Development of the Tracheal System in *Drosophila melanogaster*

An Honors Thesis (HONRS 499)

by

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The development of the tracheal system of Drosphila melanogaster is being traced by examining the impact of pattern disrupting mutations upon the normal development of the tracheal tree. The procedure depends upon a tissue specific P-element which carries the B-galactosidase producing gene, lac Z. Visualization of the tracheal system is attained through immunohistochemical staining of the embryos. The P-element, which is present in the sma strain of Drosophila melanogaster, is incorporated into the genome of flies that have pattern disrupting mutations. The resulting defects present in the tracheal system will be used to develop a sequence of gene activation and assist in the formation of a scheme of the development of the tracheal system in Drosophila melanogaster.

Introduction

In the past, the consequences of mutations in genes that control embryonic development have been examined in only two components of insect anatomy, the segmented external (cuticular) structures and the segmented central nervous system. Because it appears that majority of the development of the embryo is dependent upon several genes that lay the foundation for further development, there is reason to believe that a mutation in these genes will not only impact the cuticle and nervous systems but will also affect other systems, including the tracheal

system. Examining the consequences that mutations in these genes have upon the tracheal system will be valuable in formulating a sequence of gene expression as well as a sequence of tracheal-specific gene activation.

An "enhancer trap" insertion of a DNA fragment, which contains a portion of the Escherichia coli Beta-galactosidase gene (here referred to as lacZ) has been utilized. Because this insert, called sma, is positioned next to an enhancer of a gene which is expressed only in the developing tracheal system in Drosophila embryos, the enzyme product of lacZ is produced only in the cells of this system. This allows specific staining of the trachea as it develops by indirect immunoperoxidase reaction directed against Beta-galactosidase (here, B-gal).

Patterning mutations, which are distributed throughout each of the chromosomes of melanogaster, have historically established disruptions of embryonic segmental pattern. The mutations were selected based on this quality as well as the region of the mutation's impact recognized in the cuticular areas of Drosophila anatomy (see table 2). In addition, selection of patterning gene mutations located on the third chromosome was dependent on their absence of linkage to the sma gene, which is also located on the third chromosome. A standard, recombination (meiotic)

gene mapping technique was utilized to determine the location of sma gene.

The stocks were then crossed in various ways with sma-containing strains in order to incorporate lacZ into the genome of the stocks with patterning mutations. These strains were tested for the presence of the patterning mutation, then for the pattern of lacZ expression.

Currently, each stock is being examined for the effects of the mutations on this tracheal pattern. The embryos are tested at times beginning with the activation of the tracheal gene controlling the lacZ at five hours hours until the development's completion in the sevententh hour. This will encompass the full range of the development of the tracheal system. From there, analysis will determine the specific effects of pattern disrupting mutations on tracheal development.

Background

Extensive investigation and analysis of the genic control of the embryonic development of Drosophila has developed an understanding of the "stages" of developmental cellular committment into a concept of "tiers" of sequentially expressed genes which produce the spatial pattern

of Drosophila (Akam, 1987 and Ingham 1990). Currently, intrigue is shifting towards the interactions of these catagories of genes. These interactions produce similarities and differences that can be observed in individual segmental compartments of Drosophila. The main gene tiers in this heirarchy of control are maternal effect (coordinate) genes, zygotic effect (gap, pair rule,and segment polarity) genes, and homeotic (segment specific) genes.

Maternal effect genes are expressed during egg production. The product of some of these genes are differentially localized in the egg and subsequently impact the determination of the embryos' germ cells or generate the anterior-posterior (Mauseau and Schupbach, 1988) and dorsal ventral axes (Govind and Steward, 1991). Their products trigger the expression of specific sets of zygotic genes to initiate further patterns of segmentation (Pankratz and Jackle, 1990).

This tier of developmental control directs subsequently expressed groups of genes that begin to divide the embryo into sections along the anterior-posterior axis. These genes' activities eventually form the segments of the larva. This segmentation is controlled at at least three levels: large regions of the early embryo (gap mutations), a two-segment unit (pair-rule mutations), and individual segments (segment polarity

mutations).

The final tier of genes is known as the homeotic selector group. Although genes from this area of developmental control were not used for this experiment, this set of genes direct the development of the specific differences of each segmental compartment, i.e. differentiation (Lewis, 1978).

Tracheal Growth

The tracheal system is employed by the larva as well as the adult and functions as the respiratory sytem. A picture of its network of tubes that branch off two primary tracheal trunks , illustrates its name, the "tracheal tree". The initial formation of the trachea is activated during the fifth hour of the embyros' formation (the tenth stage according to Campos-Ortega; see table 1). Evidence of the primary structures of this network of tubes can be seen in ten segmental specializations of the lateral ectoderm known as the tracheal placodes (Campos-Ortega, 162; see fig.1). Tracheal placodes invaginate to form pits which are localized in the anterior section of each of these segments. Placement of the ten lateral pairs of pits begins in the mesothorax segments and continues

posteriorally to the ninth abdominal segment. There are no pits in the prothorax or head segments. During stage 14-15 the pits in the mesothorax and the abdominal segment a8 fuse to form the anterior and posterior spiracles (Campos-Ortega, 163). After invagination, the pits develop into small transversal tubes which are oriented perpendicular to the germ band (see fig. 2). Fusion of each single tracheal fragment with its posterior neighbor work in conjunction with simultaneous cell elongation to form the two main longitudinal tracheal tubes.

The development of the tracheal system begins in stage ten and continues elaboration of the tracheal tree until stage seventeen when it fills with air prior to hatching. Drosophila development is discussed in stages or isolated events because it facilitates comprehension of the process. In reality, however, snychronous events are interacting to affect the total development of all parts of the embryo. For this reason, I would hypothesize that the development of the tracheal system, when patterning mutations are present, will display segmental problems similar to those the cuticle exhibits. The ability to watch the effects of the mutations will allow for interpretation of the sequence of the gene activation and perhaps gene interaction that is required for normal development of the tracheal system.

Table 1

Stages of Drosophila Embryos

(after Campos-Ortega and Hartenstein)

TIME (hour)	STAGE	DEVELOPMENTAL OCCURRENCE
0-0:24	Stage 1	meiosis, fertilization, 2 cleavages
0:25-1:05	Stage 2	cleavages 3-8 retracts anterior and posterior
1:05-1:20	Stage 3	cleavage 9, polar buds form
1:20-2:10	Stage 4	syncytial blastoderm, last 4 cleavages
2:10-2:50	Stage 5	cellular blastoderm
2:50-3:00	Stage 6	mesoderm and endoderm invaginate, pole cells and dorsal plate move dorsal, cephalic furrow forms
3:00-3:10	Stage 7	gastrulation completed
3:10-3:40	Stage 8	amnioproctodeal invagination forms, germ band elongation, meso segmented
3:40-4:20	Stage 9	neuroblast segregates, ends when stomadeum invaginates
4:20-5:20	Stage 10*	stomadeum forms, max germ band elongation
5:20-7:20	Stage 11	parasegments form, germ band shorten
7:20-9:20	Stage 12	germ band shortening
9:20-10:20	Stage 13	anal plate at posterior, head involution
10:20-11:20	Stage 14	head involution, dorsal side flattens
11:20-13:00	Stage 15	dorsal closure and gut closure complete
13:00-16:00	Stage 16	dorsal segments, dorsal ridge overgrows the clypeolabrum
16:00-22:00	Stage 17	tracheal tree contains air, hatching

* tracheal tree develops from 7 hour until 17 hours

The sma gene

Only through visualization of the resulting abnormal tracheal systems will an understanding of the organization of its genic control be possible. To visualize tracheal patterns produced by each structural gene and compare it to the cuticle pattern, the sma gene is used. The function

of the sma gene is to produce B-gal specifically in the developing trachea. Because the lacZ gene was inserted into a synthetic transposon derived from the P-element, this is possible. The placement of this transposon near an enhancer element, which is active only in the tracheal system, allows for specific immunohistochemical staining for B-gal (see fig. 7; Perrimon et al, 1991).

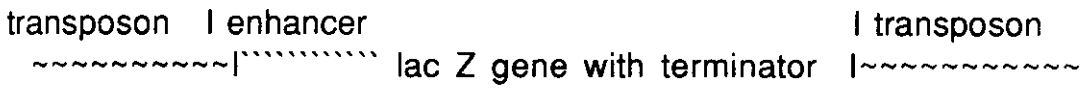


Fig. 7-Diagram of the P-element which was inserted downstream of a strong enhancer on the third chromosome in the sma strain of Drosophila melanogaster. As the genes for the trachea system are activated, B-gal is produced.

During any testing for the presence of the sma gene, positive results will be obtained only in the tracheal system, *but* in all embryos containing sma. If the tests are negative, the chromosome bearing the sma gene was not properly incorporated into the genome of that strain of flies.

Materials and Methods

Strains: Fifteen stocks carrying patterning muations were selected) display the disruption of embryonic development specifically, in the acheal system (see table 3). Additional stocks were utilized for their ıarkers or balancers (see table 2). All stocks were identified as Irosophila melanogaster and were raised on Instant Drosophila Media Carolina Biological). Majority of the stocks were obtained from the 3owling Green and Bloomington stock centers. The sma gene, however, vas kindly provided by Norbert Perrimon at Harvard. All chromosomes and nutations not specifically mentioned here are described in Lindsley and Zimm (1992).

Table 2-Stocks used primarily for their markers or balancers

FM6; CyO/bw[D]; sma/sma	rucuca
FM6; CyO/bw[D]; TM3	rucuca Pr
CyO/bw[D]; sma/sma	e sma ca
TM6b, e Tb ca/TM3, Sb e	CyO/bw[D]; TM3

Table 3- Patterning mutations and phenotype.

Mutation	abv.	type	group	Phenotype
armadillo	arm	Z		posterior 2/3 of segments replaced by mirror image of anterior
bicoid	bcd	MEL	AP-a	lack head and thorax
cut (lethal)	ct	Z	TA	posterior spiracles affected
l (1)C214	C214	Z	TA	no filzkorper
dorsal	dl	MEL	DV	lack ventral structures
empty spiracle	ems	Z	TA	no filzkorper
engrailed (lethal)	en	Z	SP	abnormalities in posterior compartment structures
Folded gastrulation	fog	Z	DV&G	lack of posterior midgut
fushi tarazu	ftz	Z	PR	deletes alternating segmental boundaries
fused	fu	MEL	SP	vein L3 and L4 in wing fuse, lacks anterior cross vein
l (1) GA41		Z	TA	gap in tracheal trunks
grained (lethal)	gran	Z	G	embryos do not elongate
hairy	h	Z	PR	deletes regions complementary to those deleted by ftz
hunchback	hb	Z	Ap-a & GAP	lack head and thorax
knirps	kni	Z	Ap-p & GAP	lack abdominal segments

MEL = Maternal Effect Lethal, Z = Zygotic Effect, AP = Anteriorposterior Pattern (a = anterior, p = posterior, t = terminal), DV = Dorsalventral Pattern, GAP = Gap Pattern, PR = Pair-rule Pattern, SP = Segment Polarity, G = Gastrulation Effect, and TA = Tracheal or Associated Structural Effect.

Mapping the sma Gene

A standard recombination gene mapping technique was used to located the approximate map units of the sma insert in relationship to the pre-established genes of ebony (70.7) and claret (100.7). The approximate location of the sma gene was vital to the incorporation of the sma gene into the genome of the stocks that carried the pattern disrupting mutations on the third chromosome. If the sma gene were located near a pattern disrupting gene, then recombination would occur at such a low probability that obtaining a patterning mutation strain that *also* carried the sma gene would be very infrequent. In order to test the frequency of recombination of the sma gene with ebony and claret, these genes were crossed with rucuca. (see appendix 1).

The recombinant forms were catagorized according to the results of the immunohistochemical staining for B-gal i.e. the sma gene. Two single crossovers and one double crossover produced six possible phenotypes (see fig.8).

Figure 8- Resulting phenotypes of recombination

Relative Position of the Markers:

ebony	+	claret
(70.7)		(100.7)
+	sma	+

On the third chromosome, ebony is located at 70.7 and claret is located at 100.7 on the standard Drosphila map. On the homologous chromosome, opposite those markers, are wild type genes which are denoted "+". Preliminary mapping had shown that the the sma gene is located somewhere between the markers.

The following are possible genotypes if recombination had occurred. If there were no recombination, then the flies would be ebony and claret or would be phenotypically wild and stain for B-gal.

Possible Recombinations

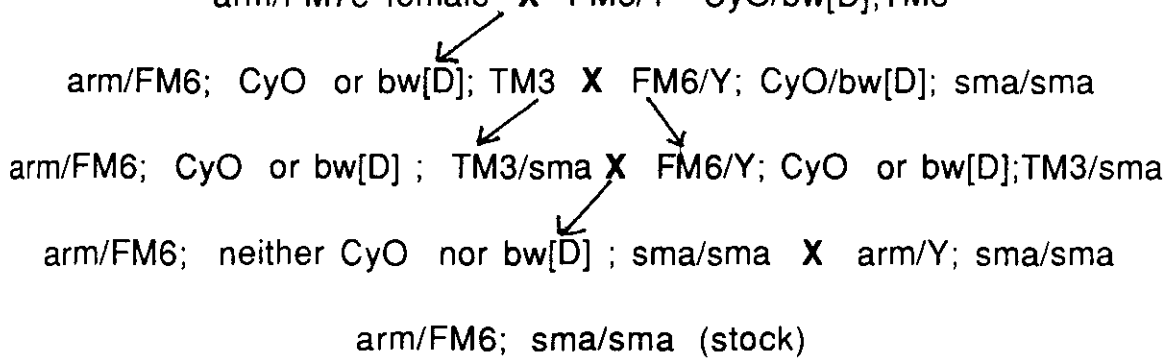
Single Crossovers	Results
e + ca / + sma +	e sma + and + + ca
e + ca / + sma +	e + + and + sma ca
Double Crossovers	**Results**
e + ca / + sma +	e sma ca and

Incorporation of the sma gene into Stocks with Pattern Disrupting Mutations

The following patterning mutations are located on the chromosome ıne and therefore are X-linked genes:

y arm[K2] (1-1.2, 1B14-1E4)
y ct[M3] (1-20, 7B3-4)
fu[E599] (59.5, 17D-E)
l(1)C214 (1-17?, 6D1-?)
l(1)GA41 (1-21?, 7C5-8)
y fog[S4] (1-65, 20A-B)

In order to be able to stain for B-gal, the sma gene, which is located ɔn the third chromosome, must be incorporated into the genome of the ıbove stocks. The general sequence of crosses is as follows:



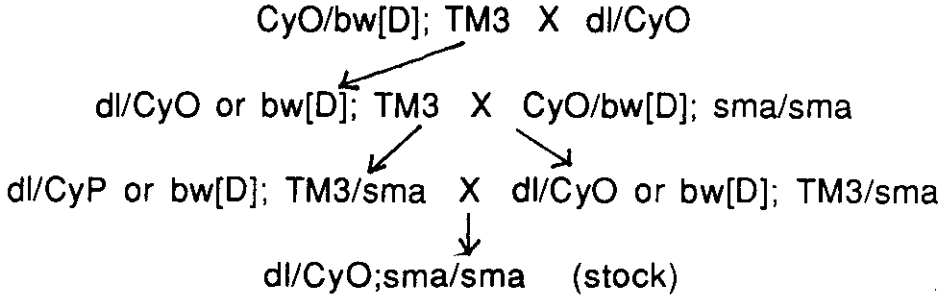
This sequence of crosses is followed for each the patterning mutation genes that are located on chromosome number one. The above

xample used the gene arm, other genes can be incorporated by changing ıe gene in the initial *arm/FM7c* stock.

Development of the second chromosome stocks is similar to the cheme used in the first chromosome example. The following patterning hutation genes are located on the second chromosome:

dl[1] (2-52.9, 36C2-9) **en[IIB86]** (2-62, 48A2)

The genes located on the second chromosome are opposite the CyO nd bw[D] balancers. The sma gene is incorporated into the genome of the dl and en stocks by completing the following crosses:



After mapping the sma gene on the third chromosome, the third chromosome patterning mutations could be recombined with the sma gene. Below are third chromosome patterning genes followed by location and other markers present.

ru **h[5H07]** (3-26.5, 66D8-12)

ru h th st **kni[5F107]** (3-47.0, 77E1-4) cu sr e ca

th st ri **bcd[E1]** (3-47.5, 84A1) roi p

ftz[W20] (3-47.5, 84B1-2) Ki p[p]

st **hb[14F21]** (3-48.3, 85A3-B1) e

ru h th st **ems[7D99]** (3-53, 88A1-10) sr e ca

ru h th st **gra** (3-47) cu sr e ca

Combining the sma gene and the patterning mutation gene onto the same chromosome required several repetitive mating schemes. Because the rate of recombination is a function of their linkage distance from sma, some crosses required more recombinants than others. The following is an example of the method for recombination of *ftz[W20]*:

1) ftz Ki p/TM3 **X** e sma or sma ca

2) ftz Ki p _____ **x** rucuca
+ + + e sma or sma ca

3a) + + + + ftz Ki p + + e sma + **x** TM6b/TM3
ru h th st + + + cu sr e + ca

3b) + + + + ftz Ki p + + + sma ca **x** TM6b/TM3
ru h th st + + + cu sr e + ca

4a) from 3a collect and mate ftz Ki p + + e sma +
e Tb ca (TM6b)

4b) from 3b collect and mate ftz Ki p + + + sma ca
e Tb ca (TM6b)

Hoyer's Mount: patterning mutations stocks.

The Hoyer's cuticle preparation removes interior organs for clear assessments of abnormalities in cuticle patterns (van der Meer, 1977). Embryos are aged twenty-four hours, dechorionated in 50% bleach, and then washed with 0.1% Triton-X in water. They are placed in glycerol/ acetic acid (1:3), and placed in a covered syracuse dish. After incubating the embryos at 65° for two to twenty-four hours, the embryos are mounted in Hoyer's medium (van der Meer, 1977) on a coverslide, and then fully cleared at approximately 65° on a slide warming table (see fig. 3).

Screens: Immunohistochemical staining for B-gal: patterning mutation stocks and recombinants.

The immunoperoxidase staining for B-gal, which tests for the sma gene, begins once the embryos have been aged six to seven hours (Klambt et al). After the embryos are dechorionated with 50% bleach and the vitelline membrane is removed, all nonspecific antigens are "blocked" with a normal serum. The embryos are incubated in mouse monoclonal antibody to Beta-galactosidase followed by a secondary antibody against mouse antibody consisting of peroxidase-conjugated antimouse IgG. A final reaction with vectastain ABC allows for the indirect staining of the

β-gal through a peroxidase substrate enzymatic reaction. (Vectastain aboratories mouse immunohistochemical stain).

Results

In mapping the approximate location of the sma gene, a total of eighty (80) recombinant stocks were tested. These stocks were then separated by phenotype to determine the genotype and as a result, the location of the crossover. This information will determine the frequency of crossing over (see table 4 below).

ebony and sma		claret and sma	
e sma +	6	e + +	16
+ + ca	20	+ sma ca	14
e sma ca	9	e sma ca	9
+ + +	4	+ + +	4
	39		43

Table 4- Indicates the total number of crossovers between the ebony and sma genes in comparison to the crossovers between the claret and sma genes. The double crossovers are counted as two single crossovers.

The frequency of crossovers between ebony and sma is .4875 or 48.75%. The frequency of crossovers between claret and sma is .5375 or

3.75%. These results indicate that the sma gene is closer to ebony. The cation of the sma gene is calculated by multiplying the frequency by the istance between ebony and claret (30 mu) and then adding that (14.63 mu) the map location of ebony (70.7 mu). The sma gene is located at pproximately 85.33 map units on the third chromosome. A 95% onfidence interval of plus or minus .184 map units has been calculated r this location.

The stocks are first tested for the patterning mutation gene, which s evaluated by the cuticle prep, and then for the sma gene, which is proven by a positive immunoperoxidase stain (see table 5).

Stocks	Hoyers	B-gal
arm		
bdc	+	+
C214		
ct		
dl	+	+
ems	+	+
en		
fog		
ftz	+	+
fu	+	+
GA41		
gra	+	-
h	+	-
hb		
kni	+	+

Table 5- The results of the Hoyer's cuticle preparation and the immunoperoxidase staining for B-gal in each stock.
Areas left blank have not been tested yet.

Of the stocks that have been tested, only six stocks have tested

positive for both procedures. The grained (gra) and hairy (h) stocks, that have been tested up to this point, do not carry the sma gene. Because both of these patterning mutations are located on the third chromosome, incorporation of the sma gene is dependent upon recombinant progeny. Locating the stocks carrying both genes will require testing additional identical mating schemes. Although majority of the chromosome one and two stocks have not been tested, positive results should be obtained for both procedures. Negative results in these stocks will most likely be attributed to a nonfunctioning balancer. While this is an unlikely occurrence, the status of the bw[D] balancer during the second chromosome matings was questionable. As a result, homozygous CyO stocks were used rather than stocks heterozygous for bw[D] and CyO.

Embryos of stocks carrying the sma gene and the patterning mutation gene will be tested for the effects of each patterning mutation on the tracheal system at times intervals between six and seventeen hours. Because of synchronous developmental events that interact to form all parts of the embryo, mutations in the patterning gene, which produce a known developmental mutation in the cuticle, should produce similar errors in the tracheal system. Anotherwords, a patterning

mutation that deletes alternating segmental boundaries should also disrupt the continuity of the tracheal system (see fig. 5 and 6). In comparison, patterning mutations that impact portions of the embryo where the trachea is not present, should not effect the tracheal development (see fig. 4). By examining a variety of these mutations which are visible in the development of the tracheal system and comparing them to the corresponding cuticular abnormalities, a sequence of gene activation can be developed.

Third Chromosome Recomination Scheme

Initial Cross: CHARACTERISTICS

$$\frac{sma}{sma} \times \frac{rucuca}{rucuca}$$

$$\frac{sma}{rucuca} \times \frac{TM3,\ Sb,\ Ser,\ e}{Tm6b,\ Tb,\ e,\ ca}$$

(virgin females) **(males)**

$$\frac{TM6b,\ Tb,\ e,\ ca}{e,\ +}$$ tubby, nonstubble, ebony, not claret

and

$$\frac{TM6b,\ Tb,\ e,\ ca}{+,\ ca}$$ tubby, nonstubble, not ebony, claret

* Collect the males and females of the above two phenotypes. Mate them individually to $\frac{sma}{TM6}$

1) $$\frac{sma}{TM6} \times \frac{TM6,\ e,\ ca}{e,\ +}$$

$$\frac{Tm6,\ e,\ ca}{e,\ +}$$ **(save)** test the wild type for sma not ebony, not claret, and not tubby

2) $$\frac{sma}{TM6} \times \frac{Tm6,\ e,\ ca}{+,\ ca}$$

$$\frac{TM6,\ e,\ ca}{+,\ ca}$$ **(save)** test wild type for sma not ebony, not claret, and not tubby

Figure Legends

Fig. 1 Normal embryo containing the sma 2 gene insert at 5.5 hours after the egg deposition stained with antibody for Beta-galactosidase. The bilateral anlagen of the tracheal system are stained for horse radish peroxidase. This embryo is oriented anterior to the left and ventral side up. 127 X magnification.

Fig. 2 Dorsal view of a normal embryo containing the sma 2 insert at about11.5 hours after egg deposition stained as in figure one. The bilateral, longitudinal tracheal trunks with dorsal and ventral segmental branches are evident. Anterior is to the left. 140 X magnification.

Fig. 3 Hoyer's cuticular preparation of a normal embryo within the vitelline membrane. The three thoracic (T1-3) and eight abdominal (A1-8) segments are indicated as are the mouth parts (M) and filzkorper (F) in the posterior spiracles of the tracheal trunks. 127 X magnification.

Fig. 4 Hoyer's cuticular preparation of an embryo produced by a female homozygous for the fs(1)Nasrat mutation. Note the absence of all structure posterior to abdominal segment seven (A7) but the tracheal trunks (T) anteriorly are still present. 130 X magnification.

Fig. 5 Hoyer's cuticular preparation of an embryo homozygous for the hairy mutation. Note the presence of first abdominal denticle belt (A1) but denticle belts of thoracic segments T1 and T3 and abdominal segments A2, A4, A6 and A8 are deleted and naked cuticle of abdominal segments A3, A5 and A7 is also deleted. 130 X magnification.

Fig. 6 Hoyer's cuticular preparation of an embryo homozygous for the runt mutation. Note that every other segment is missing starting with thoracic segment T2 so that only T1, T3, abdominal segments A2, A4, A6 and A8 are present. 130 X magnification.

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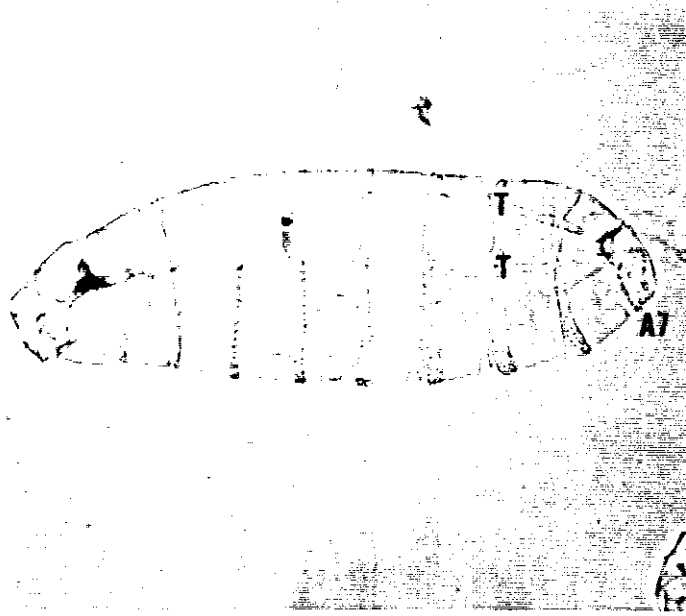

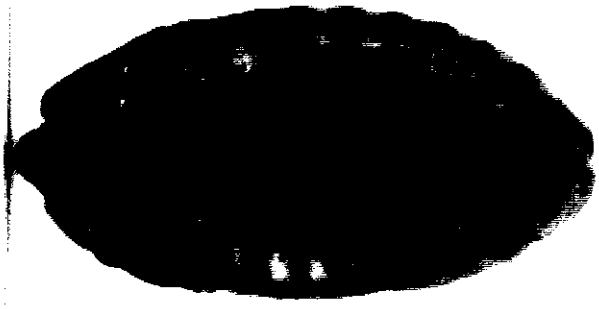

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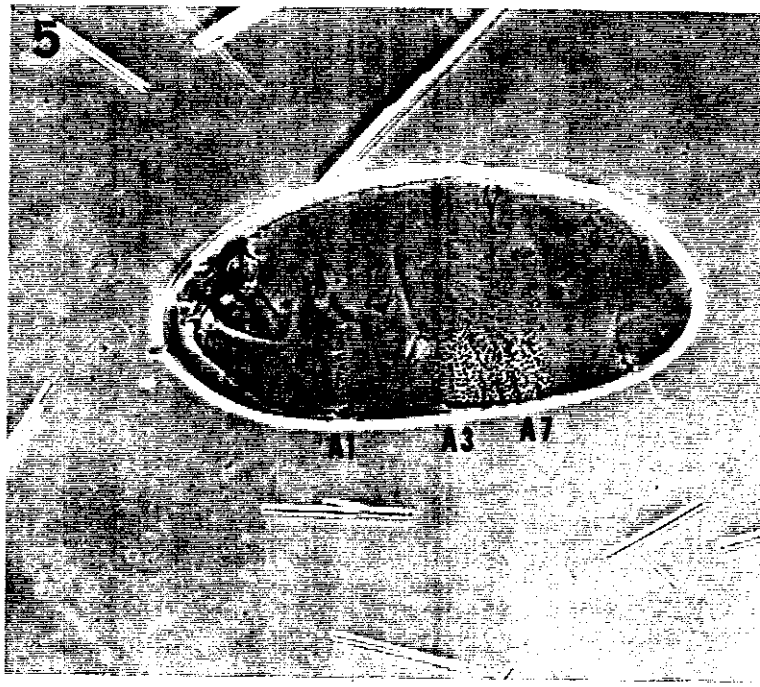

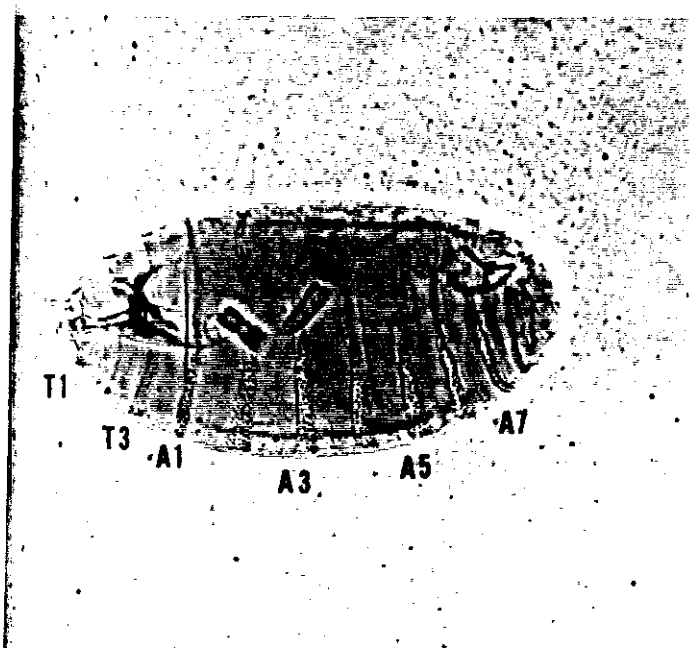

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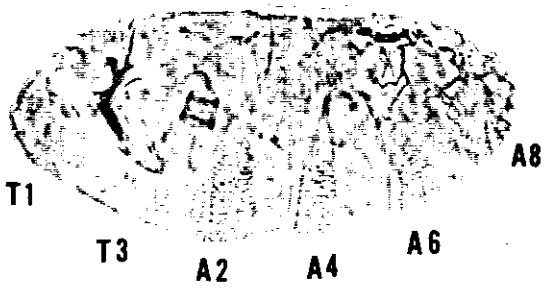

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