Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*

II. Zygotic loci on the third chromosome

G. Jürgens*, E. Wieschaus**, C. Nüsslein-Volhard*, and H. Kluding

European Molecular Biology Laboratory D-6900 Heidelberg, and
Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstrasse 37–39, D-7400 Tübingen, Federal Republic of Germany

Summary. The present report describes the recovery and genetic characterization of mutant alleles at zygotic loci on the third chromosome of *Drosophila melanogaster* which alter the morphology of the larval cuticle. We derived 12600 single lines from ethyl methane sulfonate (EMS)-treated *st e* or *rucuca* chromosomes and assayed them for embryonic lethal mutations by estimating hatch rates of egg collections. About 7100 of these lines yielded at least a quarter of unhatched eggs and were then scored for embryonic phenotypes. Through microscopic examination of unhatched eggs 1772 lines corresponding to 24% of all lethal hits were classified as embryonic lethal. In 198 lines (2.7% of all lethal hits), mutant embryos showed distinct abnormalities of the larval cuticle. These embryonic visible mutants define 45 loci by complementation analysis. For 32 loci, more than one mutant allele was recovered, with an average of 5.8 alleles per locus. Complementation of all other mutants was shown by 13 mutants. The genes were localized on the genetic map by recombination analysis, as well as cytologically by complementation analysis with deficiencies. They appear to be randomly distributed along the chromosome. Allele frequencies and comparisons with deficiency phenotypes indicate that the 45 loci represent most, if not all, zygotic loci on the third chromosome, where lack of function recognizably affects the morphology of the larval cuticle.

Key words: *Drosophila* – Larval cuticle – Pattern formation – Embryonic lethal mutations

Introduction

The sophisticated genetics of *Drosophila melanogaster* provide a rare opportunity for genetic dissection of the complex developmental processes that transform the fertilized egg cell into the spatial pattern observed in the differentiated embryo. Systematic attempts at the genetic analysis of embryogenesis require the isolation and characterization of a large number of embryonic mutants. These mutants pro-

vide a means of obtaining information about different aspects of embryonic development. The number of gene functions affected indicates how many components are specific to the process. The kinds of phenotypic change observed in mutant embryos reveal parameters of the developmental system. Insights into functional relationships between genetically defined components can be obtained by studying phenotypes of combinations of mutant genes. Finally, genetic identification and characterization of the individual functions involved in any complex developmental process are necessary prerequisites for further study by present-day techniques of molecular biology.

Our long-term research activities concern the processes establishing the basic body pattern of the *Drosophila* embryo. As a first step toward this objective, we have performed large-scale screens for zygotic mutations that distinctly alter the morphology of the embryonic cuticle. The present report describes the isolation and genetic characterization of such embryonic visible mutations located on the third chromosome. Similar screens for the other chromosomes are reported in the accompanying papers (Nüsslein-Volhard et al. 1984; Wieschaus et al. 1984).

Materials and methods

Strains. Marker mutants and balancers are described in Lindsley and Grell (1968). Deficiency stocks and marker mutants were obtained from the Drosophila Stock Centres at Caltech Pasadena, Cal, USA and Bowling Green Ohio, USA or directly from the discoverer. Flies were grown on standard medium in humidified rooms at the temperatures indicated.

Lethal-free third chromosomes of the genotypes *ru h th st cu sr e^s ca (rucuca)*, and *st e* (two different lines) were mutagenized. *In(3LR)561, DTS4 th st Sb e/Ser* was used to eliminate DTS-bearing progeny (Marsh 1978). *DTS4* is a dominant temperature-sensitive mutation which survives at low temperature; its temperature-sensitive period extends from embryogenesis until puparium formation (Holden and Suzuki 1973).

Balanced stocks of putative embryonic visible mutants were established over *TM3, Sb Ser* or *TM1, Me cu*, with the aid of *DTS4* or *DTS7, st p*. *DTS7* has a late temperature-sensitive period (Holden and Suzuki 1973).

Mutagenesis. A total of 5000 males, 0–48 h old, were fed with 25 mM ethyl methane sulfonate (EMS) in 1% sucrose solution for 24 h (Lewis and Bacher 1968). During EMS

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* *Present address:* Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstraße 37–39, D-7400 Tübingen Federal Republic of Germany

** *Present address:* Department of Biology, Princeton University, Princeton, New Jersey 08544, USA

Offprint requests to: G. Jürgens at the above address

treatment the males were kept in 30 bottles plugged with foam stoppers into which liquid scintillation vials had been inserted. The vials contained cotton wool soaked with the EMS solution (approximately 6 ml per vial). The bottoms of the vials were replaced by filter-paper disks through which the flies received the mutagen.

To determine the frequency of third-chromosomal lethals, 100 balanced lines were established for each of the three separately treated chromosomes and scored for the survival of homozygous *rucuca** or *st e** flies.

Screening procedure. The crossing scheme is illustrated in Fig. 1. We mated 5000 mutagenized males to 10000 *DTS/Ser* females and discarded them after 6 days. F1 progeny males were individually mated to 3 *DTS4/Ser* females. A total of 14000 single lines were set up and grown at 18° or 25° C so that the progeny could be tested 2 weeks later. Periods of permissive temperature were interrupted by 2 days of 29° C exposure, which was sufficient to kill developing *DTS4*-bearing progeny. Escapers were observed only rarely. They usually eclosed later and were very weak. The parental flies were removed from the vials using a vacuum cleaner after the first day at 29° C. Eggs were collected from F2 flies as previously described (Nüsslein-Volhard 1977). This was done at 29° C in the first of four series, but at room temperature in subsequent series to reduce the frequency of unfertilized eggs. Hatch rates were estimated, and eggs processed for microscopic inspection as previously described (Nüsslein-Volhard et al. 1984) if at least a quarter of the eggs remained unhatched. To recover putative mutants, 2–10 heterozygous males were mated individually to *DTS7, st p/TM3, Sb Ser* or *DTS4/TM1, Me cu* females. Their developing F1 progeny were exposed to 29° C at an early or late stage, according to the temperature-sensitive period of the *DTS* chromosome used. The surviving F1 adults carrying both the mutagenized chromosome and a balancer were used to establish stocks. For putative second-chromosomal mutants, five males were crossed with *DTS91/CyO* females and the second chromosome was isogenized in the following generation.

Characterization of mutants. Tests for putative translocations and semi-dominant maternal effect mutations, complementation tests as well as genetic and cytological localisations were performed as described by Nüsslein-Volhard et al. (1984). The embryonic visible mutations were genetically mapped using the markers present on the *rucuca* chromosome.

Results

Mutant screen

Single lines derived from individual mutagenized third chromosomes were screened for zygotic lethal mutations affecting the morphology of the embryonic cuticle. Our crossing scheme presented in Fig. 1 differed from the usual procedure in that the single lines were not balanced before the scoring of phenotypes in homozygous embryos, nor were the lines preselected for lethal mutations. This procedure was employed because all available third-chromosomal balancers carried embryonic or embryonic-larval boundary lethals, which would have interfered with the detection of embryonic lethals by hatch rate.
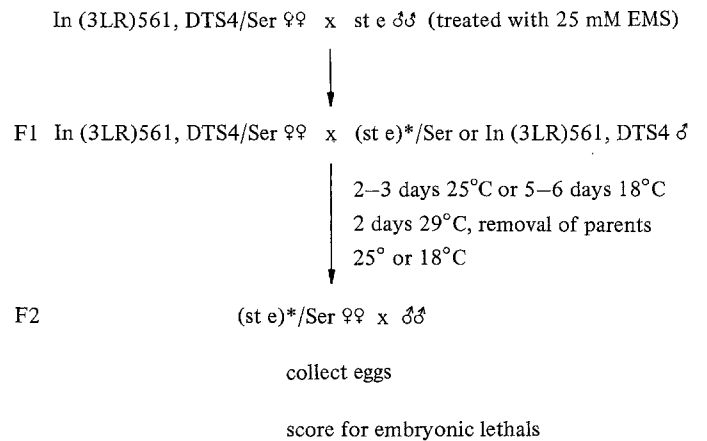
In (3LR)561, DTS4/Ser ♀♀ x st e ♂♂ (treated with 25 mM EMS)

F1 In (3LR)561, DTS4/Ser ♀♀ x (st e)*/Ser or In (3LR)561, DTS4 ♂

2–3 days 25°C or 5–6 days 18°C
2 days 29°C, removal of parents
25° or 18°C

F2   (st e)*/Ser ♀♀ x ♂♂

collect eggs

score for embryonic lethals

Fig. 1. Crossing scheme for identifying zygotic embryonic visible mutants induced in genetically marked third chromosomes. About half the single lines were derived from individual mutagenized *rucuca*, instead of *st e*, chromosomes. Eggs were collected and phenotypes were scored as described in Materials and methods. Stocks of putative embryonic visible mutants were established by crossing F2 males to appropriate females (see text)

A consequence of our screening procedure was that the efficiency of mutagenesis had to be separately assessed. To estimate the frequency of EMS-induced lethals, balanced single lines were established for each of the three mutagenized genotypes. A total of 257 lines were derived, of which 112 did not produce homozygous flies in the F2 generation. The frequency of 44% lethals corresponds to approximately 0.6 lethal hits per chromosome. This value is much lower than expected for the EMS concentration used. The low efficiency of mutagenesis may have been caused by the feeding protocol (see Materials and methods).

Approximately 14000 single lines of mutagenized third chromosomes were set up. Of these, 12600 lines produced enough progeny so that eggs could be collected from them. Cuticle preparations of embryos were made from the 7100 lines in which at least a quarter of the eggs remained unhatched. The cuticle preparations were screened for morphological abnormalities. The test lines were distributed among four broad categories according to the appearance of the embryo (Nüsslein-Volhard et al. 1984).

1. Putative embryonic viable lines. Most of the unhatched eggs were apparently unfertilized. Developed embryos did not show uniform phenotypes.

2. Putative embryonic lethal lines, apparently normal morphology.

3. Putative embryonic lethal lines, poorly differentiated or subtle defects. Most of the embryos were either not fully differentiated or showed very slight deviation from normal morphology. In contrast to the screen for embryonic visible mutations on the second chromosome, mutants in which head development appeared to be unspecifically affected were included in this category. In such cases all the head structures of a wild-type larva were present but abnormally located due to incomplete involution, or melanized material was found in the head region (see also Nüsslein-Volhard et al. 1984).

4. Putative embryonic lethal lines, distinct morphological abnormalities (putative embryonic visible mutations). The majority of the unhatched eggs displayed a distinct phenotype such as unpigmented cuticle, no cuticle, holes,

Table 1. Screen for embryonic lethal mutants on the third chromosome

	n	% of lethal hits
Lines tested	12 600	
Lethal hits[a]	7300	100
Embryonic lethal lines[b]	1772	24.3
Normal morphology[b]	1149	15.7
Subtle defects[b,c]	426	5.8
Zygotic embryonic visible mutations on third chromosome	198	2.7
In complementation groups	185	2.5
Single mutants defined[d]	5	0.1
Other single mutants	8	0.1

[a] Calculated as number of lethal hits/chromosome × number of lines tested; based on the lethal frequency of a sample of 257 balanced lines (44%) assuming a Poisson distribution of lethal hits (m = 0.58)

[b] Approximate values due to screening procedure (see text)

[c] Includes poor differentiation, slight deviation from normal morphology and unspecific head defects (see text)

[d] Either allelic to known loci or uncovered by deficiency showing the same phenotype

altered segmentation, homoeotic changes or lack of certain cuticular structures.

We saved 493 putative embryonic visible mutant lines for further analysis. Immediately after balancing all lines were retested, except 17 lines which were accidentally lost. Of the remaining lines 229 still produced phenotypes while the others were assigned to one of the other three categories. We classified 198 lines as true zygotic embryonic visible mutations on the third chromosome. Eleven lines were presumably translocations, as they produced aneuploid offspring in matings of wild-type females to heterozygous mutant males. We localized 14 zygotic mutants with interesting phenotypes, such as altered segmentation, to the second chromosome and these were identified as alleles at previously identified loci (see Nüsslein-Volhard et al. 1984). In addition, three maternal effect mutations on the second chromosome and three dominant maternal effect mutations on the third chromosome were identified. Table 1 summarizes the data, taking changes due to reclassification into account. The final number of putative embryonic lethal lines was 1772, corresponding to 24.3% of all lethal hits. Table 1 shows that the 198 confirmed zygotic mutants on the third chromosome represent a very small proportion of all lethal hits (2.7%).

Complementation analysis

One of our aims was to determine the number of loci which were represented by the 198 zygotic mutants on the third chromosome. To facilitate the analysis, mutants were grouped on the basis of phenotypic similarities (see Nüsslein-Volhard et al. 1984). Within each group, one mutant was tested for complementation with the other members of the group. Many mutants were assigned to complementation groups in this manner in the first two series of crosses. With the remaining mutants, complementation analysis was
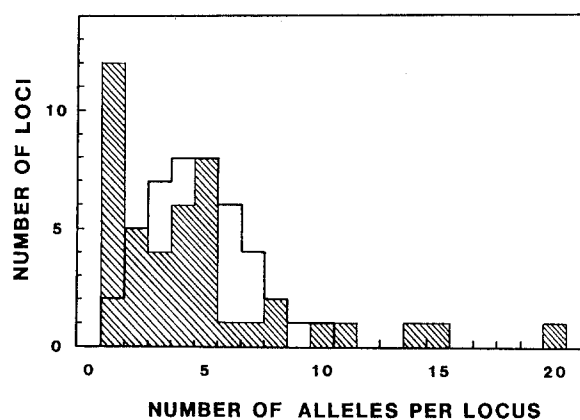

Fig. 2. Distribution of allele frequencies. The observed number of loci with the indicated number of alleles are represented by the *cross-hatched columns*. The *open columns* show the Poisson distribution of loci based on the observed mean value m = 4.5

done after studying temperature-dependent expression of mutant phenotypes or after genetic localization. We did not observe complementation between alleles at any of the loci on the third chromosome, with the exception of *tolloid (tld)*. The *tld* locus showed a complex pattern of complementation in that only one *tld* mutant did not complement all the other *tld* mutants, whereas the remaining 14 alleles partially complemented at least one other allele.

Of the 198 mutants, 185 fall into 32 complementation groups with more than one allele, with an average of 5.8 alleles per group. Two stocks proved to be mutant at two loci; 13 mutants, or 6% of all mutants, remained single. If the single mutants are included in the calculation, each group was on the average represented by 4.5 alleles. The allele frequencies are distributed among complementation groups as illustrated in Fig. 2. The Poisson distribution of allele frequencies based on the same mean value differs from the observed distribution both in the single mutants and in the large complementation groups, indicating that the embryonic visible mutants we isolated are not randomly distributed among the complementation groups. Specifically, two phenotypic classes, "homoeotics" (five complementation groups) and "dorsal holes" (seven complementation groups) are mainly represented by single mutants (seven) and loci with only two alleles (two) in our sample of mutants. Finally, four of the single mutants and four complementation groups are allelic to the previously identified loci *Pc, Antp, bxd, E(spl), h, Dl, Scr,* and *ftz*. A list of the 45 embryonic visible loci on the third chromosome is presented in Table 2.

Embryonic phenotypes

The following preliminary classification of mutant phenotypes is based mainly on cuticle preparations (Fig. 3).

Ten loci affect the anteroposterior pattern. Six of these are clearly involved in segmentation. In addition to the previously described loci *hairy-barrel, hunchback, hedgehog, knirps* (Nüsslein-Volhard and Wieschaus 1980) and *fushi tarazu* (Wakimoto and Kaufman 1981), one other segmentation locus, *odd-paired,* was identified. *Odd-paired* alleles cause deletions in a double-segmental repeat corresponding to that of the locus paired, but shifted by one segment (Nüsslein-Volhard et al. 1982). Stronger alleles of *hunchback* were isolated deleting all gnathal and thoracic seg-

Table 2. Zygotic loci on the third chromosome mutating to embryonic visible phenotypes

Locus	Phenotype	Number of alleles			Map position[a]	Cytology[b]
		total	weak	ts		
Antennapedia (Antp)	homoeotic; T2 and T3 resemble T1; no dominant "Antp" phenotype in adults	1	–	–	47.5	84B1,2[c]
bithoraxoid (bxd)	homoeotic; A1 resembles T2/T3; no dominant "Ubx" phenotype in adults	1	–	–	58.8	89E1,2[d]
branch (bch)	incomplete fusion of denticle bands	1	–	–	(46)	
canoe (cno)	dorsal open	14	4	1	49	
crumbs (crb)	many small holes in cuticle	6	1	–	82	(95E-96A)
Delta (Dl)	no ventral cuticle; hypertrophy of central nervous system; dominant "Dl" phenotype in adults	10	3	2	66.2	92A1,2[e]
disembodied (dib)	no differentiation of cuticle and head skeleton	3	–	–	12	(62D-64C)
empty spiracles (ems)	spiracles devoid of filzkörper, no antenna, head open	5	–	–	53	88A1-10
Enhancer of split (E(spl))	no ventral cuticle; hypertrophy of central nervous system	1	–	–	89	96E-F[e]
fork head (fkh)	head skeleton forked; no anal pads	1	–	–	98	98B-99A
fushi tarazu (ftz)	pair-rule segmentation defects; deletion of denticle bands of T2, A1, A3, A5, A7 and adjacent naked cuticle on either side	4	–	–	47.5	84B1,2[f]
grain (gra)	filzkörper not elongated, head skeleton defective	2	–	–	47	
hairy (h)[i]	pair-rule segmentation defects; deletion of denticle bands of T1, T3, A2, A4, A6, A8 and naked cuticle of T2, A1, A3, A5, A7	8	4	–	26.5	66D8-12[g]
haunted (hau)	only head skeleton visible; no differentiation of cuticle	4	–	–	48	
hedgehog (hh)	segment polarity mutant; deletion of naked cuticle, fusion of denticle bands	7	2	2	81	(94E)
homothorax (hth)	homoeotic; thoracic segments similar to one another	1	–	–	48	(85E-86B)
hunchback (hb)	gnathal and thoracic segments deleted	11	5	1	48	85A[h]
kayak (kay)	dorsal open	2	–	–	99	(99A-100A)
knickkopf (knk)	head skeleton defective, denticle bands narrower, embryo rarely inverted in egg case	4	1	1	49	(85E-86B)
knirps (kni)	denticle bands of A1 to A7 fused into single field	3	2	–	47.0	77E[h]
krotzkopf verkehrt (kkv)	head skeleton crumbled, denticle bands narrower, embryo sometimes inverted in egg case	20	–	–	47	
naked (nkd)	denticle bands deleted	5	3	–	47	(75D-76B)
neuralised (neu)	no ventral cuticle; hypertrophy of central nervous system	3	1	1	50.0	86C1-D8[e]
odd-paired (opa)	pair-rule segmentation defects; deletion of denticle bands of T2, A1, A3, A5, A7 and naked cuticle of T1, T3, A2, A4, A6	5	3	–	48	(82A-E)
pale (ple)	unpigmented cuticle and head skeleton	4	–	1	18	(65A-E)
pannier (pnr)	dorsal anterior open	2	–	–	58	89B4-10
pebble (pbl)	head skeleton, denticle bands and filzkörper rudimentary; posterior end on dorsal side	5	–	–	26	(66A-C)

Table 2. (continued)

Locus	Phenotype	Number of alleles			Map position[a]	Cytology[b]
		total	weak	ts		
pointed (pnt)	head skeleton pointed, deletion of median portion in all denticle bands	2	–	–	79	(94E)
Polycomb (Pc)	homoeotic transformation of head and thoracic segments towards A8	1	1	–	47.2	78D-79B
punt (put)	dorsal open	1	–	–	(58)	88C3-E2
rhomboid (rho)	head skeleton pointed, deletion of median portion in all denticle bands	1	1	–	3	(61F-62D)
serpent (spt)	posterior end of embryo remains on dorsal side	5	–	–	58	89A1-B4
Sex combs reduced (Scr)	homoeotic transformation of labium to maxilla and prothorax to mesothorax	4	–	–	47.5	84B1,2[c]
shade (shd)	no differentiation of cuticle and head skeleton	5	–	–	41	(70D-71C)
shadow (sad)	no differentiation of cuticle and head skeleton	5	–	–	51	86F6-87A7
shrew (srw)	posterior end pulled towards interior	1	1	–	(15)	(62D-64C)
shroud (sro)	no differentiation of cuticle and head skeleton	4	–	–	100	(99A-100A)
spook (spo)	no differentiation of cuticle and head skeleton	5	–	–	19	
string (stg)	number of denticle rows strongly reduced	8	2	1	99	(98A-99A)
tailless (tll)	A8 and telson missing, head skeleton defective	1	–	–	102	(100A-B)
tolloid (tld)	embryo twisted; denticle belts laterally spread; visible at gastrulation	15	9	1	85	(96A-C)
tracheales (trh)	no tracheae, filzkörper not elongated	2	–	–	–1	(61E-F)
yurt (yrt)	dorsal posterior hole	3	1	–	52	87E12-F12
l(3)5G83	dorsal open	1	–	–	(80)	
l(3)7E103	dorsal open	1	–	–	(47)	

[a] Map positions in parentheses are approximate;
[b] Cytology given in parentheses is based on segmental aneuploidy of translocations;
[c] Kaufman et al. (1980);
[d] Lewis (1978);
[e] Lehmann et al. (1983)
[f] Wakimoto and Kaufman (1981)
[g] D. Ish-Horowicz, personal communication;
[h] R. Lehmann, personal communication
[i] hairy has also been referred to as barrel by Nüsslein-Volhard and Wieschaus (1980)

ments, as well as having defects in the eighth abdominal segment. One other locus, naked, produces pair-rule defects in hypomorphic alleles, whereas almost no segmental denticle belts are found in strong alleles. Tailless affects the most anterior and the most posterior structures of the embryo. One mutant, branch, causes fusion of segments without any apparent regularity. Segmentally repeated defects of denticle belts are found in string embryos.

Five loci affect the dorsoventral pattern. The effects of tolloid (tld) are first visible at gastrulation (Frohnhöfer 1982). In strong alleles the tld phenotype approaches the ventralized phenotype of the dominant maternal effect mutations at the Toll locus seen in the deletion of dorsal pattern elements (Anderson and Nüsslein-Volhard 1983). The

mutant shrew produces a phenotype similar to moderate tld phenotypes. Two loci, rhomboid and pointed, cause reduction of denticle belts mediolaterally, producing phenotypes similar to the second-chromosomal mutants Star and spitz (Nüsslein-Volhard et al. 1984). Serpent embryos appear slightly twisted with the posterior end wound up on the dorsal side.

The ventral cuticle is missing in embryos mutant for the three neurogenic loci neuralised, Delta and Enhancer of split whose phenotypes have been studied in detail (Lehmann et al. 1981, 1983). Mutants at one locus, crumbs, show many small holes in the cuticle presumably caused by death of epidermal cells. A big gap in the dorsal cuticle results from mutations at five loci including canoe, kayak and punt.
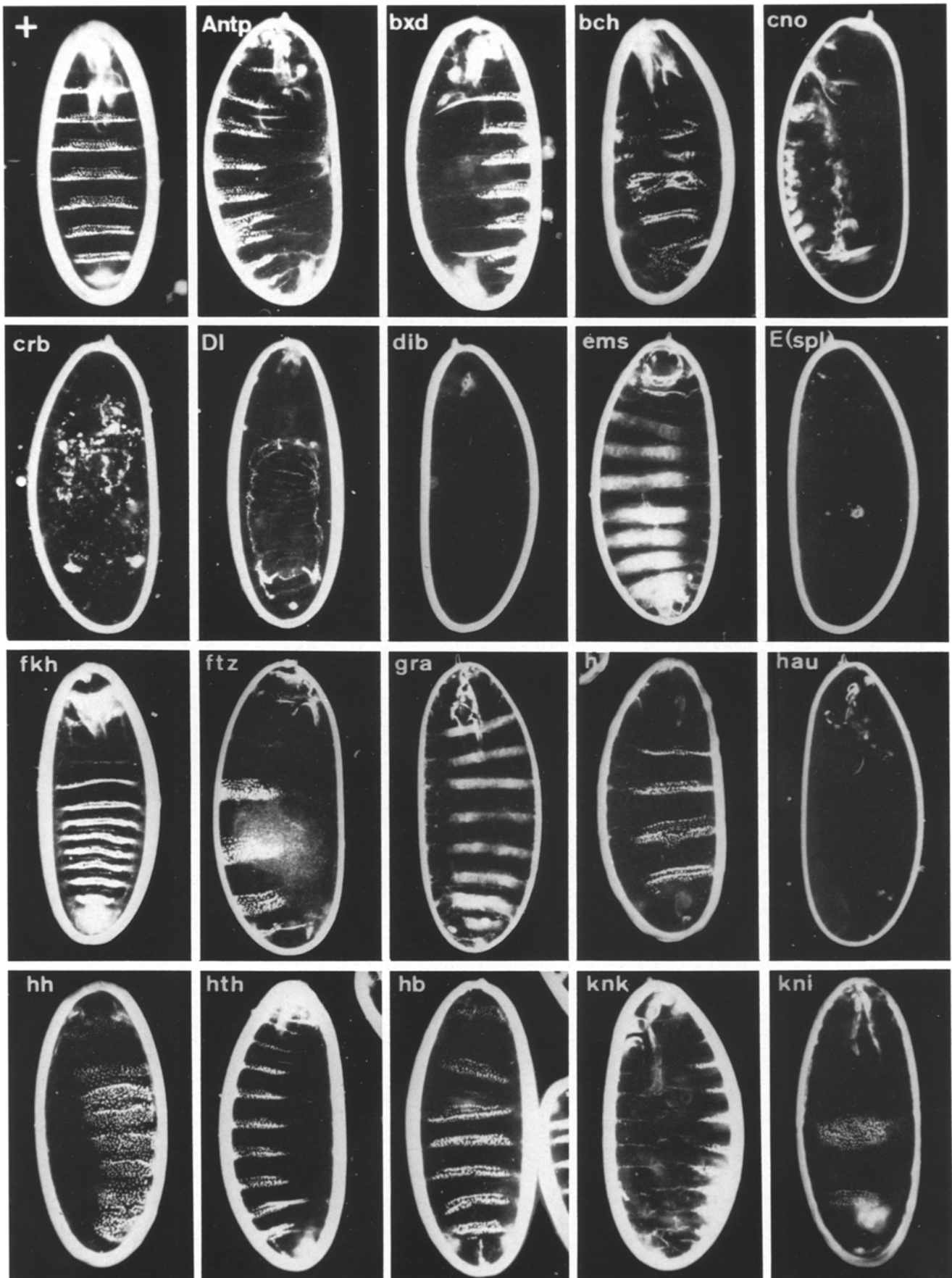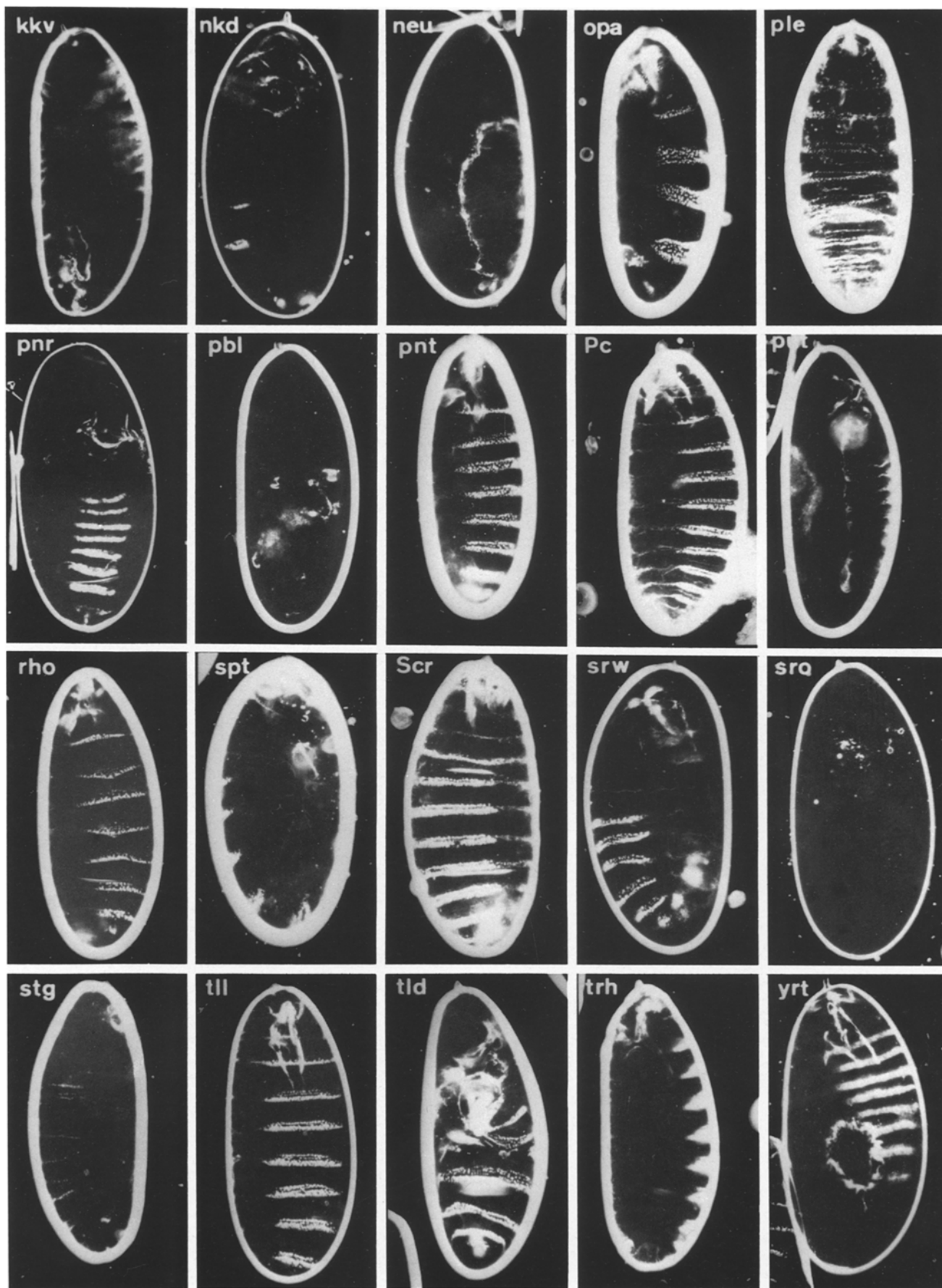
Fig. 3. Dark-field photographs of cuticle preparations of homozygous mutant embryos. For designations refer to Table 2

The dorsal cuticle is anteriorly open in *pannier* embryos, while *yurt* mutations produce a posterior hole in the dorsal cuticle.

Two loci with similar phenotypes, *krotzkopf-verkehrt (kkv)* and *knickkopf (knk)*, affect the sclerotization of the head skeleton, which is patchy in *kkv* embryos and confined to the dorsal and lateral portions in *knk* embryos. Alleles at these two loci also cause hyperactivity of the differentiated embryo which may turn around in the egg case. One locus, *pale*, affects pigmentation of the cuticle while the pattern is normal. This phenotype is very similar to the phenotypes of *Ddc, faint, faintoid* and *unpigmented* (Nüsslein-Volhard et al. 1984; Wieschaus et al. 1984). Six loci interfere with the differentiation of the cuticle. One of them, *haunted*, produces the head skeleton but no cuticle. The remaining five loci, *disembodied, shade, shadow, shroud* and *spook*, do not differentiate any cuticle specializations. Three loci affect the filzkörper. In mutants at two loci, *grain (gra)* and *tracheless (trh)*, the filzkörper appear round rather than elongated whereas *empty spiracles* lacks them (and the antennae) completely. In addition, *gra* and *trh* affect the head skeleton and the tracheae, respectively. In the mutant *fork head* the head skeleton appears forked and the anal pads are missing.

Finally, mutant alleles at five homoeotic loci have been identified. Four of them had been previously known: *Pc* and *bxd* (Lewis 1978) as well as *Scr* and *Antp* (Wakimoto and Kaufman 1981). In the single mutant *homothorax* the morphology of the thoracic denticle bands is intermediate between normal T1 and T2.

Genetic localization

Lethality of a mutant was mapped by genetic recombination with respect to the markers present on the mutant chromosome. Subsequently, the map position of the embryonic phenotype was confirmed within the relevant marker interval. As shown in Fig. 4, the embryonic visible loci appear not to be randomly distributed on the genetic map of the third chromosome but rather clustered around the centromere. Of the 45 complementation groups, 36% map within 6% of the genetic map, i.e. between *st* and *cu*. However, when the cytogenetic map is chosen as the reference system, the loci seem to be more evenly distributed, except that loci mapping in the middle region of the left arm are rare (Fig. 4, Table 2). Genes which mutate to related phenotypes do not in general map next to one another. This is exemplified by the neurogenic loci *neu, Dl* and *E(spl)* or by the "shadow" genes affecting cuticle differentiation, which are scattered along the chromosome. In contrast, some homoeotic genes are tightly linked, such as *Scr* and
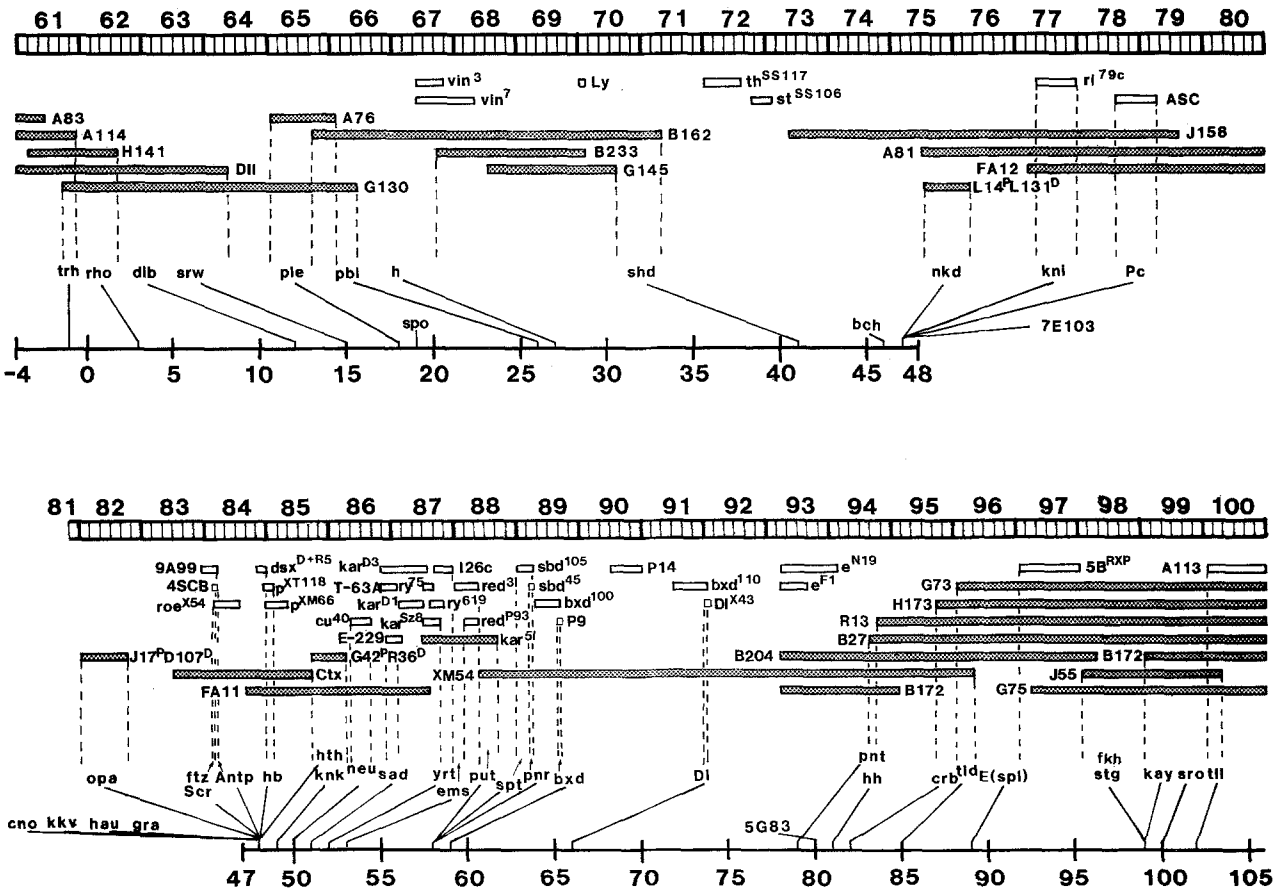

Fig. 4. A simplified map of the third chromosome indicating map positions and cytological locations of the 45 embryonic visible loci. The chromosomal aberrations used for cytological localization are represented by *open bars* (deficiencies which were analysed for their own embryonic phenotypes) and *shaded bars* (terminal or interstitial deficiencies segregated by translocations). The *vertical dashed lines* define the limits of cytological locations assigned to 37 of the 45 loci. Loci not defined cytologically are shown directly above the genetic map. For breakpoints of deficiencies see Table 3

Table 3. Third-chromosomal deficiencies used for cytological localization of embryonic visible loci

Chromosomal aberration	Cytology of deficiency	Embryonic phenotype of homozygous deficiency	Embryonic visible loci uncovered	Reference
T(Y; 3)A83	61A1; 61C	—	—	a
T(Y; 3)A114	61A1; 61F	—	trh	a
T(Y; 3)H141	61A-B; 62D	—	trh, rho	b
T(2; 3)Dll	61A1; 64B-C	—	trh, rho, dib, srw	c
T(Y; 3)G130	61E; 66C	—	trh, rho, dib, srw, ple, pbl	b,t
T(Y; 3)A76	65A; 66A	—	ple	b
T(Y; 3)B162	65E; 71A-C	—	pbl, h, shd	b
T(Y; 3)B233	67E; 70A	—	—	b
Df(3L)vin³	68C8-12; 68E3-F1	normal	—	d
Df(3L)vin⁷	68C8-12; 69B3-C1	normal	—	d
T(Y; 3)G145	68D; 70D	—	—	b
Df(3L)Ly	70A2-3; 70A5-6	normal	—	e
Df(3L)th^{SS117}	72A1; 72D5	random holes	—	f
Df(3L)st^{SS106}	72E5; 73A4	heterogeneous: head open, tail-up	—	f
T(Y; 3)J158	73C; 79D	—	nkd, kni, Pc	b
T(Y; 3)A81	75C-D; 80	—	nkd, kni, Pc	b
Df(3L)L14^{P}L131^D	75D; 76B	—	nkd	a
T(2; 3)FA12	77A-B; 80F	—	kni, Pc	g
Df(3L)ri^{79c}	77B-C; 77F-78A	"knirps"	kni	c
Df(3L)ASC	78D-E; 79B-C	"Polycomb"	Pc	c
Df(3R)J17^{P}D107^D	82A; 82E	—	opa	a
T(2; 3)Ctx	83C9-D1; 85E5-9	—	Scr, ftz, Antp, hb	s
Df(3R)9A99	83F2-84A1; 84B1,2	"fushi tarazu"	Scr, ftz	c
Df(3R)4SCB	84A6-B1; 84B2-3	"fushi tarazu"	Scr, ftz, Antp	c
Df(2R)roe^{X54}	84A6-B1; 84D4-9	"fushi tarazu"	Scr, ftz, Antp	c
T(1; 3)FA11	84D-E; 87D	—	hb, hth, knk, neu, sad	g
Df(3R)dsx^{D+R5}	84F2-3; 84F16	poorly differentiated	—	h
Df(3R)p^{XT118}	84F; 85A	"hunchback"	hb	g
Df(3R)p^{XM66}	84F-85A;85B-C	"hunchback"	hb	c
Df(3R)G42^{P}R36^D	85E; 86B	—	hth, knk	a
Df(3R)cu^{40}	86C1,2; 86D8	"neuralised"	neu	i
Df(3R)M-S31	86D1; 86D4	normal	—	i
Df(3R)kar^{D3}	86E16-18; 87D3,4	"shadow"	sad	j
Df(3R)T-63A	86F1,2; 87A4,5-7	"shadow"	sad	k
Df(3R)E-229	86F6,7; 87B1-2	"shadow"	sad	k
Df(3R)kar^{D1}	87A7,8; 87D1,2	normal	—	j
Df(3R)kar^{3Q}	87B2-4; 87C9-D3,4	normal	—	k
Df(3R)ry^{85}	87B15-C1; 87F15-88A1	undifferentiated	—	l
Df(3R)kar^{SZ8}	87C1-3; 87D14-15	normal	—	m
T(1; 3)kar^{51}	87C7-D1; 88E2-3	—	ems, put	l
Df(3R)ry^{75}	87D1,2; 87D14-E1	normal	—	o
Df(3R)ry^{1608}	87D4-6; 87E1-2	normal	—	o
Df(3R)ry^{619}	87D7-9; 87E12-F1	normal	—	o
Df(3R)l26c	87E1-2; 87F11-12	dorsal hole	yrt	o
Df(3R)red^{31}	87F12-14; 88C1-3	undifferentiated	ems	l
Df(3R)red^{P93}	88A10-B1; 88C2-3	normal	—	s
T(2; 3)XM54	88C2-3; 96B11-C1	—	put, tld	c
Df(3R)sbd^{105}	88F9-89A1; 89B9-10	"serpent"	spt, pnr	e
Df(3R)sbd^{45}	89B4; 89B10-13	"dorsal open"	pnr	n
Df(3R)bxd^{100}	89B5-6; 89E2-3	"bithoraxoid"	bxd	e
Df(3R)P9	89E1; 89E4-5	all abdominal segments transformed into thoracic ones	bxd	r
Df(3R)P14	90C2-D1; 91A2-3	normal	—	e
Df(3R)bxd^{110}	91C7-D1; 92A2-3	"Delta"	Dl	e
Df(3R)Dl^{X43}	92A	"Delta"	Dl	g
T(Y; 3)B204	93B; 98B	—	pnt, hh, crb, tld, E(spl)	b
Df(3R)e^{F1}	93B6-7; 93E1-2	normal	—	p
Df(3R)e^{N19}	93B; 94A	poorly differentiated	—	u
T(Y; 3)B172	93B; 95A and 99A; 100F5	—	pnt, hh, kay, sro	a
T(Y; 3)B27	94E; 100F5	—	pnt, hh	a
T(Y; 3)R13	94E, 100F5	—	crb	a
T(Y; 3)H173	95E; 100F5	—	crb, tld	a
T(Y; 3)G73	96A; 100F5	—	tld, E(spl)	a
Df(3R)5B^{RXP}	97A; 98A1,2	normal	—	q

Table 3 (continued)

Chromosomal aberration	Cytology of deficiency	Embryonic phenotype of homozygous deficiency	Embryonic visible uncovered	Reference
T(Y; 3)G75	97B; 100F5	—	stg, tll	a
T(Y; 3)R128	97F; 100F5	—	stg, kay	a
T(Y; 3)J55	98A; 100B	—	fkh, stg, kay, sro, tll	b
T(Y; 3)A113	100A; 100F5	"tailless"	tll	a

[a] Lindsley et al. (1972); [b] Seattle-La Jolla Drosophila Laboratories (1971); [c] Jürgens, unpublished work; [d] Akam et al. (1978); [e] Lindsley and Grell (1968); [f] Ashburner et al. (1980); [g] R. Lehmann, unpublished work; [h] Duncan and Kaufman (1975); [i] Ashburner et al. (1981); [j] Costa et al. (1977); [k] Gausz et al. (1981); [l] Hall and Kankel (1976); [m] Gausz et al. (1980); [n] Spillmann and Nöthiger (1978); [o] Hilliker et al. (1980); [p] Fortebraccio et al. (1977); [q] K. Anderson, unpublished work; [r] Lewis (1980); [s] Lewis; cytology: Jürgens, unpublished work; [t] cytology revised; [u] Garcia-Bellido et al. (1983)

Antp in band 84B1,2 (Kaufman et al. 1980) and the *BX-C* genes in bands 89E1-5 (Lewis 1978).

Cytological localization

Attempts were made to localize the loci on the polytene chromosome map by complementation tests with cytologically defined deficiencies. This was done by scoring embryonic phenotypes of trans-heterozygotes. In addition to simple deficiencies, we also used translocations segregating interstitial or terminal deficiencies for the third chromosome. The results are summarized in Fig. 4 and Table 2. Of the 45 loci, 34 were localized to chromosomal segments of the size of one numbered division or less. For three loci phenotypes were uncovered by deficiencies derived from translocations, which confine these loci to regions less than three numbered divisions long. Eight loci were not uncovered by available deficiencies.

The simple deficiencies used for cytological localization of the loci were also screened for embryonic phenotypes in cuticle preparations (Table 3). Homozygous deficiency embryos from two stocks, ry^{85} and red^{31}, did not differentiate cuticle. The remaining deficiencies spanning approximately 520 bands fell into two classes: those which do not interfere with morphologically normal development and those which themselves produce the embryonic phenotypes they uncover when in *trans* to the respective embryonic visible mutations. None of the available deficiencies causes distinct alterations of the embryonic cuticle not already identified by the mutations described here. Excluding those deficiencies that had been specifically isolated because of particular embryonic visible loci (*ASC, 9A99, roe*^{X54}, bxd^{100}), we have analysed 430 bands, or 21% of the third chromosome for embryonic phenotypes. These bands include nine loci or 20% of all these loci on the third chromosome. This suggests that few embryonic visible loci on the third chromosome have escaped detection in our screen.

Allelic series

Different mutations may change the activity of a gene in different ways. It is therefore difficult to infer the role of a gene in normal development from mutant phenotypes resulting from unspecified genetic changes. According to Muller (1932), three major classes of gene mutation can be distinguished: lack of gene function (amorph), reduced level of normal gene function (hypomorph) and abnormal gene function (hypermorph, antimorph, neomorph). Embryonic visible mutants are operationally classified as amorphs if their phenotypes do not differ noticeably from those of the corresponding homozygous deficiencies. Embryonic visible mutants are classified as hypomorphs if their phenotypes are intermediate between the deficiency phenotype and the morphology of the wild-type larva. Mutation to abnormal gene function is a rare event. This kind of genetic change is therefore most likely to be found among the single mutants.

Among the 32 loci with more than one allele, there are 17 where all alleles produce identical phenotypes. Deficiencies for 6 of the 17 loci were available and phenotypically indistinguishable from the 25 corresponding embryonic visible mutants, which were therefore considered to be amorphic mutations, e.g. *sad-Df(3R)E229* or *spt-Df(3R)sbd*^{105} (Table 3). Alleles of varying phenotypic strengths represented 15 of the loci. Phenotypic comparisons with deficiencies uncovering 5 of these 15 loci indicated that only 15 of 35 mutants were amorphs, while 19 of them showed weaker phenotypes. Examples are *kni-Df(3L)ri*^{79c} or *Dl-Df(3R)bxd*^{110}. In addition, 1 of 11 *hb* alleles was exceptional in that its phenotype appeared different from those of *hb* deficiencies and 4 presumably amorphic alleles. This *hb* allele may therefore represent an example of abnormal gene function.

Phenotypic comparison between point mutation and deficiency is particularly instructive in the case of loci defined by single mutants. Single mutants may produce their mutant phenotypes by causing abnormal gene expression in cases where the normal gene function may not be involved in the process perturbed in the mutant. Of the 13 single mutants, 4 are uncovered by deficiencies which, in the case of *Antp, bxd,* and *tll,* produce essentially the same phenotypes as the corresponding point mutations, while the phenotype of the single *Pc* allele was much weaker than that of a deficiency for *Pc* or strong *Pc* alleles. In addition, the single *E(spl)* allele that we isolated produces the same phenotype as three revertants of the original dominant *E(spl)* mutant (Lehmann et al. 1983). These five single mutants apparently represent lack of gene activity or reduced gene activity, but not abnormal gene function. The remaining eight single mutants could not be assessed by the same method.

To detect possible temperature-sensitive mutants, all mutants were assayed at 18° and 29° C for temperature-dependent differences in the expression of mutant phenotypes. We identified 11 temperature-sensitive alleles at nine loci (Table 2).

In summary, at least 95% of the 197 embryonic visible

mutants produce their phenotypes by either reducing normal gene activity or by lacking gene function, according to our operational criterion. The ratio of amorphic to hypomorphic mutations approaches 2:1. Mutation to abnormal gene function seems to be rare in our sample of mutants. Only one such mutant was positively identified.

Discussion

Assessment of the screening procedure

Our screening procedure relied exclusively on the phenotypic distinction between mutant and normal embryos as recognized in cuticle preparations. This procedure can be applied only if mutations produce uniform and distinct phenotypes. Wright (1970) summarized the earlier work on the genetics of embryogenesis in *Drosophila*. The heterogeneity of the phenotypes of many of the mutants he describes leaves the reader with the impression that many embryonic-lethal mutations display lethal phases which extend over a considerable proportion of embryogenesis and hence show heterogeneous phenotypes. In contrast, the screen for embryonic visible mutations on the second chromosome suggests that, in general, this class of mutations show uniform phenotypes in cuticle preparations, while heterogeneous phenotypes could be attributed to either chromosomal aberrations or semi-dominant maternal effects (Nüsslein-Volhard et al. 1984). Uniform phenotypes have also been noticed in cuticle preparations of embryos homozygous for deficiencies (Table 3). These observations provided the basis for the present screening procedure, in which the embryonic phenotype was directly scored without preselection for lethal mutations.

Uniformity of the mutant phenotype was one criterion for identifying an embryonic visible mutation. The other criterion was distinctness of the phenotype. About 25% of all lethal mutations are lethal to the embryo (Table 1; cf. Hadorn 1955), while we classified less than 3% of the lethal mutations as embryonic visible in cuticle preparations. Of the embryonic lethal mutations about 80% produced either normal-looking embryos or distinct phenotypes. The remaining 20% were predominantly mutations yielding poorly differentiated embryos while only a minority, and hence a small proportion of all embryonic lethal mutations, produced "subtle" phenotypes whose qualification depended on subjective judgement. In practice, we classified phenotypes as either "distinct" or "subtle" by evaluating the morphological difference between mutant and normal embryos. When this difference was marginal or regarded as the result of unspecific perturbation of development, the phenotype was classified as "subtle". We made this decision as reproducibly as possible. Thus, we classified "head defects" as subtle phenotypes and did so throughout this screen, in contrast to the second-chromosomal screen (Nüsslein-Volhard et al. 1984). This explains the apparent difference between the two chromosomes in regard to the number of loci mutating to embryonic visible phenotypes, since 14 of 61 second-chromosomal loci affect the head exclusively. It also explains why single mutants and loci with two alleles prevail among the "dorsal holes" loci in this screen; weak alleles at such loci on the second chromosome cause unspecific head defects; presumably due to perturbation of head involution (Nüsslein-Volhard et al. 1984). Whether our criterion of phenotypic distinctness was re-

sponsible for low allele frequencies at other loci, e.g. homoeotic loci, cannot be assessed.

Has saturation been achieved in our screen?

We have identified 45 zygotic loci on the third chromosome which mutate to embryonic visible phenotypes. The phenotypes result from partial or complete inactivation of the gene in most, if not all, cases as judged by allele frequency and comparison with deficiency phenotypes. The following considerations indicate that almost no gene of this class has remained undetected in our screen.

In our sample, each gene is represented on the average by 4.5 alleles. Calculation of the zero class based on a Poisson distribution (Fig. 2) would suggest that we did not miss any relevant gene. However, our sample of mutants does not conform to a random distribution and hence this argument cannot be used to infer saturation. Non-random distributions of allele frequencies have also been observed in other saturation experiments (cf. Hilliker et al. 1981). This phenomenon may relate to differences in susceptibility to the mutagen used or to selection in favour of or against particular genes or phenotypes.

Whether saturation has been achieved in our screen can be examined for a short segment of the third chromosome. Gausz et al. (1979, 1981) saturated deficiencies spanning 86F12 to 87D1,2 with EMS-induced lethal and visible mutations and determined lethal phases of complementation groups. The only candidate for an embryonic visible locus in that interval is *ck9* localized to band 87A4,5. *ck9* is in all likelihood allelic to *shadow*, the only locus we have assigned to the same cytogenetic region. The studies by Gausz et al. (1979, 1981) and the data on the *rosy* region (Hilliker et al. 1980) support the notion that there are approximately equal numbers (2000) of loci and bands on the third chromosome. These studies also suggest that 80%–90% of the genes are essential for survival to the adult stage. Our data on lethal hits (7300) and the average allele frequency of 4.5 yield a total number of 1650 essential genes, which is similar to these other estimates based on a different set of data.

Probably the best evidence for saturation is provided by phenotype comparison between point mutations and deficiencies. None of the deficiencies available to us produces an embryonic phenotype which is not represented by our collection of embryonic visible mutations. We have therefore identified all relevant loci in about one-quarter of the third chromosome by this criterion. Considering only those deficiencies which were not preselected for embryonic phenotypes, they account for about one-fifth of the chromosome and include about 20% of the loci identified in our screen. Since the loci of our sample of mutants are randomly distributed along the chromosome, the correlation between deficiency phenotypes and embryonic visible mutations seems to be valid for the entire chromosome. Taken together, these data strongly support our conviction that we have identified most, if not all, zygotic loci on the third chromosome mutating to embryonic visible phenotypes.

Limitations of the identification-by-phenotype screen

We have identified embryonic visible mutations by their cuticle phenotypes in our screen. This procedure excludes detection of mutations which only affect internal organs or mutations which cause developmental arrest before the

cuticle is secreted by the epidermal cells (see Nüsslein-Volhard et al. 1984, for discussion). In addition, mutations in a locus whose amorphic phenotype, although distinct, is compatible with hatching of the larva would be detected only fortuitously, e.g. *Ubx*. Even excluding these limitations, our collection of mutants may not represent all zygotic genes specifically involved in embryonic pattern formation.

It is conceivable that genes specific to embryonic pattern formation cannot be recognized by distinct embryonic phenotypes for any of the following reasons.

1. Two copies of the gene are required for survival to the adult stage. According to Lindsley et al. (1972), there exist about 20 such haplo-lethal loci in the entire genome.

2. More than one copy of a gene is present in the haploid genome due to duplication of an ancestral gene or chromosomal segment during evolution. Well-known examples are the heat shock loci at 87A7 and 87C1 for which point mutants have not been isolated despite large-scale screens specifically designed for that purpose (Gausz et al. 1979, 1981).

3. Genes are also difficult to identify phenotypically if the lack of one gene function can be compensated for by another gene. This possibility may explain why particular homoeotic genes mutate to inconspicuous embryonic phenotypes. *Pcl* mutants on the second chromosome and *Scm* mutants on the third chromosome, which have escaped detection in our screens because of their subtle phenotypes, can be combined to produce strong homoeotic transformation in the embryo similar to amorphic *Pc* alleles (Jürgens, in preparation). It is not known how many genes of this kind exist in the genome and whether this feature is unique to homoeotic genes.

4. Genes without mutant phenotypes have been known to biochemical geneticists working on metabolism where genetic blocks are by-passed in "shunts" so that no lethal alleles can be isolated for particular genes. Some enzymes are apparently dispensable for survival, fertility and behaviour (Voelker et al. 1981). We do not know whether "shunting" applies to pattern formation as a means for buffering against perturbation.

5. A gene is expressed during oogenesis and embryogenesis. In this case the oocyte would be supplied with a sufficient amount of gene product to support normal morphological development of the embryo. Zygotic lethal mutations with strong maternal effects causing embryonic visible phenotypes have not systematically been searched for, so we cannot estimate the number of such genes present in the genome.

Concluding remarks

The present report demonstrates that zygotic genes involved in embryonic pattern formation can be directly identified by their embryonic phenotypes as recognized in cuticle preparations. Disregarding those genes whose phenotypic effects are suppressed for special reasons, we have identified most, if not all, such genes on the third chromosome. Their number does not exceed 50 or approximately 2.5% of all third-chromosomal genes. Similar proportions have been calculated for the other chromosomes (Nüsslein-Volhard et al. 1984; Wieschaus et al. 1984). One should, however, bear in mind that about 25% of all zygotic genes are required for embryonic survival (Table 1; Hadorn 1955). The majority of these genes is presumably involved in rather

general cell functions common to most, if not all, cells such as metabolism, cell division and cell differentiation. Morphogenesis of the embryonic epidermis apparently requires only about 140 specific zygotic gene functions. This figure is surprisingly low if one considers the spatial complexity of the cuticular pattern.

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Note added in proof

One representative allele of each locus can be obtained from the following address: Mid-America Drosophila Stock Center, Department of Biological Sciences, Bowling Green State University, Bowling Green, Ohio 43403, USA