Molecular analysis of the ecdysterone-inducible 2B5 'early' puff in *Drosophila melanogaster*

Anna T.Chao and Gregory M.Guild

Department of Biology/G5, University of Pennsylvania, Philadelphia, PA 19104, USA

Communicated by M.Ashburner

The 2B5 region of the X-chromosome in *Drosophila melanogaster* plays a developmentally important role in the ecdysterone-triggered response of the late third instar salivary gland. Using a combination of transposon-tagging and chromosomal walking techniques, we have isolated 231 kb of contiguous genomic DNA sequences corresponding to this region. We have more precisely aligned this DNA to the 2B1,2 to 2B5-6 interval of the cytogenetic map by locating the position of three well-characterized chromosomal breakpoints by *in situ* hybridization and genomic DNA blotting experiments. Labeled cDNA, synthesized from poly(A)⁺ RNA isolated from hormone-induced salivary gland and imaginal disc tissues and hybridized to the cloned DNA, demonstrated that the ecdysterone-inducible sequences mapped to DNA segments corresponding to the 2B3,4 to 2B5-6 interval. Although some of these sequences were inducible in only one tissue type, many were found to be inducible in both salivary glands and imaginal discs. RNA blotting experiments have detected a major 4.5-kb RNA which is hormone inducible in the larval salivary gland and whose quantitative induction is not inhibited by cycloheximide. Thus, the 4.5-kb RNA represents at least one product from the ecdysterone-responsive 2B5 'early' puff.

Key words: *Drosophila*/salivary gland/ecdyserone/2B5 puff/transcription

Introduction

Sequential gene activation in *Drosophila melanogaster* can be observed by monitoring polytene chromosome puffing in the larval salivary gland genome. Coordinately regulated puffs reflect the developmentally specific and temporally coordinated expression of gene sets. Careful analysis of puffing patterns displayed in developing third instar larval salivary glands indicates that multiple gene sets are sequentially activated in a program of gene expression controlled, in part, by the steroid molting hormone ecdysterone (Ashburner and Berendes, 1978). The 'intermolt' puff set becomes active by the mid-third instar period and encodes some of the salivary gland glue proteins (Muskavitch and Hogness, 1980; Meyerowitz and Hogness, 1982; Guild and Shore, 1984). An increase in the hemolymph titer of ecdysterone is associated with their regression and a concomitant induction of the 'early' puff set. The 'early' puffs are active for 4 h and then regress. In concert with 'early' puff regression, the 'late' puff set becomes active. The products of the 'early' and 'late' genes are not known (Ashburner and Berendes, 1978).

The interaction between these three puff sets has been studied by culturing third instar larval salivary glands *in vitro* and by genetic analyses. These studies indicate that the 'early' puffs play

a key role in the regulation of all three puff sets. 'Early' puffs exhibit a rapid and dosage-dependent response to ecdysterone that is independent of protein synthesis since the 'early' puffing pattern is induced normally in cultures treated with the inhibitor cycloheximide. In contrast, the 'late' puffs respond in an all-or-none fashion to ecdysterone and are not inducible in the presence of cycloheximide (Ashburner, 1973, 1974). Ashburner's sequential puffing model postulates that the 'early' genes respond to the hormone signal in a primary way to produce at least one gene product that subsequently activates the 'late' puff set and represses the 'early' puff set in an autoregulatory manner (Ashburner *et al.*, 1973). An analysis of the puffing pattern in aneuploids carrying one to three copies of a chromosomal region containing the 74EF and 75B 'early' puffs supports these ideas (Walker and Ashburner, 1981).

Genetic analyses of mutations in the 2B5 'early' puff provide further evidence for the regulatory role of 'early' genes. Careful electron microscopic analysis of chromosomal rearrangements that break within the 2B1,2 to 2B9,10 cytogenetic interval identifies the 2B5 band as the focal point for an ecdysterone-inducible puff (Belyaeva *et al.*, 1980). This region includes the complementation groups *br* (*broad*), *rbp* (*reduced bristle number on palpus*), *1(1)pp1* and *1(1)pp2*. These four complementation groups are known collectively as the 'overlapping complementation complex' or *occ* because some mutations do not complement any of the four groups in the complex (Belyaeva *et al.*, 1980). When salivary glands of homozygous and hemizygous *occ* mutants are examined for their response to ecdysterone, some 'intermolt' puffs fail to regress, other 'early' puffs fail to be maximally induced and some 'late' puff sites are not induced at all. Thus, the 2B5 region disrupted by these mutations appears to be necessary for the normal ecdysterone-triggered puffing sequence (Belyaeva *et al.*, 1981; Zhimulev *et al.*, 1982). Molecular studies on the 68C 'intermolt' gene cluster also implicate the 2B5 *occ* gene product as an important regulator for glue gene expression (Crowley *et al.*, 1984).

Several *Drosophila* primary ecdysterone-responding genes have been isolated and studied at the molecular level (Moritz *et al.*, 1984; Savakis *et al.*, 1984; K.Burtis, W.Jones, W.Segraves, C.Thummel and D.Hogness, personal communication). In this paper we report on our molecular analysis of the 2B5 'early' puff.

Results

Molecular cloning of the 2B5 region

The method of transposon-tagging (Bingham *et al.*, 1981) has been widely used to obtain cloned DNA fragments adjacent to transposable elements. We used the repetitive element *gypsy* (Bender *et al.*, 1983b; Modolell *et al.*, 1983) to enter molecularly the 2B region. *In situ* hybridization of a cloned *gypsy* element to salivary gland polytene chromosome spreads showed that *gypsy* sequences are present in the 2B region of the *gt^{1-wa}* genome but are absent from this region in the Oregon-R (Ore-R) genome (W.Bender, personal communication; and unpublished data). We

Fig. 1. Composite restriction map of the 2B chromosomal walk. The restriction sites generated by the enzymes *EcoRI* (R), *HindIII* (H) and *SalI* (S) for the *D. melanogaster* (Canton-S strain) wild-type DNA are shown. The map is oriented left to right, telomere to centromere. A coordinate scale in kb below the restriction map has been set with zero as the most telomeric sequence. The order of the *HindIII* and *EcoRI* sites for the 0.1-kb fragments located at coordinates 205 and 216 is uncertain. The chromosomal walk was initiated near coordinate 35 by utilizing a *gr¹wa* genomic clone that contains sequences from the repetitive element *gypsy* (see text for details). Inserts of the overlapping Canton-S/bacteriophage λ Charon 4 clones and two Oregon-R/MUA3 cosmid clones (Dm340 and Dm341) collected during the walk are shown in the array below the composite restriction map. Thin lines connecting restriction sites above the coordinate scale starting at coordinates 62.5, 74.9, 83.1, 131.4, 187.1, 191.9 and 218.3 represent regions that contain repetitive sequences. Bold lines above the map represent the regions where the 2B1,2 $\pm$, 2B3,4/5 and 2B5/6 breakpoints in the stocks Dp(1,f)101, T(1,3)*sta*/FM3 and Df(1)S39/FM6 respectively, have been localized (see text for details).

Fig. 2. *In situ* hybridization of polytene chromosome spreads from (a) Dp(1,f)101 probed with Dm159; (b) Df(1)*sta*/Df(1)*sta*; Dp(1,3)*sta*/Dp(1,3)*sta* probed with Dm340; and (c) Df(1)S39/FM6 probed with Dm207. Thin arrows point to the control region of hybridization over the (a) wild-type X-chromosome, (b) translocated region of X-chromosome or (c) FM6 balancer chromosome. Bold arrows point to the new region of hybridization detected by the probes, indicating that these clones contain DNA spanning the chromosomal breakpoint of each stock. Autoradiographic exposure times were for (a) 25 days, (b) 3 days and (c) 5 days.

constructed a genomic DNA library from the *gr¹wa* stock and screened it with a cloned *gypsy* probe. *Gypsy*-positive clones were isolated and rescreened by *in situ* hybridization to Ore-R polytene chromosome spreads. Since the Ore-R genome does not contain any *gypsy* sequences at 2B, any *gypsy*-positive clone that hybridized to the Ore-R 2B region must contain *gypsy* DNA flanked by 2B DNA sequences. One clone was identified and its non-*gypsy* containing restriction fragments were subsequently used to initiate a chromosomal walk in a *D. melanogaster* genomic DNA

library prepared from the Canton-S stock. To expedite the collecting of overlapping genomic clones, we also probed an Ore-R cosmid library.

Approximately 231 contiguous kilobases of genomic DNA has been isolated as genomic DNA clones and mapped using the restriction enzymes *EcoRI*, *HindIII* and *SalI* (Figure 1). All of the clones have been screened for the presence of repetitive sequences either by using them as probes for whole genomic DNA blot analyses or by probing them with nick-translated genomic

Fig. 3. Whole genome DNA blot analyses. 2 μ g of DNA derived from (a) Oregon-R, males and females (Ore-R), or Dp(1,f)101, males and females, (101); (b) Oregon-R, males and females, (Ore-R), or Df(1)sta/FM3; Dp(1,3)sta/Dp(1,3)sta females (sta/FM3), or Df(1)sta; Dp(1,3)sta/Dp(1,3)sta males (sta); (c) Oregon-R, males and females, (Ore-R), or FM6 males (FM6), or Df(1)S39/FM6 females (S39/FM6) were digested with the enzymes (a) *SalI*, (b) *SalI* and (c) *HindIII*, respectively, size fractionated through a 0.7% (w/v) agarose gel and blotted to nitrocellulose. These filters were probed by hybridization with nick-translated (a) Dm156, (b) an isolated 7.1-kb fragment (Figure 1, coordinates 94.6–101.7) or (c) an isolated 3.8-kb fragment (Figure 1, coordinates 217–220.8). Marks to the right of each blot represent the positions of bacteriophage λ DNA length standards (kb) produced by *HindIII* plus *EcoRI* double digestion. Autoradiographic exposures were for (a) 4 days, (b) 8 h and (c) 4 days.

DNA using a 'reverse Southern' technique (Hall *et al.*, 1983). Seven regions contain repetitive sequences and their locations are indicated in Figure 1. The identities and sizes of the repetitive sequences within these restriction fragments have not been investigated further.

Cytogenetic alignment to the DNA map

We used several precisely mapped chromosomes containing rearrangements in the 2B region to align the cloned 231-kb DNA with the cytogenetic map. The stock Dp(1,f)101 includes a free duplication which contains the tip of the X-chromosome with a proximal breakpoint near the 2B1,2 band (Belyaeva *et al.*, 1982). Cloned DNA segments were used to probe this chromosome to localize the 2B1,2 breakpoint. *In situ* hybridizations of the cloned segment Dm159 to polytene chromosome spreads of this stock revealed two areas of hybridization. One intense area of

hybridization was localized over the 2B region on the X-chromosome, and the second lighter area of hybridization was found on the free duplication (Figure 2a). The same two hybridization sites were also seen using Dm156 and Dm153. When Dm160 and Dm168 were used, silver grains were detected only over the normal X-chromosome and not on the free duplication. These patterns of hybridization suggest that Dm159 contains DNA sequences which are located proximal to the 2B1,2 breakpoint in Dp(1,f)101 and that the breakpoint must lie within Dm159. In contrast, Dm160 and Dm168 consist of DNA sequences that are not contained on the free duplication.

This interpretation was confirmed by probing genomic DNA blots derived from stocks carrying only a wild-type X-chromosome (Ore-R) or a wild-type X-chromosome plus the free duplication [Dp(1,f)101]. These genomic DNA blots were probed with clones representing 122 kb of the genomic walk (Figure 1, coordinates 2–68 and 99–155). When Dm156 was used to probe a *SalI* digest of the genomic DNA blots, a new 6.3-kb restriction fragment was observed in the DNA from the free duplication stock which was absent from Ore-R DNA and not similar in length to any wild-type fragment covered by Dm156 (Figure 3a). It is unlikely that the fragment reflects a restriction site polymorphism between the two stocks since new fragments were also observed when other restriction enzymes were used. An *EcoRI* digest of the genomic DNA probed with Dm156 showed a 2.3-kb restriction fragment in the free duplication stock which was not found in Ore-R. The same genomic DNA blot probed with a combination of Dm159 and Dm160 also showed a 2.3-kb restriction fragment present only in the Dp(1,f)101 DNA. The 6.3- and 2.3-kb fragments were the only differences observed between Dp(1,f)101 and Ore-R DNA, suggesting that these sequences were from the free duplication and homologous to DNA contained in Dm156 and Dm159 or Dm160. The genomic blotting data and the *in situ* hybridization results are consistent with the idea that the 2B1,2 breakpoint for Dp(1,f)101 is in the region of Dm159 that is overlapped by Dm156 and extends to the part of Dm159 that is not contained in Dm160. The location of this breakpoint is probably restricted to a 4-kb region between coordinates 31.3 and 35.3 on the composite DNA restriction map (Figure 1).

The stock T(1,3)sta/FM3 is a combination of the stocks Dp(1,3)sta and Df(1)sta (Lindsley and Grell, 1968) and hence consists of two chromosomal rearrangements. Df(1)sta is a deletion for the region between 1E1,2 and 2B3,4 or the interband between 2B3,4 and 2B5 (Belyaeva *et al.*, 1980, 1982). Since this deficiency is lethal, the stock T(1,3)sta/FM3 contains a translocation [Dp(1,3)sta] of this region to the third chromosome, and is maintained over the X-chromosome balancer FM3. When the cosmid clone Dm340 was used to probe T(1,3)sta/FM3 salivary gland chromosome spreads homozygous for the Df(1)sta chromosome, two major areas of hybridization were observed. One hybridization site corresponds to the 2B region remaining on the Df(1)sta X-chromosome and the second area corresponds to the 2B region translocated to the 89B/C interval on the third chromosome of the Dp(1,3)sta chromosome (Figure 2b). *In situ* hybridizations with Dm168 showed silver grains only over the X-chromosome region translocated to the third chromosome, whereas Dm174 showed the same dual hybridization results seen with Dm340. These results suggest that the DNA sequences spanning the deficiency X-chromosome proximal breakpoint in T(1,3)sta/FM3 map between Dm168 through Dm174, excluding those sequences within Dm168 (i.e., coordinates 78–106.4).

Whole genome DNA blots from Ore-R, Df(1)sta/FM3 and

Df(1)*sta* males were probed with an isolated 7.1-kb restriction fragment located between coordinates 94.6 and 101.7. The resulting autoradiogram revealed that the hemizygous Df(1)*sta* DNA lacks two adjacent *SalI* fragments (2.1 and 0.75 kb; found between coordinates 96.8 and 99.6) when compared with the Ore-R and heterozygous Df(1)*sta*/FM3 DNA. However, the adjacent 2.3-kb restriction fragment (coordinates 99.6–101.9) is present in all lanes (Figure 3b). Both female and male adults from the T(1,3)*sta*/FM3 stock also showed three new restriction fragments (4.3, 1.4 and 1.0 kb) not found in the wild-type Ore-R DNA. These new fragments could represent polymorphisms between the stocks or fusion fragments generated by the deficiency and translocation chromosomal rearrangements. Since whole genome blot analyses cannot distinguish between the deficiency X-chromosome breakpoint and the X-to-third chromosome fusion generated by the translocation, it is impossible to interpret these results to the restriction fragment level. However, these results strongly suggest that the Df(1)*sta* breakpoint in the 2B3,4 band (or within the 2B3,4 to 2B5 interband) is located in the 7.1-kb probe fragment and probably lies within the region bounded by the *SalI* sites at coordinates 98.8 and 101.9 (Figure 1). As an aside, DNA in this region may constitute part of the *sta* gene since this breakpoint is associated with the stubarista phenotype.

The stock Df(1)S39/FM6 contains a deletion spanning from 1E1,2 to the 2B5 band or the interband between 2B5 and 2B6 [Df(1)S39] carried over the FM6 balancer. This deficiency removes the 2B5 band and does not puff in response to ecdysterone (Belyaeva *et al.*, 1980, 1982). When Dm207 was used as a probe for *in situ* hybridization to Df(1)S39/FM6 polytene chromosome spreads, two areas of hybridization were observed. One corresponds to the 2B region on the FM6 balancer chromosome and the second corresponds to the 2B region on the deficiency X-chromosome (Figure 2c). Hybridization over the Df(1)S39 X-chromosome suggested that Dm207 contained DNA sequences that extend proximally over the 2B5 to 2B6 breakpoint. *In situ* hybridizations using Dm208 show the same result confirming that these DNA sequences also extend beyond the 2B5 to 2B6 breakpoint.

When a 3.8-kb *EcoRI* fragment in Dm207 (coordinates 217–220.8) was used to probe whole genome DNA blots from Ore-R, FM6 males and Df(1)S39/FM6 females, a 15-kb fragment was seen in all lanes. A novel 22-kb restriction fragment was also detected in Df(1)S39/FM6 female DNA that was absent in FM6 balancer and Ore-R DNA (Figure 3c). This was the only new restriction fragment found among the three different genotypes despite probing with > 198 kb of DNA (coordinates 2–68 and 99–231) and probably represents those DNA sequences spanning the 2B5/6 breakpoint on the Df(1)S39 chromosome. Hence this breakpoint may be placed within the 3.8-kb segment between coordinates 217 and 220.8 on the composite DNA restriction map.

Ecdysterone-regulated transcription

To assay for hormone-regulated transcriptional activity in the cloned 231-kb region, we isolated RNA from Ore-R third instar salivary glands cultured (2 h) in the absence (control) or continuous presence (induced) of 4×10^{-6} M ecdysterone. Chromosome squashes were made to evaluate the puffing status of the glands at the end of the culture period. Typically, the control glands remained at the 'intermolt' puff stage (PS1) while induced glands developed to an 'early' puff stage (PS3–4) [see Ashburner and Berendes (1978) for a discussion of salivary gland

puff stages]. Poly(A)⁺ RNA was isolated from the glands, converted into labeled cDNA, and used to probe DNA blots containing restriction digests of genomic clones that encompassed the entire chromosomal walk. A portion of these blots is shown in Figure 4. Dm184, Dm192, Dm193 and Dm202 all showed bands of hybridization when probed with the induced cDNA, with Dm193 and Dm202 having particularly strong hybridization signals (Figure 4a). These same regions also hybridized to sequences in the control RNA population (Figure 4b) but at a much reduced level. The differential intensity of hybridization exhibited by the two cDNA populations suggests that there exist regions of this DNA which are homologous to ecdysterone-inducible RNA sequences.

These experiments were repeated using RNA derived from mass isolated imaginal discs which had been cultured *in vitro* in the presence or absence of hormone (kindly provided by J.Natzle and J.Fristrom). A portion of these blots is shown in Figure 4 (c and d). The results demonstrate the presence of long stretches of DNA homologous to both RNA populations, with the intensity of hybridization being stronger for the induced cDNA probe. This difference is even greater when it is noted that the autoradiographic exposure time of the blot probed with control cDNA is 5.7 times longer than that for the blot probed with induced cDNA. Dm191 also showed a strong hybridization signal for this imaginal disc cDNA whereas it did not hybridize to the salivary gland cDNA.

When the map positions of the hormone-inducible RNAs were determined (Figure 4e), some interesting observations were made. First, most of the hormone-inducible RNA sequences map to the 2B5 region (i.e., between the 2B3,4/5 and 2B5/6 breakpoints). Second, while many regions are complementary to RNA sequences inducible in both salivary glands and imaginal discs (e.g., coordinates 144–156) other regions are complementary to sequences inducible only in salivary glands (e.g., coordinates 110–112.7) or imaginal discs (e.g., coordinates 163–165.2). Third, some regions appear transcriptionally quiet since they are not complementary (within the limits of this detection) to any cDNA probe (e.g., coordinates 172.2–175.8).

To understand better the transcriptional nature of this region, poly(A)⁺ RNA blots were made from induced and control salivary gland cultures and probed individually with Dm191, Dm190, Dm192, Dm193 and Dm202. No hybridization was observed with Dm190. Dm191 detected a single RNA transcript (estimated to be > 10 kb) in the induced salivary glands, with the signal for this RNA being very weak. However, since Dm191 contains some repetitive sequences, this large RNA may not be unique to the 2B region. RNA blots probed with Dm192, Dm193 and Dm202 all yielded similar results, namely, a single 4.5-kb RNA was seen in the induced lane and only slightly visible in the control lane. The filter was reprobed with an actin cDNA clone to ensure that the difference in hybridization intensity was not due to an RNA loading artifact.

'Early' puff sites respond to ecdysterone and remain puffed in the presence of the protein synthesis inhibitor cycloheximide (Ashburner, 1974). To determine how closely the 4.5-kb RNA parallels the activity of an 'early' puff, Ore-R salivary glands were dissected and cultured in the presence of: (i) no additions; (ii) ecdysterone alone; (iii) cycloheximide alone; or (iv) ecdysterone plus cycloheximide. Each culture was maintained for 2, 4 and 8 h. A poly(A)⁺ RNA blot derived from these cultured glands was probed with Dm339 (a single-stranded DNA subclone of the 1.5-kb *EcoRI* fragment from coordinates 165.4 to 166.9). The results show that Dm339 is complementary to at least six

Fig. 4. Hybridization of labeled cDNA to cloned DNA. (a and b) Dm184, Dm191, Dm190, Dm192, Dm193, Dm202, a cloned genomic DNA segment from the 74EF 'early' puff (74EF), an *hsp70* heat-shock, cDNA clone (h.s.), and an actin cDNA clone (actin); or (c and d) Dm191, Dm190, Dm192 and Dm193, were digested with *Hind*III and *Eco*RI, size fractionated through 0.7% (w/v) agarose gels and blotted to nitrocellulose. These filters were probed with ^{32}P -labeled cDNA synthesized from random oligonucleotide-primed poly(A)⁺ RNA extracted from 2 h *in vitro* cultures of (a) salivary glands with 4×10^{-6} M ecdysterone (Rhoto Pharmaceuticals) added, (b) salivary glands with no hormone added, (c) imaginal discs with 2×10^{-6} M ecdysterone added or (d) imaginal discs with no hormone added. Comparison of the actin lane (which contained partial digestion products) for both the induced and control cDNA probes showed approximately the same level of hybridization for each, demonstrating that there was little difference in the actin RNA content in the RNA populations. The lack of any strong hybridization to the heat shock DNA demonstrated that there was little heat shocking of the salivary glands during the culture. The positive control clone 74EF also showed stronger hybridization to the induced cDNA probe than to the control cDNA probe, as expected (a and b). These same clones (actin, h.s. and 74EF) were also used with similar results to evaluate the imaginal disc cultures (not shown). Autoradiographic exposure times were for (a) 4 days, (b) 4 days, (c) 3 days and (d) 17 days. (e) Composite restriction map of the chromosomal walk summarizing the positions of ecdysterone-inducible restriction fragments for both salivary glands (solid lines) and imaginal discs (dotted lines) determined by cDNA hybridizations. Both oligonucleotide-primed and oligo(dT)-primed (not shown) cDNA populations were used with similar results. The entire 231 kb was probed with the salivary gland-derived cDNA. Imaginal disc cDNA was used to probe cloned DNA representing coordinates 0–186 but not from 186 to 231 (n.d.).

Fig. 5. Analysis of hormone and cycloheximide-treated salivary gland RNA. 0.85 μ g of poly(A)⁺ RNA was isolated from salivary glands cultured *in vitro* in the presence (I) or in the absence (C) of 4×10^{-6} M ecdysterone and in the presence (+cx) or in the absence of 7×10^{-5} M cycloheximide (Sigma) for 2, 4 and 8 h. The RNA was size fractionated through a 0.7% (w/v) formaldehyde-agarose gel and blotted to nitrocellulose. The filter was hybridized using a two-step procedure involving an initial probing with single-stranded M13mp19 recombinant viral DNA from (a) Dm339 (coordinates 165.4–166.9, Figure 1), or (b) Dm378 (coordinates 187.1–190.5, Figure 1) followed by hybridization with nick-translated double-stranded M13 RF DNA. Exposure time for both autoradiograms was 5 days. Marks at the side of each blot indicate the positions of the single-stranded length standards (kb) derived from bacteriophage λ DNA digested with *Hind*III and pBR322 DNA digested with *Eco*RI and *Pst*I and with *Taq*I. Bacteriophage λ DNA digested with *Eco*RI was also included as a marker (not shown).

different RNA species which were 12.0, 8.8, 6.9, 4.5, 4.2 and 1.2 kb in length, with hybridization to the 4.5-kb RNA being the most intense (Figure 5a). Comparison of the cultures with and without cycloheximide showed that although the drug appeared to induce or stabilize all of the RNAs, it did not inhibit the ecdysterone induction of the 8.8-, 6.9-, 4.5- and 4.2-kb RNA. Cycloheximide also stabilized the accumulation of these RNA species in long-term culture (without hormone) in that they were all still present in the 8-h cultures with the drug and only barely detectable in the 8-h cultures without the drug. When this RNA blot was reprobed with Dm378 (a single-stranded subclone from the 3.4-kb *Eco*RI fragment from coordinates 187.1 to 190.5), multiple RNAs homologous to this probe were also found. The most prominent hybridization bands measured 9.5, 8.8, 7.5, 6.4, 4.5 and 4.4 kb (Figure 5b). However, Dm378 contains a repetitive sequence and so these RNA species may not reflect transcription unique to this region. The 4.5-kb RNA homologous to Dm378 is the most abundant and displays a clear ecdysterone-inducible phenotype. It also appears to accumulate more stably in the presence of cycloheximide and its hormone induction is not inhibited by the drug. These characteristics of the 4.5-kb RNA detected by both Dm339 and Dm378 (which are derived from the same DNA strand) mimic the failure of 'early' puffs to regress in the presence of cycloheximide.

Discussion

In this paper, we report on the cloning of 231 kb of contiguous genomic DNA from the X-chromosome of *D. melanogaster*.

Using a combination of *in situ* hybridization and whole genomic DNA blotting experiments, we have aligned this cloned section with the cytogenetic region 2B1,2 through 2B5. Evidence that this region contains the ecdysterone-inducible 2B5 puff is based on the proximal breakpoints for two chromosomal deficiencies. Cytogenetic analyses have shown that the Df(1)sta (proximal breakpoint coordinates 94.6–101.7, Figure 1) removes the 2B1,2 band and at least a portion of the 2B3,4 band but retains the 2B5 ecdysterone-inducible puff site (Belyaeva *et al.*, 1980, 1982). Df(1)S39 (proximal breakpoint coordinates 217–220.8) removes the 2B5 band and possibly some of the interband region between 2B5 and 2B6 as well as the ecdysterone-inducible puff (Belyaeva *et al.*, 1980, 1982). These results allow us to position those sequences necessary for the 2B5 'early' puff between coordinates 94.6 and 220.8 on the DNA map (Figure 1). However, the DNA sequences important for the hormonal response of the region may extend beyond the 2B5 to 2B6 interval and hence our collection of clones may not be all encompassing. We believe that the cytogenetic coincidence of the 'early' puff and the cloned region indicates that at least some (and probably the majority) of the 'early' puff sequences have been cloned.

The *occ* locus also coincides with this region on the X-chromosome, with a left end point defined by the proximal breakpoint in Df(1)sta since this chromosome is able to complement *occ* mutants. Although Df(1)S39 does not complement *occ* mutations (Aizenon and Belyaeva, 1982; Belyaeva *et al.*, 1982), assigning this break to the right end point of the *occ* locus may be misleading because the deficiency may disrupt an important

regulatory and/or coding region and not necessarily remove all *occ* DNA sequences. Since both the Df(1)*sta* and Df(1)S39 proximal breakpoints have been localized on our 231-kb restriction map, we believe that at least some (if not the majority) of the *occ* DNA sequences are also contained within the cloned region. Given that other ecdysterone-inducible genes in *Drosophila* span large stretches of DNA (K.Burtis, W.Jones, W.Segraves, C.Thummel and D.Hogness, personal communication), it is reasonable to hypothesize that *occ* occupies a considerable portion of the long genomic region (126.2 kb) defined by the Df(1)*sta* and Df(1)S39 proximal breakpoints.

The transcriptional profile for the region appears to be quite complex. Labeled cDNA experiments have shown that ecdysterone inducible-sequences for both the salivary gland and imaginal disc tissues map to DNA segments (defined by restriction enzymes) clustered in the same 126.2-kb region also occupied by the 2B5 puff and *occ* locus. Some of these segments are complementary to inducible sequences from both tissues. It remains to be seen whether the same RNA transcripts are produced or whether there exist alternative splicing pathways in the different tissues. The existence of segments complementary to salivary gland- or imaginal disc-specific probes suggests tissue-specific transcriptional control or RNA processing in this region. The existence of segments not homologous to RNA from either tissue type supports the idea of multiple transcriptional units and/or RNA splicing patterns with long introns.

The most abundant transcript we have detected is a 4.5-kb ecdysterone-inducible RNA present in the larval salivary gland. Its induction is similar to other primary ecdysterone-inducible transcripts that exhibit a dramatic accumulation after hormone stimulation (Savakis *et al.*, 1984). The induction is rapid, occurs within 2 h of culture *in vitro* and is not inhibited by cycloheximide. These results closely parallel 'early' puffing data (Ashburner, 1974), as well as molecular data from the 74EF and 75B 'early' genes (K.Burtis, W.Jones, W.Segraves, C.Thummel and D.Hogness, personal communication). It seems likely that this RNA species is derived from a relatively long primary transcript since it can be detected using two subcloned probes of the same polarity which map 20 kb apart. Alternatively, since the set of transcripts homologous to each probe do not completely overlap, each probe may recognize a different RNA member within a 4.5-kb inducible transcript family.

Since the 4.5-kb ecdysterone-inducible transcript also maps to the DNA region occupied by the 2B5 early puff and *occ*, it can be considered a candidate for the perhaps multifunctional 2B5 'early' gene product and also for at least one of the functions encoded by the *occ* locus. By performing complementation mapping using P-element-mediated transformation (Spradling and Rubin, 1982; Rubin and Spradling, 1982), it may be possible to determine the genetics of the 4.5-kb RNA. The cloned DNA also provides the opportunity to investigate *occ* mutants to uncover further details concerning the 2B5 ecdysterone-inducible region and to establish the molecular relationship between the 'early' puff and *occ* locus.

Materials and methods

Fly stocks

D. melanogaster stocks were obtained from the Department of Biochemistry, Stanford University School of Medicine (Ore-R); the Mid-America *Drosophila* Stock Center, Bowling Green [Dp(1,f)101]; and the California Institute of Technology Stock Center [T(1,3)*sta*/FM3 and Df(1)S39/FM6]. The *gr^{1w}* stock was kindly provided by W.Bender (Harvard Medical School). Mutations and balancer chromosomes are described in Lindsley and Grell (1968).

Cloned DNA

A cloned genomic DNA segment from the bithorax-complex (*Dmbr^{34c-6a2}*) containing a *gypsy* element (Bender *et al.*, 1983b), a cloned genomic DNA segment from the 74EF 'early' puff (K.Burtis, W.Jones and D.Hogness, unpublished), and the *hsp70* heat-shock cDNA clone adm87A+C.1 (Lis *et al.*, 1981) were generously provided by W.Bender, K.Burtis (Stanford Medical School) and J.Lis (Cornell University), respectively. The actin cDNA clone adm108D11 (Wolfner, 1980), the Canton-S/bacteriophage λ Charon 4 genomic library (Maniatis *et al.*, 1978) and Oregon-R/cosmid MUA-3 genomic library (Meyerowitz *et al.*, 1980), are described in the indicated references.

The *gr^{1w}* genomic library was constructed by isolating DNA from adult flies, digesting to completion with *Hind*III, ligating to *Hind*III-digested and alkaline phosphatase-treated λ Charon 30 DNA (Rimm *et al.*, 1980), packaging *in vitro* (Hohn, 1979) and infecting *Escherichia coli* K802 (Wood, 1966).

Single-stranded DNA subclones were constructed in bacteriophage M13 mp19 vector (Norlander *et al.*, 1983) using isolated *Eco*RI fragments as outlined in Guild (1984).

Chromosomal walking was performed essentially as described in Bender *et al.* (1983a) following standard plaque and colony hybridization protocols (Maniatis *et al.*, 1982).

Preparation of DNA and RNA

Bacteriophage λ (Davis *et al.*, 1980) and cosmid (Meyerowitz *et al.*, 1980) DNA and *Drosophila* genomic DNA from adult flies (Meyerowitz and Hogness, 1982) were prepared as described in the indicated references.

DNA from restriction fragments was prepared by cutting the desired restriction fragment out of a low melting temperature agarose gel, melting the agarose at 65°C in 1 $\times$ TBE (89 mM Tris-base, 89 mM boric acid, 2.5 mM EDTA), phenol extracting the DNA three times with an equal volume of water-saturated phenol: chloroform:isoamyl alcohol (100:96:4) and precipitating the DNA with two volumes of ethanol.

RNA extraction from cultured salivary glands (Guild, 1984), poly(A)⁺ selection by oligo(dT)-cellulose chromatography (Maniatis *et al.*, 1982), electrophoretic fractionation of DNA on horizontal agarose gels and RNA on horizontal formaldehyde-agarose gels (Guild and Shore, 1984), salivary gland polytene chromosome spreads (Wolfner, 1980) and *in situ* hybridizations (Guild and Shore, 1984) were performed as described in the indicated references.

Salivary gland *in vitro* cultures

A 35-mm culture dish was floated inside a humid chamber consisting of a 100-mm Petri dish containing 20 ml of modified (Ashburner, 1972) Grace's medium. 1 ml of modified Grace's medium ($\pm 4 \times 10^{-6}$ M ecdysterone and $\pm 5 \times 10^{-7}$ M cycloheximide) was placed on the 35-mm culture dish. Third instar larval salivary glands were dissected in modified Grace's medium and immediately placed into the 1-ml drop. Typically 20–30 glands were placed in each culture and incubated in the covered humid chamber at room temperature (20–24°C) for 2, 4 or 8 h. Chromosome squashes were made in 45% (v/v) acetic acid at the beginning and end of each culture period to determine the puff stage of the glands.

DNA labeling and hybridization conditions

Labeled cDNA was synthesized from poly(A)⁺ RNA using reverse transcriptase (Lis *et al.*, 1978) in the presence of one or two [α -³²P]dNTP. The reactions were primed with either oligo(dT) or a random oligonucleotide primer (1 μ g/ μ l). Nick translations of double-stranded DNA using [α -³²P]dCTP or [methyl-³H]dTTP were done as described (Rigby *et al.*, 1977). Conditions for *in situ*, DNA blot and RNA blot hybridizations are detailed in Guild (1984). To remove hybridized probe from RNA blots prior to reprobing, the nitrocellulose filters were washed twice in a solution of 25% formamide, 0.1 mM EDTA at 100°C for 5 min and given a final rinse in the same solution at room temperature for 5 min.

Acknowledgements

We thank Lynn Zukosky for constructing the *gr^{1w}* genomic library and for expert technical and photographic assistance. We are grateful to Welcome Bender for sharing with us his original observation on *gypsy* and for *Dmbr^{34c-6a2}*; to Jeanette Natzle and Jim Fristrom for generously providing imaginal disc RNA; to Ken Burtis, Weldon Jones, Bill Segraves, Carl Thummel and Dave Hogness for sharing information and DNA; and to John Lis for the heat shock cDNA clone. We thank Greg Greisman and Fazal Manejwala for their help with the chromosomal walk. This work was supported by a grant from the National Institutes of Health (5-R01 GM28228).

References

- Aizenzon, M.G. and Belyaeva, E.S. (1982) *Drosophila Inf. Ser.*, **58**, 3-7.
- Ashburner, M. (1972) *Chromosoma*, **38**, 255-281.
- Ashburner, M. (1973) *Dev. Biol.*, **35**, 47-61.
- Ashburner, M. (1974) *Dev. Biol.*, **39**, 141-157.

- Ashburner,M. and Berendes,H.D. (1978) in Ashburner,M. and Wright,T.R.F. (eds.), *The Genetics and Biology of Drosophila*, Vol. **2B**, Academic Press, NY, pp. 315-395.
- Ashburner,M., Chihara,C., Meltzer,P. and Richards,G. (1973) *Cold Spring Harbor Symp. Quant. Biol.*, **38**, 655-662.
- Belyaeva,E.S., Aizenzon,M.G., Semeshin,V.F., Kiss,I.I., Kiczka,K., Baritcheva,E.M.,Gorelova,T.D. and Zhimulev,I.F. (1980) *Chromosoma*, **81**, 281-306.
- Balyaeva,E.S., Vlassova,I.E., Biyasheva,Z.M., Kakpakov,V.T., Richards,G. and Zhimulev,I.F. (1981) *Chromosoma*, **84**, 207-219.
- Belyaeva,E.S., Aizenon,M.G., Kiss,I.I., Gorelova,T.D., Pak,S.D., Umbetova,G.H., Kramers,P.G.N. and Zhimulev,I.F. (1982) *Drosophila Inf. Serv.*, **58**, 184-190.
- Bender,W., Spierer,P. and Hogness,D.S. (1983a) *J. Mol. Biol.*, **168**, 17-33.
- Bender,W., Akam,M., Karch,F., Beachy,P.A., Peifer,M., Spierer,P., Lewis,E.B. and Hogness,D.S. (1983b) *Science*, **221**, 23-29.
- Bingham,P.M., Levis,R. and Rubin,G.M. (1981) *Cell*, **25**, 693-704.
- Crowley,T.E., Mathers,P.H. and Meyerowitz,E.M. (1984) *Cell*, **39**, 149-156.
- Davis,R.W., Botstein,D. and Roth,J.R. (1980) *Advanced Bacterial Genetics*, published by Cold Spring Harbor Laboratory Press, NY.
- Guild,G.M. (1984) *Dev. Biol.*, **102**, 462-470.
- Guild,G.M. and Shore,E.M. (1984) *J. Mol. Biol.*, **179**, 289-314.
- Hall,L.M.C., Mason,P.J. and Spierer,P. (1983) *J. Mol. Biol.*, **169**, 83-96.
- Hohn,B. (1979) *Methods Enzymol.*, **68**, 299-309.
- Lindsley,D.L. and Grell,E.H. (1968) *Genetic Variations of Drosophila melanogaster*, Carnegie Institute of Washington, Publication 627, Washington, DC.
- Lis,J.T., Prestidge,L.S. and Hogness,D.S. (1978) *Cell*, **14**, 901-919.
- Lis,J.T., Neckameyer,W., Dubensky,R. and Costlow,N. (1981) *Gene*, **15**, 67-80.
- Maniatis,T., Hardison,R.C., Lacy,E., Lauer,J., O'Connell and Quon,D. (1978) *Cell*, **15**, 687-701.
- Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*, published by Cold Spring Harbor Laboratory Press, NY.
- Meyerowitz,E.M. and Hogness,D.S. (1982) *Cell*, **28**, 165-176.
- Meyerowitz,E.M., Guild,G.M., Prestidge,L.S. and Hogness,D.S. (1980) *Gene*, **11**, 271-282.
- Modolell,J., Bender,W. and Meselson,M. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1678-1682.
- Moritz,Th., Edstrom,J.E. and Pongs,O. (1984) *EMBO J.*, **3**, 289-295.
- Muskavitch,M.A.T. and Hogness,D.S. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 7362-7366.
- Norlander,J., Kempe,T. and Messing,J. (1983) *Gene*, **26**, 101-106.
- Rigby,P.W.J., Dieckmann,M., Rhodes,C. and Berg,P. (1977) *J. Mol. Biol.*, **113**, 237-251.
- Rimm,D.L., Horness,D., Kucera,J. and Blattner,F.R. (1980) *Gene*, **12**, 301-309.
- Rubin,G.M. and Spradling,A.C. (1982) *Science*, **218**, 348-353.
- Savakis,C., Koehler,M.M.D. and Cherbass,P. (1984) *EMBO J.*, **3**, 225-243.
- Spradling,A.C. and Rubin,G.M. (1982) *Science*, **218**, 341-347.
- Walker,V.K. and Ashburner,M. (1981) *Cell*, **26**, 269-277.
- Wood,W.B. (1966) *J. Mol. Biol.*, **16**, 118-133.
- Wolfner,M.F. (1980) Ph.D. Thesis, Stanford University, Stanford, CA.
- Zhimulev,I.F., Vlassova,I.E. and Belyaeva,E.S. (1982) *Chromosoma*, **85**, 659-672.

Received on 7 October 1985; Revised on 11 November 1985