-directed mutagenesis may prove of interest for biotechnological applications, since they will provide a source of bIAP of predictable purity and properties.

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