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Programmed cell death: dying cells synthesize a co-ordinated, unique set of proteins in two different episodes of cell death

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Intersegmental muscles of the tobacco hornworm, *Manduca sexta*, degenerate promptly after the ecdysis of the moth. Protein content of the growing, static, and degenerating muscle was evaluated by two-dimensional electrophoresis; RNA was isolated from the muscle and translated, and the translation products likewise analyzed by two-dimensional electrophoresis. Growing and static muscles synthesize predominantly myofibrillar proteins, and small cytosolic proteins constitute a vanishingly small proportion of the proteins identified even by silver staining. When the muscle begins to degenerate, a large number of smaller proteins is seen on the gels. Many of these are apparently fragments of the myofibrillar proteins, but over 30 can be recognized as new translation products. Most of the translation products are found in two regions, one ranging from 20 to 40 kDa and with a p*I* of 6.5–6.9; and the other approx. 50 70 kDa and p*I* 5.8–6.2. The pattern is identical in two separate instances of degeneration.

Cell death; Protein synthesis; Proteolysis; (Muscle, *Manduca sexta*)

1. INTRODUCTION

We have known for several years that, in many systems, protein synthesis is required in order for naturally occurring cell death to be carried out [1–5] and several groups have recently suggested that specific genes are activated to kill thymocytes [3,6] and prostate and other cells [7]. We report here that, in two independent instances of cell death involving the intersegmental muscles during the metamorphosis of an insect, a similar set of genes is activated. Thus, it appears that the cells evoke specific commands in order to destroy themselves.

2. MATERIALS AND METHODS

Eggs of *Manduca sexta* were purchased from Carolina Biological Supply (Burlington, NC) and reared on an artificial diet (no.9666, Otis modified hornworm diet, Bio-Serve, Frenchtown, NJ). Myofibrils, tropomyosin and troponin C were prepared by the method of Regenstein and Szent-Gyorgyi [8]. Highly purified myosin was prepared according to Chan and Reibling [9]. Protein was quantitated as described by Bradford [10] using bovine serum albumin as a standard. Total RNA was purified by the method of Cathala et al. [11]. Translation of RNA was carried out with an in vitro reticulocyte translation system (Promega Biotech, Madison, WI). Two-dimensional gels were run according to O'Farrell [12]. Gels were stained by a modification of the method of Wray et al. [13] in which proteins were fixed in 10% glutaraldehyde for 30 min and washed with several changes of distilled water prior to staining. Two-dimensional gels of in vitro translation proteins were treated with EnHance (New England Nuclear, Boston, MA) prior to drying and exposed at −60°C for periods of 1–3 weeks.

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3. RESULTS AND DISCUSSION

The guanidinium thiocyanate procedure for purifying RNA gave yields of 17–26 μg total RNA per 100 mg tissue (wet wt). Although the intersegmental muscle tissue mass decreased from approx. 125 mg in pharate adults to less than 20 mg in +10 h or older adults, there was no apparent difference in total RNA concentration per

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unit wt tissue. 2 μg total RNA was used in each translation, yielding an average of 24000 cpm. Translation products were analyzed by two-dimensional gel electrophoresis and compared to in vivo protein patterns. Translated myofibrillar proteins were identified by comparison with purified proteins.

The pattern of translation products can be summarized as follows: when the muscle is static or growing, the predominant products of translation are myofibrillar proteins, of which actin, paramyosin, troponin C, tropomyosin and myosin light chains 1 and 2 are easily identified (fig.1). Presumably, myosin heavy chain is also synthesized in vivo during this period but is not seen in the translation system because of the inefficient translation of the necessarily large mRNA. This situation occurs in growing 5th instar larvae as well as in pupae during the second and third weeks of development into a moth. As the muscle reaches a lytic phase, however, translation of myofibrillar proteins abruptly ceases (within a period of approx. 6 h) and is replaced by translation of a wide range of new proteins, primarily in two groups of molecular masses 20–40 and 50–70 kDa and p*I* values of 6.5–6.9 and 5.8–6.2, respectively, and which are clearly not major myofibrillar proteins (fig.2). The appearance of these translation products is sudden, since they are absent from muscles taken during the day preceding ecdysis and present at ecdysis. Several of the new products correspond to spots seen in whole muscle homogenates of similar age (fig.3). The new translation pattern remains essentially constant until well into the rapid lytic phase (+8 h post-ecdysis). The proteins originate within the muscle, since phagocytes do not enter the tissue until much later [14]. These same proteins are seen both during the first few days after pupation, when the anteriormost and posteriormost portions of the intersegmental muscles degenerate, and immediately after adult ecdysis, when the rest of the muscle degenerates (fig.4). The pattern is strikingly similar during the two independent instances of degeneration, with virtually every spot present and in approximately the same proportion in the two preparations.

At least some of the products seen in in vitro translation are synthesized in sufficient amounts to be detectable with silver staining. Presumably, those translated proteins that do not correspond to identical spots in gels of whole muscle homogenates are rare in vivo or undergo post-translational modifications, altering their molecular masses and isoelectric points. Although the rabbit reticulocyte system has been shown to be capable of efficiently translating numerous insect mRNAs [15,16], it is possible that some mRNAs present are incapable of being translated in this system. The similarity of the evidence from both in vivo and in vitro studies argues that the materials observed on the two-dimensional gels are not fragments resulting from site-specific RNases activated at the beginning of degeneration.

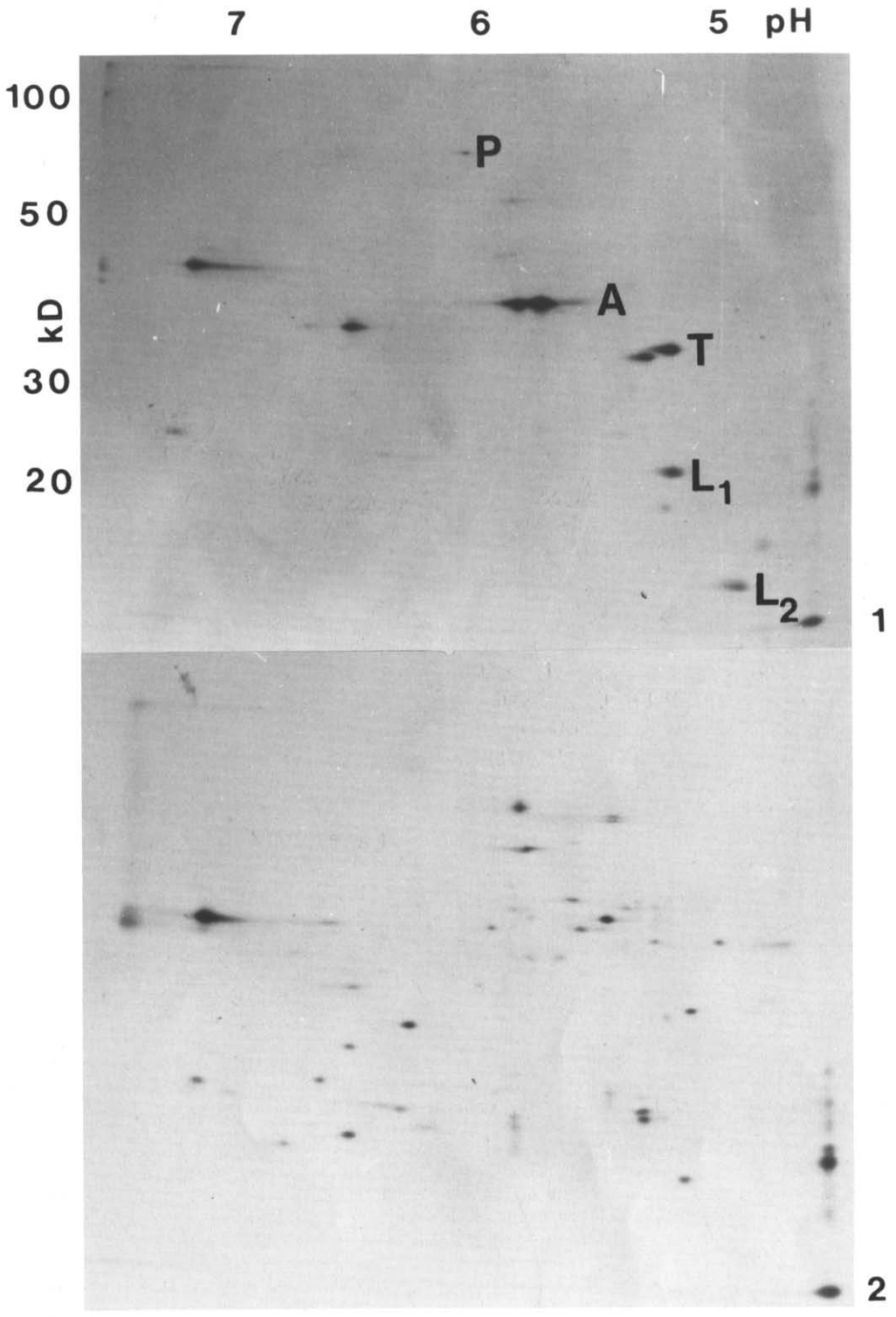
The slow lytic phase starting 3–4 days prior to ecdysis apparently does not require the later

Fig.1. Autoradiogram of translation products from muscle of an 11-day pupa. At this time the muscle is substantially in the steady state. Translation of the major myofibrillar proteins paramyosin (P), actin (A), tropomyosin (T) and light chains 1 and 2 (L_1 and L_2) is readily apparent, as the messages appear to be intact and the products identical to the native products. The protein of 35 kDa and p*I* 7.2 is currently unidentified. The streak at 40 kDa is an artifact of the translation kit.

Fig.2. Autoradiograms of 0 h adult. The messages for actin and tropomyosin remain barely detectable, and the pattern of synthesis has now changed to virtually entirely non-myofibrillar proteins. At this time the muscle is contractile and functional. The pattern of translation continues essentially unchanged from this point to the time of depolarization between 10 and 12 h after ecdysis.

Fig.3. Silver-stained preparation of proteins extracted from 10 h adult degenerating muscle, with those proteins which can be identified in autoradiograms of translations from freshly emerged adults being indicated by open arrows. Closed arrows indicate locations in which a translation product is seen on the autoradiogram, but for which the identity of the spot is possible but not certain. At least 17 and perhaps as many as 32 of the proteins produced by the translation system can be found in the degenerating muscle, arguing that the translation products are not artifactual.

Fig.4. Autoradiogram of 2-day new pupa, when the anteriormost and posteriormost portions of the intersegmental muscle degenerate, with superimposed pattern from 10 h adult. The positions of actin and tropomyosin are indicated by dashed lines. The pattern is virtually identical in both preparations.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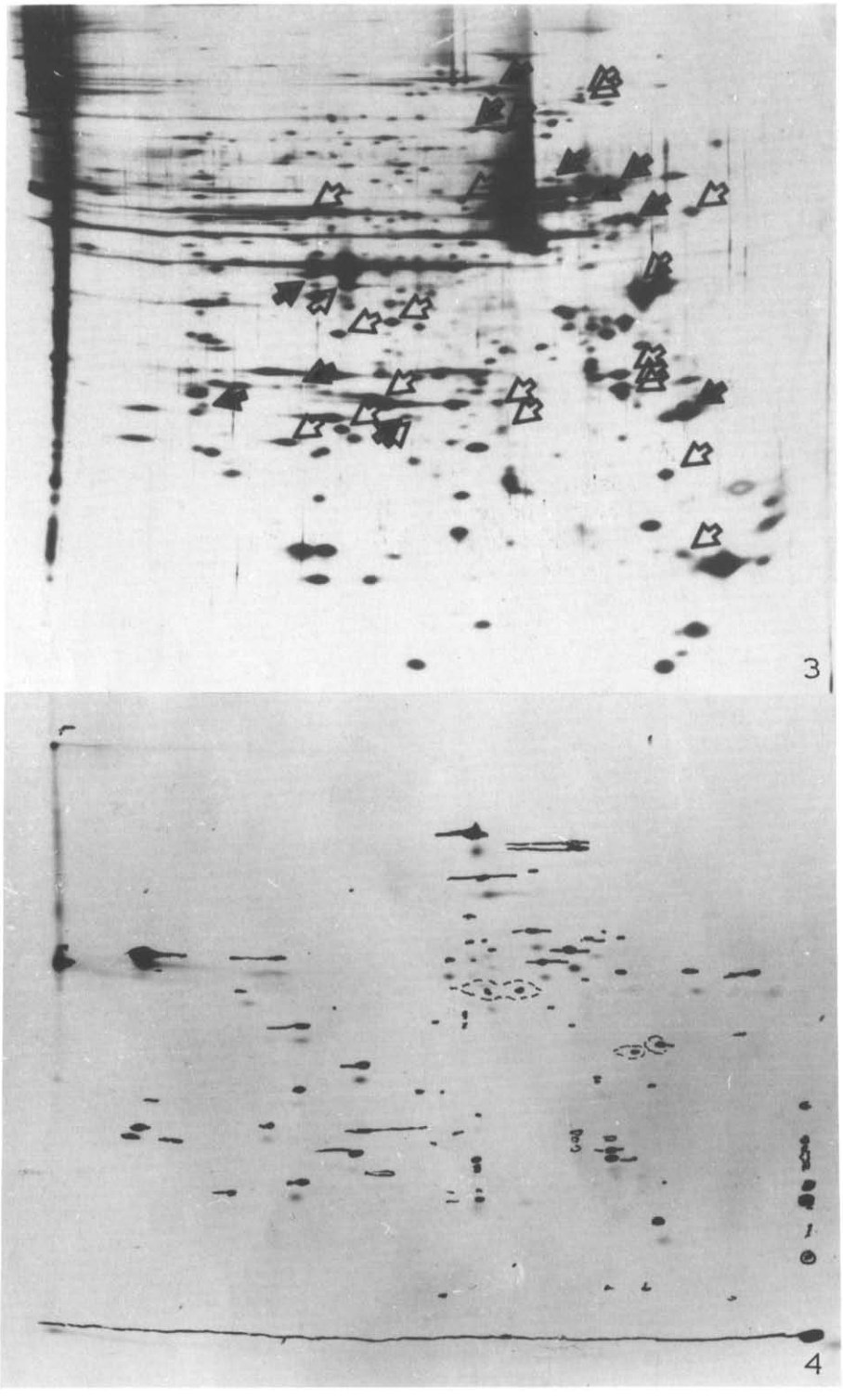
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observed burst of translatable mRNAs. However, after ecdysis and the presumed burst in protein synthesis, the involution of the muscle accelerates rapidly. It appears therefore that at least some of the new mRNAs are required for involution, since inhibitors of RNA and protein synthesis in similar Saturniid moth intersegmental muscle delay involution [2,17]. The striking similarity of the patterns at pupal ecdysis, when the anteriormost and posteriormost muscles are destroyed, and at adult ecdysis, when the abdominal muscles are destroyed, suggests that the co-ordinate synthesis of these proteins is a major factor in involution. Since the cells are under stress at this time and it is known that several factors, including both hypoxia and inhibition of oxidative phosphorylation, and even increases in calcium levels, can induce the synthesis of stress proteins [18–20], this possibility is currently being explored. The proteins appear to be too alkaline to be heat-shock proteins. Most interestingly, testosterone-repressed prostate message 2, which appears to be a ubiquitous gene, the expression of which is apparently associated with cell death (Buttyan, R., Columbia University, personal communiation) may also be present (unpublished). Otherwise the function of the genes remains unknown and awaits our ability to probe these spots with specific antibodies or to isolate and characterize the genes. This story [21] and similar accounts [22–24] have recently been reported in abstract form.

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