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Complex regulatory region mediating *tailless* expression in early embryonic patterning and brain development

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SUMMARY

***tailless* encodes a transcription factor expressed in multiple domains in the developing embryo. Early and transient expression at the posterior pole is required to establish a domain from which the eighth abdominal segment, telson and posterior gut arise. Just a few nuclear cycles later, a brain-specific domain is initiated at the anterior; expression in this domain is maintained with complex modulations throughout embryogenesis. Expression of *tailless* in this domain is required to establish the most anterior region of the brain. To understand the function and regulation of these different domains of expression, we provide a detailed description of *tailless* expression in brain neuroblasts and show that this expression is not detectably regulated by the head gap genes *buttonhead* or *orthodenticle*, by the proneural gene *lethal of scute* or by *tailless* itself. We show that approximately 6 kb of sequenced upstream regulatory DNA can drive *lacZ* expression in a pattern that mimics the full *tailless* embryonic expression pattern. Within this sequence we identify multiple modules responsible for different aspects of the *tailless* pattern. In addition to identifying additional *torso* response elements that mediate early blastoderm polar expression, we show that the complex brain expression pattern is driven by a combination of modules; thus expression at a low level throughout the brain and at a high level in the dorsal medial portion of the brain and in the optic lobe, as well as neuroblast-specific repression are mediated by different DNA regions.**

Key words: *tailless* gene, *torso* receptor, tyrosine kinase, neuroblast fate, brain development, transcription factor, regulatory module

INTRODUCTION

Expression of the *Drosophila tailless* (*tll*) gene provides an instructive example of what has now become a generally accepted principle: many developmental regulatory genes, particularly those encoding transcription factors, although initially identified on the basis of their role in a specific process, are utilized at different times and in different places during development to control a wide variety of events. Analysis of the mechanisms responsible for the requisite spatial and temporal regulation is critical for understanding the function of these multifaceted genes. In many studies, a multiplicity of regulatory modules has been identified for a single gene; each module mediates a particular aspect of the overall expression pattern (reviewed by Kirchhamer et al., 1996). The problem of the organization and function of multiple distinct regulatory modules is of particular importance for understanding the regulation of gene expression required for normal brain development, as so many regulatory genes have been recruited over the course of evolution (presumably by the addition of novel regulatory modules) to serve the formation of this most complex organ.

tll was identified in a mutant screen as a gene required for establishment of the eighth abdominal segment (A8) and telson (Jürgens et al., 1984), and subsequently shown also to be required for establishment of the posterior gut and, at the anterior, for the development of parts of the head and the most anterior portion of the brain, the protocerebrum (Strecker et al., 1986, 1988; Pignoni et al., 1990; Younossi-Hartenstein et al., 1997). Molecular analysis showed that *tll* encodes a transcription factor of the nuclear receptor family and that it is expressed in domains consistent with the mutant phenotype: transiently in a posterior cap from which A8, telson and posterior gut arise, and throughout embryogenesis in the acron primordium, then procephalic neuroblasts and finally in the developing brain (Pignoni et al., 1990; Younossi-Hartenstein et al., 1997).

The first phase of *tll* expression is relatively transient and is required for patterning of the portion of the embryo that gives rise to A8 and the posterior gut. At the beginning of the syncytial blastoderm stage, local activation of the maternally encoded *torso* (*tor*) receptor tyrosine kinase (tor RTK) (reviewed by Duffy and Perrimon, 1994) leads to transcription

of *tll* by a relief-of-repression mechanism. Specifically, two synergistically interacting regions within the upstream regulatory region mediate repression of *tll* throughout the embryo; this repression is lifted at the poles when the tor RTK pathway is locally activated (Liaw et al., 1995). Within the *tll* regulatory regions are consensus *tor* response elements (tor-REs) that mediate the *tor*-sensitive repression (Liaw et al., 1995). This earliest phase posterior cap of *tll* mRNA serves its function and disappears by the end of gastrulation (Pignoni et al., 1990).

Subsequent phases of *tll* expression are concerned almost entirely with development of the brain. The second phase of *tll* expression is initated only a few nuclear cycles later (still during the syncytial blastoderm stage), as the first phase anterior cap disappears rapidly and is quickly replaced, under control of the terminal system and of *bicoid* (*bcd*) and *dorsal* (*dl*), by a horseshoe-shaped stripe at the cellular blastoderm stage. Expression in this anterior stripe (rather than the anterior cap) is essential for normal anterior embryonic development (Pignoni et al., 1992).

A third phase of expression can be defined as the time when the anterior stripe is modulated into two roughly triangular dorsolateral domains from which the brain neuroblasts arise; portions of this expression pattern are maintained in late embryogenesis in a complex pattern in the dorsal-medial portion of the brain (Pignoni et al. 1990; Younossi-Hartenstein et al., 1997). A fourth phase consists of expression in the optic lobe primordia. The mechanism by which the complex pattern of *tll* expression in the brain primordium is established and maintained, and the requirement for this expression in normal embryonic brain development are unknown.

Analysis of *tll* regulation in the developing brain of the *Drosophila* embryo is likely to have far-reaching implications, since the existence of orthologous genes, and their similarities in spatial expression, suggest that there is a common ground plan for both the insect and the vertebrate embryonic brain (Arendt and Nübler-Jung, 1996). In both classes of organisms, proneural genes of the *achaete-scute complex* (ASC) promote commitment to the neural fate, while a set of orthologous genes defines specific regions in the anterior portion of the brain. *tll*, *orthodenticle* (*otd*) and *empty spiracles* (*ems*) are expressed in the procephalic region of the *Drosophila* embryo and are required for the development of different portions of the syncerebrum (composed of proto-, deutero- and tritocerebrum) (Pignoni et al., 1990; Hirth et al., 1995; Arendt and Nübler-Jung, 1996; Younossi-Hartenstein et al., 1997). The respective vertebrate orthologs, Tlx, Emx and Otx, are expressed in the pros- and mesencephalon, and Otx2 is required for development of these domains (reviewed by Cohen and Jürgens, 1991; Finkelstein and Boncinelli, 1994; Yu et al., 1994; Monaghan et al., 1995; Matsuo et al., 1995). These similarities compel further investigation into the roles that these genes play in brain development and into the mechanisms by which their expression is restricted to specific regions of the brain.

Like *tll*, the head gap genes *otd*, *ems* and *buttonhead* (*btd*) all encode transcription factors, are expressed in broadly overlapping anterior stripes at the blastoderm stage and are required for normal head development (Finkelstein and Perrimon, 1991; Cohen and Jürgens, 1991; Gonzalez-Gaitan et al., 1994; Schmidt-Ott et al., 1994; Hirth et al., 1995; Younossi-Hartenstein et al., 1997). Our analysis of their expression domains at the blastoderm and later stages relative to that of *tll* suggests that *otd* and/or *btd* might play a role in regulating *tll* expression in the head. Another possible *tll* regulator is *lethal of scute* (*l'sc*), a proneural gene of the ASC that encodes a bHLH transcription factor expressed throughout the brain anlage (Alonso and Cabrera, 1988; Younossi-Hartenstein et al., 1996). In spite of these suggestive expression patterns, analysis of *tll* expression in embryos mutant for these various genes, as described here, does not support a required role for *otd*, *btd* or *l'sc* in the initial expression of *tll* in the brain primordium.

To understand the regulation of and requirement for *tll* expression in the procephalic neuroectoderm, we characterize both endogenous *tll* expression and that driven by a reporter construct carrying approximately 6 kb of *tll* upstream regulatory DNA. We show that the reporter construct drives a pattern of expression essentially identical to the endogenous *tll* expression pattern in the developing central nervous system (CNS) of the embryo. We map both endogenous *tll* mRNA expression and promoter driven β-galactosidase (β-gal) expression onto a recently established (Younossi-Hartenstein et al., 1996) fate map of the *Drosophila* embryonic brain.

To begin the process of identifying regulatory modules and, ultimately, relevant interacting proteins involved in *tll* regulation, we sequenced the roughly 6 kb *tll* regulatory region and used portions of it to generate *lacZ* reporter constructs that were transformed into the germline. From the results of these experiments, we identify a region containing additional tor-REs. Most significantly, we also identify a number of regions that behave as brain-specific modules, in particular a 350 bp sequence capable of driving expression exclusively in the neuroblasts that become the dorsal medial portion of the brain. Identification of modules mediating spatially localized expression of *tll* in the brain is an important advance that should allow identification of relevant transcription factors and may ultimately have implications for understanding the regulation of the vertebrate homolog.

MATERIALS AND METHODS

Sequence analysis

Standard molecular techniques used were those of Sambrook et al. (1989). The 4.5 kb *Sal*I-*Hin*dIII fragment from the *Drosophila melanogaster tll* 5′ regulatory region (Fig. 3) was subcloned into pBluescript SK (Stratagene). Nested deletions were generated using exonuclease III (Erase-a-base, Promega). Both strands were sequenced by the chain termination method (Sambrook et al., 1989). Sequences were compiled and analyzed with the Genetics Computer Group (GCG) Wisconsin Sequence Analysis Package (1995) and analyzed with the same package, as well as with MacVector (IBI) and MatInspector (Quandt et al., 1995). Similar techniques were used to sequence the *Drosophila virilis tll* 5′ regulatory region (Liaw et al., 1993), except that sequencing was only on one strand and internal primers, rather than nested deletions, were used. The sequences of the roughly 6 kb of upstream *tll* regulatory regions of *Drosophila melanogaster* and *Drosophila virilis* have been deposited in GenBank (accession numbers AF019362 andAF019361, respectively).

Construction of *tll* fusion genes and germline transformation

Various fragments of the *tll* 5′ regulatory region (Fig. 1) were cloned into the multiple cloning site of the *lacZ* P-element vectors PwHZ16 (Liaw and Lengyel, 1992) and PwHZ128; PwHZ128 is a derivative of PwHZ16, with a modified multiple cloning site (i.e., the addition

of an M13 primer, an *Xho*I restriction site, and a single *Kpn*I restriction site) (Liaw et al., 1995). Constructs P1 through P4 have been described previously (Liaw and Lengyel, 1992). Construct TM was generated by inserting the *Bst*EII-*Xmn*I fragment of the *tll* 5′ regulatory region (Fig. 3) into the *Xba*I (blunted)-*Not*I sites of PwHZ16. For constructs K1 through K10, a single copy of each fragment was subcloned into the P-element vector; for construct K11, fragments of the *tll* promoter region were amplified using PCR, then oligomerized in tandem as described (Liaw, 1994). Oligomers containing four tandemly repeated copies of different portions of the A_4 region were inserted into the P-element vector PwHZ128. All constructs were injected into w^{1118} embryos as described (Rubin and Spradling, 1982). For each construct, at least five independent transformant lines were established and assayed for expression pattern by in situ hybridization (see below).

Localization of *tll* mRNA and *lacZ* mRNA and protein

Endogenous *btd*, *ems*, *otd* and *tll* mRNA in wild-type embryos, and *lacZ* mRNA in embryos transformed with the various *tll* promoter-*lacZ* constructs, were detected by in situ hybridization (Tautz and Pfeifle, 1989) using digoxigenin-labeled (Boehringer Mannheim Biochemicals) *btd* (Wimmer et al., 1993), *ems* (Walldorf and Gehring, 1992), *otd* (Finkelstein et al., 1990) and *tll* (Pignoni et al., 1990) cDNA, or *lacZ* DNA as a probe. β-galactosidase in embryos from these lines was detected by antibody staining, using standard techniques (Ashburner 1989) and antibodies and reagents from Jackson Laboratories. Rabbit polyclonal (primary) antibody to β-galactosidase was used at a dilution of 1:10,000, and biotin-conjugated goat anti-rabbit (secondary) antibody at 1:2,000. The Vectastain ABC kit was used with diaminobenzidine staining to detect the secondary antibody. Stained embryos were dehydrated through an ethanol series and mounted in Epon (Polysciences). Embryonic stages were determined according to Campos-Ortega and Hartenstein (1985).

Drosophila strains

P-element constructs were maintained in a w^{1118} homozygous background. To determine the effect of alterations in *tor* activity on the expression driven by particular constructs, males from a line carrying a construct insert in homozygous condition were crossed to females heterozygous for the dominant gain-of-function allele tor^{D4021} (Klingler et al., 1988). Alleles used to study effect on *tll* expression were otd^{h1}, btd^{XG} and *Df(1)scB57*, which genetically behave as nulls (Younossi-Hartenstein et al., 1997).

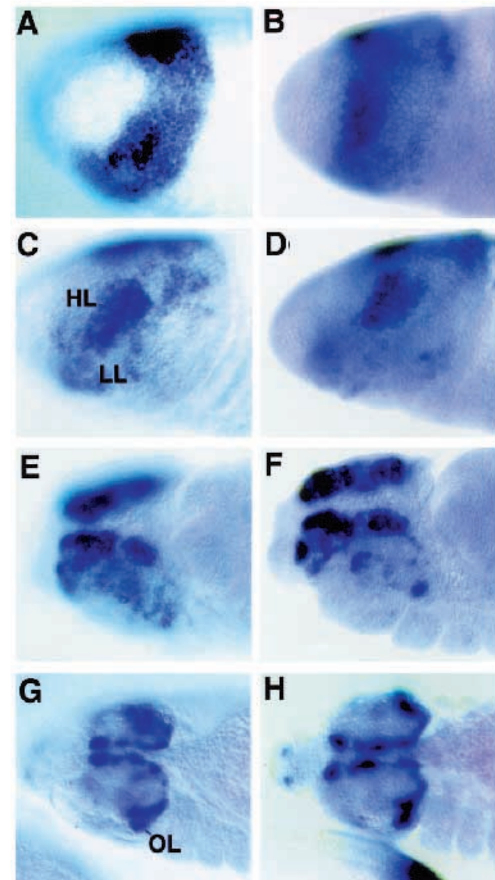


Fig. 1. *lacZ* expression driven by *tll* regulatory region mimics endogenous *tll* expression. (A,C,E,G) Endogenous *tll* mRNA expression; (B,D,F,H) *lacZ* mRNA expression driven by 5.9 kb of *tll* regulatory region (construct P1 in Fig. 3). *tll* and *lacZ* mRNAs were detected by in situ hybridization with appropriate probes (see Materials and Methods). Embryos are shown at stages 6 (A,B), 8 (C,D), 11 (E,F) and 13 (G,H). The regions of *tll* expression designated HL and LL (see Fig. 2) are indicated in C. All embryos are oriented to some degree dorsolaterally; left-right pairs of panels have the same degree of dorsolateral orientation.

RESULTS

Endogenous *tll* expression

To provide the background information needed for dissection of the *tll* regulatory region, particularly for identification of modules controlling expression in the brain neuroblasts, we characterized expression of endogenous *tll* mRNA throughout embryogenesis.

Polar blastoderm caps

Transcription of *tll* is initiated early in the syncytial blastoderm stage (stage 4) in two symmetrical caps; these two caps depend entirely on activity of the maternally encoded terminal system (Pignoni et al., 1992). Expression in the posterior cap is required for posterior patterning of the blastoderm stage embryo (Mahoney and Lengyel, 1987; Weigel et al., 1990). Although expression in both caps is transient, that in the posterior cap reaches a higher level and persists longer, i.e., through most of the cellular blastoderm stage. Expression in the posterior cap largely disappears during gastrulation and is undetectable by the end of germband extension (Pignoni et al., 1990).

Complex, brain-specific expression

By the beginning of the cellular blastoderm stage, the anterior cap is replaced by a horseshoe-shaped stripe that straddles the dorsal midline between 76 and 89% egg length (EL) (Pignoni et al., 1990, 1992). Projected onto the blastoderm fate map (Campos-Ortega and Hartenstein, 1985), this stripe covers the entire brain anlage, i.e., the procephalic neurectoderm; expression in this stripe continues in domains that will become the brain (Pignoni et al., 1990; Younossi-Hartenstein et al., 1997).

tll expression in the procephalic neurectoderm continues, with complex modulations, from the blastoderm stage into late stages of embryogenesis. We followed this expression pattern (by in situ hybridization to whole-mount embryos) and correlated it with the recently defined (on the basis of expression patterns of proneural and neurogenic genes) procephalic

proneural domains from which the neuroblasts of the brain are derived. The nomenclature used below to refer to these domains, and the neuroblasts that delaminate from them, is that given by Younossi-Hartenstein et al. (1996). Confirming and extending earlier work (Younossi-Hartenstein et al., 1997), our analysis shows that *tll* mRNA is present in all proneural domains of the protocerebrum; furthermore, the level of *tll* mRNA in a domain is highest shortly before and during the stage at which neuroblasts delaminate from that domain.

During gastrulation, the horseshoe-shaped stripe of *tll* expression becomes tilted backwards and undergoes a process of internal differentiation into regions with different levels of expression. A group of cells in the dorsal midline gradually ceases expressing *tll*, resulting in a split of the horseshoe into two dorsolateral domains (Fig. 1A,C). Within each of these domains, a roughly triangle-shaped, anterodorsal region shows the highest level of expression and is designated HL; posteroventral to HL is a domain with a lower level of expression designated LL (Figs 1A,C, 2).

The HL domain during stages 7 and 8 covers the dorsocentral part of the protocerebral neurectoderm. From within this region, the first groups of protocerebral neuroblasts (Pc2, 3) delaminate during late stage 8 (Younossi-Hartenstein et al., 1996). During late stage 9 (Figs 1C, 2 and data not shown), the HL domain becomes restricted to the dorsoanterior portion of the procephalic region; this position corresponds to the anterior protocerebral domain from which neuroblast groups Pa3-4 delaminate during stage 10 (Younossi-Hartenstein et al., 1996). From stage 11 (Fig. 1E) onward, high levels of *tll* are found primarily in a bilaterally symmetric arc close to the dorsal midline; this arc largely overlaps the dorsomedial *l'sc* expression domain Pdm of Younossi-Hartenstein et al. (1996), but extends posteriorly to it.

tll is expressed in a spotty pattern in the LL domain, which surrounds the HL domain anteriorly, ventrally and posteriorly. LL covers the entire protocerebral neurectoderm, including the domains from which the Pc1/2 neuroblasts delaminate during stage 9, the Pa1/2 and Pp1/2 neuroblasts during stage 10, and the Pp3, 4 and 5 neuroblasts during stage 11. The relationship of the HL to the LL domain at stage 9, and to the position of the delaminating neuroblasts, is shown schematically in Fig. 2.

During stage 12, *tll* is expressed at a high level in a new region: the primordium of the optic lobe. This structure arises by invagination of the posterior procephalic ectoderm, a region that corresponds to the proneural domains Pp3-5 (Younossi-Hartenstein et al., 1996). *tll* expression remains high in the optic lobe throughout late embryonic development and can also be seen in the optic lobe in the late third instar larva (Fig. 1G and data not shown).

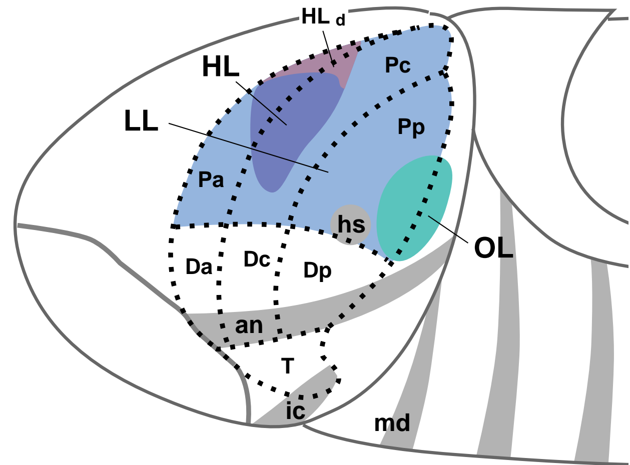


Fig. 2. Summary of *tll* expression projected onto the stage 9 embryo. Schematic diagram of anterior of the stage 9 *Drosophila* embryo, showing the domains of *tll* expression superimposed on the regions in the procephalon from which portions of the brain arise. Regions of *tll* expression are indicated in color, and labeled HL (for **H**igh **L**evel of expression; dark blue [note that the HL domain includes the HL_d domain, which is described below]), LL (for **L**ow **L**evel of expression; light blue) and OL (for **O**ptic **L**obe; turquoise; although indicated in the figure, expression in the OL region does not begin until late stage 11). Consistent with the notation of Younossi-Hartenstein et al. (1997), the anterior, central and posterior regions from which protocerebral neuroblasts will arise are labeled Pa, Pc and Pp; the anterior, central and posterior regions from which the deutocerebral neuroblasts will arise are indicated by Da, Dc and Dp; and the region from which the tritocerebrum will arise is labeled T. Expression domains of *engrailed*, which define the different regions along the anterior-posterior axis of the brain (Schmidt-Ott and Technau, 1992; Hirth et al., 1995), are gray and are labeled hs (head spot), an (antennal), ic (intercalary) and md (mandibular). The dorsomedial region of HL, where brain-specific portions of the *tll* regulatory region drive expression (designated HL_d in Fig. 5) is indicated in purple.

Reporter construct reflecting endogenous *tll* expression

To identify regulatory elements responsible for the complex expression pattern described above, we began with reporter construct P1, which contains all of the 5′ *tll* regulatory region (5.9 kb) necessary to rescue a null *tll* mutation (Fig. 3; Pignoni et al., 1990). This construct has already been shown to express *lacZ* in a pattern that mimics endogenous *tll* expression at the blastoderm stage (Liaw and Lengyel, 1992). As described below, *lacZ* expression driven by P1 also mimics brain-specific *tll* expression.

During gastrulation, P1 drives expression at high levels in the HL domain, and lower levels in the LL domain, essentially coincident with the endogenous *tll* pattern (Fig. 1B,D). During later embryonic development, the dynamics of P1-driven *lacZ* mRNA expression within the HL and LL domains do not significantly differ from the pattern described above for the endogenous *tll* transcript (Fig. 1F,H). Thus the 5.9 kb of *tll* 5′ regulatory region drives expression transiently in all protocerebral neuroblasts (with the possible exception of the posteriormost groups Pp3-5), and in the clusters of neural precursors that segregate from the dorsomedial procephalon during stages 12-13 by mass delamination and invagination (domains defined by Younossi-Hartenstein et al., 1996). In addition to mimicking the endogenous *tll* expression in the HL and LL regions, the 5.9 kb region also drives expression at stage 12 in the optic lobe primordium. Like endogenous *tll* expression, P1-driven expression in the dorsomedial brain hemispheres and optic lobe persists until late embryonic stages (Fig. 1H).

These results indicate that the 5.9 kb of DNA immediately 5′ to the *tll* transcription unit has within it essentially all of the stage- and region-specific enhancers necessary to drive the endogenous *tll* expression pattern. In what follows, therefore,

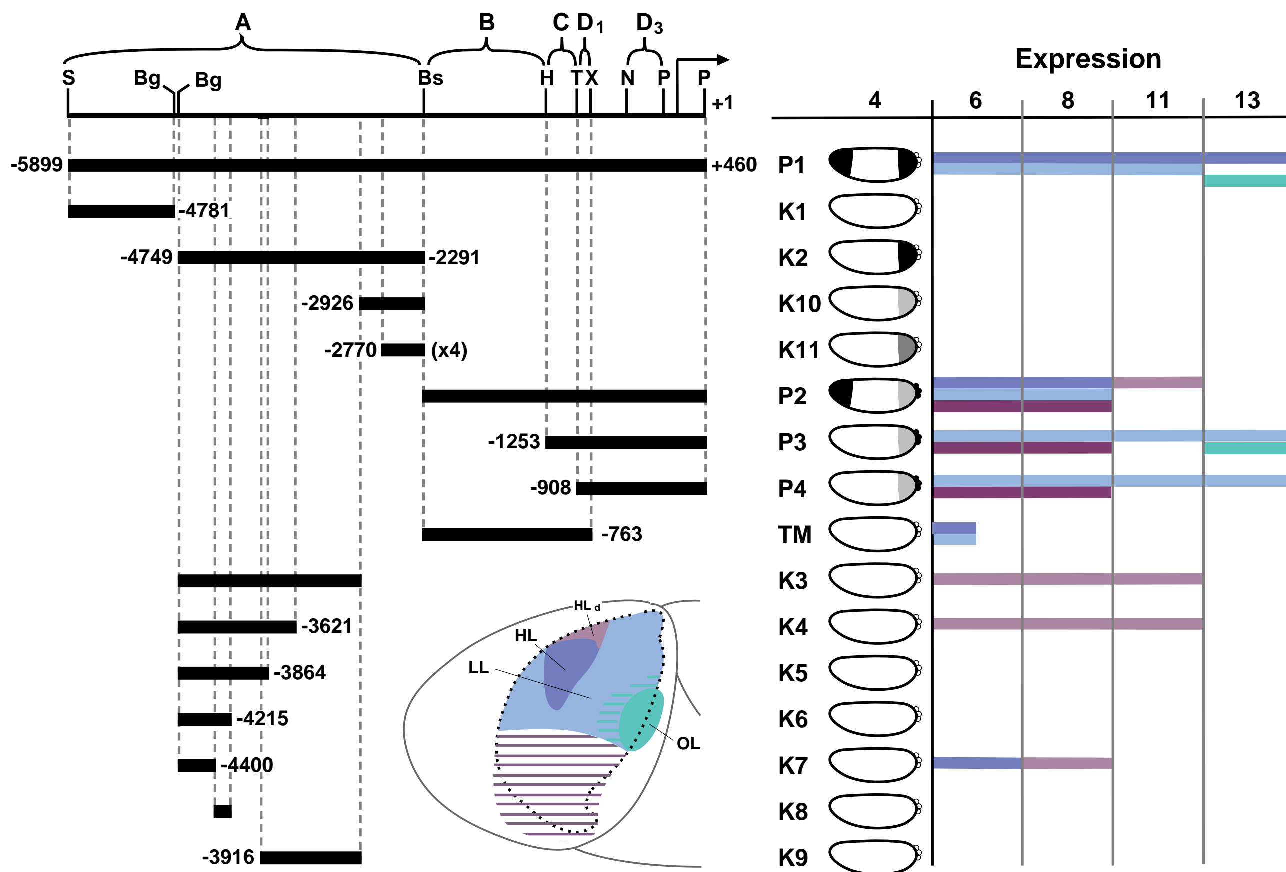


Fig. 3. Regulatory elements that drive distinct portions of the *tll* expression pattern. A partial restriction map of the 5.9 kb 5′ *tll* regulatory region is shown in the top line (S, *Sal*I; Bg, *Bgl*II; Bs, *Bst*EII; H, *Hin*dIII; T, *Sst*I; X, *Xmn*I; N, *Nco*I; P, *Pst*I; only restriction sites used to prepare constructs are shown). The transcription start site (+1) is at the initiation position of the rightward-pointing arrow; regions A,B,C,D1 and D3 and constructs P1 through P4 have been described previously (Liaw and Lengyel, 1992; Liaw et al., 1993). Constructs TM and K1-K10 were generated as described in Materials and Methods. The nucleotide positions of the endpoints of each fragment are given, except where these coincide with endpoints of other fragments (dotted lines). The right side of the figure summarizes the *lacZ* expression driven by each construct at different stages of embryogenesis. Expression at stage 4 is shown by a cartoon of the blastoderm stage embryo; for constructs P1 through P4, this is the pattern described previously (Liaw and Lengyel, 1992). For later stages, expression in the brain domains described in Fig. 2 (see insert) is indicated by colored bars (HL, dark blue; LL, light blue; OL, turquoise; HL_d, purple). Ectopic expression on the ventral side of the head is indicated by dark purple bars and dark purple hatching in the inserted figure; expression continuous with and adjacent to the optic lobe is indicated by turquoise hatching.

we refer to this DNA, contained within the P1 construct, as the ‘complete regulatory region’ and to embryos carrying the construct as [P1] embryos.

Genetic control of *tll* expression in the brain

Antibody staining of [P1] embryos detects β-gal in essentially the same cells as *lacZ* mRNA (above), but present for longer (cf. Fig. 1F,H with Fig. 4A,B). This strong P1-driven β-gal expression pattern (which we refer to as *tll* P1 expression) allows us to test specific genes for their effect on expression of *tll* in the head.

Likely candidate regulators of *tll* are the head gap genes *otd*, *ems* and *btd*. These genes, in addition to their required role in patterning the gnathal segments and procephalic region (reviewed by Cohen and Jürgens, 1991), have each recently been shown to be required for the establishment of different specific neuroblast populations of the brain (Hirth et al., 1995; Younossi-Hartenstein et al., 1997). To evaluate their possible roles in regulation of *tll* brain expression, we review previous results and provide additional descriptions of the expression patterns of these head gap genes. At the cellular blastoderm stage, *otd*, *ems* and *btd* are expressed in broad overlapping stripes along the anterior-posterior axis of the embryo at positions on the dorsal midline of approximately 70-90, 69-75 and 67-75% egg length (EL), respectively (Dalton et al., 1989; Finkelstein and Perrimon, 1990; Wimmer et al., 1995). Note that, as described above, the anterior horseshoe domain of *tll* lies within the *otd* blastoderm expression domain.

Beginning at gastrulation (stage 6), expression of each of the head gap genes undergoes a different set of modulations. *otd* expression retreats from the ventral midline, but persists in a major portion of the deuterocerebral and protocerebral neurectoderm where it forms a horseshoe domain that overlaps with *tll* expression (Fig. 5A). *btd* expression becomes divided into two domains, the most anterior of which is a bilateral, anterior dorsomedial region overlapping partially with the *tll* HL domain (Fig. 5C). Both *btd* and *ems* are expressed in stripes just anterior to the cephalic furrow; these stripes are not part

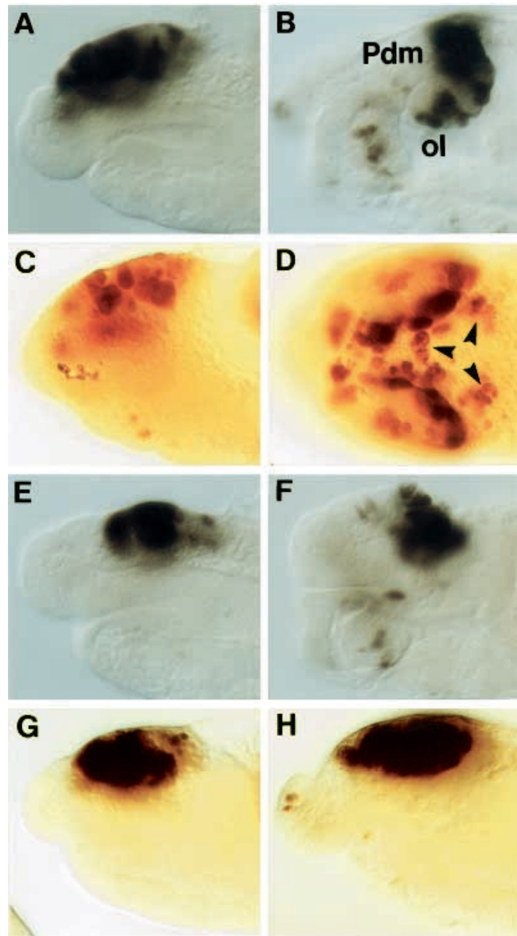


Fig. 4. *tll* expression in *l'sc*, *otd*, *btd* and *tll* mutant embryos. Expression driven by the complete *tll* regulatory region (construct P1 in Fig. 3) was detected by staining with anti-β-gal antibody. *tll* P1 expression in a wild-type background is shown in A at stage 12, and in B at stage 14. Note expression in the dorsal medial portion of the protocerebral domain (Pdm) and in the optic lobe (ol) at stage 14. *tll*P1 expression was examined in embryos homozygous for null mutations in the genes *l'sc* (C,D), *otd* (E,F) and *btd* (G,H). From stage 11 onward, specific morphological deformities allow unambigous recognition of homozygous mutant embryos. Embryos in A,C, and G are at stage 12; those in B,D,F at stage 14. All views are lateral, except for D, which is a dorsal view. In embryos lacking the *l'sc* gene at stage 14, there are many labeled cells that have been engulfed by macrophages; these are labeled with arrowheads (D). In *btd* mutants there is no optic lobe and hence no reporter expression in this domain in these embryos (G,H).

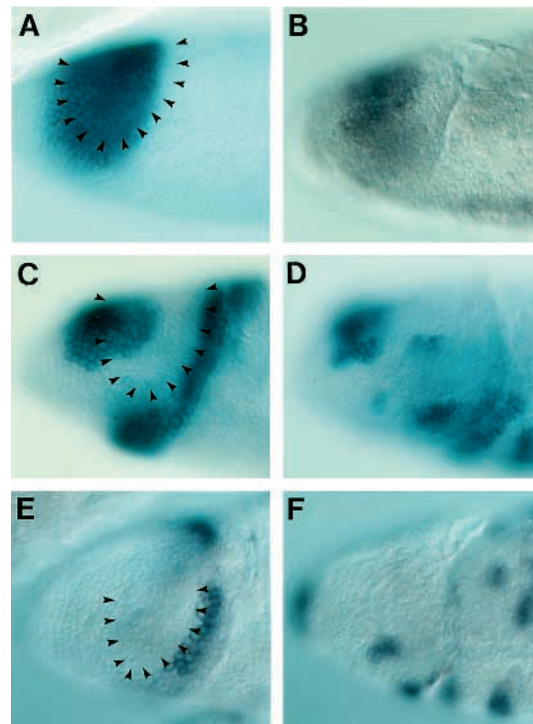


Fig. 5. Expression of *otd*, *btd* and *ems* in the brain region. *otd*, *btd* and *ems* mRNA were detected by in situ hybridization with appropriate probes. Expression of *otd* is shown in A and B, *btd* in C and D, and *ems* in E and F. Embryos in A, C and E are at stage 6; those in B, D and F at stage 9. In A, C and E, the expression domain of *tll* at stage 6 (Pignoni et al., 1990; Fig. 1A) is outlined by arrowheads. All views are lateral, with the exception of E, which is dorsolateral.

of the protocerebral neurectoderm, however, and lie posterior to the domain of *tll* expression (Fig. 5C,E). By stage 9, there is little or no expression of *btd* or *ems* in the protocerebral domains of the head, while *otd* expression persists in a fairly broad domain that covers most of the protocerebral domain of the head ectoderm (Fig. 5B,D,F).

From the preceding description of head gap gene expression, it is evident that only the *otd* and *btd* domains overlap with the *tll* protocerebral expression domain and might therefore affect *tll* expression. To investigate the role of *otd* or *btd* in establishing or maintaining *tll* expression in the brain primordium, the P1 construct was crossed into null *otd* and *btd* backgrounds.

In *btd* mutant embryos, development of the protocerebrum proceeds essentially normally (described in more detail by Younossi-Hartenstein et al., 1997), and the domain of *tll* P1 expression is initiated normally and remains normal in size and level into later stages (Fig. 4G,H). The smaller expression domain of *tll* at stage 14 is due to the absence of the optic lobe from *btd* embryos; therefore, we cannot assess the effect of *btd* on *tll* expression in this domain. We conclude that *btd* expression, even though partly overlapping with *tll* expression at stage 6, is not required for establishing or maintaining most of the *tll* expression pattern.

In *otd* mutant embryos, *tll* P1 expression is normal at stages 9 and 10 (i.e., no abnormal patterns were seen among progeny from the cross of *otd* heterozygous parents), indicating that *otd* is not required for *tll* expression until at least stage 10. Defects are first detectable in the brain primordium of *otd* embryos beginning at stage 11; these ultimately result in a protocerebrum that is severely reduced in size (Younossi-Hartenstein et al., 1997). Correspondingly, after stage 11, the *tll* P1 domain is reduced in size; the expression level within this domain, however, is similar to that seen in wild-type embryos (Fig. 4E,F). Thus although we cannot assess the role of *otd* in parts of the protocerebrum at later stages, we can say that *otd* is not required for *tll* expression prior to stage 11, or for *tll* expression in the portion of the protocerebrum that remains in older *otd* embryos.

Additional candidate genes for regulation of *tll* are the proneural genes within the ASC; all four of these genes encode bHLH transcription factors (Alonso and Cabrera, 1988). Of these four genes, however, only *l'sc* shows significant expression in the procephalic neurectoderm; this expression is seen in a broad domain in the protocerebrum between stages 7 and 11 that overlaps the domain of *tll* expression (Younossi-Hartenstein et al., 1996). Elimination of the activity of *l'sc* (as well as that of the three other genes of the ASC) by use of the deficiency *Df(1)B57* did not, however, result in a loss of *tll* expression. At early stages, *tll* P1 expression in homozygous ASC mutants appears indistinguishable from expression in wild-type embryos. Beginning during stage 13 in embryos lacking *l'sc*, there is an increased amount of apoptotic cell death in the *tll* P1-expressing cells of the protocerebral ectoderm of *l'sc* embryos, as indicated by the appearance of numerous macrophages containing β-gal-positive cellular debris at the dorsal aspect of the developing brain. This excess cell death leads to a decrease in the size of the *tll*-expressing cell population (Fig. 4C,D). We conclude that *l'sc*, although required to maintain viability of certain *tll*-expressing cells in the protocerebrum, is not required to establish or maintain *tll* expression per se.

Finally, since *tll* itself encodes a nuclear receptor transcription factor (Pignoni et al., 1990), we can ask if *tll* regulates its own expression. Prior to stage 11, no difference is seen among embryos from crosses between heterozygous parents carrying a null *tll* allele (Pignoni, 1991). By stage 12, homozygous *tll* embryos are seen to lack a significant portion of the brain, the protocerebrum (Younossi-Hartenstein et al., 1997); these embryos still express *tll*, however, in a round placode-like domain that appears to constitute the uninvaginated optic lobe (Fig. 7L). These results indicate that the *tll* gene does not autoregulate.

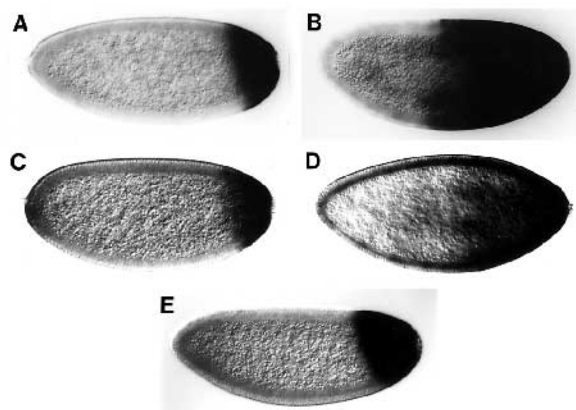
We conclude that, even though their domains of expression in the blastoderm stage embryo overlap partially with the anterior domain of *tll*, the head gap genes *btd* and *otd* are not required for establishing or maintaining *tll* expression in the anterior stripe. Further, neither these genes nor *l'sc* is required to maintain *tll* expression in the brain neuroblasts; in particular, these genes do not regulate *tll* via the 5.9 kb regulatory region.

Modular structure of the 5.9 kb *tll* regulatory region

Distal module mediating *tor*-dependent *tll* activation by relief-of-repression

Previous analysis identified an 11 bp *tor*-responsive element (tor-RE) in the proximal region D3 (Fig. 3). D3 functions as a minimal regulatory element, or module; a module with similar function and interacting synergistically with D3 was inferred in the distal (upstream of −2291) region (Liaw and Lengyel, 1992; Liaw et al., 1993, 1995; see Fig. 3).

To localize the synergistically interacting distal tor-RE, constructs K1-K10 (Fig. 3) were generated. Of these, only K2 and K10 (which both carry the most proximal 635 bp of the distal regulatory region) drive polar expression (Figs 3, 6A,C). When



F

```
-2770                                                                                              T CTCGGGAAAT AACCATGGAA
-2700  ATAGGGTCGG TCTTCCCTCG AAGAGTTTAC TCAATGCTCA ATGAATTCGG AGCGGCGGCG GCAACCTTTG GGCCATAAAA GCGAAGAGCG GGATTTTTCC
-2600  ACCTCCAGCAT GCAAATGAA GTGAATTTCC CAGCTATTTA ACCCTTTCAT TTCGCTCGAA CCTCGAATTG CCATTAGCCG TGTGGCGTCT TAACCCTTTT
-2500  CTCCGAGCCGT CGAGCAATT CATAAAAACG CAGCGAAAGT GCCATAAAAA TCGAAGCCAA CCCAAACGGT AGCACCTGCA TTCTTCCAAC ACAAAAAGGT
-2400  CCTAACTCGTA ATTAAGACG AATAATCGCT ACCTAGATCC TTTCGCTTTT CGTCCTCAAG GAACTTTGTA AAAAAGTCGC AAAAATTCTG GGAACTTTTT
-2300  ATTCACTTTTA TTCATCGGC GACCTTGAAT GGCCGTTTTG ATGGCGGTGG GTGGTTACC  -2291
```

tor-RE consensus: TGCTCAATGAA
Bicoid consensus: TCTAATCCC
Caudal half site: CCATAA

Fig. 6. Distal regulatory region mediating *torso*-dependent posterior cap expression. *lacZ* expression driven by the different constructs is shown; all embryos are at the syncytial blastoderm stage (stage 4), and are shown with anterior to the left and dorsal up. Region −2291 to −4749 (construct K_2), the proximal subregion −2291 to −2926 (construct K10), and the even smaller subregion −2291 to −2770 oligomerized fourfold (construct K11), all drive expression in a posterior cap (A,C,E). Expression driven by constructs K2 and K10 is expanded in tor^{D4021} embryos (B,D, respectively). The sequence of the 479 bp, four-fold oligomerized region of construct K11 is shown in F. Numbering of the sequence relative to the transcriptional start site (+1) is per Liaw et al. (1993). Within the sequence are two *torso* response elements (Liaw et al., 1995; (tor-REs, black background), two Bicoid consensus binding sites (Driever et al., 1989; boxed) and three Caudal consensus half-sites (Dearolf et al., 1989; shaded). While there are also Caudal-binding sites in the D_3 region, and this similarity might suggest a role for Caudal in activating *tll* transcription at the blastoderm stage, the normal *tll* expression in embryos from females carrying *cad* germline clones does not support this possibility (L. H. Wu, A. J. Courey and J. A. Lengyel, unpublished).

K2 and K10 are introduced into embryos in which Torso is ectopically active, the posterior cap of expression is expanded dramatically (Fig. 6B,D), indicating that both constructs contain an element(s) that responds to activated Torso. Sequence comparison with the D3 region (Liaw et al., 1993) revealed two tor-REs in region −2291 to −2770 (Fig. 6F); this region, four-fold oligomerized to generate construct K11, drives strong polar expression (Figs 3, 6E). K11 drives only posterior and not anterior polar expression, perhaps due to direct repression by Bicoid via consensus binding sites in the −2291 to −2770 region (Fig. 6F); consistent with this interpretation, the D_3 region lacks Bicoid-binding sites and drives expression at both poles (Liaw and Lengyel, 1992).

Multiple modules controlling 'brain-specific' neuroblast expression

To determine how *tll* expression is controlled in the HL, LL and optic lobe (OL) procephalic neuroblast domains, we examined expression driven by both previously and newly generated promoter-*lacZ* constructs (Liaw and Lengyel, 1992; Fig. 3). No single portion of the regulatory region was capable of driving the entire endogenous *tll* brain pattern through stage 13; rather, various brain expression modules are dispersed throughout the *tll* regulatory region.

Three regions were identified that drove some expression in the HL domain, starting at stage 6 (beginning gastrulation). One of these, deduced from the pattern driven by the P2 but not the P3 construct, lies in region B (−1253 to −2291, Fig. 3). Since expression in the HL domain driven by the P2 construct is only maintained to stage 11 (Fig. 7A-C), and since construct TM, containing region BCD_1 (−1253 to −2291) drives expression in the HL domain only early and transiently, there must be elements proximal to −763 that function synergistically with element(s) in BCD_1 to establish and maintain HL expression throughout embryogenesis.

Two brain-specific elements were identified in the distal regulatory region. Constructs K3 and K4, but not K5, drive expression in a portion of the HL domain, localizing one brain-specific enhancer to a 243 bp region (Fig. 3; −3621 to −3864). Expression driven by K3 and K4 begins during stage 6 as a dorsal medial stripe (Fig. 7I) that overlaps with the portion of the HL domain where *tll* expression is strongest (cf. Fig. 1A,B). This expression by stage 8 has become a chevron-shaped domain that constitutes the most dorsal-medial portion of the HL domain (thus lying within the most dorsal part of the Pa domain and the dorsal portion of the Pc domain); we refer to this expression domain as HL_d (Fig. 7J). Expression driven by K3 and K4 continues in the HL_d domain into stage 11 (Fig. 7K) but not beyond. An important brain-specific enhancer is present in the 349 bp fragment carried by construct K7 (Fig. 3, −4400 to −4749). Construct K7 drives expression throughout the HL region at stage 6, in the HL_d region by stage 8, but drives little expression thereafter (data not shown). The fact that construct K9, which contains the HL enhancer between −3621 and −3864, cannot drive HL expression on its own, while construct K7 does, indicates that region −4400 to −4749 is the stronger of the two HL enhancers; these enhancers probably interact synergistically to drive brain neuroblast-specific expression.

A region containing an enhancer for the LL region can be deduced from the fact that constructs P2, P3 and P4 all drive expression in the LL region; the smallest of these constructs, P4, which contains the D region (Fig. 3, −908 to +460), drives expression during stages 6 through 11 in a pattern that overlaps the LL domain (cf. Figs 1A,C,E, 7E-G).

An optic lobe-specific enhancer within region −908 to −1253 is deduced from the fact that construct P3, but not P4, drives expression in the optic lobe as well as in immediately adjacent cells in the protocerebrum (Figs 3, 7D). No other elements

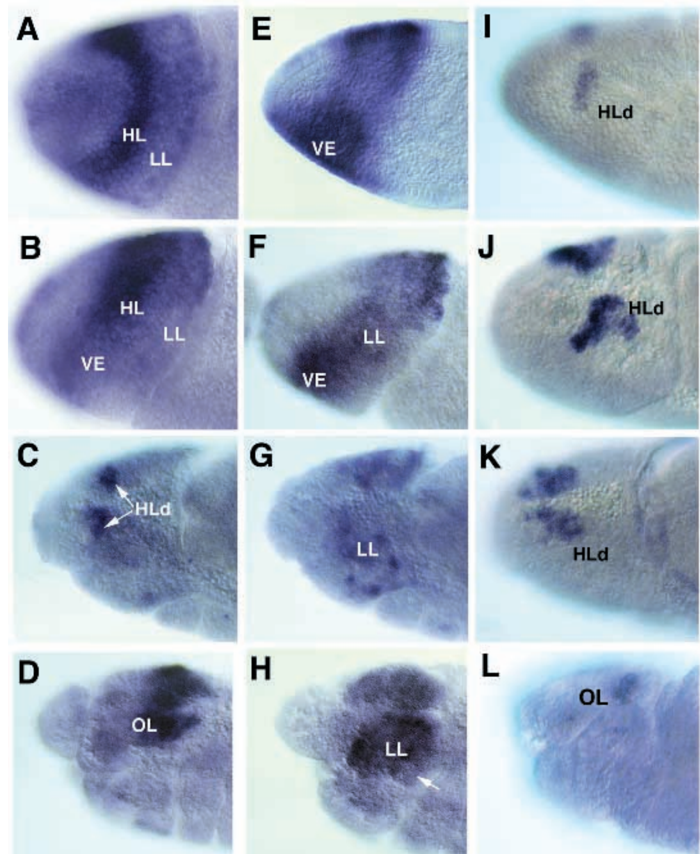


Fig. 7. Brain neuroblast expression driven by various portions of the *tll* regulatory region. *lacZ* mRNA expression in embryos from lines carrying the constructs indicated in Fig. 3 is shown detected by in situ hybridization. P2 (−2298 to +460) drives expression in both HL and LL domains at stages 6 (A) and 8 (B), but only in the anterior portion of the dorsal medial domain (HL_d) at stage 11 (C). P4 drives expression throughout the LL domain in stages 6 (E) and 8 (F), and in discrete groups of cells dispersed throughout the brain at stage 11 (G); a similar pattern is driven by construct P3 (not shown). Note that P2 and P4 drive ectopic expression on the ventral side of the head (VE in B,E,F); the same is true for construct P3 (not shown). At stage 13, P4 drives diffuse expression throughout the brain (H), while P3 drives expression in the posterior portion of the brain and in the optic lobe (D). Construct K4 drives expression only in the HL_d domain, starting at stage 6 (I) and continuing through stages 8 (J) and 11 (K); expression driven by construct K3 is similar (not shown). Expression driven by the smallest brain-specific construct, K7, is initiated in both the HL and LL domains at stage 6, becomes restricted to the HL_d domain by stage 8, and virtually disappears by stage 11 (data not shown). In embryos homozygous for a null *tll* allele (tll^{l49}), *tll* expression at stage 11 is seen in a non-invaginating placode corresponding to the optic lobe (OL) (L).

mediating optic lobe expression were identified; specifically, the HL-specific enhancers identified above were not found to drive expression in the optic lobe (OL) domain.

Two head-specific repression elements can be inferred from comparing expression driven by K3 to K2, and K7 to K6. In each case, removal of a proximal fragment from a construct that does not drive anterior expression (K2 and K6) results in a construct that drives expression in the HL domain (K3 and K7) (Figs 3, 7). The regions mediating repression appear to be neuroblast-specific, since the K2 construct, which contains both of these regions, drives polar expression at the blastoderm stage. In addition, since they are immediately adjacent to HL-specific enhancers (described above) and the presence of one of them (−4215 to −4400) does not affect the enhancing effect of a more distant region (−3621 to −3864) (Fig. 3, constructs K3 and K4), these repression elements may only affect activation driven by an immediately adjacent enhancer. Since its addition to the P3 construct reduces ability to drive expression in the optic lobe but not at the poles during the blastoderm stage (Fig. 3), region −1253 to −2291 probably also contains a neuroblast-specific repression element.

Sequence analysis of *tll* regulatory region

Sequences conserved in the regulatory regions of distantly related species can provide suggestions of possible regulatory sites. Thus similarities between *Drosophila melanogaster* and *Drosophila virilis* in the −1 to −450 region of the *tll* 5′ regulatory region contributed to the identification of elements involved in regulation of the blastoderm polar caps (Liaw et al., 1993, 1995). To allow this rationale to be extended to the brain-specific modules, we obtained sequence for approximately 6 kb upstream of the *tll* transcription initiation sites for both *Drosophila melanogaster* and *Drosophila virilis* (GenBank accession numbers AF019362 and AF019361, respectively) and compared these to each other.

In addition to 450 bp promoter-proximal region of similarity, three additional regions of *melanogaster tll* regulatory DNA with similarity to *virilis tll* DNA were identified: −1300 to −1900, −2100 to −2900, and −3200 to −3650. How are these four regions of similarity related to the regulatory elements defined by promoter dissection? The −1 to −450 region overlaps with the LL element and one of the HL elements; the specific similar sequence elements in this region have been described (Liaw et al., 1993). The −1300 to −1900 region overlaps with a module that can independently drive early and transient expression in the HL and LL domains (Fig. 3); it is noteworthy that this region contains five 40 to 80 bp sequences that are highly conserved between *melanogaster* and *virilis*. The −2100 to −2900 region overlaps with the proximal neuroblast specific repressor. The −3200 to −3650 region does not overlap with regulatory elements defined here.

Surprisingly, for the two distal HL modules (−4400 to −4749; −3621 to −3864), one of which is capable of independently driving brain-specific expression, no region of sequence similarity was identified in the *virilis tll* regulatory DNA. In another approach, searching for repeated motifs within the *melanogaster* HL modules, we identified three repeats of the sequence TCTGG between −4611 and −4465 and four between −3763 and −3866. A number of these sites were identified in the *virilis* upstream sequence. While this repeat and the sequences in which it is embedded does not fit consensus binding sites of described *Drosophila* transcription factors, it does fit the consensus for a heterodimer of the two bHLH proteins E47 (the vertebrate homolog of the neurogenic Daughterless gene product) and Thing1 (Hollenberg et al., 1995).

DISCUSSION

Regulation of *tll* in the posterior of the *Drosophila* embryo under control of the tor RTK has been investigated in detail and has led to the definition of a tor-RE in the proximal *tll* regulatory region (Liaw et al., 1993; 1995). Dissection of the distal regulatory region described here has has allowed identification of additional, synergistically interacting tor-REs. In contrast to our relatively more detailed understanding of the early, *tor*-dependent regulation of *tll*, little is known about the regulation of *tll* in the developing brain. The work presented here provides a detailed description of *tll* expression throughout embryonic brain development, examines (and discards) possible regulators of *tll*, and most significantly, identifies specific enhancer elements (modules) that drive expression in different portions of the developing *Drosophila* brain.

tll expression and regulation in brain neuroblasts

We have mapped *tll* expression to a recently generated map of procephalic neuroblasts (Younossi-Hartenstein et al., 1996), and expanded an earlier description of *tll* expression in the brain (Younossi-Hartenstein et al., 1997). The earliest brain-related *tll* expression is in a horseshoe domain that covers the anlage of the entire brain; this domain then becomes split into two lateral expression domains containing high level (HL) and low level (LL) expressing regions. Later, *tll* is expressed most strongly in the earliest delaminating Pc2,3 and the later delaminating Pa3,4 protocerebral neuroblasts. Still later, *tll* is strongly expressed in the invaginating optic lobe. Neuroblasts that continue to express *tll* throughout embryogenesis are those in the most dorsal-medial protocerebrum and the optic lobe. Both the early horseshoe expression, as well as expression in the HL and LL domains (which together cover the entire protocerebral neurectoderm) are consistent with the *tll* mutant phenotype, which is the absence of the entire protocerebrum (Younossi-Hartenstein et al., 1997). The requirement for the later strong expression in the dorsal-medial protocerebrum and in the optic lobe remains to be addressed.

A number of genes that are expressed in the head, encode transcription factors and give brain phenotypes in mutant embryos were tested for their effect on *tll* expression, as assessed by in situ hybridization or expression of the P1 reporter construct. Neither the head gap genes *otd* and *btd* nor the genes of the ASC (the most relevant of which is *l'sc*) appeared to affect *tll* expression in the procephalic region, at least through stage 10. Furthermore, as *tll* mRNA is still seen in regions of the brain (optic lobe) that remain in a null *tll* mutant, it appears that *tll* does not regulate its own expression.

Regulation of *tll* in the embryonic brain thus remains something of a conundrum. Although it might be proposed that *tll* expression, once activated in the anterior horseshoe domain, is maintained (like the homeotic genes) by a complex of proteins of the Polycomb and Trithorax group (Orlando and Paro, 1995), the dynamic modulation of the *tll* expression domain into the HL and LL domains, and the appearance of *tll*

expression at stage 11 in the OL domain renders such a mechanism improbable. Two other possibilities, not mutually exclusive, seem more likely: that additional genes regulating *tll* later during embryogenesis remain to be discovered, and/or that a constellation of partially redundantly functioning transcription factors, removal of any one of which does not have a strong effect, is required to generate the complex *tll* brain expression pattern.

Enhancers and repression elements in the *tll* regulatory region

Work described here, taken together with previous work primarily on the proximal region, provides a detailed map of the 5.9 kb *tll* regulatory region. Since this DNA can drive the endogenous expression pattern and also, as part of a construct containing the *tll* coding region (plus 2 kb of 3′ DNA of untested significance), rescue the null *tll* phenotype (Pignoni et al., 1990), it most likely contains all of the essential *tll* regulatory sequences. As well as identifying additional tor-REs mediating early expression in polar caps under control of the terminal system, we have identified both positive and negative elements mediating the HL, LL and OL domains of *tll* expression in the developing brain. The modules identified by our promoter dissection and genetic crosses are summarized in Fig. 8.

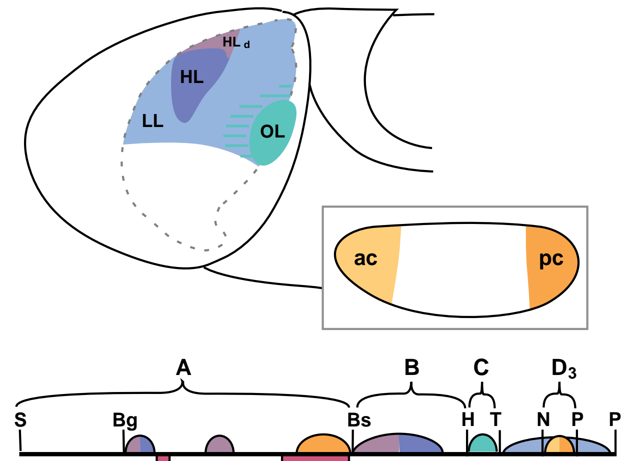


Fig. 8. Elements of *tll* regulatory region correlated with spatial expression domains. The schematic summarizes the mapping of modules that have an activating (rounded, above line) or repressing (square, below line) activity. Regions responding to *tor* activation are yellow (both anterior and posterior poles) or orange (posterior pole only), regions driving brain neuroblast expression are shaded according to the schematic in Fig. 2, i. e., optic lobe (turquoise), LL (light blue), HL (dark blue) and HL_d (purple). Regions repressing expression in the brain are cherry red.

tor-REs

Like the previously described proximal region D_3 (yellow/orange in Fig. 8; Liaw et al., 1993), the distal region −2291 to−2770 (orange in Fig. 8) mediates polar expression in response to activation of the terminal system. Furthermore, this distal region contains two sequences similar or identical to the tor-RE identified in the proximal *tll* promoter region (D_3) (Liaw et al., 1995; Fig. 6F) and drives an expanded domain of expression in embryos with an ectopically active Torso receptor.

brain-specific enhancers and repression elements

The novel result from our regulatory region dissection is the identification of elements (modules) that mediate different portions of the *tll* expression pattern in the procephalic neurogenic ectoderm. As the portion of the *tll* expression pattern that is conserved between *Drosophila* and vertebrates is the anterior, brain-specific expression (see below), identification of modules specifically mediating portions of this pattern could have more general significance. This is, to our knowledge, the first identification in either *Drosophila* or vertebrates of a brain-specific enhancer based on assays in the whole organism.

The fact that different DNA sequence elements drive different portions of the anterior pattern demonstrate that there is a complex basis for the pattern of *tll* expression in the procephalic region. Integration of results from regulatory region dissection with mapping of expression patterns allows us to conclude that there are at least three independently regulated domains of *tll* expression: HL, LL and OL. The phenotype of *tll* mutant embryos reveals that complete absence of *tll* activity starting from the syncytial blastoderm stage results in absence of the entire protocerebrum (Strecker et al., 1988; Pignoni et al., 1990; Younossi-Hartenstein et al., 1997). It is possible that continued expression in the distinct LL, HL and OL subdomains during later stages of embryogenesis is also required, and that absence of any one of these might result in more subtle defects in brain development.

The LL region encompasses most of the procephalic neurogenic area and is characterized by a relatively low and somewhat uneven expression of *tll*. The proximal regulatory region (+475 to −908) drives this expression (light blue, Fig. 8) throughout the procephalic neurogenic region, indicating that the HL domain is contained within the LL domain.

Strong expression in the triangular, dorsomedial HL region persists throughout much of embryogenesis (stage 6 to stage 13). Does the high level of *tll* expression in the HL domain make the HL cells different in some way from the surrounding LL cells? Since it covers only a portion of the protocerebral anlage, HL expression cannot function to define the (putatively) segmental protocerebrum as a unit. Furthermore, HL expression does not appear to result in an immediately obvious difference in cell behavior, since the morphology of neuroblast delamination in stages 10 and 11 is not noticeably different in the HL as compared to surrounding LL domain (V. H., unpublished observations). A third possibility is that HL expression plays a role in establishing a portion of the larval brain, the anlage for which has not yet been mapped in the embryonic procephalic neurogenic ectoderm. A candidate for such a brain region is the mushroom bodies, since they arise from a dorsomedial position in the protocerebrum (reviewed by Younossi-Hartenstein et al., 1996).

The ability to drive expression in the HL domain was localized to three different regions (two by subtraction); one of these regions, −4400 to −4749, is capable of independently driving expression in the HL domain. The lack of sequence similarity between *Drosophila melanogaster* and *Drosophila virilis* genomic DNA for these distal modules makes identification of significant sequences difficult. Multiple repeats of the

sequence TCTGG, however, were found in the *melanogaster* HL elements (and also in the distal *virilis* genomic DNA). While TCTGG is not a described consensus binding site for a known *Drosophila* transcription factor, it is a site for a bHLH protein, one component of which is E47, the vertebrate homolog of the *Drosophila* Daughterless protein. It may be relevant that *daughterless* is required during *Drosophila* embryogenesis for commitment to the proneural fate, and that *daughterless* mutant embryos display brain defects (Caudy et al., 1988). The independently functioning −4400 to −4749 module provides a reagent for further investigation of the molecular and genetic basis of *tll* expression in the HL domain in the embryonic brain.

Implications for gene regulation in the vertebrate brain

The Tlx protein of chickens and mice is 81% identical to the Tailless protein in the DNA-binding domain, and 41% identical in the 'ligand binding' (although no ligand is known for either) domain (Yu et al., 1994). These high levels of sequence identity, as well as the fact that more closely related genes have not been identified, indicate that *Drosophila tll* and vertebrate Tlx are orthologous genes.

In addition to this apparent common ancestry, related expression patterns suggest that the *Drosophila* Tailless and vertebrate Tlx proteins function in homologous regions of the brain. *tll* is expressed in the protocerebrum, the most anterior part of the primordial insect brain; similarly, Tlx is expressed in the most anterior portion of the vertebrate embryonic brain, the telencephalon and diencephalon (Yu et al., 1994; Monaghan et al., 1995). An additional similarity is that both *tll* and Tlx are expressed in visual centers of the brain. *tll* is expressed in the optic lobe of the stage 11/12 embryo and 4 days later in the optic lobe of the third instar larva (data not shown). Tlx is expressed in the optic vesicle and eye, which originate from the diencephalon; in the newborn mouse, Tlx expression is seen in in the optic tract and the portion of the brain giving rise to it (Monaghan et al.,1995).

Dissection of the *tll* regulatory region has identified multiple modules controlling expression in specific portions of the *Drosophila* embryonic brain. This information should pave the way for identification of brain-specific regulatory factors that could be studied both molecularly and genetically. Since, corresponding to the genes *tll*, *otd* and *ems* expressed in the *Drosophila* brain, there are orthologs Tlx, Otx and Emx similarly expressed in anterior regions of the vertebrate brain (Arendt and Nübler Jung, 1996), it is conceivable that insights gained into regulation of *tll* may increase our understanding of pathways of gene activity controlling development of the vertebrate brain.

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