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Regulation of proteinase levels in the nematode *Caenorhabditis elegans*

Preferential depression by acute or chronic starvation

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Acute starvation of the wild-type of the nematode *Caenorhabditis elegans* depresses the level of cathepsin D by 65% within 4–8 h and the level of the thiol cathepsins Ce1 and Ce2 to about the same extent after 24 h. There is no parallel loss of lysosomal β-glucosidase or β-hexosaminidase activities. In strains which are chronically starved as a result of mutations which compromise feeding behaviour (*unc-52*) or nutrient uptake into the intestinal cells (*daf-4*), cathepsin D levels are decreased to about 15% of the level in fully fed wild-type animals. We suggest that the decline in the cathepsin D level results from autodigestion when alternative protein substrates are depleted in the lysosomes.

INTRODUCTION

Despite the wealth of biochemical information on intracellular proteinases, we know relatively little about which proteinases are involved in specific functions (reviewed in [1–3]), or about the genetic and metabolic factors which regulate the synthesis and degradation of these enzymes.

As a model system for exploring these questions, we have been using the soil nematode *Caenorhabditis elegans*, principally because of the relative ease with which mutant strains can be isolated and characterized genetically [4,5]. *C. elegans* has a highly developed lysosomal system [6,7] which contains a complement of phosphatases, glycosidases [6] and proteinases [8] with many resemblances to mammalian lysosomal enzymes. Sarkis *et al.* [8] have shown the presence of a pepstatin-sensitive aspartic proteinase (cathepsin D), two leupeptin-sensitive thiol proteinases (cathepsins Ce1 and Ce2), and a thiol-independent endoproteinase (cathepsin Ce3). Cathepsin D is the only one of these proteinases which inactivates target enzymes *in vitro* under conditions (pH 4.5–5) which approximate to the intralysosomal milieu (J. Ashcom & L. Jacobson, unpublished work). Nonetheless, structural gene mutations [10] which reduce cathepsin D activity by 90% have little obvious effect on the animals.

In the present paper we show that the levels of several lysosomal proteinases are depressed by 4–5-fold within 6–24 h after the imposition of acute starvation on wild-type *C. elegans* and that even lower proteinase levels (about 15% of fully fed wild-type controls) are observed in strains which are chronically starved by virtue of mutations which compromise feeding behaviour or nutrient uptake. We show that cathepsin D autolyses *in vitro* and suggest that the starvation-induced decline in cathepsin D level results from self-digestion.

MATERIALS AND METHODS

C. elegans strain N2 (wild-type) was used unless otherwise specified. Strain CB669 *unc-52(e669)II* and strain CB1364 *daf-4(e1364)III* were obtained from the *Caenorhabditis* Genetics Center, University of Missouri, Columbia, MO, U.S.A. Strain PJ603, homozygous for both these mutations, was constructed by standard genetic techniques. Animals heterozygous for the *unc-52* or *daf-4* mutations were F_1 progeny obtained by crossing homozygous mutant hermaphrodites with wild-type males.

Growth conditions on nematode-growth (NG) agar with lawns of *Escherichia coli* OP50 were as given by Bolanowski *et al.* [6]. Starvation was initiated by washing the nematodes from growth plates and replating on NG agar containing 200 μg of streptomycin/ml and no *E. coli* lawns. Control animals were replated on *E. coli* lawns.

At the indicated intervals, ten-worm samples were hand-picked into buffer (0.1 M-sodium acetate, pH 5; 0.1% Nonidet P40) and lysed by six cycles of freezing in liquid N_2 and thawing at 10 °C. Assays for β-glucosidase and β-hexosaminidase were as previously described [6]. The measurement of cathepsin D activity by hydrolysis of fluorescein isothiocyanate-treated haemoglobin, and of cathepsin Ce1 + Ce2 activity by hydrolysis of benzyloxycarbonyl Phe-Arg-7-amino-4-methylcoumarin (Z-Phe-Arg-NMec) were as described by Sarkis *et al.* [8]. Methods for SDS polyacrylamide-gel electrophoresis and detection of cathepsin D by immunoblotting with rabbit anti-(cathepsin D) IgG and ^{125}I-labelled goat anti-rabbit IgG were as described by Jacobson *et al.* [10]. Band intensities on autoradiograms were quantified by densitometric scanning and integration with a Hewlett–Packard integrator [10].

Abbreviations used: NG, nematode-growth; Z, benzyloxycarbonyl; NMec, 7-amino-4-methylcoumarin.

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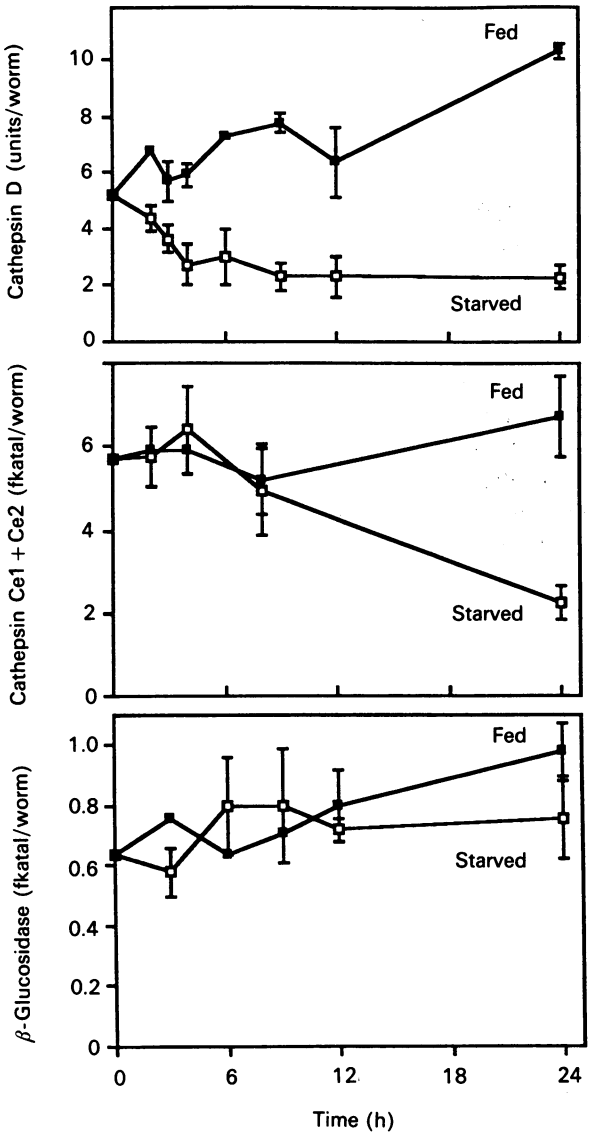

Fig. 1. Effect of acute starvation on the levels of cathepsin D (*a*) cathepsins Ce1 and Ce2 summed activity assayed with Z-Phe-Arg-NMec (*b*) and β-glucosidase (*c*)

Animals were grown for 65 h at 25 °C prior to the onset of starvation. Results are means ± S.D. of seven assays from three independent experiments (cathepsin D), eight assays from two independent experiments (cathepsin Ce1 + Ce2) or four assays from two independent experiments (β-glucosidase).

RESULTS

Effect of acute starvation

We observed, by chance, an unusually low level of cathepsin D activity (expressed as total enzyme units per animal) when *C. elegans* on a Petri plate had completely consumed the *E. coli* lawn. As shown in Fig. 1(*a*), the level of cathepsin D declined quite rapidly when fully fed nematodes were washed and transferred to fresh plates without *E. coli* lawns. Over a 24 h starvation period, the amount of cathepsin D activity per animal declined to about one-third of its initial level; most of the decrease occurred within the first 4–8 h. After correction for the fraction of activity which appears non-labile, the loss of cathepsin D in the first 8 h is reasonably well described by a first-order process, with an indicated half-life of 2.4 h.

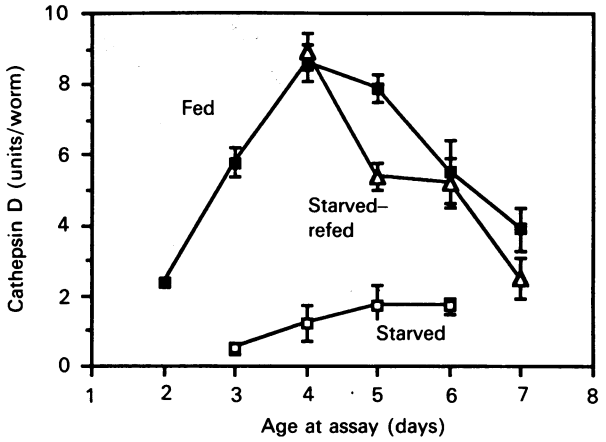

Fig. 2. Effect of starvation and refeeding on cathepsin D levels

Animals were either fed continuously (■), starved for 24 h (□) or refed for 24 h after 24 h of acute starvation (△). For example, the refed animals assayed at day 4 were starved from day 2 to day 3, and refed from day 3 to day 4. Results are means ± S.D. for quadruplicate assays on ten-worm samples.

The activities of other lysosomal cathepsins did not decline so rapidly. The combined activity of the thiol cathepsins Ce1 and Ce2 remained approximately constant for 8–12 h, but by 24 h declined to about one-third of the initial level (Fig. 1*b*). Although there is no available assay which separately quantifies cathepsins Ce1 and Ce2 in crude extracts, we have measured their activities after separation by non-denaturing isoelectric focusing [8]. Preliminary results indicate that the activities of Ce1 and Ce2 decline to the same extent after 24 h of starvation.

In contrast with the declines in cathepsin levels, starvation had little or no effect on the total content of lysosomal glycosidases. β-Glucosidase activity (the sum of two distinct enzymes, cf. [6]) remained constant during 24 h of acute starvation (Fig. 1*c*). β-Hexosaminidase activity also remained unchanged (results not shown).

Effect of re-feeding

The data in Fig. 2 show the effects upon cathepsin D level of starvation and refeeding initiated at different points in the life cycle. The fractional loss of cathepsin D activity upon acute starvation is approximately constant; about 70–80% of the activity present on any given day is lost after 24 h of starvation. Because the cathepsin D level in continuously fed controls (Fig. 2; cf. [9]) is markedly age-dependent, however, the absolute number of activity units lost during 24 h of acute starvation varies considerably with the time of initiation of starvation.

A 24 h refeeding after 24 h starvation restores cathepsin D activity to almost the same level observed in continuously fed animals of the same age (Fig. 2). That is, the enzyme rapidly returns to the level which would have been attained by continuously fed animals at that point in the life cycle.

It follows that more cathepsin D accumulates in a given interval in the starved–refed animals than in

continuously fed controls (Fig. 3). For example, from day 3 to day 4, each continuously fed animal accumulates about 3 units of cathepsin D activity. Over the same period (from day 3 to day 4), an animal starved from day 2 to day 3 accumulates about 8.5 units upon refeeding. This suggests that cathepsin D accumulation at any given chronological age is not controlled by an intrinsic limit upon the absolute amount of enzyme synthesized.

In the period after day 4, the cathepsin D level in continuously fed controls suffers a decline which represents a net loss of cathepsin D protein, defined antigenically [9]. Nonetheless, starved–refed animals show positive enzyme accumulation over the same time period (Fig. 3). Thus older animals remain capable of net synthesis of cathepsin D, at least under the special conditions of recovery from acute starvation.

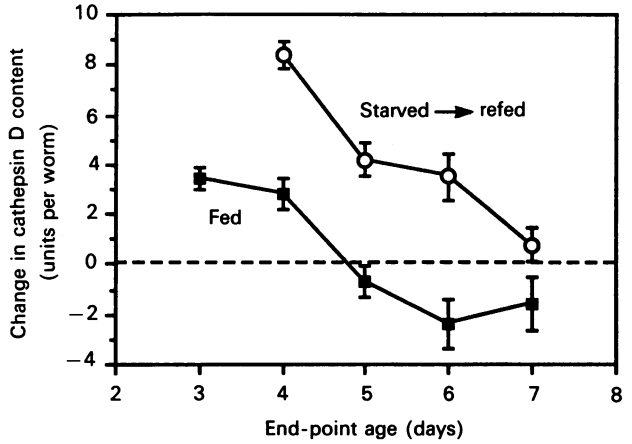

Fig. 3. Change in cathepsin D activities in continuously fed animals (■) and during refeeding after 24 h starvation (○)

The change in activity in each 24 h period for the continuously fed animals is the activity at the plotted day minus the activity on the previous day. For the starved–refed animals, the activity after 24 h starvation is subtracted from the activity after 24 h of refeeding. Data are taken from Fig. 2, with appropriate error propagation.

Genetically induced chronic starvation

The nutritional status of *C. elegans* is chronically compromised by a variety of mutations. For example, *unc-52* mutants become paralysed as a result of dystrophic muscle [11] and so quickly exhaust the supply of *E. coli* in their immediate vicinity. Such paralysed animals have only about 15% of the cathepsin D activity found in fully fed wild-type animals (Table 1). The effect of *unc-52* mutations on muscle structure is genetically recessive [4,11], and *unc-52*/+ heterozygotes have a normal cathepsin D level (Table 1). We have previously reported [10] that various mutations in other genes which produce an 'unco-ordinated' phenotype also produce variable extents of deficiency in cathepsin D, approximately commensurate in each case with the extent of behavioural deficit.

The reduced cathepsin D activity in *unc-52* mutants reflects a reduced content of cathepsin D antigen. We separated equal amounts of crude extract protein from wild-type and *unc-52* mutant animals by electrophoresis on SDS/polyacrylamide gels and measured the amount of cathepsin D antigen by immunoblotting (see the Materials and methods section). We found that the cathepsin D antigen content of the *unc-52* extracts in two independent experiments was 15 and 13% of that in the wild-type extracts. Thus the reduced content of cathepsin D antigen entirely accounts for the reduced activity in the *unc-52* mutant.

Animals homozygous for mutations in the *daf-4* gene are deficient in the uptake of macromolecules from the intestinal lumen into the intestinal lysosomes (G. V. Clokey, O. J. Bashor & L. A. Jacobson, unpublished work). These mutants are also markedly deficient (13% of normal) in cathepsin D (Table 1). Although the predisposition of *daf-4* mutants to form 'dauerlarvae' [12] is temperature-dependent [13], neither the endocytosis deficiency (G. V. Clokey, O. J. Bashor & L. A. Jacobson, unpublished work) nor the characteristic depression of cathepsin D activity depends upon growth temperature. Heterozygotes (*daf-4*/+) have normal nutrient uptake and also have normal cathepsin D activity (Table 1).

We therefore hypothesize that the very different *unc-52* and *daf-4* mutations depress cathepsin D levels by the same mechanism, i.e. by physiological starvation. If this

Table 1. Effect of genetically-induced starvation on cathepsin D levels

All animals were grown at 16 °C and were 5 days old at the time of assay. Results are means ± S.D. for quadruplicate assays on ten-worm samples.

Genotype	Phenotype	Cathepsin D activity (units/worm)	Cathepsin D activity (% of wild-type activity)
+/+	Wild-type	6.0±1.6	100
daf-4(e1364) III	Endocytosis-deficient	0.8±0.1	13
daf-4/+	Wild-type	6.2±0.5	103
unc-52(e669) II	Paralysed	0.9±0.1	15
unc-52/+	Wild-type	6.1±0.6	102
daf-4 III; unc-52 II	Paralysed; endocytosis-deficient	0.7±0.06	12

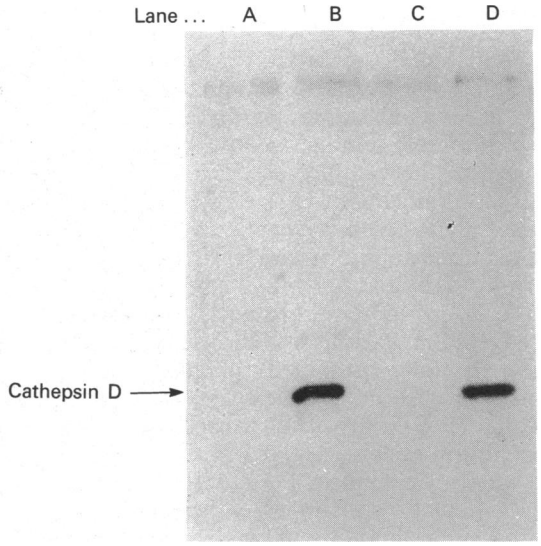

Fig. 4. *In vitro* degradation of cathepsin D in crude extracts

Fully fed wild-type nematodes were sonicated in buffer (0.2 M-sodium formate/2 mM-dithiothreitol/1.5 mM-EDTA, pH 4) and debris removed by centrifugation for 10 min at 12000 ***g***. Extracts were incubated for 1 h at 25 °C without further additions (lane A), with 10 nM-pepstatin (lane B), 100 nM-leupeptin (lane C), or with both pepstatin and leupeptin (lane D). Samples were separated on a 10% (w/v)-polyacrylamide/SDS gel and cathepsin D was detected by immunoblotting [10]. The identity of the cross-reacting material at the top of the gel (> 205 kDA) is unknown. It does not appear in affinity-purified cathepsin D, and fractions enriched in this material are devoid of cathepsin D activity.

is correct, then double-mutant animals should show no further deficit of cathepsin D activity. On the other hand, if two mutations depress cathepsin D levels by different mechanisms, their effects in double mutants should be additive or multiplicative.

Table 1 shows that animals homozygous for both the *unc-52* and *daf-4* mutations are no more deficient in cathepsin D than either single mutant homozygote, indicating that the two mutations indeed affect the cathepsin D level by the same mechanism.

By contrast, additive effects are observed in double-mutant animals homozygous for the *j01* mutant allele of the *cad-1* gene (believed to be the structural gene for cathepsin D; [10]) and mutant alleles of either *unc-52* or *daf-4*. Such double mutants have significantly lower enzyme levels (2–5% of normal; results not shown) than any of the single mutant homozygotes. In these cases it is clear that the mutation in the *cad-1* structural gene directly affects the catalytic capacity of the enzyme [10], whereas the mutations which phenocopy the starvation effect only depress the *amount* of cathepsin D protein.

Autodegradation of cathepsin D *in vitro*

The rapid loss of cathepsin D activity in acutely starved animals implies that the enzyme turns over rapidly *in vivo*. This degradation is readily observed *in vitro* (Fig. 4). When crude extracts of fully fed animals were incubated at pH 4, cathepsin D antigen disappeared entirely within 1 h. Degradation was completely prevented by the addition of the specific cathepsin D inhibitor pepstatin, but not by leupeptin (which inhibits cathepsins Ce1 and Ce2). Cathepsin D degradation was slightly slower at pH 6 (near the pH optima of cathepsins Ce1 and Ce2 [8]), and was still not retarded by the addition of leupeptin (results not shown).

These observations suggest that autolysis is the predominant mode of degradation of cathepsin D. We have purified cathepsin D to homogeneity by affinity chromatography on immobilized pepstatin (L. Jacobson, L. Jen-Jacobson, G. P. Owens, J. D. Ashcom, M. A. Bolanowski & M. V. Shah, unpublished work) and verified that the pure enzyme undergoes self-digestion *in vitro*. Comparable autolysis has been observed for mammalian cathepsin D [14].

DISCUSSION

The starvation effect and its mechanism

The rapid decline in cathepsin D during acute starvation, as well as the slower decline in thiol-proteinase levels, must represent the net degradation of pre-existing enzymes. The further depression of cathepsin D activity in chronically starved animals is entirely accounted for by a lower content of cathepsin D antigen and probably involves superposed effects from reduced synthesis and increased degradation. There is no evidence to indicate that either acute starvation or mutations which produce chronic starvation have any specific effects on the expression of proteinase genes.

We have found that cathespin D can be degraded by autolysis *in vitro*, both in crude extracts and as purified enzyme. Because pepstatin but not leupeptin protects cathepsin D *in vitro*, we infer that the leupeptin-sensitive thiol cathepsins Ce1 and Ce2 cannot by themselves degrade cathepsin D. Although the partially purified Ce1 and Ce2 can degrade some particular proteins (e.g. casein), they have little ability to degrade a mixture of *C. elegans* proteins in the absence of cathepsin D activity [8] and do not inactivate a variety of test enzymes (J. Ashcom and L. Jacobson, unpublished work). There is evidence that autolysis contributes to the inactivation of lysosomal thiol proteinases *in vivo* [15]. We propose that autolysis is also the predominant mode of inactivation of cathepsin D *in vivo*.

About 70% of the cathepsin D in *C. elegans* is localized in the 32–34 intestinal epithelial cells (J. M. Hawdon, S. W. Emmons & L. A. Jacobson, unpublished work). Thus the rapid onset of proteinase inactivation upon starvation may be triggered by a change in the availability of competing substrates. In fully fed animals, exogenous proteins entering the lysosomes provide an adequate supply of proteinase substrates, such that autolysis would be improbable. When the supply of alternative substrates is depleted in the lysosomes of starved animals, however, cathepsin D molecules may be degraded by autolysis. It is not yet known whether autolysis proceeds by an intermolecular or an intramolecular mechanism.

The lysosomal glycosidases, by contrast, show no net activity decline during acute starvation, indicating that they are metabolically stable. The data suggest that starvation only inhibits their continued synthesis.

Regulation of cathepsin D levels

Our data indicate the nutritional status is an important modulator of the level of lysosomal proteinases. Furthermore, the starvation–refeeding data (Fig. 2) have

several interesting implications for the regulation of cathepsin D levels in normally fed animals.

The most striking observation is that animals subjected to a regime of starvation and refeeding rapidly re-establish the cathepsin D level characteristic of animals of that chronological age. In so doing, they accumulate more enzyme during refeeding than unstarved controls over the same time interval (Figs. 2 and 3).

Because cathepsin D is metabolically unstable even in continuously fed animals [9], both the normal age-dependence and the starvation–refeeding data can be understood as follows. The steady-state cathepsin D level at any time is determined by the relative rates of synthesis and degradation. The age-dependence curve (Fig. 2 and [9]) thus results from a 'developmentally programmed' decrease in the synthesis rate, an increase in the degradation rate, or both. Starvation probably inhibits synthesis, but it is not clear whether it also increases the rate of degradation. Upon refeeding, both synthesis and degradation resume at the characteristic rates for animals of a given age, such that the 'normal' steady-state level is re-established within 24 h.

Cathepsin D levels and protein catabolism

The marked decline in proteinase levels in response to acute or chronic starvation is somewhat unexpected in view of the general observation that rates of protein degradation *increase* during nutritional deprivation [16]. Lysosomal proteinases probably play a significant role in such increased degradation [17], so one might have expected their activities to remain constant or to rise in support of an increased rate of protein degradation.

The fact that they do not may be interpreted in several ways. One possibility is that the loss of cathepsin D upon acute starvation (Fig. 1*a*) occurs only in the intestinal cells, whereas cathepsin D in other cells remains available to catalyse intracellular protein degradation in those cells. It may be only coincidental, but the fraction of cathepsin D which is labilized by acute starvation corresponds quite closely to the fraction of total activity located in the intestinal cells.

A second possibility is that the rate-limiting step in degradation is not proteolysis *per se*, but rather the entry of proteins into the lysosome compartment. There is considerable evidence that the rate of autophagocytosis increases during starvation [16]. In addition, the level of any particular proteinase may be greater than is actually required. For example, fully-fed *C. elegans* appear to contain an excess of cathepsin D activity. Structural gene mutations (*cad-1*) which decrease cathepsin D activity by 10-fold in fully fed animals have little obvious effect on growth rate, development, morphology or behaviour [10]. Thus the 3–10-fold lower cathepsin D levels in starved wild-type animals may still be more than adequate to sustain increased rates of intracellular protein catabolism.

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REFERENCES

1. Hershko, A. & Ciechanover, A. (1982) Annu. Rev. Biochem. **51**, 335–364
2. Holzer, H. & Heinrich, P. C. (1980) Annu. Rev. Biochem. **49**, 63–91
3. Pontremoli, S. & Melloni, E. (1986) Annu. Rev. Biochem. **55**, 455–481
4. Brenner, S. (1974) Genetics **77**, 71–94
5. Sternberg, P. W. & Horvitz, H. R. (1984) Annu. Rev. Genet. **18**, 489–524
6. Bolanowski, M. A., Jacobson, L. A. & Russell, R. L. (1983) Mech. Ageing Dev. **21**, 295–319
7. Clokey, G. V. & Jacobson, L. A. (1986) Mech. Ageing Dev. **35**, 79–94
8. Sarkis, G. J., Kurpiewski, M. R., Ashcom, J. D., Jen-Jacobson, L. & Jacobson, L. A. (1988) Arch. Biochem. Biophys. **261**, 80–90
9. Sarkis, G. J., Ashcom, J. D., Hawdon, J. M. & Jacobson, L. A. (1988) Mech. Ageing Dev. **45**, 191–201
10. Jacobson, L. A., Jen-Jacobson, L., Hawdon, J. M., Owens, G. P., Bolanowski, M. A., Emmons, S. W., Shah, M. V., Pollock, R. A. & Conklin, D. S. (1988) Genetics **119**, 355–363
11. Mackenzie, J. M., Jr., Garcea, R. L., Zengel, J. M. & Epstein, H. F. (1978) Cell (Cambridge, Mass.) **15**, 751–762
12. Cassada, R. C. & Russell, R. L. (1975) Dev. Biol. **46**, 326–342
13. Swanson, M. M. & Riddle, D. L. (1981) Dev. Biol. **84**, 27–40
14. Lah, T. & Turk, V. (1982) Hoppe-Seyler's Z. Physiol. Chem. **363**, 247–254
15. Kominami, E., Tsukahara, T., Bando, Y. & Katunuma, N. (1987) Biochem. Biophys. Res. Commun. **144**, 749–756
16. Mortimore, G. E. & Mondon, C. E. (1970) J. Biol. Chem. **245**, 2375–2383
17. Mortimore, G. E. & Ward, W. F. (1981) J. Biol. Chem. **256**, 7659–7665

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