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The BTB domain, found primarily in zinc finger proteins, defines an evolutionarily conserved family that includes several developmentally regulated genes in *Drosophila*

(bric à brac/tramtrack/transcriptional regulation/amino-terminal domain/embryogenesis)

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ABSTRACT **The *Drosophila* bric à brac protein and the transcriptional regulators encoded by tramtrack and Broad-Complex contain a highly conserved domain of ≈115 amino acids, which we have called the BTB domain. We have identified six additional *Drosophila* genes that encode this domain. Five of these genes are developmentally regulated, and one of them appears to be functionally related to bric à brac. The BTB domain defines a gene family with an estimated 40 members in *Drosophila*. This domain is found primarily at the N terminus of zinc finger proteins and is evolutionarily conserved from *Drosophila* to mammals.**

Many conserved protein domains that mediate specific molecular functions have been identified. In addition to clarifying the mechanisms underlying a variety of biological processes, the characterization of such domains has led to the classification of genes into families, helping to assign function to other genes and facilitating the identification of interesting new genes.

We have recently identified the *Drosophila* gene bric à brac (*bab*), which is required for pattern formation along the proximal–distal axis of the leg and antenna (1). Mutations in *bab* cause homeotic transformation and fusion of tarsal segments. The graded expression pattern in tarsal primordia, together with analysis of *bab* mutants, suggest that the concentration of the nuclear bab protein determines tarsal segment specification. In addition, *bab* is required during morphogenesis of the ovary (D.G., unpublished data).

bab contains a motif of ≈115 amino acids also found in the zinc finger proteins encoded by the *Drosophila* genes tramtrack [*ttk* (2)] and Broad-Complex [*BR-C* (3)]. The ttk protein binds transcriptional regulatory elements of the segmentation genes fushi tarazu and even-skipped. It is required during embryogenesis, presumably to repress inappropriate transcription of these genes, and possibly other segmentation genes, including odd-skipped, hairy, and runt (4–7). ttk is also required during cell fate determination in the eye (8). *BR-C* is an early response locus in the ecdysone-induced regulatory cascade that initiates metamorphosis, and its products are thought to activate downstream regulatory genes (3). The transcripts of both *ttk* and *BR-C* are alternatively spliced, producing proteins in which the motif they have in common with bab is at the N terminus, fused to different sets of zinc fingers near the C terminus (3, 7). We have called this motif the BTB domain, for BR-C, ttk, and bab (1).

In this study, we used PCR to search for additional BTB domain-encoding genes in *Drosophila*. We have identified and characterized six additional members of this family.‖ Five of them are transcribed in spatially and temporally regulated patterns, suggesting that these genes have specific and distinct functions during development. We estimate that the BTB domain family contains as many as 40 members in *Drosophila*.

MATERIALS AND METHODS

Equal amounts of *Hin*dIII- or *Eco*RI-digested *Drosophila* genomic DNA were loaded onto multiple lanes of an agarose gel and transferred to nylon. The filter was then cut into strips and hybridized with a ^{32}P-labeled probe encoding the bab BTB domain in 0.25 M NaH_2PO_4/0.25 M Na_2HPO_4, pH 7.2/7% SDS/1 mM EDTA/1% bovine serum albumin, at 55°C. Each strip was washed at a different stringency. The final wash conditions were as follows: 0.1% SDS/0.1× standard saline/citrate (SSC) at 65°C, 0.1% SDS/0.1× SSC at 60°C, 0.1% SDS/0.5× SSC at 60°C, 0.1% SDS/1× SSC at 60°C, or 0.1% SDS/2× SSC at 55°C.

Degenerate PCR primers incorporated all possible codons for the N-terminal sequence FCLRWNN [1: 5′-tagtctagaagcttTTYTGYYTNMGNTGGAAYAA-3′, where M is A or C] and for the central sequence ACSPYF [2: 3′-CGNACRAGNGGNATRAARcttaagcacgtctgt-5′ and 3: 3′-CGNACRTCRGGNATRAARcttaagcacgtctgt-5′]. Primers 2 and 3 differ at the serine codon. Coding sequences are capitalized; restriction enzyme sites are underlined. Reaction conditions were as follows: 2 μg of genomic DNA, 1 μM each oligonucleotide, 0.2 mM each dNTP, 10 mM Tris·HCl (pH 8.8), 50 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, and 2.5 units of AmpliTaq DNA polymerase, in 100 μl. In one experiment, conditions were as follows: initial denaturation, 95°C for 5 min; followed by cycles of denaturation, 95°C for 1 min; annealing, 55°C for 2 min; and extension, 72°C for 2 min. In another experiment, annealing was done at 55°C, 46°C, or 37°C for the first three cycles and subsequently at 55°C to allow for imperfect matches between templates and primers. Cycles were repeated ≈30 times. The PCR products were digested with *Hin*dIII and *Eco*RI, gel-purified, and subcloned into M13mp18 for sequencing. From all PCR reactions described above, a total of 144 independent subclones was sequenced.

To obtain DNA encoding full-length BTB domains, the PCR products were used to probe a genomic library (Stratagene *Drosophila* genomic library made from Canton-S embryos, in the λ FIX II vector) at high stringency. Subfrag-

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Abbreviation: CNS, central nervous system.

‖The sequences encoding the proteins reported in this paper have been deposited in the GenBank data base (accession nos. U14399–U14404).

ments encoding each BTB domain were then identified by Southern analysis.

Sequence analyses were done with programs from the Genetics Computer Group package (9), using default settings in all cases. Sequence searches were performed at the National Center for Biotechnology Information using the BLAST network service.

In situ hybridization to polytene chromosomes and to whole-mount embryos was done as described in Godt *et al.* (1). The following genomic DNA fragments encoding the corresponding BTB domains were used as probes: BTB-II, a 2.4-kb or a 0.75-kb fragment; BTB-III, two overlapping 8-kb fragments or a 0.7-kb *Sac* I–*Hin*dIII fragment; BTB-IV, a 15-kb fragment or a 0.6-kb *Bam*HI–*Xho* I fragment; BTB-V, a 15-kb fragment or a 0.6-kb *Eco*RI–*Xba* I fragment; BTB-VI, a 7-kb fragment or a 1.6-kb *Hin*dIII–*Not* I fragment; BTB-VII, a 1.4-kb *Sal* I–*Sac* I fragment. For each BTB domain, the same expression pattern was observed with all probes used. Embryonic stages were assigned according to Campos-Ortega and Hartenstein (10). Cytological positions were determined according to the chromosome maps of G. Lefevre in ref. 11.

RESULTS

Identification of Additional BTB Domains. A set of equivalent *Drosophila* genomic Southern blots was probed with a sequence encoding the bab BTB domain, under conditions of various stringencies. High-stringency conditions resulted in a single major band; additional bands appeared with decreased stringency. At the least stringent conditions used, the probe hybridized to at least 40 DNA fragments (Fig. 1). In a similar experiment, Southern blots were hybridized at high stringency with probes encoding each of the newly identified BTB domains (see below). The fragment that hybridized specifically to each of these probes corresponded to one of the minor bands seen at low stringency with the bab BTB probe, supporting the interpretation that these minor bands encode other BTB domains (data not shown). This analysis provides a rough estimate of 40 for the number of BTB domain-encoding genes in the *Drosophila* genome.

Additional members of the BTB domain family were identified using PCR with degenerate primers based on two sequences that are completely conserved in the BTB domains of bab, ttk, and BR-C. Of the 144 PCR products sequenced, 84 encoded BTB domains, including those of BR-C and ttk, but not that of bab, indicating that the screen was not saturating. Six additional BTB domains were identified and are called BTB-II, -III, -IV, -V, -VI, and -VII. The full-length BTB domain sequences were then obtained after isolating the corresponding genomic clones (Fig. 2).

The BTB domain is highly conserved among ttk, BR-C, bab, and BTB-II to -VII. Amino acid identities range from 41% between BTB-III and -VI to 77% between bab and BTB-II, with an average identity of 53%. Several BTB domain-encoding sequences, including that of *ttk*, appear to contain introns. The BTB-IV to -VII sequences presented in Fig. 2 are those predicted after putative introns are removed, according to the consensus splice sites present. In all cases except the first putative intron of BTB-V, failure to splice the intron would result in translation termination, a frame-shift relative to the BTB consensus, or both. Splicing within BTB domain-encoding sequences appears to be common, and it is possible that alternative splicing, in which downstream exons produce BTB domain variants, may also occur.

Embryonic Expression and Chromosomal Localization of BTB-II to -VII. ***BTB-II (map position 61F 1–2).*** The BTB-II transcript is mostly, but not exclusively, expressed in specific portions of the mesoderm and its derivatives. It is present at high levels in the primordia of the visceral muscles of the foregut, hindgut, midgut, and dorsal vessel and is present at lower levels in the somatic muscles (Fig. 3 *A*, *B*, and *D*). The transcript was not detected in other mesodermal structures, such as the fat body and the somatic portion of the gonads. There is also transient expression in the ventral epidermis (stages 11 and 12; Fig. 3*C*), in specific portions of the ectodermal and endodermal gut rudiments, as well as in some structures in the head and tail of the embryo (Fig. 3*D*).

BTB-II appears to be functionally related to *bab*. They map to the same cytological position, and their BTB domains are, by far, the most similar of those identified. BTB-II is expressed in all tissues that express *bab*, including specific somatic cells of the ovary (D.G., unpublished data) and a subdistal domain in the epithelium of leg and antenna imaginal discs (Fig. 3*E*; ref. 1). In addition, BTB-II is expressed in tissues where bab RNA was not detected, such as the embryo and the adepithelial tissue of imaginal discs (Fig. 3 *A–E*).

BTB-III (map position 60F). Expression starts at stage 4, with a distribution of transcript between 10% and 60% egg length, excluding the anlage of the mesoderm (Fig. 3*F*). This distribution resolves first into two (syncytial blastoderm) and later five (cellular blastoderm) transverse stripes (Fig. 3*G*), which disappear at the onset of gastrulation. In addition, a wedge-shaped dorsal expression domain (50–90% egg length) appears in the syncytial blastoderm and becomes confined to the procephalic region after gastrulation (Fig. 3*H*), where it persists during germ band extension. From the late extended germ band stage onward, the transcript is associated with the developing nervous system. RNA can be detected in the midline (stages 11–13; Fig. 3*I*), the sensory organs (stages

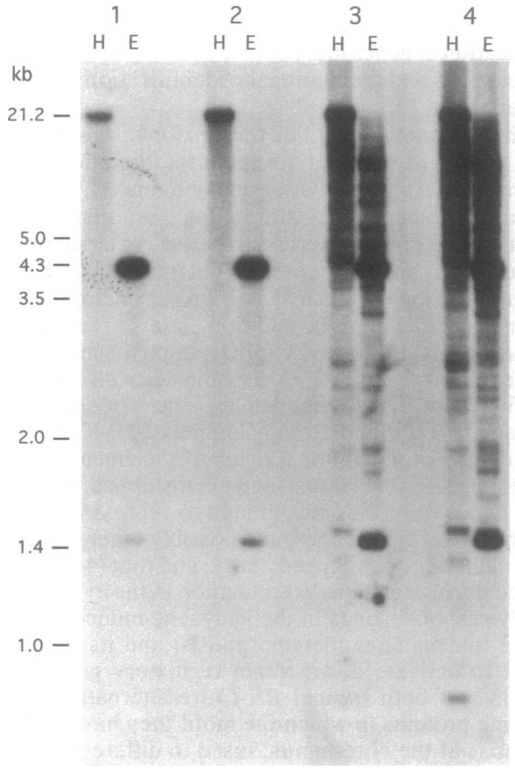

FIG. 1. Estimation of the size of the BTB domain family in *Drosophila*. Equivalent Southern blots containing *Hin*dIII (H)- and *Eco*RI (E)-cut *Drosophila* genomic DNA were hybridized with a probe encoding the bab BTB domain and washed at a range of stringencies. Lanes: 1, highest stringency; 2–4, decreasing stringency. At the lowest stringency, the probe hybridized to at least 40 DNA fragments. Further reduction in stringency did not yield additional bands (data not shown). The 1.5-kb *Eco*RI band that persists at high stringency corresponds to BTB-II.

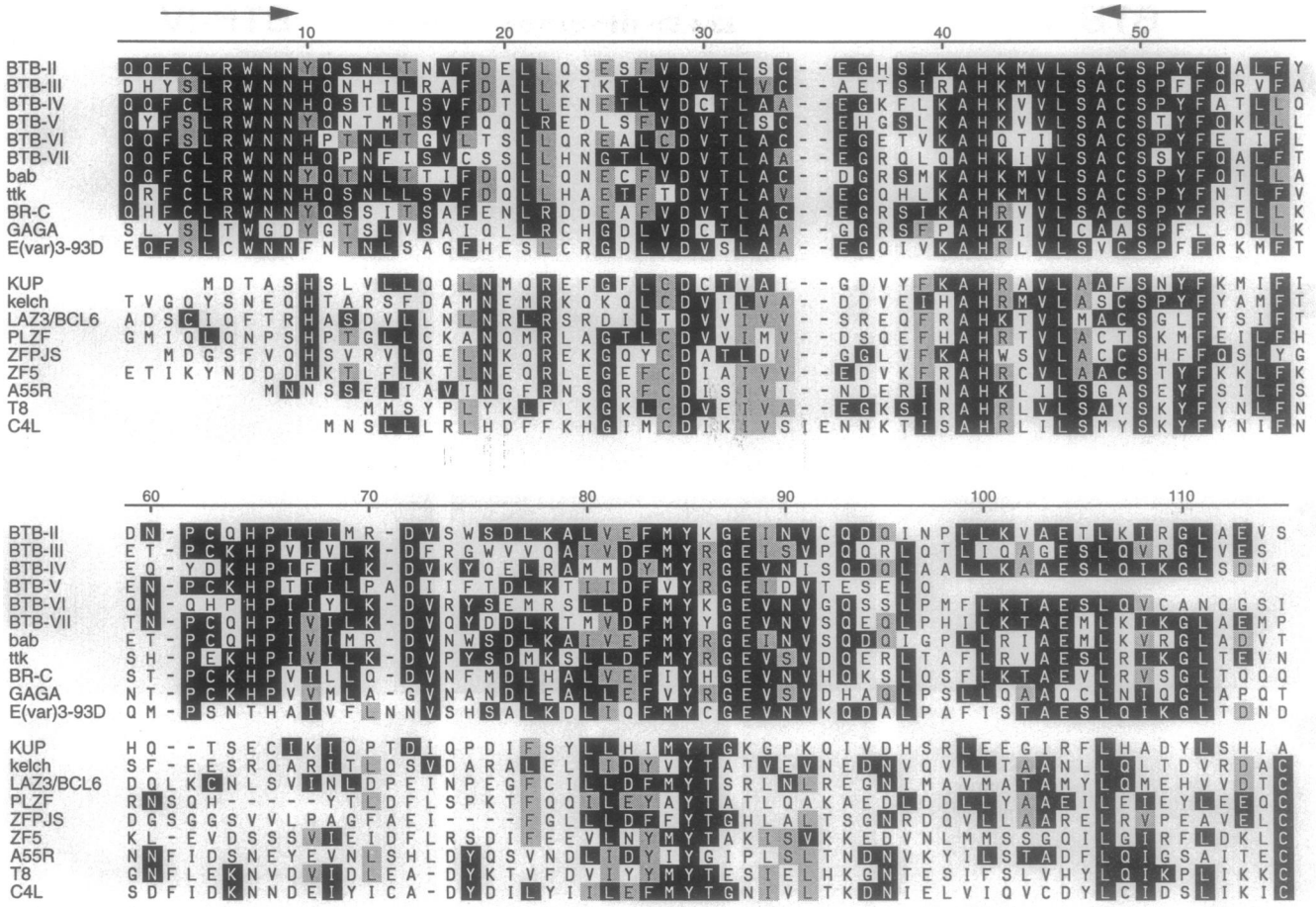

FIG. 2. Alignment of BTB domain sequences. Only identities are shaded: residues found at a position at least five times are shaded black; if there is a second consensus at that position, it is shaded gray. Positions 34 and 81 have three such consensus sequences. The arrows indicate the sequences used to design PCR primers. Numbering is based on the BTB domain of ttk. Sequences shown are those predicted after putative introns are removed, according to the splice sites present. The predicted intron in BTB-IV, located between positions 54 and 55, is 64 nt long. The first predicted intron in BTB-V is between positions 43 and 44; if it is not spliced, the following in-frame insertion would result: VKYLDICIYTIVMKLIKFLIRKLQ. The second predicted intron site in BTB-V (between positions 97 and 98) contains a 5′ splice sequence, followed by several termination codons. No 3′ splice acceptor was found within >0.7 kb that would result in a continuation of the consensus sequence. BTB-VI contains a putative 72-nt intron between positions 40 and 41, and based on the presence of a 5′ splice sequence and subsequent divergence from the consensus, there may be a second intron after residue 107. A putative intron of 61 nt appears in BTB-VII between positions 54 and 55. The DNA sequences corresponding to BTB-II to -VII, including the predicted introns, have been deposited in GenBank. In ttk, BR-C, GAGA, and E(var)3-93D, the BTB domain begins within 6 residues of the predicted N terminus. The other BTB domains are also predicted to be very close to their N termini, except for kelch, where it is predicted to start at residue 129; this has not been determined for bab (1). For BTB-IV, -V, -VI, and -VII, a methionine is predicted within seven residues of the domain start. These methionines are all preceded by sequences that satisfy the *Drosophila* translational start site consensus (12).

14–16), and the central nervous system (CNS; from stage 12 onward), where it is expressed in a dynamic pattern associated with specific subsets of neural cells (Fig. 3 *J* and *K*). The BTB-III RNA distribution pattern is very similar to that of the *Drosophila Tkr* gene, which encodes a tyrosine kinase-related protein and also maps to position 60F (13). The published sequence of *Tkr* does not, however, encode a BTB domain, and one of the BTB-III probes that was used encodes little more than the BTB domain, suggesting that these are different genes.

BTB-IV (map position 47A). The BTB-IV transcript is maternally provided and is present throughout the syncytial and cellular blastoderm, except in the pole cells. During gastrulation, the RNA level drops significantly in the posterior and anterior midgut primordia, where it is no longer detected after stages 8 and 9, respectively. The RNA persists in the mesoderm and ectoderm at a gradually decreasing level throughout germ band extension (until stage 10), and expression becomes restricted to the neural primordium (stage 11). High levels of transcript are associated with neuroblasts and later with the ganglion mother cells and neurons of the developing CNS (Fig. 3 *M* and *N*). BTB-IV is also expressed in the precursors of the peripheral nervous system (stages 11–13; Fig. 3*O*) and in precursors of the stomatogastric nervous system (stage 12). From stage 13 onward, staining is restricted to the CNS (Fig. 3*P*).

BTB-V (map position 47A). BTB-V has a strong maternal component in the early embryo. The transcript is ubiquitous throughout embryogenesis, present at high levels until gastrulation and at lower levels afterward (data not shown).

BTB-VI maps to position 91AB, and BTB-VII maps to position 63BC. Their transcripts were not detected during embryogenesis; however, BTB-VI is expressed during imaginal development in portions of the CNS such as the optic lobes, as well as during oogenesis, in the centripetally migrating follicle cells of stage 10 egg chambers (data not shown).

DISCUSSION

We have isolated six additional *Drosophila* genes that encode a BTB domain, five of which are developmentally regulated. Recently, several other genes have been identified, in *Drosophila* and in other species, that encode a BTB domain (Fig.

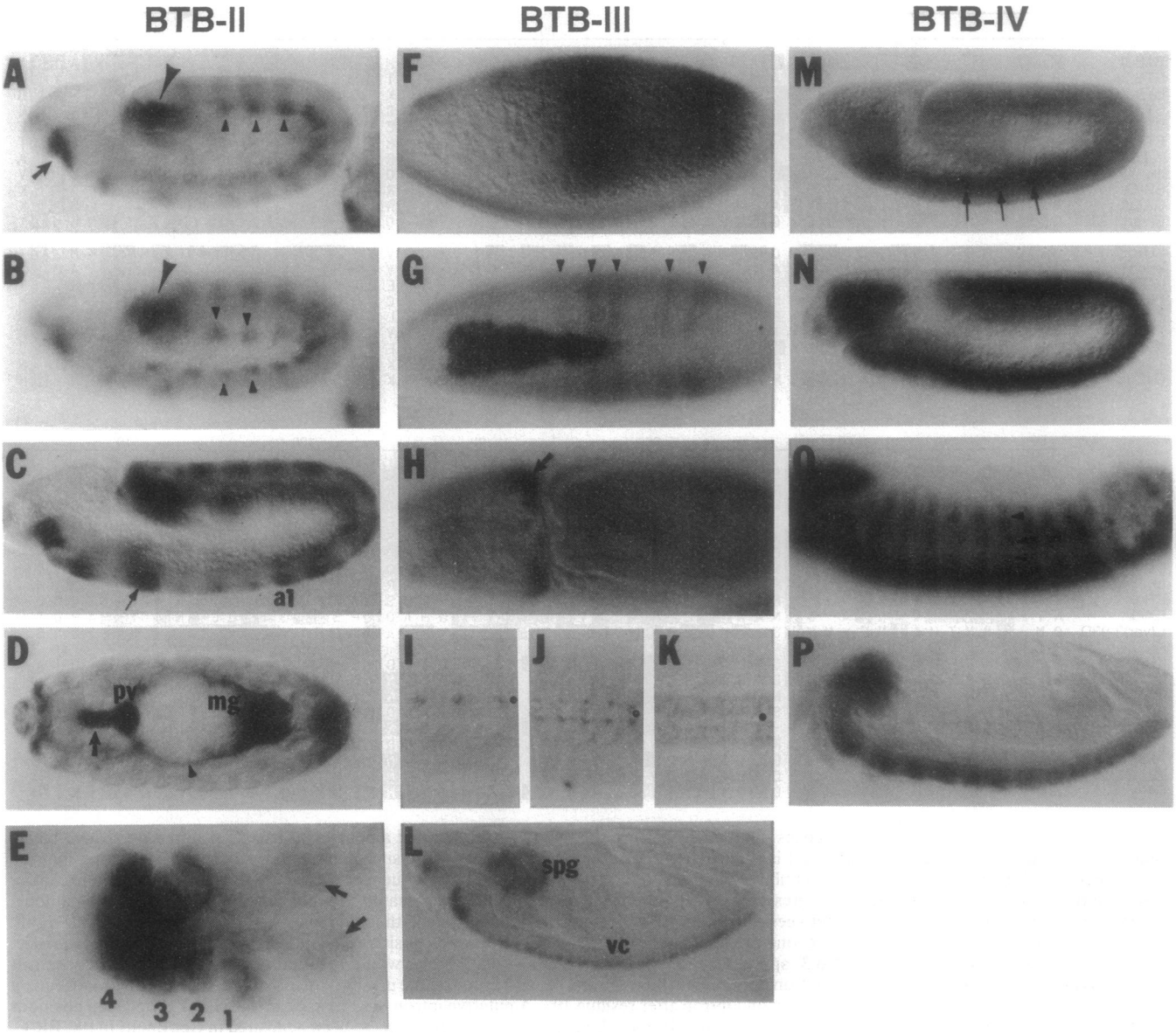

FIG. 3. Distribution of BTB-II, -III, and -IV transcripts during wild-type embryonic development. (*A–E*) BTB-II expression: (*A*) Stage 10 embryo with staining in the visceral mesoderm around the stomodeum (large arrow) and proctodeum (large arrowhead) and in ventral segmental clusters of mesodermal cells that give rise to somatic muscles (small arrowheads). (*B*) Same embryo as in *A* with focus on staining in 11 dorsal clusters of mesodermal cells (small arrowheads) and the visceral mesoderm of the proctodeum (large arrowhead). (*C*) Stage 11 embryo with staining in the mesoderm and in segmental stripes of ventral ectoderm. There are high RNA levels in the mandibular, labial (arrow), and eight abdominal segments (a1, first abdominal segment) and lower levels in the three thoracic segments. (*D*) Stage 14 embryo (dorsal view) with staining in the visceral muscles of the esophagus (large arrowhead) and of the midgut (mg, small arrowhead). Staining is also seen in the proventriculus (pv) and posterior midgut epithelium, and in some head and tail structures. (*E*) Prepupal leg imaginal disc with a graded distribution of BTB-II transcript in the epidermal primordium of tarsal segments 1–4 (denoted by numbers), as well as staining of the adepithelial tissue (arrows). (*F–L*) BTB-III expression: (*F*) Early stage 5 embryo with a dorsolateral expression domain between 10% and 60% egg length and a middorsal stripe. (*G*) Cellular blastoderm (dorsal view), where transcript is present in a wedge-shaped domain and in five transverse stripes (arrowheads). (*H*) Stage 9 embryo (dorsal view), with staining concentrated in the procephalic ridge (arrow) anterior to the amnioserosa. (*I*) Localization of RNA in the midline at stage 12. (*J*) Localization of RNA in neural clusters adjacent to the midline at stage 13. (*K*) Localization of RNA in a distinct subset of cells in the CNS at stage 15 (ventral view; midline is marked by dot). (*L*) RNA expression in the nervous system at stage 15 (vc, ventral cord; spg, supraesophageal ganglion). (*M–P*) BTB-IV expression: (*M*) Stage 9 embryo with high levels of transcript in neuroblasts (arrows) and lower levels in the ectoderm. (*N*) Stage 11 embryo with staining restricted to the neural primordium. (*O*) Stage 13 embryo with expression in cells of the peripheral nervous system (arrowheads). (*P*) Stage 14 embryo with transcript throughout the CNS. (Dorsal is up unless otherwise noted; anterior is at left.) (*A–D* ×120; *E* ×300; *F–P* ×120.)

2). These include the zinc finger-encoding genes BCL-6, also known as *LAZ3*, a putative protooncogene whose disruption is associated with a human lymphoma (14, 15), human *ZNF145* (formerly *PLZF*), which was altered by a chromosomal translocation associated with a promyelocytic leukemia (16), human ZFPJS, which encodes a transcription factor that regulates the major histocompatibility complex II promoter (GenBank accession no. L16896), human *ZNF46* (formerly *KUP*), whose function is unknown (17), murine ZF5, which represses transcription from the c-*myc* and thymidine kinase promoters (18), the *Drosophila* GAGA transcription factor, which activates transcription of several genes, including engrailed, Ultrabithorax, fushi tarazu, even-skipped, and Krüppel (19); as well as several genes that do not encode zinc fingers, including *Drosophila* enhancer of position-effect variegation *E(var)3-93D* (20), *Drosophila* kelch, which en-

codes a cytoplasmic protein required for the formation of ring canals in developing egg chambers (21), and a related group of poxvirus genes, including vaccinia virus A55R (22), myxoma virus T8 (23), and swinepox virus C4L (24). For all of these genes except kelch, the BTB domain is located very close to the predicted N terminus.

Within the BTB-domain family, one particularly conserved subgroup is apparent, the "ttk group," that includes BR-C, bab, BTB-II to -VII, the GAGA factor, and E(var)3-93D, as well as ttk, the first of these to be identified. The ttk group aligns with just one gap (position 70 to 71) and contains several highly conserved sequences, such as the first 10 residues, that are not found in the other BTB domains. The average identity within the ttk group is 49%, compared with an average identity of 24% between the ttk group and the other BTB domains. The average identity among the other BTB domains is 29%. These relationships are probably not simply due to differences among species. Although the members of the ttk group are all from *Drosophila*, this group does not include *Drosophila* kelch; in addition, the mammalian BTB domains identified so far do not form a closely related group. There are several positions in the BTB domain at which there is one consensus for the ttk group and another for the other BTB domains; for example, the ttk group has a conserved Val-28, whereas the others have a conserved cysteine. This does not necessarily define these other BTB domains as a distinct group. The consensus in these BTB domains may represent the predominant motif in the family. The progenitor of the ttk group might have varied from this consensus at several positions, and this would have been amplified as the progenitor underwent the numerous duplications that formed the ttk group. Although the predominance of the ttk group within the family may be due, in part, to the bias of our PCR screen, five of these genes were identified independently. As more BTB domains are identified, other distinct subgroups may become apparent, and it will be interesting to determine the functional significance of the diversity within this family.

The BTB domain defines a large family whose members function in a variety of biological processes. Its frequent association with putative transcriptional regulators that contain zinc finger motifs suggests a role in transcriptional regulation. Because the zinc finger is itself a DNA-binding motif, it seems unlikely that the BTB domain directly binds DNA; however, it may be involved in modulation of DNA binding. It may mediate protein–protein interaction, perhaps in multimerization with other BTB domains or with another protein or family of proteins. One possibility is a role in transcriptional regulation involving the alteration of chromatin condensation. Several transcriptional regulators are thought to function this way (25), and some of the BTB-domain proteins have been implicated as modulators of chromatin structure. *E(var)3-93D* codes for chromatin-associated proteins thought to contribute to the establishment or maintainance of an open, and thus transcriptionally accessible, chromatin conformation (20). The GAGA factor acts as an antirepressor of histone H1-mediated repression of transcription from the Krüppel promoter and may also be required for an open chromatin conformation (26). It also maps to the same position as gene *62*, an enhancer of position-effect variegation (20). In addition, the leg phenotype of *bab* mutants is very similar to that of some Polycomb-group mutants (27), and Polycomb-group proteins are thought to regulate transcription by inducing local heterochromatinization within the regulatory regions of target genes (28). Further characterization of these genes will determine the relationship between the BTB domain and chromatin structure.

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