Purification and characterization of a dipterecin homologue from *Sarcophaga peregrina* (flesh fly)

Mami ISHIKAWA, Takeo KUBO and Shunji NATORI*

Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

A protein with a molecular mass of 8 kDa was found to be synthesized specifically when the fat-body from injured *Sarcophaga peregrina* larvae was cultured *in vitro*. This protein was purified from the haemolymph of the injured larvae to near-homogeneity. Partial amino acid sequencing revealed that this protein is a dipterecin homologue. It showed bactericidal activity on growing, but not resting *Escherichia coli* cells. *E. coli* cells become elongated on treatment with this protein.

INTRODUCTION

Lepidopteran and dipteran insects are known to respond to bacterial challenge and to injury by producing various humoral defence proteins [1–6]. Two types of defence protein have been found in *Sarcophaga peregrina* (flesh fly) larvae. One type is a galactose-binding lectin named *Sarcophaga* lectin [7–9] and the other is four groups of heat-stable antibacterial proteins [10–15]. *Sarcophaga* lectin was found to participate in elimination of foreign cells introduced into the abdominal cavity [16]. Of the antibacterial proteins, sarcotoxins I, II and III are effective for Gram-negative bacteria, whereas sapecin is effective for Gram-positive bacteria [10,12,14,15]. These defence proteins are synthesized in response to injury, sapecin by haemocytes and the others by the fat-body, and are secreted into the haemolymph.

Northern-blot analysis revealed that the genes for the defence proteins were activated transiently and simultaneously in the fat-body when the body wall of third-instar larvae was pricked with a hypodermic needle [17–19]. Thus the induction of defence proteins occurs at the transcriptional level under these conditions. To identify proteins synthesized by the fat-body in response to injury, we previously established a system for culture of the fat-body *in vitro* [20]. Using this system, we newly identified an 8 kDa protein synthesized exclusively by fat-body isolated from injured larvae. We then found that the same protein was actually present in the haemolymph of injured larvae.

This paper describes the purification and characterization of this 8 kDa protein from the haemolymph of injured larvae. Judging from the sequence of the first 37 amino acid residues from the *N*-terminus of the purified protein, this protein is a member of the dipterecin family, a group of antibacterial proteins found in immunized larvae of the dipteran *Phormia terranova* [21,22]. This is the first demonstration of *Sarcophaga* dipterecin.

MATERIALS AND METHODS

Animals and collection of haemolymph

Third-instar larvae of *Sarcophaga peregrina* were used throughout. When kept in a plastic container with a small amount of water at room temperature, the larvae could be used for isolation of haemolymph for up to 5 days after removal from their food. Haemolymph was collected by cutting off the anterior tip of the larvae with fine scissors and collecting the drop of haemolymph that exuded in an ice-cold test tube. For induction

of 8 kDa protein, larvae were anaesthetized on a glass board on ice and their body wall was pricked with a hypodermic needle. Usually 1 ml of haemolymph was collected from about 150 larvae. The haemolymph was centrifuged at 700 *g* for 5 min to remove haemocytes and stored at -80°C .

Collection of fat-body culture medium containing labelled protein

The fat-body was excised from third-instar larvae and rinsed well in insect saline (130 mM-NaCl/5 mM-KCl/1 mM- CaCl_2). It was then incubated in 300 μl of methionine-free Grace's insect medium (Gibco) containing 30 μCi of [^{35}S]methionine and incubated at 27°C . After 1 h, the fat-body was removed, and the culture medium was centrifuged at 700 *g* for 5 min and the resulting supernatant containing labelled protein was stored at -80°C .

Purification of 8 kDa protein from the haemolymph of injured larvae

For purification of the protein from haemolymph, radioactive 8 kDa protein synthesized *in vitro* was mixed with the haemolymph as a marker. Haemolymph collected from third-instar larvae 48 h after injury of the body wall (2 ml) was mixed with 3 ml of fat-body culture medium containing labelled 8 kDa protein (1.5×10^5 c.p.m.). The mixture was centrifuged for 15 min at 16000 *g*, and the resulting supernatant was loaded on a gel-filtration column of Sephacryl S-200 (2.5 cm $\times$ 112 cm). The column was developed with buffered insect saline (10 mM-Tris/HCl, pH 7.5, containing 130 mM-NaCl, 5 mM-KCl and 1 mM- CaCl_2). Each fraction of eluate was subjected to SDS/PAGE followed by fluorography, and fractions containing radioactive 8 kDa protein were pooled.

The pooled fraction was subjected to h.p.l.c. on a Synchropak (Gilson) RP-P (C_{18}) reversed-phase column, and material was eluted with a linear gradient of 0–37.5% acetonitrile in 0.05% trifluoroacetic acid. Fractions containing radioactive 8 kDa protein were combined, dried to remove solvent, and resubjected to h.p.l.c. twice on the same column. Material was eluted with a linear gradient of 0–37.5% acetonitrile in 0.05% trifluoroacetic acid. Radioactive 8 kDa protein was eluted as a single peak coinciding with a peak of material with absorbance at 220 nm. The 8 kDa protein was almost homogeneous at this stage as shown by SDS/PAGE.

* To whom correspondence should be addressed.

SDS/PAGE

Electrophoresis on SDS/polyacrylamide slab gel was carried out by the method of Laemmli [23]. Samples were denatured by heating them for 20 min at 75 °C in 1 % (w/v) SDS and 2 % (v/v) 2-mercaptoethanol. After electrophoresis, the gels were stained by the method of Fairbanks *et al.* [24]. For the estimation of the relative amount of the 8 kDa protein, the gels were dried and subjected to fluorography. The autoradiograms were scanned at 580 nm with a Shimadzu CS-9000 scanner.

Radioimmunoassay of the 8 kDa protein

Antibody against the 8 kDa protein was obtained by injecting the purified 8 kDa protein (20 µg) mixed with complete Freund's adjuvant into a male albino rabbit. A booster injection of 20 µg of the protein in incomplete Freund's adjuvant was given 10 days later. The animal was bled 10 days after the booster injection. Radioiodination of the 8 kDa protein was performed by the method of Hunter *et al.* [25]. The specific radioactivity of the labelled protein was about 10^8 c.p.m./µg and the purity of the labelled protein was more than 95 %, as judged by SDS/PAGE followed by autoradiography. Radioimmunoassay of the 8 kDa protein using the antiserum and labelled 8 kDa protein was performed as follows. Labelled 8 kDa protein (10^5 c.p.m.), antiserum and the sample were incubated in 120 µl of 10 mM-sodium phosphate buffer (pH 7.4) containing 137 mM-NaCl, 2.7 mM-KCl, 0.02 % NaN_3 , 0.1 % gelatin and 1 % Triton X-100 for 14 h at 4 °C. Then 100 µl of 10 % *Staphylococcus aureus* Cowan I solution prepared by the method of Kessler [26] was added. The reaction mixture was centrifuged at 12000 *g* for 5 min, and the resulting pellet was washed successively with buffer I [20 mM-Tris/HCl buffer (pH 7.4) containing 1 M-NaCl and 0.1 % Triton X-100], buffer II [50 mM-Tris/HCl buffer (pH 7.4) containing 150 mM-NaCl, 5 mM-EDTA, 1 % Triton X-100, 0.02 % NaN_3 and 0.3 % SDS] and buffer III [10 mM-Tris/HCl buffer (pH 7.4) containing 0.1 % Triton X-100]. Then the radioactivity in the pellet was measured. The amount of 8 kDa protein in the sample was determined from a standard curve obtained with purified 8 kDa as a competitive inhibitor.

RESULTS

Specific synthesis of an 8 kDa protein by fat-body isolated from injured larvae and its purification from haemolymph

Two fat-body samples were prepared: one from normal third-instar larvae, and the other from larvae which had been pricked with a hypodermic needle 6 h previously. These fat-bodies were cultured for 1 h at 27 °C in the presence of [^{35}S]methionine, and labelled protein secreted into the culture medium was analysed by SDS/PAGE followed by autoradiography. As is evident in Fig. 1, a protein indicated by an arrow was synthesized specifically by fat-body itself or haemocytes attached to it from injured larvae. These results suggested that this protein is an acute-phase protein and is probably present in the haemolymph of injured larvae. Thus we tried to purify this protein from the haemolymph of injured larvae. For this, we mixed culture medium containing labelled 8 kDa protein with haemolymph from injured larvae and monitored this radioactivity during purification of the 8 kDa protein. Haemolymph was first loaded on a column of Sephacryl S-200 and fractionated. As shown in Fig. 2, radiolabelled 8 kDa protein was eluted as a sharp single peak. The elution position of the 8 kDa protein corresponded to its monomer molecular mass. The fractions containing this radioactive 8 kDa protein were pooled and subjected three times to h.p.l.c. In this way, we obtained labelled 8 kDa protein in a sharp peak fraction, as shown in Fig. 3. As the absorbance of the major peak and the

Fig. 1. Specific synthesis of the 8 kDa protein by fat-body from injured larvae

Fat-bodies from normal and injured larvae were cultured *in vitro* in the presence of [^{35}S]methionine for 1 h at 27 °C, and labelled protein secreted into the medium was analysed by SDS/PAGE followed by autoradiography. Lane 1, fat-body from normal larvae; lane 2, fat-body from injured larvae. Sizes of molecular-mass markers are given in kDa. The arrow indicates the position of the 8 kDa protein.

radioactivity of the 8 kDa protein always behaved in the same way on h.p.l.c., and as the band of the major protein stained by Coomassie Brilliant Blue and that of radioactivity on autoradiography coincided well on electrophoresis under denaturing conditions, we concluded that the proteins in the major peak and the radioactive 8 kDa protein are identical. Typical results on purification of the 8 kDa protein from 2 ml of haemolymph of injured larvae are shown in Table 1.

Change in the content of the 8 kDa protein in the haemolymph

As the 8 kDa protein seemed to be an acute-phase protein, we examined whether its content in the haemolymph increased when

Fig. 2. Elution profile of labelled 8 kDa protein on Sephacryl S-200

Haemolymph from injured larvae was mixed with culture medium of fat-body containing labelled 8 kDa protein and subjected to gel filtration on Sephacryl S-200. Each fraction was subjected to SDS/PAGE followed by autoradiography. Bands of the 8 kDa protein on autoradiograms were scanned by densitometry and relative contents were determined from the weight of chart paper corresponding to the peak. ---, A_{280} ; ●, relative content of the 8 kDa protein. Arrows indicate the elution positions of molecular-mass makers in kDa.

Fig. 3. Reversed-phase h.p.l.c. profile of the 8 kDa protein
(a) Fractions from the first reversed-phase h.p.l.c. were pooled, concentrated and rechromatographed on the same reversed-phase h.p.l.c. —, A_{220} ; ----, acetonitrile. (b) The peak fraction was subjected to SDS/PAGE with various molecular-mass markers and then autoradiographed. Lane 1, Coomassie Brilliant Blue staining; lane 2, autoradiogram. The arrow indicates the 8 kDa protein.

the body wall of third-instar larvae was injured. For this, we raised antibody against the 8 kDa protein and measured the content of the protein in the haemolymph by radioimmunoassay. As shown in Fig. 4, the amount of the 8 kDa protein in the larval haemolymph was found to increase markedly with time after injury to the body wall, reaching a maximum after about 10 h. The maximum concentration was about 20 times the basal level in normal larvae, which was about 8 $\mu\text{g}/\text{ml}$, and this maximum concentration was maintained for at least 3 days. These results support the idea that this 8 kDa protein is an acute-phase protein like various other antibacterial proteins of this insect, and that it is secreted from the fat-body in response to body injury.

Partial amino acid sequencing and determination of the amino acid composition of the 8 kDa protein

To obtain more information about the structure of this protein, we determined the sequence of 37 amino acid residues from its *N*-terminus. We found a significant similarity (43.3 %) between the *N*-terminal sequences of the 8 kDa protein and diptericin A [21], as shown in Fig. 5. Diptericsins are a group of antibacterial proteins with molecular masses of about 8 kDa found in *Phormia terranova*, another dipteran insect. The 8 kDa protein is probably a *Sarcophaga* diptericin because it contains a pentaglycine

Fig. 4. Change in the amount of the 8 kDa protein in the haemolymph with time after injury of the body wall
Larvae were pricked at time 0 and kept for 3 days. At the indicated times, the amount of the 8 kDa protein in the haemolymph was determined by radioimmunoassay. Bars represent deviation of duplicate measurements, and points are averages. ○, Injured larvae; ●, normal larvae.

Table 1. Summary of purification of the 8 kDa protein

Protein was determined by the method of Lowry *et al.* [27]. The units for the ^{35}S -labelled 8 kDa protein are arbitrary units based on the intensity of the band of the 8 kDa protein on autoradiograms containing 5×10^4 c.p.m.

Fraction	Protein (mg)	^{35}S -labelled 8 kDa protein (unit)	Specific content (units/mg)	Recovery (%)	Purification (fold)
Haemolymph	300	100	0.33	100	1
Sephacryl S-200	5.0	91	18	91	55
H.p.l.c.	0.036	15	410	15	1200

Fig. 5. Sequence similarity of the *N*-terminal region of the 8 kDa protein to that of diptericin A [20]

A sequence of 37 amino acid residues from the *N*-terminus is compared with that of the corresponding region of diptericin A. The sequences are aligned to achieve maximum similarity. Identical residues are boxed.

Table 2. Comparison of amino acid compositions of the 8 kDa protein and diptericin A

The values for diptericin A are taken from ref. [21].

Amino acid	8 kDa protein		Diptericin A	
	(Residues/ mol)	(mol %)	(Residues/ mol)	(mol %)
Asp,Asn	10	12.8	10	12.2
Thr	2	2.6	2	2.4
Ser	4	5.1	7	8.5
Glu,Gln	7	9.0	4	4.9
Gly	15	19.2	15	18.3
Ala	4	5.1	3	3.7
Val	2	2.6	5	6.1
Met	1	1.3	0	0
Ile	6	7.7	3	3.7
Leu	3	3.8	5	6.1
Tyr	2	2.6	5	6.1
Phe	2	2.6	2	2.4
Lys	5	6.4	4	4.9
His	4	5.1	3	3.7
Arg	3	3.8	3	3.7
Trp	2	2.6	1	1.2
Pro	6	7.7	10	12.2
Cys	0	0	0	0
Total	78	100	82	100.1

stretch characteristic of *Phormia* dipterics in an equivalent position.

We determined the amino acid composition of the purified 8 kDa protein. As shown in Table 2, the content of glycine was significantly higher than those of other amino acids and no cysteine was found, as in diptericin A [21]. However, the 8 kDa

Fig. 6. Loss of viability of *E. coli* cells in the presence of the 8 kDa protein

E. coli cells were incubated in the presence or absence of 25 µg of the 8 kDa protein/ml in antibiotic medium 3 (Difco) or 0.9% NaCl at 37 °C. Numbers of viable cells were determined by plating samples taken at intervals. ●, With 8 kDa protein in medium 3; ○, without the 8 kDa protein in medium 3; ▲, with 8 kDa protein in 0.9% NaCl; △, without the 8 kDa protein in 0.9% NaCl.

protein contained methionine, whereas the four dipterics from *Phormia* so far sequenced do not [21,22]. This is the only significant difference between the amino acid compositions of dipterics and the 8 kDa protein, which could be labelled with [³⁵S]methionine *in vitro*.

Fig. 7. Scanning-electron-microscopic appearance of *E. coli* treated with the 8 kDa protein

E. coli cells were incubated in antibiotic medium 3 (Difco) with 25 µg of the 8 kDa protein/ml at 37 °C for 140 min, and then collected on a Millipore filter (GSWP, 0.22 µm pore size). (a) Untreated bacteria; (b) bacteria treated with the 8 kDa protein. Bars represent 5 µm.

Antibacterial activity of the 8 kDa protein

As the 8 kDa protein seemed to be a protein of the dipterecin family, we tested its antibacterial activity. For this, we added various amounts of the 8 kDa protein to the medium of bacterial cultures at 37 °C, and measured their turbidity after incubation for appropriate periods as an index of bacterial growth. Of the Gram-negative bacteria tested, only *Escherichia coli* and *Shigella sonnei* were susceptible to the 8 kDa protein, showing significant growth inhibition. The minimum inhibitory concentrations of the 8 kDa protein for these bacteria were 6.25 and 12.5 µg/ml respectively. *Proteus vulgaris*, *Proteus rettgeri* and *Pseudomonas aeruginosa* were resistant to this protein. As Gram-positive bacteria, we tested *Bacillus subtilis*, *Staph. aureus*, *Micrococcus luteus*, *Bacillus megaterium*, *Corynebacterium bovis* and *Enterobacter cloacae*, but all these bacteria could grow in the presence of more than 100 µg of the 8 kDa protein/ml. Therefore, like dipterecin, this protein seems to inhibit the growth of a few species of Gram-negative bacteria [28].

The response of *E. coli* to the 8 kDa protein is shown in Fig. 6. During incubation with 25 µg of the 8 kDa protein/ml under growing conditions, the number of viable cells decreased rapidly, scarcely any viable cells being detectable after 2 h. Thus the 8 kDa protein had a bactericidal effect. However, this bactericidal effect was not observed on its addition to *E. coli* cells in 0.9% NaCl, indicating that it is toxic only to growing *E. coli*, as is dipterecin [21]. In this respect it differs from other antibacterial proteins of *Sarcophaga* such as sarcotoxin I, II and sapecin, which are toxic to bacteria irrespective of their physiological state.

We examined the morphology of *E. coli* cells cultured in the presence of 25 µg of the 8 kDa protein/ml for 140 min. As seen in Fig. 7, scanning electron microscopy showed that, in its presence, the cells became elongated. Possibly, the 8 kDa protein inhibits septum formation and results in formation of elongated cells with no colony-forming ability.

DISCUSSION

We found that the fat-body from injured *Sarcophaga* larvae, but not from normal larvae, synthesizes and secretes an 8 kDa protein when cultured *in vitro*. We also purified an 8 kDa protein from the haemolymph of injured larvae. Furthermore, we concluded that the purified 8 kDa protein and the 8 kDa protein synthesized by fat-body *in vitro* are the same protein because when the former protein was labelled *in vitro* and added to the haemolymph, the two proteins were copurified.

The protein was purified from haemolymph as a single component, as judged by sequencing, and was found to be a dipterecin homologue. Dipterecins are a group of antibacterial proteins first found in the haemolymph of immunized *Phormia terranova* larvae, and the primary structures of at least four related proteins have been determined from their cDNA sequences [21,22]. All these proteins contain a characteristic pentaglycine stretch near their N-terminus. We did not determine the complete amino acid sequence of the 8 kDa protein, but we concluded that it is a member of the dipterecin family because we found that it also has a pentaglycine stretch and a similar molecule mass to other dipterecins. Recently a dipterecin cDNA was also cloned from *Drosophila* [29]. Therefore this protein is probably ubiquitous in dipteran insects.

We obtained only one protein by our purification procedure. Therefore it is unknown whether the haemolymph of injured *Sarcophaga* larvae contains multiple dipterecin proteins as in *Phormia* larvae. Possibly other dipterecin proteins in the haemolymph do not contain methionine and so were not labelled with [³⁵S]methionine when fat-body from injured larvae was

cultured *in vitro*. Previously we isolated an antibacterial protein named sarcotoxin III from the same haemolymph [14]. The molecular mass of sarcotoxin III is 7 kDa, and so it is a little smaller than the 8 kDa protein. Moreover, sarcotoxin III does not contain a methionine residue. As the 8 kDa protein is a dipterecin, sarcotoxin III may also be a dipterecin. For examination of this possibility, cDNA clones for *Sarcophaga* dipterecin must be isolated and analysed.

Of the various bacterial strains tested, only *E. coli* and *S. sonnei* were sensitive to the 8 kDa protein. However, although it was bactericidal, its minimum inhibitory concentrations for these bacteria were about 6–13 µg/ml, which are more than 50 times that of sarcotoxin I. Therefore the 8 kDa protein is unlikely to be an effective antibacterial substance. Possibly, this protein has some other biological function necessary in the acute-phase response.

The mode of action of the 8 kDa protein on *E. coli* or *S. sonnei* is unknown. We found that *E. coli* cells became elongated on treatment with the 8 kDa protein. This effect is similar to that of sarcotoxin II [30], but we could not detect any unusual projections or bulges on the surface of elongated cells like those seen on treatment of *E. coli* cells with sarcotoxin II.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and by Grant-in-Aid BMP 92-IV-2-4 (Bio Media Program) from the Ministry of Agriculture, Forestry and Fishery of Japan.

REFERENCES

- Natori, S. (1977) *J. Insect. Physiol.* **23**, 1169–1173
- Natori, S. (1987) in *Molecular Entomology* vol. 49 of *UCLA Symposia on Molecular and Cellular Biology*, New Series (Law, J. H., ed.), pp. 369–378, Alan R. Liss, New York.
- Boman, H. G., Faye, I., Hofsten, P. V., Kockum, K., Lee, J.-Y., Xanthopoulos, K. G., Bennich, H., Engstrom, A., Merrifield, B. R. & Andreu, D. (1986) in *Immunity in Invertebrates* (Brehelin, M., ed.), pp. 63–73, Springer-Verlag, Berlin and Heidelberg.
- Boman, H. G. & Hultmark, D. (1987) *Annu. Rev. Microbiol.* **41**, 103–126
- Boman, H. G., Faye, I., Gudmundsson, G. H., Lee, J.-Y. & Lidholm, D.-A. (1991) *Eur. J. Biochem.* **210**, 23–31
- Boman, H. G. (1991) *Cell* **65**, 205–207
- Komano, H., Mizuno, D. & Natori, S. (1980) *J. Biol. Chem.* **255**, 2919–2924
- Komano, H., Nozawa, R., Mizuno, D. & Natori, S. (1983) *J. Biol. Chem.* **258**, 2143–2147
- Takahashi, H., Komano, H., Kawaguchi, N., Kitamura, N., Nakanishi, N. & Natori, S. (1985) *J. Biol. Chem.* **260**, 12228–12233
- Okada, M. & Natori, S. (1983) *Biochem. J.* **211**, 727–734
- Okada, M. & Natori, S. (1985) *J. Biol. Chem.* **260**, 7174–7177
- Ando, K., Okada, M. & Natori, S. (1987) *Biochemistry* **26**, 226–230
- Ando, K. & Natori, S. (1988) *Biochemistry* **27**, 1715–1721
- Baba, K., Okada, M., Kawano, T., Komano, H. & Natori, S. (1987) *J. Biochem. (Tokyo)* **102**, 69–74
- Matsuyama, K. & Natori, S. (1988) *J. Biol. Chem.* **263**, 17112–17116
- Komano, H. & Natori, S. (1985) *Dev. Comp. Immunol.* **9**, 31–40
- Takahashi, H., Komano, H. & Natori, S. (1986) *J. Insect Physiol.* **32**, 771–779
- Nambu, R., Nakajima, Y., Ando, K. & Natori, S. (1988) *Biochim. Biophys. Res. Commun.* **150**, 540–544
- Matsuyama, K. & Natori, S. (1988) *J. Biol. Chem.* **263**, 17117–17121
- Takahashi, H., Komano, H., Kawaguchi, N., Obinata, M. & Natori, S. (1984) *Insect Biochem.* **14**, 713–717
- Dimarcq, J.-L., Keppi, E., Dunbar, B., Lambert, J., Reichhart, J.-M., Hoffmann, D., Rankine, S. M., Fothergill, J. E. & Hoffmann, J. A. (1988) *Eur. J. Biochem.* **171**, 17–22
- Rechhart, J.-M., Essrich, M., Dimarcq, J.-L., Hoffmann, D., Hoffmann, J. A. & Lageaux, M. (1989) *Eur. J. Biochem.* **182**, 423–427
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

24. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617
 25. Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495–496
 26. Kessler, S. W. (1975) *J. Immunol.* **115**, 1617–1624
 27. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
 28. Keppi, E., Pugsley, A. P., Lambert, J., Wicker, C., Dimarcq, J.-L., Hoffmann, J. A. & Hoffmann, D. (1989) *Arch. Insect Biochem. Physiol.* **10**, 229–239
 29. Wicker, C., Reichhart, J.-M., Hoffmann, D., Hultmark, D., Samakovlis, C. & Hoffmann, J. A. (1990) *J. Biol. Chem.* **265**, 22493–22498
 30. Ando, K. & Natori, S. (1988) *J. Biochem. (Tokyo)* **103**, 735–739
-

Received 18 February 1992/14 April 1992; accepted 21 April 1991