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

III. Zygotic loci on the X-chromosome and fourth chromosome

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Summary. In order to identify X-chromosomal genes required in *Drosophila* for early patterning and morphogenesis, we examined embryos hemizygous for EMS-induced lethal mutations to determine which of those mutations cause gross morphological defects. Embryos from 2711 lethal lines, corresponding to 3255 lethal point mutations were studied. Only 21% caused death during embryogenesis and of these, only one-sixth, or 3% of the total lethals, were associated with defects visible in the final cuticle pattern. Of the 114 point mutants causing visible cuticle defects, 76 could be assigned to 14 complementation groups. An additional 25 mutations mapping to regions of the X-chromosome not covered by male fertile duplications were assigned to six complementation groups based on similarities of map position and phenotype. Thirteen mutations could not be assigned to complementation groups. All mutations allowed normal development through the cellular blastoderm stage, the first defects associated with the earliest acting loci being observed shortly after the onset of gastrulation. The phenotypes of the various loci range from alterations in segment pattern or early morphogenetic movements to defects in final pigmentation and denticle morphology.

Cuticle preparations were also examined for 63 deletions spanning in total 74% of the X-chromosome, as well as for 8 deletions and point mutations derived in saturation mutagenesis screens of the fourth chromosome (Hochman 1976). With the exception of defects in head morphology and defects in cuticle differentiation, none of the hemizygous deletions showed phenotypes other than those predicted by point mutations known to lie in those regions. No deletion caused new or unknown alterations in gastrulation, segmentation or cuticle pattern. These results suggest that the number of genes required zygotically for normal embryonic patterning is small and that most, if not all such loci, are represented by point mutations in our collection.

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

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Introduction

Embryonic development depends on gene products deposited in the egg during oogenesis, as well as products made by the embryo itself. Distinguishing between maternal and zygotic contributions to a given developmental process is difficult based solely on molecular or embryological techniques, largely because specific gene products cannot always be followed and their relative importance during development is difficult to assess. In *Drosophila*, genes whose products must be supplied by transcription in the embryo itself can be identified using mutations. If such products are required for normal embryonic development, elimination of the wild-type allele will result in death or abnormalities in homozygous embryos. Although the majority of lethal mutations still allow embryonic viability (Hadorn and Chen 1952), a number have been described which cause specific defects in the development of homozygous embryos. The effects of many of these loci have been reviewed by Wright (1970), the classic example being the neural hypertrophy phenotype associated with deletions of the *Notch* locus (Poulson 1940).

We have recently undertaken large scale mutagenesis screens aimed at identifying all zygotically active genes, which when mutated cause visible alterations of the embryonic pattern (Nüsslein-Volhard et al. 1984; Jürgens et al. 1984). The following paper represents the last in that series of experiments and describes the isolation and characterization of mutants on the X-chromosome, as well as a brief analysis of the effect of the fourth chromosome on embryonic development.

Materials and methods

Strains. New mutations were induced on a X-chromosome marked with recessive viable mutations *yellow* or *white*. The *yellow* chromosome had been isogenized immediately prior to the mutagenesis; the *white* chromosome, although not isogenized, had been shown to contain no lethal mutations affecting male viability. *FM7* is a balancer chromosome carrying the mutations $y^{31d}w^{a}$ sn^{x2} v and B (Merriam and Duffy 1972). It is viable and fertile in males. *FM7-TW9* is a lethal variant of *FM7* constructed by Wright. *FM7/FM7-TW9* females are sterile due to homozygosity for singed^{x2}. *Notch*$^{264.47}$ is an embryonic lethal used in this

experiment to kill the males in the F_1 generation, thus simplifying the collection of the F_1 females as virgins. The *y* $N^{264.47}$/*FM7*, *TW9* females were obtained from a stock containing the w^+ *Y*-chromosome, which allows *y* $N^{264.47}$ males to survive. *C(4)RM*, *ci* ey^R, *gvl* sv^n is an attached fourth chromosome used to generate embryos nullosomic for chromosome four. Most of the fourth chromosomal lethal mutations and deletions used were isolated by Hochman (1976) and obtained from the *Drosophila* stock center at Bowling Green. The vast majority of the X-chromosomal deletions are from a collection induced and cytologically characterized by George Lefevre (Craymer and Roy 1980). They were obtained from the *Drosophila* stock center at Pasadena. More detailed descriptions of the mutations used can be found in Lindsley and Grell (1968).

Isolation of X-linked embryonic lethal mutations. X-linked lethal mutations were isolated using the crossing scheme outlined in Fig. 1 a. Males carrying a non-lethal X-chromosome marked with either *yellow* or *white* were fed 0.025 M ethyl methane sulfonate (=EMS) in sugar solution following the procedure of Lewis and Bacher (1968). The EMS-treated males were then mated to $N^{264.47}$/*FM7*, *TW9* females and transferred to bottles of standard *Drosophila* medium. The mutagenized males were removed after 5 days at 25° C. Individual F_1 daughters carrying a mutagenized X-chromosome and *FM7*, *TW9* were mated to *FM7* males and stocks established from each female. X-linked lethal mutations were detected in the following generation, stocks being classified as such if no males carrying the mutagenized chromosome survived. Non-lethal stocks were discarded.

To determine whether the mutation causes lethality in the embryonic stage, new balancer males (*FM7* or *M5*) were added and the stock transferred to plastic tubes for egg collection (Nüsslein-Volhard 1977). All fertile females in each stock will be heterozygous for the mutagenized X-chromosome. Thus, if the newly induced mutation causes death during embryogenesis, one-quarter of the progeny (the *y**/*Y* or *w**/*Y* hemizygous males) will die before hatching. The unhatched eggs from all stocks showing 25% or more nonhatch were dechorionated, fixed and mounted in Hoyer's medium (van der Meer 1977) for microscopic examination using dark field and phase-contrast optics (Nüsslein-Volhard et al. 1984). When abnormal embryos were found, their number was compared to the recorded hatch rates to determine whether they represented one-quarter of the total developed progeny and thus whether they could be the zygotic lethal class. Putative zygotic lethals were retested in the following generation for viability of hemizygous males and for production of the embryonic phenotype. Stocks which produced phenotypes in the expected frequencies were re-isogenized and balanced with *FM7*.

Phenotypic classification and assignment to complementation groups. After their isolation and re-isogenization, the mutants were classified into groups according to their phenotype in cuticle preparations. The goal of this classification was to simplify the complementation analysis, in that initially only mutants with similar phenotypes were to be crossed with each other. Mutations which caused head defects or large holes in the dorsal or ventral region of the cuticle were very common and it was difficult, based on cuticle patterns alone, to assign them into subgroups small enough to allow complementation analysis. Embryos from 42 such stocks were examined under Voltalef oil early during gastrulation and germ-band elongation to distinguish those causing early morphological abnormalities from those in which the abnormalities only became apparent during germ-band shortening, head involution or dorsal closure. Eighteen stocks with early morphogenetic abnormalities were found and, based on their phenotypes, were subsequently assigned to three subgroups.

After the mutants were assigned to phenotype groups, representative lines were selected from each class and the lethality mapped relative to the visible markers (*y*) *sc* (*w*) *ec cv ct v g f* (Lindsley and Grell 1968). Once a preliminary map position had been obtained, all mutants in a phenotypic class were mated to males carrying a duplication for that chromosomal region. Survival of F_1 mutant males indicated that the mutation was located in the cytologically defined region covered by the duplication. Such males were mated to attached-X females carrying the appropriate autosomal balancers (*CyO*, *TM3* or ey^D) to allow following the duplication during subsequent crosses. In complementation tests (Fig. 1 b), mutant males heterozygous for the duplication and the balancer were mated to females from the other stocks showing a similar phenotype. The lethality of the F_1 transheterozygous daughters not carrying the duplication was used as the criterion for assigning mutants to the same complementation group. Complementation tests were also performed in a similar manner with previously identified lethal and visible mutations obtained from other laboratories as well as with deletion chromosomes in order to obtain cytological localization for the individual complementation groups.

Phenotypic characterization of overlapping deficiencies. Cuticle preparations were made of embryos hemizygous for 63 X-linked deficiencies to determine whether elimination of cytologically defined chromosomal regions caused phenotypes equivalent to the EMS-induced point mutations already identified in those regions. In most cases, deficiency embryos were obtained as progeny of heterozygous mothers; in a small number of cases, as progeny from attached-X females. In at least one experiment for each deficiency, a more accurate hatch rate was obtained not by estimations, but by counting the number of empty chorions, the number of undeveloped (unfertilized) eggs and the number and phenotypes of the differentiated embryos. Most deficiency embryos were also scored under Voltalef 3S oil during the blastoderm stage and at gastrulation and germ-band elongation. Although deletion breakpoints were not confirmed cytologically in polytene chromosomes, the stocks were controlled by complementation tests performed with other mutations, deficiencies and duplications in the region.

Determination of onset of developmental abnormalities. Phenocritical stages were determined for hemizygous embryos of each complementation group by examining eggs under Voltalef 3S oil at various times during embryonic development. About 30–40 embryos from each stock balanced over *FM7* were selected at the cellular blastoderm stage and examined for abnormalities at approximately 2-h intervals thereafter. Deviations from normal development were recorded and the embryos were scored as mutant or

wild-type after they had completed development. Internal organs and final differentiation cannot be easily scored in living embryos under Voltalef 3S oil and the procedure is mainly useful for identifying surface abnormalities and defects in morphogenetic movements. Therefore, for individual mutations, the phenocritical stage may be variably underestimated and it is likely that many of the genes are active earlier in development than the stages when mutant embryos are first detected.

Results

The crossing scheme used to detect X-linked lethal mutations is outlined in Fig. 1 a. It differs slightly from standard schemes developed by Muller (1928; see also Spencer and Stern 1948) principally in the use of parental females carrying a lethal balancer chromosome (*FM7, TW9*) and a recessive lethal ($N^{264.47}$). Due to the two lethals, no males survive in the F_1 generation, and in lines where a lethal had been induced on the mutagenized chromosome, only females will emerge in the F_2 generation. The absence of males simplifies the collection of virgins. The subsequent out-crossing of the females at each generation to unmutagenized *FM7* males reduces the background of unhatched eggs due to autosomal lethals, dominant maternal effects, and chromosomal aberrations. This step was necessary given that in the initial analysis, each lethal stock was tested only once and embryonic lethals were distinguished from other types of lethal stocks only by the failure of one-fourth of the eggs from the F_2 females to hatch.

In four separate experiments, 8614 lines were established. In the F_2 generation, 2711 of these lines were found to contain at least one lethal mutation causing hemizygous males to die before adult eclosion. Calculations from the Poisson distribution indicate that the probability of a lethal mutation per chromosome is 0.38. The total number of lethal mutations in the 8614 lines can, therefore, be estimated at 3255. In order to identify those mutations which cause death during embryogenesis associated with altered pattern or morphology, eggs were collected from each of the 2711 lethal stocks. When 25% or more of the eggs from a given stock did not hatch, the unhatched eggs were fixed, mounted in Hoyer's mounting medium, and examined using phase-contrast optics at 400×. Most (67%) of the 1719 embedded lines showed substantial numbers of undeveloped, evidently unfertilized eggs. Only 653 lines (21%) produced numbers of unhatched developed embryos consistent with zygotic lethality. In most of these cases, the lethal embryos were normal in cuticle pattern or showed only slight, variable abnormalities suggesting defects during late differentiation. Only 294 stocks produced a sufficient number of morphologically abnormal embryos to merit retesting. The 168 lines which produced phenotypes on the retest were re-isogenized and tested several times over the course of the following year. In 38 lines, the phenotype became less strong or less frequent, and the stocks were eventually discarded. Since these lines had been isogenized and balanced for the X-chromosome, it is likely that the original phenotype was due at least in part to mutations elsewhere in the genome. Eight lines were lost prior to completion of complementation tests. The remaining 122 lines were the subject of further genetic analysis. The data are summarized in Table 1.

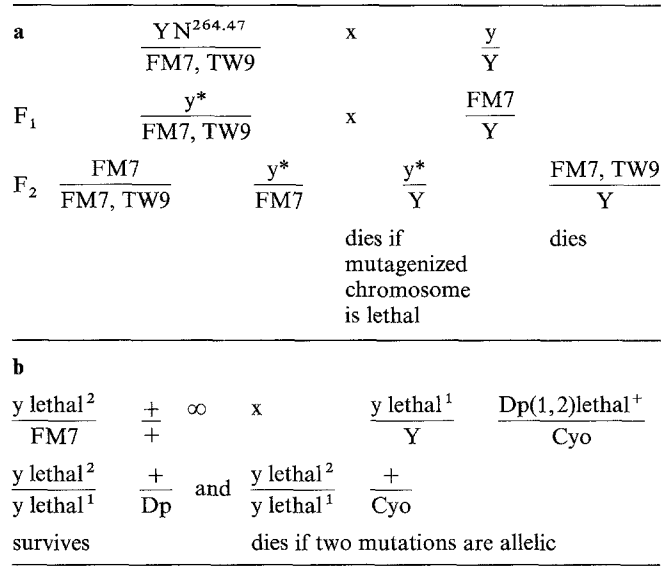

Fig. 1 a, b. Cross schemes for isolation of X-linked lethal mutations and subsequent complementation tests. **a** Crossing scheme for isolation of X-linked lethal mutations. If an X-linked lethal mutation has been induced by the EMS treatment, no males will survive in the F_2 generation. **b** Crossing scheme for complementation tests. In the example shown, the lethal mutation is covered by a wild-type duplication on the second chromosome. Mutant males which carry the duplication survive. In the complementation test, the duplication is balanced with *CyO*, a dominantly marked second chromosome balancer such that the male gives his F_1 daughters either the duplication or the *CyO*. Since the *CyO* daughters are transheterozygous for the two lethals, they will only survive when the two lethals are not in the same complementation group. The cross also yields *FM7* heterozygous females, *FM7* males, and sometimes mutant males. These are not relevant to the complementation test, but they provide a useful control for survival. For duplications on the third and fourth chromosomes, the *TM3* and ey^D balancers were used. For duplications on the Y chromosome, no balancer was needed since none of the F_1 daughters receive the duplication following normal segregation

Table 1. Screens for embryonic lethal mutations on the X-chromosome

Lines tested	8614		
Lethal lines	2711		
Calculated lethal hits	3255		
Embryonic lethal lines[a]	653		
a) normal		278	
b) poorly differentiated		97	
c) phenotype not confirmed		148	
d) lost before completion of complementation test		8	
e) genetically deviant (not simple zygotic lethals)		8	
f) zygotic visible phenotype		114	
1) mutants assigned to complementation groups			76
2) mutants assigned to putative complementation groups			25
3) mutants not in complementation groups			13

[a] The number of embryonic lethal hits was calculated using the Poisson distribution from the frequency of embryonic lethal lines and found to be 679. The probability of a lethal mutation causing death during embryogenesis is thus 20.9% (i.e., 679/3255). When the phenotype of a putative embryonic lethal line could not be confirmed, the line was discarded without retesting whether it was in fact an embryonic lethal. Many of these discarded lines may have been larval or pupal lethals. Given this uncertainty, the total number of embryonic lethals is probably an overestimate

Mapping and complementation analysis

After the lethal mutations had been classified to groups according to their phenotype in cuticle preparations, representative mutants from each group were mapped and the available X-chromosomal duplications were tested to identify those which would allow mutant males to survive to fertile adults (Table 2). When such duplications were found, complementation tests were carried out among mutants with similar phenotype following procedures outlined in Materials and methods. Of the 122 mutants, 76 could thus be assigned to 14 complementation groups. Four of these loci [*cut, giant, Notch, l(1)mys*] were known previously due to their lethality or to the recessive viable phenotype of weak alleles. Duplications which allow viability and fertility in males are available for about 70% of the X-chromosome. An additional 25 mutants with clear distinct phenotypes were mapped to regions of the chromosome not covered by duplications. These have been assigned to six putative complementation groups based on similarity of phenotype and map position. The remaining 21 mutants could not be assigned to complementation groups. Out-crossing these stocks to autosomal balancers showed that 2 of these mutants segregated non-randomly with respect to the second or third chromosome, suggesting that the abnormal embryos produced in those stocks may be aneuploid segregants of X-autosomal translocations. High frequencies of non-disjunction (>10% patroclinous males) were observed in 2 other mutant lines which showed normal segregation with respect to the major autosomes. Of the remaining stocks, four produced high frequencies of abnormal embryos incompatible with simple zygotic lethality (>40% rather than 25%). The 13 remaining stocks showed no deviation from the expected behaviour of zygotic lethals. These 13 mutants were mapped and only four were found to contain more than one lethal. This is very close to that expected for randomly chosen mutant lines given that the frequency of lethal hits was 38%. The high frequency of single lethal sites makes it unlikely that the embryonic abnormalities in the majority of these 13 lines were due to cumulative effects of multiple lethals. The embryonic phenotypes associated with these mutations are restricted to head defects, incomplete dorsal closure and cuticle differentiation. Although some of these mutations may identify genes for which only one mutant allele was obtained, we think it likely that most can be explained by other means (see Discussion). These 13 single mutations were not characterized in greater detail.

Table 2. Duplications and translocations used

Genetic designation	X-chromosome breakpoints	Lethal complementation groups covered by duplication	References
T(1,2) Bld	tip; 1C3	(*arm*$^-$)	a
y^{2}Y^{67g}	tip; 2B17	*arm*$^+$	b
Dp(1,3)w^{vco}	2B17-2C1; 3C4-5	*gt*$^+$, (*arm*$^-$)	b
w$^+$Y	2D1-2; 3D1-2	*gt*$^+$, *N*$^+$	a
T(1,2)w^{+64b13}	3C2; 5A1-2	*N*$^+$, *hnt*$^+$, *svb*$^+$	b
T(1,2)rb^{+71g}	3F3; 5E8	*hnt*$^+$, *svb*$^+$	b
Dp(1,3)sn^{13a1}	6C11; 7C9	*ct*$^+$	b
T(1,2)sn^{+72d}	7A8; 8A5	*ct*$^+$, *l(1)mys*$^+$, *sdt*$^+$, *otd*$^+$	b
Dp(1,2)v^{+75d}	9A2; 10C2	*rtv*$^+$, (*btd*$^-$)	b
Dp(1,2)v^{63i}	9E1; 10A11	*rtv*$^+$, (*btd*$^-$)	b
v$^+$B^s$^-$Y	9F3; 10E3-4; 20B; base	*rtv*$^+$	b
Dp(1,2)v^{65b}	10A1; 11A7	*rtv*$^+$, *ftd*$^+$, *tsg*$^+$, (*btd*$^-$)	b
Dp(1,4)r$^+$f$^+$	13F; 16A2	*baz*$^+$, *exd*$^+$, (*sog*$^-$)	b
Dp(1,4)81h24b	13F; 14B5-18; 14E1-4; 16A2	*exd*$^+$	d
Dp(1,4)82b26c	13F; 14D1-2; 15A3-5; 16A2	*exd*$^+$	d
y$^+$Ymal	18F; base	*run*$^+$, *fog*$^+$	c
y$^+$Ymal^{126}	20A1; base	*fog*$^+$	c
y^{2}Y67g	20A3; base	*fog*$^+$	c
B^{s}Y	20B; base	(*fog*$^-$)	c
v$^+$Yy$^+$	9F3; 10C1; 20B; base	(*fog*$^-$)	c

a) Lindsley and Grell 1968; b) Craymer and Roy 1980; c) Schalet and Lefevre 1976; d) D. Falk, personal communication

Table 3 summarizes the relevant genetic data and phenotype for each complementation group or putative complementation group. The map positions are randomly dispersed along the X-chromosome (Fig. 2). The range of phenotypes (Fig. 3) is similar to that described for zygotic lethal mutations on the autosomes (Nüsslein-Volhard et al. 1984; Jürgens et al. 1984). Six loci have striking effects on segment pattern (*runt, armadillo, giant, unpaired, orthodenticle* and *extradenticle*). Nine new alleles were obtained of the previously identified pair rule mutation *runt* (Nüsslein-Volhard and Wieschaus 1980), including three alleles producing only partial pattern deletions and one allele which is temperature-sensitive in phenotype. The newly identified *armadillo* locus causes mirror-image duplications of each denticle band, resulting in embryos very similar to those produced by the *wingless* mutation (Nüsslein-Volhard and Wieschaus 1980). Two loci (*giant* and *unpaired*) cause defects in the segment pattern not repeated at homologous intervals along the length of the larva. Mutants for *unpaired* have defects predominantly in the fifth abdominal segment and metathorax, although occasional defects are also observed in the second and eighth abdominal segments. Embryos mutant for lethal alleles of *giant* have defects in the fifth through seventh abdominal segments, as well as a defect in the head region. The regional specificity of this phenotype was originally described for deficiency and amorphic mutations by Campos-Ortega and Jimenez (1980). All the denticles in the anterior abdomen of *orthodenticle* embryos point posteriorly (Fig. 4b). The denticle bands are thinner than wild-type, particularly at the ventral midline, and lack the first and fourth denticle rows, which normally are the only rows in denticle bands which point anteriorly. The mutation *extradenticle* identifies the strongest candidate for a homoeotic locus detected among our embryonic lethal mutations. In mutant embryos, the first abdominal segment has the additional denticle rows characteristic of the more posterior abdomen and the meso- and metathoracic segments have thick denticles which might be described as prothoracic or abdominal in character (Fig. 4c).

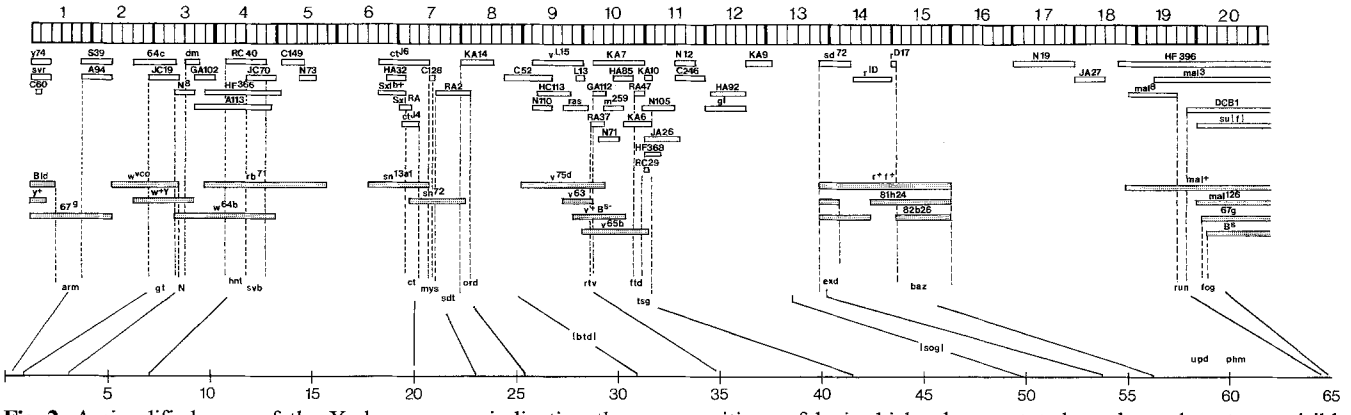

Fig. 2. A simplified map of the X-chromosome indicating the map positions of loci which when mutated produce phenotypes visible in late embryonic cuticle preparations. The diagram also shows the location of the various deletions and duplications used in this study. The deficiencies are represented by *open bars*, duplications and translocations by *shaded bars*. More detailed cytological descriptions are available in Tables 2–4

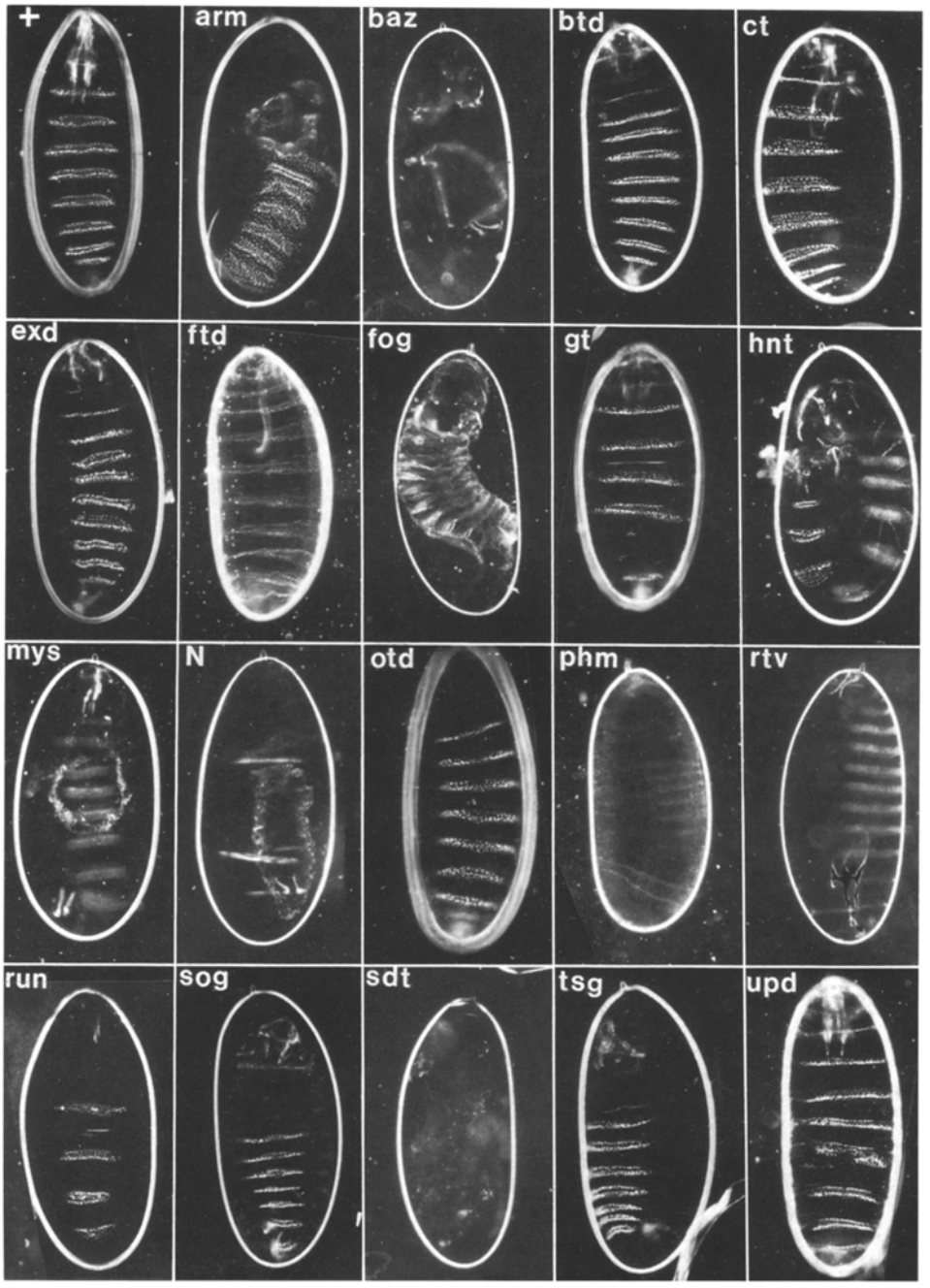

Fig. 3. Dark field photographs of cuticle preparations of late wild-type and mutant embryos. The identity of each mutant is indicated by the allele designation in the upper left of each photograph

Table 3. Genetic data and phenotype for complementation groups or putative complementation groups

Name	Phenotype	Number of alleles			Map position	Cytological localization
		Total	Weak (not ts)	ts		
armadillo (*arm*)	segment polarity mutant, mirror-image duplication of denticle band	4	1	0	1	(1C3-1E3, 2B15-17)
bazooka (*baz*)	large dorsal and ventral hole	(3)	0	0	57	(15A5-16A2)
buttonhead (*btd*)	head involution incomplete	(6)	0	1	31	(8A5-9A1)
cut (*ct*)	posterior defects in spiracles, no Keilin's organs, abnormal maxillary complex	3	1	0	20	7B3-4*
extradenticle (*exd*)	meso- and metathoracic segments like prothorax, first abdominal segment like posterior abdom	(2)	1	0	54	13F1-14B1
faintoid (*ftd*)	larva unpigmented	7	0	0	nd	10F1-10F10
folded gastrulation (*fog*)	gastrulation defective, extensive folds in the germ band, ventral holes in cuticle, particularly at anterior and posterior end	9	0	(1)	nd	20A3-20B
giant (*gt*)	defect in head and in fifth through seventh abdominal segment	8	4	0	1	3A1-4*
hindsight (*hnt*)	no germ-band retraction, embryo U-shaped with head facing posterior end	(3)	0	0	7	4B1-4C15
lethal-myospheroid (*l(1)mys*)	dorsal closure defective	4	0	0	23	7D1-5
Notch (*N*)	hypertrophy of ventral nervous system, ventral cuticle absent	12	2	3	3	3C7*
orthodenticle (*otd*)	all denticles in anterior abdomen point posteriorly, defects at ventral midline, head defect	3	1	0	26	7F1-8A5
phantom (*phm*)	cuticle not well differentiated, contracted posteriorly	(5)	0	0	64	(17A-18A)
retroactive (*rtv*)	mouth part darkly sclerotinized (embryo sometimes reversed in egg case, due to hyperactivity at late stages?)	3	0	0	38	10A7-10A11
runt (*run*)	pair-rule segment defects, mirror-image duplication of abdominal denticle bands 2, 4, 6 and 8	9	3	1	nd	19E1-19F1
shavenbaby (*svb*)	denticles shorter and reduced in number, dorsal hairs absent	2	0	0	nd	4C15-4F1
short gastrulation (*sog*)	band extension incomplete, head and spiracles defective in posterior cuticle	6	–	1	53	nd
stardust (*sdt*)	hypoderm almost totally absent, small remains of cuticle	6	2	1	23	7D10-7F2
twisted gastrulation (*tsg*)	gastrulation abnormal, head and posterior spiracles defective in larval cuticle, ventral nervous system split anteriorly	3	0	0	42	11A1-11A7
unpaired (*upd*)	defects predominantly in mesothorax and 5th row, head defects, eigth segment defects	3	1	0	59	nd
l(1)XC46	head defect	1	–	6, 20	nd	
l(1)XC78	head defect	1	–	–	23	nd
l(1)XD03	poor cuticle differentiation, normal head	1	–	–	61	nd
l(1)Xi08	poor cuticle differentiation, head skeleton tilted	1	–	–	33	nd
l(1)XN75	head defect, anterior open	1	–	–	60, 13	nd
l(1)XN82	head defect, anterior open	1	–	–	59, 20	nd
l(1)XN52	like *buttonhead*	1	–	–	57	nd
l(1)YA13	holes, cuticle degenerate, like *baz*	1	–	–	57	nd
l(1)YD23	holes, cuticle degenerate, like *baz*	1	–	–	0.1, 56	nd
l(1)YE17	head defect, anterior open	1	–	–	67	nd
l(1)Yi29	dorsal hole	1	–	–	57	nd
l(1)YL21	head defect, anterior open	1	–	–	26	nd
l(1)YQ09	like *buttonhead*	1	–	–	59	nd

Allele numbers for putative complementation groups in parentheses. Map positions for these groups were computed by averaging values obtained for individual alleles. The individual map positions for three *hindsight* alleles were 6.6, 6.1 and 8.3; for the *extradenticle* alleles 50 and 56.7; for the *short gastrulation* alleles 50, 50, 55, 56, 57; for the *unpaired* alleles 58.7 and 59.3 (P. Gergen, personal communication); for the *phantom* alleles 59, 70, 65, 61 and 62; for the *buttonhead* 31, 32, 31, 31, 33, and 39.

Lethal lines not assigned to complementation groups or putative complementation groups are designated only by laboratory code names. When such lines contain more than one lethal, the map positions of both lethals are given.

Cytological localizations with asterisk (*) were taken from Lindsley and Grell (1968); *nd*, not determined. Localizations in parentheses are tentative due to the lack of appropriate deficiencies or duplications for complementation tests

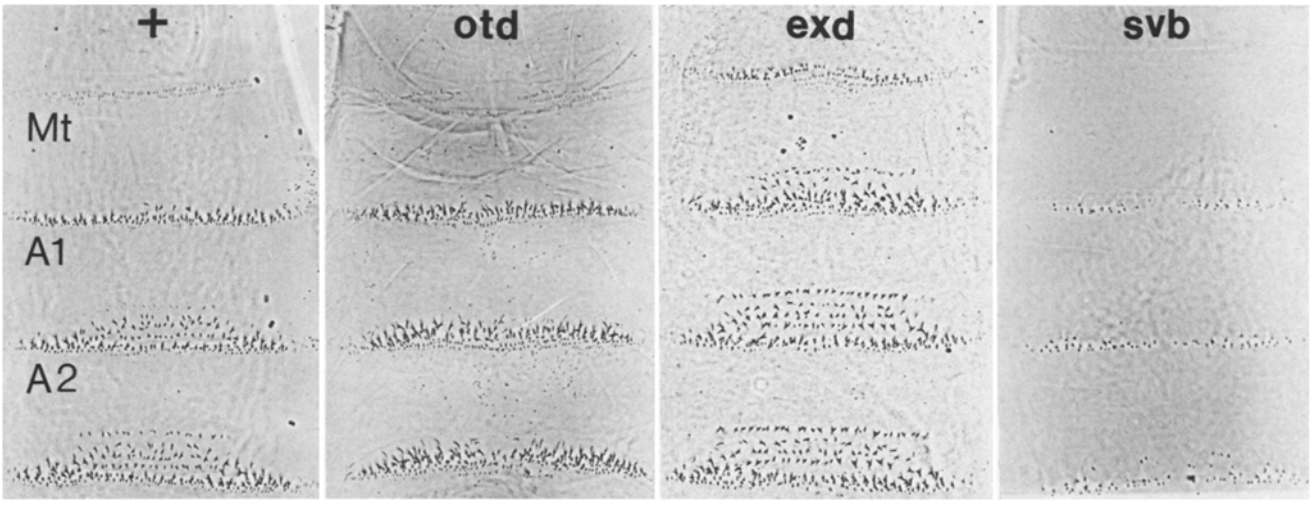

Fig. 4a–d. Phase contrast photographs of denticle morphology in the metathoracic and anterior abdominal region of **a** wild-type, **b** *orthodenticle*, **c** *extradenticle*, and **d** *shavenbaby* late embryos. *Mt* methathorax; *A1* first abdominal segment; *A2* second abdominal segment

In addition to their effects on final cuticle morphology, alleles from each complementation group were studied in living embryos under Voltalef oil to define the developmental onset of the mutant abnormalities. All mutations allow completion of a normal cellular blastoderm. Three complementation groups interfere with the early morphogenetic movements associated with gastrulation and germband extension (*short gastrulation*, *twisted gastrulation* and *folded gastrulation*). The exact alterations are locus-specific, and in each case mutant embryos continue to develop, yielding highly disorganized first-instar larvae. Most of the remaining mutants caused defects during the middle period of embryogenesis. These include two complementation groups (*stardust* and *bazooka*) which caused holes or degeneration of the future epidemis at about 8 h of development, as well as the previously described *Notch* (Poulson 1940) and *lethal myospheroid* (Wright 1960) loci. In summary, 4 of the 20 loci on the X-chromosome cause defects visible within 1 h after the completion of the cellular blastoderm (*runt* and the three gastrulation defective mutants), and mutants at five additional loci (*armadillo*, *bazooka*, *stardust*, *Notch*, *hindsight*) are defective by 4 h later, at germ-band shortening. Mutants at three loci show generally normal morphology until late in development and have their most obvious effect on late cuticle differentiation (*shavenbaby* (Fig. 4d); *faintoid* and *retroactive*).

The average number of mutants in each genetically defined complementation group is 5.4 and ranges from 2 for *shavenbaby* to 12 for *Notch*. The comparable figure for the putative complementation groups is similar (4.2). The high frequency of alleles per locus indicates that most loci on the X-chromosome should be represented by at least one point mutant in our collection. Most loci have their own distinct phenotypes, and with rare exceptions the final assignment of a mutant to a complementation group could be predicted based solely on its morphology in cuticle preparations. On the other hand, the strength of the phenotype sometimes varied among the different alleles at a given complementation group. About half of the complementation groups were represented by weak as well as strong alleles (Table 3). The weaker alleles were less frequent and represent less than one-fifth of the total mutations in the collection. Comparison of the cuticle phenotype of embryos raised at 29° C and 18° C identified alleles at five loci whose phenotypes were weaker at 18° C (Table 3, column 6). In no case, however, did genetically mutant embryos develop to fertile adult progeny at the permissive temperature.

Cytological localization

The map position and size of the duplication used in the complementation tests give a rough localization for each complementation group. More precise localizations were obtained in complementation tests with cytologically defined deficiencies and translocations (Table 4). For most complementation groups, this analysis allowed localization to intervals the size of one numbered subunit or less (Table 3, Column 5).

Cuticle preparations were made of embryos hemizygous for each of the deficiencies tested in Table 4, regardless of whether they contained one of the known complementation groups identified in the EMS mutagenesis screen. The goal of this analysis was to determine whether elimination of the wild-type genes would produce a phenotype other than that observed in embryos hemizygous for the strongest point mutation obtained for each locus. Such might be the case if the mutations identified in our screens did not totally eliminate the functional gene product or if the screens had not identified all zygotically active loci in a deficiency region which have effects on embryonic development. The overlapping deficiencies used in this study involve 757 of the 1028 bands described in Bridges's map (1938). Most deficiencies (49/64) produced either no phenotype at all or produced phenotypes indistinguishable from point mutations known to lie in the cytologically deleted region.

Several large deficiencies allowed only poor differentiation in hemizygous embryos. One large deficiency (*Df(1)N19*) blocked the formation of cuticle entirely, a phenotype which may be related to the cluster of lethals mapped to that region which cause poor cuticle differentiation and which we grouped together in the *phantom* putative complementation group. All the other unpredicted pheno-

Table 4. Deletion chromosomes examined

Deficiencies	X-chromosome breakpoints	Gastrulation	Homozygous cuticle phenotype	Complementation tests	Reference
Df(1)svr	tip; 2B10-13	+	head defect	*arm*$^{+}$	a, c
Df(1)y$^{-74K\ 24.1}$	tip; 2B9-10		head defect		b
Df(1)C60-1	1A4; 1A7	+	normal		c
Df(1)Bld	tip; 1C3	+	head defect, pale	*arm*$^{+}$	a, c
Df(1)A94	1E3; 2B15	+	normal, poorly differentiated		b, c
Df(1)S39	1E4; 2B11-12	+	normal, pale		b, c
Df(1)64c18	2E1-2; 3C2		giant	*gt*$^{-}$	b, c
Df(1)JC19	2F3; 3C5		giant	*gt*$^{-}$	b
Df(1)N^{8}	3B4-C1; 3D6-E1	+	Notch	*N*$^{-}$	a, c
Df(1)dm^{75e}	3C11; 3E4	+	normal		b, c
Df(1)GA102	3D5; 3F7-8	+	normal	*hnt*$^{+}$	
Df(1)A113	3D6-E1; 4F7-8		hindsight, shavenbaby		b
Df(1)HF366	3E8; 5A7	+	hindsight, shavenbaby		b, c
Df(1)RC40	4B1; 4F1	+	hindsight, shavenbaby	*svb*$^{-}$	b
Df(1)JC70	4C15-16; 5A1-2	+	shavenbaby		b
Df(1)C149	5A8-9; 5C5-6	+	normal		b, c
Df(1)N73	5C2; 5D5-6	+	normal		b, c
Df(1)ct^{J6}	6E1; 7C1		head defect, differentiation		b
Df(1)HA32	6E4-5; 7A6	+	head defect, differentiation		b
Df(1)Sx1^{bt}	6E2; 7A6		head defect, differentiation		e
Df(1)Sx1^{ra}	7A1; 7B3		head defect, differentiation		e
Df(1)ct^{J4}	7A2; 7C1	+	head defect, differentiation, cut	*l(1)mys*$^{+}$, *rtv*$^{+}$	b, c
Df(1)sn^{72}	7A8; 8A5	+	stardust	*l(1)my*$^{s-}$, *sdt*$^{-}$	
Df(1)C128	7D1; 7D5	+	l(1)mys	*l(1)mys*$^{-}$	b
Df(1)RA2	7D10; 8A45	+	stardust	*sdt*$^{-}$, *otd*$^{-}$	b, c
Df(1)KA14	7F1-2; 8C6	+	orthodenticle, differentiation	*otd*$^{-}$, *sdt*$^{+}$	b, c
Df(1)C52	8E4; 9D	+	head defect, differentiation		b, c
Df(1)C52/Dp(1,2)v^{75d}	8E4; 9A2		head defect		
Df(1)v^{L15}	9B1; 10A1	+	normal (pale)		b, c
Df(1)HC133	9B9; 9E7		normal	*ftd*$^{+}$, *rtv*$^{+}$	b, c
Df(1)N110	9B3-4; 9D1-2		not embryonic lethal	*ftd*$^{+}$, *rtv*$^{+}$	b, c
Df(1)ras-v^{17Cc8}	9E3-4; 10A4-5	+	normal (pale)		b
Df(1)v^{-L3}	9F6-7; 10A6	+	not embryonic lethal	*rtv*$^{+}$	b, c
Df(1)GA112	10A11-B1; 10C2	+	head defects	*rtv*$^{+}$	b, c
Df(1)RA37	10A7; 10B17	+	retroactive head defects	*rtv*$^{-}$	b, c
Df(1)KA7	10A9; 10F10	+	head defect, poor differentiation	*rtv*$^{-}$, *ftd*$^{-}$, *tsg*$^{+}$	b, c
Df(1)HA85	10C1; 10F		faintoid	*ftd*$^{-}$, *rtv*$^{+}$	b
Df(1)N71	10B5; 10D4	+	normal	*rtv*$^{+}$	b
Df(1)m$^{259.4}$	10C12; 10E1-2		normal	*rtv*$^{+}$	b
Df(1)KA6	10E1; 11A7	(+)	head defect, differentiation	*tsg*$^{-}$, *ftd*$^{-}$, *rtv*$^{+}$	
Df(1)RA47	10F1; 10F10	+	faintoid	*ftd*$^{-}$, *rtv*$^{+}$	b
Df(1)N105	10F7; 11C4-D1	tsg	twisted gastrulation	*tsg*$^{-}$	b, c
Df(1)JA26	11A1; 11D-E	tsg	twisted gastrulation	*tsg*$^{-}$	b, c
Df(1)KA10	11A1; 11A7	tsg	twisted gastrulation	*tsg*$^{-}$	b, c
Df(1)RC29	11A	tsg	twisted gastrulation		
Df(1)HF368	11A2; 11B9	tsg	twisted gastrulation		b
Df(1)N12	11D1-2; 11F1-2	+	slight head defect		b, c
Df(1)C246	11D, 12A1-2	+	slight head defect		b, c
Df(1)HA92	12A6-7, 12D3	+	normal		b
Df(1)g^{l}	12A; 12E	+	normal		a, c
Df(1)KA9	12E1; 13A5		normal		b, c
Df(1)sd^{72b26}	13F1; 14B1	+	extradenticle, pale		b
Df(1)r^{ID}	14B6; 15A2	+	normal		
Df(1)r^{D17}	15A1; 15A5	+	normal		
Df(1)N19	17A1; 18A2	+	no cuticle		b, c
Df(1)JA27/y^{+Ymal}	18A; 18D1-2	+	normal	*runt*$^{+}$	b, c
Df(1)HF396	18E1-2; 20	fog	folded gastrulation, (runt) differentiation		b, c
Df(1)HF396/y^{+}Ymal	18E1-2; 18F	+	differentiation		
Df(1)mal^{3}	19A1; 20	fog	folded gastrulation, runt		d
Df(1)mal^{8}	18F4-5; 19E1	+	pale		d
Df(1)DCB1-35b	19F1, 20F	fog	folded gastrulation	*runt*$^{+}$, *fog*$^{-}$	b, c, d
Df(1)mal/y^{+}Ymal^{126}	19A, 20A1	runt	runt	*runt*$^{-}$	d
Df(1)su(f)^{4b}	20A; 20	fog	folded gastrulation	*fog*$^{-}$	

a) Lindsley and Grell 1968; b) Craymer and Roy 1980; c) Campos-Ortega and Jiminez 1980; d) Schalet and Lefevre 1976; e) Nicklas and Cline 1983

types found in deficiency embryos involved defects in head morphology or cuticle pigmentation. In some cases, these defects may correspond to single point mutations or putative complementation groups which we identified but could not assign a cytological localization due to the absence of suitable duplications. In other cases where duplications were available, the head phenotypes may identify late acting genes not represented by alleles obtained in our mutagenesis screens. This may be the more likely explanation for the head phenotypes associated with deficiencies involving 7A or 10B. The defects observed in embryos hemizygous for any of the deficiencies described above were detected only late in development. Deficiency embryos from 50 of the deletion stocks were examined under Voltalef oil during early development. These deficiencies span 71% of the length of the X-chromosome. No regions were identified which were required zygotically for the formation of a cellular blastoderm and with the exception of those deficiencies which deleted previously identified loci affecting gastrulation, none caused alterations in early morphogenetic movements. No new loci were identified among the deficiencies which zygotically altered segment number or pattern.

The effect of fourth chromosomal lethals on embryonic development

Hochman (1976) has carried out extensive mutagenesis on the fourth chromosome and has identified alleles in most, if not all, fourth chromosomal lethal complementation groups. Prior to our present study, we had described the embryonic phenotype of one lethal mutation on the fourth chromosome (ci^D) which causes mirror-image duplications of each denticle band (Nüsslein-Volhard and Wieschaus 1980). Since ci^D fails to complement a number of lethal complementation groups on the fourth chromosome, the specific gene responsible for this pattern abnormality was unknown. To identify this locus and to determine whether the small fourth chromosome has any other loci affecting embryonic pattern, we examined cuticle preparations of embryos homozygous for fourth chromosomal deficiencies and those recessive lethal mutations reported to cause embryonic lethality. Homozygosity for *Df(4)M* or for *l(4)13* produce the same phenotype observed in ci^D homozygous embryos. Both mutations are lethal in transheterozygotes with ci^D, and it is likely that the relevant complementation group causing the ci^D embryonic phenotype is defined by the point mutation *l(4)13*. The other embryonic lethal mutations on the fourth chromosome we have tested (*Df(4)M*^{63a}, *l(4)bt*^{D}, *l(4)10*, *ey*^{D}, *l(4)5*, *l(4)2*) cause no phenotype visible in cuticle preparations. Embryos which had no fourth chromosome at all were generated by mating individuals carrying attached fourth chromosomes. Nullo-4 embryos show the same phenotype as *l(4)13* homozygotes. This indicates that if other loci on the fourth chromosome have effects on embryonic development, none of the effects are strong enough to be detectable in the only moderately abnormal *l(4)13*. Thus, it seems likely that *l(4)13* may be the only gene required zygotically on the fourth chromosome for normal cuticle pattern.

Discussion

The original goal of the mutagenesis experiments described in this paper was genomic saturation. We wanted to identify all loci on the X-chromosome required for differentiation of a morphologically normal larva. Our basic assumption was that such genes, when mutated, would result in zygotic lethality. Hemizygous mutant embryos should not only fail to hatch but should show distinct reproducible alterations which distinguish them from their heterozygous siblings. On the assumption that all genes on the X-chromosome have the same average mutability and that the total number of bands observed in polytene chromosomes (1028, Bridges 1938) roughly equals the number of lethal complementation groups (Judd et al. 1972; Lefevre 1974), the 3255 lethal mutations we have scored should be sufficient to identify at least 96% of the genes. The level of saturation we have actually achieved is more difficult to estimate. Several observations suggest that we are at least close to saturation for mutations which produce their phenotypes by elimination or reduction of wild-type gene activity.

1. Most of our mutations were assignable to complementation groups or probable complementation groups. The average number of alleles per defined locus was 5.4 and ranged from 2 to 12. This suggests that isolation of more lethal mutations would only produce additional alleles in complementation groups already identified. Although the mutagenized lines were screened initially for embryonic lethality, our mutagenesis experiments would also have identified mutations causing pattern phenotypes in embryonic cuticle not resulting in lethality. We mounted embryos from more than half of all lethal stocks. Most of these preparations contained reasonable numbers of late differentiated embryos, although in only 21% of the lines were the relative numbers of such embryos high enough that the late embryonic lethality might have been due directly to loss of a zygotically active vital gene. If mutations causing non-lethal pattern alterations were randomly distributed among the 8614 mutagenized lines, we would have embedded embryos from about one-fifth (=1719/8614) such stocks. If a striking pattern had been observed, the mutant lines would have been kept. Since the number of lines scored for viable pattern defects (1719) is much less than the number scored for embryonic lethals (8614), the level of saturation is certainly much lower. Still, assuming equal mutability and a 96% saturation for embryonic lethals, 1719 lines should be sufficient to identify about half the loci on the X-chromosome causing viable embryonic pattern alterations. Of the loci we did detect, *shavenbaby* and *unpaired* alleles are in fact seldom embryonic lethal. Two mutations at each locus were identified due to the morphology of embryos observed in the first egg collections. The embryos from these stocks had been embedded because more than 25% of the eggs had failed to hatch, apparently for some other reason.

The strength of the saturation arguments based on allele frequencies per complementation group is undermined by the relatively large number of mutations (13) which could not be assigned to complementation groups. If these mutations identify genes necessary for embryonic development, the sensitivity of such loci to EMS mutagenesis must be much lower than that of the average complementation group we have detected. It is impossible to estimate the level of saturation or the number of such hyposensitive loci. On the other hand, the abnormal embryos produced in many of these stocks may not reflect a direct requirement for zygotic transcription. They can be explained equally

well as partially penetrant dominant maternal effect mutations (Garcia Bellido and Moscoso del Prado 1979), aneuploid segregants of undetected translocations, or additive effects of closely linked point lethals or deficiencies. This would be consistent with the observation that most of the phenotypes produced in these stocks involved relatively late processes such as head involution, dorsal closure or cuticle differentiation.

2. We have identified new alleles at most if not all of the previously described embryonic lethal loci on the X-chromosome. These included 12 *Notch* alleles, 8 *giant* alleles, 5 *lethal myospheroid* alleles and 3 lethal alleles of *cut*. In 1956, Ede described a number of X-linked lethals with very striking effects on embryonic pattern. Although all of these mutations have subsequently been lost, we can guess from phenotypes and mapping positions that our *twisted gastrulation* corresponds to his *Lff 11* (*Ede* 1956a), *folded gastrulation*, to his *X2* (*Ede* 1956b), and *runt*, perhaps to his *X27* (*Ede* 1956c). We have identified no mutation which showed the vacillation between neural and epidermal defects he describes for lethal *X20* (*Ede* 1956d). That mutation had been mapped to the tip of the X-chromosome near *scute* before being lost. Analysis of deficiencies and point mutations in that region (Jimenez and Campos-Ortega 1979; White 1980) have repeatedly failed to identify a locus responsible for that phenotype. It is possible that the original *X20* was a neomorphic "gain of function" mutation, perhaps at the *scute* locus itself. A variable over- or under-production of that gene product might result in loss of epidermal cells or neuroblasts respectively.

3. We have examined the phenotypes of 63 deficiency chromosomes deleting about 74% of the total X-chromosome length. Most of these deficiencies showed no phenotype in cuticle preparation or showed phenotypes and patterns consistent with point mutations which map to those regions. These observations provide the strongest support that we are indeed close to saturation for mutations causing pattern defects in embryos.

Because our screens of deficiency chromosomes and point mutations were limited to early morphogenesis or cuticle phenotypes, our survey says very little about loci which affect exclusively internal organs. When a single locus affects the patterning of both internal and external structures (Jan and Jan 1982), cuticle preparations will be sufficient to identify mutations which eliminate the wild-type gene. On the other hand, at least one region on the X-chromosome (*12A-13A*, *Df(1)KA9*) has been identified (Campos-Ortega and Jimenez 1980), which shows striking effects on the pattern of the embryonic nervous system with no corresponding effect on embryonic cuticle. Large deficiencies for the tip of the X-chromosome cause a degeneration of the nervous system as well as heterogeneous head defects in the late cuticle. These phenotypes can be interpreted as an additive effect, due to a number of genes in the region, each of which has a more minor effect on development (Jimenez and Campos-Ortega 1979; White 1980). Our failure to detect any point mutations in this region would be consistent with this interpretation. If other examples of cumulative effects occur on the X-chromosome in *Drosophila* and result in visible cuticle phenotypes, we would not have detected the corresponding point mutations for these loci either, given that no single locus would be expected to produce a phenotype visible in cuticle preparations.

Onset of gene activity during embryogenesis

Major RNA synthesis in the *Drosophila* embryo begins at the syncytial blastoderm stage (Lamb and Laird 1976; McKnight and Miller 1976; Zalokar 1976). The small amount of transcription detected prior to that stage may be developmentally significant. On the other hand, much of it is localized in the mitochondria (Zalokar 1977; Anderson and Lengyel 1979) and of the sequences known to be of nuclear origin, most are small in size and different in other respects from those found at the blastoderm stage (McKnight and Miller 1976; Sina and Pellegrini 1982). Attempts to resolve the onset of zygotic gene activity using genetic techniques have yielded ambiguous results. Although no lethal mutations have been identified which have reproducible, zygotically dependent effects prior to formation of the cellular blastoderm, many gross chromosomal aberrations cause death at earlier stages. The best-studied example of this phenomenon is the abnormal cleavage and syncytial blastoderm formed by embryos having no X-chromosome (Poulson 1940; Scriba 1964). Similar or even earlier abnormalities are reported in embryos deficient for half of either of the two major autosomes (Scriba 1967, 1969). Since such aneuploid embryos are derived from mothers which are wild-type, at least with respect to total chromosome content, the early embryonic defects must depend on the zygotic genotype of the embryo. Thus, they are often interpreted as being due to the loss of some essential gene whose transcriptional activity is required during cleavage (Hadorn 1955; Scriba 1969; see Wright 1970).

All point mutations we have identified on the X-chromosome allow development to a normal cellular blastoderm. The earliest defects we have observed became apparent only at the onset of gastrulation. It is arguable that we may have failed to detect genes whose absence causes earlier death, since our initial characterization was based on cuticle phenotypes and such stocks would be indistinguishable from lines showing high frequencies of unfertilized eggs. The analysis of deficiency chromosomes, however, tends to rule out this possibility. All deficiencies we examined allow formation of a normal cellular blastoderm and many, even relatively large ones, develop to late embryonic stages showing phenotypes the same or similar to point mutations which map in those regions. This implies that the regions surrounding our identified point mutations contain no lethal loci causing early death. A similar argument can be made using those deficiencies which allow development to morphologically normal larvae, although when chromosomes are not marked, the conclusion that the homozygotes are indeed the normal larvae and not the apparently undeveloped eggs depends on our careful determination of hatch rates. Any undetected, early lethals on the X-chromosome would have to be limited to those 25% of the bands not scored in deficiency homozygotes. If, on the other hand, none of the genes on the X-chromosomes are actually required prior to cellular blastoderm, one is left to explain the early lethality of embryos which lack the entire chromosome. These abnormalities are not due to a "structural" requirement during cleavage for at least one copy of each chromosome, since the small fourth chromosome can be deleted entirely with no effect on development, other than that associated with lethal *(4)13*.

Comparison of X-chromosomal and autosomal loci

When the results of the mutagenesis experiments on the X-chromosome are compared with those obtained for autosomal loci (Nüsslein et al. 1984; Jürgens et al. 1984), it is remarkable how similar the three sets of data are (Table 5). For all three major chromosomes, about one-quarter of the lethal mutations caused homozygous embryos to die before hatching and only about 3% of the vital gene functions are required zygotically in the embryo for a normal gross morphology, at least at the level detected in cuticle preparations. Since the X-chromosome is only one-fourth the size of the autosomal complement, the absolute number of X-linked loci affecting embryonic pattern is much smaller. The range of phenotypes produced by such loci, however, is very similar to that found among the autosomal mutations. Moreover, the probability of obtaining phenotypes of a particular kind is about that expected from the frequency of those phenotypes on the autosomes. For example, at least four of the X-chromosomal loci have a dramatic effect on segment number or polarity (*run, arm, gt, upd*), compared to 18 such loci on the autosomes. Most of the broad phenotypic classes found on the autosomes are also represented on the X-chromosome. This includes, for example, one homoeotic locus, and loci affecting pigmentation and denticle morphology, as well as those affecting early and late morphogenetic movements.

The similarity of the phenotypes associated with the X-chromosome and those of the autosomes is of both theoretical and practical importance. X-chromosomal genes are present in different doses in males and females, and are consequently subject to dosage compensation. The similarity in phenotype regardless of chromosomal location might argue either that embryonic processes requiring zygotic gene activity are insensitive to doubling or halving the normal number of wild-type genes, or that the dosage compensation mechanism acts very early to equalize the imbalance. From the practical standpoint, our observation that loci on the X-chromosome are required for the same embryonic processes affected by genes on the autosomes means that it may be possible to extrapolate from detailed analysis of X-chromosomal mutants to understanding more about the potential role of the autosomal genes in the same embryonic processes. The X-chromosome offers special advantages for such an analysis, due to the ease with which embryonic mosaics can be constructed for X-linked mutations.

Table 5. Comparison of mutagenesis screens for X-, second and third chromosomal lethals

	X-chromosome	Second chromosome[a]	Third chromosome[b]
Number of bands on chromosomes polytene	1028	1944	2062
Number of lethal hits	3255	7581	7300
Embryonic lethal hits (% of lethal hits)	679 (21%)	1907 (25%)	1772 (24%)
Embryonic visible hits (% of lethal hits)	114 (3.5%)	274 (3.6%)	198 (2.7%)
Number of complementation groups	20	48	32
Average number of alleles per complementation group[c]	5.1	5.4	5.8
Number of single mutations not assigned to complementation groups	13	13	13

[a] Data from Nüsslein-Volhard et al. 1984
[b] Data from Jürgens et al. 1984
[c] Complementation groups defined by more than one allele

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