The *Drosophila* gene **pointed** encodes two ETS-like proteins which are involved in the development of the midline glial cells

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SUMMARY

Glia-neuronal cell interactions at the ventral midline are necessary for the proper elaboration of commissures in the embryonic CNS of *Drosophila*. In particular, migrating midline glial cells are required for the separation of segmental commissures. During this process the glial cells recognize specific neuronal cells at the midline, they migrate posteriorly along their cell processes and thereby separate the segmental commissures. The gene **pointed** (*pnt*) is required for this glia-neuronal cell interaction, as loss of function mutations lead to a change in the migration behavior of the midline glial cells. As a consequence, anterior and posterior commissures do not become separated and appear fused. Molecular analysis of **pointed** has revealed two differently spliced types of transcripts, which are encoded in a region extending over 55 kb of genomic sequence. In the CNS both transcript classes are expressed in cells of the midline, including the midline glial cells. Sequence analysis of cDNA clones corresponding to both transcript types reveals two different **pointed** proteins which share an ETS domain common to a number of transcription factors related to the vertebrate *ets* oncogene. Furthermore, one **pointed** protein form contains an additional domain of homology of approx. 80 amino acids in length, which is shared by only a subset of the ETS protein family.

Key words: *Drosophila* CNS, midline glia, **pointed**, ETS

INTRODUCTION

A general theme in the organization of higher nervous systems is their bilaterally symmetrical appearance. In the spinal cord of vertebrates, as well as in nerve cord of insects, commissural axons interconnect the two halves of the nervous system. In both systems, formation of these nerve tracts is tightly linked to a special set of cells located in the symmetry axis, the midline of the nervous system. Commisural growth cones first grow towards the midline, but then they cross the midline and turn to extend rostrally or caudally in one of the contralateral longitudinal axon tracts. This is remarkable, since the growth cones show no affinity for the homologous tracts on the ipsilateral side, but change their behavior as they cross the midline. Not surprisingly, the cells located at the midline play an important and instructive role in the establishment of these commissural axon tracts, in that they guide commissural growth cones towards and across the midline. What are the main features that midline cells need to perform these functions? For the vertebrate nervous system in vitro experiments indicate that the midline, or floor plate, cells are capable of chemotropically attracting commissural growth cones (Tessier-Levigne et al., 1988; Placzek et al., 1990). However, direct cell-cell interactions with the floor plate occur as the commissural growth cones cross it (Boloventa and Dodd, 1990).

Similar considerations apply for the *Drosophila* system. Here the midline comprises a small number of neuronal and glial cells (Jacobs and Goodman, 1989a; Klämbt et al., 1991) that have unique morphological properties, which prompted Poulsom (1950) to coin the term mesectoderm for this cell group. During the blastoderm stage two stripes flanking the mesodermal anlage, each a single cell wide, become determined to form the future midline as they start to express the gene **single minded** (*Crews et al., 1988*). This gene encodes a transcription factor of the HLH type and serves as the master gene for midline development (Nambu et al., 1990, 1991). As gastrulation proceeds, the two cell rows expressing the **single minded** gene are brought together at the midline, where they intermingle to form a single row comprising eight precursor cells per segment. These cells divide, forming a distinct mitotic domain (Foe, 1989), to produce about 26 midline cells per segment. Of these six ventral unpaired median (VUM) neurons and three pairs of midline glial cells are known to have important functions during commissure formation (Klämbt et al., 1991). Commisural connections begin to form soon after the completion of cell divisions that result in the 26 midline cells. Initially the anterior and posterior commissures are found in very close proximity in each segment. The final appearance of the ladder-like axon pattern of the insect central nervous system (CNS) is subsequently attained by the action of the midline glial cells. During the develop-
ment of commissures these glial cells recognize cell processes of midline neurons belonging to the VUM cluster, along which the glial cells migrate posteriorly, thereby separating anterior and posterior commissures (Klämbt et al., 1991).

*Drosophila* provides the unique opportunity to dissect genetically the glial-neuronal cell interactions that lead to migration of midline glial cells and hence to the separation of commissures. Fortunately a number of mutants that affect different aspects of midline development are already known (Jürgens et al., 1984; Wieschaus et al., 1984; Nüsslein-Volhard et al., 1984; Mayer and Nüsslein-Volhard, 1988; Thomas et al., 1988; Bier et al., 1990; Finkelstein et al., 1990; Klämbt et al., 1991; Raz and Shilo, 1992; Klämbt et al., 1992; Rutledge et al., 1992). Based on defects seen in the ventral cuticle a subset of these genes has been classified as the ‘spitz group’ (Mayer and Nüsslein-Volhard, 1988). In addition to their epidermal phenotype, all members of the ‘spitz group’ show a CNS phenotype, which have as a common factor, midline cells affected in various ways (Thomas et al., 1988; Mayer and Nüsslein-Volhard, 1988; Klämbt et al., 1991). If for example, the midline glial cells are absent or impaired in their function, as can be seen in certain mutant backgrounds (Star, spitz, rhomboid and certain faint little ball alleles), no separation of the segmental commissures occurs and they appear fused (Klämbt et al., 1991; Raz and Shilo 1992). Here I report on the characterization and molecular isolation of pointed, a member of the spitz group. In addition to its role during pattern formation of the ventral ectoderm, pointed is needed for glial-neuronal interactions in the CNS. In homozygous pointed embryos midline glial cells do not migrate posteriorly, but instead move out laterally along the width of the commissures. As a consequence, the anterior and posterior commissures appear fused. In addition to the epidermal phenotype, at least two other organs, the trachea and certain muscles, require pointed expression. Molecular analysis of the pointed gene reveals a complex transcription pattern. Two promoter regions, which are separated by some 50 kb, drive two temporally and spatially different transcription patterns. cDNA clones corresponding to the different transcript types were isolated and sequenced. They code for two different proteins that share a common region with a high degree of homology to the ETS domain, a DNA binding domain found in a family of proteins related to the vertebrate ets oncogene (Karim et al., 1990). In one of the two deduced pointed proteins, an additional stretch of homology to this family of transcription factors is found, defining a new sequence domain conserved during evolution.

**MATERIALS AND METHODS**

**Isolation of new pointed alleles**

The following pointed alleles were used in this study: two EMS alleles pointed$^{8874}$ and pointed $^{9331}$ (Jürgens et al., 1984) and pointed$^{M254}$, which was isolated in an enhancer trap screen carried out in the laboratory of C.S.Goodman, Berkeley, USA. (C.Klämbt, A.Nose and C.S.Goodman, unpublished data). All these alleles are homozygous lethal and the phenotypic traits associated with the different pointed mutations are indistinguishable from each other (see below). The embryonic lethality associated with pointed$^{M254}$ can be reverted by mobilizing the P-element insertion (Robertson et al., 1988), thus pointed$^{M254}$ represents a P-element induced lethal pointed allele. Moreover in pointed$^{M254}$ no pointed message could be detected (data not shown), so that this mutant allele probably represents a null (see below). Additional pointed alleles were recovered as revertants of the pointed$^{M254}$ allele. 25 independent excision chromosomes were tested. 11 lines were viable, suggesting an exact excision of the element and 14 lines remained lethal and failed to complement pointed EMS alleles. Therefore pointed$^{M254}$ is a P[+Z; rosy]1 induced lethal pointed allele. All pointed alleles were kept over a TM3 Sb balancer chromosome carrying a P[elav-lacZ] construct in order to allow the unambiguous identification of mutant embryos.

**Antibody staining**

Immunohistochemistry was performed as described previously (Klämbt et al., 1991).

**DNA techniques**

Initially, pointed DNA sequences were isolated by inverse PCR. 1 µg pointed$^{M254}$ DNA was digested with MboII to completion and religated at 0.2 µg/ml. Two oligo nucleotides designed to prime within the 3’ end of the P-element were used to perform a standard PCR reaction (1 minute at 95°C, 1.5 minutes at 55°C, 1 minute at 72°C, for 35 cycles). The resulting PCR product of 820 bp was purified on an agarose gel and subsequently used to screen a genomic EMBL4 library, kindly provided by M.Noll Zürich. Chromosomal walking was performed according to standard procedures. Several genomic phages were hybridized to polytenic chromosomes to verify the cloning of the correct chromosomal region at 94E/F. cDNA clones were isolated from a λgt10 library, kindly provided by K.Zinn (Zinn et al., 1988). Nested deletions were constructed after DNAase I treatment (Sambrook, 1989) and subsequently sequenced using a T7 based sequencing kit (Phar

**In situ hybridization**

DNA probes were labeled by random priming. Non-radioactive in situ hybridization experiments using digoxigenin-labeled probes were performed as described (Tautz and Pfeifle, 1989). The stained embryos were embedded in GMM (Ashburner, 1989) and photographed using a Zeiss Axiophot microscope.

**RESULTS**

pointed function is required in several different tissues

In addition to the epidermal phenotype, which lead to the isolation of pointed (Jürgens et al., 1984; Mayer and Nüsslein-Volhard, 1988), other phenotypes are associated with this mutation. Inspection of homozygous pointed embryos with antibodies that outline the trachea (antibodies to erythrocytes; Tepass et al., 1990 and antiseraum no. 84, kindly provided by B.Shilo) reveals that the tracheal dorsal trunk fails to form its segmental connections, resulting in a tracheal phenotype similar to that associated with hypomorphic alleles of breathless, the gene encoding the FGF receptor 1 (Klämbt et al., 1992). As with weak breathless alleles, the tracheal cells in pointed mutant embryos start to grow normally in homozygous pointed embryos, but migration stalls when the cell reach the ventral oblique musculature (Fig. 1B). A second defect can be seen in pointed
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Embryos by using the muscle specific antibody 1B7 (kindly provided by A. Nose and C.S. Goodman, Berkeley, USA). A mature wild-type embryo contains 31 muscle fibers per abdominal hemisegment A1-A7 (Bate, 1990). Comparison of the muscle patterns in wild-type and homozygous mutant embryos revealed that in homozygous pointed embryos several muscle fibers are missing (fiber no. 4 = p12, 11 = deo2, 19 = deo1, 20 = peo1; nomenclature according to Crossly,
Muscles 15, 16 and 17, which attach at the ventral epidermis appear small and displaced (Fig. 1E, arrows). These defects however may be a reflection of the epidermal phenotype of pointed embryos, in which the ventral epidermal cells are affected (Mayer and Nüsslein-Volhard, 1988).

CNS phenotype of pointed embryos

The terminal CNS phenotype of homozygous pointed embryos, as it becomes manifest in a stage-16 embryo consists of fusion of anterior and posterior commissures and reduction in thickness of axon bundles in the longitudinal connectives (Fig. 2E). This differs from the CNS phenotype described by Mayer and Nüsslein-Volhard (1988), who observed a disruption of both the commissures and the connectives. The fusion of commissures can be interpreted as being due to a disruption of midline glial cell function.

During development of the wild-type CNS the two segmental commissures are initially formed in close proximity and are then separated by migration of the middle pair of midline glial (MGM) cells (Klämbt et al., 1991). For this migration to take place, the midline glial cells must recognize cell processes of the VUM neurons, along which they are able to move. The enhancer trap line AA142 shows lacZ expression specifically in the midline glial cells (Klämbt et al., 1991). To follow the fate of the midline glial cells in homozygous pointed embryos a recombinant of AA142 and pointed8B74 was isolated. Double labelling experiments using X-gal to show midline cells and immunohistological detection of the monoclonal antibody BP 102 to show CNS axon tracts, revealed that the midline glial cells are still present in homozygous pointed embryos. However, these glial cells do not migrate posteriorly along the cell processes of the VUM neurons (Fig. 2C,D). Although the

**Fig. 2.** Commissures appear fused in pointed embryos. Frontal views of dissected embryonic CNS. The midline glial cells are labeled with X-gal staining of the enhancer trap insertion AA142 in a pointed8B74 background (blue), CNS axons are visualized using the monoclonal antibody BP102 and HRP immunochemistry (brown). (A) In a late stage-12 wild-type embryo the anterior and middle pair of midline glial cells have migrated towards the ‘fusion point’ of anterior and posterior commissure. (B) The middle pair of midline glial cells migrates further posteriorly and thereby separates anterior and posterior commissure. These glial cells are always in close contact to the axons of the VUM cluster (arrowhead). In a homozygous pointed8B74 embryo as shown in C,D the midline glial cells are found distributed over the entire width of the commissures. Contrary to wildtype, where the middle pair of midline glial cells separates the segmental commissures (B), in homozygous pointed embryos the midline glial cells fail to migrate and remain unfunctional. Therefore the segmental commissures stay fused (D). The glial cells become misplaced dorsally and finally degenerate (D, compare the glial cells marked by arrows in the different segments). Abbreviations: arrowhead, axons of the VUM cluster; ac, anterior commissure; pc, posterior commissure.
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midline glial cells apparently fail to recognize the cell surfaces of the VUM neurons, they still seem to be able to recognize axonal membranes, because in the mutant they are now found associated with axons of commissural neurons as they cross the midline. This results in all midline glial cells lining up along the entire width of the commissural connections (compare Fig. 2A and C). The ultimate consequence of the missrouting of midline glial cells is that anterior and posterior commissures are not separated into two distinct commissures but appear fused (compare Fig. 2B and D). In later stages of embryonic development the misplaced midline glial cells degenerate and eventually die (see Fig. 2D).

Is the aberrant behavior of the midline glial cells due to a lack of pointed function in the glial cells themselves or in their target cells, the VUM neurons? The AE60 enhancer trap line expresses β-galactosidase in the six VUM neurons and the two MP1 neurons at the midline (Klämbt et al., 1991). Analysis of the AE60 staining pattern in homozygous pointed8B74 embryos, showed the normal number of VUM neurons in the correct position (data not shown). In addition, staining with the monoclonal antibody 22C10

Fig. 3. CNS phenotype of double mutants. Axons of dissected embryonic CNS (stage 14) are visualized using the monoclonal antibody BP102 and HRP immunochemistry. A shows the CNS of a wild-type embryo. Note the regular appearance of anterior and posterior commissure in each segment. B shows the CNS of a pointed, rhomboid double mutant embryo. Note the reduction in commissural axon tracts. C shows a CNS of a pointed; Star double mutant embryo. Note the reduction in commissural axons are missing.

Fig. 4. Genomic organization of the pointed locus. Schematic drawing of the genomic organization of pointed at the cytological location 94E/F. The insertion site of the P[lacZ, rosy] enhancer trap element in pointed8M254 is indicated by a triangle. The c4 cDNA corresponds to the P1 transcript type and spans about 5 kb of genomic sequence. Exons (1, 2, 3, 4) were identified by hybridization and comparison of genomic and cDNA sequences. A genomic piece of DNA containing only exon 1 was used as a P1-specific probe. Exons 2,3,4 are also contained within the c5 cDNA, which represents the P2 transcript type. This transcript contains five additional P2-specific exons (I, II, III, IV, V) and covers 55 kb of genomic sequences with two large introns of 16 kb and 21 kb. A c5 cDNA restriction fragment containing exons I and II was used as a P2-specific probe. Exons are not drawn to scale, for the exact size of individual exons see Fig. 5. The extend of the deletion pointedΔ114 was determined by Southern blot analysis and is indicated by a black bar, shaded regions are uncertain.
(Fujita et al., 1982), which labels the cell surfaces of the VUM neurons from stage 14 onwards, detected no defects associated with these neurons (data not shown). Of the six muscles fibers normally targeted by the VUM motor neurons only one is missing in pointed embryos (muscle fiber 19, Sink and Whitington, 1991).

To address the question of which midline cell type (glial or neuronal) requires pointed function, I analyzed the CNS phenotype of double mutant combinations. Loss of rhomboid or Star gene function leads to the specific loss of the midline glial cells or of midline glial cell function and to the same axon phenotype - fused commissures - as pointed (Fig. 1F; Klämbt et al., 1991). If the functional requirement for pointed gene activity is restricted to the midline glial cells, double mutant embryos should exhibit the same CNS phenotype as either mutation alone. However, double mutant combinations of rhomboid and pointed show a severe reduction in the number of commissural fibers crossing the midline, with only one commissure per segment (Fig. 3B). Double mutant combinations of Star and pointed lead to an even more severe phenotype in which no commissures are formed (Fig. 3C). These results indicate that some pointed function is needed in the VUM cluster, but cannot address the question of whether pointed is needed in the midline glial cells.

In summary, pointed mutations lead to a number of different defects in all three germ layers. For the CNS, pointed function is needed at least in the VUM cluster of midline cells but we cannot exclude a requirement for pointed in the midline glial cells.
Cloning of pointed sequences

To understand better the function of pointed, it is necessary to clone the gene and determine the spatial distribution of corresponding transcripts. The location of the P element in the lethal P[lacZ, rosy]-induced pointed mutation pointedrM254 was determined by hybridizing lacZ probes to polytene chromosomes (not shown), to be 94E/F and corresponds well with the meiotic location of pointed at 3-79 (Jürgens et al., 1984). Genomic sequences flanking the pointedrM254 insertion were isolated by inverse PCR (see Materials and methods) and about 90 kb of genomic sequences were subsequently cloned by standard techniques (not shown, Fig. 4). The P element in the pointedrM254 mutation was inserted in the 5′ region of a transcription unit that gives rise to a 4.2 kb long mRNA (data not shown). Correspondence of pointed to this transcription unit is based on the following observations: (i) transcription of this locus is eliminated in homozygous pointedrM254, (ii) this transcription unit is absent in the excision line pointedΔ114, which behaves phenotypically like pointedrM254 (Fig. 4, H.Scholz and Klämbt, unpublished). In addition the transcription pattern of this gene corresponds well to all phenotypic traits of the pointed mutation (see below). DNA flanking the P-element insertion site was therefore used to screen a cDNA library (Zinn et al., 1988). The longest cDNA clone (c4, 3178 bp in length, corresponding to the so called P1 transcript, see Fig. 4) was further analyzed and used to search for more cDNAs. This led to the isolation of the 3193 bp clone c5, a representative of a different set of splice variants of pointed (the P2 transcript). Neither of the cDNA clones isolated represents a full length cDNA, since northern blot analysis revealed a size of 4.2 kb for the P1 transcript and 4.0 kb for the P2 transcript. However, a complete coding region is included in these cDNA clones (Chen et al., 1992). As judged from in situ hybridization and northern blot data the P2 class is about 1/10 as abundant as the P1 transcript in embryos (not shown, Chen et al., 1992). Both cDNA types were hybridized to phages comprising the cloned region to determine the exon-intron structure of the pointed gene. Some exon-intron boundaries were further verified by comparing genomic and cDNA sequences (Figs. 4, 5). This analysis showed that the P2 transcript initiates at least 45 kb upstream of the P[lacZ, rosy] insertion in pointedrM254 (Fig. 4). It shares a minimum of 3 exons with the P1 transcript, but has at least 5 different 5′ exons spreading over 50 kb. The genomic organization of pointed is summarized in Fig. 4.

pointed encodes two proteins containing an ETS domain and a novel domain

The sequence of the longest cDNA clone for each transcript class was determined. The c4 clone, corresponding to the P1 transcript class, is 3177 bp in length. At base 1047 there is an ATG in frame with a long open reading frame (ORF) coding for a hydrophilic protein of 623 amino acids, with...
a deduced relative molecular mass of 66.8×10^3. The c5 clone corresponds to the P2 transcript class and is 3147 bp in length. It contains a long ORF starting at base 776 with an ATG followed by an ORF of 718 amino acids, the deduced M_r 77.6×10^3 (see Fig. 5). Both translational start sites fit a Drosophila consensus sequence (Cavener, 1987). The two transcripts contain specific sequences at their 5′ ends, while the sequence spanning positions 1746-3147 in c5 is also found in c4 (1733-3134; see Fig. 5 for details). The protein sequences deduced for both cDNA clones were compared with those in the EMBL database using the FASTA program (Pearson and Lipman, 1988). No significant homology could be detected in the N-terminal 2/3 of the c4 ORF, but a region of high homology was found in the C-terminal third of the deduced pointed protein. This region, which is identical in both classes of pointed proteins, is homologous to a number of proteins related to the vertebrate ets oncogene. This region of homology represents an ETS domain, for which DNA binding activity was recently demonstrated (Karim et al., 1990). When compared to the murine ets2 or ets1 sequences, homology is not restricted to the approximately 85 amino acids that define the DNA binding domain, but spreads into flanking sequences (Fig. 5: underlined sequences are compared in Fig. 6. The ETS domain is highlighted by double underlining). Sequence identity to the murine or human ets1 protein sequence (Watson et al., 1988a; Gunther et al., 1990) is 55% over a stretch of 200 amino acids. In the central region containing the ETS domain, homology is higher, here 95 out of 100 amino acids are identical (Fig. 6C). Other members of the ETS family, like Erg, Elk, E74, D-elg; GABPα, Fli1, PEA3 (Reddy et al., 1987; Rao et al., 1989; Burtis et al., 1990; Pribyl et al., 1991; LaMarco et al., 1991; Ben-David et al., 1991; Xin et al., 1992) show weaker homology to pointed sequences ranging from 90% to 60% in the central domain (not shown, see also Chen et al., 1992). In pointed the ETS domain and its flanking sequences are distributed over at least 4 exons, with one intron position (position 2996 in c4 sequence) being conserved from insects to vertebrates (Watson et al., 1988b).

Comparison of sequences derived from the c5 cDNA, specific for the P2 transcript class, with those of the EMBL database revealed an additional homology with some members of the ETS family of transcription factors. This second

**Fig. 6.** Sequence homology to members of the ETS family. Sequences of pointed, murine ets1, ets2, GABPα, erg, fli1 are compared to show the location of POINTED and ETS domains. (A) Schematic view to show the location of POINTED and ETS domains. (B) Amino acid sequence comparison of the POINTED domains shown in A. Only 103 amino acids are shown. The identity in this region is 47.5% for ets2; 51.5% for ets1; 27.2% for GABPα; 26.2% for Fli1; 24.3% for erg2. If only the core region of 72 amino acids is compared, homology is higher: 52.8% for ets2; 58.3% for ets1; 36.1% for GABPα; 34.7% for Fli1; 33.3% for erg2. (C) Amino acid sequence comparison of the ETS domains. Note the conserved tryptophan residues. The position of conserved exon boundaries are indicated by arrowheads. Only amino acids identical to POINTED sequences are indicated. ets1: Gunther et al., 1990; ets2: Watson et al., 1988a; GABPα: LaMarco et al., 1991; Fli1: Ben-David et al., 1991; erg2: Reddy et al., 1987.
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domain of homology is situated in the N-terminal region and spans some 120 aa when compared with ets1 or ets2 (Fig. 6). Remarkably, the central region of homology, which spans some 70 amino acids is contained in a distinct exon in both pointed and chicken ets (Watson et al., 1988). Thus the exon structure in this region has also been conserved during evolution. The murine ets2 and the P2 protein sequences are 46.3% identical over a 123 aa long stretch and murine ets1 sequences share 44.4% identical amino acids over a stretch of 133 amino acids with the deduced pointed c5 protein. This sequence domain is found to be slightly less conserved in some other members of the ETS family: the GA binding proteinc (GABAα; LaMarco et al., 1991) with 23.5% identity over 149 amino acids; the human erg proteins (Reddy et al., 1987) with 34.3% identity over 70 amino acids and Fli1 (Ben-David et al., 1991) with 31% identity over 84 amino acids (see Fig. 6 for details). As in the case of the ETS domain (Karim et al., 1991) or myb-related proteins (Anton and Frampton, 1988) several tryptophan residues are conserved. This region of homology thus defines a second domain conserved during the evolution of ETS-type transcription factors, which, for want of a better name, was designated POINTED domain. Since this domain is found in only a subset of the ETS-like proteins, it divides the ets family of transcription factors into two groups. Some members have only the ETS domain, others have both the ETS and the POINTED domain. The functional significance of this finding remains to be determined.

**Differential distribution of P1 and P2 transcripts during embryogenesis**

To address the question of which cells in the midline express pointed, we determined the temporal and spatial distribution of transcripts by whole-mount in situ hybridization of digoxigenin- (DIG) labeled DNA probes specific to the two different transcript classes (Exon I as P1-specific, exons I, II, III and IV as P2 specific, see Fig. 4). The first pointed P1-type transcripts can be detected during the cellular blastoderm stage in two broad stripes in the lateral neurogenic region, which are modulated in a pair rule pattern. At the beginning of gastrulation (stage 6, according to Campos-Ortega and Hartenstein, 1985) a gradient of

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**Fig. 7.** pointed transcription pattern in young embryos. Transcript distribution was visualized in whole-mount embryos hybridized with transcript-specific, DIG-labeled DNA probes and subsequent alkaline phosphatase detection. The embryo shown in A was hybridized with P2-specific DNA, embryos shown in B-F were hybridized with P1-specific DNA probes. Embryos are oriented with anterior to the left. (A) P2-specific pointed transcripts can first be detected at the anterior pole of a precellular blastoderm embryo. (B) At the onset of gastrulation a broad band of P1 expression can be detected in the lateral neuroectoderm. (C) Double hybridization experiments using snail and pointed c4 DNA probes show that the mesectodermal precursor cells do not express pointed RNA at this stage. (D-F) As gastrulation proceeds, P1 transcription is progressively restricted to a row of ectoderm at first three and later one cell wide, flanking the midline cells. (F) In the lateral ectoderm, segmentally arranged groups of cells begin to express the P1 transcript. These cell groups belong to the tracheal anlage.
pointed P1 transcripts is apparent, with higher levels at the dorsal edge of the neurogenic region and lower levels towards the mesectodermal anlage (Fig. 7B). As gastrulation proceeds, pointed expression refines from a region of initially 10-11 cells broad to a three-cell wide stripe in the lateral neuroectoderm, directly flanking the mesectodermal cells (Fig. 7C, D, E). To test whether the initial lateral domain of P1 expression includes the mesectodermal anlage, whole-mount in situ hybridization experiments were performed using snail and P1-specific DIG-labeled probes simultaneously. At the cellular blastoderm stage, snail is specifically expressed in the mesodermal anlage and not expressed in the directly adjacent mesectodermal cells (Nambu et al., 1990, Kosman et al., 1991; Leptin, 1991). In the stage-6 embryo shown in Fig. 7C a row of ventral cells that are directly adjacent to the mesoderm and correspond to the mesectodermal anlage expresses neither snail nor pointed. Thus, during gastrulation pointed is not expressed at detectable levels in the mesectoderm.

As the germband extends, expression in the ventral neu-

Fig. 8. pointed transcription pattern in older embryos. Transcript distribution was visualized in whole-mount embryos hybridized with transcript-specific DIG-labeled DNA probes and subsequent alkaline phosphatase detection. The embryo shown in B and H was hybridized with P2-specific DNA, embryos shown in A and C-G were hybridized with P1-specific DNA probes. Embryos are oriented with anterior to the left. G, H are frontal views of dissected CNSs, after hybridization with P1-specific DIG-labeled probes (G) and after hybridization with P2-specific DIG-labeled probes (H). (A) P1-type transcripts are found only in the ectoderm, whereas P2-type transcripts are localized mostly in the mesoderm (B). (C, D, E) Expression in the tracheal system becomes detectable at stage 11 and continues throughout tracheal outgrowth. Also P1 expression becomes visible in the mandibular, maxillary and labial segment. (D, F) Expression in the nervous system is found in the longitudinal glial cells (asterisk) and a subset of midline cells, possibly the support cells of the VUM neurons (arrows). (G) Dissected stage-13 embryo. The longitudinal glial cells and cells from the VUM cluster (different focal plane) express P1-type RNA. (H) Dissected stage-14 embryo. P2-type transcripts are found specifically in the midline glial cells (arrowheads indicate the location of commissures).
roectoderm becomes further restricted, first to a row of two cells and subsequently to a single cell row flanking the midline cells (Fig. 7E,F). During germband retraction no more pointed P1 transcripts can be detected in the ventral ectoderm. When segmentation becomes obvious (stage 11) a group of cells surrounding the future tracheal pits start to express the pointed P1 transcript (compare Fig. 8A and C). Transcription in the tracheal cells is maintained during their migration and stops when the tracheal system is fully formed (data not shown). Additional P1 transcripts can be detected in the head region and the lateral body wall (Fig. 8C,D,E). In the CNS, pointed P1 expression first becomes detectable during germband retraction at stage 12. The longitudinal glial cells (Jacobs et al., 1989) express pointed most prominently (Fig. 8F,G asterisk). Expression in the longitudinal glial cell lineage continues until stage 14 (data not shown). A second set of identified CNS cells that express pointed during this time interval are found at the midline. Here two cells per abdominal segment and four cells per thoracic segment in the VUM cluster express pointed P1 transcripts (Fig. 8F, arrows).

The first P2 transcripts are detectable in the precellular blastoderm stage localized at the anterior tip of the embryo (Fig. 7A). Transcription of the P2 promoter however is then shut off and resumes during gastrulation (see below). In contrast to the P1-type transcripts, which are found exclusively in ectodermal derivatives, P2-type transcripts are found predominantly in the mesoderm (Fig. 8B). Here expression appears more or less uniformly and no pronounced expression of P2 transcripts in muscle precursor cells can be detected. As the germband retracts (stage 12) and formation of commissures starts (Klämbt et al., 1991) P2-driven pointed expression is shut off in the mesoderm. At the same time P2 transcripts become detectable in a few cells located at the dorsal roof at the midline of the CNS. These cells correspond to the midline glial cells, in which expression continues until the end of embryogenesis (Fig. 8H).

In summary, both midline glial cells and cells of the VUM cluster express the pointed gene. Moreover, expression in these two cell groups is differentially regulated by the use of two promoters. The P1 promoter drives expression in a subset of cells in the VUM cluster, while the P2 drives expression in the midline glial cells.

DISCUSSION

Gli-neuronal cell interactions are an important element in the construction of axonal networks, as has been elegantly illustrated by ablation experiments in the embryonic grasshopper CNS (Bastiani and Goodman, 1986). Previous work has demonstrated that the ventral midline of the Drosophila CNS presents a useful model system to dissect these processes at the genetic and molecular levels. The existence of a number of mutations that affect the midline cells of the CNS in various ways is helpful in understanding mutual relationships of different midline cells and neuronal growth cones at the midline (Thomas et al., 1988; Crews et al., 1988; Mayer and Nüsslein-Volhard, 1988; Finkelstein et al., 1990; Rothberg et al., 1988, 1990; Bier et al., 1990; Klämbt et al., 1991; Raz and Shilo, 1992; Klämbt et al., 1992).

The most striking CNS phenotype associated with homozygous pointed embryos is manifested in the fusion of segmental commissures. This particular axon pattern phenotype is seen for mutations in a number of genes (Star, rhomboid, spitz, DER/flb ts alleles) and has been shown to be due to a failure of midline glial cell function (Klämbt et al., 1991; Raz and Shilo, 1992). In homozygous pointed embryos however, the midline glial cells show the unique phenotype of an altered substratum affinity. Instead of recognizing and binding to their correct target cells at the midline, the midline glial cells of homozygous mutant pointed embryos now adhere to other neuronal membranes, the axons of the commissural neurons. This phenotype could be due either to the lack of a specific receptor on either cell type or to the fact that the target cells at the midline, the VUM neurons, are abnormal or even absent. The VUM neurons, however, are present in homozygous pointed embryos and no defects can be seen in their axon tracts in later embryos. Nevertheless, inspection of the phenotype of double mutant combinations of pointed with rhomboid or Star indicates a contribution of the VUM neurons to the final phenotype. Given these results, we therefore expect that pointed has a function in the differentiation of cells of the VUM cluster and not the determination of these cells. A direct requirement of pointed for the differentiation of the midline glial cells, which are phenotypically affected in homozygous pointed embryos cannot be proved so far, but seems very likely, based on the analysis of the temporal and spatial distribution of the different pointed transcripts. The function of pointed in the CNS is needed for correct differentiation and not determination of individual cells and is therefore similar to its role in epidermal development as discussed by Mayer and Nüsslein-Volhard (1988). A similar function of pointed in regulating cell differentiation seems to apply for the tracheal system. The tracheal anlage forms normally in homozygous pointed embryos, but halfway through formation of the tracheal system, the tracheal cells stop their migration. This phenotype, which is reminiscent of the phenotype shown by weak hypo-morphs of the breathless locus (Klämbt et al., 1992), indicates that pointed might be needed to activate breathless in concert with other transcription factors like Cfla, which are also expressed in the tracheal cells (Johnson and Hirsh, 1990; Billin et al., 1991).

Several lines of evidence can be adduced to show that the genomic sequences cloned here indeed correspond to pointed. The isolation of a lethal P-element induced pointed mutation, pointedM254 located at 94E/F, which disrupts transcription of both RNAs, P1 and P2, provided the first direct indication that this locus corresponds to pointed. The subsequent isolation of a chromosomal deletion, pointedM114, in which coding region sequences common to both transcript classes are deleted, constitutes further evidence that this region includes pointed sequences. Phenotypically this mutation behaves identically to the original P-induced pointed allele, which indicates that both pointed alleles are null mutations (Scholz and Klämbt unpublished). In addition, the transcription pattern of the gene hit by the P-element in the pointedM254 allele corresponds well to all
aspects of the pointed phenotype. Indeed, it had already been predicted by Mayer and Nüsslein-Volhard (1988) that pointed, like rhomboid, should be expressed in a longitudinal stripe in the early embryo.

The deduced pointed sequences encode two different proteins sharing a common sequence including an ETS domain. The high degree of homology (>90% with the vertebrate ets1 and ets2 sequences) suggests that the pointed encoded proteins like other ETS proteins, are localized in the nucleus and bind sequences specifically to DNA. The sequence presented here contains the D-ets2 sequence isolated by Pribyl et al., (1988), which had been incorrectly mapped to 58A/B. Chen et al. (1992) have recently described the isolation of several ETS-like sequences in Drosophila by PCR techniques, one of them is identical to the ETS sequence shown here. In the deduced pointed P2 protein sequence we find a second domain of homology conserved between pointed and members of the ets family. This domain spans some 70 amino acids and is found in a distinct exon in flies as well as in chicken (exon IV; Watson et al., 1988b). Thus, the finding of the POINTED domain allows the partition of members of the ETS family into two groups: one group sharing only the ETS domain, the other sharing both the ETS and POINTED domains. The POINTED domain presents a new motif with no strong structural resemblance to known protein motifs in other transactivation factors. It is interesting to note that the POINTED domain is contained within the regulatory domain of the ets1 protein (Schneickert et al., 1992).

Analysis of the pointed transcript distribution revealed that different transcript forms, encoding related but different proteins, are expressed in the different cell groups found at the midline of the CNS. They are found in the midline glial cells and cells of the VUM cluster during the exact time period when the CNS phenotype emerges and the midline glial cells exhibit their changed substratum affinity. From our in situ hybridization experiments we cannot determine precisely which cells of the VUM cluster express pointed. The VUM cluster consists out of six neurons and two or four glial support cells, depending on the segment (Jacobs and Goodman, 1989a; Klämbt and Goodman, 1991). The fact that four cells per thoracic segment and only two cells per abdominal segment seem to express pointed suggests that these are the glial support cells of the VUM cluster. Moreover in the pointed-M254 enhancer trap line, β-galactosidase activity is also found in the support cells of the VUM cluster as well as in the longitudinal glial cells (which also express pointed P1 RNA). Interestingly, in connection with the expression of pointed in the longitudinal glial cells, a defect can be seen in the formation of longitudinal connectives of homozygous pointed embryos. Thus, if pointed is indeed expressed in the support cells of the VUM cluster, it might act as an important glial-specific transcription factor regulating glial-neuronal interaction in the CNS. The function of the support cells of the VUM cluster remains to be determined. They may also be involved in the glial-neuronal cell interaction at the midline or alternatively they may exert different and independent cellular functions. The latter possibility might explain the deterioration of the CNS axon pattern phenotype as seen in double mutants of pointed and rhomboid or pointed and Star.

Which genes could represent possible targets for pointed?
So far only a limited number of genes is known to be expressed in the midline glial cells (Zak et al., 1990; Freeman et al., 1992; Kretzschmar et al., 1992); their expression can now be analyzed in a pointed mutant background. But since no alteration in substratum affinity of the midline glial cells (as it is the case in homozygous pointed embryos) is associated with mutations in any of these genes, we rather expect genes coding for specific receptor or adhesion molecules, (such as astrotactin, which is involved in certain glial-neuronal interactions in vertebrates, Stitt and Hatten, 1990) to be primary target genes controlled by pointed.

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REFERENCES
pointed encodes two ETS-like proteins


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NOTE ADDED IN PROOF

The accession numbers for the sequences reported in this paper are X69166 and X69167.