Biochem. J. (1989) 257, 685–689 (Printed in Great Britain)

β-Adrenergic induction of a cysteine-proteinase-inhibitor mRNA in rat salivary glands

Phyllis A. SHAW* and Tibor BARKA*†

Departments of *Anatomy and †Pathology, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, NY 10029, U.S.A.

Transcripts encoding the cysteine-proteinase inhibitor rat cystatin S are induced in submandibular and parotid glands by the β-adrenergic agonist isoproterenol (isoprenaline). High levels of cystatin S mRNA persist in glands of chronically treated animals for 6 days after discontinuation of the catecholamine, indicating a long half-life of the mRNA. Post-transcriptionally the size of the mRNA decreases, owing to a shortening of the poly(A) tail.

INTRODUCTION

β-Adrenergic agonists, particularly isoproterenol (isoprenaline, IPR), have long been known to influence the function, differentiation and growth of salivary glands. IPR stimulates DNA synthesis in the submandibular and parotid glands of rats and mice and, when given repeatedly, produces conspicuous enlargements of these organs [1–9]. This rapid induced growth is accompanied by changes in the structure and composition of the glands, as well as the saliva which they secrete. IPR apparently induces the expression of a number of genes in salivary glands, but only a few of these genes have been characterized. These include the family of proline-rich proteins which have been described in the salivary glands of mice, rats and the subhuman primate *Macaca fascicularis* [10–13]. A protein that is IPR-inducible to high concentrations in rat submandibular glands and is subsequently secreted into the saliva has been described by Menaker *et al.* [14,15] and characterized by Naito and his co-workers [16–18]. Recently, we have cloned and sequenced cDNA that encodes this protein (termed 'LM protein' by Menaker *et al.* [14,15]) and established that it is a cysteine-proteinase inhibitor belonging to family 2 of the cystatin superfamily [19]. In the present paper we describe the β-adrenergic-receptor-mediated induction of this inhibitor, rat cystatin S. This demonstration of the induction of the expression of a cystatin gene by catecholamines will permit an analysis of the regulation of cystatin genes by catecholamines and their role in the growth and development of salivary glands.

EXPERIMENTAL

Materials

The chemicals and their sources were as follows: guanidinium thiocyanate, Fluka AG; nitrocellulose sheets, Schleicher und Schuell; DNA polymerase I (endonuclease-free) and Random Primed DNA Labeling Kit, Boehringer-Mannheim; RNAase H, Bethesda Research Laboratories; α-^{32}P-labelled deoxynucleoside triphosphates, New England Nuclear; agarose (ultra pure), International Biotechnologies; DL-isoproterenol (isoprenaline) hydrochloride, Sigma.

Methods

Treatment of animals. Female Sprague–Dawley rats with an average weight of 150 g were kept in a temperature- and humidity-controlled environment (12 h light/12 h dark cycle) with free access to water and standard laboratory chow. Before they were killed by exsanguination under diethyl ether anaesthesia, the food was removed for 4 h. DL-IPR, dissolved in 0.1% sodium metabisulphite/0.85% NaCl, was given intraperitoneally. Four groups of rats were treated as follows. Group I: control, no treatment; Group II: 0.1 μmol of IPR/g body wt. twice daily (with the exception of day 6, when only one injection was given at 09:00 h) for 6 days; the rats were killed 24 h after the last injection of IPR; Group III: 0.1 μmol of IPR/g body wt. twice daily for 6 days, followed by a 6-day recovery period; Group IV: one injection of 0.1 μmol of IPR/g body wt. The submandibular and parotid glands were removed at 0.5, 1, 4, 18, 24, 48 and 72 h later. There were at least four rats in Groups I, II and III, and at least four rats at each of the time points in Group IV. The submandibular and parotid glands were removed and immediately frozen in liquid nitrogen.

RNA isolation. Total cellular RNA was purified by the method of Chirgwin *et al.* [20]. Poly(A)$^+$ RNA was isolated by oligo(dT)–cellulose chromatography [21].

Northern and slot-blot hybridization. Hybridization probes were prepared by nick-translation [22] or random prime labelling [23,24] of the plasmids containing cDNA encoding rat cystatin S [19]. All RNA samples were analysed by at least three separate Northern-blot hybridizations; the RNA samples were judged to be intact by examination of the ethidium bromide staining of RNA before blotting to nitrocellulose. Total RNA (20 μg) and poly(A)$^+$ RNA (1 μg) from submandibular glands of untreated and IPR-treated rats were electrophoresed through 1.5 or 2.0% agarose/6% formaldehyde gels [25,26]. Nitrocellulose blots of the RNA were prepared and hybridized (10^6 c.p.m./lane) in 50% (v/v) formamide at 42 °C for 16 h to ^{32}P-labelled nick-translated or random-primed rat cystatin S cDNA probes (1.6×

Abbreviations used: IPR, isoproterenol (isoprenaline; 4-{1-hydroxy-2-[(1-methylethyl)amino]ethyl}benzene-1,2-diol) hydrochloride; poly(A)$^+$, polyadenylated.

10^8 c.p.m./μg and 1.3×10^9 c.p.m./μg respectively). The blots were washed as previously described [19] and exposed to X-ray film at −70 °C for approx. 18 h.

Slot-blot analyses were performed at least three times with two different probes: one cDNA encoding the *N*-terminal of rat cystatin S from position 1 to position 53 of the mature protein, and the other encoding the *C*-terminus of rat cystatin S from position 48 to position 101; the size of the mature protein is 118 amino acids. For slot-blots, equivalent amounts of each RNA sample were dissolved in sterile distilled water and a dilution series was prepared. Adult brain RNA, a negative control since it does not hybridize to rat cystatin S mRNA, was added to the samples to equalize the amount of RNA. The RNA samples were made 50% with respect to formamide, and 2 × in respect of SSC (1 × SSC = 0.15 M-NaCl/0.015 M-sodium citrate). The samples were then heated for 10 min at 65 °C, cooled on ice, applied to nitrocellulose sheets using a Schleicher und Schuell (Keene, NH, U.S.A.) slot-blotting apparatus, and hybridized at 42 °C to ^{32}P-labelled nick-translated rat cystatin S cDNA. After a 12–18 h exposure to X-ray film, the film was scanned with a densitometer (EC Corp., St. Petersburg, FL, U.S.A.). Three different areas of each slot were scanned, and the relative peak heights were compared for each area; the means and S.D. values were calculated for each concentration of RNA.

RNAase H assays. A 20 μg portion of total cellular RNA extracted from submandibular glands at 18 and 72 h after a single injection of IPR was hybridized to 1 μg of oligo(dT) in 1 × /RNAase H buffer [20 mM-Tris/HCl (pH 7.5) / 10 mM-$MgCl_2$ / 0.1 mM-DTT / 5% sucrose] for 15 min at room temperature. One unit of RNAase H (from *Escherichia coli*; BRL) was added and the samples were incubated at 37 °C for 15 min [27–29]. The samples were extracted with phenol/chloroform (1:1, v/v) twice, precipitated with ethanol in the presence of 2 M-ammonium acetate on solid CO_2 for 30 min, and the RNA was pelleted at 4 °C in an Eppendorf centrifuge. The precipitates were washed with 70% (v/v) ethanol, freeze-dried, dissolved in Northern gel-loading buffer, and electrophoresed in 2% (w/v) agarose/6% (v/v) formaldehyde gels as described above. Controls consisted of RNA samples that were hybridized to oligo(dT) but not RNAase H-treated, since it is known that RNAase H 'nibbles' at the poly(A) tail in the absence of oligo(dT) [28]. Nitrocellulose blots of electrophoretically separated RNAs were hybridized to random-primed ^{32}P-labelled cDNA probes overnight at 42 °C. The blots were washed and exposed to X-ray films for 12–18 h.

RESULTS

In accord with our previous findings [19], administration of IPR for 6 days caused a marked increase in the concentration of cystatin S mRNA in both the submandibular and parotid glands. After daily injections of IPR to adult rats for 6 days and discontinuation of the catecholamine for 6 days, at the time when the glands reverted to their normal size, relatively high levels of steady-state cystatin S mRNA were still observable in submandibular-gland RNAs (Fig. 1, lanes 3 and 4). The Northern gel is overexposed to demonstrate that this mRNA was also present in the parotid gland after a 6-day recovery period (Fig. 1, lane 7), albeit at a much

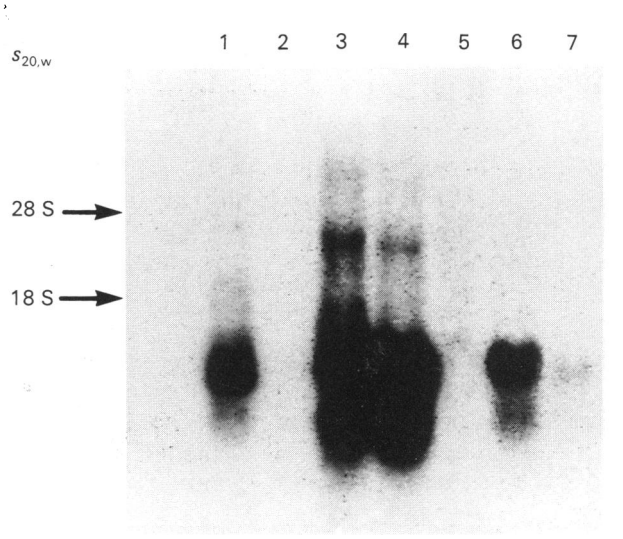

Fig. 1. Northern-blot analysis (as described in the Experimental section) of rat cystatin S mRNA

Lane 1, a positive control, i.e. 1 μg of poly(A)$^+$ RNA extracted from submandibular glands of chronically IPR-induced (6 days of IPR, twice each day) adult rats; lane 2, 20 μg of total RNA from untreated submandibular glands; lane 3, 20 μg of total RNA from 6-day-IPR-induced submandibular glands; lane 4, 20 μg of total RNA from 6-day IPR-induced (plus a 6-day recovery) adult-rat submandibular glands. Steady-state levels of cystatin S mRNA in parotids are also represented: lane 5, unstimulated; lane 6, 6-day-IPR-induced; lane 7, 6-day-IPR-induced plus a 6-day recovery. This blot is overexposed to show the levels of parotid cystatin S mRNA.

lower concentration. Rat cystatin mRNA was not detectable in total RNA extracted from the submandibular or parotid glands of untreated rats (Fig. 1, lanes 2 and 5). The steady-state levels of cystatin S mRNA in the chronically IPR-stimulated parotid gland are approx. 20-fold lower than in the similarly stimulated submandibular gland. However, the size of the message is the same, about 900 bases in length.

Quantitative slot-blots were used to determine the relative concentraitons of cystatin S mRNA in the submandibular glands of chronically IPR-stimulated rats, and in submandibular glands after 6 days of IPR treatment followed by a 6-day recovery period. The relative concentration of cystatin S mRNA declined by about 32% during a 6-day recovery period after a 6-day regimen of IPR administration (Table 1).

Expression of the rat cystatin S gene was also induced by a single injection of IPR. Groups of adult female rats were given a single injection of 0.1 μmol of IPR/g body wt., and total RNA was isolated from the submandibular and parotid glands at different times after the injection of the agonist and analysed by Northern-blot hybridization. A single dose of IPR greatly increased the steady-state levels of cystatin S mRNA as early as 4 h in the submandibular gland (Fig. 2*a*, lane 4) and at 18 h in the parotid gland (Fig. 2*b*, lane 6). The level of cystatin S mRNA remained elevated in both tissues at 48 h after treatment (Fig. 2*a*, lane 7; Fig. 2*b*, lane 8). Since these data suggested that rat cystatin S mRNA is long-lived, the experiment was repeated to include a longer time interval. The levels of cystatin S mRNA

Table 1. Relative concentration of rat cystatin S mRNA

Relative-peak-height values are absorbances in arbitrary units (mean ± S.D., $n = 3$); they are different for the same probe and same concentration of RNA because of different exposure times. Abbreviations: SUBM, submandibular; PAR, parotid gland. Rats in the 'Chronic IPR' group were given six daily injections (0.1 μmol/g body wt.) of IPR. In the 'Chronic IPR plus recovery' group the 6-day IPR regime was followed by a 6-day recovery period. The *N*-terminal probe is from position 1 to position 53 of the mature protein, whereas the *C*-terminal probe is from position 48 to position 101.

Gland	RNA (μg)	Probe	Relative peak height		$\frac{(b)}{(a)} \times 100$
			(*a*) Chronic IPR	(*b*) Chronic IPR plus recovery	
SUBM	5.00	*N*-Terminal	7.13 ± 0.32	Not done	
	2.50	*N*-Terminal	3.17 ± 0.12	2.03 ± 0.12	64
	1.25	*N*-Terminal	1.12 ± 0.03	0.77 ± 0.03	69
PAR	5.00	*N*-Terminal	0.33 ± 0.06	Not measurable	
SUBM	2.50	*C*-Terminal	4.85 ± 0.73	3.39 ± 0.65	70
	1.25	*C*-Terminal	1.97 ± 0.40	1.33 ± 0.34	68

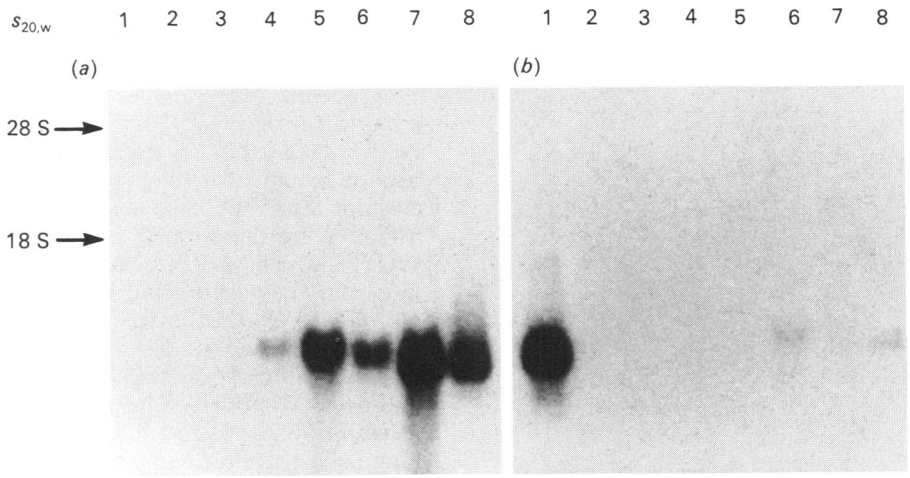

Fig. 2. Northern blots depicting the induction of cystatin S mRNA in adult-rat submandibular (*a*) and parotid (*b*) glands after a single injection of IPR

A 20 μg portion of total RNA was electrohoresed, except where noted. (*a*) Total RNA from rat submandibular gland. Lane 1, no injection of IPR; lane 2, 30 min after IPR injection; lane 3, 1 h after IPR injection; lane 4, 4 h after IPR injection; lane 5, 18 h after IPR injection; lane 6, 24 h after IPR injection; lane 7, 48 h after IPR injection; lane 8, poly(A)$^+$ RNA from submandibular glands of rats chronically treated with IPR (6 days of IPR, twice each day, as a positive control). (*b*) Lane 1, 1 μg of poly(A)$^+$ RNA from submandibular glands of rats chronically treated with IPR (6 days of IPR, twice daily, as a positive control); lanes 2–8, the same time course as listed for (*a*) i.e. 0, 0.5, 1, 4, 18, 24 and 48 h after IPR, except that the RNA is from the parotid gland.

remained elevated in submandibular (Fig. 3, lane 6) and parotid glands [Fig. 3, lane 11 (the blot is overexposed to show the steady-state levels of rat cystatin S in the parotid)] even 72 h after a single injection of IPR; the concentration of rat cystatin S mRNA was approx. 20-fold higher in the submandibular gland than in the parotid gland. It was undetectable, however, in the submandibular (Fig. 3, lane 2) and parotid glands (Fig. 3, lane 7) of untreated adult rats.

The kinetic study of IPR induction of rat cystatin S mRNA revealed a change in the size of the message with time. Thus, at 18 h after the injection of the drug, the message was approx. 980 bases in length, whereas at 72 h it was shorter, namely about 900 bases. Furthermore, the size of rat cystatin S mRNA 72 h after IPR administration, as observed by hybridization to total RNA, was the same size as its mature poly(A)$^+$ RNA (Fig. 3, lane 1). RNAase H experiments were performed to determine whether the change in size was due to a decrease in the poly(A) tail length or due to some other mRNA-processing event. The total RNA samples from rat submandibular glands at 18 and 72 h after IPR stimulation were chosen to examine poly(A) processing, since they represented the longest and shortest cystatin S mRNAs respectively. The controls consisted of the same samples of total RNA, but with oligo(dT) alone (Fig. 4, lane 1, 18 h; lane 4, 72 h). When the experimental samples were hybridized to oligo(dT), treated with RNAase H, and electrophoresed in a 2.0% agarose gel, the observed size was the same at 18 and 72 h (Fig. 4, lanes 2 and 3). Thus the difference in size was due to a change in the poly(A) tail length.

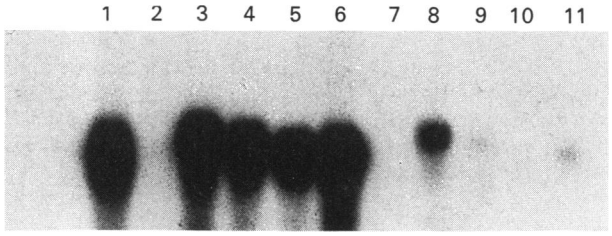

Fig. 3. Northern blot of 20 μg of total RNA from submandibular and parotid glands of uninduced and IPR-induced adult rats

Lane 1, 1 μg of poly(A)$^+$ RNA from submandibular glands of chronically IPR-treated (6 days of IPR, twice each day) adult rats (positive control). The animals were given one injection of IPR, and submandibular glands were removed and RNA extracted at 18 (lane 3), 24 (lane 4), 48 (lane 5) and 72 (lane 6) h later; lane 2 is total RNA from submandibular glands of untreated rats. The parotid glands were also removed, and total RNA extracted at 18 (lane 8), 24 (lane 9), 48 (lane 10) and 72 (lane 11) h after a single injection of IPR; lane 7 is total RNA from parotid glands of untreated rats. This blot has been overexposed to show the difference of steady-state rat cystatin levels in the submandibular gland and the parotid.

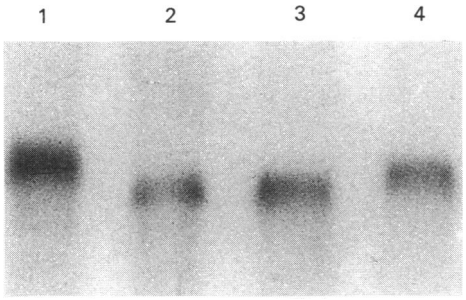

Fig. 4. Northern blot of 20 μg of total RNA extracted from submandibular glands 18 h (lane 1) and 72 h (lane 4) after a single injection of IPR, and electrophoresis in a 2% agarose/6% formaldehyde gel

A 20 μg portion of total RNA from each of these two time points was hybridized with oligo(dT) and treated with RNAase H as described in the Experimental section; lane 2 is the 18 h total RNA sample hybridized with oligo(dT) and treated with RNAase H; lane 3 is the 72 h total RNA sample handled in the same fashion.

DISCUSSION

Low-molecular-mass cystatins, belonging to family 2 of the cystatin superfamily [30–33], have been isolated from several tissues and body fluids of humans and animals. Human saliva contains several cystatins, which are presumably synthesized in the salivary glands, since cystatin is demonstrable immunocytochemically in the submandibular and parotid glands [34]. Neither the function of cystatins in the saliva nor the regulation of salivary-gland cystatin genes is known. It is surmised, however, that saliva cystatins play a protective role in the oral cavity against bacterial or cellular proteinases. However, neither experimental evidence nor data showing changes in saliva cystatin levels in pathological conditions is available to support this supposition.

The seminal finding presented here is that the expression of the rat cystatin S gene in the submandibular and parotid glands is stimulated by the β-adrenergic agonist IPR, most likely mediated by cyclic AMP. At 4 h after the administration of a single injection of IPR, cystatin S mRNA levels were markedly elevated and remained so for at least 72 h. Induction was observed also in the parotid gland, but at any given time the concentration of parotid cystatin S mRNA was far below that of the submandibular gland. Since both DNA synthesis and growth are stimulated by IPR equally well, or even more, in the parotid gland than in the submandibular gland, the difference in induction of cystatin S mRNA in the two glands suggests organ-specific regulatory mechanisms.

The most pronounced accumulation of rat cystatin S mRNA was observed in the submandibular glands of rats treated with IPR for several days. This increase in mRNA concentration parallels the increase in the level of the protein cystatin S, previously described as 'LM protein' [16].

Rat cystatin S mRNA seems to have an unusually long half-life. This is suggested by the high level of the mRNA 72 h after one injection of the drug, but even more by its slow decline during a 6-day recovery period after the administration of IPR for 6 days. Since IPR is rapidly metabolized and/or excreted, a persistent stimulus cannot account for the prolonged increased level of rat cystatin S mRNA. The steady-state levels of functional mRNAs are determined in part by their decay in the cytoplasm. The mRNAs of transiently expressed genes frequently have short half-lives. The apparent long half-life of rat cystatin S mRNA and the structural features which determine it deserve further study. This is of particular interest, since changes in the rates of synthesis and decay of mRNAs during hypertrophic/hyperplastic growth and regression may contribute to the observed long persistence of the high concentration of the cystatin S message.

A single injection of IPR stimulates DNA synthesis and cell replication in both the parotid and submandibular glands, and with repeated administration of the drug these organs become greatly enlarged [1–9]. An increased rate of protein synthesis and a decrease in degradation may contribute to this enlargement. It is tempting to speculate that the induction of a proteinase inhibitor by IPR may be causally related to its growth-promoting effect. However, whether or not rat cystatin S, which is a secretory protein, has an intracellular function is not known. Our preliminary data suggest that rat cystatin S may not be the only cysteine-proteinase inhibitor that is induced by IPR. Characterization of such inhibitors and determination of their functions await further studies.

We thank Ms. H. van der Noen, Dr. Edward Gresik, Dr. Beth Schachter and Dr. Jim Cox for criticism of the manuscript. This work was supported by Grants DE08714 (to P.A.S.) and DE07637 (to T.B.) from the National Institutes of Health.

REFERENCES

1. Brenner, G. M. & Wulf, R. G. (1981) J. Pharmacol. Exp. Ther. **218**, 608–612
2. Barka, T. (1965) Exp. Cell Res. **37**, 662–679

3. Barka, T. (1965) Exp. Cell Res. **39**, 355–364
4. Baserga, R. (1970) Fed. Proc. Fed. Am. Soc. Exp. Biol. **29**, 1443–1446
5. Brown-Grant, K. (1961) Nature (London) **191**, 1076–1078
6. Schneyer, C. A. (1972) in Regulation of Organ and Tissue Growth (Goss, D. R. J., ed.), p. 211, Academic Press, New York
7. Schneyer, C. A. & Shackelford, J. M. (1963) Proc. Soc. Biol. Med. **112**, 320–324
8. Seyle, H., Veilleux, R. & Cantin, M. (1961) Science **133**, 44–45
9. Barka, T. & van der Noen, H. (1976) Lab. Invest. **35**, 507–514
10. Fernandez-Sorensen, A. & Carlson, D. M. (1974) Biochem. Biophys. Res. Commun. **60**, 249–256
11. Mehansho, H., Clements, S., Sheares, B. T., Smith, S. & Carlson, D. M. (1984) J. Biol. Chem. **260**, 4418–4423
12. Ziemer, M. A., Swain, W. F., Rutter, W. J., Clements, S., Ann, D. K. & Carlson, D. M. (1984) J. Biol. Chem. **259**, 10475–10480
13. Belford, H. S., Triffleman, E. S., Offner, G. D., Troxler, R. F. & Oppenheim, F. G. (1984) J. Biol. Chem. **259**, 3977–3984
14. Menaker, L., Cobb, C. M. & Taylor, R. E. (1974) Ala. J. Med. Sci. **11**, 356–359
15. Menaker, L., Sheetz, J. H., Cobb, C. M. & Navia, J. M. (1974) Lab. Invest. **30**, 341–349
16. Naito, Y. (1981) Chem. Pharm. Bull. **29**, 1365–1372
17. Naito, Y. & Saito, M. (1982) Chem. Pharm. Bull. **30**, 1063–1065
18. Naito, Y. & Suzuki, I. (1981) Chem. Pharm. Bull. **29**, 1373–1376
19. Shaw, P. A., Cox, J. L., Barka, T. & Naito, Y. (1988) J. Biol. Chem., in the press
20. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) Biochemistry **18**, 5295–5299
21. Aviv, H. & Leder, P. (1972) Proc. Natl. Acad. Sci. U.S.A. **69**, 1408–1412
22. Maniatis, T., Jeffrey, A. & Kleid, D. G. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 1184–1188
23. Feinberg, A. P. & Vogelstein, B. (1983) Anal. Biochem. **132**, 6–13
24. Feinberg, A. P. & Vogelstein, B. (1984) Anal. Biochem. **137**, 266–267
25. Rave, N., Crkvenjakov, R. & Boedtker, H. (1979) Nucleic Acids Res. **6**, 3559–3567
26. Thomas, P. S. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 5201–5205
27. Stavrianopoulos, J. G. & Chargaff, E. (1973) Proc. Natl. Acad. Sci. U.S.A. **70**, 1959–1963
28. Sippel, A. E., Stravianopoulos, J. G., Schutz, G. & Feigelson, P. (1974) Proc. Natl. Acad. Sci. U.S.A. **71**, 4635–4639
29. Donis-Keller, H. (1979) Nucleic Acids Res. **9**, 179–192
30. Barrett, A. J. (1980) Biomed. Biochim. Acta **45**, 1363–1374
31. Okada, Y., Teno, N., Itoh, N. & Okamoto, H. (1985) Chem. Pharm. Bull. **33**, 5149–5152
32. Abrahamson, M., Barrett, A. J., Salvesen, G. & Grubb, A. (1986) J. Biol. Chem. **261**, 11282–11289
33. Salvesen, G., Parkes, C., Rawlings, N. D., Brown, M. A., Barrett, A. J., Abrahamson, M. & Grubb, A. (1986) in Cysteine Proteinases and Their Inhibitors (Turk, V., ed.), pp. 413–428, Walter de Gruyter, New York
34. Barka, T., Yagil, C., van der Noen, H. & Naito, Y. (1986) Lab. Invest. **54**, 165–171

Received 24 August 1988/3 November 1988; accepted 11 November 1988