Translation of rat intestinal RNA yields two alkaline phosphatases

Norman L. SUSSMAN,[†] Shakuntla SEETHARAM,* Mark C. BLAUFUSS[‡] and David H. ALPERS*[§]

*Division of Gastroenterology, Washington University, St. Louis, MO 63110, U.S.A., [†]Division of Gastroenterology, Baylor College of Medicine, Houston, TX 77030, U.S.A., and [‡]Department of Pediatrics, University of North Dakota, Grand Forks, ND 58201, U.S.A.

After translation of total rat intestinal RNA, immunoprecipitation using monospecific antiserum against rat intestinal alkaline phosphatase yielded two polypeptides in the adult duodenum and jejunum (molecular masses 62 and 65 kDa). Immunoprecipitation of both bands was blocked by a single purified alkaline phosphatase. In the adult ileum and in the entire small intestine of suckling pups, only the 62 kDa translation product was found. After fat feeding, translated alkaline phosphatase increased by an amount proportionate to the increase in enzyme activity previously seen in the serum. A small fraction of nascent alkaline phosphatase was translocated into microsomal vesicles, producing peptides of 65 and 69 kDa. Tunicamycin-treated membranes demonstrated a different signal peptide for each translation product. *N*-Terminal sequencing of the translation products showed leucine residues at similar positions, but overlap with the mature protein sequence was not demonstrated. On the basis of these data, we propose the presence of two mRNAs encoding alkaline phosphatase in the rat intestine.

INTRODUCTION

Intestinal alkaline phosphatase (AP) is distinguishable from the other forms of the enzyme by its peptide map (McKenna *et al.*, 1979; Seargeant & Stinson, 1979), lectin affinity (Lehmann, 1980), absence of sialic acid, resistance to heat and urea, and sensitivity to L-phenylalanine inhibition (Harris, 1982). Purification of AP from rat intestine has shown that the enzyme in the tissue exists in a membrane and a soluble form, the latter also being secreted into the bloodstream (Young *et al.*, 1981a; Yedlin *et al.*, 1981). The presence of both a membrane and a secreted form of a protein is unusual, but has been described in pheochromocytoma cells (Sabban *et al.*, 1982). However, among the intestinal brush-border enzymes this finding is unique to AP.

The rate of turnover of the intestinal brush-border enzymes is more rapid than that of the entire brush border (enterocyte), but is still relatively long (12–14 h) compared with the lifespan of the cell (48 h) (James *et al.*, 1971). Therefore these proteins are represented by low-abundance mRNA species (Wacker *et al.*, 1982; Danielsen *et al.*, 1983). In addition, their size is large, and sequence data are available for only the *N*-terminus of sucrose-isomaltase (Hauri *et al.*, 1982).

In the absence of cloned cDNA sequences encoding AP, and with limited protein-sequence data, we chose to study AP first by translational analysis. Our results suggest that two mRNAs are present, and demonstrate that an increase in translatable RNA occurs in response to fat feeding.

EXPERIMENTAL

Materials

Sprague–Dawley rats were obtained from Chappell Breeders, Bel Ridge, MO, U.S.A. Swiss–Webster mice

were obtained from Laboratory Supplies, Indianapolis, IN, U.S.A. Labelled amino acids of the highest specific radioactivity were obtained from New England Nuclear (Boston, MA, U.S.A.) or Amersham (Lincoln Heights, IL, U.S.A.). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise specified.

RNA isolation

Rats maintained on standard lab chow or a 30% (w/v) triacylglycerol diet (modified from 45%; U.S. Biochemical Corp.) were killed by decapitation, and small-bowel-epithelial RNA was extracted as described by Gordon *et al.* (1982).

Cell-free translation

Untoasted wheat germ was a gift from General Mills (Vallejo, CA, U.S.A.). A lysate was prepared by the method of Roberts & Paterson (1973) as modified by Gordon *et al.* (1977). For translation *in vitro*, conditions were as described by Gordon *et al.* (1977) with the addition of 1200 units of placental ribonuclease inhibitor (RNasin; Promega-Biotec, Madison, WI, U.S.A.)/ml and altered K⁺ (164 mM) and Mg²⁺ (1 mM) concentrations.

Ribosomes, microsomal membranes and a ribosome-free supernatant were prepared from Krebs II ascites-tumour cells by the method of Szczesna & Boime (1976). Tunicamycin-treated membranes were prepared by the method of Bielinska *et al.* (1978). Translation *in vitro* utilized the method of Szczesna & Boime (1976), except that 25 μ M unlabelled amino acids and 20 μ Ci of the labelled amino acid per 50 μ l of translation mixture were substituted. In many cases trypsin was added post-translationally (3 μ g/100 μ l of translation mixture) to digest the non-translocated proteins and thus to identify the translocated products (Hortin & Boime, 1981). After

Abbreviation used: AP, alkaline phosphatase (EC 3.1.3.1).

[§] To whom correspondence should be addressed.

incubation at 31 °C for 45 min, a 1.5-fold excess of soya-bean trypsin inhibitor was added and immunoprecipitation was performed as usual.

Total protein synthesis in the cell-free system was assessed by hot-trichloroacetic acid precipitation (Gordon *et al.*, 1977). Immunoprecipitation was done by the method of Gordon *et al.* (1977), except that the reaction mixture was not centrifuged at the end of the incubation period, as this resulted in the loss of all precipitable AP. An additional modification was the boiling of the reaction mixture in 2.6% (v/v) SDS before addition of immunoprecipitation buffer. This resulted in lower background radioactivity.

Only 0.1 µl of monospecific anti-rat AP antiserum (fully characterized by Yedlin *et al.*, 1981) was necessary for complete precipitation of AP from a 50 µl translation. Proteins were separated by the method of Laemmli (1970). To assess the fraction of mRNA encoding AP, the translation product was immunoprecipitated and subjected to disc-gel electrophoresis. Slices (1 mm) were soaked overnight at 37 °C in a 0.1 mM-phenylmethane-sulphonyl fluoride/1 mM-benzamidine solution, and a portion was counted for radioactivity in 8 ml of 3a70 scintillation cocktail (Research Products International, Mount Prospect, IL, U.S.A.). The AP radioactivity (c.p.m.) was then expressed as a fraction of the total incorporated (trichloroacetic acid-precipitated) radioactivity.

Protein sequencing

Labelled AP (from cell-free translation) was sequenced by sequential Edman degradation with a Beckman

sequencer and a previously described program (Grant & Bradshaw, 1978). Unlabelled soluble AP (1 nmol) was sequenced by an Applied Biosystems 470A Gas Phase Sequencer (Hewick *et al.*, 1981). Purity of the unlabelled AP was assessed by SDS/polyacrylamide-gel electrophoresis and silver staining (Merril *et al.*, 1981).

RESULTS

Identification and quantification of alkaline phosphatase

The results of wheat-germ cell-free translation and immunoprecipitation are shown in Fig. 1. The pattern of total translation (labelled T) has been published (Gordon *et al.*, 1982) and is shown in Fig. 1(c). Immunoprecipitation with specific anti-AP antiserum demonstrated a single polypeptide of 62 kDa in the entire intestine of the suckling animal (Fig. 1a) and in the ileum (labelled I) of the adult (Figs. 1b and 1c). A second, larger, polypeptide of 65 kDa was seen faintly in the adult jejunum (labelled J) and more prominently in the duodenum, labelled D (Figs. 1b and 1c).

Both the soluble and membrane fractions of intestinal AP have previously been shown to be immunologically identical (Yedlin *et al.*, 1981). Accordingly, purified secreted AP (Yedlin *et al.*, 1981) was used to block the immunoprecipitation of AP from a cell-free translation mixture. Lane 2 in Fig. 2 shows the effect of 400 µg of purified AP added to 40 µl of a translations mixture immediately before the addition of specific antiserum. As shown, the precipitation of both polypeptides is blocked. The control lane of pre-immune rabbit serum showed no precipitated polypeptide (lane 3).

Fig. 1. Immunoprecipitation of AP after translation of intestinal RNA in a wheat-germ cell-free system

The [³⁵S]methionine-labelled immunoprecipitated peptides were separated in 12.5% -polyacrylamide gels containing 0.1% SDS. Exposure time was 10 days for panels (a) and (b), and 3 days for panel (c). Lanes: D, duodenum; J, jejunum; I, ileum. Lane T shows the pattern of total polypeptide synthesis in a wheat-germ lysate programmed by intestinal RNA (300 µg/ml). The positions of pre-apolipoprotein A-IV (48.5 kDa) and pre-pro-apolipoprotein A-I (27.5 kDa) are marked. The arrowheads show the positions of the immunoprecipitated 62 kDa and 65 kDa polypeptides. The smaller bands in panels (a) and (b) are contaminating proteins, which become more prominent with prolonged exposure times.

Fig. 2. Blocking of immunoprecipitated polypeptides by unlabelled purified soluble AP

Immunoprecipitation was performed in the absence or presence of 400 μ g of soluble AP, added immediately before the addition of 0.5 μ l of specific antiserum. Lane 1, AP immunoprecipitated after translation of duodenal RNA. Lane 2, soluble AP added. Lane 3, as in lane 1, except with pre-immune serum instead of specific antiserum.

The abundance of AP was shown by translational analysis to range from 0.049% in the adult duodenum to 0.003% in the adult ileum. Table 1 shows the comparison of AP abundance in suckling, adult and fat-fed adult animals. In the adult, separation of the two polypeptides was not sufficient to allow for their individual quantification. Abundance of AP in these organs is therefore expressed as the sum of radioactivity in both bands (Fig. 1). The highest amount in the suckling animals is in the jejunum, with approximately equal abundance in the duodenum and ileum. In fat-fed animals the increase in translatable AP mRNA was 3-fold in the duodenum, 4-fold in the jejunum and 2.5-fold in the ileum. The mean increase after fat feeding was 3.2-fold for the entire mucosa.

Table 1. Concentration of alkaline phosphatase mRNAs in suckling, adult and fat-fed adult rats by anatomic region

Radiolabelled APs synthesized in wheat-germ lysates were eluted from disc gels, and the radioactivity (c.p.m.) was expressed as a percentage of total protein synthesis. Data shown are the means $\pm$ S.E.M. for three values. In the adult duodenum and jejunum, both precipitated polypeptides were included in the analysis.

	Concn. of alkaline phosphatase mRNA (% of translatable mRNA)		
	Duodenum	Jejunum	Ileum
Suckling	0.029 $\pm$ 0.007	0.036 $\pm$ 0.001	0.020 $\pm$ 0.001
Adult	0.049 $\pm$ 0.001	0.018 $\pm$ 0.003	0.003 $\pm$ 0.001
Fat-fed	0.147 $\pm$ 0.017	0.070 $\pm$ 0.006	0.008 $\pm$ 0.001

Processing

Two events frequently associated with microsomal processing are cleavage of the signal peptide and core glycosylation of the synthesized polypeptide (Katz *et al.*, 1977; Lingappa *et al.*, 1978). To elucidate the pathway by which AP is processed, we utilized the Krebs II system (Szczesna & Boime, 1976). Membranes from this system have been shown to process and glycosylate a number of proteins efficiently (Gordon *et al.*, 1982; Rogers *et al.*, 1982).

The results of co-translational processing are shown in Fig. 3. The two polypeptides of 62 and 65 kDa are shifted up to positions of 65 and 69 kDa. The upward shift in mass is not unexpected, in view of the fact that AP is known to be a glycoprotein. Translocation of the two larger polypeptides into the interior of the membranes was confirmed by their resistance to trypsin added post-translationally (Hortin & Boime, 1981) (Fig. 3, lane 4; Fig. 4, lanes 3 and 5). Addition of a detergent before addition of trypsin resulted in the degradation of all translation products (results not shown).

Because the above results did not exclude the possibility of an internal signal sequence, the experiment was repeated with tunicamycin-treated membranes. These have been shown to retain their translocating and signal-peptidase activities while lacking the ability to glycosylate nascent polypeptides (Hortin & Boime, 1981). Lanes 3 and 4 of Fig. 3 show that the translocated proteins were trimmed to 59 and 61 kDa.

In order to clarify the exact nature of the processed polypeptides, we performed the same set of experiments, but with RNA from suckling animals. The single translation product allowed us to identify the position of the 62 kDa polypeptide. Fig. 4 shows the 62 kDa polypeptide increasing to 65 kDa in the presence of normal membranes, and decreasing to 59 kDa in the presence of tunicamycin-treated membranes.

By this analysis, the two APs were initially synthesized with *N*-terminal pre-peptides of fairly large and dissimilar size. The 62 kDa polypeptide had a pre-peptide of 3 kDa, and the 65 kDa polypeptide had one of 4 kDa.

N-Terminal sequence of alkaline phosphatase

Unlabelled secreted and membrane APs were purified to homogeneity, as demonstrated by silver-stained SDS/polyacrylamide gels (apparent masses of 58 and

Fig. 3. Processing of alkaline phosphatase by microsomal membranes

Total duodenal RNA was translated in the ascites lysate system with or without microsomal membranes, and immunoprecipitated as described in the Experimental section. Where incomplete translocation took place, the untranslocated fraction was digested post-translationally by trypsin (3 µg/100 µl). A 1.5-fold excess of soya-bean trypsin inhibitor was added before immunoprecipitation. (a) SDS/12.5%-polyacrylamide-gel electrophoresis of immunoprecipitated AP processed by native microsomal membranes. Lane 1, without membranes; lane 2, with membranes (3 A_{280} units/100 µl of translation mixture). (b) SDS/10%-polyacrylamide-gel electrophoresis of immunoprecipitated AP processed by tunicamycin-treated microsomal membranes. Lanes 3 and 4, both with tunicamycin-treated membranes (4 A_{280} units/100 µl of translation mixture). In lane 4, post-translational incubation with trypsin was carried out to remove untranslocated AP.

68 kDa respectively; results not shown). Both forms were subjected to sequential Edman degradation. The *N*-terminal sequence of the secreted form was as follows:

All peaks were greater than 3 times background, except for residues 9 and 14, the peaks of which were greater than 2 times background. Positions 1 and 2 could not be assigned because of Tris contamination of the first two cycles despite electro dialysis. Two separate attempts to sequence the membrane form of AP were unsuccessful, despite the demonstration of the sample by silver-stained gel and amino acid analysis, leading us to believe that the *N*-terminus was blocked.

Sequencing of the synthesized 62 kDa polypeptide synthesized *in vitro* demonstrated leucine residues at positions 7, 8, 9, 10, 11, 13 and 17. A similar pattern was shown for the 65 kDa protein, although rising background radioactivity made assignment of positions 14 and 15 less certain (Fig. 5). Methionine was examined only in the 62 kDa protein and was found only at position 1.

Fig. 4. Processing of the 62 kDa alkaline phosphatase by microsomal membranes

Methods were identical with those in Fig. 3, except that jejunal RNA from 14-day-old suckling rats was used. Acrylamide concentration in gels was 10%. Lane 1 had no membranes added. Lanes 2-5 depict immunoprecipitations from translations done in the presence of membranes. In lanes 3 and 5 trypsin was added to digest untranslocated AP. Lane 1 was exposed for 4 days. Lanes 2-5 were exposed for 3 weeks.

Sequencing with [3 H]lysine was done because this amino acid was found at positions 4 and 7 in the mature soluble enzyme. Owing to low incorporation, sequencing was carried out to only 20 amino acids, but lysine was not found in any of these positions. Since the signal peptide was estimated to be 3 kDa, we were not able to determine the precise size of the pre-peptide by this analysis. Because of difficulty with isolating sufficient material, sequencing with other radiolabelled amino acids was not done for the 65 kDa polypeptide.

Because of the low efficiency of translocation across membranes (Fig. 3, lane 4), attempts to purify sufficient quantities of the processed polypeptides for *N*-terminal sequencing were unsuccessful, even when the highly incorporated [3 H]leucine was used.

DISCUSSION

The origin of the secreted form of AP is not clear. The major cellular form is the membrane type, which is located in the brush border, with most of its mass facing

the intestinal lumen. Studies using [125 I]iodosulphanilic acid (Young *et al.*, 1981b) have confirmed that this enzyme does not enter the bloodstream. Rather, the circulating form seems to have an independent rate of synthesis, as measured by [3 H]leucine incorporation (Young *et al.*, 1981c). Furthermore, the paradoxical responses that these two enzymes show in response to dietary manipulation suggests that their expression is independently controlled. The present data are relevant to the biology of the two isoforms and the level at which their expression is regulated.

Our cell-free data suggest the presence of two mRNA species. Artfactual proteolysis or early termination of translation is an unlikely explanation, because of the following factors: (a) the presence of identical translation products in two independent cell-free systems, (b) a

Fig. 5. *N*-Terminal sequence analysis of the primary translation products of AP

The two labelled APs were eluted from disc gels and subjected to automated Edman degradation. The sequences of both the 62 kDa (●) and the 65 kDa (○) polypeptides are shown. The percentage recovery (—) is a hypothetical curve based on a 96% repetitive yield and normalized to the highest peak. The amounts of radiolabelled AP subjected to sequencing were 11000 d.p.m. for [³⁵S]methionine, and, for [³H]leucine, 62000 d.p.m. for the 62 kDa polypeptide and 39000 d.p.m. for the 65 kDa polypeptide.

reproducibly unique pattern in each area of the intestine, (c) no alteration in the pattern in cases where the incubation was continued for 4 h beyond the normal end-point, and (d) unique pre-peptides, suggesting that some differences exist at the *N*-terminus, which accounts for only part of the difference in the size of the translated products. We are unable to say at this stage whether the two proposed RNA species are the product of differential splicing or are transcribed from two separate genes.

We have tentatively equated the more abundant, smaller, product with the secreted AP, and the larger with the membrane enzyme. Several pieces of evidence support this assignment.

(a) As discussed in the Introduction, brush-border enzymes turn over rather slowly, whereas secreted AP has a short half-life (25 min) in plasma. Hence we would expect the more abundant mRNA to encode the soluble AP. In areas where the membrane enzyme is much less abundant than in the adult duodenum (i.e. the adult ileum or the entire small bowel of suckling pups), it is conceivable that mRNA amounts might be low enough to escape detection by our methods. This would explain the absence of the larger polypeptide in translations from these regions.

(b) The size of the larger cell-free product after processing is 69 kDa. This is close to the size of the mature brush-border enzyme (68 kDa). The smaller polypeptide (65 kDa) would have to undergo further processing before secretion to reach the mature size of 58 kDa.

(c) The most compelling evidence is the correlation of

translation with the response to fat feeding. As shown by Young *et al.* (1981a), rats fed on triacylglycerols demonstrate a 3.5-fold increase in newly synthesized circulating AP while showing a small response in tissue amounts of the membrane form, and no increase in tissue amounts of the soluble form. This suggests that the gene for expression of the soluble type is independently regulated, and an efficient system of export from the cell exists for this protein. The 3.2-fold increase in translatable mRNA after fat feeding closely matches the expected rise in circulating activity. Furthermore, Fig. 1(c) clearly shows that the major increase in radioactivity was in the 62 kDa band.

The two APs have rather large prepeptides (3 and 4 kDa), which are clearly seen in Figs. 3 and 4. The lack of overlap in the *N*-terminal sequences of the soluble enzyme and the cell-free product, and the blocked sequence of the membrane type, support the concept of a cleavable signal sequence. The abundance of leucine residues fits the model of the hydrophobic core thought necessary for a functioning signal peptide. In this regard, the membrane enzyme is different from the other brush-border enzyme studied, γ -glutamyl transpeptidase, which appears to have an 'internal' signal sequence (Finidori *et al.*, 1984).

If the secreted enzyme is indeed the more abundant form, then the same may be true for other tissues which secrete alkaline phosphatase. In fact, two polypeptides have been identified in cell-free translation of rat liver (Seetharam *et al.*, 1986) and human placental polyadenylated RNA (C. Ovitt, personal communication). Organ and tissue culture may be used to study the relative production rates of the secreted and membrane forms of intestinal alkaline phosphatase.

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REFERENCES

- Bielinska, M., Grant, G. A. & Boime, I. (1978) *J. Biol. Chem.* **253**, 7117–7119
- Danielsen, E. M., Noren, O. & Sjostrom, H. (1983) *Biochem. J.* **216**, 37–42
- Finidori, J., Laperche, Y., Haguenaer-Tsapir, R., Barouki, R., Guellaen, G. & Hanoune, J. (1984) *J. Biol. Chem.* **259**, 4687–4690
- Gordon, J. I., Deeley, R. G., Burns, A. T. H., Paterson, B. M., Christmann, J. L. & Goldberger, R. F. (1977) *J. Biol. Chem.* **252**, 8320–8327
- Gordon, J. I., Smith, D. P., Andy, R., Alpers, D. H., Schonfeld, G. & Strauss, A. W. (1982) *J. Biol. Chem.* **257**, 971–978
- Grant, G. & Bradshaw, R. A. (1978) *J. Biol. Chem.* **253**, 2727–2731
- Harris, H. (1982) *Harvey Lect.* **76**, 95–123
- Hauri, H. P., Wacker, H., Rickli, E. E., Bigler-Meier, B., Quaroni, A. & Semenza, G. (1982) *J. Biol. Chem.* **257**, 4522–4528
- Hewick, R. M., Hunkapiller, M. W., Hood, L. E. & Dreyer, W. J. (1981) *J. Biol. Chem.* **256**, 7990–7997

- Hortin, G. & Boime, I. (1981) *J. Biol. Chem.* **256**, 1491–1494
- James, W. P. T., Alpers, D. H., Gerber, J. E. & Isselbacher, K. J. (1971) *Biochim. Biophys. Acta* **230**, 194–203
- Katz, F. N., Rothman, J. E., Lingappa, V. R., Blobel, G. & Lodish, H. F. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3278–3282
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lehmann, F. G. (1980) *Biochim. Biophys. Acta* **616**, 41–59
- Lingappa, V. R., Katz, F. N., Lodish, H. F. & Blobel, G. (1978) *J. Biol. Chem.* **253**, 8667–8670
- McKenna, M. J., Hamilton, T. A. & Sussman, H. H. (1979) *Biochem. J.* **181**, 67–73
- Merril, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. (1981) *Science* **211**, 1437–1438
- Roberts, B. E. & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2330–2334
- Rogers, G., Gruenebaum, J. & Boime, I. (1982) *J. Biol. Chem.* **257**, 4179–4186
- Sabban, E. L., Greene, L. A. & Goldstein, M. (1982) *J. Biol. Chem.* **258**, 7812–7818
- Seargeant, L. E. & Stinson, R. A. (1979) *Nature (London)* **281**, 152–154
- Seetharam, S., Sussman, N. L., Komoda, T. & Alpers, D. H. (1986) *Hepatology*, in the press
- Szczesna, E. & Boime, I. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1179–1182
- Wacker, H., Jaussi, R., Sonderegger, P., Dokow, M., Ghersa, P., Hauss, H. P., Christen, P. & Semenza, G. (1982) *FEBS Lett.* **136**, 329–332
- Yedlin, S. T., Young, G. P., Seetharam, B., Seetharam, S. & Alpers, D. H. (1981) *J. Biol. Chem.* **256**, 5620–5626
- Young, G. P., Friedman, S., Yedlin, S. T. & Alpers, D. H. (1981*a*) *Am. J. Physiol.* **241**, G461–G468
- Young, G. P., Yedlin, S. T. & Alpers, D. H. (1981*b*) *Biochim. Biophys. Acta* **676**, 257–265
- Young, G. P., Yedlin, S. T. & Alpers, D. H. (1981*c*) *Biochem. J.* **200**, 645–654

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