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# PROJECT ADMINISTRATION DATA SHEET



[X] ORIGINAL [ ] REVISION NO.

Project No. G-32-624 (R5994-0A0) GTRC/~~GIT~~XXX DATE 7/31/85

Project Director: S. M. Politz School/~~Lab~~XXXX Biology

Sponsor: National Science Foundation

Type Agreement: Grant DCB-8510567

Award Period: From 8/1/85 To 7/31/88* (Performance) 4/30/88 (Reports)

| Sponsor Amount: | This Change | Total to Date |
|---|---|---|
| Estimated: | $ | $ 138,000 |
| Funded: | $ | $ 138,000 |

Cost Sharing Amount: $ 6,795 Cost Sharing No: G-32-339

Title: Genetic Specifications of Nematode Surface Antigens

## ADMINISTRATIVE DATA

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Defense Priority Rating: N/A Military Security Classification: N/A

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## RESTRICTIONS

See Attached NSF Supplemental Information Sheet for Additional Requirements.

Travel: Foreign travel must have prior approval — Contact OCA in each case. Domestic travel requires sponsor approval where total will exceed greater of $500 or 125% of approved proposal budget category.

Equipment: Title vests with GIT

## COMMENTS:

*Includes 6 month unfunded flexibility period.

No funds may be expended after 1/31/88.



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## SPONSORED PROJECT TERMINATION/CLOSEOUT SHEET

Date 10/14/88

Project No. G-32-624 / R5994-0A0 School/~~Unit~~ Applied Biology

Includes Subproject No.(s) N/A

Project Director(s) S. M. Politz GTRC/~~GTRI~~

Sponsor National Science Foundation

Title Genetic Specifications of Nematode Surface Antigens

Effective Completion Date: 7/31/88 (Performance) 10/31/88 (Reports)

Grant/Contract Closeout Actions Remaining:

- [X] None
- [ ] Final Invoice or Copy of Last Invoice Serving as Final
- [ ] Release and Assignment
- [ ] Final Report of Inventions and/or Subcontract:
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- [ ] Govt. Property Inventory & Related Certificate
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Progress Report
Samuel M. Politz, Principal Investigator

The following is a summary of research progress supported by my NSF grant DCB-8510567 "Genetic Specification of Nematode Surface Antigens" from 8/85 to date. Early results on adult-specificity of rabbit antisera were obtained during a prior postdoctoral in R. S. Edgar's laboratory at UC Santa Cruz and are mentioned here for clarity.

1. Evidence for adult-specific surface antigens. Rabbit antisera were prepared against a total mixture of adult cuticle proteins (21). Antisera were tested for stage-specific binding to live nematodes by immunofluorescence and radioimmunoassay . Briefly, nematodes were incubated sequentially with anti-cuticle antibodies followed by either fluorescein (FITC) anti-rabbit antibody or iodine-125 labeled protein A. FITC labeled nematodes were observed microscopically; total bound radioactivity in a population of radiolabeled nematodes was measured in a gamma counter as a more quantitative confirmation of antibody binding. Crude antiserum cross-reacted with the larval surface, but could be rendered adult-specific by adsorption with large numbers of live L4 (fourth larval stage) animals . These adult-specific antibodies are now our standard reagents in immunofluorescence assays of genetically determined surface antigen phenotypes; experiments proposed here rely on them extensively.

2. Discovery of a gene that causes surface antigenic differences between C. elegans varietal strains.

Twelve independently-isolated varietal strains of C. elegans were tested for surface immunofluorescence using an adult-specific antiserum prepared against antigens isolated from the standard wild-type strain, N2. This antiserum did not bind to adults of three varietal strains, PA-1, DH424, or Bergerac BO. Thus these strains appeared to lack the adult-specific surface antigens detected on the wild-type surface.

Dominance and genetic linkage of the surface antigen phenotype were analyzed in crosses between N2 and the antigen-negative strain PA-1. Results were as follows:

a. The antigen-positive phenotype of N2 was dominant over the negative phenotype of PA-1 in heterozygous cross progeny .

b. A surprisingly simple linkage pattern emerged from crosses between PA-1 and N2 strains marked with standard visible linkage markers. The genetic determinant of the adult surface antigen phenotype was a single genetic locus on linkage group II. Markers on all 5 autosomal linkage groups were tested; three different visible markers on II showed significant linkage to the surface antigen marker . Markers on the other autosomes showed no significant linkage.

c. We have mapped the locus, now designated srf-1 on linkage group II . Two three-factor crosses placed srf-1 on the right arm of II near the marker rol-1. Deficiency mapping experiments further clarified the position of srf-1 and supported the suggestion from linkage data that a single gene or cluster of genes is involved. To our knowledge, this is the first characterization of a genetic locus specifically affecting surface antigen in a nematode.

d. We attempted to determine if the antigen-negative strain DH424 expresses an antigenic type different from, and perhaps allelic to, the one expressed by N2. Rabbit antibodies were raised against adult cuticle proteins isolated from DH424. After adsorption with N2 adults to remove cross-reacting antibodies, residual antibodies bound to neither the DH424 nor the N2 surface. Although we cannot eliminate the possibility that DH424 makes an allelic

antigen that is non-immunogenic, the results suggest that the gene carried by DH424 may be an inactive allele of srf-1.

3. Isolation of putative heterochronic mutants using adult-specific antibodies. Immunofluorescence of large populations of live nematodes viewed under a specially adapted epifluorescent stereomicroscope was used to isolate 16 C. elegans mutants that apparently express adult-specific surface antigens at inappropriate developmental times. Mutants were isolated after ethyl methane sulfonate (EMS) mutagenesis; mutagenized Po populations were allowed to segregate mutations in the homozygous condition by two generations of self-fertilization prior to antibody screening. Populations were screened in thin liquid layers and interesting individuals were picked with a microcapillary pipet under the microscope. Only mutants with independent post-mutagenesis pedigrees were picked. Mutants were isolated in three procedures that select for potentially different phenotypes.

a. Reversion of lin-4 retarded mutants. lin-4 animals express a larval cuticle as an adult (1), and do not bind our adult-specific rabbit antibodies . Revertant animals were easily identified as the only animals in the lin-4 background that bound adult-specific antibody and "lit up" in indirect immunofluorescence assay. Three mutants have been isolated in this way. Two have an adult multi-vulva phenotype (yj46, yj48), and one has a hermaphrodite tail defect (yj42).

b. Isolation of precocious mutants developing through the normal developmental pathway. Populations of nematodes in the wild-type genetic background were synchronized by harvesting L1 animals that hatched from previously laid eggs in a one hour period. Synchronously developing larvae were screened with adult-specific antibodies by immunofluorescence to identify mutants that bound adult-specific antibody as larval stages. Six mutants have been isolated in this way. All the mutants bind adult-specific antibodies as larval animals, with high penetrance. Three mutants appear to express the adult-specific antigen at all larval stages, while three appear to express it only at late larval stages (e.g, L3-L4 sized animals with no vulva). Four mutants have no visible abnormalities; of the other two, one has a hermaphrodite "club tail" (yj10). The other (yj2aa) has several defects including an uncoordinated (Unc) phenotype and pharygeal abonormalities. The latter two mutants were backcrossed to wild-type and the visible phenotypes resegregated; precocious adult antigen expression co-segregated with the visible phenotypes through two backcrosses. By linkage testing with standard genetic markers, the club tail mutation was assigned to linkage group III and the Unc-pharyngeal mutation to linkage group I.

c. Isolation of precocious mutants developing through the dauer developmental pathway. Under conditions of starving or overcrowding, L2 animals molt into a specially adapted arrested stage called the dauer larva (24). Upon refeeding, dauers molt into L4's and continue development into adults. Large populations can be synchronized by refeeding dauers. The adults and L4's developing from dauers may be distinct from those developing from L3's, however. For example, adult sqt-2 mutants are squat if they develop from L3's, but are rollers if they develop from dauers (25). In addition, most known heterochronic cell lineage mutants show wild-type rather than heterochronic phenotypes after passage through dauer (V. Ambros, personal communication). The only known exceptions are lin-29 mutants, which have an heterochronic phenotype after either pathway.

We have isolated seven precocious mutants by refeeding dauer larvae and screening the synchronously developing population to identify mutants that bind adult-specific antibody in immunofluorescence assay as L4's. By the

above argument, these may identify a class of mutants functionally distinct from those obtained via the L3 developmental pathway.

Precocious mutants obtained after dauer formation show the precocious surface antigen phenotype with high penetrance. Five appear to express it at all larval stages, and two appear to express it only at late larval stages (L3-L4). Four mutants have no visible phenotype. Of the other three, all have mutiple, partially penetrant visible defects. One has vulvaless (Vul) adults, and another has both Vul and multi-vulva (Muv) adult phenotypes. The third has a "club tail" hermaphrodite phenotype.

To summarize, we have isolated 16 independent heterochronic mutants. We can estimate by observed mutation frequency the number of genes that can be mutated to give these phenotypes. In all three selection pathways described above, mutation frequencies were in the same range, i.e., ca 5 x 10 mutants found per F1 mutagenized progeny individual. This is about half the frequency expected if a single gene were capable of mutating to give all the different phenotypes we see. An alternative explanation for the low frequency is that many mutations that change the timing of postembryonic events are lethal; about 50% of the mutants initially picked are, in fact, inviable. However, it is clear that the selection method is capable of identifying rare individuals with interesting and relevant phenotypes.

4. Isolation of cuticle-specific mouse monoclonal antibodies. In collaboration with Dr. Ed Hedgecock of the Roche Institute of Molecular Biology, Nutley, NJ, we isolated mouse monoclonal antibodies with specificity for the C. elegans cuticle. Two separate hybridoma fusions and screens were conducted.

a. An L1-specific monoclonal antibody was elicited by in vitro immunization of a mouse splenocyte primary culture with a mixture of embryonic (egg) antigens. Hybridoma clones were screened by indirect immunofluorescent staining of whole nematode "squashes" on microscope slides. Two hybridoma clones (M37 and M38) expressed antibodies that labeled the L1 cuticle (this is reasonable, because the L1 cuticle is made by the embryo prior to hatching). My laboratory has partially characterized the antibody and antigen. The M38 antibody, which appears to be of the IgM class, binds to the surface of live L1's in indirect immunofluorescence assays. No binding to the surface of other live stages or to other stages or structures in squashes was observed. Initial attempts to identify the M38 antigen in detergent extracts of L1 cuticles used a "dot blot" immunoassay in which antigen mixtures are immobilized in small spots on nitrocellulose. The antigen is detected in an SDS extract of L1 cuticles. However, the cuticle collagens are not extracted by SDS alone; extraction with SDS plus 2-mercaptoethanol (BME) is required for the majority (90%) of the cuticle mass to be solubilized. Thus the M38 antigen is held into the cuticle less strongly than the characterized cuticle collagens. Protease K digestion of the SDS extract containing the antigen destroys the antigenicity measurable by dot blotting, but collagenase digestion does not. Biochemical characterization of the M38 antigen will be useful if we are able to select "retarded" mutants that express it at an inappropriately late developmental stage, as proposed below.

b. More recently, cuticle-specific monoclonal antibodies were deliberately elicited by immunizing mice with a total mixture of adult cuticle proteins (an SDS-BME extract of adult cuticles). Hybridoma screening of worm squashes identified three clones expressing cuticle-specific antibodies, M45, M49, and M51. In contrast to the M38 results, these antibodies showed little stage-specificity and did not bind to the surface of live nematodes. We will use these antibodies to select mutants with alterations in the organization of the cuticle layers such that new antigenic determinants are exposed on the cuticle surface.

Annual Progress Report
NSF Grant #DCB-8510567
Project Title: Genetic Specification of Nematode Surface Antigens
Effective date: August 1, 1985
Expiration Date: January 31, 1988

1. Summary of overall progress. In the first grant year, a specific genetic locus in C. elegans that affects surface antigen expression in the adult nematode was identified. Because this discovery was not predicted in the original proposal, it will be described first. Then other significant progress will be described, including a comparison of accomplishments with proposed goals. Finally, proposed goals for the second grant year will be set.

A. Discovery of a gene that causes surface antigenic differences between C. elegans varietal strains. Twelve independently-isolated varietal strains of C. elegans were tested for surface antibody binding using an adult-specific antiserum prepared against antigens isolated from the standard wild-type strain, N2. This antiserum did not bind detectably to adults of three varietal strains, PA-1, DH424, and Bergerac BO (hereafter referred to as srf strains). Thus these strains appeared to lack the adult-specific surface antigens detected on the N2 surface. We analyzed dominance and genetic linkage in crosses between N2 and the srf strain PA-1. The results were as follows: a. The antigen-positive phenotype expressed by N2 was dominant over the negative phenotype of PA-1 in heterozygous outcross progeny; b. A surprisingly simple linkage pattern emerged from crosses between PA-1 and N2 strains visibly marked with standard linkage markers. The major genetic determinant of adult surface antigen expression (as detected by our antibodies) was a single genetic locus on linkage group II. Markers on all 5 autosomal linkage groups were tested; three different markers on II showed significant linkage to the srf marker. Markers on the other autosomal linkage groups showed no significant linkage; a marker on the X chromosome showed weak linkage, but sex-linkage was not confirmed by the results of reciprocal crosses between PA-1 and N2. We have continued to map the srf gene located on linkage group II. A three-factor cross gave the order dpy-10 unc-4 srf, indicating that the srf gene is located on the right arm of linkage group II. This may be the first characterization of a genetic locus specifically affecting surface antigen expression in a nematode.

We attempted to determine if one of the antigen-negative strains expresses an antigenic type different from, and perhaps allelic to, the one expressed by N2. We prepared antibodies against adult cuticle proteins isolated from strain DH424. After subjection to cross-adsorption with N2 adults to remove cross-reacting antibodies, residual antiserum bound to neither the DH424 nor the N2 surface. Although we cannot eliminate the possibility that DH424 makes an allelic molecule that is non-immunogenic, the results suggest that the gene carried by DH424

may simply be a null allele of the gene expressed by N2.

B. Isolation of cuticle-specific mouse monoclonal antibodies. In collaboration with Dr. Ed Hedgecock and Ms Harshida Bhatt of the Roche Institute of Molecular Biology, Nutley NJ, we isolated mouse monoclonal antibodies with specificity for the cuticle surface. In March, 1986, I visited Dr. Hedgecock's lab for five days to help screen the first batch of hybridomas for cuticle binding. The most interesting antibody to emerge so far (M38) has specificity for the L1 (first larval stage) surface. The M38 antibody was characterized here at Georgia Tech. In indirect immunofluorescence assay, M38 binds only to the L1 surface when tested against live nematodes or nematode squashes on microscope slides. No binding to the surface of other stages or to other structures was observed. Initial attempts to identify the M38 antigen in detergent extracts of L1 cuticle preparations used a "dot blot" immunoassay. The antigen is detected strongly in an SDS extract of purified L1 cuticles; the non-ionic detergent Triton X-100 does not extract the antigen. However, most of the cuticle proteins (collagens) are not extracted by SDS alone; 2-mercaptoethanol is required for complete solubilization (90%) of the cuticle. Thus the L1-specific antigen is held into the cuticle less strongly than most cuticle proteins, and is extracted into a relatively specific biochemical fraction by SDS. The antigen is probably a protein; protease digestion of the fraction containing the antigen eliminates antibody binding in the "dot blot" assay.

C. Isolation of putative heterochronic mutants using adult-specific antibodies. We used adult-specific rabbit antibodies to isolate C. elegans mutants that express adult-specific surface antigens at inappropriate developmental times. These mutants may identify regulatory genes involved in developmental stage transitions. We isolated mutants after EMS mutagenesis by two procedures. In the first, we searched for revertants of a lin-4 mutant. lin-4 animals express a larval cuticle as an adult. Revertant animals were easily identified as the only animals in the lin-4 background that bound adult-specific antibody and "lit up" in immunofluorescence staining assay. Three mutants were obtained in this way; two have an adult multi-vulva phenotype, and one has hermaphrodites partially transformed into males. These mutants are being genetically and phenotypically characterized.

In the second mutant screen, synchronous populations of developing larvae were screened with adult-specific antibodies by immunofluorescence for identification of mutants that express adult-specific antigens precociously (as larvae). Two mutants were obtained in this way; both appear to initiate vulval formation precociously and have a partial egg-laying defect. One of these was examined in more detail; all developmental stages bound adult-specific antibody; some larvae had lateral alae (normally an adult-specific trait), and the alae expressed in sexually mature adults were morphologically defective. These mutants are being genetically characterized.

2. Comparision of actual accomplishments with proposed goals.

A. Biochemical identification of antigens. An attempt was made to radiolabel the surface of live nematodes and identify extracted antigens by immunoprecipitation of labeled molecules. While labeled surface proteins could be separated electrophoretically and identified by autoradiography, the labeled proteins did not precipitate with rabbit anti-cuticle antisera. We therefore have not been able to use this method to identify surface antigens. More progress was made with the L1-specific monoclonal antibody described above; it appears to recognize a protein antigen extracted from the adult cuticle by SDS.

B. Monoclonal antibody reagents. Progress in obtaining and characterizing these antibodies was more rapid than we had expected. One of the assays we proposed for screening hybridoma supernatants was immunofluorescence staining of worm squashes on microscope slides; this proved to be a rapid and reliable method for identifying hybridomas with cuticle specificity. We found 4 monoclonal antibodies in initial screens. Because we already have tested one of these successfully for binding to the surface of live L1 animals, we know that our assay systems work; this should facilitate future characterization of the three other monoclonal antibodies that we now have available.

C. Selection of surface antigen cDNA clones. Two *C. elegans* DNA libraries cloned in the *E. coli* phage lambda gt11 were obtained from Barbara Meyer, MIT. We screened these for antigen-producing clones using the plaque-replica immunoblot method of Young and Davis. One library was 90% recombinant; one million plaques from this library were screened, but no positives were found. The other library was only 5% recombinant, and only about one hundred thousand plaques were screened. Again, no positives were found. The assay method worked with a positive control chicken ovalbumin gene cloned in lambda gt11, so presumably the problem was not the assay technique.

D. Identification of genes affecting timing of surface antigen expression. Initially, we had to solve a technical problem; how to view immunofluorescently stained nematodes so that individual mutants could be picked. At first, stained nematodes were plated onto agar plates so that they could be picked under a low-power fluorescent microscope; staining could not be observed for the requisite time before movement of nematodes rubbed off the antibody stain. The method that worked well involves spreading the stained nematodes in buffer onto a glass Petri dish; mutants were picked under the microscope using a drawn-out capillary pipet and a mouth tube. Once this problem was solved, we immediately began to find mutants. We used two of the mutant screens described in our original proposal and found several mutants with interesting phenotypes using each method.

3. Proposed goals for the second grant year.

A. Genetic characterization of the locus on linkage group II affecting surface antigen expression. The locus will be more precisely mapped. A three-factor cross gives the order dpy-10 unc-4 srf. The srf locus will be bounded on the other side by appropriate three-factor crosses, and will be further localized by deletion mapping. A number of deficiencies with known end-points on linkage group II are available; heterozygotes of genotype Df/srf will be constructed and screened for the presence or absence of the adult-specific surface antigen marker. If a deficiency covers the srf locus, heterozygotes will be antigen-deficient. Because the antigen-negative phenotype is recessive to wild-type, heterozygotes for deficiencies not covering the srf locus will be antigen-positive.

Once localized to a specific interval on the genetic map of chromosome II, molecular genetic analysis of the srf locus may become feasible. While completion of such an analysis is outside the scope of the present grant, an indication of the possibilities and the rationale are given here.

1. Discovery of the srf locus and observation of the varietal strains' phenotypes raises a new question. What are the genetic differences between wild-type and srf strains at the molecular level? Wild-type expresses an adult-specific surface antigen, but the antigen is missing from adults of three other varietal strains. Our inability to identify an alternative antigen expressed by one of these strains suggests that it does not express an antigen modified from the wild-type condition. A simple explanation of the difference is that the srf strains carry a "null" allele of the srf locus. Interestingly, two of the three antigen-negative strains carry high copy numbers of the DNA sequence of the transposable element TC1. One of these strains has an active form of TC1 that transposes at observable frequencies in both somatic and germ line DNA; a mutator strain derived from Bergerac BO is currently being used by a number of investigators to tag genes by transposon mutagenesis. Although the correlation between high TC1 copy number and srf phenotype may be coincidental, it suggests an attractive hypothesis to explain the genetic basis of the srf phenotype. The srf locus carried by antigen-negative strains may be interrupted by a TC1 insertion that functionally inactivates the srf gene. The TC1 insertion may be genetically relatively stable; Emmons et al have shown that TC1 excision from known genetic sites occurs at a much lower frequency in the germ line than the somatic cells. This in turn might explain our observation of occasional srf$^+$ adults in populations of antigen-negative strains; these variant phenotypes were not demonstrably heritable in cloned progeny.

Because the aforementioned mutator strain is in the antigen-negative Bergerac BO genetic background, a simple test of the hypothesis would be to screen the strain for antigen-positive

variants, collect variants and screen their progeny for genetic inheritance of the variant phenotype. If we find a positive genetic revertant in this background, it presumably would have arisen from excision of TC1 from the srf gene and concomitant activation of the srf gene activity.

If we obtain a genetic variant in this way, it will be worthwhile and feasible to pursue molecular genetic analysis of the difference between positive and negative strains. Several C. elegans genes have been identified and cloned by TC1 insertional tagging; transposon tagging may be the quickest way to clone C. elegans genes. The srf gene on chromosome II may already contain a TC1 insertion in antigen-negative strains; if so, a series of backcrosses to N2 with reselection of the antigen-negative phenotype after each backcross will isolate the srf associated TC1 element in the N2 genetic background. Then comparison of the genomic DNA fragments detected by a TC1-specific hybridization probe by Southern blotting of N2 and backcrossed strain DNA should identify the TC1-containing DNA fragment linked to the srf gene. We estimate that this line of investigation should occupy most of the second grant year; if successful, it should lead to molecular cloning of the srf gene.

2. If it does not appear that a TC1 element is inserted into the srf locus, there may be a closely linked TC1 element or other DNA structural polymorphism that can serve as the start point of a chromosomal walk. For example, the srf strains DH424 and Bergerac BO contain about 250 copies of TC1 per haploid genome, so that on the average, a TC1 element occurs every 250,000 base pairs. Thus, the backcrossed strains described above are potentially useful for cloning the srf locus even if a TC1 element does not interrupt the srf gene, as long as there is an identifiable DNA structural polymorphism nearby.

B. Isolation of cuticle-specific mouse monoclonal antibodies. We will continue to characterize the L1-specific surface antigen using the M38 monoclonal antibody. Now that we have some evidence from dot blotting that the antigen is a protein, we will further characterize the antigen by Western blotting or gel filtration. Gel filtration column fractions can be assayed by dot blotting for the presence of antigen. We anticipate that a detailed molecular characterization of the M38 antigen will require most of the second grant year.

The M38 antigen is part of the L1 cuticle. This is the only cuticle that is synthesized during the embryonic part of the life cycle. Using established protocols for fluorescent antibody staining of embryos, we will study the time of synthesis and incorporation of the M38 antigen. The developmental age of an embryo is easily determined by observation of its cell lineages; these will serve as markers to correlate the time of appearance of the M38 antigen with other developmental events.

Three other new monoclonal antibodies will be characterized for stage-specificity and cuticle specificity by immunofluorescence. The antigens will be characterized by identification of the cuticle protein fraction containing the antigen and by various immunoassays including Western blotting.

C. Isolation of heterochronic mutants using adult-specific antibodies. The selection methods described above will be used to isolate more mutants with alterations in the timing of adult surface antigen expression. Now that we have an L1-specific monoclonal, we can also begin to look for mutants that express the M38 antigen at an inappropriately late developmental time. Once we have a number of mutants, these will be characterized genetically. Linkage will be established and mutations mapped by genetic crosses with visible marker strains. Complementation between our new mutants and between our mutants and known heterochronic mutants characterized by others will be assessed in heterozygous complementation tests. Complementation tests will allow us to estimate the number of genes that can be mutated to give a precocious expression of adult surface antigens, and will also give information about the range of phenotypes possible in mutants with different alterations in a single gene. Epistasis assessed in double mutants will give information about possible regulatory hierarchies. Null phenotypes will be identified by testing mutants over a deficiency and by identifying nonsense mutants with a nonsense suppressor.

Mutant phenotypes will be characterized initially using adult-specific antibodies to determine which developmental stages besides the adult express the adult-specific antigen. Cell lineage patterns will be examined for deviations from the wild-type pattern. Morphology and ultrastructure of mutant cuticles will be examined for characteristic stage-specific cuticle features such as alae and pattern of layers. Mutant cuticles will be examined biochemically to determine if the heterochronic phenotype extends to the cuticle collagens.

NATIONAL SCIENCE FOUNDATION
Washington, D.C. 20550

# FINAL PROJECT REPORT
NSF FORM 98A

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**PART I—PROJECT IDENTIFICATION INFORMATION**

| 1. Institution and Address | 2. NSF Program | 3. NSF Award Number |
|---|---|---|
| School of Applied Biology<br>Georgia Institute of Technology<br>Atlanta, GA 30332 | Developmental Biology | DCB-8510567 |
| | 4. Award Period<br>From 2/1/85 To 7/31/88 | 5. Cumulative Award Amount<br>$145,500 |

6. Project Title

Genetic Specification of Nematode Surface Antigens

**PART II—SUMMARY OF COMPLETED PROJECT** *(FOR PUBLIC USE)*


The primary objective of the project was twofold: 1. to identify genes controlling post-embryonic developmental stage transitions in the nematode Caenorhabditis elegans; and 2. to identify genes controlling the expression of stage-specific surface molecules in C. elegans. The proposed study was designed to help explain how genes control animal differentiation and development.

Genes were identified by a combination of immunofluorescence tagging of live nematodes and formal genetic analysis of mutations. The standard tools for detection of antigenic differences between nematodes were antibody probes that recognized molecules on the surface of specific developmental stages.

The findings were that a single gene or a well-defined cluster of genes on C. elegans chromosome II controls the expression of an adult-specific class of surface molecules. The implications of these findings were that small, well-defined genetic differences can cause distinct patterns of antigenic variation in a nematode. These findings may have implications for understanding the role of surface antigenic variation in avoiding the host immune response in nematode parasitism.


**PART III—TECHNICAL INFORMATION** *(FOR PROGRAM MANAGEMENT USES)*

| 1. ITEM *(Check appropriate blocks)* | NONE | ATTACHED | PREVIOUSLY FURNISHED | TO BE FURNISHED SEPARATELY TO PROGRAM | |
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| b. Publication Citations | | X | | | |
| c. Data on Scientific Collaborators | | X | | | |
| d. Information on Inventions | X | | | | |
| e. Technical Description of Project and Results | | X | | | |
| f. Other *(specify)* 2 copies each of 2 publications | | X | | | |

| 2. Principal Investigator/Project Director Name *(Typed)* | 3. Principal Investigator/Project Director Signature | 4. Date |
|---|---|---|
| Samuel M. Politz | | 9/21/88 |

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# Genetic and Immunological Studies of Surface Antigens of the Nematode *Caenorhabditis elegans*

A THESIS

Presented to

The Faculty of the Division of Graduate

Studies and Research

By

Daniel Lee Herman

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in the School of Applied Biology

Georgia Institute of Technology

August, 1987

# ABSTRACT

Cuticle proteins were extracted from Caenorhabditis elegans adults (strain DH424) and used to produce a polyclonal rabbit antiserum. This antiserum was then used to study strain and developmental-stage differences in DH424 animals.

Genetic studies were also undertaken to further understand the regulation and expression of nematode surface antigens. These studies involved heterozygous complementation tests and complementation tests with C. elegans chromosomal deficiency mutants.

# IDENTIFICATION AND PARTIAL CHARACTERIZATION OF A STAGE-SPECIFIC SURFACE ANTIGEN IN C.ELEGANS

A THESIS
Presented to
The Faculty of the Division of Graduate Studies

By

Steven Glenn Donkin

In Partial Fulfillment
of the Requirements for the Degree
Master of Science in Applied Biology

Georgia Institute of Technology
August, 1988

## SUMMARY

The nematode Caenorhabditis elegans has a life cycle consisting of several precisely-timed stages, each characterized by expression of a new cuticle while the old cuticle is shed. Two monoclonal antibodies, M37 and M38, were found to bind only to the surface of the L1 larval stage cuticle. L1 larval cuticles were isolated and the solubilized components subjected to various biochemical assays including dot immunobinding, SDS-polyacrylamide gel electrophoresis, and Western blotting, in order to isolate and characterize as a molecular species the L1 cuticle antigen or antigens to M37 and M38.

The antigen was found to be in the SDS-soluble fraction of isolated cuticles, and was identified by Western blotting as consisting of several closely-spaced bands. The antigen was subjected to proteinase K and collagenase digestion and appeared to be resistant to both, though it is sensitive to complete acid hydrolysis. Both M37 and M38 appear at this point to recognize the same antigen. The potential for using such stage-specific monoclonal antibodies in further characterizing cuticle antigens, and in screening for heterochronic mutants, is discussed.

B. PUBLICATION CITATIONS

Politz, Samuel M., Politz, Joan C., and Edgar, Robert S. Small collagenous proteins present during the molt in Caenorhabditis elegans. J. Nematol. 18, 303 (1986).

Politz, Samuel M., Chin, Karl J., and Herman, Daniel L. Genetic Analysis of Adult-Specific Surface Antigenic Differences between Varieties of the Nematode Caenorhabditis elegans. Genetics 117, 467 (1987).

C. Data on Scientific Collaborators

1. Graduate research assistants supported wholly or in part by the grant:

   a. Steven G. Donkin, MS 1988
   b. Daniel L. Herman, MS 1987

2. Laboratory Technician supported by the grant:

   a. Karl J. Chin

3. Undergraduate research assistants supported by the grant:

   a. Miguel Estevez
   b. Annette Estevez
   c. J.T. Singh
   d. Daniel Heithold

E. Technical Description of Project and Results

The following is a summary of research progress supported by my NSF grant DCB-8510567 "Genetic Specification of Nematode Surface Antigens" from 8/85 to date. Early results on adult-specificity of rabbit antisera were obtained during a prior postdoctoral in R. S. Edgar's laboratory at UC Santa Cruz and are mentioned here for clarity.

1. Evidence for adult-specific surface antigens. Rabbit antisera were prepared against a total mixture of adult cuticle proteins (21). Antisera were tested for stage-specific binding to live nematodes by immunofluorescence and radioimmunoassay (for details, see Appendix 1). Briefly, nematodes were incubated sequentially with anti-cuticle antibodies followed by either fluorescein (FITC) anti-rabbit antibody or iodine-125 labeled protein A. FITC labeled nematodes were observed microscopically; total bound radioactivity in a population of radiolabeled nematodes was measured in a gamma counter as a more quantitative confirmation of antibody binding. Crude antiserum cross-reacted with the larval surface, but could be rendered adult-specific by adsorption with large numbers of live L4 (fourth larval stage) animals (Appendix 1). These adult-specific antibodies are now our standard reagents in immunofluorescence assays of genetically determined surface antigen phenotypes; experiments proposed here rely on them extensively.

2. Discovery of a gene that causes surface antigenic differences between C. elegans varietal strains. These genetic results are described in detail in Appendix 1. Twelve independently-isolated varietal strains of C. elegans were tested for surface immunofluorescence using an adult-specific antiserum prepared against antigens isolated from the standard wild-type strain, N2. This antiserum did not bind to adults of three varietal strains, PA-1, DH424, or Bergerac BO. Thus these strains appeared to lack the adult-specific surface antigens detected on the wild-type surface.

Dominance and genetic linkage of the surface antigen phenotype were analyzed in crosses between N2 and the antigen-negative strain PA-1. Results were as follows:

a. The antigen-positive phenotype of N2 was dominant over the negative phenotype of PA-1 in heterozygous cross progeny (Appendix 1, Table 1).

b. A surprisingly simple linkage pattern emerged from crosses between PA-1 and N2 strains marked with standard visible linkage markers. The genetic determinant of the adult surface antigen phenotype was a single genetic locus on linkage group II. Markers on all 5 autosomal linkage groups were tested; three different visible markers on II showed significant linkage to the surface antigen marker (Appendix 1, Table 2). Markers on the other autosomes showed no significant linkage.

c. We have mapped the locus, now designated srf-1 on linkage group II (Appendix 1, Fig. 8). Two three-factor crosses placed srf-1 on the right arm of II near the marker rol-1. Deficiency mapping experiments further clarified the position of srf-1 and supported the suggestion from linkage data that a single gene or cluster of genes is involved. To our knowledge, this is the first characterization of a genetic locus specifically affecting surface antigen in a nematode.

d. We attempted to determine if the antigen-negative strain DH424 expresses an antigenic type different from, and perhaps allelic to, the one expressed by N2. Rabbit antibodies were raised against adult cuticle proteins isolated from DH424. After adsorption with N2 adults to remove cross-reacting antibodies, residual antibodies bound to neither the DH424 nor the N2 surface. Although we cannot eliminate the possibility that DH424 makes an allelic antigen that is non-immunogenic, the results suggest that the gene carried by DH424 may be an inactive allele of srf-1.

3. Isolation of putative heterochronic mutants using adult-specific antibodies. Immunofluorescence of large populations of live nematodes viewed under a specially adapted epifluorescent stereomicroscope was used to isolate 16 C. elegans mutants that apparently express adult-specific surface antigens at inappropriate developmental times. Mutants were isolated after ethyl methane sulfonate (EMS) mutagenesis; mutagenized Po populations were allowed to segregate mutations in the homozygous condition by two generations of self-fertilization prior to antibody screening. Populations were screened in thin liquid layers and interesting individuals were picked with a microcapillary pipet under the microscope. Only mutants with independent post-mutagenesis pedigrees were picked. Mutants were isolated in three procedures that select for potentially different phenotypes. Mutants described here are summarized in tabular form in Appendix 2.

a. Reversion of lin-4 retarded mutants. lin-4 animals express a larval cuticle as an adult (1), and do not bind our adult-specific rabbit antibodies (Appendix 1, Fig. 5). Revertant animals were easily identified as the only animals in the lin-4 background that bound adult-specific antibody and "lit up" in indirect immunofluorescence assay. Three mutants have been isolated in this way. Two have an adult multi-vulva phenotype (yj46, yj48), and one has a hermaphrodite tail defect (yj42).

b. Isolation of precocious mutants developing through the normal developmental pathway. Populations of nematodes in the wild-type genetic background were synchronized by harvesting L1 animals that hatched from previously laid eggs in a one hour period. Synchronously developing larvae were screened with adult-specific antibodies by immunofluorescence to identify mutants that bound adult-specific antibody as larval stages. Six mutants have been isolated in this way. All the mutants bind adult-specific antibodies as larval animals, with high penetrance. Three mutants appear to express the adult-specific antigen at all larval stages, while three appear to express it only at late larval stages (e.g, L3-L4 sized animals with no vulva). Four mutants have no visible abnormalities; of the other two, one has a hermaphrodite "club tail" (yj10). The other (yj2aa) has several defects including an uncoordinated (Unc) phenotype and pharygeal abonormalities. The latter two mutants were backcrossed to wild-type and the visible phenotypes resegregated; precocious adult antigen expression co-segregated with the visible phenotypes through two backcrosses. By linkage testing with standard genetic markers, the club tail mutation was assigned to linkage group III and the Unc-pharyngeal mutation to linkage group I.

c. Isolation of precocious mutants developing through the dauer developmental pathway. Under conditions of starving or overcrowding, L2 animals molt into a specially adapted arrested stage called the dauer larva (24). Upon refeeding, dauers molt into L4's and continue development into adults. Large populations can be synchronized by refeeding dauers. The adults and L4's developing from dauers may be distinct from those developing from L3's, however. For example, adult sqt-2 mutants are squat if they develop from L3's, but are rollers if they develop from dauers (25). In addition, most known heterochronic cell lineage mutants show wild-type rather than heterochronic phenotypes after passage through dauer (V. Ambros, personal communication). The only known exceptions are lin-29 mutants, which have an heterochronic phenotype after either pathway.

We have isolated seven precocious mutants by refeeding dauer larvae and screening the synchronously developing population to identify mutants that bind adult-specific antibody in immunofluorescence assay as L4's. By the above argument, these may identify a class of mutants functionally distinct from those obtained via the L3 developmental pathway.

Precocious mutants obtained after dauer formation show the precocious surface antigen phenotype with high penetrance. Five appear to express it at all larval stages, and two appear to express it only at late larval stages (L3-L4). Four mutants have no visible phenotype. Of the other three, all have mutiple, partially penetrant visible defects. One has vulvaless (Vul) adults, and another has both Vul and multi-vulva (Muv) adult phenotypes. The third has a "club tail" hermaphrodite phenotype.

To summarize, we have isolated 16 independent heterochronic mutants. We can estimate by observed mutation frequency the number of genes that can be mutated to give these phenotypes. In all three selection pathways described above, mutation frequencies were in the same range, i.e., ca $5 \times 10^{-4}$ mutants found per F1 mutagenized progeny individual. This is about half the frequency expected if a single gene were capable of mutating to give all the different phenotypes we see. An alternative explanation for the low frequency is that many mutations that change the timing of postembryonic events are lethal; about 50% of the mutants initially picked are, in fact, inviable. However, it is clear that the selection method is capable of identifying rare individuals with interesting and relevant phenotypes.

4. Isolation of cuticle-specific mouse monoclonal antibodies. In collaboration with Dr. Ed Hedgecock of the Roche Institute of Molecular Biology, Nutley, NJ, we isolated mouse monoclonal antibodies with specificity for the

*C. elegans* cuticle. Two separate hybridoma fusions and screens were conducted.

a. An L1-specific monoclonal antibody was elicited by in vitro immunization of a mouse splenocyte primary culture with a mixture of embryonic (egg) antigens. Hybridoma clones were screened by indirect immunofluorescent staining of whole nematode "squashes" on microscope slides. Two hybridoma clones (M37 and M38) expressed antibodies that labeled the L1 cuticle (this is reasonable, because the L1 cuticle is made by the embryo prior to hatching). My laboratory has partially characterized the antibody and antigen. The M38 antibody, which appears to be of the IgM class, binds to the surface of live L1's in indirect immunofluorescence assays. No binding to the surface of other live stages or to other stages or structures in squashes was observed. Initial attempts to identify the M38 antigen in detergent extracts of L1 cuticles used a "dot blot" immunoassay in which antigen mixtures are immobilized in small spots on nitrocellulose. The antigen is detected in an SDS extract of L1 cuticles. However, the cuticle collagens are not extracted by SDS alone; extraction with SDS plus 2-mercaptoethanol (BME) is required for the majority (90%) of the cuticle mass to be solubilized. Thus the M38 antigen is held into the cuticle less strongly than the characterized cuticle collagens. Protease K digestion of the SDS extract containing the antigen destroys the antigenicity measurable by dot blotting, but collagenase digestion does not. Biochemical characterization of the M38 antigen will be useful if we are able to select "retarded" mutants that express it at an inappropriately late developmental stage, as proposed below.

b. More recently, cuticle-specific monoclonal antibodies were deliberately elicited by immunizing mice with a total mixture of adult cuticle proteins (an SDS-BME extract of adult cuticles). Hybridoma screening of worm squashes identified three clones expressing cuticle-specific antibodies, M45, M49, and M51. In contrast to the M38 results, these antibodies showed little stage-specificity and did not bind to the surface of live nematodes. We will use these antibodies to select mutants with alterations in the organization of the cuticle layers such that new antigenic determinants are exposed on the cuticle surface.

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Genes that can be mutated to reveal hidden antigenic determinants in the cuticle. Samuel M. Politz, Miguel Estevez, Karl J. Chin, and Peter J. O'Brien, School of Applied Biology, Georgia Institute of Technology, Atlanta, Georgia 30332.

In our initial attempts to isolate mutants that express stage-specific surface antigenic determinants at inappropriate stages, we've turned up some mutants with a novel, unexpected phenotype. In immunofluorescence tests, these mutants stain uniformly with a rabbit antiserum that does not stain the surface of wild-type animals. This antiserum was prepared by treating an anti-wild-type cuticle serum (by adsorption with wild-type adults) to remove antibodies capable of binding to the wild-type surface. Thus, antigenic determinants that are not available on the wild-type surface are exposed in the mutants. We describe here isolation and preliminary genetic characterization of the mutants.

Mutants were isolated by screening synchronous populations of $F_2$ progeny of animals mutagenized with EMS. Populations were synchronized by refeeding $F_2$ dauer larvae, or by gently washing plates to remove egg-laying $F_1$ adult hermaphrodites while leaving $F_2$ eggs stuck in the *E. coli* lawn. In the latter case, eggs were allowed to hatch for 1-2 hours and then L1's were washed off and transferred to fresh plates for growth. The egg-hatching and transferring steps were usually repeated to obtain a second "wave" of synchronous $F_2$'s. Populations were harvested at the L4 stage or younger and screened with L4-adsorbed anti-adult serum (Genetics 117:467 1987) by indirect FITC immunofluorescence. Samples in 100-200 ul PBS were spread in thin layers onto Pyrex Petri dishes; up to 10,000 animals per sample were screened at a time. Samples were screened using an epifluorescent stereomicroscope (Leitz-Wild). Immunofluorescent larvae were observed at low frequency; these were picked using a mouth tube and a drawn-out 100 ul capillary micropipet. Clones were established and cloned stocks were rescreened by immunofluorescence to eliminate false positives. Viable mutants were obtained at a frequency of about $5 \times 10^{-4}$ per $F_1$.

Of 12 mutants that we have analyzed, 11 fit the pattern described in the first paragraph above; the remaining one produces an incompletely penetrant "small adult" phenotype that may be of interest, but this is not its story. Initially, we looked at which stages besides adult stained with the L4-adsorbed antibody. Eight of the 11 mutants stained at all stages, while the remaining three stained only at late larval stages. We looked at several of the mutants to see if they expressed adult lateral alae at earlier stages, but surprisingly, found no larvae that carried alae. *yi10* is the only mutant in the set that has a readily apparent visible phenotype; it is somewhat scrawny and has a cold-sensitive weak Left Roller phenotype. These results led us to consider that the mutant phenotypes might result from cuticle structural defects rather than, or in addition to, precocious expression of an adult-specific cuticle type. The 11 mutants were then tested for staining with the same parent antiserum after it had been adsorbed with wild-type adults to remove all antibodies capable of reacting with the wild-type surface. All of the mutants were antigen-positive; wild-type was antigen-negative (this is the result summarized in the first paragraph). The 8 mutants that light up at all stages with this serum are termed "super-bright" in our lab jargon and the other three that

exhibit apparent stage-specificity stain much more weakly.

We considered the possibility that known apparent cuticle phenotypes like Sqt and Rol might share this phenotype with our new mutants. We obtained a set of ten sqt and rol mutants from Bob Edgar and tested them with adult-adsorbed antibodies. None were antigen-positive. Thus our mutants have a new phenotype that is not shared with the classical morphological cuticle mutants we have tested.

We have made some progress in analyzing the "super-bright" mutants genetically. It has been possible to use the difference between wild-type and mutant antigen phenotypes to do linkage, complementation, and mapping using methods similar to those described for mapping of the srf-1 antigenic polymorphism (Genetics paper again). All mutants were backcrossed to wild-type twice and an antigen-positive (mutant) segregant picked after each backcross. Penetrance of the surface antigen phenotype is complete; it is very unusual to find a non-staining individual in a stained population of one of the homozygous mutant stocks. This is in contrast to the srf-1 phenotype which we found to be incompletely penetrant both in wild-type and srf-1 mutant strains. All of the mutant antigen phenotypes are recessive; the best evidence for this is that in crosses with unlinked unc markers, 25% or less of Unc segregants issuing from an unc +/+ srf parent are antigen-positive.

Linkage was determined by using unc markers on the autosomes as selected markers and staining for antigen-positive Unc segregants of the double heterozygote. Absence of the double homozygous recombinant in a small population was taken as evidence for linkage. For complementation testing, srf-a/+ males were mated with srf-b hermaphrodites. F1 male progeny were collected and stained. Failure to complement was indicated by presence of antigen-positive heterozygous males. So far there are two complementation groups. At least 5 non-complementing mutations are linked to unc-13(e51)I, and yj10 shows linkage to unc-24(e138)IV. We are presently designating the chromosome I mutations as srf-2 and yj10(IV) as srf-3. We have mapped srf-2(yj262) by 2 and 3 factor crosses; it is on IR in the vicinity of lin-11 and unc-75. A 3 factor cross with these two markers is in progress.

Mutant phenotypes were compared serologically by the adsorption method. Antiserum adsorbed with srf-2(yj262) adults binds to yj10 adults but not to yj262 or any of the other srf-2 mutants. The same parent antiserum adsorbed with yj10 adults doesn't bind yj10 or any of the srf-2 mutants. Thus the two complementation groups correspond to two distinct serological phenotypes, and the results fit a model in which the antigens exposed in srf-2 mutants are a subset of those exposed in srf-3(yj10).

To summarize, all mutants in this set share the general characteristic of expressing surface antigenic determinants that are not on the wild-type surface. The fact that the antisera were raised against a wild-type cuticle immunogen indicates that the antigens exposed in the mutants are probably wild-type antigens and not novel structures. The fact that the mutant phenotypes are recessive, even though they constitute an apparent gain of antigenicity, suggests that the mutant lesions are defects in cuticle structure that uncover normally hidden antigenic determinants. Our hope is that these mutants and the genes that they identify will contribute to understanding genetic control of the layered organization of the cuticle in a way that is complementary to what can be learned from morphological mutants.

# Monoclonal antibodies with specificity for the L1 surface.

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In a screen for monoclonals with specificity for embryonic antigens, H.B. and E.H. fortuitously found two hybridoma lines that produce antibodies that bind specifically to the L1 cuticle in squashes of mixed stages. S.D. and S.P. have partially characterized the binding to live worms and antigens in extracts of L1 cuticles.

Antibodies were elicited by in vitro immunization of a primary mouse splenocyte culture with a mixture of embryonic antigens. Hybridomas were screened by indirect immunofluorescence of Bristol strain worms in squashes fixed on microscope slides (Albertson, Sulston, and Hedgecock, WBG Vol. 7, #1, p. 73). Two hybridomas were found that stained L1 cuticles specifically. Only small larvae with L1 type alae stained; other structures or stages never stained. After cloning by limiting dilution and rescreening, these cell lines were grown in quantity and immunoglobulins isolated from culture supernatants by 0-50% saturated ammonium sulfate precipitation. Resuspended and dialyzed ammonium sulfate fractions are the standard antibody solutions used in the experiments described here.

Immunoglobulin class was determined by immunodiffusion (Ouchterlony). Both antibodies (M37 and M38) precipitated specifically with sheep anti-mouse IgM serum. In subsequent indirect antibody binding experiments, goat anti-mouse IgM secondary antibodies were used.

Binding of the antibodies to live L1's in immunofluorescence tests is strongly temperature-dependent. When antibody incubations with live animals are done at room temperature, little binding is observed. If antibody incubations are done at $4^{\circ}$ or at $0^{\circ}$, binding is quite uniform initially, but the antibody stain "flakes off" in big flakes like paint flaking off a wall as the slide warms up. After flaking has occurred, the same sample can be washed and restained, indicating that some antigen is still present on the surface. We do not know whether the flaking involves dissociation of the antibody without antigen removal, or whether antigen comes off too. However, heat-killed L1's can also be restained after flaking, indicating that restaining does not require active replacement of the antigen by living worms. Antibody binding to worms fixed in squashes exhibited no similar temperature dependence.

L1's were obtained in large numbers (up to $10^6$) for isolation of cuticle proteins. Eggs obtained by Cloroxing were hatched overnight in M9. L1's were harvested and cuticles and cuticle proteins were isolated after sonication by standard procedures. Sonication supernatant, SDS extract, and SDS-BME extract were tested for antibody binding in a "dot blot" assay. Cuticle extracts were dotted onto nitrocellulose and incubated sequentially with M37 or M38, alkaline phosphatase- or horseradish peroxidase-conjugated goat anti-mouse IgM, and enzyme substrate. Antigen was detected predominantly in the SDS extract of L1 cuticles. Antigen was not detected in similar extracts of adult cuticles. Antibody binding to the SDS extract showed a distinct optimum of pH 5-6 for the primary antibody incubation.

Cuticle extracts were separated on 12% Laemmli SDS-PAGE slab gels and electrotransferred to nitrocellulose. Gels were incubated with antibody using conditions optimized in the dot blot. As before, antibody binding was observed only in an SDS extract of L1 cuticles. The predominant

antigenic species formed a "ladder" of about ten sharp, equally spaced bands centered around 20,000 daltons in MW. Less reproducibly, higher molecular weight antigenic bands appear. Appearance of the lower molecular weight ladder is insensitive to predigestion of the sample with protease K. Currently, our working hypothesis is that the antigen recognized by M37-M38 (both produce similar patterns in the Western blot experiment) is a stable, non-protein moiety that may be attached to a labile cuticle surface protein molecule.