1990

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Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 6594–6598, September 1990
Developmental Biology

Gene activation is required for developmentally programmed cell death

(cloning/*in vitro* translation /degeneration/muscle/ecdysteroid)

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Communicated by Igor B. Dawid, June 11, 1990 (received for review March 28, 1990)

ABSTRACT **The intersegmental muscles of the tobacco hawkmoth *Manduca sexta* die during the 36-hr period after metamorphosis. The trigger for cell death is a fall in the ecdysteroid titer. Commitment of the intersegmental muscles to degenerate involves selective repression and activation of ecdysteroid-responsive genes. When the pattern of gene expression is altered after injection of either 20-hydroxyecdysone or actinomycin D, the muscles persist. cDNA clones have been isolated for four genes that become abundantly expressed coincident with the commitment to degenerate. The data presented here indicate that programmed cell death is not due to the cessation of macromolecular synthesis in condemned cells but rather is due to the activation of a differentiative pathway.**

During both embryonic and post-embryonic development, many cells die by a process that appears to be independent of age-related senescence (1). This developmentally programmed cell death serves a variety of functions in different systems. For example, much of the sculpting of the body form involves the removal of unneeded cells, such as in the formation of the digits during embryogenesis (2). Cell death can also serve to adjust the number of neurons innervating either central or peripheral targets (3). As well, cell death can remove obsolete tissues during development, as occurs during metamorphosis in amphibia (4) and insects (5, 6).

Despite the ubiquity of programmed cell death, there is relatively little information available regarding either the triggers or mechanisms that mediate this process. In the embryonic chicken nervous system, motor neuron degeneration appears to be regulated by competition among neurons for peripheral muscle targets (3). A muscle-derived "trophic" factor is currently being purified for characterization (7). In the nematode *Caenorhabditis elegans*, the trigger(s) responsible for programmed cell death are unknown, although several mutations have been identified that block normal patterns of neuron loss (8, 9). The molecular isolation and characterization of the genes containing these mutations may provide considerable insight into the mechanisms of cell death.

Another system that has proven useful for the analysis of cell death is the intersegmental muscles (ISMs) of *Lepidoptera* (10). Each fiber within these muscles is approximately 5 mm long and 1 mm in diameter. The fibers are organized in sheets that span each of the abdominal segments in the larva. After pupation, the ISMs in the first two and last two segments degenerate, leaving four segments of intact muscle remaining. The ISMs are then retained until 3 days before adult eclosion (day 15 of development) at which point they begin to atrophy. Atrophy results in a loss of 40% of muscle mass, without any change in resting potential, force/cross sectional area, or skinned-fiber calcium sensitivity (L.M.S., unpublished data). The ISMs are used during the performance of the eclosion (emergence) behavior on day 18, the last day of pupal/adult development. After eclosion, the ISMs are no longer needed by the animal and undergo a rapid degeneration during the subsequent 36-hr period (11).

In the tobacco hawkmoth *Manduca sexta*, the trigger for ISM atrophy and degeneration is a decline in the insect molting hormone, 20-hydroxyecdysone (20-HE) (12). When the ecdysteroid titer falls below a specific threshold on day 15 of development, the muscles begin to atrophy. If the ecdysteroid titer is artificially maintained at this intermediate level by injection of 20-HE, the muscles do not degenerate but instead continue to atrophy. During normal development, there is a natural decline in the ecdysteroid titer early on day 18, which commits the ISMs to degenerate. Once committed, the muscles cannot be rescued with exogenous hormone.

One plausible mechanism for initiating ISM degeneration could be the cessation of transcription and/or translation. Blockage of either process might then allow degradation of cellular constituents to outstrip synthesis, resulting in the loss of cellular mass. We have examined transcriptional and translational activity of ISMs during development in the hawkmoth *M. sexta*. We report here that the commitment of the muscle to die does not involve metabolic shutdown but rather does involve selective regulation of gene expression in the muscles.

EXPERIMENTAL PROCEDURES

Animals. The tobacco hawkmoth, *M. sexta*, was reared as described (12). Comparably sized animals were staged during development according to specific cuticular markers. In some experiments, animals were injected with pharmacological agents [actinomycin D (ActD) and 20-HE; Sigma] or radioisotopes. A Hamilton syringe was used to introduce 10–25 μl into the dorsal thorax.

Insects were dissected under saline (12) and the lateral ISMs were removed for assay. Where muscle mass was determined, the ISMs were placed on preweighed foil, dried at 60°C overnight, and then reweighed. The mass of the muscle is expressed as {[mg of ISMs (dry weight)/gram of body weight] × 1000}.

Protein Gels. Individual staged insects were injected with 500 μCi (1 Ci = 37 GBq) of Trans ^{35}S-Label ([^{35}S]methionine; ICN) and maintained at room temperature for 5.5 hr. The lateral ISMs were rapidly dissected free and all adhering fat body and trachea were removed. The samples were homogenized in loading buffer, boiled, and fractionated in one dimension with 10% polyacrylamide gels containing SDS (13). Equal amounts of protein were loaded per lane. Gels were incubated in EN^{3}HANCE (NEN), dried, and fluorographed with Kodak XAR x-ray film.

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Abbreviations: ISM, intersegmental muscle; 20-HE, 20-hydroxyecdysone; ActD, actinomycin D.

[^{35}S]Methionine-labeled ISM proteins were also fractionated by two-dimensional polyacrylamide gel electrophoresis as described by O'Farrell (14) and Garrels (15). *In vivo*-labeled samples were solubilized in 2% (wt/vol) SDS/50 mM Tris·HCl, pH 6.7/5% (vol/vol) 2-mercaptoethanol/0.005% butylated hydroxytoluene and boiled for 2 min. The SDS was removed by adding Nonidet P-40 nonionic detergent in an 8-fold excess to the SDS (wt/wt) (16). Samples were then focused on narrow-range ampholines (pH 5–7) and size-fractionated on a 10% polyacrylamide gel.

RNA. The ISMs have high levels of RNase (L.M.S., unpublished data), and so great care was required in isolating RNA. Animals were rapidly dissected under ice-cold saline and the ISMs were frozen in liquid nitrogen. Groups of muscles from approximately 20 animals were homogenized with a Tissuemizer (Tekmar, Cincinnati, OH) in 4 M guanidinium isothiocyanate extraction buffer as described by MacDonald *et al.* (17). Total RNA was isolated by centrifugation through a 5.7 M CsCl pad. The pellet was resuspended in 5% 2-mercaptoethanol/0.5% sarkosyl/5 mM EDTA, phenol/chloroform-extracted, and ethanol-precipitated. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (18).

For Northern blots, 15 μg of total RNA was denatured in formaldehyde and separated by size in 1.5% agarose (19) prior to transfer to Zeta-Probe membranes (Bio-Rad). Blots were hybridized overnight at 65°C under stringent conditions in 1 mM EDTA/7% SDS/0.5 M NaH_2PO_4, pH 7.2. The ^{32}P-labeled DNA probes are described below. Blots were autoradiographed with Kodak XAR film.

RNA was translated *in vitro* with a rabbit reticulocyte lysate from Stratagene using [^{35}S]methionine (Amersham). Protein products were analyzed as above.

Library Construction. cDNA was synthesized from day 18 ISM poly(A)$^+$ RNA with a cDNA synthesis kit (Boehringer Mannheim), ligated to *Eco*RI linkers, and cloned into λgt10. The library had approximately 86,000 original recombinants, with an average insert size of 600 base pairs and was amplified once before use. High specific activity single-stranded ^{32}P-labeled cDNA was synthesized with avian myeloblastosis virus reverse transcriptase (International Biotechnologies) and oligo(dT) as described by Gerard (20). Individual plaques were picked and transferred to duplicate *Escherichia coli* lawns and lifts were taken with nylon filters (Micron Separation, Westboro, MA). Clones were screened by differential hybridizations with radioactive cDNAs from day 15 (pre-commitment) and day 18 (post-commitment) mRNA (plus/minus screening). Recombinants that displayed differential labeling with the two probes were rescreened to verify the initial observation. Inserts were then isolated by polymerase chain reaction amplification using primers that flanked the *Eco*RI sites in λgt10 (21). Inserts were nick-translated (22) and used to probe Northern blots (above).

RESULTS

Patterns of Protein Expression. To determine the synthetic capacity of the ISMs, individual insects were injected with [^{35}S]methionine at various stages of pupal–adult development. After a 5.5-hr equilibration period, the ISMs were isolated and the cellular proteins were fractionated by SDS/polyacrylamide gel electrophoresis. As can be seen in Fig. 1, the ISMs displayed active protein synthesis at all stages examined, with little apparent quantitative difference.

Careful examination of this figure reveals several protein bands that are apparent only at select stages of development. To obtain better resolution of the patterns of protein expression associated with the commitment of the muscles to degenerate, radio-labeled proteins were fractionated by two-dimensional polyacrylamide gel electrophoresis. The times

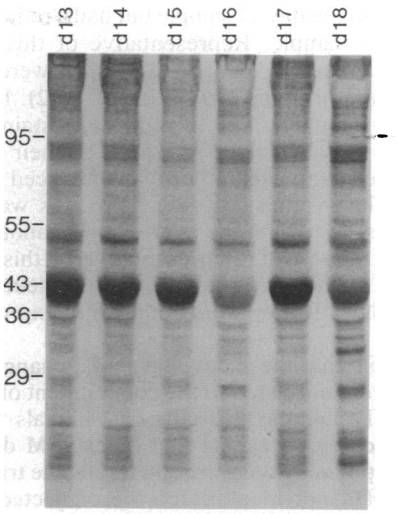

FIG. 1. Patterns of ISM protein synthesis during development. Individual insects were injected with [^{35}S]methionine. After a 5.5-hr equilibration period, the ISMs were removed and homogenized, and protein extracts were fractionated on a 10% polyacrylamide gel. Newly synthesized radioactive proteins were detected by fluorography. d, Day of development. Molecular masses in kDa are indicated to the left.

selected for examination were days 17 and 18 of development, since the ISMs become committed to degenerate early on day 18 (12). The majority of the ISM proteins were expressed at both stages of development (Fig. 2). However, subsets of proteins were developmentally regulated, in that they were substantially more abundant at one stage of development relative to the other. In one group, proteins

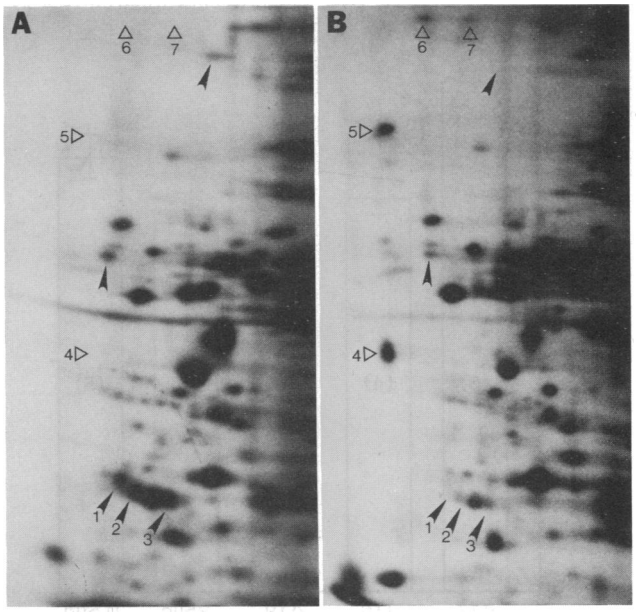

FIG. 2. Patterns of newly synthesized ISM proteins before and after the commitment to degenerate. ISM proteins were labeled as in Fig. 1 and then fractionated by two-dimensional polyacrylamide gel electrophoresis. Ampholines used in the first dimension were from pH 4 to pH 6 (left-to-right on the gel). The second dimension was fractionated on a 10% polyacrylamide gel. (*A*) Day 17 of development. (*B*) Day 18 of development. Solid arrowheads denote proteins that were abundantly expressed on day 17 (prior to commitment to degenerate) but reduced or absent on day 18 (subsequent to commitment). Open triangles identify proteins that were abundant on day 18 but not on day 17. Proteins 1–3 are representative of the former class; proteins 4–7 are representative of the latter.

were present in the day 17 sample but had a reduced intensity in the day 18 sample. Representative of this group were proteins designated 1, 2, and 3, which were acidic low molecular mass proteins (≈25 kDa; pI ≈5.2). Interestingly, these proteins could still be seen on silver-stained gels from day 18 (data not shown), suggesting that their absence was due to reduced synthesis rather than enhanced degradation.

Whereas the synthesis of some proteins was decreased between days 17 and 18, the expression of another group of proteins was increased. Representative of this group were proteins 4–7. Although all four of these proteins were undetectable on day 17, they were abundantly expressed on day 18.

It is possible that these developmental changes in protein expression were unrelated to the commitment of the ISMs to degenerate. To address this concern, animals were treated with pharmacological agents that block ISM degeneration. The two compounds used were 20-HE and the transcriptional inhibitor ActD. Individual insects were injected late on day 17 of development with either 20-HE at 5 μg/g (12) or ActD at 0.1 μg/g. When examined on day 19, the ISMs of control animals were degenerate and noncontractile, but those of treated animals were comparable to that of preeclosion animals (Fig. 3). Grossly, the ISMs from treated animals appeared to be identical to muscles from control preeclosion animals. Once the ecdysteroid titer declined in 20-HE-treated animals, the ISMs did subsequently degenerate (12). However, the ISM degeneration was not observed in ActD-treated animals, even when examined 6 days after drug injection (L.M.S., unpublished data).

Since 20-HE and ActD blocked ISM degeneration at the normal time in development, we examined their effects on *de novo* protein synthesis. Individual animals were injected on day 17 with 20-HE or ActD and then reinjected on day 18 with [^{35}S]methionine, and the ISM proteins were fractionated on two-dimensional gels as above. These agents apparently were not toxic to the muscles, since the ISMs were biosynthetically active 24 hr after treatment. The patterns of expression seen on these gels were similar to that seen in day 18 control ISMs (Fig. 4). For example, the normal reduction in proteins 1, 2, and 3 was still observed in these muscles. However, pretreatment did block the appearance of some of the newly synthesized day 18 proteins. For instance, proteins 4 and 7 were expressed in the steroid-treated muscles, but proteins 5 and 6 were not. In the ActD-injected animal, proteins 4, 5, and 6 were present in the ISMs, but protein 7 was absent. Therefore, subtle perturbations in the patterns of proteins made by the ISMs had a profound effect on their development.

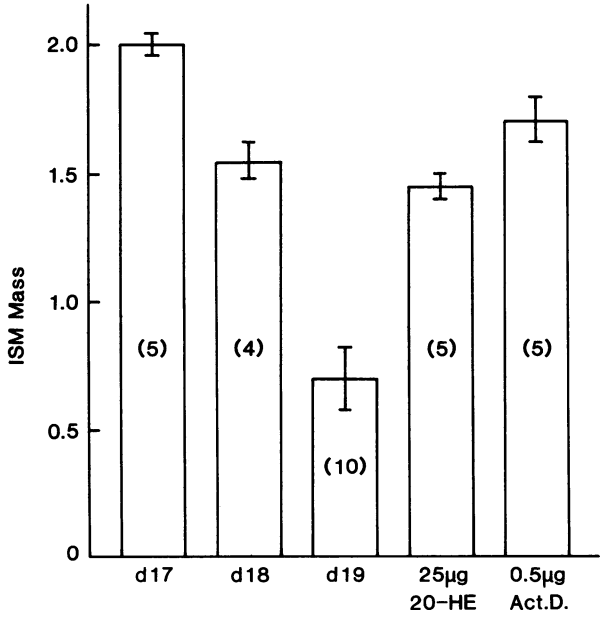

FIG. 3. Effects of ActD and 20-HE on ISM degeneration. The mass of the ISMs was determined after treatment and then normalized to the weight of the insect {[mg of ISMs (dry weight)/g of body weight] × 1000}. Histogram bars for d17–d19 show the normal decline in ISM weight after the completion of the atrophy program and the initiation of degeneration. Test animals were injected on day 17 and their muscle mass was measured on day 19, 16 hr after the time of eclosion. Test insects received either ActD at 0.1 μg/g or 20-HE at 5 μg/g. Numbers in parentheses are the number of insects tested. Values are mean ± SEM.

Changes in RNA Expression. The data presented above suggest that ISM degeneration is associated with both the loss and appearance of several proteins. We next sought to determine whether these changes were regulated at the level of transcription or translation. RNA was isolated from muscles at eight developmental stages, ranging from prior to atrophy to well into the degeneration phase. In addition, one group of animals was treated with 20-HE on day 17 and RNA was isolated from their ISMs 5 hr after the time of normal eclosion on day 18. Each sample was translated *in vitro* in the presence of [^{35}S]methionine, and the protein products were fractionated by one-dimensional polyacrylamide gel electrophoresis (Fig. 5). At each stage examined, there was a full size range of proteins synthesized, suggesting that the RNA was intact and translatable. The majority of the synthesized proteins were comparably expressed at each developmental stage, which supports their role as "housekeeping" sequences. However, there were a small number of developmentally regulated transcripts as well. Most dramatic were the protein products that disappeared coincident with commitment. Seven such RNAs were abundantly expressed between days 14 and 17 but were reduced or missing at the time of commitment. Conversely, products from an additional seven of the RNAs were apparent on day 18 but were not observed at earlier stages of development. Pretreatment

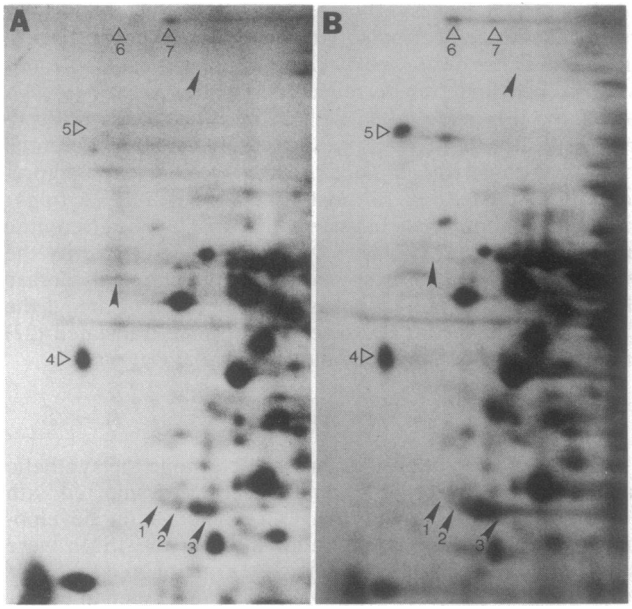

FIG. 4. Effects of ActD and 20-HE on *in vivo* protein expression. Animals were treated with either drug on day 17, as described in Fig. 3, and injected on day 18 with [^{35}S]methionine, and the proteins were fractionated as in Fig. 2. (*A*) 20-HE. (*B*) ActD. Solid arrowheads identify the location of the proteins that were down-regulated between days 17 and 18 in control animals. Open triangles identify the location of the proteins that normally appear coincident with muscle commitment to degenerate (see Fig. 2).

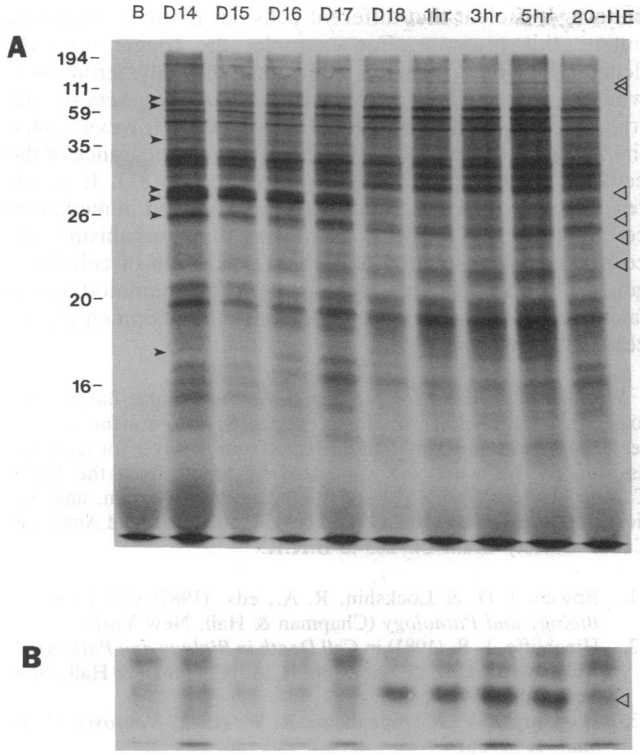

FIG. 5. *In vitro* translation of ISM RNA during development. Equal amounts of RNA for each stage of development were translated *in vitro* with a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The newly synthesized proteins were fractionated in 10% polyacrylamide gel (*A*) or in 12% polyacrylamide gel (*B*). (The molecular mass for the up-regulated protein in *B* is not known, since this was in a nonlinear region of the gel.) Solid arrowheads identify proteins from RNAs that were expressed primarily before commitment (day 18); open triangles show proteins from RNAs that appeared after commitment. D, day of development; 20-HE, animals pretreated on day 17 with 20-HE and examined 5 hr after the normal time of eclosion; hr, time (hr) after adult eclosion (emergence). Molecular masses in kDa are indicated to the left in *A*.

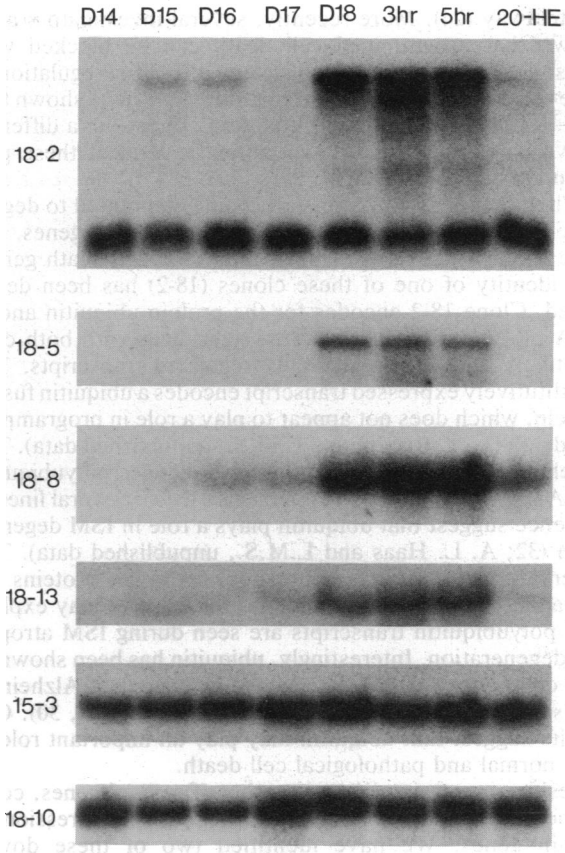

FIG. 6. Northern blots labeled with select cDNA clones. ISM RNA was isolated from animals at various stages of development and from animals pretreated with 20-HE. Total RNA (15 μg) from each stage was fractionated in 1.5% agarose under denaturing conditions and transferred to Zeta-Probe membranes. Ethidium bromide was included in the RNA samples, so that rRNA could be visualized after fractionation to ensure equal loading of RNA. Blots were probed with ^{32}P-labeled cDNA from individual clones isolated by plus/minus screening of a day 18 cDNA library. Blots were exposed for comparable lengths of time and, in most cases, were stripped of probe and reprobed with another labeled clone. Day 18 ISM RNA was included from animals pretreated on day 17 with 20-HE.

with 20-HE greatly reduced the developmental changes in the coding potential of the ISM RNA, producing a pattern that was intermediate between patterns at days 17 and 18.

Isolation of cDNA Clones. From the findings above, we suspected that a number of the newly expressed ISM proteins were encoded by differentially expressed mRNAs. To characterize these sequences and their presumptive protein products, we used mRNA from day 18 of development to construct a cDNA library in λgt10. The library was screened with single-stranded ^{32}P-labeled cDNA made from poly(A)$^+$ RNA isolated from the muscles on days 15 (precommitment) and 18 (post-commitment). After several rounds of screening, 40 clones were isolated that were labeled more strongly with the cDNA from day 18 than day 15. After screening for duplicate recombinants, four unique clones were isolated, 18-2, 18-5, 18-8, and 18-13. In addition, two clones that gave equal signals with each probe were selected as controls (clones 18-10 and 15-3).

RNA was isolated from the ISMs at several developmental stages and from animals pretreated with 20-HE. The RNA was fractionated in agarose and blotted to membranes. These Northern blots were then hybridized with radio-labeled probes from each of the recombinants described above (Fig. 6). Clones 15-3 and 18-10 labeled constitutively expressed transcripts that were unaffected by developmental stage or steroid treatment. In contrast, all four of the recombinants isolated by plus/minus screening labeled transcripts whose abundance changed during development. The mRNA corresponding to clone 18-5 was undetectable until day 18, at which point it became an abundant transcript. RNAs labeled by clones 18-8 and 18-13 were present prior to commitment, although at markedly reduced levels. Clone 18-2 labeled members of the multigene family within the ISMs. One transcript was constitutively expressed, whereas others were developmentally regulated. The transiently expressed transcripts were present at low level during the early phases of atrophy (days 15 and 16), disappeared on day 17, and then were abundant coincident with commitment. In all cases, expression of the developmentally regulated transcripts was repressed by pretreatment with 20-HE, but constitutively expressed transcripts were unaffected. These data support the contention that cell death is associated with increased expression of certain genes.

DISCUSSION

Programmed cell death has been observed during the development of virtually all metazoan organisms (1). In the few cases that have been characterized, cells appear to die either in response to a specific trigger (4, 11) or by the disappearance of a required trophic factor (7, 12, 23). Historically, the general assumption has been that once a cell is fated to die, macromolecular synthesis is terminated and the cells simply

waste away (24). More recently, several investigators have shown that programmed cell death can be blocked with transcriptional inhibitors, suggesting a role for regulation of gene expression (4, 25–27). In this study, we have shown that ISM degeneration requires activation of genes in a differentiative pathway and we have cloned several of these presumptive cell-death genes.

When the ISMs of *Manduca* become committed to degenerate, they express a complement of cell-death genes. We have isolated cDNA clones for four such cell-death genes. The identity of one of these clones (18-2) has been determined. Clone 18-2 encodes for the protein ubiquitin and in RNA blotting experiments cross-hybridizes with both constitutively and developmentally regulated transcripts. The constitutively expressed transcript encodes a ubiquitin fusion protein, which does not appear to play a role in programmed cell death (S. T. Bishoff and L.M.S., unpublished data). The developmentally regulated transcript encodes polyubiquitin (32; A. Myer and L.M.S., unpublished data). Several lines of evidence suggest that ubiquitin plays a role in ISM degeneration (32; A. L. Haas and L.M.S., unpublished data). The observation that ubiquitin functions to target proteins for degradation by a nonlysosomal proteinase (28) may explain why polyubiquitin transcripts are seen during ISM atrophy and degeneration. Interestingly, ubiquitin has been shown to be a component of the cytoplasmic inclusions of Alzheimer and several other neurodegenerative diseases (29, 30). Our results suggest that ubiquitin may play an important role in both normal and pathological cell death.

Besides increasing the expression of several genes, commitment is also associated with the apparent repression of certain genes. We have identified two of these down-regulated transcripts as the products of the actin and myosin genes (L.M.S. and L.K., unpublished data). The identities of the other regulated genes are unknown. Some may encode other contractile proteins. It is interesting to speculate that other regulated genes may encode products that play a negative role in the commitment process, such as repressing cell-death gene expression. Alternatively, they may encode ecdysteroid-responsive sequences that were used by the ISMs at earlier stages of development but are unrelated to the cell-death process. We have made a cDNA library from precommitment ISM poly(A)$^+$ RNA, so that recombinants encoding these repressed genes can be isolated for characterization.

It appears that 20-HE modulates these developmental changes in ISM gene expression. Between days 17 and 18 of development, there is a decline in the circulating ecdysteroid titer that commits the ISMs to degenerate (12). During this time interval, a number of specific mRNAs and proteins either increase or decrease in abundance in the ISMs. When 20-HE is injected into day 17 animals, cell death can be delayed and macromolecular synthesis in the ISMs is perturbed. The ISMs do subsequently degenerate, presumably due to a decline in the titer of the injected hormone.

We interpret the inhibitory effect of ActD on the ISM cell-death program to be at the level of transcription. Administration of ActD to day 17 animals prevents the loss of the ISMs for up to 6 days after eclosion. This pharmacological block of ISM degeneration is accompanied by the absence of specific proteins that normally appear in the condemned ISMs. Interestingly, a few proteins do appear *de novo* in the ISMs after ActD treatment, which suggests that there may be translational as well as transcriptional control in this system. However, expression of these proteins appears to be insufficient to direct cell death, as other regulatory gene products may be necessary.

In the various metazoa examined to date, programmed cell death is initiated in a reproducible temporal and spatial pattern. It is clear that different cells use distinct triggers to initiate this program. For example, the trigger for ISM degeneration in *Manduca* is a decline in the ecdysteroid titer, whereas in a related moth, *Antheraea polyphemus*, the trigger is the peptide eclosion hormone (5). However, once the ISMs are committed to degenerate, the appearance of the cells seems to be the same in both species (31). It seems reasonable to speculate that the genes involved in mediating cell death in these moths, as well as other organisms, are conserved. The isolation and characterization of cell-death genes in simpler systems, such as moths and nematodes, will facilitate the examination of these basic developmental processes in other organisms.

We thank L. Gilbert for providing *Manduca* pupae during pilot experiments, J. Buckner for *Manduca* eggs, S. Robinson for a critical reading of the manuscript, and S. Bishoff and A. Myer for technical assistance. This work was supported by grants from the North Carolina Molecular Biology and Biotechnology Program, and National Institutes of Health Grant GM40458 to L.M.S., and American Cancer Society Grant CD-263 to B.K.K.

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