

# Molecular Organization of a *Drosophila* Puff Site That Responds to Ecdysone

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## Summary

The 68C locus in the polytene chromosomes of *Drosophila melanogaster* salivary glands is puffed during the last half of the third larval instar and harbors the structural gene for *sgs3*, one of the glue polypeptides synthesized by the glands during this period. This puff regresses in response to the steroid hormone ecdysone. We have isolated a set of overlapping cloned segments that define approximately 50 kb of DNA at the 68C puff locus. Three polysomal poly(A)<sup>+</sup> RNAs that are abundant in the salivary glands during the intermolt-puff stage are transcribed from three genes (II, III and IV) that map near the center of the cloned DNA in a 5 kb cluster region. These are the only transcripts from the 50 kb of 68C DNA detectable in these glands, and they are undetectable in other larval tissues at this stage, or in whole animals at other stages of development. Correlative criteria indicate that gene IV, which yields an RNA of 1.1 kb, is the structural gene for *sgs3*. Genes II and III, which yield RNAs of 0.36 kb and 0.32 kb, respectively, are oppositely oriented so that their promoters are adjacent, suggesting that this pair may form a single regulated unit, a suggestion that is enhanced by the fact that the pair is bounded by an inverted repeat of 0.3 kb elements. The possible, but as yet unidentified, functions of this gene pair are discussed. A 9.2 kb element belonging to a family of transposable elements called *roo* is inserted adjacent to the 5 kb cluster region in some but not other *D. melanogaster* strains. This insertion has no obvious effect on the transcription of genes II, III and IV. Although *roo* elements yield a 9 kb poly(A)<sup>+</sup> RNA in embryos, no *roo* transcripts were detected in intermolt salivary glands, whether they do or do not contain an element at the 68C puff site.

## Introduction

The larval phase of *Drosophila melanogaster* development is punctuated by two molts that divide larval life into three stages or "instars," the first two lasting one day each and the third almost two days. During the terminal half of the third instar, the larval salivary glands become factories for the synthesis of a half-dozen or so glue polypeptides that account for 30% of the total gland protein by the end of the third instar,

when synthesis ceases and the polypeptides are secreted to form a glue that attaches the pupal case to solid surfaces (Beckendorf and Kafatos, 1976; Korge 1977a; also see review by Berendes and Ashburner, 1978). This period of glue polypeptide synthesis correlates with the period when the half-dozen or so "intermolt" puffs can be observed in the polytene chromosomes of the salivary glands; in particular, these puffs of transcription regress near the end of third instar in response to the dramatic increase in the concentration of the steroid hormone ecdysone that occurs at this time (reviewed by Ashburner and Berendes, 1978). Furthermore, recombination and cytogenetic mapping, as well as gene dosage studies, indicate that the structural genes for three of the glue polypeptides are located at chromosomal positions occupied by three of the intermolt puffs (Korge, 1975, 1977b; Akam et al., 1978; Velissariou and Ashburner, 1980). The postulate that transcription within the intermolt puffs provides the mRNAs coding for at least three, and perhaps all, of the glue polypeptides is therefore current.

We are concerned here with the DNA and the derivative transcripts that are responsible for one of the largest of the intermolt puffs, that located at 68C on the left arm of chromosome 3. The mapping experiments of Korge (1975) and Akam et al. (1978) indicate that this puff site harbors the *Sgs3* structural gene that codes for the *sgs3* glue polypeptide (fraction 3 of Korge, 1975, 1977a; fraction P1 of Beckendorf and Kafatos, 1976). This site is transcribed when puffed (Bonner and Pardue, 1977; Bonner et al., 1977), and that puff regresses rapidly in response to ecdysone, not only in vivo as noted above, but also in vitro as observed in isolated glands exposed to the hormone (Ashburner, 1973; Ashburner and Richards, 1976; Bonner and Pardue, 1977). Furthermore, recent evidence (Gronemeyer and Pongs, 1980) that the hormone binds to the 68C puff at the time of its regression suggests that transcription at this locus is directly repressed by an ecdysone-receptor complex. The 68C locus is thus transcribed at a known developmental time in a known tissue, yields a known gene product and is a likely candidate for direct regulation by known steroids.

The molecular analysis of the 68C locus that is presented here has its origins in the isolation of a set of cDNA clones representing salivary gland poly(A)<sup>+</sup> RNAs that are abundant if the intermolt puffs are present and rare if they have regressed (Muskavitch, 1980; Wolfner, 1980; M. Wolfner, D. J. Kemp, M. B. T. Muskavitch, G. M. Guild and D. S. Hogness, in preparation). Cross-hybridization tests divided this set of intermolt cDNAs into five nonoverlapping homologous sequence groups, and in situ hybridization mapping showed that the sequences in these groups derive from chromosomal sites occupied by intermolt puffs. Group I corresponds to the puff at 3C11-12 and

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group 5 corresponds to the puff at 90BC, whereas the remaining three groups (II, III and IV) all map at the 68C puff site. The group I cDNAs have been used to isolate and analyze the 3C11-12 genomic DNA whose single transcript codes for the *sgs4* glue polypeptide (Muskavitch and Hogness, 1980). Here we similarly use the cDNAs of groups II, III and IV to initiate our studies on the more complex 68C genomic DNA and its three transcripts.

## Results

### New Clones for Old

The cDNA clones originally used to define groups II, III and IV of the 68C locus consist of duplex cDNA segments inserted at the Bam HI site of pSC105 plasmid vector by (dA)<sub>n</sub>-(dT)<sub>n</sub> joints; their cDNA segments derive from the poly(A)<sup>+</sup> RNA of wild-type (Oregon-R) salivary glands in the intermolt-puff stage (Wolfner, 1980; M. Wolfner et al., in preparation). These clones suffer the disadvantage that their cDNA segments cannot be neatly excised without inclusion of vector DNA. New group II, III and IV cDNA clones were therefore isolated that are free of this defect (Experimental Procedures). These new clones contain the same sort of cDNA segments, but differ from the old in that these segments are inserted at the Pst I site of the pBR322 plasmid vector by (dG)<sub>n</sub>-(dC)<sub>n</sub> joints that regenerate Pst I sites to each side of the segment, thereby allowing its excision (Bolivar et al., 1977). One clone was chosen from among those in each homologous sequence group to represent the sequences characteristic of that group. The reference clones for groups II, III and IV contain Pst I-excisible inserts that are, respectively, 0.37 kb, 0.30 kb and 0.67 kb long, and will subsequently be referred to as clones II, III and IV, respectively (Experimental Procedures).

### The cDNA Clones Define Three Polysomal RNAs in Larval Salivary Glands at the Intermolt-Puff Stage of Development

Total RNA from Oregon-R salivary glands in the intermolt-puff stage ("intermolt salivary glands") was fractionated by gel electrophoresis, transferred to diazobenzyloxymethyl paper (DBM paper; Alwine et al., 1980), and then hybridized with <sup>32</sup>P-labeled Pst I-excisible inserts from the cDNA clones II, III and IV. Figure 1 indicates that the cDNA insert from each clone hybridized to a single RNA and that these RNAs are of different lengths. Clones II, III and IV therefore derive from poly(A)<sup>+</sup> RNAs with lengths of 0.36 kb, 0.32 kb and 1.1 kb, respectively. The transcripts will subsequently be referred to as RNAs II, III and IV. The lengths of RNAs II and III closely approximate the lengths of the Pst I-excisible insert of the respective cDNA clones, whereas RNA IV is significantly longer than the clone IV insert. Since all three of the inde-

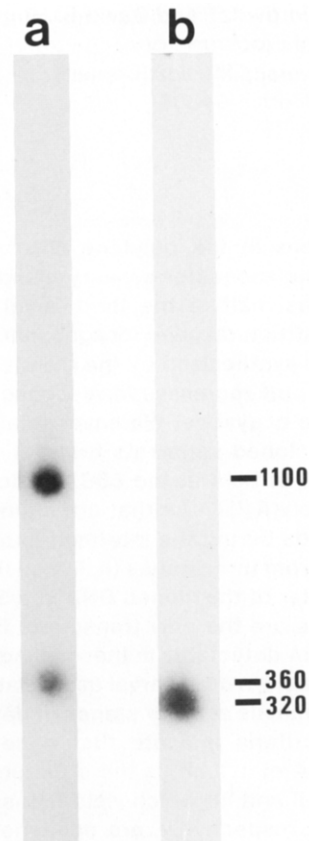


Figure 1. Lengths of RNAs II, III and IV

Total RNA from intermolt salivary glands was denatured, glyoxal-treated, fractionated by electrophoresis in a 1.75% agarose gel and then transferred to DBM paper (Alwine et al., 1980). (Lane a) Hybridization of a mixture of the <sup>32</sup>P-labeled cDNA inserts from clones II and IV to homologous RNAs. (Lane b) Same filter after elimination of the previously hybridized cDNA by boiling in 0.1 × SSC and subsequent hybridization with the labeled cDNA insert from clone III. The RNA lengths given on the right were determined from the mobilities of length standards consisting of the single strands of pBR322 restriction fragments (Experimental Procedures), and are in nucleotides. That the 360 and 1100 nucleotide RNAs registered in lane a are homologous, respectively, with clones II and IV was shown in separate experiments.

pendently isolated cDNA clones in group IV have Pst I-excisible inserts of 0.67 kb (Experimental Procedures), we suspect that elongation of the cDNA strand from its oligo(dT)-primer by reverse transcriptase is blocked at a point about 0.67 kb from the 3' end of the RNA IV template.

Each of the three RNAs appears to be associated with polysomes. This is shown in Figure 2 for RNAs II and IV, where the polysomes of intermolt salivary glands were fractionated by zone sedimentation in a sucrose gradient and the resulting fractions then analyzed for the presence of these RNAs. Both RNAs cosediment with particular polysome fractions and, as would be expected, the longer RNA IV cosediments with the larger polysomes. A separate experiment of

the same sort (not shown) demonstrated that RNA III cosedimented with the same polysome fraction as RNA II, as expected from the similar lengths of these two RNAs.

The three RNAs have been detected only in salivary glands, and not in any other tissue of third instar larvae at the intermolt-puff stage of development. Total RNA extracted from whole larvae, from salivary glands and from the carcass remaining after removal of the salivary glands were tested for the presence of RNAs II, III and IV by the gel-blot assay illustrated in Figure 1. All three RNAs were detected in whole larvae and in the salivary glands; none of the RNAs was detected in carcasses deprived of their salivary glands, even after autoradiographic exposures that would have detected 1% of that found in whole larvae.

This gel-blot assay was also used to test for RNAs II, III and IV in whole animals at different stages of the development. None of the three RNAs was detected in embryos, in first and second instar larvae, or in early (~one day) pupae. Thus we have found these RNAs only in salivary glands of late third instar larvae (climbing stage), when the 68C and other intermolt puffs are expressed. A similar developmental profile was obtained for the *sgs4* mRNA from the 3C11-12

intermolt puff by Muskavitch and Hogness (1980), who observed that this RNA first appeared in larvae that had completed more than 20% and less than 40% of the third-instar period and had disappeared by the time of the larval-to-prepupal transition. Indeed, when the same samples of third instar and prepupal RNAs were probed with our cDNA clones II, III and IV, the same third-instar profile was observed for the three RNAs from 68C (Muskavitch, 1980).

#### Mapping Genes II, III and IV within the Chromosomal DNA

Genes II, III and IV are defined by the chromosomal DNA that is transcribed to yield RNAs II, III and IV, respectively. The cDNA clones II, III and IV were therefore used as hybridization probes to screen libraries of cloned segments of *D. melanogaster* chromosomal DNA (Dm segments) for those that contain these genes. Two sorts of genomic libraries were used, both of which consist of sheared segments of embryonic DNA cloned in bacteriophage  $\lambda$  vectors. They differ in that one represents the DNA in our stock of Oregon-R (Experimental Procedures), whereas the other represents the DNA in the Caltech stock of Canton-S, another wild-type strain (Maniatis et al., 1978). Seven Oregon-R and nine Canton-S clones were isolated by screening these libraries—first with the cDNA clones to obtain Dm segments carrying the three genes and then with restriction fragments from these segments to extend the chromosomal regions covered by the genomic clones. Figure 3 shows the 16 overlapping Dm segments and a restriction map of the more than 50 kb of chromosomal DNA that they define. In situ hybridization of  $^3\text{H}$ -labeled  $\lambda\text{Dm1501-10}$  DNA to salivary gland polytene chromosomes (not shown) indicates that the Dm1501-10 segment derives from the region including the bands 68C3-5 and occupies the central region of the 68C puff.

Genes II, III and IV were located relative to this restriction map by hybridization of  $^{32}\text{P}$ -labeled Pst I-excised inserts of cDNA clones II, III and IV to blots of gels containing the electrophoretically separated fragments from restriction endonuclease digests of the genomic clones (Southern, 1975). These analyses showed that the three genes are clustered in a single 5 kb region (the "cluster region") in the order, II-III-IV, as shown in Figure 3. In addition,  $^{32}\text{P}$ -labeled single-stranded cDNAs, made from the total poly(A)<sup>+</sup> population of intermolt salivary glands, hybridized only to those restriction fragments that exhibit hybridization with the cloned cDNAs. This result indicates that RNAs II, III and IV are the only abundant salivary gland poly(A)<sup>+</sup> RNAs that derive from the cloned part of the 68C3-5 locus at this stage of development. This conclusion was confirmed and extended by examination of both the poly(A)<sup>+</sup> and poly(A)<sup>-</sup> fractions of the intermolt gland RNA according to the method shown in Figure 1, except that the  $^{32}\text{P}$ -labeled probes con-

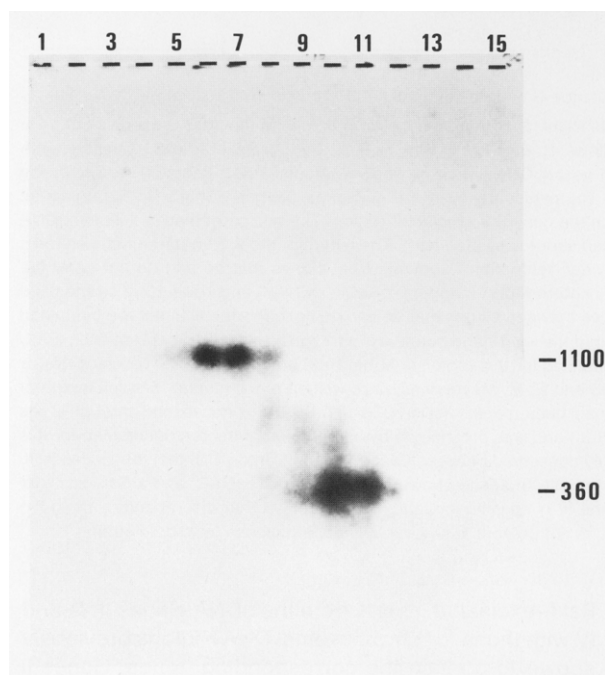


Figure 2. Location of RNAs II and IV in a Polysome Gradient

Polysomes from 30 third instar salivary glands were extracted and sedimented in a sucrose gradient (Experimental Procedures). RNAs purified from each of the 15 fractions of the gradient were glyoxylated, subjected to electrophoresis in 15 adjacent tracks of a 1.75% agarose gel and then treated as described for Figure 1, lane a to assay for RNAs II and IV in each track. The fractions from the top of the sucrose gradient are to the right. Lane 13 contained only 28S ribosomal RNA and lane 14, only 18S, while lanes 1 through 12 showed rRNA from both ribosomal subunits. RNA sizes shown in nucleotides.

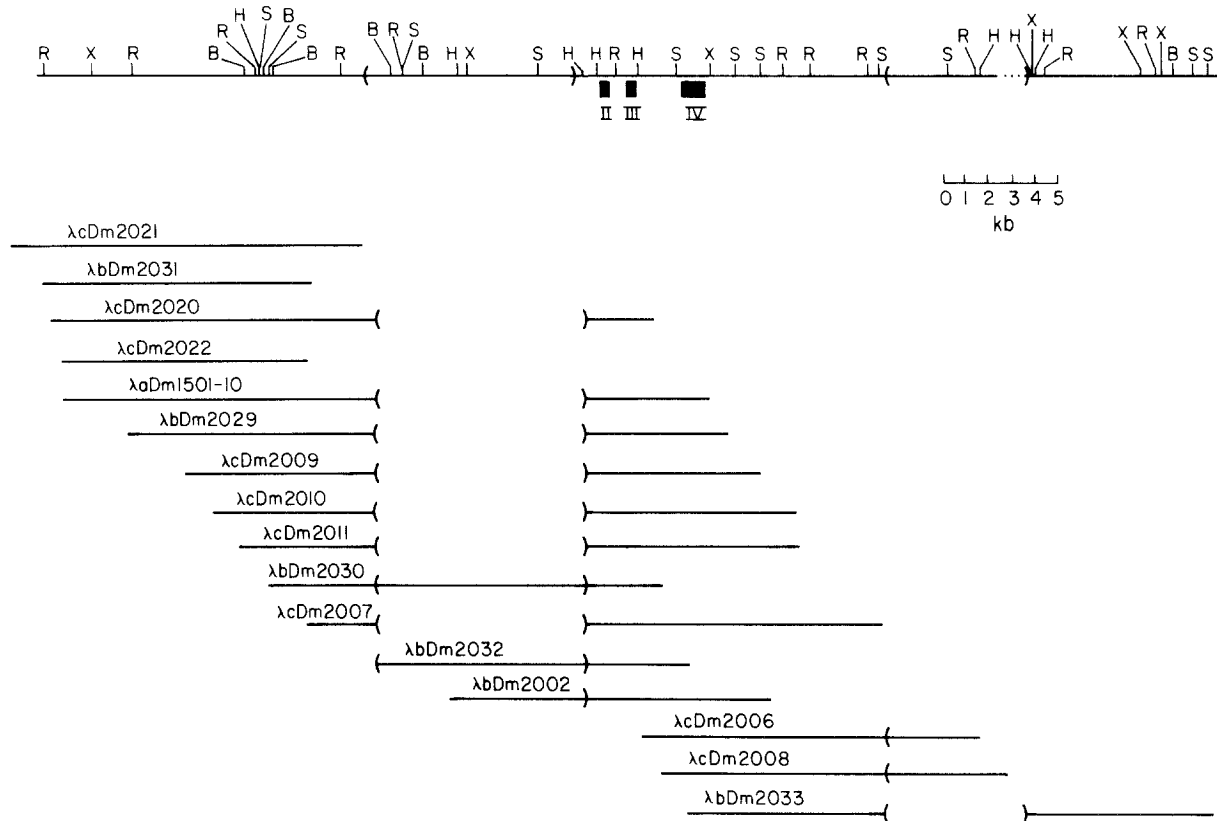


Figure 3. Cloned Dm Segments from the 68C3-5 Locus in Oregon-R and Canton-S and Their Combined Restriction Map

The restriction map (top) derives from the maps of the cloned Dm segments in the overlapping set shown below. Mapping was carried out as in Experimental Procedures. Restriction endonucleases are abbreviated as follows: B: Bam HI, H: Hind III, R: Eco RI, S: Sal I, X: Xho I. Dm segments are designated according to the λDm clones from which they derive, the λ vector DNA not being shown. Oregon-R Dm segments are from the λaDm (vector λ647) and λbDm (vector λSep6) clones, while Canton-S segments are from λcDm clones (vector λCharon4; Experimental Procedures). Solid bars just below map: Position of genes II, III and IV within the central 5 kb cluster region. The two parentheses delineate DNA present in some clones but absent in others, consisting of dispersed repeated sequences (see text). The length of the 9.2 kb parenthetical element to the left of the cluster region was determined by length differences between restriction fragments from clones that do and do not carry the element. Its indicated right-hand boundary was approximately determined by heteroduplex mapping of λaDm1501-10 and λbDm2002 strands and its other boundary then placed 9.2 kb to the left. All restriction site differences between clones that do and do not carry the element are contained within these boundaries, with the exception of the Hind III site just to the right of the right-hand boundary, which is present in the λbDm2002, 2030 and 2032 clones but not in any others. A comparison of the nucleotide sequences for this region in λbDm2002 and λaDm1501-10 demonstrates that this site is not, however, included in the element (M. Garfinkel, R. Pruitt and E. M. Meyerowitz, unpublished experiments). The full length of the parenthetical DNA to the right of the cluster region cannot be determined because the λcDm2006 and 2008 do not extend through it; its insertion site is indicated by the position of the open parentheses in λbDm2033 and was determined by comparison of the restriction map for this clone with those of the previous two clones. The nonparenthetical DNA is highly conserved between Oregon-R and Canton-S in that the clones from each strain yield the same restriction map except for only two sites: the left-most Xho I site shown is specific to Oregon-R; and a Sal I site (not shown) located 1.0 kb to the right of the first Eco RI site to the right of gene IV is specific to Canton-S. The only other discrepancy among the clones is that λbDm2029 contains a Xho I site between genes III and IV that is not present in several overlapping clones from both strains.

sisted of genomic clones representing the entire cloned region of the 68C3-5 locus. The only RNAs detected by these probes exhibit the same lengths as RNAs II, III and IV, and these were detected only with those genomic clones that contain the respective genes. These RNAs are found almost exclusively within the poly(A)<sup>+</sup> fraction.

Genes II, III and IV were more precisely located within the 5 kb cluster region by a combination of fine structure restriction mapping and S1 nuclease protection experiments (Berk and Sharp, 1978). Figure 4 compares the fine structure restriction maps for the

Pst I-excisable inserts from the cDNA clones II, III and IV with those for chromosomal DNA regions previously shown to contain the corresponding genes. Genes II and III can be localized with fair precision by this comparison because the cDNA insert lengths closely approximate the lengths of RNAs II and III. However, the 0.67 kb cDNA insert of clone IV lacks approximately 0.4 kb of sequences present in RNA IV—sequences that required S1 nuclease protection mapping for their localization. Figure 5, lane a shows that hybridization of the 1.1 kb RNA IV with the strands of the Sal I fragment containing gene IV, and subsequent

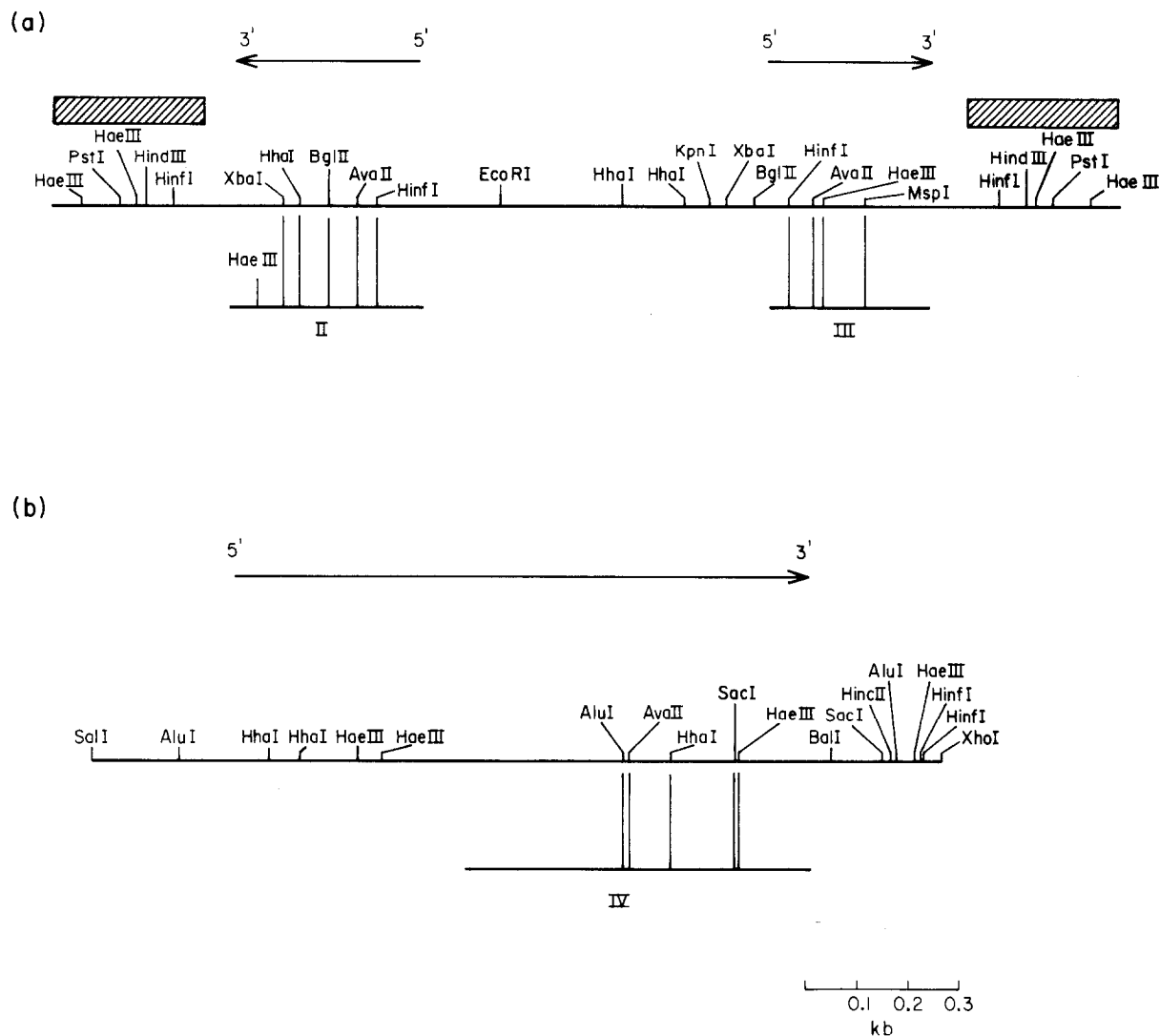


Figure 4. Fine Structure Restriction Maps for the Cluster Region and for the cDNA Inserts of Clones II, III and IV

(a) Restriction maps for the 0.37 kb and 0.30 kb cDNA inserts from clones II and III are aligned with that for the 2.0 kb at the left end of the cluster region. The Hae III site that is in the group II cDNA clone but not in the corresponding genomic DNA could arise either from polymorphism in the Oregon-R stock or from mutation during cloning in *E. coli*. Striped blocks: positions of the 0.3 kb elements of an inverted repeat (see text). (b) Restriction map for the 0.67 kb cDNA insert from clone IV is aligned with that for the 1.6 kb at the right end of the cluster region.

Maps in (a) and (b) are oriented as in Figure 3, and those from the cluster region derive from subcloned restriction fragments of  $\lambda$ Dm2002. All restriction sites were determined as in Experimental Procedures, except for two in the cluster region shown in (b), which were revealed by nucleotide sequencing (the first Hae III site to the left of the DNA represented in clone IV and the furthest right of the Hinf I sites; M. Garfinkel et al., unpublished experiments). Arrows above the maps: 5'-to-3' directions and extents of the sequences homologous to RNAs II, III and IV (see text).

digestion by S1 of any single-stranded regions in the hybridization product, yields an RNA-DNA duplex that exhibits the same approximate length as RNA IV. Gene IV must therefore either lack introns or restrict them to positions very near its ends; most of the missing 0.4 kb must then lie adjacent to the sequence present in the cDNA insert. Recent experiments indicate that gene IV, as well as genes II and III, contain a small intervening sequence near their 5' ends (M. Garfinkel, R. Pruitt and E. M. Meyerowitz, unpublished; K. Burtis and D. S. Hogness, unpublished). Figure 5, lane c shows that gene IV extends approxi-

mately 1.0 kb to the left of its single Sac I site, which is shown in Figure 4b to lie near its right end. This result localizes gene IV to the position of the arrow in Figure 4b.

#### Genes II and III Form an Oppositely Oriented Pair Bounded by an Inverted Repeat

The direction of the arrows in Figure 4 defines the direction of transcription of the respective genes. These directions were established by the experiments shown in Figure 6 and described in its legend. In summary, the strands of genomic DNA segments spe-

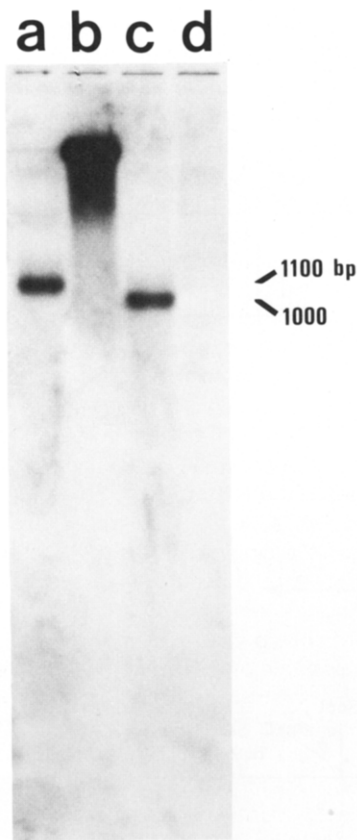


Figure 5. S1 Protection Mapping of Gene IV

Total RNA (3.5  $\mu$ g) from intermolt salivary glands was hybridized under R-loop conditions (Thomas et al., 1976) and in a total vol of 25  $\mu$ l to 0.5  $\mu$ g of either Sal I-digested (lane a) or Sac I-digested (lane c)  $\lambda$ bDm2002 DNA that was denatured prior to hybridization. The resulting RNA-DNA duplexes were treated with 400 units of S1 nuclease for 30 min at 37°C to remove single-stranded regions and then fractionated by electrophoresis in a 2% neutral agarose gel. The duplexes in the gel were denatured and blotted to a nitrocellulose filter (Southern, 1975), which was then hybridized with the  $^{32}$ P-labeled 2.4 kb Sal I fragment that includes gene IV and that was subcloned from  $\lambda$ bDm2002 (Figure 3). (Lane b) Control for lane a, RNA omitted. (Lane d) Control for lane c, RNA omitted. The lengths of the RNA-DNA duplexes registered in lanes a and c were determined from the mobilities of duplex DNA standards (Experimental Procedures).

cific to each gene were separated by gel electrophoresis; identified in respect to their 5'-to-3' directions relative to the maps in Figure 4; and tested for their ability to hybridize with  $^{32}$ P-labeled single-stranded cDNA synthesized by reverse transcriptase from the poly(A)<sup>+</sup> RNA population of intermolt salivary glands. Hence the direction of transcription equals the 5'-to-3' direction of the genomic DNA strand that hybridizes with the labeled cDNA. Figure 6A shows that this direction is rightward for gene IV, 6B shows that it is rightward for gene III and Figure 6C shows that it is leftward for gene II.

Genes II and III therefore constitute an oppositely oriented pair whose promoters lie within the short region separating them. This gene pair exhibits the additional interesting feature that it is bounded by the

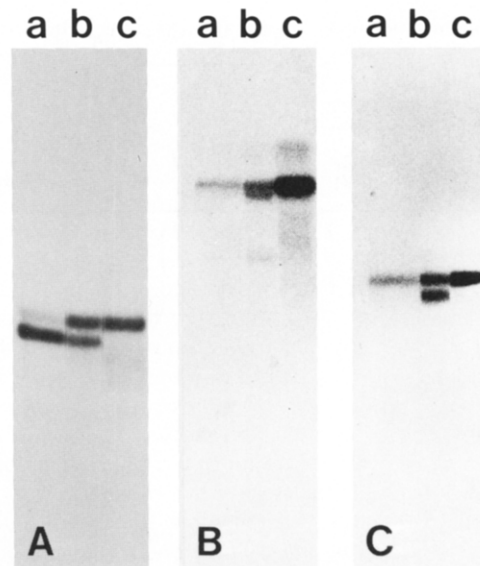


Figure 6. Orientation of Transcription

(A) Gene IV. Strands of  $\lambda$ cDm2008 (Figure 3) were separated by electrophoresis in a 0.4% agarose gel (2 mM EDTA, 40 mM Tris-acetate [pH 8.3]) at 0.75 Vcm<sup>-1</sup> for 23 hr (McDonnell et al., 1977). Since this  $\lambda$  clone contains gene IV but not any other sequences homologous to detectable RNAs in intermolt salivary glands (see text), the orientation of gene IV can be obtained by determining which of the separated strands of  $\lambda$ cDm2008 hybridizes with a cDNA probe synthesized from the poly(A)<sup>+</sup> RNA of intermolt glands as in Experimental Procedures. This was accomplished by blotting the wide gel lane containing the separated strands to a nitrocellulose filter and then cutting the filter into three lanes, each of which was hybridized with a different  $^{32}$ P-labeled probe. (Lane c) Hybridized with the cDNA probe to register the strand whose 5'-to-3' direction equals that of gene IV transcription. (Lane b) Hybridized with nick-translated  $\lambda$  DNA to mark the positions of both strands. (Lane a) Hybridized with a probe that registers only that strand of  $\lambda$ cDm2008 whose 5'-to-3' direction is from right to left according to the orientation of  $\lambda$ cDm2008 given in Figure 3. Since this probe and the cDNA probe hybridize to opposite strands, transcription of gene IV must be from left to right (see Figure 4). The strand-specific probe used for lane a was constructed as described by Muskavitch and Hogness (1980; see their Figure 3). It consists of a terminal fragment of  $\lambda$  DNA that was isolated after 3'-end labeling in the cohesive sites with reverse transcriptase, and which was shown by restriction mapping to comprise the right end of  $\lambda$ cDm2008 as oriented in Figure 3.

(B) Gene III. Strands of the 1.0 kb Eco RI-Hind III fragment of  $\lambda$ bDm2002 that is specific for gene III (Figure 3) were separated in a 1.7% agarose gel and transferred to nitrocellulose paper that was cut into three lanes as in (A). (Lane c) Hybridized with the cDNA probe used above; (lane b) with the nick-translated 1.7 kb Hind III fragment of  $\lambda$ bDm2002 that contains both genes II and III. Since the probe used for lane a registers only that strand of the Eco RI-Hind III fragment whose 5'-to-3' direction is from left to right, and since that is the strand hybridized by the cDNA probe, transcription of gene III is from left to right. The strand-specific probe was prepared by 5'-end labeling of the above 1.7 kb Hind III fragment with polynucleotide kinase, followed by Eco RI cleavage of this fragment and isolation of the resulting 0.7 kb Hind III-Eco RI and 1.0 kb Eco RI-Hind III fragments, of which the latter was used as the probe.

(C) Gene II. Identical to (B), except the strands of the 0.7 kb Hind III-Eco RI fragment of  $\lambda$ bDm2002, which is specific to gene II, were separated by electrophoresis, and the probe used in lane a registers only that strand whose 5'-to-3' direction is from right to left, which must then be the direction of transcription in gene II. This probe is the 0.7 kb Hind III-Eco RI fragment of the 5'-end labeled 1.7 kb Hind III fragment described in (B) above.

0.3 kb members of an inverted repeat, whose positions are indicated by the striped blocks in Figure 4. This inverted repeat was first detected and mapped by electron microscopic observation of the hairpin structure it forms in the single-stranded Dm regions of heteroduplexes between  $\lambda$ Dm1510-10 and wild-type  $\lambda$  DNAs, or between the DNAs of  $\lambda$ Dm1510-10 and  $\lambda$ bDm2002 (Figure 3), whose Dm segments are inserted into their  $\lambda$  vectors in opposite orientations (data not shown). The positions determined by electron microscopy were confirmed by detection of restriction sites repeated in inverted order at these positions (Figure 4) and more recently by nucleotide sequence determination to be reported elsewhere (M. Garfinkel et al., unpublished results).

The two 0.3 kb elements of the inverted repeat at 68C3-5 belong to a family of five repeated elements, whose other three members are located at or very close to the nearby 68C10 polytene band, where they form a series of commonly oriented elements separated by different DNA sequences (E. M. Meyerowitz, unpublished experiments). This was shown by cloning and mapping the three additional elements within a single Dm segment, by *in situ* hybridization of polytene chromosomes with subcloned sequences from this clone and by gel-blot hybridization of such sequences to separated fragments from restriction digests of total embryonic DNA. *In situ* and quantitative gel-blot hybridization (Lis et al., 1978) with probes representing the entire 5 kb cluster region for genes II, III and IV indicate that the only DNA of this region repeated elsewhere in the genome is that of the inverted repeat.

#### Transposable Elements at the 68C3-5 Puff Site

Quantitative gel-blot hybridization of restriction fragments representing the whole Oregon-R and Canton-S genomes with probes consisting of cloned sequences lying to the left and right of the cluster region reveal that this flanking DNA is not repeated elsewhere in the genome, with the exception of the DNA within the two parentheses shown in Figure 3. These parenthetical DNAs are repeated many times in the genomes of both strains and appear to represent two different families of transposable elements, a member of each having been inserted at the 68C3-5 puff site of one strain or the other.

Consider first the 9.2 kb element delineated by the parenthesis located just to the left of the cluster region in Figure 3. This element was originally defined by a discrepancy in the restriction maps generated by the Canton-S and Oregon-R clones containing DNA lying to the left of the cluster region. The Canton-S clones generate a single, consistent restriction map for the 17 kb of "left flank" DNA that they define. By contrast, the Oregon-R clones generate two maps. One of these is virtually identical to the Canton-S map, while the other differs from this common map by the insertion of the 9.2 kb of DNA within the parenthesis.

The two types of Oregon-R clones (that is, those

that do and do not carry DNA from the 9.2 kb element) derive from two alleles at the 68C3-5 locus, both of which are present in the Oregon-R stock from which the libraries of Oregon-R clones derive. Figure 7 demonstrates this point. Two strains were constructed, each homozygous for a single 68C region derived from an individual third chromosome from our Oregon-R stock. The total DNA from each of these strains, and from our Oregon-R stock, was digested with Hind III, and after electrophoretic fractionation and blot transfer to nitrocellulose paper (Southern, 1975), the resulting fragments were hybridized with a  $^{32}$ P-labeled 5.6 kb Hind III fragment that spans the insertion site and derives from a clone lacking the element ( $\lambda$ aDm1501-10). This fragment extends from the leftmost Hind III site shown in Figure 3 to the Hind III site immediately to the left of gene II (see Figure legend 3). Strains that lack the element at 68C3-5 should therefore yield a single hybridization band at 5.6 kb, as is the case for the homozygous strain represented in lane a. Strains homozygous for the allele carrying the element should yield 8.6 kb and 0.55 kb Hind III fragments capable of hybridization with the probe, of which only the 8.6 kb fragment is registered by the assay (see Figure 7). This is the case for the other homozygous strain represented in lane b. As expected, both fragments are present in our polymorphic Oregon-R stock (lane c). In the course of constructing the two strains represented in Figure 7, a total of five different third chromosomes from our Oregon-R stock were examined, of which two contained the 68C3-5 element and three did not.

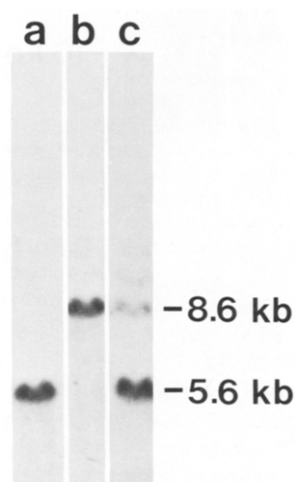


Figure 7. The 9.2 kb Element Is Inserted at 68C3-5 in Some Oregon-R Third Chromosomes But Not in Others

Fractionated Hind III digest of total adult DNA isolated from homozygous strains OR8a (lane a) and OR16f (lane b), defined in text, and from our Oregon-R stock (lane c). Electrophoresis was in 0.5% agarose gels, and the 5.6 kb Hind III fragment used as the probe (see text) was isolated from  $\lambda$ aDm1501-10 by subcloning in pBR322 (subclone aDm2003) and was  $^{32}$ P-labeled by nick translation (Experimental Procedures). The 0.55 kb hybridization band expected in lanes b and c (see text) would not be seen in this experiment because fragments of this length were lost during electrophoresis.

In situ hybridization of the two types of Oregon-R clones to polytene chromosomes shows that clones carrying DNA from the element, such as  $\lambda$ bDm2002, label approximately 100 sites spread over all five major chromosome arms, whereas clones lacking the element, such as  $\lambda$ aDm1501-10, hybridize only to the 68C locus. Hence the element contains dispersed repeated sequences.

Figure 8 indicates that this repetition results from dispersion of the entire element. Gel blots containing fragments from Bam HI, Hind III and Sal I digests of total Oregon-R DNA were probed with  $^{32}$ P-labeled DNA from a central 4 kb region of the element that is defined by the left end of the Dm2002 segment and by the first Sal I site to its right. This probe sequence is entirely contained within the 5.9 kb Sal I fragment of the element shown in Figure 3 (the "68C element"). If the element is largely conserved at the different chromosomal loci, the probe should reveal a major 5.9 kb hybridization band in a Sal I digest of total DNA; it does. That band has been overexposed in lane c to allow registration of the minor bands, which we ascribe to a small number of variant elements with Sal I site distributions different from the consensus sequence for this family of elements. By contrast, the probe sequence is not included within any Bam HI fragment contained in the 68C element, both Bam HI sites lying to the left of the probe (Figure 3). Conse-

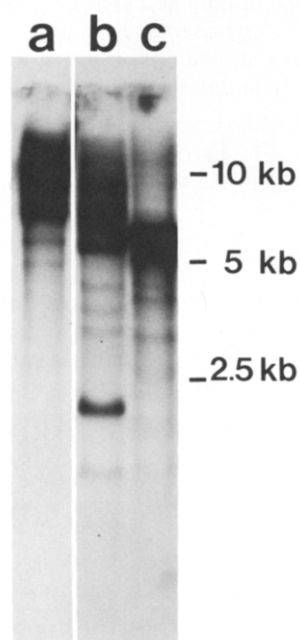


Figure 8. Conservation of the Repeated 9.2 kb Element

Oregon-R embryonic DNA (5  $\mu$ g) was digested with Bam HI (lane a), Hind III (lane b) and Sal I (lane c), fractionated by electrophoresis in a 0.7% agarose gel and transferred to a nitrocellulose filter (Southern, 1975). The hybridization probe was labeled by nick translation of a 4.8 kb Sal I fragment isolated from  $\lambda$ bDm2002 and extending leftward from the right-most Sal I site in the 9.2 kb element to the first Sal I site in the  $\lambda$ Sep6 vector DNA of this hybrid phage.

quently, the fragments in a Bam HI digest capable of hybridization with the probe should extend rightward from the right-most of these Bam HI sites, through 7 kb of the element, to the first Bam HI site in the DNA flanking the right end of the element at the different chromosomal loci. That is, they should form a heterogeneous set ranging in length from an undefined maximum down to a minimum or cut-off value of 7 kb; they do (lane a). The two minor bands representing lengths less than 7 kb are again ascribed to variant elements. The single Hind III site of the 68C element is located within the 4 kb probe sequence such that 90% of the probe lies to its right and 10% to its left. The larger right-hand fraction should hybridize with a heterogeneous set of fragments in the Hind III digest that is analogous to the set of hybridizing Bam HI fragments, except that the cut-off value should be 1.4 kb less, given that the Hind III site lies 1.4 kb to the right of the nearest Bam HI site (Figure 3). Such a set is indeed represented by the upper part of the hybridization pattern shown in lane b. The smaller left-hand fraction of the probe should hybridize to another set of Hind III fragments with a cut-off value just under 4 kb. This set is not observed; rather, the lower part of the hybridization pattern is characterized by a single major band at 2.3 kb. This suggests that the consensus sequence contains two Hind III sites: one corresponding to that in the 68C element and another located 2.3 kb to its left. Apparently, the 68C element is itself a variant in respect to the distribution of Hind III sites.

The well defined families of transposable elements in *D. melanogaster*, such as *copia*, exhibit all of the characteristics we have thus far described for the family defined by the 9.2 kb element: moderate repetition frequency, high but imperfect conservation at multiple chromosomal sites and presence at a particular site in some but not other strains (Finnegan et al., 1978; Potter et al., 1979; Strobel et al., 1979; Young, 1979). In addition, these transposable elements generally produce poly(A)<sup>+</sup> transcripts that are coextensive with the element. This also appears to be the case for this family, which we call *roo* (Milne, 1926). Thus, when poly(A)<sup>+</sup> and poly(A)<sup>-</sup> fractions of Oregon-R embryonic RNA were probed according to the method of Figure 1 with the 5.5 kb Hind III fragment subcloned from the 68C element in  $\lambda$ bDm2030 (Figure 3), a 9 kb RNA was detected in the poly(A)<sup>+</sup> but not in the poly(A)<sup>-</sup> fraction. An identical hybridization band was also observed when the probe consisted of the subcloned Hind III fragment lying immediately to the left of the 5.5 kb fragment in Dm2030, indicating that the 9 kb RNA contains sequences from both the left and right halves of the 68C element.

Curiously, no transcripts containing sequences homologous to the 68C element were detected by the above method in the RNA of intermolt salivary glands from Oregon-R strains homozygous for the presence

or absence of the 68C element. Apparently the roo elements are repressed in intermolt salivary glands, although we cannot say whether this repression applies to the element at the 68C3-5 puff site because we have no evidence that it can be transcribed under any condition. We also probed such RNA preparations with the cDNA clones II, III and IV and found no repeatable difference between the two homozygous strains in the amounts of RNAs II, III and IV that they produce. This observation that flanking sequences more than a few hundred base pairs to the left of the cluster region can be displaced 9.2 kb further to the left without affecting expression of genes II, III and IV suggests that these sequences are not required for that expression.

The parenthetic DNA lying to the right of the cluster region is also defined by a difference in the restriction maps generated by the Oregon-R and Canton-S clones and by its dispersed repetition. In this case, the Oregon-R clones define 16 kb of "right flank" DNA that is not repeated elsewhere in the genome, as judged by whole genome blots. The 13 kb of right flank DNA defined by the Canton-S clones mimics that in Oregon-R only for the first 8 kb, the remaining 5 kb (the parenthetic DNA) exhibiting a different restriction map and containing dispersed repeated sequences, again as determined by whole genome blots. We know less about this parenthetic DNA than that comprising the roo element. In particular, its full length has not been determined, and consequently we do not know whether it represents one or more than one repeated element. However, the characteristics of dispersed repetition and presence at a given chromosomal site in one but not another strain clearly make it a likely candidate for a transposable element from another family.

## Discussion

The chromosomal DNA defined by the segments cloned from the 68C3-5 loci present in two wild-type strains can be viewed as a 38 kb backbone of "stable" DNA that is common to all loci and into which blocks of "unstable" DNA have been inserted at some but not other loci. Thus our Oregon-R stock contains two kinds of loci: one with a backbone uncontaminated by unstable DNA and one whose backbone has suffered the insertion of a 9.2 kb element from the roo family of transposable elements. By contrast, none of the backbones in the Canton-S loci is uncontaminated by insertion; all appear to contain a block of at least 5 kb of dispersed repeated sequences, perhaps resulting from insertion of a transposable element from another family. All of the unstable DNA therefore consists of dispersed repeated sequences. While almost all of the stable backbone DNA consists of single-copy sequences, a small fraction does not fit this rule. The exception consists of the two 0.3 kb elements of

the inverted repeat at the center of the 38 kb backbone—a structure that is conserved among the 68C3-5 loci yet consists of elements from a small dispersed family.

The central region is also exceptional in that it contains the only DNA in the backbone that is transcribed to yield detectable RNAs in the intermolt salivary glands. Genes II, III and IV comprise that DNA and it is their transcription that appears to be responsible for the 68C puff and to provide the messenger RNA encoding the *sgs3* glue polypeptide; that at least is the strong indication of the following correlations. First, these genes and the center of the puff are both localized by *in situ* hybridization to the same polytene locus, and cytogenetic mapping places the structural gene for *sgs3* within a small region that includes this locus (Korge, 1975; Akam et al., 1978). Second, RNAs II, III and IV first appear just before 40% completion of third instar and disappear by the end of that period, while *sgs3* was first detected at approximately 45% completion of third instar (Korge, 1977a), and its rate of synthesis drops precipitously during the third instar–prepupal transition (Beckendorf and Kafatos, 1976); similarly, the 68C puff can be seen at the time puffs are first mapable (just before *sgs3* was first detected; Korge, 1977a), and regresses just before the end of third instar (Ashburner, 1967, 1973; Korge, 1977a). Third, both *sgs3* (Korge, 1977a) and RNAs II, III and IV are restricted to the salivary glands among the tissues of late third-instar larvae. Finally, the observation that the 68C puff is a major site of transcription (Bonner and Pardue, 1977; Bonner et al., 1977) correlates with the high abundance of *sgs3* (Beckendorf and Kafatos, 1976; Korge, 1977a) and of the polysomal RNAs II, III and IV in the intermolt glands.

Given that one of genes II, III and IV codes for *sgs3*, which is it? The apparent molecular weight of *sgs3* determined from its electrophoretic mobility in SDS–acrylamide gels is of limited use in this identification because its glycosylation is expected to result in anomalously low mobilities that inflate the apparent molecular weight (Beckendorf and Kafatos, 1976; Korge, 1977a). This is clearly the case for the glycosylated *sgs4*, where the apparent molecular weights determined by the above authors are three to four times that allowed by the 0.95 kb *sgs4* mRNA (Muskavitch and Hogness, 1980). The apparent molecular weight obtained for *sgs3* is somewhat greater than that for *sgs4*, and would be compatible with the 1.1 kb RNA IV if the inflation ratio is approximately the same as that for *sgs4*, but would be incompatible with the small RNAs II and III unless one is willing to consider inflation ratios of ten or more. In this regard, it should be noted that Beckendorf and Kafatos (1976) carried out pulse–chase experiments that indicated that the mobilities of *sgs3* and *sgs4* changed with time from early values yielding apparent molecular weights

in the  $40,000 \pm 10,000$  range to those yielding the threefold greater apparent molecular weights of the modified mature polypeptides. The early values were postulated to derive from nonglycosylated precursors, and their apparent molecular weights are directly compatible with the coding capacities of RNA IV and the *sgs4* mRNA, which are 40 and 35 kilodaltons (kd) of polypeptide, respectively. Again, the coding capacities of RNAs II (13 kd) and III (12 kd) are too small.

That RNA IV is equivalent to the *sgs3* mRNA is also suggested by our observation that the RNA IV obtained from the Hikone-R wild-type strain is 0.1 kb shorter than the 1.1 kb RNA IV from Oregon-R, since this correlates with the observation of Beckendorf and Kafatos (1976) that the apparent molecular weight of the *sgs3* from Hikone-R is less than that from Oregon-R. Different wild-type strains also yield *sgs4* mRNAs of different lengths. This has been shown to be due to variation in the number of tandemly repeated 21 base pair sequences found in the region of the *Sgs4* gene that codes for the amino-terminal half of the *sgs4* polypeptide (Muskavitch, 1980; Muskavitch and Hogness, 1980). A recent determination of the nucleotide sequence of gene IV (K. Burtis and D. S. Hogness, unpublished experiments) reveals that it contains tandemly repeated 15 base pair sequences that are similarly located within this gene, and which we suppose are similarly varied to account for the variation both in the lengths of RNA IV and in the apparent molecular weights of *sgs3* observed in different wild-type strains. Taken together, the above observations provide a strong basis for the conclusion that gene IV is equivalent to *Sgs3*, the structural gene for the *sgs3* glue polypeptide.

What then of genes II and III? The structure of this gene pair suggests that it may constitute a single regulated unit independent of and isolated from gene IV. Thus genes II and III are oppositely oriented so that their promoters are adjacent, and the pair is bounded by an inverted repeat of 0.3 kb elements (Figure 4). Both genes yield polysomal poly(A)<sup>+</sup> RNAs, and recent *in vitro* translation studies show that each of these RNAs encodes a small polypeptide (T. Crowley and E. M. Meyerowitz, unpublished experiments). What might be the functions of these polypeptides? One possibility derives from the observation of Gronemeyer and Pongs (1980) that the puff at 68C, but not that at 25AC, binds the steroid hormone ecdysone at the time when both of these intermolt puffs are regressing. Since the 25AC locus also harbors a glue polypeptide gene (Velissariou and Ashburner, 1980) and since both puffs regress when intermolt glands are exposed to ecdysone (Ashburner, 1973), this observation suggests that genes II and III may act at the top of a regulatory hierarchy triggered by ecdysone and serving to coordinate the expression of the glue polypeptide genes, or at least a subset of these genes.

A less intriguing but perhaps more likely possibility is that the polypeptides encoded by genes II and III are represented among the smaller glue polypeptides. Of these, the best candidate consists of the polypeptides (or polypeptide) in the P6 fraction of Beckendorf and Kafatos (1976), which do not appear to be glycosylated, exhibit an apparent molecular weight of 8,500 and are encoded by genes whose chromosomal loci are presently unknown. The notion that the gene II-III pair is differently regulated from gene IV would not necessarily be at odds with this possibility, since the developmental profile for the synthesis of the P6 fraction is somewhat different from that of *sgs3* (Beckendorf and Kafatos, 1976). While this possibility does not offer so nice an explanation of the differential binding of ecdysone to the intermolt puffs, the significance of these binding studies has not yet been well enough established to be determinative in choosing between the two possibilities. Clearly the most direct experimental approach toward effecting such a choice is to determine whether the glue polypeptides do or do not include those encoded by genes II and III. Experiments directed toward this determination are currently underway in the laboratory of E. Meyerowitz.

#### Experimental Procedures

##### Materials

Restriction endonucleases were purchased from New England Biolabs or Bethesda Research Labs. *E. coli* DNA polymerase I was a gift from S. Scherer, *E. coli* alkaline phosphatase (prepared from Worthington BAPF) was a gift from T.-S. Hsieh and S1 nuclease was a gift from J. Widom. DNAase I, T4 polynucleotide kinase, <sup>32</sup>P-labeled nucleoside triphosphates and <sup>3</sup>H-labeled nucleoside triphosphates were purchased from Worthington, P-L Biochemicals, Amersham and New England Nuclear. AMV reverse transcriptase was from a preparation by J. Beard (Life Sciences). The Oregon-R strain of flies was that kept at the Department of Biochemistry, Stanford University; Canton-S flies were from the Caltech Stock Center.

##### Nucleic Acid Preparations

Plasmid and total *D. melanogaster* DNAs were purified as described by Meyerowitz et al. (1980), except that in some cases the *D. melanogaster* DNA was obtained by a rapid procedure in which adult flies were first homogenized in a solution of 0.12 M sucrose, 0.075 M EDTA, 0.15 M Tris-HCl (pH 8.0), 0.75% SDS and 0.025% diethylpyrocarbonate; the SDS was subsequently precipitated by addition of potassium acetate to 0.1 M and the nucleic acid then isolated by equilibrium centrifugation in an isopycnic CsCl-ethidium bromide gradient, after which it was butanol-extracted, ethanol-precipitated and washed in 70% ethanol. This preparation yields an average of 170 ng of high molecular weight DNA per mg of flies.  $\lambda$  DNA was prepared either by the rapid procedure of Cameron et al. (1977) or as described in Thomas and Davis (1975).

Salivary gland RNA was obtained by hand dissecting third-instar larval salivary glands and vortexing the glands in equal volumes of phenol and 1% (w/v) Sarkosyl, 100 mM NaCl, 100 mM Tris-HCl (pH 8.5) and 20 mM EDTA. The aqueous phase was phenol-extracted several times further, and then ethanol-precipitated to obtain the nucleic acid. Embryonic RNA was prepared by a similar procedure using 0–19 hr embryos. Poly(A)<sup>+</sup> RNA was isolated by passing the total RNA through an oligo(dT)-cellulose (Collaborative Research T3) column in 0.5 M NaCl and 10 mM Tris-HCl (pH 8.0), washing with several column vol of 0.25 M NaCl, 10 mM Tris-HCl (pH 8.0) and then eluting the bound poly(A)<sup>+</sup> RNA with water. Salivary gland

polysomes were isolated from hand-dissected glands as described by McKenzie et al. (1975), with minor modifications.

#### Gel Electrophoresis, Restriction and Heteroduplex Mapping

Electrophoresis of nucleic acids in agarose gels was carried out as described by Meyerowitz et al. (1980), with use of the same length standards that they employed. Acrylamide gels were run in Tris-borate EDTA buffer (Peacock and Dingman, 1968), with use of restriction fragments of pBR322 (Sutcliffe, 1978) or Col E1 as length standards. Restriction maps were derived by a combination of complete, double and partial digests followed by gel electrophoresis of the resulting fragments. Heteroduplex mapping was carried out as described by Lis et al. (1978).

#### DNA Labeling and Hybridization

DNA was radioactively labeled by nick translation (Rigby et al., 1977) or with polynucleotide kinase as described by Meyerowitz et al. (1980). <sup>32</sup>P-labeled cDNA to poly(A)<sup>+</sup> RNA was made as described by Lis et al. (1978). Hybridization of labeled DNA to recombinant colonies (Grunstein and Hogness, 1975) and to recombinant  $\lambda$  plaques (Benton and Davis, 1977) was carried out as described in the indicated references. DNA blots (Southern, 1975) were hybridized as described by Meyerowitz et al. (1980), and the preparation and hybridization of RNA blots followed the glyoxal gel procedure of Alwine et al. (1980). Quantitative genome blot hybridizations were performed as described by Lis et al. (1978). In situ hybridization to polytene chromosomes was performed by using <sup>3</sup>H-labeled DNA probes under the conditions described in Lis et al. (1978).

#### Cloned Dm Segments and cDNAs

Libraries of  $\lambda$ dDm and  $\lambda$ bDm clones were prepared by inserting Dm segments obtained from sheared Oregon-R embryonic DNA into  $\lambda$ 647 (Murray et al., 1977) and into  $\lambda$ Sep6 (constructed by E. M. Meyerowitz and D. Kemp; defined in Davis et al., 1980), respectively, with (dA)<sub>n</sub>:(dT)<sub>n</sub> or (dG)<sub>n</sub>:(dC)<sub>n</sub> joints (Wensink et al., 1974). The library of  $\lambda$ cDm clones was prepared by Maniatis et al. (1978) and consists of sheared Canton-S embryonic DNA inserted into  $\lambda$ Charon4 by Eco RI linkers.

The cDNA clones originally used to represent the three homologous sequence groups of *intermolt* cDNAs from 68C are p<sub>kdm</sub>2G6 (group II), p<sub>kdm</sub>2C7 (group III) and p<sub>kdm</sub>1H2 (group IV; Wolfner, 1980; Muskavitch, 1980). These were used as probes to isolate  $\lambda$ dDm1501-10 (Figure 3) and to identify restriction fragments from this clone containing sequences homologous to each cDNA clone. These restriction fragments were then used as probes to screen a library of cDNAs for additional clones belonging to groups II, III and IV. This library was a gift from G. Guild and M. Wolfner and was constructed by insertion of cDNA segments synthesized from the poly(A)<sup>+</sup> RNA of Oregon-R third instar salivary glands into the pBR322 plasmid at its Pst I site with (dG)<sub>n</sub>:(dC)<sub>n</sub> joints (Wolfner, 1980). Three clones belonging to group IV were obtained; each had a Pst I-excisable cDNA insert 0.67 kb long, and one of these, adm124E8, is the reference clone IV. Of the five clones belonging to group III, four had Pst I-excisable cDNA inserts, each of which was approximately 0.3 kb long; one of these four, adm127C8, is the reference clone III. Both group II clones that were obtained had Pst I-excisable cDNA inserts, one of which, with a 0.37 kb insert, is adm109F4, the reference clone II. Hybrid plasmids or phage were propagated in EK1 host-vector systems under P2 containment conditions, as defined in the National Institutes of Health guidelines.

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