The EMBO Journal Vol.2 No.11 pp.2027–2036, 1983

Genomic and cDNA clones of the homeotic locus *Antennapedia* in *Drosophila*

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Communicated by W.J.Gehring
Received on 18 July 1983

Homeotic genes are involved in the control of developmental pathways: dominant mutations at the *Antennapedia* locus of *Drosophila*, for example, lead to replacement of the antennae on the head of the fly by mesothoracic legs. Using a combination of chromosome walking and jumping, we have cloned a DNA region from *Drosophila* containing *Antennapedia*. Five DNA inversion rearrangements which are associated with the *Antennapedia* mutant phenotype were localized within a 25-kb region. Genomic DNA sequences from this area were used as hybridization probes to screen cDNA libraries prepared from *Drosophila* embryonic and pupal poly(A)$^+$ RNA. A 2.2-kb cDNA sequence (903) was isolated which appears to derive from at least four non-contiguous chromosomal regions that span 100 kb. It includes the positions of the inversion breakpoints. A second cDNA of 2.9 kb (909) is composed of sequences from at least three chromosomal regions, two of which are similar or identical to sequences contained in the 903 clone but the third is derived from genomic DNA within a putative 903 intron. The unusual size and complexity of this locus are discussed.

Key words: homeotic genes/chromosome walking and jumping/cDNA clones/mutation changes in DNA/genome organization

Introduction

The development of multicellular organisms is based on a program of differential expression of genetic information. Recently, considerable progress has been made towards understanding the genome organization of eukaryotes. So far, the molecular analysis of gene expression has largely been confined to genes whose products are involved in the basic housekeeping functions of all cells or to genes coding for products that appear only in specialized cell types. These differentially expressed genes, however, are active in cells already committed to particular functions. Little is known about how the activity of such genes is spatially and chronologically controlled and how commitments to certain developmental pathways are made.

One developmental process which requires such basic determinative decisions is body segmentation. A number of loci in *Drosophila* are necessary for the subdivision of blastoderm cells into groups that are each committed to becoming a different segment (Nüsslein-Volhard and Wieschaus, 1980). Each segment goes on to develop characteristic structures, for example hairs and sensilla or organs like antennae or wings. The homeotic mutations define genes which are involved in specifying the distinct structures associated with each of the body segments (for a review see Ouweneel, 1976). Because homeotic mutations generally lead to the transformation of one normal body part into another, it is deduced that the normal gene (from now on called a homeotic gene) functions to promote the formation of one normal structure or set of structures over another.

The most extensive genetic analyses of homeotic genes have been carried out on the *bithorax* gene complex, a cluster of loci which promote development of the posterior fly segments (Lewis, 1963, 1978; Duncan and Lewis, 1982). A second cluster of homeotic genes appears to be responsible for promoting the development of anterior segments in a manner similar to the *bithorax* gene complex (Kaufman *et al.*, 1980; Lewis *et al.*, 1980), and one member of this cluster is named *Antennapedia (Antp)*. The *Antp* locus was initially identified by a number of dominant mutations which show a transformation of the adult antennae into second legs in heterozygous (*Antp/Antp*$^+$) individuals (Le Calvez, 1948; Hannah and Strömnaes, 1955; Lewis, 1956; Falk, 1964).

Several lines of evidence indicate that the dominant phenotype represents a new function for the altered gene. Deletion of the *Antp* locus does not result in the dominant mutant phenotype (deletion/*Antp*$^+$ flies have normal antennae). Secondly, *Antp* is dominant in triploids (*Ant*/+/+ flies have the transformed phenotype; Tottoli, 1977; Bulyzhenkov and Ivanov, 1980). Finally, the dominant mutant phenotype can be reverted by deletion (Denell, 1972, 1973; Duncan and Kaufman, 1975; G.Jürgens, personal communication). From this evidence, *Antp* is a gain of function mutation in the terminology of Lewis (1978). A second phenotype, also characteristic of *Antp* alleles, is associated with a recessive lethality and appears to be a loss of function mutation. *Antp* mutants are generally recessive lethals at late embryonic or early larval stages and show a partial transformation of the mesothoracic and metathoracic segments towards the prothoracic segment (Wakimoto and Kaufman, 1981).

Mutation of the *Antp* gene need not result in a stock with both the dominant phenotype and the recessive lethality. Several mutants have been isolated (*Antp* recessive) which share the *Antp* recessive lethality but have no dominant adult transformation (Wakimoto and Kaufman, 1981; Jürgens *et al.*, in preparation). Conversely, two dominant mutations called *Nasobemia* (*Ns*, Gehring, 1966) and *Antp*^{72j} (Baker, 1974) have been found which show an antenna to leg transformation but are homozygous viable. Finding these mutations indicates that the two *Antp* functions, one dominant and one recessive, are separable. Two other dominant homeotic mutations, *Extra sex combs (Scx)* and *Cephalothorax (Ctx)*, share the *Antp* recessive lethality but transform completely different body parts than does *Antp* (Hannah-Alava, 1958; Duncan and Lewis, 1982). Here we have used the original designations for each of the mutants rather than make them all *Antp* alleles. Our judgement rests on the fact that *Antp* and *Ns* complement fully for the *Antp* recessive lethality (Tottoli, 1977), that there is no conclusive allelism test for dominant mutations, and that phenotypic revertants of *Ns* have been isolated which are not *Antp*$^-$. A summary

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Table I. Mutations associated with the *Antennapedia* locus and their phenotypes

Mutation	Symbol	Dominant adult transformation	Recessive embryo transformation	Viability[b]	Reference
Antennapedia	Antp	antenna → leg 2	T2 + T3 → T1[a]	L	1
Extra sex combs	Scx	leg 2 + leg 3 → leg 1	T2 + T3 → T1	L	1
Cephalothorax	Ctx	head → mesopleura; eye → wing	not studied	–	2
(Antennapedia) recessive lethal	$Antp^{rec}$	none	T2 + T3 → T1	L	1,3
Nasobemia	Ns	antenna → leg 2; head[c] → sterneopleura	none	V	4
Humeral	Hu	Hu/+ → extra bristles; Hu/Hu → new bristles	none	V	5

[a]T1, T2 and T3 are the pro-, meso- and metathoracic segments.
[b]L = lethal, V = viable.
[c]Prefrons and vibrissae.
References: (1) Wakimoto and Kaufman (1981); (2) Duncan and Lewis (1982); (3) Jürgens *et al.* (1983); (4) Gehring (1966); (5) Lewis *et al.* (1980).

of the mutations associated with the *Antp* locus is presented in Table I.

From the genetic studies, it can be concluded that the *Antp* locus is complex and can be mutated to generate a variety of distinct phenotypes that are homeotic in nature. To elucidate the structure of this locus and to understand how homeotic loci specify the development of distinct anatomical segments, we have cloned a chromosomal DNA region including the *Antennapedia* gene.

Results

Strategy for cloning Antennapedia

Antennapedia (Antp) mutations define a gene for which nothing is known about final products or modes of action. What is known is the cytogenetic location of *Antp*. *D. melanogaster* fly stocks genetically mutant at the *Antp* locus and which have grossly rearranged chromosomes consistently show lesions of the 84B1-2 chromomere on the right arm of chromosome 3 (Kaufman *et al.*, 1980). When this project was initiated, no cloned sequences coming directly from 84B1-2 were available. To obtain these sequences, we began with clones derived from the 84F1-2 chromomere, at least 10 cytogenetic bands or several thousand kilobase pairs away from *Antp* (Figure 1). While this distance is too great for reasonable chromosome walking (Bender *et al.*, 1983), the gap was greatly shortened by making use of the inversion mutation *In(3R)Hu* which joines 84F sequences directly to 84B sequences. This mutation is associated with the phenotype *Humeral (Hu)* and is the result of the simultaneous inversion of two adjacent regions of chromosome 3 having breakpoints localized to 84B2-3, 84F2-3 and 86BC (Lindsley and Grell, 1968). As shown in Figure 1, we collected a series of overlapping clone sequences from the 84F region until sequences corresponding to the 84F2-3 end of the first *Hu* inversion were reached. This DNA was used as a probe to screen a genomic DNA library prepared from *In(3R)Hu* DNA. In this way we isolated phages which contained the DNA breakpoint fragment in which 84F sequences were fused to those from 84B. The 84B portion of such hybrid phages provided a probe which was used to start a second chromosome walk that eventually included the *Antp* region.

Cloning and characteristics of the 84F region

Two previously cloned sequences were used to initiate the project. p146E6 was selected from a plasmid gene library using an embryonic poly(A)$^+$ mRNA probe (Steward, 1978), and p14C4 was isolated from the same library with a tRNA probe (Steward, 1978; Gehring, 1978; Dudler *et al.*, 1980). Both were localized by *in situ* hybridization to the 84F1-2 band and inside the first *Hu* inversion (see Figure 1). Because hybridizations including both plasmid probes gave a single signal, we could not determine which plasmid lay closer to the inversion breakpoint at 84F2-3. Therefore, chromosome walking was carried out in both directions from each of these plasmids until two of the four walks joined. The walk was then continued from the two remaining ends until ~250 kb were collected (Figure 2). Except where mentioned, we used the lambda library derived from *Drosophila* Canton-S strain DNA (Maniatis *et al.*, 1978). In total, this walk encompassed

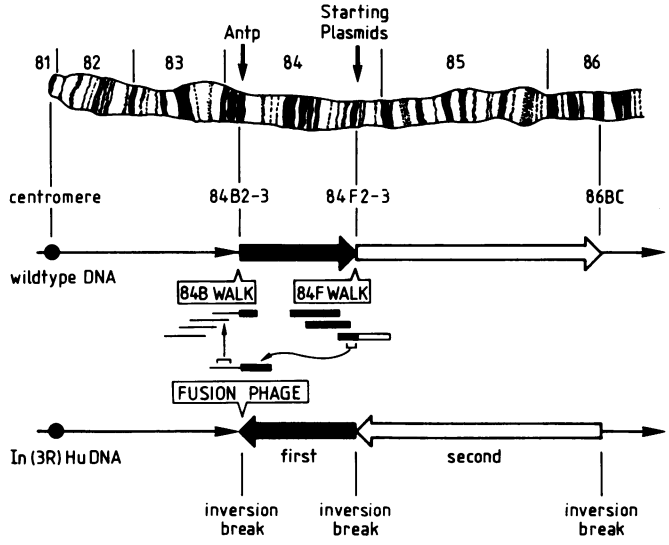


Fig. 1. Strategy for cloning *Antennapedia*. Starting with plasmids mapping to 84F1-2, a chromosome walk yielded overlapping DNA segments until the 84F2-3 breakpoint for the inversion *In(3R)Hu* was reached. A probe from the 84F clone just to the left of the breakpoint was used to select hybrid phage from a library constructed from homozygous *In(3R)Hu* DNA. A new probe derived from the 84B portion of a fusion phage was then used to select 84B clones. From these a chromosome walk in 84B was initiated until *Antp* was reached. The upper drawing represents the banding pattern of the proximal portion of chromosome 3R. The orientation of two stretches of DNA are shown in the wild-type configuration (upper) and in the doubly-inverted *In(3R)Hu* configuration. The three *In(3R)Hu* breakpoints are marked at the bottom.

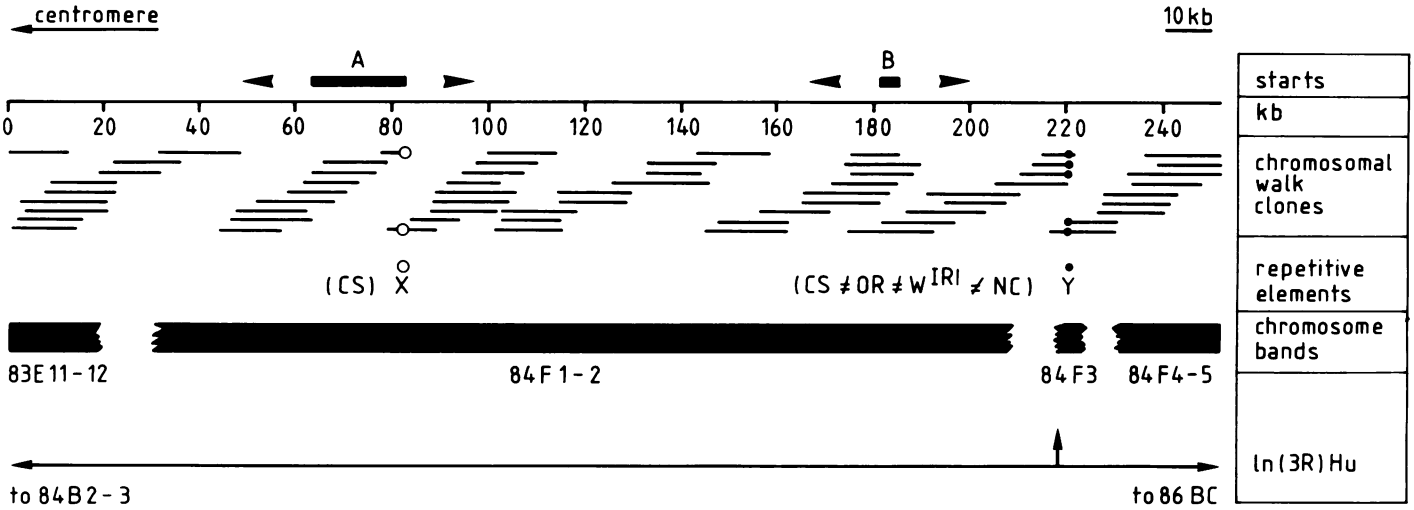


Fig. 2. The 84F chromosome walk. Starting with the plasmids **A** (p14C4) and **B** (p146E6), ~250 kb of genomic DNA lambda clones were collected by chromosome walking. Two regions (X and Y) are insertion sites for mobile repetitive elements in Canton-S DNA. The X element is B104 or roo (Scherer *et al.*, 1982; Meyerowitz and Hogness, 1982) by DNA–DNA hybridization with sequences provided by these authors. The Y position is filled by a different element in each of four strains tested (Canton-S, Oregon-R, w^{IR1} and a North Carolina wild isolate). It was jumped by isolating a cosmid clone from a library constructed in the laboratory of D.Ish-Horowicz. The 84F walk spans the 84F1-2 chromosome doublet band (which contains ~170 kb of DNA) and extends into 84E11-12 on the left and through 84F3 into 84F4-5 on the right. The interbands represent only 10–15 kb. The *In(3R)Hu* inversion breakpoint is located very close to the Y repetitive element insertion site at 84F3.

the 84F1-2 band, one very small band (84F3) and a little of each of the next adjacent bands on both sides. Two repeated element sequences were found in the 84F DNA of the Canton-S strain. Using labeled cDNA as probes, 12 different transcription units were detected within the sequences from the 84F1,2 band (data not shown). As only the most abundant units are detected by this method, the figure is a minimum estimate.

Jumping from 84F to 84B

Mapping the *In(3R)Hu* breakpoint at 84F on the DNA map permitted the isolation of 84B sequences as described below. A new lambda library was constructed incorporating DNA from homozygous *In(3R)Hu* flies, and it was screened with the 84F sequence containing the inversion breakpoint (Figure 1). The probe, a *Sal-Kpn* fragment from phage 157 of the Canton-S library (Figure 3), selected phage 331 from the *In(3R)Hu* library. Restriction enzyme sites in the right three-fourths of 331 align (although there are strain differences) with the Canton-S sites of phage 157 and 154, but in the left portion the sites are new. Thus, clone 331 was presumed to contain the inversion fusion fragment, and the lefthand sequences were expected to come from 84B. The 2-kb *Eco*RI fragment from 331 was used to screen the Canton-S library, and among the phage isolated was 339 which maps to 84B by *in situ* hybridization.

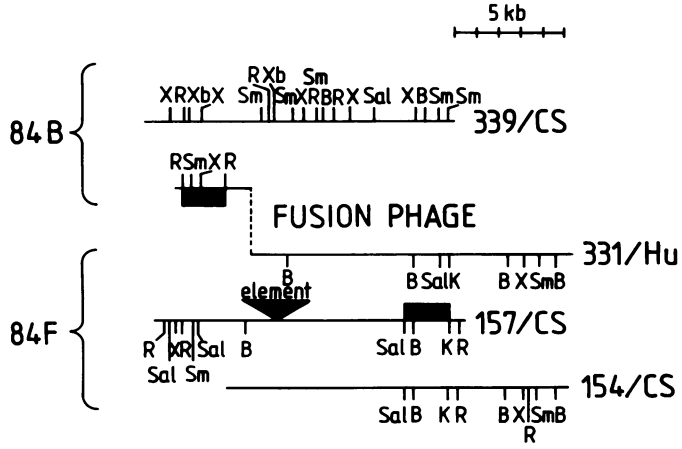


Fig. 3. The jump from 84F to 84B. The phages involved in the cloning jump from 84F to 84B are diagrammed. Overlapping Canton-S phages 154 and 157 derive from 84F sequences. The 2-kb *Sal*-K fragment from 157 (marked as a heavy bar) was used to select the phage 331 from the *In(3R)Hu* library. 331 contains the 84F2-3/84B2-3 breakpoint fusion near its left end. Its leftmost sequences do not align with the 84F restriction enzyme sites of 157, suggesting an 84B origin. The R-R fragment of 331 was used to select phage 339 from the Canton-S library. By *in situ* hybridization to chromosomes, 339 was shown to come from 84B. R = *Eco*RI, X = *Xho*I, B = *Bam*HI, Sm = *Sma*I, Sal = *Sal*I, K = *Kpn*I, Xb = *Xba*I. CS = Canton-S, *Hu* = *In(3R)Hu*.

Cloning the 84B region

Starting with the 339 Canton-S clone from 84B, walking was continued in the wild-type DNA library in order to find *Antp* (Figures 4 and 5). Phage 339 (at position 0) was found by *in situ* hybridization to be to the right of the 84B breakpoints of four *Antp* alleles which are associated with inversions, $Antp^{73b}$, $Antp^{B}$, $Antp^{R}$ and $Antp^{LC}$. Thus, the main walk was directed towards the centromere. However, because flies with the *In(3R)Hu* mutation were shown to interact with *Antp* mutants (Lewis *et al.*, 1980), indicating that the inversion might affect part of the *Antp* gene, a small walk was continued to the right from clone 339 for 40 kb. These sequences were tested for homology with a radiolabeled probe of DNA complementary to unfertilized egg poly(A)$^+$ RNA made available by B.Mechler and E.Hafen. Two extremely strong hybridization signals were mapped near position −40 kb (Figure 4). By cross-hybridization with an identified α-tubulin gene obtained from J.Natzle and B.J.McCarthy (clone pα3-3), one strong signal was found to correspond to the α-tubulin gene previously localized to 84B-C (Mischke and Pardue, 1980, 1982; Sanchez *et al.*, 1980; Kalfayan and Wensink, 1981). As reported by Kalfayan and Wensink (1981) and Mischke and Pardue (1982), there is a repetitive element located adjacent to this α-tubulin gene in Canton-S

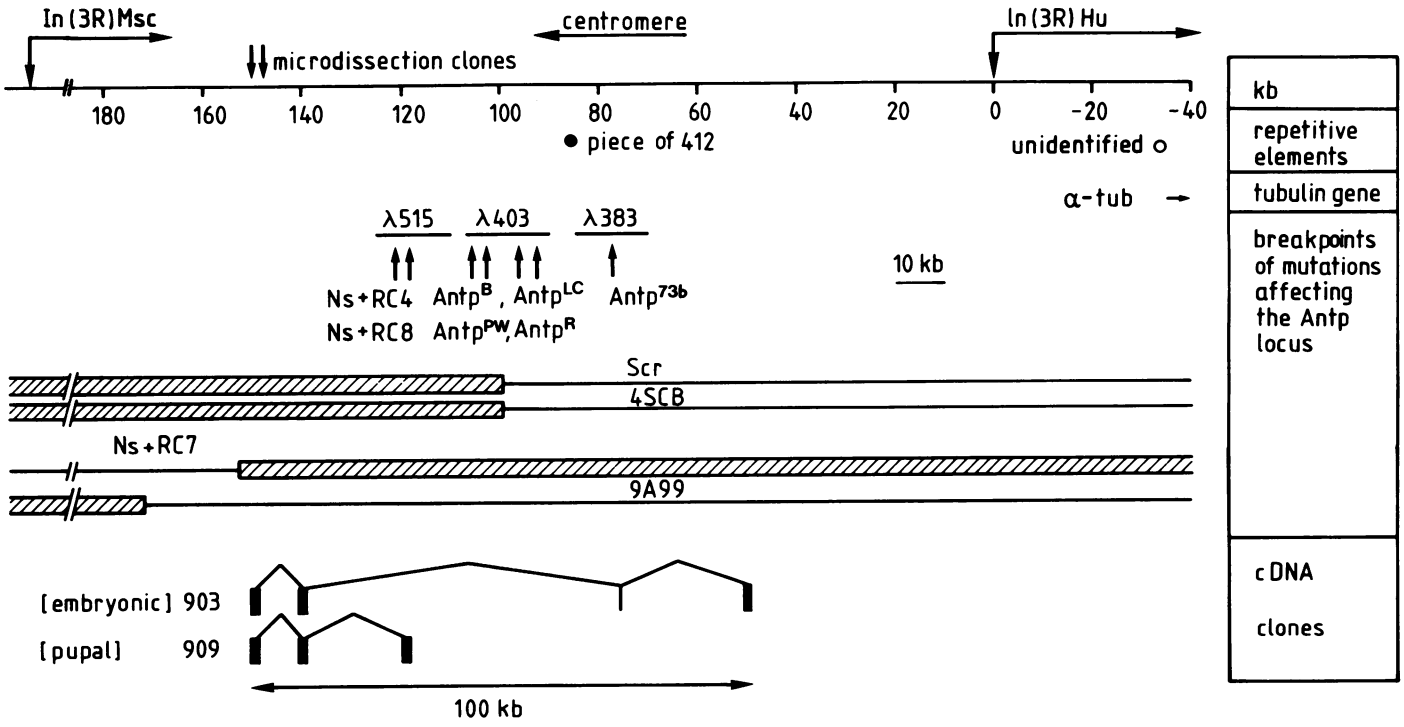


Fig. 4. The 84B chromosome walk. The jump from 84F with *In(3R)Hu* landed at position 0 in 84B1-2. To the right at −35 kb is an unidentified repeated element in Canton-S DNA followed closely by an α-tubulin gene. DNA segments isolated by walking extended leftward from the *In(3R)Hu* breakpoint were connected with walks initiated from clones obtained by microdissection until ~240 kb were collected. A partial 412 repetitive element is inserted in Canton-S but not Oregon-R DNA at position 85. Clone 403, from position 100, spans the 84B breakpoints for four Antp inversions ($Antp^B$, $Antp^{LC}$, $Antp^{PW}$ and $Antp^R$). Clone 383 spans the left $Antp^{73b}$ inversion breakpoint. The breakpoints for two *Ns* phenotypic revertants occurs at position 118 (*Ns+RC4* is an inversion and *Ns+RC8* is a translocation). The extents of three deletions affecting *Antp* were mapped, and solid lines indicate sequences remaining. *Scr* and *4SCB* delete sequences to the left while *Ns+RC7* removes sequences to the right. Deletion *9A99* genetically complements all *Antp* alleles tested and its endpoint defines the maximum leftward extent of *Antp*. The genomic sequences which are found joined together in two cDNAs are drawn showing the putative exon (blocks) and intron portions. Clone 903 derives from poly(A)$^+$ RNA of embryos and 909 of pupae. Our walk ends at ~190 kb, still to the right of the *In(3R)Msc* breakpoint.

DNA. This is the second transcript we see.

The walk leftwards from the *In(3R)Hu* breakpoint towards the *Antp* inversion breakpoints was joined by walks from several new 84B entry points. These new sequences, derived by microdissection of the 84B1-2 band and subsequent insertion of the DNA into a lambda vector (as described generally in Scalenghe *et al.*, 1981), were made available by V.Pirrotta. Several mapped by *in situ* hybridization between the *Antp* inversion breakpoints and the next lethal complementation group to the left for which we had a chromosomal rearrangement. The latter locus was defined for our analysis by *Multiple sex combs (Msc)*, a mutation associated with a chromosome inversion. Joining these independent walks yielded a total of ~230 kb of sequenced clones from the 84B region (Figures 4 and 5). As five different *Antp* inversion breakpoints lay in between the microdissected clones and the *In(3R)Hu* breakpoint, joining these segments resulted in the cloning of at least a portion of the *Antp* locus. The leftmost end of the walk still lies to the right of the *In(3R)Msc* breakpoint.

A second repetitive element was found in the 84B region at position 86 (Figures 4 and 5). By DNA−DNA hybridization it was shown to have sequence homology with plasmids cDm2046 and cDm2066 (Young, 1979) and cDm412 (Rubin *et al.*, 1976; Will *et al.*, 1981) all of which contain repeated elements in the 412 family. As it is smaller than the complete element and lacks many of the characteristic restriction enzyme sites, we consider this to be a partial 412 element.

Localization of Antp

A crude localization of the *Antp* locus was made by *in situ* hybridization of clones from the walk to chromosomes of five *Antp* alleles, all identified by cytological examination as inversions. Figure 6 lists the chromosome mutations used. Note that some of the rearrangements are newly described or represent new determinations of breakpoints. The allele $Antp^{LC}$, previously thought to be lost (Lindsley and Grell, 1968), was rediscovered during the cytological examination of each fly stock obtained from our various sources. An allele not previously reported in the literature, $Antp^{PW}$, was recovered from a methoxydiethylnitrosamine mutagenesis experiment and made available for our work by S.Pinchin.

Different phage clones from the walk were tested as to whether they originated from sequences to the left of, to the right of, or spanning each of the *Antp* inversion breakpoints. Around position 100 of the walk, a single lambda clone (403, see Figure 4) was identified which spans breakpoints for four *Antp* mutants assayed: $Antp^B$, $Antp^{LC}$, $Antp^{PW}$ and $Antp^R$. Clone 383, ~20 kb away from 403, spans the $Antp^{73b}$ inversion breakpoint. The *in situ* hybridization result for $Antp^R$ is shown in Figure 7A. The two positive visible signals correspond to each end of the $Antp^R$ inversion. The balancer chromosome was not paired with the *Antp* chromosome, and its 403 homology region was not photographed. Also shown are two *in situ* hybridizations to $Antp^{73b}$. One is with clone 383, a probe which spans the 84B breakpoint of this inversion (Figure 7B). The second is with clone 355, which is to the

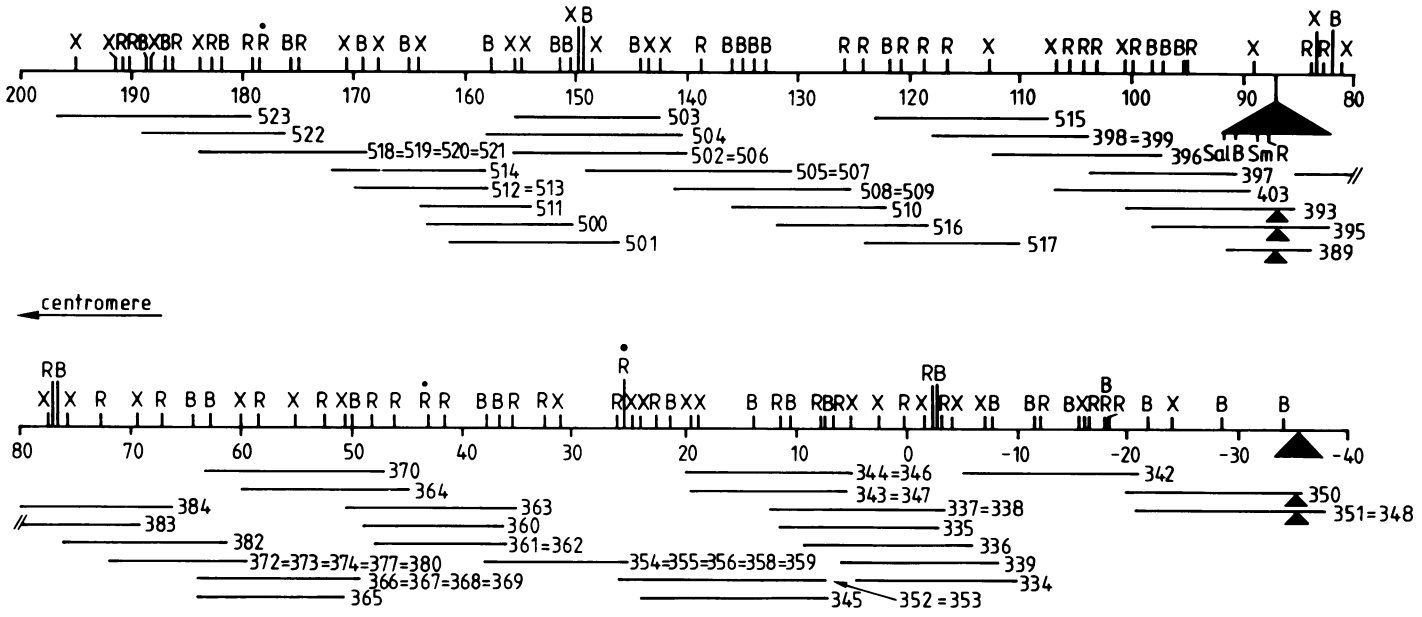
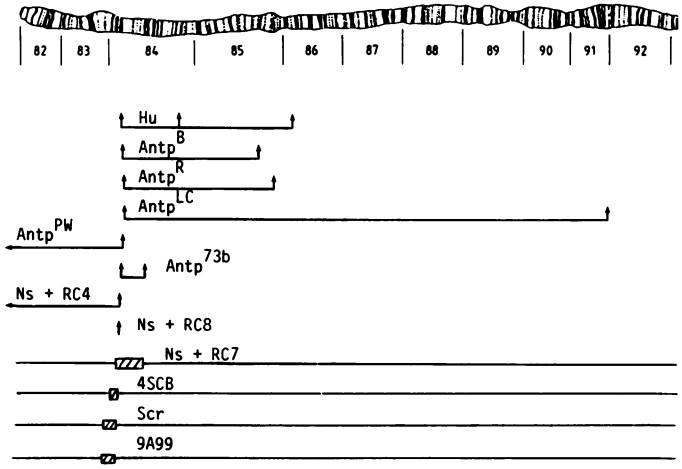


Fig. 5. The 84B walk. The base line indicates the restriction enzyme sites in Canton-S DNA positioned on the kilobase map for the 84B1-2 walk, and the individual phages cloned are indicated below. Two repetitive element sequences are indicated, one at position −35 and the other at ~86. In the 140 to 170 kb region the *Eco*RI sites were too numerous to be mapped. B = *Bam*HI, X = *Xho*I, R = *Eco*RI, R̂ = *Eco*RI site is ambiguous and not mapped further. Small fragments <0.5 kb were not mapped.



MUTATION	CYTOLOGY	REFERENCES
Hu	In(3R)84B2-3;84F2-3;86B4-C1	1
$Antp^{B}$	In(3R)84B1-2;85E	2
Ant^{R}	In(3R)84B1-2;85F	3
$Antp^{LC}$	In(3R)84B1-2;91F-92A	3
$Antp^{PW}$	In(3LR)71F;84B1-2	3
$Antp^{73b}$	In(3R)84B1-2;84D1-2	3
Ns+RC4	In(3LR)74F-75A;84B1-2	3
Ns+RC8	T(2;3)41;84B1-2	3
Ns+RC7	Df(3R)84B1-2;84D	3
4SCB	Df(3R)84B1-84B2	3
Scr	Df(3R)84A1-84B2	2
9A99	Df(3R)84A1-84B2	3

Fig. 6. Chromosome rearrangements used in this study. Below the drawing of a portion of chromosome 3R are indicated the mutations we used plus a table giving the type of rearrangement and its cytology. In = inversion; T = translocation; Df = deletion. The references are (1) Lindsley and Grell (1968); (2) Kaufman *et al.* (1980); (3) Determinations reported for the first time or corrected from previous publications in this paper. See Figure 9 for cytological preparations.

right of 383 on the DNA map and which falls within the inversion (Figure 7C). The cytology of $Antp^{73b}$ has been difficult to determine conclusively (Kaufman *et al.*, 1980; Lewis *et al.*, 1980), but the nature of the rearrangement as an inversion is clearly indicated by the *in situ* hybridization assay.

Using the lambda clones from the walk and the method of *in situ* hybridization to chromosomes, rough localizations were made for the breakpoints of other mutations which affect the *Antp* locus. Two phenotypic revertants of *Ns*, designated *Ns+RC4* and *Ns+RC8* (Struhl, 1981), have been determined to be an inversion and a translocation, respectively (Figure 6). Both map on the chromosomal DNA 10−20 kb to the left of the main *Antp* inversion breakpoint region (Figure 4).

The extents of several deletion (Df) mutations which fail to complement the *Antp* recessive lethality were also determined. *Df(3R)4SCB* (G.Jürgens, unpublished data; Struhl, 1981) and *Df(3R)Scr* (Sinclair, 1977; Kaufman *et al.*, 1980) also delete several genes to the left of *Antp*. As seen in Figure 4, both end around the clone 403 *Antp* inversion breakpoint region. *Df(3R) Ns+RC7* is a phenotypic revertant of *Ns* isolated by Struhl (1981) which by genetic assay removes *Antp* and genes to the right of it. This deletion terminates within clone 501 at position 154 kb on the DNA map. Thus, 10 lesions which in some way disrupt *Antp* activity all have chromosome breaks in the 84B region we have cloned and they fall between adjacent genetic loci to the left and right which are different from *Antp* (*Msc* and α-tubulin, respectively).

By hybridization of walk clones to genomic DNA blots, we also mapped an X-ray induced deficiency which leaves *Antp* activity intact but removes genes to the left of *Antp*. *Df(3R)9A99* (G.Jürgens, unpublished data; see Figures 6 and 9) ends within clone 518 between positions 170 and 180 kb on the walk, leaving most of the 84B chromosome walk DNA present (Figure 4). The end of this deletion presently defines the maximum extent of the *Antp* locus on the left side of our map.

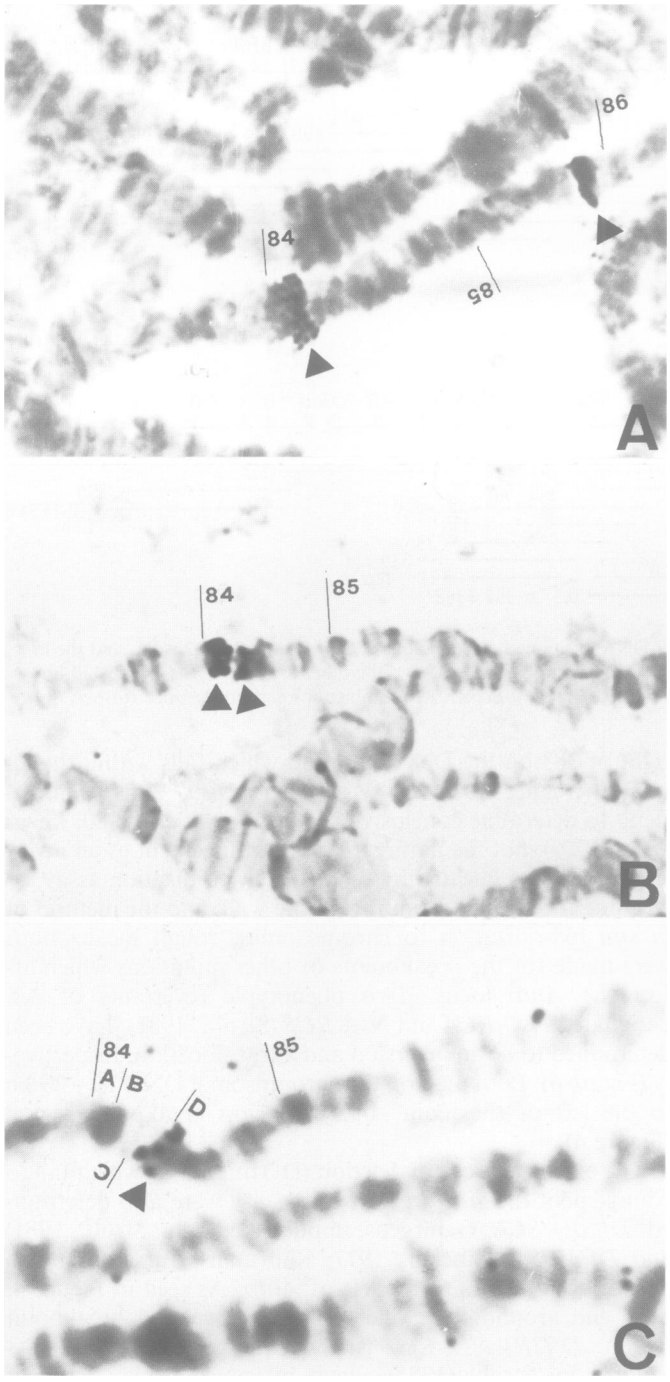


Fig. 7. *In situ* hybridizations of chromosomal DNA clones to *Antp* inversions. Hybridization of clone 403 to both ends of the *In(3R)Antp*^{R} rearrangement **(A)** and clone 383 to *Antp*^{73b} **(B)**. Hybridization of clone 355 to *Antp*^{73b} **(C)**. The **(B)** and **(C)** results indicate that *Antp*^{73b} contains an inversion between 84B and 84D1-2. The probes in **(A)** and **(B)** were labeled with biotinylated nucleotide, hybridized to chromosomes and detected by an antibody procedure involving a final horseradish peroxidase enzyme reaction yielding dark deposits (see Materials and methods). The **(C)** probe was labeled with tritiated nucleotides and detected by autoradiography.

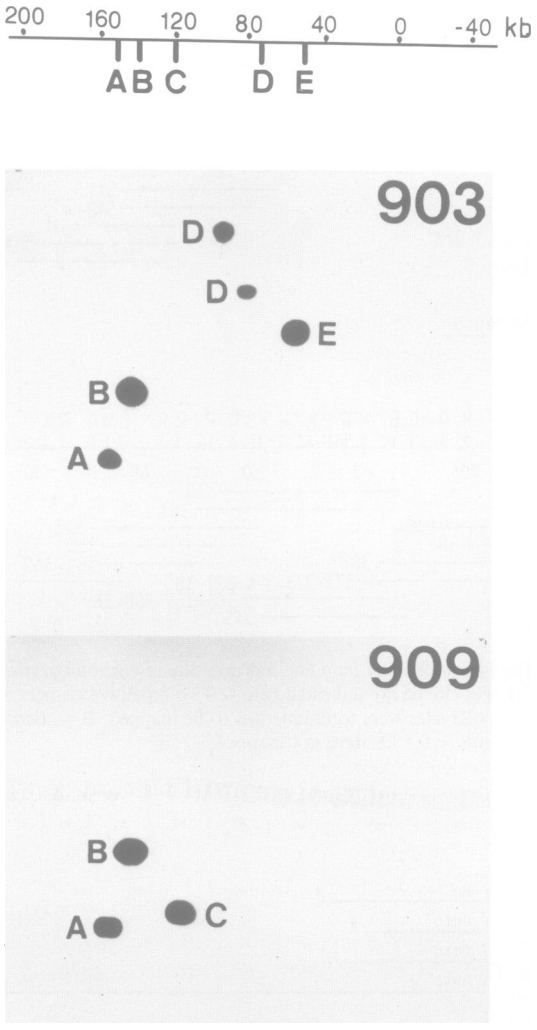


Fig. 8. Mapping of the *Antp* cDNA sequences on the chromosomal DNA. Twenty-one λ genomic DNA clones, representing the entire 240-kb walk in 84B, were digested with restriction enzymes and run on gels. The insert portions of λ cDNA clones 903 **(upper)** and 909 **(lower)** were labeled by nick-translation and hybridized to nitrocellulose blots of these gels. 903 hybridized mainly to four regions (A,B,D and E). A and E are separated by ~100 kb. The two signals labeled D result from positive hybridization to the same sequence contained in two overlapping λ clones. 909 hybridized to three genomic regions (A,B and C) spanning ~37 kb. The kilobase scale at the top corresponds to that used in Figure 4 and 5.

cDNA clones to Antp

To define better the dimensions of the *Antp* locus, we searched for sequences within the 84B walk that specify putative *Antp* transcripts. This was done by screening two cDNA libraries constructed from poly(A)$^{+}$ RNA of embryos (1.5–5 h) and pupae (1 day) by M.Goldschmidt-Clermont. Hybridization probes were derived from the genomic walk clones starting with sequences adjacent to the *Antp* inversion breakpoints. Several cDNA clones were isolated and two will be described here. Both were digested with *Eco*RI endonuclease to excise the cDNA insert (*Eco*RI linkers join the cDNA to the lambda vector), and the fragment was purified by gel electrophoresis and labeled by nick-translation. These probes were hybridized to restriction endonuclease digestion fragments of the 84B walk DNAs following agarose gel electrophoresis and blot transfer to nitrocellulose paper. By this procedure the cDNA sequences, presumably representing *Antp* transcripts, were mapped on the chromosomal DNA (Figure 8).

The lower portion of Figure 4 summarizes the genomic mapping data for the embryonic and pupal cDNA clones. The 2.2 kb long embryonic clone (903) will be discussed first. Within the 240-kb walk at 84B, 903 has strong homology with

four non-contiguous genomic DNA fragments located at positions 50, 76, 141 and 150 kb on the map. Thus, the most distant fragments with homology to the 903 cDNA are separated by ~100 kb, and all the *Antp* mutation inversions break within the 903 unit. The four genomic blocks with sequence homology to 903 are not closely related. Probes were made from three plasmid subclones which represent the left, central and right thirds of 903, and each shows a unique hybridization pattern to the genomic clones and fails to cross-hybridize with the other subclones. Two pieces of evidence support the co-linearity of the cDNA clone and cloned genomic sequences. The three cDNA subclones, left, central and right, have genomic homology sequences in the identical order. In addition, the order of characteristic restriction enzyme sites in the 903 cDNA clone matches the order of the corresponding sites in the homologous genomic fragments. We propose that the 903 cDNA clone corresponds to an RNA derived from at least four genomic exons that are separated by large introns. The transcription unit would appear to be at least 100 kb.

The 909 cDNA clone was isolated from the pupal cDNA library. As depicted in Figure 4, it also originates from several separate genomic regions. Based on DNA-DNA hybridization and a comparison of restriction enzyme sites, the 909 clone contains sequences which are very similar or identical to the left two exons found in 903 (at 141 and 150 kb). A third 909 exon comes from a new region (at 120 kb), and lies within a putative intron of the 903 transcript. The transcription unit from which 909 derives appears to be at least 37 kb in length. The 909 cDNA sequence described here is 2.9 kb long and has *Eco*RI ends (the cDNAs were treated with *Eco*RI methylase and then inserted into the vector with *Eco*RI linkers). The original 909 clone we isolated is actually 3.5 kb long and consists of two *Eco*RI fragments. Although the second fragment has homology at the 120 kb position of the 84B walk, other properties prevent us from concluding whether it is artifactual or not. The detailed study of this fragment is in progress.

Thus far, the *Antp* gene is associated with a region of at least 100 kb. Within this unit the cDNA clones 903 and 909 identify five exons. Our mapping of the exon blocks has been limited to a resolution of 1–2 kb, and each could represent several small blocks of homology. Under the hybridization conditions of our chromosome *in situ* hybridization method, the 903 clone hybridizes only to the 84B1-2 band (M.Levine, personal communication). However, small homology regions outside the 240-kb walk would not have been detected. Under stringent hybridization conditions, weak homology with both the 903 and 909 cDNA probes was detected at position 190 kb. In addition, there is sequence homology between internal portions of the 909 clone at positions 120 and 150. These findings are being investigated further.

Discussion

We have described the isolation of a large region of *D. melanogaster* genomic DNA which encompasses most if not all of the homeotic gene *Antennapedia*. Initially, *Antp* was defined as a phenotype common to certain inversions which break in the 84B1-2 chromosome band. The breakpoints of five *Antp* inversion mutations have been located in an area of 20–30 kb, larger than most normal genes. One possibility for the generation of an *Antp* phenotype is that each inversion breaks within the *Antp* gene and disrupts normal function. Another is that each inversion might have the property of bringing the *Antp* locus into the proximity of new DNA sequences which alter its activity. We also found a piece of a 412 transposable element within the chromosomal region affected by the *Antp* inversions but located in the genome of Canton-S, a phenotypically wild-type fly strain. Therefore, not all DNA changes in this area disrupt *Antp* function.

To define the locus more precisely, transcription products originating from genomic DNA sequences located near to the *Antp* inversion breakpoints were isolated as cDNA clones. Characterization of cDNA clones 903 and 909, originating from embryos and pupae, respectively, has revealed two exciting aspects of the *Antp* locus. The major finding is that they represent RNAs that are encoded by several blocks of unique genomic sequences spread over 100 kb, and these genomic regions (at least in the case of 903) are found on both sides of the inversion breakpoint area. In fact, all gross DNA rearrangements which genetically alter *Antp* function, and that we have examined, also disrupt the 100-kb DNA region in some way: by deletion, inversion or translocation. We believe this is strong evidence for the 903 and 909 cDNA clones representing *Antp* RNA species. Thus, at least one transcription unit of the *Antp* locus appears to be 100 kb, this being a minimum estimate because the cDNA copy may not be full length.

A number of possibilities exist to account for a processed RNA originating from blocks of genomic sequences scattered over 100 kb. The primary transcript could be 100 kb, but it will be difficult to detect by conventional methods because the gel electrophoresis and blot transfer methods do not have the capacity to handle such a large RNA. Also, if processing begins before transcription is completed, then a 100-kb RNA would never exist even though the RNA polymerase travels over this distance. Compatible with such a large transcript are the observations that extremely long nascent RNAs ranging up to an estimated 33 kb do exist in *Drosophila* (Laird and Chooi, 1976; Beyer *et al.*, 1981). A second way to generate the 903 RNA would be to rearrange genomic DNA sequences, at least in the cells which express the *Antp* gene, and this would need to occur very early in development in embryonic cells. At the present time it is difficult to rigorously exclude somatic genome rearrangements in only a small fraction of the fly's cells, because no technique exists to fractionate embryo tissues for the separate DNA isolations needed to make such comparisons. However, differences have not been seen in the *Antp* region of total DNA isolated from embryos and adults of several strains (our unpublished results). A final possibility which maintains somatic DNA constancy and does not require the transcription of 100 kb would have more than one separate transcript made of portions of the 100-kb region. These could later be spliced into an RNA from distinct donor molecules.

The structure of the 909 pupal cDNA clone adds a second interesting dimension to our observations of *Antp* expression. 909 contains between 1 and 2 kb of sequence homology with the 903 clone in the left two blocks, but is distinct in another region. The new region, which is by definition an exon for 909, corresponds to a region which is an intron for 903. If 903 originates by transcription of a somatically rearranged genomic DNA block, then the cells producing the 909 RNA must rearrange their genomic DNA in a second pattern. If, however, the *Antp* locus is always expressed in the appropriate cells as a 100-kb precursor, then the 903 and 909

RNAs could be derived from the same primary transcript by differential RNA splicing. Transcription could also initiate at different sites to yield the 903 and 909 RNAs, but differential splicing would still be required to join the common sequence blocks to the unique ones. Finding the 909 clone suggests that there is more RNA coding-capacity in the 100 kb than the 903 clone alone defines, and preliminary Northern blot analysis of RNA supports this notion (A.Kuroiwa, unpublished data). While the 903 and 909 cDNA clones were constructed from RNA of different stages in the fly's life, it is not known if each is restricted to its respective stage. Thus, to generate RNAs which correspond to 903 and 909, different splicing mechanisms could be active at different developmental stages, in different cells at the same stage or for different *Antp* primary transcripts of the same cell.

We must insert a word of caution about both of the cDNA clones characterized here. Because the cDNA clones were synthesized using total cellular poly(A)$^+$ RNA as a template, the possibility exists that a partially-processed RNA may have been copied by reverse transcriptase. While Northern blot analyses of the structures of accumulated *Antp* RNAs are being performed, a relatively stable intermediate could also be interpreted as a mature RNA by this method. cDNA cloning artifacts could also have occurred.

The genetic data indicate that the *Antennapedia* locus can be mutated in a variety of ways to generate a number of distinct phenotypes (see Introduction and Table I). In addition, there are several mutants which have no associated recessive lethality (and therefore cannot be tested for allelism by a *trans* complementation test), but which do show similar phenotypes to *Antp (Ns)* or show evidence for interaction in a *trans* configuration with known *Antp* alleles *(Hu)*. All of these mutations affect segment identity at some stage of the *Drosophila* life cycle and are therefore homeotic. The molecular analysis has shown that the genomic DNA sequences, in the region where rearrangements affecting *Antp* occur, encode several RNA species. Two of these may have been isolated in the form of cDNA clones, and more could exist. There might, for example, be distinct transcripts for each of the functions altered in *Antp, Scx, Ctx, Ns* and *Antp recessive*. Mutations in common blocks would affect all the transcripts using those blocks, and thus several functions. This could explain the shared recessive lethality of several of the mutations. The various mutations could also represent different ways of altering the same transcript. Due to the genetic complexity of this locus and the lack of a complementation test for dominant mutations, our criteria for allelism of the different mutations discussed in the paper will therefore require a correlation of the phenotypes and the transcripts affected.

Our cloning of the *Drosophila* gene *Antennapedia*, a locus involved in determining segment identity, has revealed that it is an unusually large gene. Very large gene units have also been reported by D.Hogness and collaborators for the *bithorax* gene complex, a group of genes that are also homeotic (Bender *et al.*, 1983). In addition, parallel studies have been carried out on *Antennapedia* by M.Scott and T.Kaufman with very similar results. At this time we place the

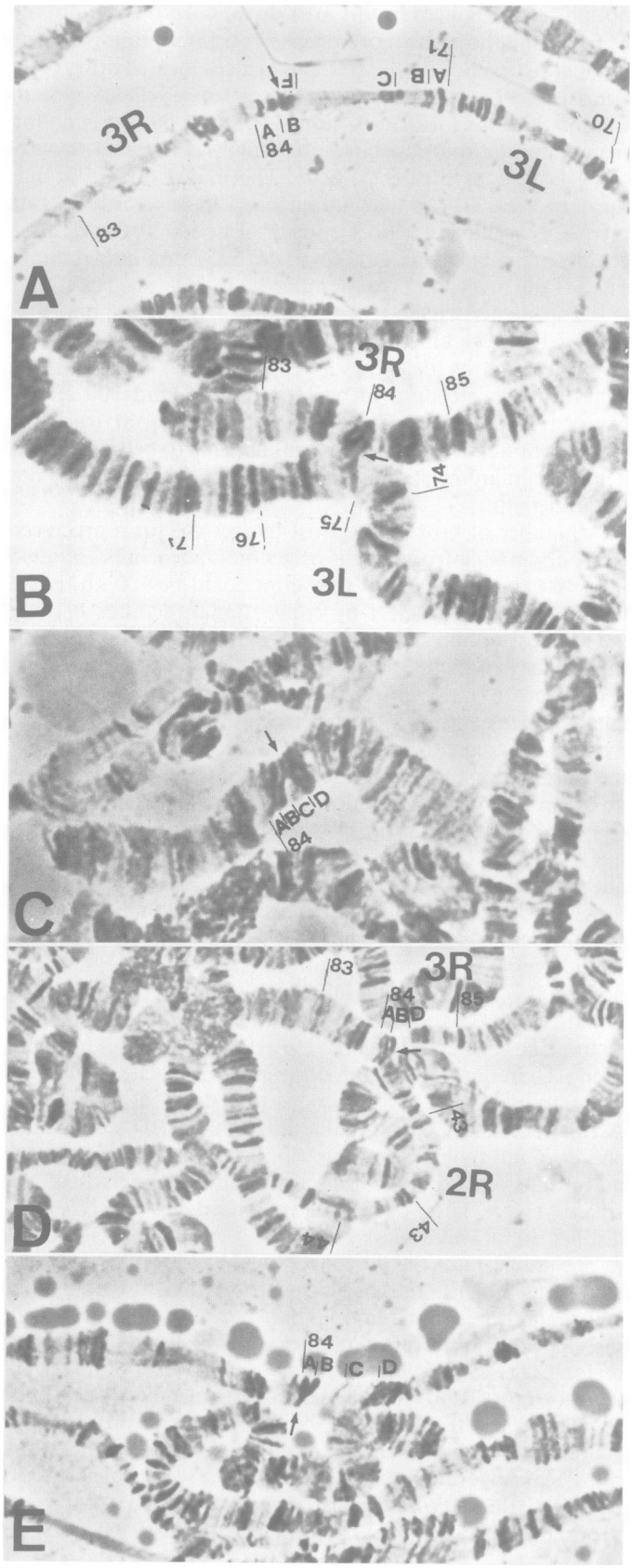


Fig. 9. Cytology of new mutant chromosomes. Salivary gland chromosomes were stained by a standard lacto-acetic acid-orcein procedure. **(A)** The *In(3R)Antp*^{PW} homologue is viewed alone because it does not pair well with the *CxD* balancer chromosome. The 84B1-2 breakpoint is indicated. The other mutant chromosomes are shown paired with an essentially normal chromosome *(W Sb)*. **(B)** *In(3R)Ns + RC4/W Sb*. The 3R-3L arm exchange position is indicated. **(C)** *Df(3R)Ns + RC7/W Sb*. The upper homologue is missing the 84B to 84D region. **(D)** *T(2;3)Ns + RC8/W Sb*. The upper homolog follows the normal 83, 84, 85 sequences of chromosome arm 3R, while the lower homologue shows the 3R-2R translocation junction of 84B to 42. **(E)** *Df(3R)9A99/W Sb*. The lower chromosome homologue is missing the 84A1 to 84B2 region.

limits on sequences necessary for *Antp* function at ~210 kb, between the end of the 9A99 deletion on the left and the α-tubulin gene on the right. The cDNA clones we have isolated define a region that is transcribed into *Antp* RNA which is ~100 kb. The structure of two isolated cDNA clones suggests that there is a complex pattern of transcription of this region. A major goal of our efforts to understand *Antennapedia* function will be to study each transcript from the gene separately in order to relate mutations and the RNAs affected. One way to alter *Antennapedia* expression without changing RNA structure would be to activate the gene in tissues where it is normally inactive. The accompanying paper (Levine *et al.*, 1983b) deals with the direct localization of wildtype *Antannapedia* gene activity by *in situ* hybridization to tissue sections. The fact that the transcription unit defined by the cDNA clones is interrupted by most if not all *Antp* mutations, and the results of localizing the RNA homologous to these cDNAs, strongly support our conclusion that the cDNA clones do represent *Antp* locus products.

Materials and methods

Nucleic acid preparations

Bacteriophage λ DNA was purified using a procedure which combines methods described by Blattner *et al.* (1977) and Vande Woude *et al.* (1979). One to three plaques are eluted over several hours in 1 ml of phosphate-buffered saline (10 mM Tris-HCl pH 7.5, 0.1 M NaCl, 10 mM $MgCl_2$, 0.05% gelatin). 0.7 ml of this is mixed with 0.5 ml of *Escherichia coli* K802 (saturated culture) and 0.5 ml of 10 mM $MgCl_2$, 10 mM $CaCl_2$, incubated 10 min at 37°C, and used to inoculate 400 ml of NZY broth containing 10 mM $MgCl_2$. When lysed, the culture is shaken for 15 min with $CHCl_3$ and debris removed by centrifugation. NaCl and PEG 6000 are added to 1 M and 10% (w/v), respectively. Phage are precipitated for 30 min to overnight in an ice water bath, then collected by centrifugation for 30 min at 7000 r.p.m. After draining off the remaining liquid, the pellet is suspended in 7 ml of buffer A [0.5% Nonident P-40 (NP-40), 3.6 mM $CaCl_2$, 30 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 125 mM KCl, 0.5 mM EDTA, 0.25% Na deoxycholate, 6 mM mercaptoethanol] prewarmed to 30°C. This suspension is treated with DNase I (23 μg/ml) and RNase A (60 μg/ml) at 30°C for 30 min, then extracted with an equal volume of $CHCl_3$. The 7 ml of phage suspension is layered onto a glycerol step gradient (1 ml of 40% glycerol in Buffer B, 3 ml of 5% glycerol in Buffer B). Buffer B is 0.5% NP-40, 30 mM Tris-HCl pH 7.5, 125 mM KCl, 0.5 mM EDTA, 6 mM mercaptoethanol. After centrifugation in a SW40 rotor at 35 000 r.p.m. and 4°C for 1 h, the liquid is poured off, the tube is wiped clean and the pellet is dissolved in 2 ml of 32 mM Tris-HCl pH 7.5, 80 mM EDTA, 2% SDS. Proteinase K is added to 0.5 mg/ml and the mixture digested for several hours to overnight at 55°C until the phage completely dissolves. The DNA is extracted sequentially with equal volumes of neutralized phenol, phenol:$CHCl_3$ (1:1) and $CHCl_3$. Two volumes of cold ethanol are overlaid and the tube gently mixed. The DNA is pulled out as a clump on a Pasteur pipet (end fused by flaming), washed by dipping in cold 70% ethanol and dissolved in 10 mM Tris-HCl pH 8, 1 mM EDTA at 55°C. Yields are usually 100–600 μg from the 400 ml culture and the procedure can be completed in one day.

Plasmid DNA was isolated using the alkaline-SDS procedure (Ish-Horowicz and Burke, 1981). After adding CsCl and ethidium bromide, the solution was spun in a desktop centrifuge to remove debris, loaded into Beckman Quick-Seal tubes and centrifuged at least 14 h in the Beckman VTi50 rotor at 47 000 r.p.m. The DNA band was extracted with n-butanol three times, diluted 3-fold with water and precipitated with an equal volume of 2-propanol, and finally reprecipitated with ethanol.

Drosophila embryo or adult DNA was isolated as described in Bingham *et al.* (1981).

Lambda libraries

Genomic libraries were constructed from Oregon-R embryo DNA and *th st Hu* adult DNA using the vector λ1059 essentially as described by Karn *et al.* (1980). DNA from six different *Sau*3A digestion conditions were pooled and fractionated by horizontal preparative agarose gel electrophoresis. Fragments of 18–22 kb were electroeluted into a trough (Wienand *et al.*, 1979; Yang *et al.*, 1979), ethanol precipitated and ligated into *Bam*HI-digested λ1059. This DNA was *in vitro* packaged using extracts prepared as in Scherer *et al.* (1982) and plated on the P2 lysogen Q359 for spi^+ selection against reformed vector. Each pool contained 5 x 10^5 different phages.

Other *Drosophila* genomic libraries in lambda vectors used were as follows: Canton-S DNA in Charon 4 obtained from J.Lauer and T.Maniatis (Maniatis *et al.*, 1978), Oregon-R DNA in Sep 6 from E.Meyerowitz and D.Kemp (Meyerowitz and Hogness, 1982) and w^{IR1} from R.Paro and M.L.Goldberg (unpublished data).

Drosophila culturing and chromosome preparations

Flies were raised on a cornmeal, sucrose, dried yeast and agar medium at 25°C for normal crosses and mass growth and at 18°C for obtaining larval salivary glands to prepare polytene chromosomes. For polytene chromosome analysis, males of mutant stocks were crossed with *CxD,D/W Sb* virgins. F1 stocks of mutant chromosome/*CxD,D* and mutant chromosome/*W Sb* were established. Salivary gland squashes of both types were stained with lacto-aceto-orcein (Ising and Block, 1980) and examined by brightfield microscopy. The combination with *CxD* usually prevented pairing at 84B since this chromosome is multiply inverted with one breakpoint in 84. Description of most mutations used in this work can be found in Lindsley and Grell (1968).

Cloned DNA sequences were mapped to cytological positions of normal and mutant polytene chromosomes by *in situ* hybridization (Pardue and Gall, 1975). Initially we used a standard protocol involving tritiated-DNA probes hybridized in the presence of dextran sulfate, and autoradiographic detection (Spierer *et al.*, 1983). Recently we have employed a method utilizing DNA probes containing biotinylated-dUTP and immunological detection of the hybridized biotin group (Langer-Sofer *et al.*, 1982; Levine *et al.*, 1983a).

Molecular analyses

Restriction enzymes were purchased from New England Biolabs and Boehringer Mannheim and used as recommended by the suppliers. Maps were constructed by digesting DNAs with various enzymes first alone, then in pairs, and deducing the distances between sites from the resulting fragment sizes.

Directional chromosome walking was achieved from a given lambda in the following manner. A map of the lambda insert was made using several restriction enzymes. A preparative DNA digestion of 10–30 μg followed by gel electrophoresis was used to isolate one DNA fragment, coming from as near as possible to the end in which direction the walk will proceed, plus a second fragment several kilobase pairs back from the first one. Both were nick-translated. The end fragment probe was used to screen 8–10 genome equivalents (~1.5 x 10^4 phage on a single 132 mm diameter plate is one equivalent) by the Benton-Davis procedure (1977). Positive regions were eluted and replated at three plaque densities. The most concentrated one was screened with the second probe (back screened), while the better density of the other two plates was used to purify a single plaque by rescreening with the end fragment probe. New clones which extend the furthest are end probe-positive but backscreen probe-negative. Only these were used to prepare DNA for restriction enzyme site mapping and the next walk step.

The nick-translation protocol of Maniatis *et al.* (1975) was used, and unincorporated nucleotides were removed by centrifugation through Sephadex G50 fine columns (Ish-Horowicz and Burke, 1981). Hybridizations to DNA on filters was performed using the conditions of Hanahan and Meselson (1980). For gel blot analysis, 0.7 μg of lambda DNA digests were fractionated on 0.7% agarose gels and transferred to nitrocellulose by the procedure of Smith and Summers (1980).

Acknowledgements

We would like to acknowledge the participation of Rachel Mohun, Manfred Steinemann and Michael Levine in the cloning project. We are grateful to Tom Maniatis and Joyce Lauer for the essential *Drosophila* DNA lambda library, David Ish-Horowicz for access to his cosmid library and for suggestions on the manuscript, Michael Goldschmidt-Clermont for aliquots of his excellent cDNA library, Michael Young for repeated element clones, and Vincenzo Pirrotta for the accurately microdissected clones. For fly stocks we thank the *Drosophila* stock centers at Umea, Bowling Green and California Institute of Technology and Gary Struhl, Gerd Jürgens, Christine Nüsslein-Volhard, Donald Sinclair, Sheena Pinchin, Raphael Falk and Douglas Cavender. Our progress benefited greatly from discussions with Gerd Jürgens, Gary Struhl, David Ish-Horowicz and our colleagues at the Biocenter of the University of Basel. Finally, our appreciation goes to Danièle De Trey for technical assistance and Erika Wenger-Marquardt for preparation of the manuscript. This work was supported by grants from the Swiss National Science Foundation and the Kanton Basel-Stadt.

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Note added in proof

Similar but distinct results on the distribution of *Ultrabithorax* transcripts have been obtained by M. Akam (see paper in this issue).