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Cloning of the *extra sex combs* gene of *Drosophila* and its identification by P-element-mediated gene transfer

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A large region containing the *extra sex combs (esc)* gene of *Drosophila* has been cloned by microdissection from polytene chromosomes and chromosomal walking. Within this DNA, the segment comprising the *esc* gene has been narrowed down in several steps. First, a deletion of the esc^+ gene, $Df(2L)esc^{10}$, defined a region of 380 kb. Then, a duplication carrying the esc^+ gene, *Dp(2;2)GYL*, and a deletion not eliminating esc^+, $Df(2L)prd^{1.7.20}$, further reduced this region to 160 kb. Finally, because *esc* transcripts are expected to be present in follicles but absent in adult males, mapping of follicle and adult male transcripts limited the *esc* region to ~10 kb. Rigorous proof that this DNA contained the esc^+ gene was obtained by P-element-mediated transformation. We conclude that the 12 kb of DNA used for transformation include the esc^+ gene as well as all flanking sequences required for its proper regulation in the female germ line.

Key words: homeotic gene/chromosomal walking/activity of Dp(2;2)GYL/transcript mapping/P-element-mediated transformation

Introduction

In the *Drosophila* embryo cell lineage becomes restricted when the first cells are formed at cellular blastoderm and primordial segments are established (Wieschaus and Gehring, 1976; Steiner, 1976; Lawrence and Morata, 1977; Lohs-Schardin *et al.*, 1979; Szabad *et al.*, 1979). Subsequent cell lineage restrictions divide primordial segments into anterior and posterior compartments which are further subdivided into subcompartments (Garcia-Bellido *et al.*, 1973, 1975; Crick and Lawrence, 1975). The development of segments during embryogenesis and larval life is governed mainly by homeotic genes belonging to the bithorax complex (BX-C) (Lewis, 1978) and the Antennapedia complex (ANT-C) (Kaufman *et al.*, 1980; Lewis *et al.*, 1980a, 1980b). Thus, the developmental fate of a particular segment or compartment is determined by a specific combination of active and inactive BX-C and ANT-C genes (Lewis, 1978; Duncan and Lewis, 1982; Struhl, 1982; Lawrence and Morata, 1983). A change in activity of any of these genes during development leads to an alteration of the determined state and a switch in segmental or compartmental identity. For example, in embryos in which all genes of the BX-C have been deleted, segments posterior to the anterior mesothoracic compartment develop like composite segments consisting of a posterior pro- and an anterior mesothoracic compartment (Lewis, 1978; Morata and Kerridge, 1981). Since no change is observed in segments anterior to the posterior mesothoracic compartment, genes of the BX-C are thought to be inactive in these segments and to determine only the development of thoracic and abdominal segments (Lewis, 1978). Similarly, it was shown that genes of the ANT-C are active predominantly in thoracic and head segments (Kaufman *et al.*, 1980; Wakimoto and Kaufman, 1981; Denell *et al.*, 1981; Struhl, 1982, 1983).

This spatial restriction of the realm of the BX-C and ANT-C genes is regulated by a small number of genes which probably respond to signals of differential spatial information deposited in the oocyte or perhaps generate themselves such signals (Lewis, 1978; Stuhl, 1981; Duncan and Lewis, 1982; Duncan, 1982; Ingham, 1983, 1984). Therefore it is not suprising that most of these genes exhibit a maternal effect (Struhl, 1981; Duncan and Lewis, 1982; Duncan, 1982; Ingham, 1984). One of them, the *extra sex combs (esc)* gene, has been shown to act as a negative regulator of the BX-C genes (Struhl, 1981) and of at least some of the ANT-C genes (Struhl, 1983). Thus, in esc^-/esc^- embryos derived from esc^-/esc^- mothers, all thoracic and abdominal segments as well as some of the head segments are transformed into eighth abdominal segments (Struhl, 1981). Since, according to the model of Lewis (1978), the eighth abdominal segment is the only segment in which normally all genes of the BX-C are active, this finding suggests that all BX-C genes are activated in all segments of such esc^- embryos (Struhl, 1981, 1983). The *esc* gene shows a maternal effect as evident from the observation that embryos obtained from homozygous esc^- mothers develop into first instar larvae which fail to hatch and die even if they possess a paternal esc^+ gene, whereas embryos of esc^+/esc^- mothers develop normally (Struhl, 1981). The maternal effect might hint at an early requirement for the esc^+ gene product during embryogenesis. Consistent with this view is the discrete temperature-sensitive period of the esc^+ gene product between blastoderm and the germ-band stage (Struhl and Brower, 1982). Thus, *esc* exhibits the characteristics of an activator gene expressed for a short time to initiate the differential activity of selector genes (Garcia-Bellido, 1975, 1977) at the BX-C and ANT-C in individual segments of the embryo. However, there is also a late requirement for the esc^+ gene which is most obvious from the appearance of sex combs on the meso- and metathoracic legs of homozygous esc^- males (Slifer, 1942; Tokunaga and Stern, 1965).

To elucidate the action of the esc^+ gene product at the molecular level, we decided, as a first step, to clone the *esc* gene. We describe here the isolation, by microdissection from polytene chromosomes (Scalenghe *et al.*, 1981) and by chromosomal walking (Bender *et al.*, 1983), of a large region containing the *esc* gene. Within this region, we have identified the *esc* gene by P-element-mediated transformation (Spradling and Rubin, 1982; Rubin and Spradling, 1982).

Results

Cloning of DNA sequences deleted by the deficiency $Df(2L)esc^{10}$

The *esc* gene is uncovered by the small deficiency $Df(2L)esc^{10}$

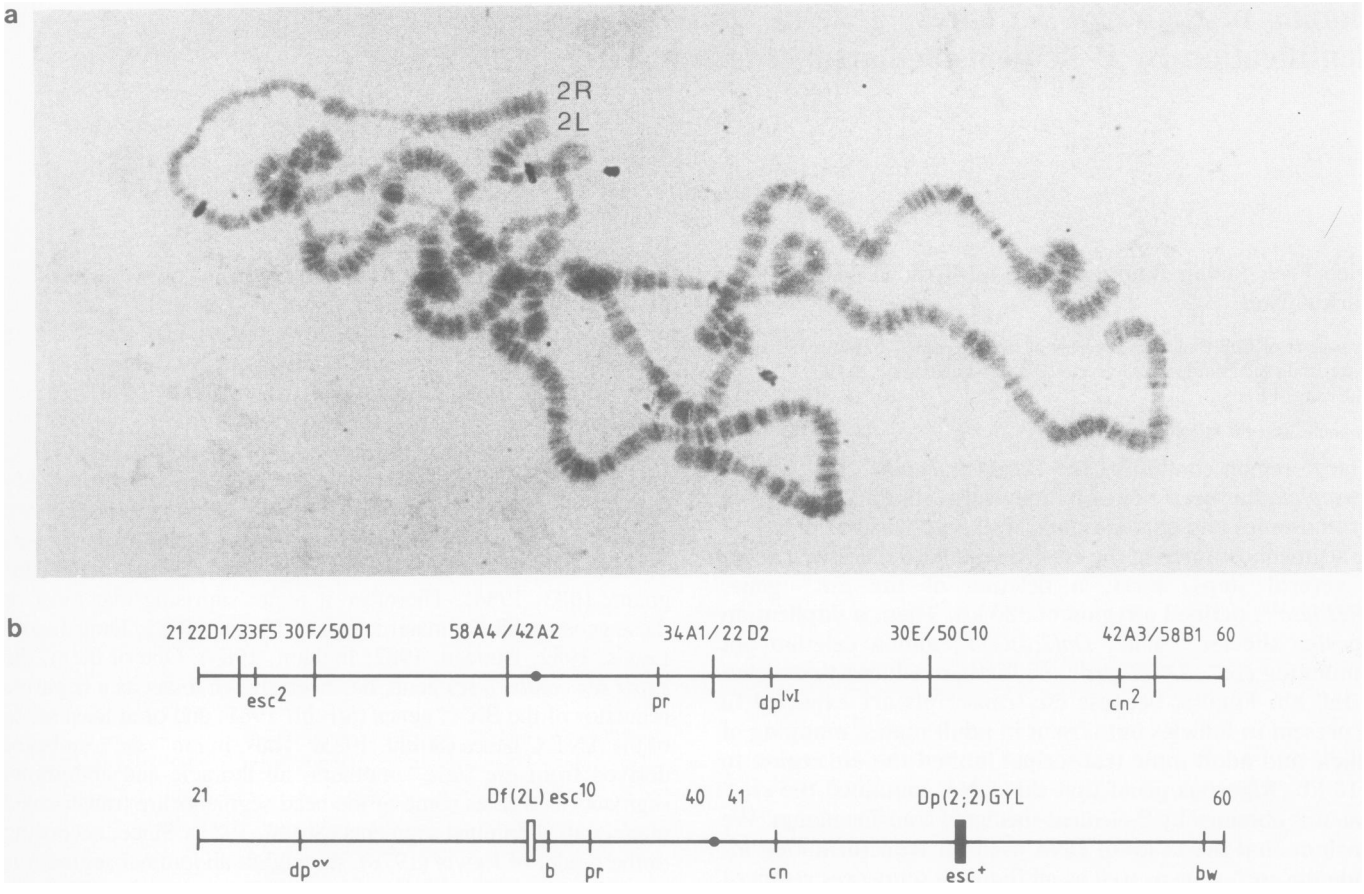

Fig. 1. *In situ* hybridization of 1F10.1 DNA to salivary gland chromosomes of *CyO, esc²/Df(2L)esc¹⁰ Dp(2;2) GYL* larvae. Biotinylated DNA of phage 1F10.1 was hybridized *in situ* to polytene chromosomes of *CyO, esc²/Dfesc¹⁰ DpGYL* larvae **(a)**. The two second chromosomes are schematically illustrated in **(b)**. Inversion breakpoints are marked by long vertical lines and their locations are indicated on the *CyO, esc²* balancer chromosome above, the positions of the deficiency *Df(2L)esc¹⁰* and of the duplication *Dp(2;2)GYL* are represented by an open and filled bar, respectively, on the other second chromosome below.

which, in polytene chromosomes, deletes the band 33B1,2 on the left arm of the second chromosome (Struhl, 1981). Hence, in a first step to clone the *esc* gene, we microdissected this band from salivary gland chromosomes of giant larvae and established a minilibrary as described by Scalenghe *et al.* (1981). From eight microdissected chromosomes ~400 recombinant phages were obtained. Inserts that contained only non-repetitive *Drosophila* DNA sequences were removed from the DNA of 23 phages by *Eco*RI digestion and were used to screen a Canton S library (Maniatis *et al.*, 1978; cf. Materials and methods). DNA of the first phage isolated from the Maniatis pool, 1F10.1, was hybridized to salivary gland chromosomes of *CyO, esc²/Df(2L)-esc¹⁰ Dp(2;2)GYL* larvae to test whether its insert consisted of DNA deleted in *Dfesc¹⁰*. The second chromosome with the deficiency *esc¹⁰* carries a duplication of the *esc⁺* region on its right arm. This duplication, *Dp(2;2)GYL*, extends from chromosomal bands 33B1,2 to 35C1,3 and is inserted at 50A,B (Ashburner, 1982; Yannopoulos *et al.*, 1982). As evident from Figure 1a, two hybridization signals are observed. One is on the *CyO* balancer chromosome close to the left telomere just outside the telomeric region where the two homologues are paired and hence corresponds to the location of the *esc²* allele (Figure 1b). The other signal is found on the right arm of the other second chromosome where *Dp(2;2)GYL* is located. No more than these two signals were ever observed which suggests that the isolated DNA lies entirely within the *esc¹⁰* deletion.

The inserts of the λ-phages isolated from the Canton S library originated from four contiguous regions of which two nearly touched (Figure 2). This became apparent from whole genome Southern analysis which revealed that the approaching ends of the two larger regions covered 15 kb and 20 kb, respectively, of the two ends of an unusually large genomic *Eco*RI fragment of ~40 kb (Figure 3). The distal end of this *Eco*RI fragment was arbitrarily chosen as zero point of the DNA map (Figure 3). Whereas these two regions, which together comprised ~110 kb, were obviously very close to each other, the distance to the other two relatively small regions was unknown. Despite this uncertainty, the ends of the isolated regions were used to initiate chromosomal walks because screening of the Canton S library with the microdissected inserts repeatedly led to the isolation of phages from the large 110-kb region already obtained. The chromosomal walks very soon united the 110-kb region with the small region located around 90-100 kb on the DNA map (Figure 2).

From *in situ* hybridizations to salivary gland chromosomes it became evident that at least one of the two phages B2 and B2.3, whose inserts bridged the gap between the 110-kb and the small region to the right, contained the proximal breakpoint of the *esc¹⁰* deletion (Figure 3). Therefore subclones of these two phages were used to map the breakpoint more precisely by whole genome Southern analysis (Figure 4). Clearly, the 4.2-kb *Eco*RI fragment of B2.3 was entirely within the deletion, whereas the 2.2-kb fragment of B2 was completely outside the deficiency, because the signal of the 4.2-kb fragment is reduced in *Dfesc¹⁰/b pr* DNA

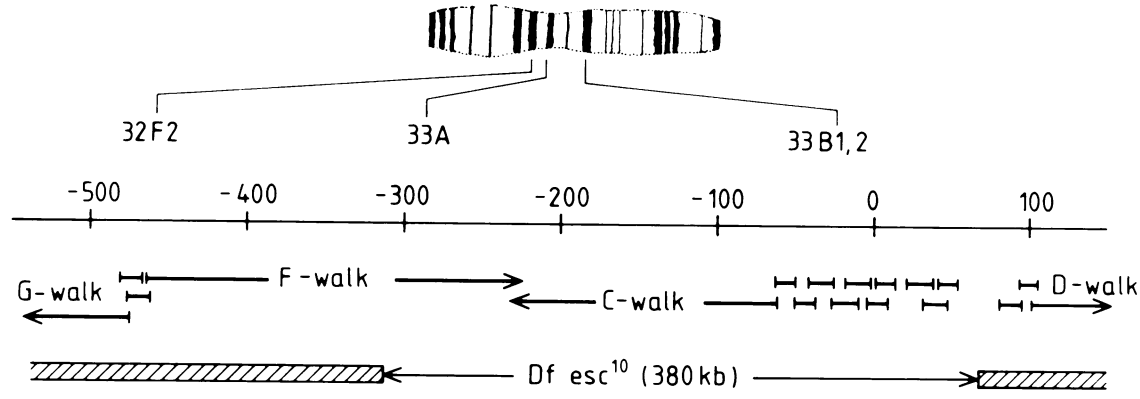

Fig. 2. Cloned DNA within polytene chromosome segments 32F to 33B. About 700 kb of cloned DNA from chromosomal bands 32F to 33B are shown. Only those bands that were easily visible in a light microscope are indicated in the polytene chromosome segments drawn schematically at the top. Cloned DNA of recombinant phages isolated from the Canton S library by screening with microdissected DNA is represented by horizontal lines whereas the regions cloned by, and the directions of, chromosomal walking are indicated by arrows (the scale refers to DNA lengths in kb). At the bottom the breakpoints and the extent of the deletion *Df(2L)esc*^{10} are shown.

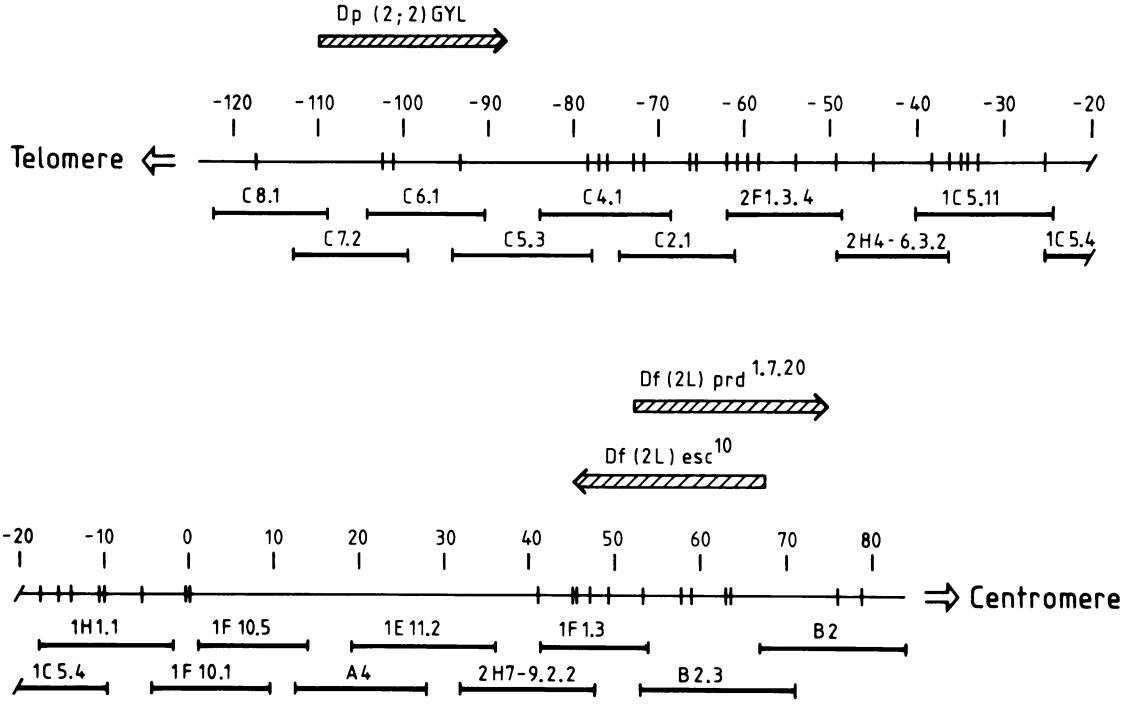

Fig. 3. Cloned DNA and *Eco*RI restriction map at the *esc* locus between the distal breakpoints of *Dp(2;2)GYL* and *Df(2L)prd*$^{1.7.20}$. A map of *Eco*RI sites (scale in kb) and the locations of corresponding cloned DNA segments of recombinant phages isolated from the Canton S library (horizontal lines) are shown. The coordinates and the vertical marks indicate the distances of *Eco*RI sites from the distal end of the 41-kb *Eco*RI fragment. The names of recombinant phages isolated by screening with microdissected DNA start with the number 1 or 2, those of phages isolated by chromosomal walking begin with the letters A, B or C. The breakpoints and orientations of the duplication *Dp(2;2)GYL*, the deficiency *Df(2L)esc*^{10}, and the deficiency *Df(2L)prd*$^{1.7.20}$ are marked by cross-hatched arrows.

as compared with homozygous *b pr* DNA, yet the signals of the 2.2-kb fragments exhibit the same intensity in both DNAs (left and right panels of Figure 4). The two central panels of Figure 4 show that both the 7.5-kb and the 9.2-kb end-fragments of B2.3 and B2, respectively, overlap with the *Eco*RI fragment containing the *Dfesc*^{10} breakpoint. This is obvious from the appearance in the *esc*^{10} lanes of a 9.4-kb *Eco*RI fragment which crosses the breakpoint while the 12.5-kb fragment originates from the *b pr* DNA. A comparison of the relative intensities of the 9.4-kb and 12.5-kb fragments indicates that the *Dfesc*^{10} breakpoint maps about in the middle of the 7.5-kb fragment and very close to the left end of the B2 insert (Figure 4).

Whereas the G-walk was interrupted as soon as *in situ* hybridizations to chromosomes proved that it moved away from the *esc*^{10} deficiency, the F-walk and C-walk were continued until they joined at −230 kb on the DNA map (Figure 2). Also the D-walk was extended to clone the *paired* gene which maps proximal to *esc* (C. Nüsslein-Volhard, personal communication). The F-walk contained the left *Dfesc*^{10} breakpoint which was mapped by *in situ* hybridization to chromosomes, whole genome Southern analysis, as well as by isolating the recombinant phage crossing the deletion from a *Dfesc*^{10}*/b pr* library (not shown). The *esc*^{10} deficiency removes ~380 kb, corresponding to chromosomal bands 33B1,2 and most of 33A, as apparent from *in situ* hybridizations of its ends to *b pr* salivary gland chromosomes (not shown).

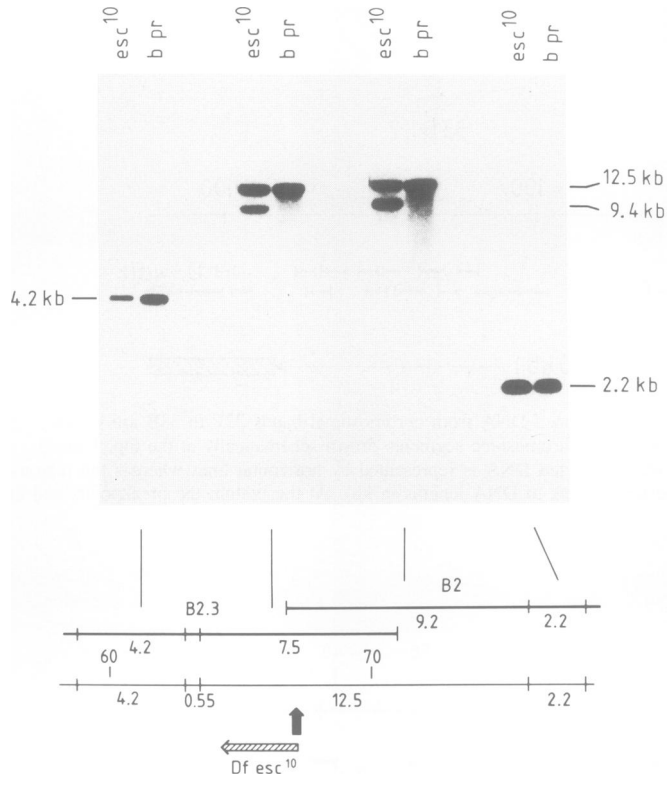

Fig. 4. Mapping of the proximal breakpoint of the deficiency *Df(2L)esc*^{10} by whole genome Southern analysis. DNAs isolated from homozygous *b pr* (lanes labeled b pr) and from *Df(2L)esc*^{10}*/b pr* flies (lanes labeled esc^{10}) were digested with *Eco*RI, and 4 μg per lane were run in a 0.6% agarose gel in TBE-buffer (Maniatis *et al.*, 1982). Each pair of lanes shows Southern blots probed with the subcloned *Eco*RI fragments of phage B2.3 and B2 indicated below. At the bottom, the corresponding section of the *Eco*RI map is shown and the approximate location of the proximal breakpoint of *Dfesc*^{10} is marked by the vertical arrow.

Mapping the breakpoints of Dp(2;2)GYL and of Df(2L)prd$^{1.7.20}$ *within the esc*^{10} *deficiency*

In an attempt to reduce the region containing the *esc* gene, we mapped the distal breakpoints of two deficiencies of the *paired* gene, *Df(2L)prd*$^{1.7.20}$ and *Df(2L)prd*$^{1.25.40}$, which is located to the right of *esc* (the mutant strains, which are both homozygous *esc*$^{+}$, were kindly provided by C. Nüsslein-Volhard). Only the breakpoint of *Df(2L)prd*$^{1.7.20}$ mapped within the *esc*^{10} deletion, by ~15 kb (Figure 3) as determined by *in situ* hybridizations to chromosomes (B2.3 is entirely within, 1F1.3 mostly outside this *paired* deficiency; not shown). However, the largest diminution of the region containing the *esc* gene was obtained when the left breakpoint of the duplication *Dp(2;2)GYL*, which carries a functional *esc* gene, was mapped. The *in situ* hybridization of phage C8.1 DNA to salivary gland chromosomes of *CyO,esc*^{2}*/Df(2L)esc*^{10} *Dp(2;2)GYL* larvae reveals two signals (arrows in Figure 5b), one of which is very weak. The strong signal occurs close to the left telomere on the *CyO* chromosome and therefore originates from the region of the *esc*^{2} allele. The location of the weak signal may be identified in Figure 5 in band 33A of the duplication which is inserted at 50B on the right arm of the other second chromosome, and which is *esc*$^{+}$ (cf. Figure 1b). Because this signal was always much weaker than that of the *esc*^{2} region, the C8.1 insert overlaps with the duplication only slightly, locating the duplication breakpoint at about −110 kb on the DNA map (Figure 3). No signal was detected on the duplication when cloned DNA distal to C8.1 was hybridized to chromosomes (not shown). It is noteworthy that *Dfesc*^{10}*/Dfesc*^{10} *DpGYL* flies are viable and fertile, although only if not crossed with each other, despite the homozygous deletion of 200 kb of DNA.

Examination of the banding pattern on the second chromosome in Figure 5 shows that, immediately proximal to the duplication, an inversion has occurred, the breakpoints of which have been tentatively assigned to chromosomal bands 44F and 50A. The duplication itself comprises the bands 33A to 34A,B. Since this is a considerably shorter region than that carried by the duplication of the original *Dp(2;2)GYL* chromosome, which extended to the chromosomal bands 35C1,3 (Ashburner, 1982), it is likely that this duplication is not inert but induces spontaneous deletions of itself (Ashburner, 1982 and personal communication; Yannopoulos *et al.*, 1982) as well as large inversions in neighboring chromosomal regions.

Localization of sequences homologous to transcripts in follicles and adult males

The breakpoints of the duplication *GYL* and of the *paired* deficiency limit the region containing the *esc* gene to 160 kb. An *Eco*RI restriction map of this region and the phages isolated from the Canton S library covering it are shown in Figure 3.

Because whole genome Southern analysis of *esc*^{1}, *esc*^{2}, and *esc*^{6} mutants did not detect any significant differences with respect to a number of wild-type DNAs in this region, another test was needed to narrow down further the *esc* region. One such test could be derived from the known developmental profile of *esc*$^{+}$ gene expression. As *esc* exhibits a maternal effect (Struhl, 1981) and is expressed cell-autonomously in the germ line (Lawrence *et al.*, 1983), *esc* mRNA is present in follicles at some stage of oogenesis. On the other hand, it seems likely that adult males do not transcribe the gene because the late requirement of the gene probably ends during late larval or pupal life (Tokunaga and Stern, 1965). Therefore radioactive cDNA of poly(A)$^{+}$ RNA from follicles was hybridized to blots of *Eco*RI digests of all recombinant phage DNAs shown in Figure 3. The signals were compared with those obtained from identical blots hybridized with cDNA of adult male poly(A)$^{+}$ RNA. As evident from Figure 6, transcripts are detected in follicles only in one region that appears to remain silent in adult males. This region, which is contained entirely within phage C2.1 (lane 6 of Figure 6), comprises three contiguous *Eco*RI fragments of 5.3 kb, 0.6 kb and 3.7 kb. There is one additional 2.9-kb fragment of phage C6.1 that is labeled only by follicle cDNA (lanes 3 of Figure 6). However, since this DNA fragment is actually the left end of the 15-kb *Eco*RI fragment within phage C5.3 which labels strongly with follicle cDNA but also weakly with adult male cDNA (lanes 4 of Figure 6), it was considered to be a less-promising candidate for harboring the *esc* gene. Also the quantitative differences between transcripts to the left of C2.1 which predominate in follicles were disregarded because they are synthesized in appreciable amounts in adult males. Other differences in Figure 6 concern mainly a region of 20 kb immediately to the right (on the second chromosome as well as in the panels of Figure 6) of the labeled C2.1 fragments. Transcripts in this region, however, are abundant in males and appear to be absent in follicles.

Identification of the esc gene by P-element-mediated gene transfer

The results shown in Figure 6 suggest that the *esc* gene is part of, or entirely within, the DNA cloned in phage C2.1. To test this supposition, an attempt was made to rescue the *esc*$^{-}$ phenotype by P-element-mediated gene transfer (Spradling and

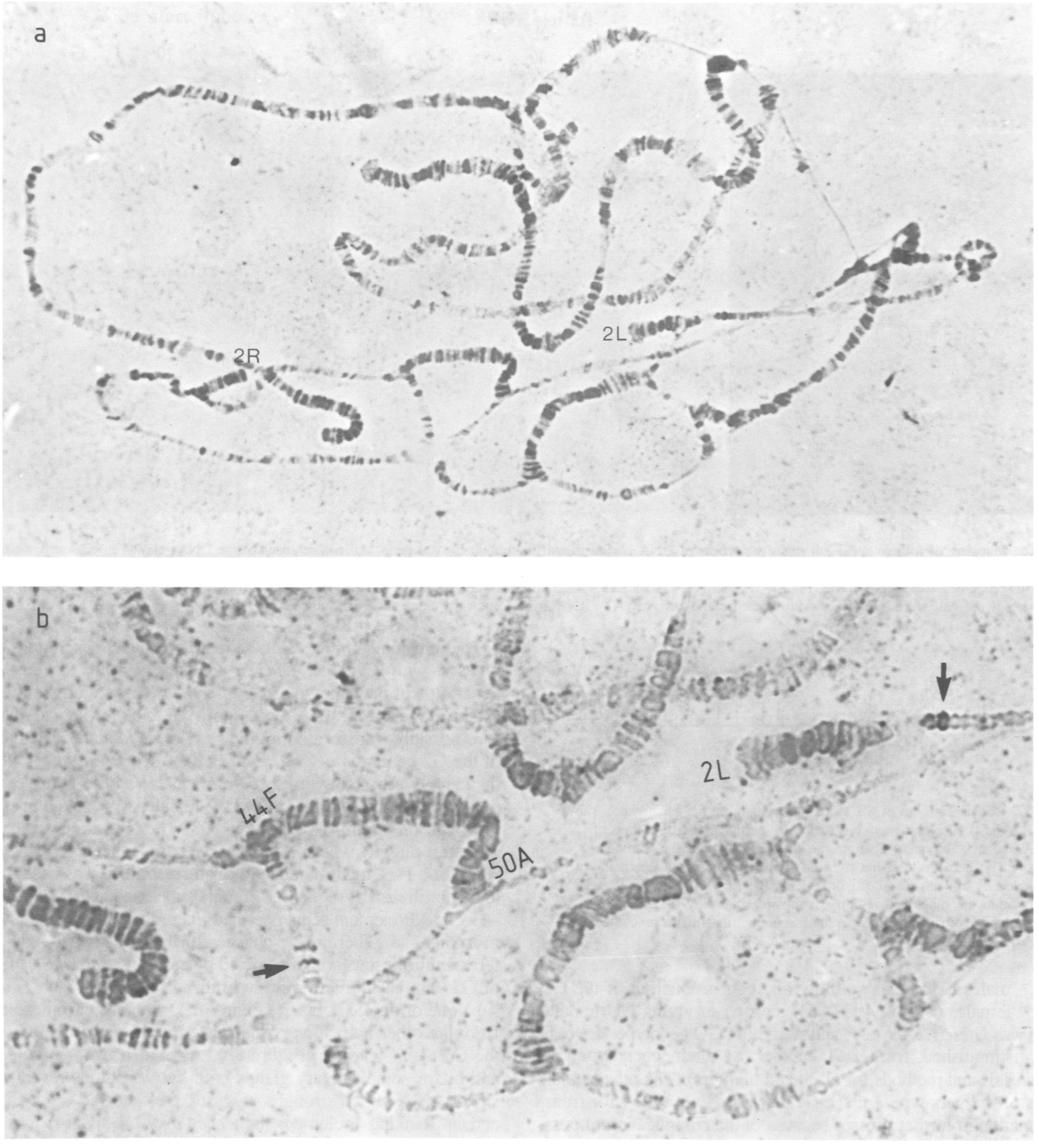

Fig. 5. *In situ* hybridization of C8.1 DNA to salivary gland chromosomes of *CyO, esc²/Df(2L)esc¹⁰ Dp(2;2)GYL* larvae. An overview of the polytene chromosomes hybridized *in situ* with biotinylated C8.1 DNA is shown in **(a)** in which the left (2L) and the right telomeres (2R) of the second chromosomes are marked. The hybridization signals of the C8.1 DNA (arrows) and the inversion between 44F and 50A on the *Dfesc¹⁰ DpGYL* chromosome are shown at higher magnification in **(b)**.

Rubin, 1982; Rubin and Spradling, 1982), using the insert of phage C2.1. To this end, most of the inserted DNA of phage C2.1 was subcloned into the *Eco*RI site of Carnegie 4, a non-autonomous P-element vector (Rubin and Spradling, 1983). A restriction map of this subclone C2.1.5(Car4) is shown in Figure 7. Subclone C2.1.5(Car4) DNA was co-injected with DNA of the helper P-element plasmid pπ25.7wc (Karess and Rubin, 1984) into the posterior pole of cleavage-stage embryos where the primordial germ cells form. Of 30 injected embryos, derived from *CyO, esc²/DpGYL, b dp^ov cn bw* mothers and *esc¹ sp c/Dfesc¹⁰, b pr* fathers, seven survived to first instar larvae and six developed into adult flies, two females and four males. Mothers carrying two copies of *esc⁺* (one on the duplication *GYL*) were used in the parental cross to enhance viability and fertility of the GO

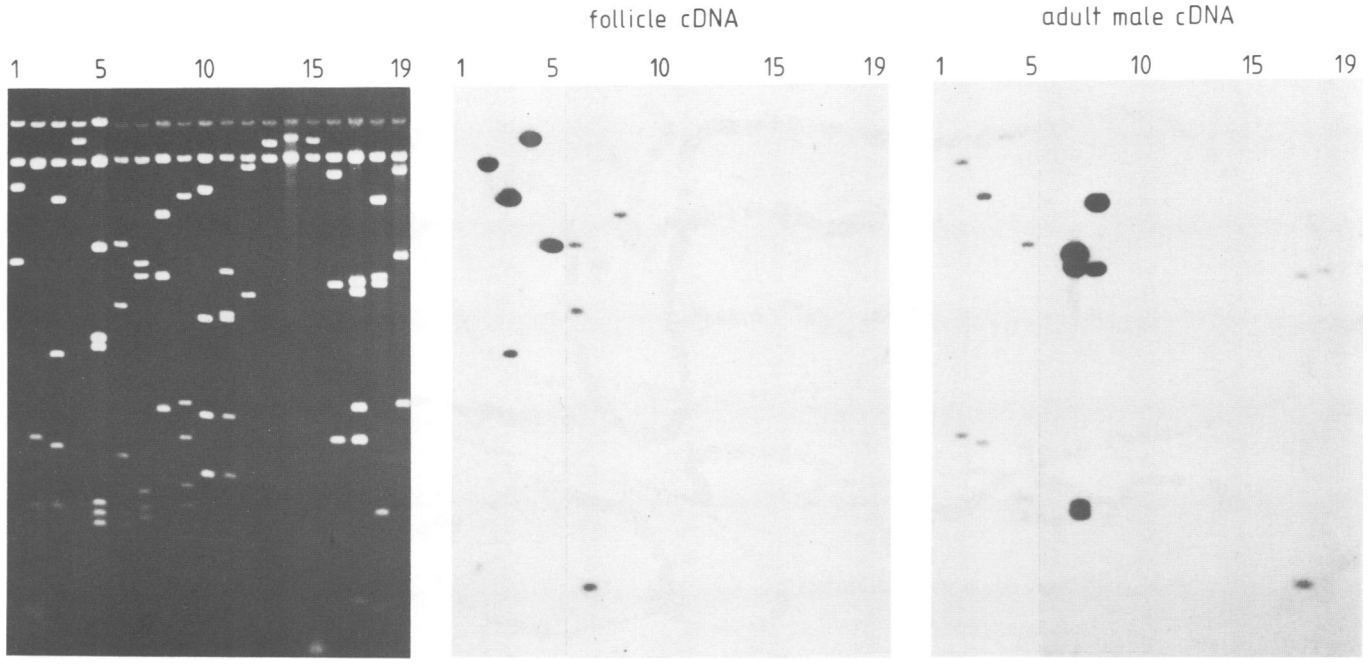

Fig. 6. Mapping of follicle and adult male transcripts in DNA region shown to contain the *esc* gene. All recombinant phage DNAs shown in Figure 3 were digested with *Eco*RI and run in a 0.6% agarose gel in TBE-buffer (Maniatis *et al.*, 1982). **Lanes 1 – 19** contained C8.1 to B2 DNAs loaded in the order of their inserts along the chromosome (Figure 3). The gel was stained with ethidium bromide (left panel) and two Southern blots, obtained by a bidirectional transfer of the gel, were hybridized with labeled cDNA of poly(A)$^+$ RNA from follicles or adult males as indicated.

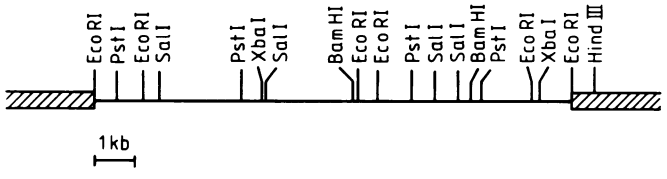

Fig. 7. Restriction map of C2.1.5(Car4) containing the *esc*$^+$ gene. The C2.1.5(Car4) subclone was constructed by partial *Eco*RI digestion of C2.1 DNA and its subsequent ligation into the *Eco*RI site of the Carnegie 4 vector. The *Eco*RI site at the right end of the insert is not genomic but produced by a synthetic *Eco*RI linker. *Eco*RI, *Bam*HI, *Sal*I, *Xba*I and *Pst*I sites within the subcloned region are shown while only the *Hind*III site in the vector was mapped to determine the orientation of the subcloned DNA in Carnegie 4.

esc$^-$ males as well as viability and egg production of the GO *esc*$^-$ females (Struhl, 1983). Of the two surviving GO females one was *Dfesc*^{10}*/CyO, esc*^{2} and sterile (all GO genotypes are easily distinguished from one another by their phenotype, cf. Materials and methods for all linked markers). The other female was of the genotype *esc*^{1}*/DpGYL* and was not tested for transformation in further crosses because of the complication of possible meiotic recombination. Of the four surviving GO males two were *CyO,esc*^{2}*/esc*^{1} [of which one had misformed genitalia, a lesion frequently observed as a consequence of the injection at the posterior pole (Dan Hultmark, personal communication), and hence was sterile], while the two others were *CyO,esc*^{2}*/Dfesc*^{10} and *Dfesc*^{10}*/DpGYL,* respectively. The fertile *CyO,esc*^{2}*/esc*^{1} male and the *CyO,esc*^{2}*/Dfesc*^{10} male were examined for germ-line transformation by *esc*$^+$ in further crosses. All *trans*-heterozygous *esc*$^-$ females obtained from the two crosses *CyO,1(2)100*^{DTS}*/esc*^{6} x CyO, esc^2*/esc*^{1} and *CyO,1(2)100*^{DTS}*/esc*^{1} x *CyO, esc*^{2}*/Dfesc*^{10} (distinguishable by different phenotypes, cf. Materials and methods) were sterile and therefore not transformed by *esc*$^+$.

The remaining *Dfesc*^{10}*/DpGYL* male of the GO generation was crossed with *CyO,esc*^{2}*/DpGYL* females. Virgins of the G1 generation of the genotype *CyO, esc*^{2}*/Dfesc*^{10} were crossed with *esc*^{1}*/esc*^{6} males and some of them proved to be fertile and therefore possibly transformed by the *esc*$^+$ gene. Balanced females of the G2 generation (*CyO, esc*^{2}*/esc*^{1} and *CyO, esc*^{2}*/esc*^{6}) were then singly crossed with *esc*^{1}*/Dfesc*^{10} males and in many cases proved to be fertile. Similarly, fertile females were found in each genotype of the third generation, G3, after injection.

To test directly whether the observed *esc*$^+$ phenotype was the result of a P-element-mediated transformation, it was necessary to demonstrate that sequences of C2.1 subcloned in C2.1.5(Car4) as well as P-element sequences had integrated at the same chromosomal site. Therefore, squashes of half a salivary gland of female third instar larvae, obtained by crossing *esc*^{6}*/Dfesc*^{10} flies of G3 with each other, were hybridized with the 5.3-kb *Eco*RI subclone of the C2.1 insert (Figure 8a), whereas chromosomes from the other half salivary gland were hybridized *in situ* with the Carnegie 4 vector (Figure 8b). As evident from Figure 8a and b, for some salivary glands both hybridizations revealed a signal at 7A which strongly suggests that both the C2.1 DNA and its flanking P-element sequences in C2.1.5(Car4) had integrated at 7A on the X-chromosome. Because only one of the two homologues, which are partly separated in Figure 8a, shows hybridization at 7A, this larva was heterozygous for the transformed X-chromosome. As expected, in Figure 8a an additional signal was observed at 33B, the original locus of the C2.1 insert, and in Figure 8b at 3C, the white locus, due to the presence of white sequences in the Carnegie 4 vector (Rubin and Spradling, 1983).

As we (Figure 5) and others (Ashburner, 1982; Yannopoulos *et al.*, 1982) had observed that the duplication *GYL* could induce inversions and deletions, we had to consider the possibility that this duplication had been induced to transpose and integrate together with P-element sequences into 7A and thus mimic a suc-

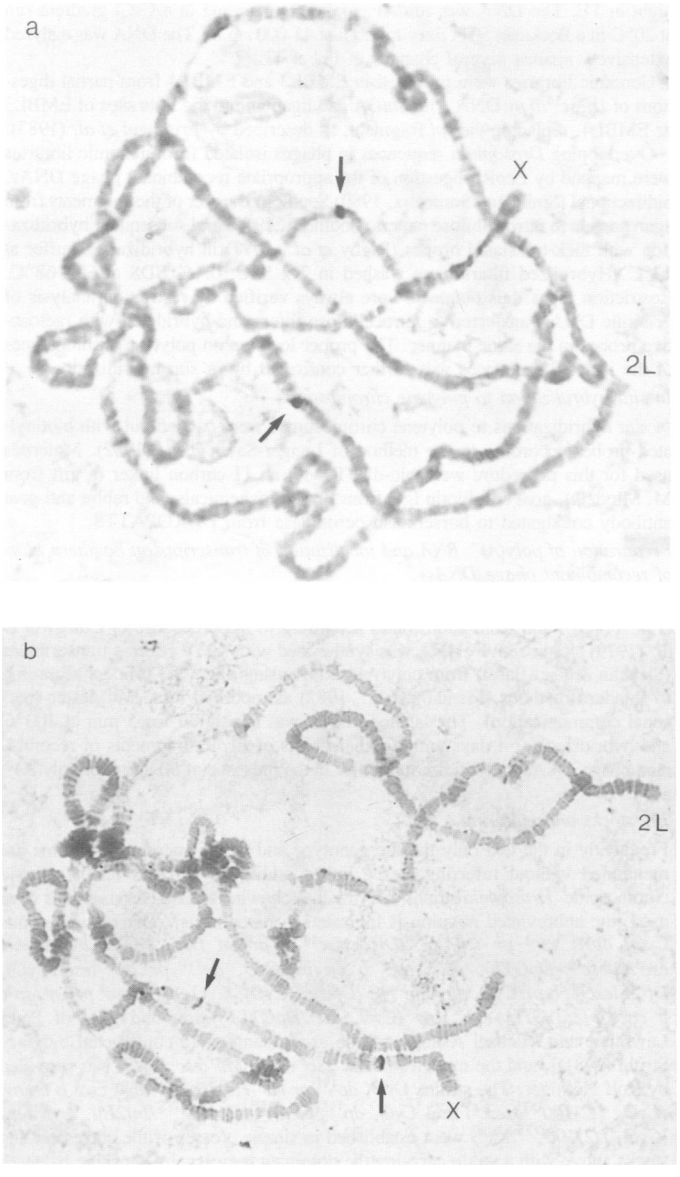

Fig. 8. *In situ* hybridizations to salivary gland chromosomes of esc^6/esc^6 or $esc^6/Dfesc^{10}$ larvae transformed with C2.1.5(Car4). Salivary gland chromosomes of transformed esc^6/esc^6 or $esc^6/Dfesc^{10}$ female larvae were hybridized *in situ* with the following biotinylated DNAs: one half of a salivary gland with pAT153 containing the 5.3-kb *Eco*RI fragment of C2.1 DNA **(a)**, the other half with the Carnegie 4 vector **(b)**. Only female larvae were used for the squashes to facilitate detection of a possible transposition of *DpGYL* into the X-chromosome. The left telomere of the second chromosome (2L) and the telomere of the X-chromosome (X) are indicated. The signals (arrow heads) are at 33B1,2 and 7A in **(a)** and at 7A and 3C in **(b)**.

cessful transformation with esc^+ of the injected $Dfesc^{10}/DpGYL$ male. Although no insertion of the duplication was visible at 7A (Figure 8b), it was still possible that only part of it, carrying the esc^+ gene yet cytologically undetectable, was present at 7A. This possibility was tested by hybridizing C2.1.5(Car4) again to chromosomes of only half a salivary gland, whereas chromosomes of the other half were hybridized either with a 15-kb subclone of C5.3 or with subclones of 2F1.3.4 and 2H4-6.3.2 which are immediately to the left or right of the C2.1 DNA (cf. Figure 3). No signal at 7A but only one at 33B was detectable after hybridization with these subclones to the left and right of C2.1, although *in situ* hybridizations of C2.1.5(Car4) with the other halves of the corresponding salivary glands revealed signals at 33B as well as at 7A and 3C (not shown). Therefore we concluded that transformation with C2.1.5(Car4) had indeed occurred and that these sequences contained the entire esc^+ gene. This conclusion was corroborated by whole genome Southern analysis of transformants which, in comparison with wild-type or *esc* mutant DNA revealed an additional 1.0-kb *Eco*RI fragment (not shown). This fragment which is located at the right end of the insert in C2.1.5(Car4), is bounded at its right limit by a synthetic *Eco*RI linker and hence occurs only in the DNA of transformants.

Discussion

We have cloned a large region containing the *esc* gene and identified its location within this region by P-element-mediated transformation of the germ line. Flies that carry only a copy of esc^+ at the ectopic chromosomal site do not exhibit the esc^- phenotype. Hence, this ectopic esc^+ gene is correctly regulated during development. This includes its proper expression in the female germ line in a cell-autonomous fashion (Lawrence *et al.*, 1983). We would, therefore, expect that transformed GO females are mosaic in their germ line with respect to esc^+ and hence probably fertile. Since we do not know whether the germ line of the only surviving sterile GO female has been transformed with esc^+, it remains to be shown whether such fertile esc^+ GO females may be obtained. In addition to the rescue of embryos derived from transformed mothers, we found that the phenotype of esc^- adult males also was rescued. Thus, when G3 females, which were heterozygous for the transformed X-chromosome, were crossed with esc^- males, about half of the male offspring did not exhibit sex combs on their meso- and metathoracic legs. This indicates that the P-element-mediated integration did not induce a hemizygous lethality on the X-chromosome and that all sequences necessary for expression of the late function of *esc* (Tokunaga and Stern, 1965), which does not necessarily differ from its early function, had integrated.

Since we have obtained only one transformant, it was crucial to prove that rescue of the phenotype was indeed a consequence of transformation rather than caused by transposition of DNA carrying esc^+ sequences from its original locus at 33B to another chromosomal site. Evidence for transformation came from two independent experiments. First, *in situ* hybridizations with C2.1 or P-element sequences to corresponding halves of salivary glands showed that both seqeunces were present at 7A. Second, whole genome Southern analysis of transformants revealed a 1.0-kb *Eco*RI fragment which is characteristic for the sequence subcloned in C2.1.5(Car4) and whose size differs in non-transformed genomes. Therefore, rescue of the esc^- phenotype has occurred by transformation of the germ line with C2.1.5(Car4) DNA and not by any other mechanism.

Materials and methods

Solutions

TE is 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, chloroform is always used as 24:1 mixture of chloroform with isoamylalcohol, and phenol/chloroform as a 1:1 mixture of the latter with phenol. Hybridization buffer consisted of 50% deionized formamide, 5 x SSC, 5 x Denardt's solution, 50 mM Na-phosphate, pH 6.5, 0.05% $Na_4P_2O_7$. 10 H_2O, 0.1% SDS, 200 μg/ml denatured herring sperm DNA (McGinnis *et al.*, 1984).

Microdissection of polytene chromosomes and preparation of a minilibrary

Squashed salivary glands were prepared from gt^{x11}/gt^1 larvae and polytene chromosomes were selected that appeared most suitable for microdissection of

band 33B1,2. Microdissection of eight polytene bands, microextraction, digestion with *Eco*RI, and ligation of the DNA into the *Eco*RI site of the *cI* gene of λNM641 (Murray *et al.*, 1977) followed by *in vitro* packaging and plating on the *hfl* strain POP 13b (r_K^-, m_K^-) were all carried out as described by Scalenghe *et al.* (1981).

Screening of genomic libraries and chromosomal walking

Phages of the minilibrary were screened with ^{32}P-labeled Oregon R DNA according to Benton and Davis (1977). Phages that were negative in the screen and hence considered to contain unique *Drosophila* DNA sequences were grown up and DNA was prepared as described below. Inserts were removed by digestion with *Eco*RI and subcloned into pAT153 according to standard procedures (Maniatis *et al.*, 1982). Most inserts (>90%) of the microdissected DNA were shorter than 300 bp. This finding is best explained by *Eco*RI* activity during digestion with *Eco*RI of the microdissected DNA. Consistent with this interpretation is the observation that many inserts in λNM641 could not be cleaved at both ends with *Eco*RI and that several inserts from within the large genomic 41-kb *Eco*RI fragment were obtained. To improve the sensitivity of screening the Canton S library of Maniatis *et al.* (1978) with such small inserts, these were isolated as *Pst-Bam* fragments from the subclones by electroelution into dialysis bags (Maniatis *et al.*, 1982), nick-translated (Rigby *et al.*, 1977), denatured and hybridized overnight at 37°C with library filters prepared according to Benton and Davis (1977) in hybridization buffer. Filters were washed at 50°C in 2 x SSC, 0.1% SDS. By this method screening with inserts as short as 65 bp was successful.

The procedure for chromosomal walking has been described in detail by Bender *et al.* (1983). When repetitive sequences were encountered in the Canton S library, we switched to a *Ns/Ns* library in λ1059 (kindly provided by A. Kuroiwa) or to a *b pr/Dfesc*^{10} library we prepared in EMBL3 and EMBL4 according to Frischauf *et al.* (1983). For walking, *Eco*RI fragments of the phage inserts were subcloned into pAT153 and recombinant plasmid DNAs were prepared in minipreps as described below.

Preparation of plasmid and phage DNA

Minipreparations of plasmid DNA were carried out according to the alkaline extraction procedure of Birnboim and Doly (1979) as modified by Ish-Horowicz and Burke (1981), except that the DNA was extracted once with phenol/chloroform and once with chloroform before precipitation with ethanol. In addition, RNase digestion was carried out and the DNA was reprecipitated with 0.4 vol. of 5 M NH_4Ac and 2 vol. of isopropanol (Maniatis *et al.*, 1982) before the DNA was used for nick-translation (Rigby *et al.*, 1977) or digestion with restriction enzymes.

When large preparations of plasmid DNA were required, the following modification of the method of Ish-Horowicz and Burke (1981) considerably improved the yield. The cells of a 250 ml culture, grown overnight in L-broth and ampicillin (200 μg/ml) at 37°C with vigorous shaking, are harvested at 4°C by centrifugation for 5 min at 7000 r.p.m. in a Sorvall GS-3 rotor and suspended in 20 ml of 50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA. The cells are lysed by mixing the suspension well with 40 ml of a freshly made 0.2 N NaOH, 1% SDS solution. After 5 min on ice, 10 ml of cold 3 M KAc, 2 M Ac are added and mixed well. After 15 min on ice, 5 ml of H_2O are added to the mixture which is centrifuged as before. The supernatant is poured carefully through filter paper, to remove small debris that did not pellet, into a measuring cylinder. The plasmid DNA is precipitated by mixing 48 ml of isopropanol with 80 ml of the filtered supernatant in a 250 ml bottle and pelleted as before in a Sorvall GSA rotor. After pouring off the supernatant, the pellet is dissolved in 10 ml of 10 x TE, 100 μg/ml RNase A and gently agitated for 30 min at 37°C. The DNA is precipitated again by adding 4 ml of 5 M NH_4Ac and 20 ml of isopropanol. After 10 min, the precipitate is pelleted for 10 min as before and the supernatant is discarded. The pellet, which is now much smaller due to the loss of RNA and protein, is dried for 5 min *in vacuo* and taken up in 3.8 ml of TE. For banding in a CsCl gradient, 4.0 g of CsCl are dissolved in this DNA solution and 0.2 ml of ethidium bromide (10 mg/ml) is added. The solution is transferred entirely to VTi65 quick seal tubes, which are filled to the neck with TE, sealed, and centrifuged at 20°C for 18 h at 47 000 r.p.m. in a Beckman VTi65 rotor. The DNA band of supercoiled plasmid DNA (which contains ~1–2 mg of DNA in the case of pAT153 plasmids) is removed with a syringe, separated from ethidium bromide according to standard methods (Maniatis *et al.*, 1982), precipitated with ethanol and dissolved in TE.

Phages were grown and phage DNA was prepared, as described by Maniatis *et al.* (1982).

Isolation of Drosophila DNA, preparation of genomic libraries and Southern blot analysis

Drosophila DNA was isolated from adults of the appropriate genotype according to the procedure of McGinnis *et al.* (1983) which was modified as follows. After the nuclei had been lysed with Sarkosyl and Proteinase K, the solution was extracted once with chloroform. The aqueous phase was removed and the DNA was precipitated by adding 0.4 vol. 5 M NH_4Ac and 2 vol. isopropanol. The precipitate was spun out, washed once with ethanol, dried and dissolved overnight in TE. The DNA was further purified by banding in a CsCl gradient run at 20°C in a Beckman 50Ti rotor for 50 h at 44 000 r.p.m. The DNA was dialyzed extensively against several changes of 0.1 x TE.

Genomic libraries were prepared in EMBL3 and EMBL4 from partial digestions of *Dfesc*^{10}*/b pr* DNA with *Sau*3A and ligation into the *Bam* sites of EMBL3 or EMBL4, replacing the *spi* fragment, as described by Frischauf *et al.* (1983).

Overlapping *Drosophila* sequences in phages isolated from genomic libraries were mapped by *Eco*RI digestion of the appropriate recombinant phage DNAs, bidirectional (Smith and Summers, 1980) Southern transfer of the fragments from agarose gels to nitrocellulose papers (Southern, 1975) and subsequent hybridization with nick-translated probes (Rigby *et al.*, 1977) in hybridization buffer at 42°C. Hybridized filters were washed in 2 x SSC, 0.1% SDS at 65–68°C. Restriction maps thus obtained were always verified by restriction analysis of genomic DNA transferred to nitrocellulose filters and hybridized with radioactive probes in the same manner. The proper location on polytene chromosomes of the isolated seqeunces was further confirmed by *in situ* hybridization.

In situ hybridization to polytene chromosomes

In situ hybridizations to polytene chromosomes were carried out with biotinylated probes according to the method of Langer-Safer *et al.* (1982). Materials used for this procedure were bio-dUTP with an 11-carbon linker (a gift from M. Mlodzik), goat anti-biotin IgG from Enzo Biochemicals, and rabbit anti-goat antibody conjugated to horseradish peroxidase from DAKOPATTS.

Preparation of poly(A)$^+$ RNA and localization of transcripts on Southern blots of recombinant phage DNAs

Poly(A)$^+$ RNA was prepared from follicles, mass-isolated as described by Petri *et al.* (1976), and from adult males according to the procedure of Chirgwin *et al.* (1979). Radioactive cDNA was synthesized with AMV reverse transcriptase (Stehelin & Cie, Basel) from poly(A)$^+$ RNA using oligo(dT) primers according to standard methods (Maniatis *et al.*, 1982) as modified by G.McMaster (personal communication). The labeled cDNA was denatured for 5 min at 100°C and hybridized for 4 days with Southern blots of *Eco*RI fragments of recombinant phage DNAs in hybridization buffer in the presence of 50 μg/ml of poly(A)$^+$ at 42°C.

Fly stocks and culturing

Frequently in the text only the *esc* genotype and the balancer chromosome are mentioned without referring to the linked additional markers on the second chromosome. *Drosophila* strains carrying the following second chromosomes were used (the abbreviated notation is indicated in parenthesis). The mutant strains *CyO, dp*^{lvl} *esc*^{2} *pr cn*^{2}*/dp*^{ov} *Df(2L)esc*^{10} *b pr cn Dp(2;2)GYL bw (CyO, esc*^{2}*/Dfesc*^{10} *DpGYL); dp*^{ov} *esc*^{6} *b cn bw/CyO, dp*^{lvl} *pr cn*^{2} *(esc*^{6}*/CyO); Df(2L)esc*^{10}*, b pr/CyO, dp*^{lvl} *pr cn*^{2} *(Dfesc*^{10}*/CyO); CyO, dp*^{lvl} *esc*^{2} *pr cn*^{2}*/dp*^{ov} *b cn Dp(2;2)GYL, esc*$^+$ *bw (CyO, esc*^{2}*/DpGYL)* were kind gifts of Peter Lawrence and Michael Ashburner [the *esc* mutants were constructed by Gary Struhl (1981)], and the mutant *In(2L)t, esc*^{1} *c sp/SM5 (esc*^{1}*/SM5)* was provided by Rolf Nöthiger. The strains *CyO, dp*^{lvl} *pr cn*^{2} *1(2)100*^{DTS}*/dp*^{ov} esc^6 *b cn bw (CyO, 1(2)100*^{DTS}*/esc*^{6}*)* and *CyO, dp*^{lvl} *pr cn*^{2} *1(2)100*^{DTS}*/In(2L)t, esc*^{1} *c sp (CyO, 1(2)100*^{DTS}*/esc*^{1}*)* were established in simple crosses of the corresponding stocks above with a strain carrying the dominant temperature-sensitive balancer chromosome *CyO, 1(2)100*^{DTS} received from Walter Gehring. The two *paired* deficiency mutants *Df(2L)prd*$^{1.7.20}$ *b pr cn sca/CyO* and *T(2L;Y) Df(2L)prd*$^{1.25.40}$ *b pr cn sca* (in which the entire left arm of the second chromosome is translocated to the Y-chromosome) were generously supplied by Christiane Nüsslein-Volhard.

Flies were maintained at 18–25°C on a cornmeal, sucrose, dried yeast, agar medium supplemented with Nipagin and seeded with a suspension of live yeast.

P-element-mediated gene transfer

Microinjection of C2.1.5(Car4) DNA (400 μg/ml) together with the helper P-element pπ25.7wc (100 μg/ml) (Karess and Rubin, 1984) into the posterior pole of cleavage-stage embryos was carried out as described by Spradling and Rubin (1982).

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