Genetic Dissection of *pointed*, a Drosophila Gene Encoding Two ETS-Related Proteins

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ABSTRACT

The Drosophila gene *pointed* (*pnt*) is required for the differentiation of a number of tissues during embryogenesis, including the ventral ectoderm, the nervous system, the tracheal system and certain muscle fibers. The phenotypes associated with strong *pointed* alleles are reflected by a complex *pointed* expression pattern during embryogenesis. Two promoters, P1 and P2, separated by some 50 kb of genomic sequences, direct the transcription of two different transcript forms, encoding two different proteins related to the ETS family of transcription factors. To assess the individual functions of the two different *pointed* protein forms, we have generated new *pointed* alleles affecting either the P1 or the P2 transcript, termed P1 and P2 alleles, respectively. Genetic analysis reveals partial heteroallelic complementation between certain *pointed* P1 and P2 alleles. Surviving *trans*-heterozygous flies have rough eyes, abnormal wings and halteres, suggesting a requirement for *pointed* function during their imaginal disc development. Further genetic analysis demonstrates that expression of a given *pointed* P2 allele depends on *trans*-acting transcriptional regulatory sequences. We have identified two chromosomal domains with opposite regulatory effects on the transcriptional activity of the *pointed* P2 promoter, one *trans*-activates and the other *trans*-represses *pointed* P2 expression. By deletion mapping we were able to localize these control regions within the 5′ region of the *pointed* P2 transcript.

THE gene *pointed* was first identified based on defects found in the cuticle of homozygous *pointed* embryos (JÜRGENS *et al.* 1984). Subsequently, *pointed* was placed together with *Star*, *spitz*, *sichel*, *single minded* and *rhomboid* in the "spitz" group, members of which are required for pattern formation in the ventral ectoderm as well as in the nervous system (MAYER and NÜSSLEIN-VOLHARD, 1988; THOMAS, CREWS and GOODMAN 1988; BIER, JAN and JAN 1990; KLÄMBT, JACOBS and GOODMAN 1991; RUTLEDGE *et al.* 1992; KLÄMBT 1993). *Star* and *rhomboid* are known to be required during later stages of development as well, where they function during compound eye and wing development (HEBERLEIN and RUBIN 1991; FREEMAN, KIMMEL and RUBIN 1992; DIAZ-BENJUMEA and GARCÍA-BELLIDO 1990; KLEIN and CAMPOS-ORTEGA 1992; STURTEVANT, ROARK and BIER, 1993).

Like other "spitz" group genes, *pointed* function is needed for a number of other processes during embryonic development, including the differentiation of the tracheal system, the development of certain muscle fibers, PNS development, and the formation of the axon pattern in the embryonic CNS (MAYER and NÜSSLEIN-VOLHARD 1988; BIER, JAN and JAN 1990; KLÄMBT 1993). Since *pointed* is required for midline glial cell differentiation we undertook a molecular analysis of the gene (KLÄMBT 1993). The *pointed* locus gives rise to two overlapping transcripts driven by two promotors, P1 and P2, separated by 50 kb of genomic sequence. The embryonic phenotypes described for strong *pointed* alleles correlate well with the transcription pattern driven by the two *pointed* promoters. The P1 promoter directs expression in the ventral ectoderm, the tracheal system and in CNS glial cells. The P2 promoter drives transcription in the mesoderm and in the midline glial cells, which are phenotypically affected in homozygous *pointed* embryos (KLÄMBT 1993). Both *pointed* transcripts are also expressed during larval and adult stages (CHEN *et al.* 1992; C. KLÄMBT, unpublished), but nothing is known about the requirement for *pointed* function during later development. The *pointed* RNAs encode two putative transcription factors, which share a common region with a high degree of homology to the ETS domain, a DNA-binding domain found in a growing family of proteins related to the vertebrate *ets* oncogene (KARIM *et al.* 1990; BEN-DAVID *et al.* 1991; LA MARCO *et al.* 1991; PRIBYL *et al.* 1991; DALTON and TREISMAN 1992; CHEN *et al.* 1992; LAI and RUBIN 1992; TEI *et al.* 1992; XIN *et al.* 1992; KLÄMBT 1993) (for review, see WASYLYK, HAHN and GIOVANE 1993). A second domain, the pointed box, which is present only in the P2 transcript, has been conserved as a distinct exon from chicken to fly (KLÄMBT 1993). This exon lies within a domain which has been implicated in the regulation of transcriptional activation in vertebrate

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ETS proteins (SCHNEIKERT, LUTZ and WASYLYK 1992).

To assess the individual functions of the two different *pointed* protein forms, it was necessary to isolate mutations that affect only one of the two transcripts. Starting from enhancer trap insertions into the 5′ regions of the P1 and the P2 transcript, we have generated deletion mutations by imprecise excision of the *P* element insertions and by X-ray mutagenesis. Heteroallelic complementation revealed that *pointed* P2 function is required during eye and wing development. Further genetic analysis showed a complex regulation of the *pointed* P2 promoter. Activity of this promoter depends in part on *trans*-acting regulatory DNA domains, where transcription of a given allele is influenced in trans by regulatory domains on the other chromosome.

Here we show that two different and distinct *trans*-acting DNA domains control the *pointed* P2 promoter activity. One domain activates *pointed* transcription in *trans*, whereas the other domain represses *pointed* transcription in *trans*. In addition, we present evidence that enhancers directing tissue-specific *pointed* P2 expression are located 3′ to the first P2 exon within intronic sequences.

MATERIALS AND METHODS

Genetics: The *pointed* allele pnt^{1277} was isolated in an enhancer trap screen carried out in Köln (A. BEERMANN, C. SCHULZ and J. A. CAMPOS-ORTEGA, unpublished). The *pointed* allele pnt^{rM254} was isolated in an enhancer trap screen carried out in the laboratory of C. S. GOODMAN in Berkeley, California (KLÄMBT 1993). To induce new *pointed* alleles, pnt^{rM254} (or pnt^{1277})/*Ki* p^p Δ*2-3* males were crossed to appropriate balancer flies (*Ki* p^p Δ*2-3* carries a stable source of P-transposase; ROBERTSON *et al.* 1988). Independent reversion events were selected on the basis of the loss of the eye color marker carried by the different *P* elements. Homozygous lethal lines were crossed to the EMS induced *pointed* allele pnt^{6B74} (JÜRGENS *et al.* 1984). All crosses were performed on standard fly food at 25°. To obtain X-ray-induced *pointed* P2 alleles, 3–4-day-old pnt^{1277}/pnt^{1277} males were irradiated with 4,000 rad and crossed to appropriate balancer flies (ASHBURNER 1989a). The chromosomes of all three reversion alleles, pnt^{T5}, pnt^{T6} and pnt^{T9} were analyzed in salivary gland squashes (ASHBURNER 1989b). Only pnt^{T5} was found to carry a complex chromosomal aberration, including a inversion breaking in 94E/F, the location of the *pointed* gene. In these polytene chromosome preparations pairing of the homologous chromosomes was often disrupted.

DNA methods: All DNA work was carried out according to standard procedures (SAMBROOK, FRITSCH and MANIATIS 1989). For the isolation of genomic DNA, 50–200 flies were homogenized in 0.25 M sucrose, 0.05 M Tris-HCl, pH 7.4, 1 mM EDTA. Nuclei were then spun out by centrifugation for 5 min at 2,000 × *g*. The resulting pellet was resuspended in 0.1 M EDTA, 0.25 M NaCl, 20 mM Na-borate, pH 9.6 and 10 μg RNAase/ml. Sodium dodecyl sulfate (SDS) was added to 2% and the DNA was purified by subsequent phenol extraction. To determine the site of integration of the *P* element genomic sequences flanking the P insertion in pnt^{1277} were isolated by plasmid rescue. Subsequent sequence analysis showed that the insertion had occurred at position +365, in the middle of the first exon. Southern blot analyses were performed according to CHURCH and GILBERT (1984).

***In situ* hybridization:** Hybridization was carried out according to TAUTZ and PFEIFLE (1989).

Antibody staining: CNS axons were visualized with the monoclonal antibody BP102 (kindly provided by N. PATEL and C. S.GOODMAN) and the tracheal pattern was visualized with the antiserum #84 (kindly provided by B. SHILO). Staging of embryos was according to CAMPOS-ORTEGA and HARTENSTEIN (1985). Antibody staining and CNS dissections were performed as described previously (KLÄMBT, JACOBS and GOODMAN 1991).

Histology: Wings were mounted in DePeX. Thorax preparations were embedded in Hoyer's medium (ASHBURNER 1989b). For scanning electron microscopy heads were dehydrated through an ethanol series and by subsequent critical point drying. Heads were mounted and sputter-coated with a 200-Å thick gold coat and viewed on a Hitachi S520 scanning electron microscope.

RESULTS

***P* element insertions into the *pointed* gene:** The genomic organization of *pointed* has been reported recently (KLÄMBT 1993) and is summarized in Figure 1. Two different transcript forms have been detected. The P1 transcript covers 8 kb of genomic DNA and is found most prominently in the ectoderm and the cells of the tracheal anlage. In the CNS *pointed* P1 expression is restricted to glial cells. The longitudinal glial cells and the glial support cells of the VUM cluster express the *pointed* P1 RNA most prominently. Its promoter region is tagged by a P[$rosy^+$, *lacZ*] enhancer trap element, resulting in the homozygous lethal allele pnt^{rM254} (KLÄMBT 1993).

The P2 transcript consists of at least 8 exons, distributed over some 55 kb of genomic DNA (Figure 1). *pointed* P2 transcripts are found most prominently in the mesoderm (Figure 2A). In addition, expression can be detected in the midline glial cells as well as in presumptive imaginal disc anlagen (Figure 2, A and B). In a screen for enhancer trap lines that express β-galactosidase in the dorsal epidermis we have isolated the P[$white^+$, *lacZ*] enhancer trap insertion line 1277. The integration of the *P* element occurred in the middle of exonI at position +365 resulting in a weak, homozygous viable *pointed* allele: pnt^{1277} (see MATERIALS AND METHODS). Strikingly, the presence of the 12-kb P[*lacZ*, $white^+$] transposon (BIER *et al.* 1989) in the middle of exonI does not seem to interfere very much with the P2 mRNA transcription. P2 transcription is only very slightly reduced in homozygous pnt^{1277} embryos as shown by whole mount *in situ* hybridization using P2 specific probes (Figure 2C).

Isolation of *pointed* P1 alleles: Mobilization of *P* elements often results in imprecise excision, leading to small deletions of DNA sequences flanking the insertion sequence (DANIELS *et al.* 1985). In order to obtain deletions that affect either one or both of the

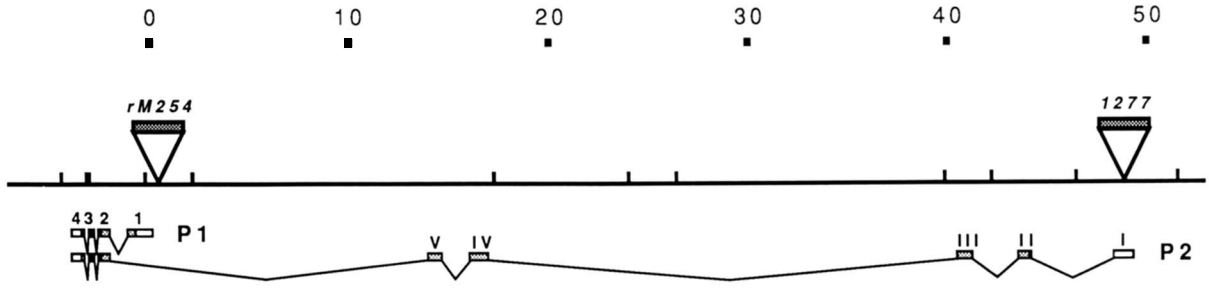

FIGURE 1.—Enhancer trap insertions in the *pointed* locus. Schematic summary of the *pointed* locus. *Eco*RI sites are indicated as (⊥), dots above the map indicate 10-kb intervals. The gene *pointed* encodes two transcript classes (P1 and P2) which are spread over 55 kb of genomic sequence. Transcription is from right to left. Exons 2, 3 and 4 are identical for both transcript classes, the location of coding sequences are indicated by shading and the ETS domain is indicated in black. The locations of the *P* element enhancer trap insertions *rM254* P[*rosy*$^+$, *lacZ*] and *1277* P[*white*$^+$, *lacZ*] are indicated by triangles.

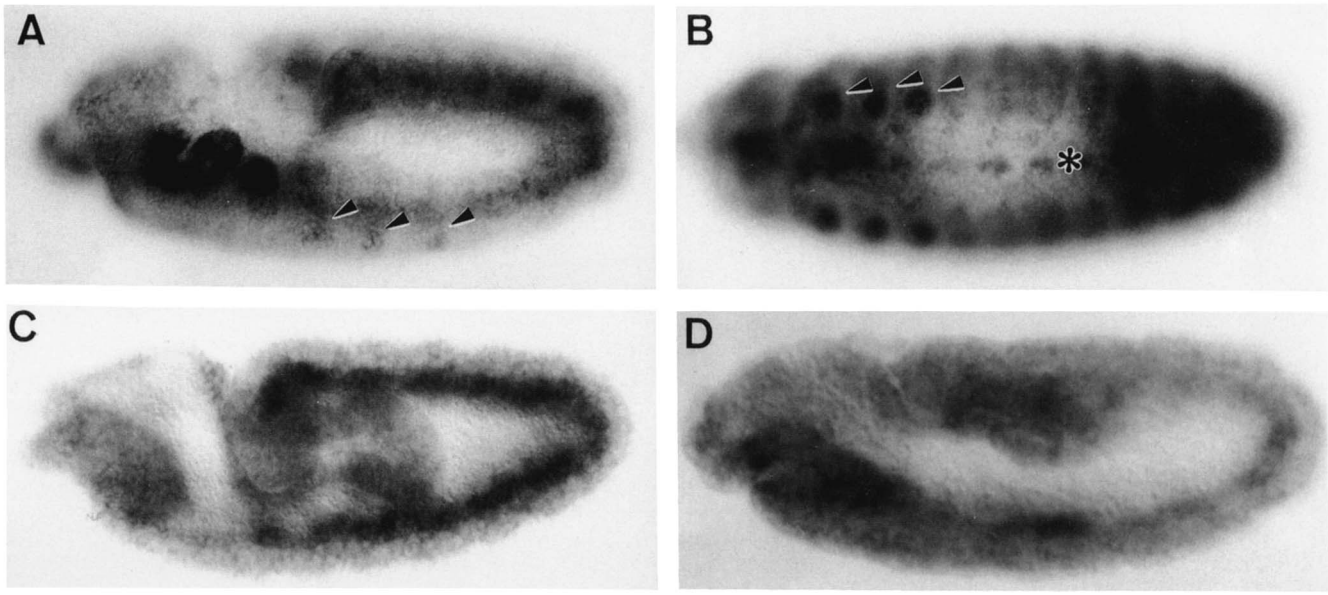

FIGURE 2.—*pointed* P2 expression. *pointed* P2 transcript distribution was visualized in whole mount embryos hybridized with P2 transcript-specific DIG-labeled DNA probes and subsequent alkaline phosphatase detection. Anterior is to the left. Wild-type embryos of stage 12 (A) and stage 13 (B) show P2 transcript accumulation in the mesoderm as well as in the imaginal disc anlagen (arrowheads). Asterisk in (B) indicates midline glial cells expressing P2 RNA. (C) In a stage 11 homozygous pnt^{1277} embryo P2 transcription appears unchanged. (D) In a stage 11 homozygous pnt^{T6} embryo, however, P2 transcription is greatly reduced.

pointed transcripts, we have mobilized the *P* element insertions in both enhancer trap lines mentioned above. The *P* element insertion in pnt^{rM254} 5′ to the P1 transcript causes an embryonic lethal *pointed* phenotype (Figure 1; KLÄMBT 1993). Of 200 independent *rosy*$^-$ excision lines, 112 (56%) remained homozygous lethal and were classified as P1 alleles. Their chromosomal organization was subsequently analyzed on Southern blots. In most of these lines, both termini of the *P* element are still present and the deletions affect only internal *P* element sequences.

Among the 112 lines tested we identified three deletions that affect exons common to both transcript forms: $pnt^{\Delta 81}$, $pnt^{\Delta 88}$, $pnt^{\Delta 114}$. The deletion in $pnt^{\Delta 81}$ breaks in the *P* element and extends beyond position −9 kb. In $pnt^{\Delta 88}$ the entire *P* element and flanking genomic sequences on both sides are deleted. Since in these two alleles the entire P1 transcript as well as all 3′ exons of the P2 transcript, encoding about one-half of the P2 protein are removed, we assume that $pnt^{\Delta 88}$ and $pnt^{\Delta 81}$ represent amorphic *pointed* mutations.

Deletion $pnt^{\Delta 114}$ removes the entire *P* element and adjacent genomic sequences from position −3 kb to +1.5 kb. Thus, in $pnt^{\Delta 114}$ exons 1 and 2 are removed. Since exon 2 can be removed from the *pointed* P2 RNA without affecting the open reading frame, the *Δ114* deletion should result in a *pointed* P2 protein with an internal deletion of 321 amino acids. Indeed, as shown below complementation analysis revealed a small amount of P2 function still present in $pnt^{\Delta 114}$ (despite the fact that 321 of the 718 amino acids of the P2 protein, including the N-terminal half of the ETS domain, are deleted).

Unfortunately, we could not identify deletions which eliminate only the P1-specific exon 1 and

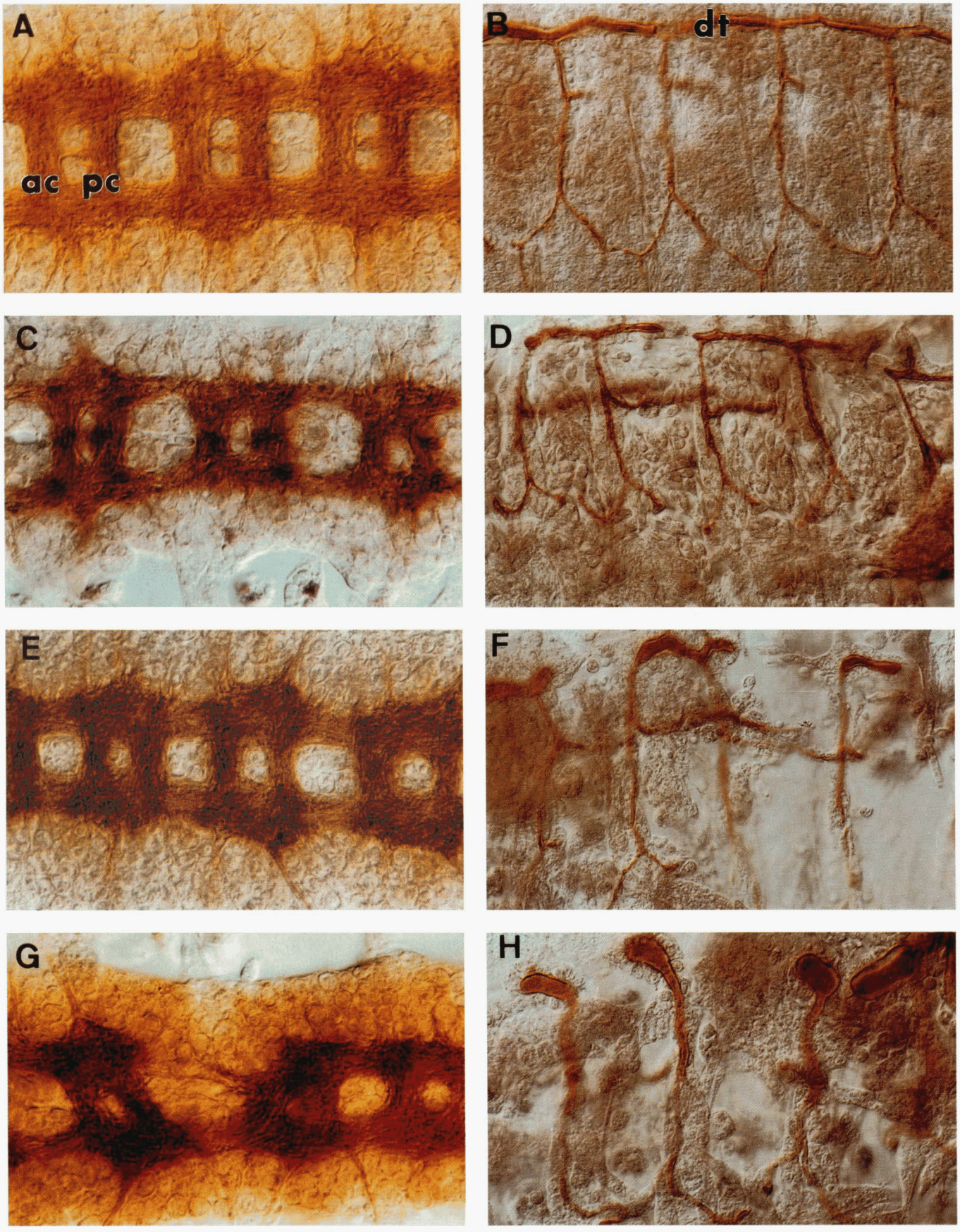

FIGURE 3.—Phenotypes of different *pointed* P1 alleles. Dissected preparations of stage 15/16 embryos showing the CNS axon pattern (A, C, E, and G) and tracheal (B, D, F, and H) pattern in wild-type and homozygous *pointed* embryos, visualized with antibody BP102 (CNS axons) or the antiserum #84 (trachea) and subsequent HRP immunohistochemistry. (A) Wild-type CNS axon pattern, anterior commissure

thereby lead to a loss of solely the P1 function. In all excision lines that affect only exon 1, some *P* element sequences remained, which interfere with P2 function. *In situ* hybridization experiments using P2-specific digoxygenin probes show no obvious reduction in the level of P2 transcription in homozygous $pnt^{\Delta 18}$ and $pnt^{\Delta 56}$ embryos. Based on the complementation behavior, however, different amounts of P2 function are present in these two alleles (see below). Therefore we expect that the remaining *P* element sequences located in $pnt^{\Delta 18}$ and $pnt^{\Delta 56}$ interfere with the splicing of the primary P2 RNA to a different extend in each allele.

Embryonic phenotypes define an allelic series of the P1 alleles: The embryonic CNS axon pattern and the tracheal system of several alleles were inspected following antibody staining. Embryos homozygous for $pnt^{\Delta 81}$, $pnt^{\Delta 88}$, pnt^{rM254}, $pnt^{\Delta 114}$ or pnt^{8B74} (an EMS-induced allele; JÜRGENS *et al.* 1984) show identical CNS and tracheal phenotypes [see KLÄMBT (1993) for more detailed phenotypic description]. Other alleles display less severe CNS or tracheal phenotypes. In homozygous $pnt^{\Delta 18}$ and $pnt^{\Delta 56}$ embryos, which both carry internal deletions in the *rM254 P* element, anterior and posterior commissures are not separated completely, which results in a very similar CNS axon pattern (Figure 3, C and E). The degree of pattern formation in the tracheal system however is quite different in the two mutant alleles, with $pnt^{\Delta 56}$ showing a more severe tracheal phenotype. The formation of the dorsal trunk (RÜHLE 1932) seems the most sensitive way to measure the relative allelic strength of a given *pointed* P1 allele. Examples of phenotypes of the different P1 alleles are shown in Figure 3 and resemble the phenotypic series obtained for *breathless* mutations (KLÄMBT, GLASER and SHILO 1992).

Isolation of *pointed* alleles affecting the *pointed* P2 function: Starting from the homozygous viable P[*white*$^+$, *lacZ*] insertion line pnt^{1277}, we have isolated a number of new X-ray- as well as transposase-induced lethal *pointed* alleles, classified as P2 alleles.

Out of 50,000 mutagenized chromosomes analyzed, we identified three *white*$^-$ X-ray-induced revertants, *T5*, *T6* and *T9*. They appear to uncover at least two lethal complementation groups close to the *P* element insertion. pnt^{T5} and pnt^{T9} both fail to complement strong *pointed* alleles and therefore represent true *pointed* alleles. pnt^{T6} can complement lethality of the strong *pointed* alleles ($pnt^{\Delta 88}$), albeit producing a rough eye phenotype. A similar rough eye phenotype is observed in flies *trans*-heterozygous for $pnt^{\Delta 88}$ and pnt^{1277} (see Figure 5B). Thus, with respect to *pointed*, pnt^{T6} behaves like pnt^{1277}. The lethality associated with pnt^{T6} is not the result of a second X-ray hit elsewhere on the third chromosome, because pnt^{T6} fails to complement the independently isolated X-ray induced allele pnt^{T9}. Therefore the lethality of pnt^{T6} does not seem to be due to loss to *pointed* function at the first glance. Interestingly, however, *in situ* hybridization experiments with P2-specific digoxygenin labeled DNA probes showed that *pointed* P2 transcription is greatly reduced in homozygous pnt^{T6} (Figure 2D) as well as in homozygous pnt^{T9} embryos (data not shown). P1-specific transcription is not affected in either allele (data not shown).

In a second mutagenesis screen for P2 alleles we have generated 100 independent transposase-induced excision lines. Twenty-five lines were identified as homozygous lethal, and again could be grouped into two classes with respect to their ability to complement the lethality associated with strong *pointed* alleles. We have used the following five lethal chromosomes, pnt^{1202}, pnt^{1208}, pnt^{1216}, pnt^{1218} and pnt^{1230} all of which, with the exception of pnt^{1218}, fail to complement the lethality associated with strong *pointed* alleles. This latter allele complements *pointed* but is essentially lethal over pnt^{T6} (and pnt^{T9}) and thus a member of the "second" lethal complementation group uncovered by pnt^{T6}.

The P2 alleles can be grouped into an allelic series: To determine the relative allelic strength of the different *pointed* P2 alleles, we performed a detailed complementation analysis (see Table 1). As mentioned above, strong *pointed* alleles, like $pnt^{\Delta 88}$, fail to complement the lethality of all *pointed* P2 alleles (except *T6* and *1218*). P1 alleles of intermediate strength as judged by their tracheal phenotypes, complement some, but not all, of the P2 alleles. Weak P1 alleles complement lethality associated with all P2 alleles (see Table 1). Based on the complementation analysis we can therefore arrange the P2 alleles into the following allelic series, in order of increasing allelic strength: $(pnt^{1218} = pnt^{T6}) = pnt^{1277} < pnt^{1202} < pnt^{1208} < pnt^{1230} \leq pnt^{T9} < pnt^{T5}$ (see Table 1).

This allelic series reflects in part the phenotypes we observe in the different homozygous mutant embryos. The strongest P2 allele pnt^{T5} leads to embryonic lethality. The pnt^{T5} chromosome is haplo-insufficient and about 50% of pnt^{T5}/wild-type embryos die. Based on the fact that pnt^{T5} carries a complex inversion with

(ac) and posterior commissure (pc) are separated and the connectives are well developed. (B) Wild-type tracheal system, the dorsal trunk has formed a continuous lumen. (C and D) homozygous $pnt^{\Delta 18}$ and (E and F) homozygous $pnt^{\Delta 56}$ show an intermediate phenotype in the CNS axon pattern as well as in the tracheal system. (G and H) show the CNS and tracheal phenotypes associated with the *pointed* null allele $pnt^{\Delta 88}$. Note the increasingly severe disruptions in the formation of the dorsal trunk (D, F and H). Abbreviations: ac, anterior commissure; pc, posterior commissure; dt, dorsal trunk

TABLE 1

Complementation Analysis reveals intragenic complementation of P1 and P2 alleles

	1277	*T6*	*1202*	*1208*	*1230*	*T9*	*T5*	EMS
♦*18*	v	v	v	v	v	v	esc,r	l
55	v	v	v	v	v	esc,r	esc,r	l
104	v	v	v	esc,r	v	v	esc,r	l
91	v	v	v	l	l	l	l	l
♦*56*	v	v	v	l	l	l	l	l
97	v	v	l	l	l	l	l	l
114	v	v	l	v	l	l	l	l
♦*35*	v	v	l	v	l	l	l	l
88	r	r	l	l	l	l	l	l
rM254	r	r	l	l	l	l	l	l

P1 alleles are listed on the *y*-axis, P2 alleles and one EMS induced (amorphic) *pointed* allele are listed on the *x*-axis. The alleles are ordered in increasing allelic strength. The results of the individual crosses are indicated by the following symbols: l, lethal; v, viable, *trans*-heterozygotes eclose in the expected frequency; esc,r, escaping *trans*-heterozygous flies were observed at a low frequency. The escaping flies exhibit wing and/or eye phenotypes. r, rough eye, *trans*-heterozygous flies emerge in the expected frequency, but have rough eyes. EMS, we have used the EMS induced *pointed* allele $pnt^{88.74}$. ♦ indicates an internal deletion of *P* element sequences.

possible breaks in several genes we did not further characterize the phenotype associated with this allele. The alleles pnt^{1218}, pnt^{1202}, pnt^{1230} and pnt^{T9} lead to larval lethality, with no obvious defects during embryogenesis. pnt^{1208} leads to pupal lethality. Interestingly the pnt^{1208} chromosome is also haplo-insufficient, as about 50% of pnt^{1208}/wild-type pupae die. The X-ray-induced allele pnt^{T6} leads to embryonic lethality. About 5% of the homozygous mutant pnt^{T5} embryos show a fused commissure phenotype, which could be a consequence of the lack of *pointed* P2 function or alternatively might be evoked by a second X-ray hit on the chromosome.

Molecular characterization of P2 alleles: To correlate the genetic complementation data with the chromosomal lesions associated with the different alleles, we determined their genomic organization by Southern blot experiments. The X-ray induced pnt^{T5} allele is a complex inversion which breaks in the *1277* enhancer trap element rendering the *white* mini-gene defective but the *lacZ* gene intact (see MATERIALS AND METHODS). This inversion leads to a loss of specific P2 transcription as shown by whole mount *in situ* hybridization experiments using P2 specific DNA probes (data not shown). Interestingly, the β-galactosidase activity is up-regulated in pnt^{T5} but appears unchanged in its temporal and spatial expression pattern (data not shown). This indicates, that enhancer elements governing the stage- and tissue-specific expression of P2 RNA must be situated on the left of exonI, since genomic sequences to the right are physically separated from the *lacZ* gene by the inversion event in pnt^{T5} (see Figures 4 and 8).

Polytene chromosomes of the other X-ray induced

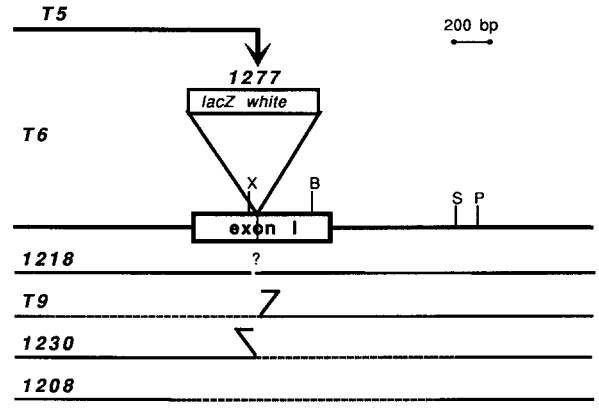

FIGURE 4.—Molecular characterization of some P2 alleles. The genomic organization around exonI of the *pointed* P2 transcript is shown as an enlargement of the region +50 to +55 of Figure 1. The *1277 P* element inserted at position 365 in exonI, with *lacZ* sequences to the left. Transcription of P2 RNA is from right to left. The following alleles are indicated. The X-ray-induced *T5* allele carries a break in the *white* gene of the *1277* enhancer trap. The *1218* allele looks like a precise excision of the *1277* enhancer trap element with a possible small deletion of less then 50 bp. The deletion found in *T9* breaks in the *P* element and remove the left exon intron boundary. In *1230* part of the *P* element and flanking genomic sequences are deleted and in *1208* the entire *P* element and flanking sequences into both directions are removed. In *T6* no restriction site polymorphisms were detected. Abbreviations: X, *Xho*I; B, *Bgl*II; S, *Spe*I; P, *Pst*I.

reversion alleles pnt^{T6} and pnt^{T9} did not show any cytological abnormalities. Southern blot experiments revealed that the deletion associated with pnt^{T9} breaks within the *P* element and extends to the left possibly removing one exon-intron boundary. No genomic alterations were found for the pnt^{T6} allele by Southern blot analysis, when compared to the original *1277* enhancer trap line. The entire *P* element was found to be intact at the original insertion site. In spite of this, the *white* mini-gene is inactivated in *cis* and *pointed* P2 transcription is greatly reduced in homozygous pnt^{T6} embryos (Figure 2D).

The extent of the chromosomal deletion associated with the transposase induced alleles *1208*, *1218* and *1230* is summarized in Figure 4. The deletion associated with pnt^{1230} breaks within the *P* element and extends to the right. In pnt^{1208} the entire *P* element and flanking DNA sequences on either side of the integration site are deleted. Surprisingly, pnt^{1218}, which like pnt^{T6} complements *pointed*, is a precise excision of the *1277* enhancer trap element as determined by Southern blot experiments. If a deletion is associated with this chromosome, we estimate it to be smaller than 50 bp, the resolution limit of our Southern blots. None of the deletions analyzed alters exon II, which contains the translational start site of the *pointed* P2 RNA.

In summary, although no DNA alterations have been detected in pnt^{T6}, lethality associated with this chromosome is still uncovered by the small deletion

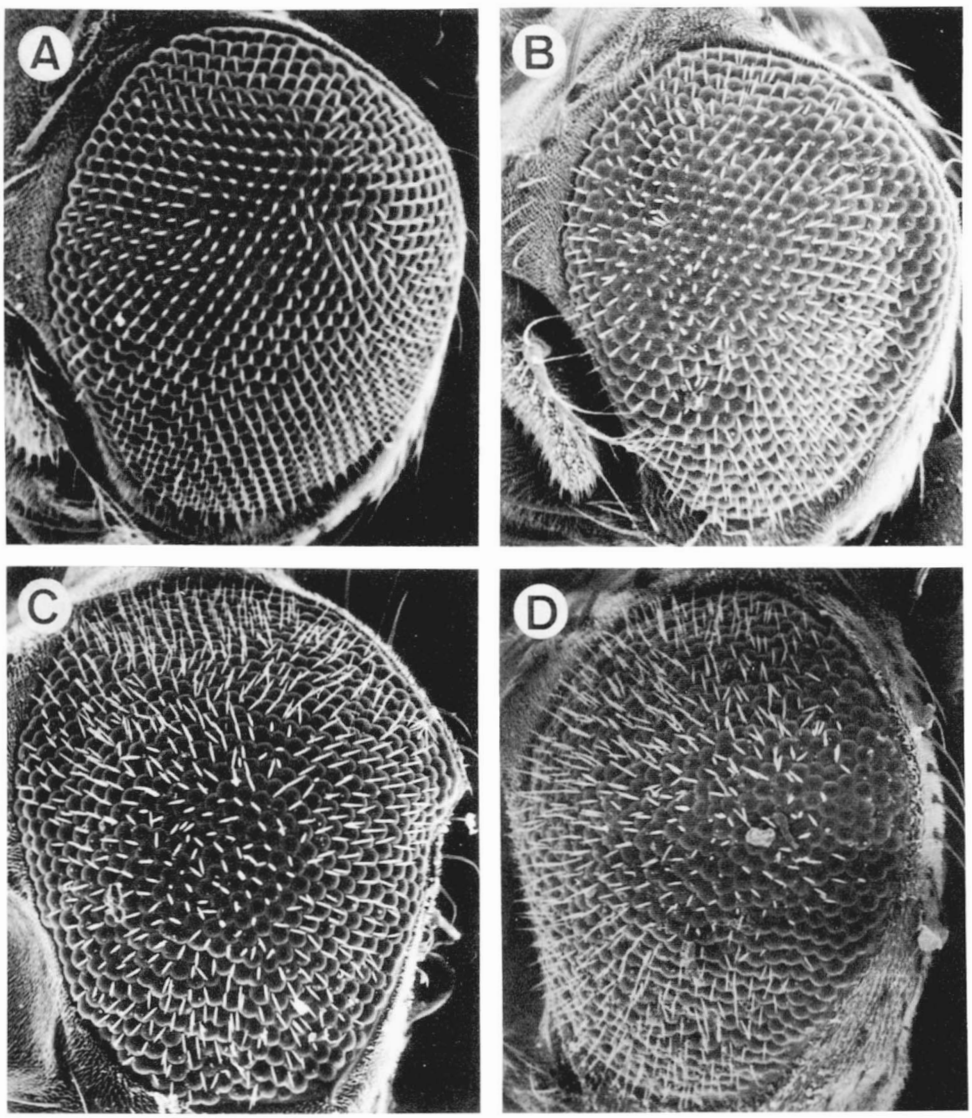

FIGURE 5.—eye Phenotypes of different *pointed* allelic combinations. Scanning electron microscopy of compound eyes from a (A) wild-type fly, (B) $pnt^{\Delta 88}/pnt^{1277}$, (C) pnt^{1230}/pnt^{T6} and (D) pnt^{T9}/pnt^{1235} *trans*-heterozygous flies. Anterior is to the left. (A) Wild-type flies exhibit a regular arrangement of the ommatidial lenses and mechanosensory bristles. (B) In P1/P2 *trans*-heterozygous flies the external eye surface appears irregular, in addition the mechanosensory bristles are sometimes duplicated. (C) A similar phenotype is detected in escaping P2/P2 allelic combinations. (D) pnt^{T9}/pnt^{1235} *trans*-heterozygous flies show slightly more bristle duplications.

found in pnt^{T9}. pnt^{1218} was determined to be a "precise" excision with a possible genomic deletion of less than 50 bp. Again, lethality associated with this allele is uncovered by the small deletion found in pnt^{T9}. This places the second lethal complementation group in the immediate vicinity of the left side of the *1277 P* element insertion site, within the first *pointed* P2 exon. Paradoxically, although the lesion in pnt^{1208} physically uncovers this complementation group as well, pnt^{1208} complements the lethality associated with pnt^{1218} and pnt^{T6}.

Heteroallelic complementation reveals *pointed* function during eye development: In various *trans*-heterozygous combinations of different P1 and P2 alleles escapers eclosed (see Table 1 for details). These flies had rough eyes with frequent duplications of the mechanosensory bristles in the compound eye (Figure 5). In general, a similar rough eye phenotype was observed in combinations of strong P1 and weak P2 or weak P1 and intermediate P2 alleles. In $pnt^{\Delta 88}/pnt^{1277}$ flies *pointed* P2 function is absent from the *Δ88* chromosome and only slightly reduced in the *1277* chromosome resulting in an reduction of P2 function by probably little more than 50%. In $pnt^{\Delta 18}/pnt^{T5}$ flies *pointed* P2 function is only slightly reduced from the *Δ18* chromosome but absent from the *T5* chromosome, again resulting in an overall reduction of P2 function by about 50%. If the overall P2 function is reduced only slightly, as in allelic combinations of weak P1 and weak P2 alleles, no rough eye phenotypes were detected (*e.g.*, *1208/Δ18*). This indicates that the level of *pointed* P2 function is crucial for proper eye development.

Heteroallelic complementation indicates *pointed* function during wing and haltere development: Some of the *trans*-heterozygous escapers showed various defects in wing development. We observed two, in a sense contrary, phenotypes–in some *trans*-heterozygous flies, wing disc development seemed to be blocked entirely, since we observed flies with only one

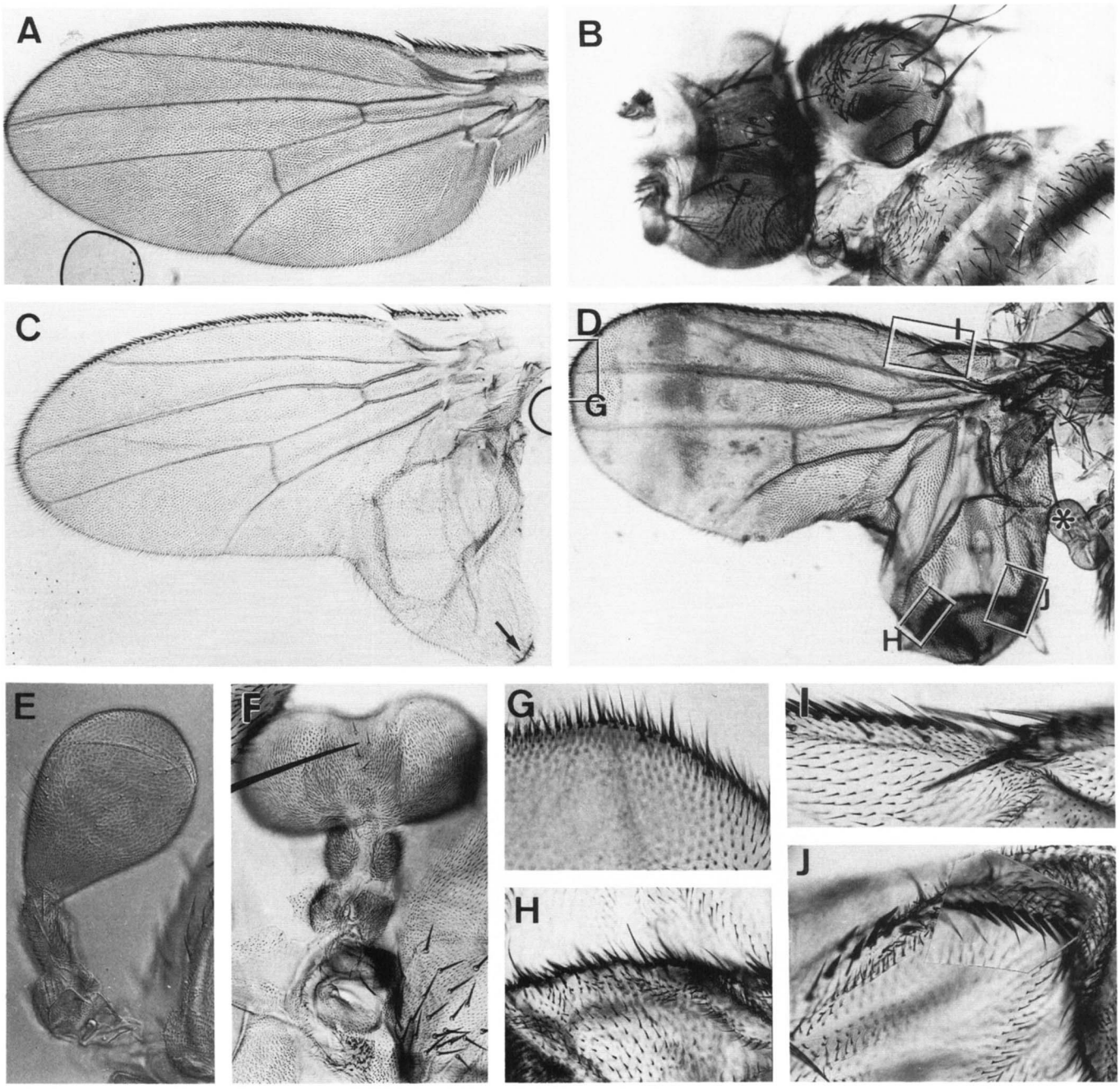

FIGURE 6.—Wing and haltere phenotypes of different *pointed* allelic combinations. The figure compares wing and haltere structures from wild-type flies (A and E) with those of different *trans*-heterozygous *pointed* flies (B, C, D and F). In certain allelic *pointed* combinations, rare escapers emerge. They often exhibit the halbling phenotype with only one wing and half a notum. (B) A dorsal view of a pnt^{T9}/pnt^{1208} halbling fly. (C) Wing of a *trans*-heterozygous P2/P2 (pnt^{T9}/pnt^{1230}) fly and (D) wing of a P1/P2 *trans*-heterozygous ($pnt^{\Delta 104}/pnt^{1208}$) fly. (C and D) Extra wing material forms posterior to vein LV. Arrow in (C) points to bristles characteristic for the anterior wing margin. Similarly, the extra wing material in (D) shows a typical anterior wing margin. The boxed areas labeled G, H, I and J are enlarged for better comparison. Note the similar bristle pattern in (G and H), where the tip of the anterior wing margin is compared with the tip of the extra wing material, and in (I and J) where the wing margins near the wing base are compared. Note also the apparent lack of wing venation in the extra wing material formed posterior to vein LV (C and D). In (D) both, the haltere and the wing are similarly affected (asterisk). This larger haltere (F) also appears to consist of mirror duplicated anterior structures, when compared to wild type (E).

wing and half of a notum ("halbling" phenotype, Figure 6B). Interestingly, flies showing the halbling phenotype eclosed in essentially all crosses where rare escapers were observed. In other cases, *trans*-heterozygous flies developed larger wings. Dorsal and ventral wing surfaces often appeared blistered. The larger wing phenotype constitutes of additional wing material devoid of extra veins added to the posterior wing (Figure 6, C and D). It appears that the larger wing phenotype arises by the addition of duplicated anterior wing portions in mirror image. This is evident by comparing the characteristic triple row bristle pattern

TABLE 2

Complementation analysis of *pointed* P2 alleles

	1218	*T6*	*T9*	*1208*	*1230*	*T5*	EMS
1218	l	esc,r	esc,r	v	v	v	v
T6		l	l	var	r	r	r
T9			l	esc,r	esc,r	esc,r	l
1208				l	esc,r	esc,r	l
1230					l	l	l
T5						l	l
EMS							l

The results of complementation crosses of the P2 alleles inter se are indicated by the following symbols: l, lethal; v, viable, *trans*-heterozygotes eclose in the expected frequency; esc,r, escaping *trans*-heterozygous flies were observed at a low frequency, ranging from 1 to 5% of the expected numbers. The escaping flies exhibit wing and/or eye phenotypes. r, rough eye, *trans*-heterozygous flies emerge in the expected frequency, but have rough eyes. var, *trans*-heterozygous flies show an occasional activation of the white mini-gene located in the *1277* enhancer trap element inserted in the first P2 exon in pnt^{T6} (see Figure 7C). EMS, we have used the EMS induced *pointed* allele $pnt^{88.1}$.

found at the anterior wing margin with the bristle pattern seen at the margin of the newly formed wing tissue (compare Figure 6, G and H, with I and J). Addition of mirror image duplicated material is restricted to a small part of the posterior wing. The alula is still present in these larger wings. No alterations were detected in the anterior portions of the wing.

Similar phenotypes were observed for the halteres: some of the *trans*-heterozygous escapers lacked halteres, while others showed enlarged or similarly duplicated halteres (Figure 6, D and F). In summary, strikingly different wing and haltere phenotypes can be found in the progeny of the same cross.

Complementation analysis of the P2 alleles: To further characterize the nature of the two complementation groups identified next to the *1277 P* element insertion, we have carried out a complementation analysis of the P2 alleles inter se (Table 2). *1208*, *1230* and *T5* uncover *pointed*, whereas *T6* and *1218* uncover a second lethal complementation group. *T9* uncovers both complementation groups. In many crosses we observed *trans*-heterozygous escapers with phenotypes very similar to those found in flies *trans*-heterozygous for different P1 and P2 alleles (compare Figure 5, B, C and D, with 6, C and D). Again wing phenotypes of opposite quality were detected.

With respect to the size of the associated deletions, several results from the P2 complementation analysis were unexpected. (1) Although the deletion associated with *1208* is larger than the *1230* deletion, *1208* behaves as the weaker *pointed* P2 allele, as it is only lethal over the strong *pointed* alleles. (2) The deletion associated with *1218* is contained within the *1208* deficiency, but nevertheless fully complements *1208*. On the other hand the *1218* chromosome is essentially lethal in *trans* to the *T9* deficiency, which uncovers the *1218* deletion as well (see Figure 4 for details). (3) The *T9* chromosome behaves as a strong *pointed* allele (see Table 1) as it fails to complement most P1 alleles. Nevertheless, when crossed to weaker *pointed* P2 alleles rare escaping *trans*-heterozygous flies emerge–except when crossed to *T6*. (4) Although *T6* as well as *T9* homozygous embryos have similarly reduced levels of P2 transcription, only *T9* fails to complement *pointed*.

Transvection-like effects may be involved in the transcriptional control of the P2 promoter: *Transcription in the pointed P2 promotor region can be activated in trans:* How can these complementation results be explained? Since the P2/P2 phenotypes resemble those found for P1/P2 allelic combinations it appears that they originate from an increase of *pointed* P2 activity. In other words, the escaping *trans*-heterozygous flies have more *pointed* P2 function compared to their parents and survive. Certain P2 alleles can therefore activate P2 transcription in trans from the homolog chromosome. This also explains the complementation results shown by the X-ray-induced allele *T6*. Homozygous *T6* embryos have reduced levels of P2 transcription and are lethal. When in *trans* to a strong P1 allele, this chromosome can now *trans*-activate P2 transcription from the *T6* chromosome. When in *trans* to *T5*, from which due to the inversion break no P2 RNA can be transcribed, P2 expression from the *T6* chromosome becomes *trans*-activated and heterozygous flies survive. The rough eye phenotype seen in *T5/T6* flies stems from the reduction of P2 transcription by the *1277* enhancer trap insertion, which is unchanged in *T6*. When *T6* is placed over *T9*, no *trans*-activation of P2 transcription occurs and *T6/T9* animals remain lethal.

Similar transvection effects were also observed for the activity of the *white* mini-gene and the *lacZ* gene, both located in the *1277* enhancer trap element. The *white* mini-gene in the 1277 enhancer trap line gives rise to a yellowish eye color when heterozygous (Figure 7A) and to an orange eye color when homozygous. In the homozygous lethal reversion allele pnt^{T9} the *white* mini-gene is destroyed. In flies *trans*-heterozygous for pnt^{1277} and pnt^{T9} the activity of the *white* mini-gene present on the pnt^{1277} chromosome is greatly reduced resulting in white eyed flies (Figure 7B). This negative *trans*-effect is not restricted to the activity of the *white* mini-gene, as the function of the *pointed* P2 promoter seems also to be reduced. This is apparent from the rough eye phenotype seen in pnt^{1277}/pnt^{T9} flies (Figure 7B). Similarly the activity of the *lacZ* gene present in pnt^{1277} is reduced by the pnt^{T9} chromosome (data not shown). A *trans*-activating DNA domain should therefore be uncovered by the T9 deletion

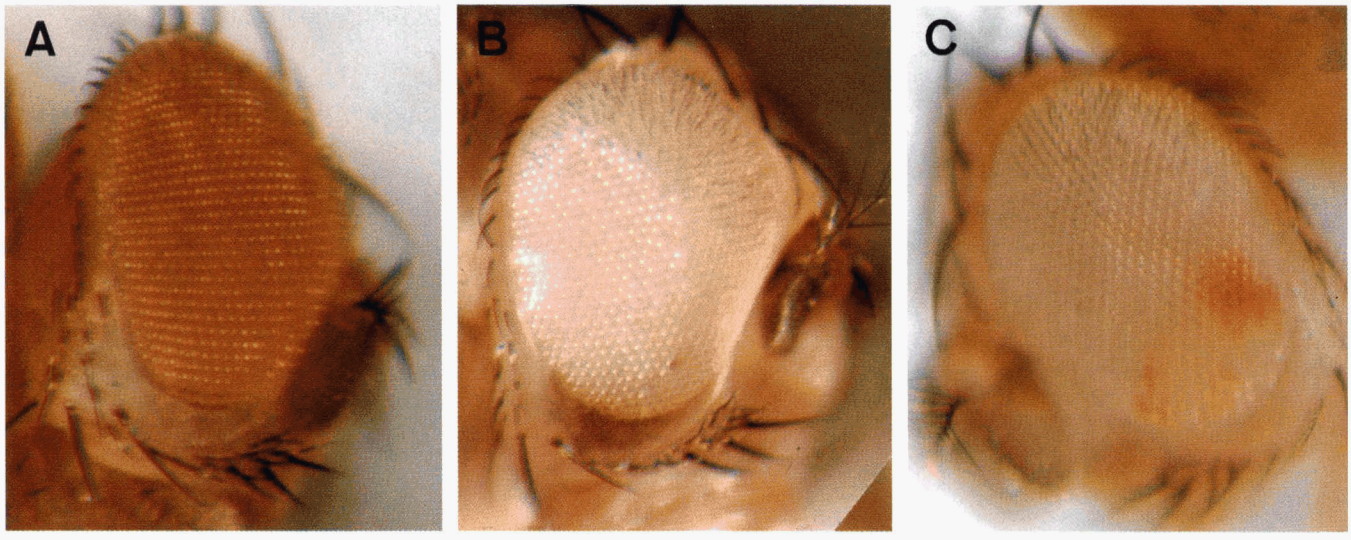

FIGURE 7.—P2 allelic combinations show a variegated eye phenotype. Compound eyes of the following genotypes are shown: (A) pnt^{1277}/*TM6*, (B) pnt^{T9}/pnt^{1277}, (C) pnt^{1208}/pnt^{T6}. Anterior is to the left in (A and B) and to the right in (C). The orange eye color associated with the 1277 enhancer trap element (A) is changed to white when *trans* to the pnt^{T9} chromosome (B). Note also the slight rough eye phenotype in (B). (C) When the X-ray-induced, $white^-$ allele pnt^{T6} is in *trans* to the $white^-$ pnt^{1208} chromosome a sporadic *trans*-activation of the *white* mini-gene in pnt^{T6} is observed, resulting in a variegated eye phenotype.

and thus resides to the left of the *1277 P* element insertion.

Transcription in the pointed P2 promotor region can be repressed in trans: The existence of additional *trans*-acting sequences is indicated by the following result. As described above, we were unable to detect any genomic alterations in pnt^{T6}, indicating that the *1277* enhancer trap element remained intact in this line. When pnt^{T6} is crossed to pnt^{1208}, *trans*-heterozygous flies have a variegated eye color phenotype (Figure 7C). This implies that the pnt^{1208} chromosome is not only able to *trans*-activate *pointed* P2 expression (*trans*-heterozygous escapers emerge) but also expression of the *white* mini-gene in pnt^{T6}. Since activation of the *white* gene in *T6* is observed only in this particular cross, a DNA sequence capable of repressing transcriptional activity in *trans* is likely to be located within the 1-kb genomic DNA stretch upstream of exon I, which is removed by the pnt^{1208} deletion but not in pnt^{1230} or pnt^{T9} (Figure 4). Removal of this *trans*-repressing sequence results in the activation of the *white* gene inserted in the P2 exon on the homologous chromosome (see Figure 8).

In summary, two *trans*-acting sequences with opposite effects control the activity of the *pointed* P2 promoter. Using X-ray- and Δ2-*3*-induced deletions we have located the approximate positions of these control sequences relative to the first P2 exon.

DISCUSSION

In this report we have presented a genetic dissection of the *pointed* locus and showed that, in addition to being required for embryogenesis, *pointed* function is needed during eye and wing development. Interestingly, like *pointed*, other members of the spitz group are required in these same tissues during embryonic and imaginal disc development (MAYER and NÜSSLEIN-VOLHARD 1988; BIER, JAN and JAN 1990; KLÄMBT, JACOBS and GOODMAN 1991; HEBERLEIN and RUBIN 1991; FREEMAN, KIMMEL and RUBIN 1992; DIAZ-BENJUMEA and GARCÍA-BELLIDO 1990; KLEIN and CAMPOS-ORTEGA 1992; STURTEVANT, ROARK and BIER, 1993).

***pointed* P2 function is not solely dependent on the ETS domain:** In order to understand the function of each of the two transcript forms expressed from the *pointed* locus, we have begun to isolate transcript specific mutations. Starting from the *rM254 P* element insertion into the 5′ region of the P1 transcript (which interferes with transcription of both P1 and P2 RNAs; KLÄMBT 1993) we have generated a set of deletion mutants.

Among the alleles with a strong embryonic *pointed* phenotype one is of particular interest. The deletion *Δ114* removes both the P1-specific exon 1 and the exon 2, which are shared by both transcript forms. Based on this and the embryonic phenotype associated with $pnt^{\Delta 114}$, this allele was initially classified as amorphic (KLÄMBT 1993). Genetic data, however, prove a residual amount of P2 function still present in $pnt^{\Delta 114}$ (see Table 1). The deletion of exon 2 from the P2 RNA does not affect the open reading frame and therefore should result in a modified *pointed* P2 protein of almost half of the normal size, lacking the N-terminal half of the ETS domain. How can we explain the residual P2 function associated with $pnt^{\Delta 114}$? DNA binding by the *pointed* ETS domain is probably destroyed by the truncation, as similar N-

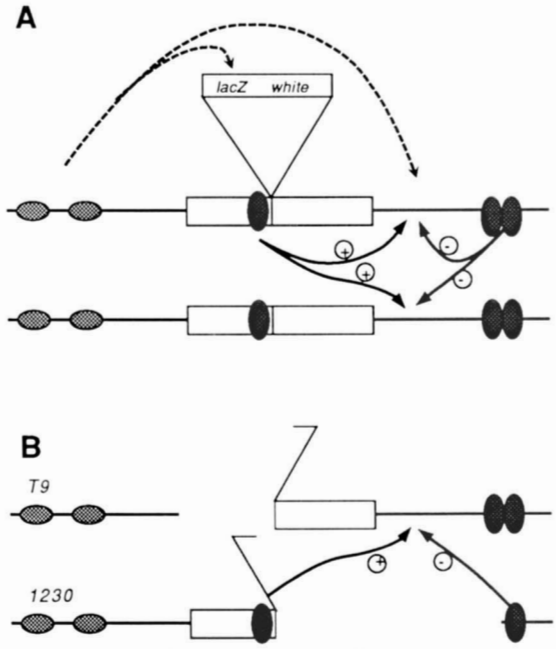

FIGURE 8.—Model for the regulation of the *pointed* P2 promotor activity. The *pointed* P2 promotor region is depicted. Exon I is boxed and the *1277* enhancer trap insertion is indicated by a triangle. Transcription is from right to left. (A) On the left of exon I, presumably within intronic sequences, enhancer elements reside that govern the tissue specific expression of the *pointed* P2 RNA as well as the expression of the *lacZ* gene in the pnt^{1277} enhancer trap line. In addition to these enhancer elements two *trans*-acting domains have been identified. One is able to *trans*-activate RNA expression, the other domain, which extends over a larger stretch of DNA leads to *trans*-repression of transcriptional activity. A *cis* action was shown only for the activation domain. (B) shows a possible explanation for the emergence of surviving *trans*-heterozygote flies at the example pnt^{T9}/pnt^{1230}. In pnt^{T9} the *trans*-activation domain is deleted, which leads to lethality in homozygotes. In pnt^{1230} the presumptive promotor region plus part of the *trans*-repression domain is deleted, which results in lethality when homozygous. However, in pnt^{1230}/pnt^{T9} flies the activation domain of *1230* acts in trans on the promotor region in *T9*. Subsequently, P2 RNA is made and escaping *trans*-heterozygous flies eclose.

terminal deletions in the human ETS1 domain, which is 96% identical to the *pointed* ETS domain, abolish DNA binding completely (WASYLYK, KERCKAERT and WASYLYK 1992). The residual *pointed* P2 function in $pnt^{\Delta 114}$ may indicate that the *pointed* P2 product functions not only via binding to DNA alone, but may accomplish its tasks by interacting with other proteins, analogous to the homoebox independent activity of the *fushi tarazu* protein (FITZPATRICK *et al.* 1992). Since the remainder of the *pointed* P2 protein is unchanged, possible protein-protein binding motifs may still be functional and could allow cooperative interaction with other proteins. Such interactions have been described for the ETS1 protein and other related transcription factors of the ETS family (WASYLYK *et al.* 1990; HIPSKIND *et al.* 1991; THOMPSON, BROWN and MCKNIGHT 1991; DALTON and TREISMAN 1992). The residual P2 function in $pnt^{\Delta 114}$ might therefore point to a dual mechanism by which *pointed* function is mediated, one via direct DNA binding and one involving cooperative interaction with other transcriptional regulators.

Two *trans*-acting domains control the *pointed* P2 expression: Reversion of the *1277 P* element insertion located in the first P2 exon yielded a number of alleles which we initially placed into two independent lethal complementation groups. Based on our genetic and molecular analysis we now classify both of these complementation groups as *pointed* alleles, with members of one lethal complementation group affecting a *trans*-activation domain.

Trans-acting or *trans*-sensing effects (TARTOF and HENIKOFF 1991) comprise phenomena of allelic *trans*-interaction that depend on chromosome pairing (for reviews see: JUDD 1988; WU and GOLDBERG 1989; TARTOF and HENIKOFF 1991). *Trans*-acting effects were first discovered by LEWIS in 1954 during the analysis of the *Bithorax* gene complex. He found that chromosomal rearrangements modified the phenotype of a specific *bithorax* allelic combination, a phenomenon which he defined as transvection (LEWIS 1954). Since then transvection and related *trans*-acting effects have been reported for a few other genes, including *decapentaplegic* (GELBART 1982), *brown* (HENIKOFF and DREESEN 1989), *white* and *zeste* (JACK and JUDD 1979), *vestigial* (WILLIAMS, BELL and CARROLL 1991), *rhomboid* (STURTEVANT, ROARK and BIER 1993), and the SGS4 locus (KORGE 1977; KORNHER and BRUTLAG 1986).

Beside enhancer elements that govern the tissue- and stage-specific expression of the P2 promoter, we have identified two sequence domains that mediate opposing effects on transcriptional activity of the *pointed* P2 promoter region in trans. This can be monitored by the *pointed* P2, the *white* mini-gene and the *lacZ* activity of the *1277 P* element. If the activation domain is affected, a reduction of *pointed* P2 and/or *white* activity occurs as seen in the alleles *T9* or *T6*. Evidence for *trans*-inactivation can be seen for example in pnt^{T9}/pnt^{1277} flies. Here the *pointed* P2 as well as the *white* mini-gene activity is reduced resulting in rough, white eyes. Similarly *lacZ* expression is reduced during embryogenesis. *Trans*-activation of *pointed* P2 function is evident in the frequent emergence of *trans*-heterozygous escapers as well as in the *trans*-activation of the *white* mini-gene as seen in pnt^{T6}/pnt^{1208} *trans*-heterozygotes. It is important to note that transcriptional activity can be activated to a different extent. pnt^{1208} and pnt^{1230} are both able to *trans*-activate *pointed* P2 transcription and thereby allow the emergence of heterozygous escapers. However only pnt^{1208} is able to *trans*-activate the *white* mini-gene in pnt^{T6} at higher levels that result in the variegated eye color phenotype. This may indicate that the *trans*-repressing domain is spread over some distance, as it has

been reported for the *brown* gene (DREESEN, HENIKOFF and LOUGHNEY 1991).

In the X-ray-induced pnt^{T6} allele *pointed* P2 function is reduced and therefore homozygous animals are lethal. This is in part due to *cis* effects, the presence of which is revealed by the white eye phenotype of heterozygous T6/balancer flies. However, if pnt^{T6} is placed in *trans* to alleles that still have the *trans*-activation domain but are unable to transcribe P2 RNA themselves (as for example the inversion allele pnt^{T5}), *pointed* P2 expression becomes *trans*-activated from the pnt^{T6} chromosome and and *trans*-heterozygous flies eclose. *Trans*-activation is not restricted to *pointed* P2 but again also affects the activity of the *white* mini-gene on the *T6* chromosome, which results in the variegated eye phenotype seen in pnt^{T6}/pnt^{1208} flies. It appears paradoxical that lethality is complemented when pnt^{T6} is *trans*-heterozygous to alleles in which both the *trans*-activation and the *trans*-repressing domains have been deleted (*e.g.*, *1208*). This might reflect a state of deregulated chromatin condensation resulting in a deregulated level of RNA expression.

***pointed* P2 functions during imaginal disc development:** Complementation analysis allowed us to place the P2 alleles into a specific order. However even in the strongest allele, pnt^{T9}, we still detect P2-specific transcription. pnt^{T9}, which does not lead to an embryonic phenotype, therefore represents a hypomorphic P2 allele. Although we cannot determine the embryonic requirement for *pointed* P2 function, our genetic analysis has revealed novel functions during the development of several imaginal discs. The phenotypes shown by different P1 and P2 allelic combinations indicated that the dose of P2 function is crucial for proper eye development. This has been independently demonstrated by clonal analyses of different *pointed* alleles (H. SCHOLZ and C. KLÄMBT, manuscript in preparation).

In addition to the rough eye phenotype wing and haltere phenotypes of strikingly different quality were detected–flies either lacked one wing and half of the notum or they had abnormally large wings. The emergence of flies lacking one wing and half of the notum suggests that one wing imaginal disc has not developed. Similar interpretations are true for the halteres. This "halbling" phenotype might be due to a reduction of *pointed* function in the imaginal disc or their anlage, which also correlates with *pointed* P2 expression (Figure 2B). The "halbling" phenotype resembles the *vestigial* phenotype, where wing and haltere formation are affected. Interestingly, expression of the *vestigal* gene, which is also expressed in the imaginal disc anlagen, is regulated by transvection like effects as well (WILLIAMS, BELL and CARROLL 1991).

Pattern duplications as seen in the wing and haltere might be due to reduction of P2 activity below a certain threshold. The additional wing material could result from local cell death and subsequent regeneration processes (BRYANT 1975; FRENCH, BRYANT and BRYANT 1976) or could be due to a change in positional information similar to the pattern duplications evoked by *costal-2* allelic combinations (SIMPSON and GRAU 1987) or the $su(f)^{ts}$ allele (JÜRGENS and GATEFF 1979). In the light of the transcriptional control mechanisms discussed above, however, it is conceivable that the presence of extra wing material is the result of an overexpression or even ectopic expression of *pointed* P2 in the wing disc.

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