MOLECULAR AND CELLULAR BIOLOGY, Mar. 1996, p. 932–942 Vol. 16, No. 3
0270-7306/96/$04.00+0

Drosophila Homologs of the Proto-Oncogene Product PEBP2/CBFβ Regulate the DNA-Binding Properties of Runt

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Received 26 September 1995/Returned for modification 10 November 1995/Accepted 28 November 1995

The *Drosophila runt* gene is the founding member of the Runt domain family of transcriptional regulators. Mammalian Runt domain genes encode the α subunit of the heteromeric DNA-binding factor PEBP2/CBF. The unrelated PEBP2/CBFβ protein interacts with the Runt domain to increase its affinity for DNA. The conserved ability of the *Drosophila* Runt protein to respond to the stimulating effect of mammalian PEBP2/CBFβ indicated that flies were likely to have a homologous β protein. Using the yeast two-hybrid system to isolate cDNAs for Runt-interacting proteins, we identified two *Drosophila* genes, referred to as Brother and Big-brother, that have substantial sequence homology with PEBP2/CBFβ. Yeast two-hybrid experiments as well as in vitro DNA-binding studies confirmed the functional homology of the Brother, Big-brother, and PEBP2/CBFβ proteins and demonstrated that the conserved regions of the Runt and Brother proteins are required for their heterodimeric interaction. The DNA-bending properties of Runt domain proteins in the presence and absence of their partners were also examined. Our results show that Runt domain proteins bend DNA and that this bending is influenced by Brother protein family members, supporting the idea that heterodimerization is associated with a conformational change in the Runt domain. Analysis of expression patterns in *Drosophila* embryos revealed that *Brother* and *Big-brother* are likely to interact with *runt* in vivo and further suggested that the activity of these proteins is not restricted to their interaction with Runt.

The *Drosophila* Runt protein is a member of the recently identified Runt domain family of transcriptional regulators (20). The *runt* gene was initially characterized from its vital role in segmentation, in which it acts as a primary pair-rule gene (10, 11, 19). Subsequently, *runt* was found to have independent functions in the developmental pathways of sex determination and neurogenesis (7, 8). In each of these three pathways, *runt* plays a role in the specification of cell fates. The nuclear localization of the Runt protein as well as the altered transcriptional regulation of downstream genes in the sex determination and segmentation pathways suggested that Runt could function as transcription factor even though it had no homology with other known transcription factors (22).

Recent studies have identified several mammalian genes that have a highly conserved 128-amino-acid region in common with Runt, called the Runt domain. The first of these to be identified was the human acute myeloid leukemia 1 gene (*AML1*) (6). This gene is expressed in a number of lymphoid cell lines and maps to the breakpoint of t(8;21) translocations associated with acute myeloid leukemia (28). These results suggest that *AML1* is necessary for normal hematopoietic development. Two other human Runt domain genes, *AML2* and *AML3*, have subsequently been identified (25). Although little is known about these two genes, they also map near translocation breakpoints that are associated with different forms of leukemia (35, 41).

Significant insights on the functions of Runt domain proteins come from studies on a DNA-binding transcription factor referred to as the polyomavirus enhancer-binding protein (PEBP2) or as core binding factor (CBF). This factor was initially identified by its interaction with core enhancer elements of both the polyomavirus DNA tumor virus (21) and mammalian type C retroviruses (42). Subsequently, this factor was implicated as an important transcriptional regulator for a variety of genes that are expressed in T cells, including, for example, the T-cell receptor α (TCRα), TCRβ, TCRγ, and TCRδ genes (14, 18, 33, 34). Purified PEBP2/CBF is in fact a heteromeric complex of two unrelated proteins (30, 31, 43). The PEBP2/CBFα subunit proteins have been identified as Runt domain proteins. PEBP2/CBFβ is an unrelated protein which we will refer to as mammalian beta (mBeta) in this paper. The human gene for mBeta is disrupted by a pericentric inversion, inv(16)(p13q22), that is characteristic of the M4Eo subtype of acute myeloid leukemia (26). Thus, the genes for both subunits of the PEBP2/CBF transcription factor have been identified as proto-oncogenes. Intriguingly, the gene for mBeta appears to be much more widely expressed than the mammalian Runt domain genes (30), suggesting that mBeta function is not restricted to interactions with Runt domain proteins during hematopoiesis.

The PEBP2/CBFα proteins bind to DNA as monomers, while the unrelated mBeta protein does not bind DNA but instead stabilizes the interaction between the PEBP2/CBFα subunit and DNA (30, 43). The Runt domain is responsible for the DNA-binding properties of the PEBP2/CBFα proteins and also mediates interaction with mBeta (31). The DNA-binding activity of the *Drosophila* Runt protein is greatly enhanced by mBeta (20, 32). This cross-species interaction not only indicates an evolutionarily conserved role for the Runt domain but also predicts that *Drosophila melanogaster* will have a homolog of mBeta. Here we describe the isolation and characterization of two *Drosophila* proteins, Brother (Bro) and Big-brother (Bgb), that are structurally and functionally homologous to mBeta. In this paper, we demonstrate that these proteins' ability to enhance DNA binding by Runt domain proteins is conserved and map the regions required for these conserved functions. We also show that DNA binding by Runt domain proteins is associated with DNA bending and that the severity

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of the bend is influenced by interaction with the Bro-related proteins. Finally, analysis of the expression of Bro and Bgb indicates that widespread expression is an evolutionarily conserved feature of the this novel family of proteins.

MATERIALS AND METHODS

Molecular biology. DNA sequence information was obtained by using double-stranded DNA templates with conventional [^{35}S]dATP dideoxynucleotide chain termination protocols. The sequence in a few compression areas was confirmed by electrophoresis in acrylamide gels containing 50% formamide. Oligonucleotide primers 5′ACT (5′-TACCACTACAATGGATGA-3′) and 3′ACT (5′-AGA TGGTGCACGATGCACAG-3′) were used to sequence the 5′ and 3′ junctions, respectively, of cDNA inserts in the pACT vector. The T3 and M13(−20) primers were used to obtain sequence from fragments subcloned into the pBluescript vector (Stratagene; minus orientation with KS polylinker). Internal sequence was obtained both by using oligonucleotide primers to extend sequence obtained from the junctions for the initial clones and also from subclones generated by restriction digestion. Both strands of pACT:Bro$^{5.2}$ and pACT:Bgb$^{25.1}$ were sequenced in their entirety. Additional sequence information was obtained from subcloned segments of *Drosophila* genomic DNA that were isolated based on their cross-hybridization to mBeta. The congruence of the sequence from the cDNA clones and genomic clones indicates that there are no introns in the *Bro* gene.

Yeast plasmids. Yeast expression plasmids were based on pGBT9 (*GAL4* DNA-binding domain/*TRP1* marker) and pGAD10 (*GAL4* activation domain/*LEU2* marker; described in reference 3). pGBT9:lamin was a gift from Stan Fields. pGBT9:mAML1 contains a subcloned segment of a pPEBP2αB1A cDNA (2) inserted as an *Eco*RI fragment into pGBT9. For pGBT9:Runt, an *Eco*RI fragment from pB:ED(Bam-8)[15] (22) was inserted into the *Eco*RI site of pGBT9. The resulting plasmid allows constructs to be cassetted in as in-frame *Bam*HI fragments from pQE30 bacterial expression vectors. This frameshifted pGBT9 vector, referred to as pGBD, was used for the following constructs. The pGBD:ΔRd deletion, which lacks amino acids 111 to 225 of Runt (all but the first five and last six amino acids of the Runt domain), was generated by digestion of the *runt* cDNA subclone pB:ED(Bam-8, ΔKS) with *Pst*I and *Sal*I, followed by treatment with T4 DNA polymerase and T4 DNA ligase. For pGBD:Rd+, PCR was used to generate a segment that contains amino acids 91 to 273 of Runt (spans the entire Runt domain) flanked by *Bam*HI sites. The pGBD:αA clone contains a subcloned segment of a full-length PEBP2αA type 1 cDNA (31).

GAD10 fusion constructs were cloned into the *Bam*HI site. The Bro deletion constructs were cut out of pQE30 vectors (see below) as *Bam*HI-*Eco*RI fragments and cloned into pGAD10:Bro digested with *Bam*HI and *Eco*RI.

Bacterial expression plasmids. Bacterial expression constructs were made as N-terminal hexahistidine fusions in the pQE30 vector (Qiagen). The pQE30:Bro construct was created by PCR with T3 and GG1 primers on pDBBTN#2 template DNA. The pDBBTN#2 plasmid contains a segment of *Bro* genomic DNA that lacks the five C-terminal codons. This segment was isolated as a *Bst*XI-*Not*I fragment from a subclone of a *Bro* genomic DNA lambda phage. The *Bst*XI site was filled in by T4 DNA polymerase prior to *Not*I digestion, and then the purified fragment was cloned into *Sma*I/*Not*I-digested pBluescript.

The GG1 primer contains the sequence 5′-CGC GGA TCC AAG ATG CCC CGC GTG G-3′; the underlined nucleotides indicate the newly generated *Bam*HI site. The PCR product generated with this primer and the T3 primer with the pDBBTN#2 template was digested with *Bam*HI and *Hin*dIII and then cloned into pQE30 cut with *Bam*HI and *Hin*dIII. To create pQE30:Bgb, PCR was carried out on clone 15.3 (from the two-hybrid screen) with the GG1 primer and the 3′ACT 3 primer. This full-length Bgb PCR product was then digested with *Bam*HI and *Bgl*II and ligated to pQE30 digested with *Bam*HI.

To create pQE30:NΔ, a PCR product was generated by using primer PG75 (5′-AA GGA TCC GAC CAG AGG TCC AAG-3′) and T3 on a Brother *Bam*HI-*Eco*RI genomic fragment from pGAD10:Bro cloned into BMKS. The resulting product was then digested with *Bam*HI and *Cla*I and ligated to pQE30:Bro cut with *Bam*HI and *Cla*I. Bro has an internal *Cla*I site, which enables us to clone in only the newly deleted region of the PCR product. pQE30:CΔ1 was generated by using the PG76 primer (5′-GG AAG CTT GAA TTC AGC ATC CTC CTG CTG-3′) and GG1 on a pGAD10:Bro template in a PCR. The two underlined sequences in PG76 represent *Hin*dIII and *Eco*RI sites, respectively. This PCR product was digested with *Hin*dIII and *Cla*I and cloned into pGAD10:Bro cut with *Hin*dIII and *Cla*I. pQE30:CΔ2 was made similarly to pQE30:CΔ1 except that the PG77 primer (5′-GG AAG CTT GAA TTC GGC TCG TTG TTC ATC-3′) was used with GG1 on pGAD10:Bro.

Hexahistidine constructs of Runt and PEBP2/CBFβ are described by Pepling and Gergen (32). pQE9:PEBP2αA was a gift from Y. Ito.

Two-hybrid library screen. All transformations in *Saccharomyces cerevisiae* were performed on strain Y153 (from S. Fields) by the lithium acetate-TE protocol of Schiestl and Gietz (36). The library transformation was done on 300 ml of cells grown to an optical density at 600 nm (OD_{600}) of 0.8 with 500 μg of pGBT9:Runt and 80 μg of a *Drosophila* embryonic 0- to 6-h cDNA library fused to the *GAL4* activation domain (generous gift from L. Pick). These cells were plated in 300-μl aliquots onto synthetic complete (SC) medium without leucine, tryptophan, and histidine (SC −Leu, −Trp, −His) and containing 33 mM 3-aminotriazole (3-AT) and then incubated at 30°C. Colonies that grew well were then restreaked onto fresh SC −Leu, −Trp, −His medium with 33 mM 3-AT and then tested for activation of the *lacZ* reporter with a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) filter assay (4). Plasmid DNA from transformants that were both *HIS*$^+$ and *lacZ*$^+$ was isolated by the method described by Hoffman and Winston (17). This DNA was transformed into the *leuB* mutant *Escherichia coli* strain MH4 and plated on M63 minimal medium with ampicillin (50 μg/ml) to select for the library plasmid. Isolates were retransformed into Y153 singly with pGBT9-Runt and pGBT9:lamin to retest activation of the reporter genes. In this case, the lamin fusion construct serves as a control for those library proteins which activate the reporter genes in a nonspecific manner. For yeast two-hybrid protein interaction assays, yeast transformants were initially grown on SC −Leu, −Trp minimal medium and later streaked onto plates of SC −Leu, −Trp, −His medium with 33 mM 3-AT. The plates were then incubated at 30°C for 7 to 9 days. Confirmation of protein-protein interactions in this growth assay was done by using the X-Gal filter assays described above.

DNA-binding and DNA-bending experiments. Electrophoretic mobility shift assays and preparation of bacterially expressed proteins were performed as described by Pepling and Gergen (32). For quantitation of binding following autoradiography of the gel, bands containing the shifted complexes were excised and counted in a liquid scintillation counter. The average fold stimulation of DNA binding was calculated from three independent experiments.

For the DNA-bending experiments, overlapping oligonucleotides that allowed insertion of a PEBP2/CBF binding site in the polylinker of pBend2 were synthesized (23). The final sequence in plasmid pLL01, from the *Xba*I site to the *Sal*I sites used for insertion, reads 5′-CTAGCTGCGGTTAGTCGA-3′. The underlined bases are from pBend2. A panel of circularly permuted labeled DNA probes was generated by using T4 polynucleotide kinase to end label pLL01 DNA digested with each of the following seven restriction enzymes: *Bgl*II, *Xho*I, *Eco*RV, *Pvu*II, *Stu*I, *Kpn*I, and *Bam*HI. Electrophoretic mobility shift assays were run as described above except that 8% instead of 10% polyacrylamide was used and the running buffer contained 0.05% Nonidet P-40. The relative mobilities of the different probes were used to calculate a bending angle as described by Kim et al. (23). The values shown in Table 1 represent averages from five (PEBP2αA), two (PEBP2αA plus mBeta), five (Runt plus Bro), two (Runt plus Bgb), and three (Runt plus mBeta) independent experiments. When addition of partner protein gives both monomeric and heteromeric complexes, the bending angles were calculated by using the relative mobilities of the upper, heteromeric complexes.

Embryo in situ hybridization. The pDBBTN#2 plasmid and a cDNA of *Bgb* (25.1) cloned into pBluescript were used to synthesize antisense digoxigenin RNA probes for *Bro* and *Bgb*, respectively. Plasmids were linearized with *Sac*I for use as templates. Approximately 1 μg of each template was transcribed with T3 RNA polymerase with the Genius 4 RNA DIG labeling kit (Boehringer Mannheim). Reactions were incubated for 2 h at 37°C and precipitated with 0.4 M LiCl–0.2 mg of tRNA–3 volumes of ethanol. The *runt* probe was prepared as described by Tsai and Gergen (39). In situ analysis of 0- to 18-h *Drosophila* embryos was done as described by Klingler and Gergen (24).

Nucleotide sequence accession numbers. The *Bro* and *Bgb* sequences have been deposited in GenBank (accession numbers U22176 and U22177, respectively).

RESULTS

Isolation of *Drosophila* homologs of mBeta. *Drosophila* homologs of the mBeta protein were identified by using the yeast two-hybrid system (9). As a pilot for this screen, this assay system was used to first test for interactions between the murine mBeta protein and three different Runt domain proteins. The first is the full-length *Drosophila* Runt protein. The second, which we refer to here as mAML1, is the full-length, 451-amino-acid isoform of the mouse homolog of human AML1 (this protein is referred to as PEBP2αB1 by Bae et al. [2]). The third Runt domain protein is the full-length, 513-amino-acid isoform of murine PEBP2αA (31). In our two-hybrid experiments, the different Runt domain proteins are expressed as fusions to the GAL4 DNA-binding domain, and the mBeta protein is expressed as a fusion to the GAL4 activation domain. Plasmids that express these different proteins were cotransformed into a yeast strain that contains GAL4-driven *HIS3* and *lacZ* reporter genes (3). From the growth of transformants on medium lacking histidine (Fig. 1) and expression of *lacZ* (not shown), a positive two-hybrid signal is obtained between mBeta and both Runt and mAML1. The interaction between mBeta and these two Runt domain proteins is specific, as no signal is detected in transformants that express

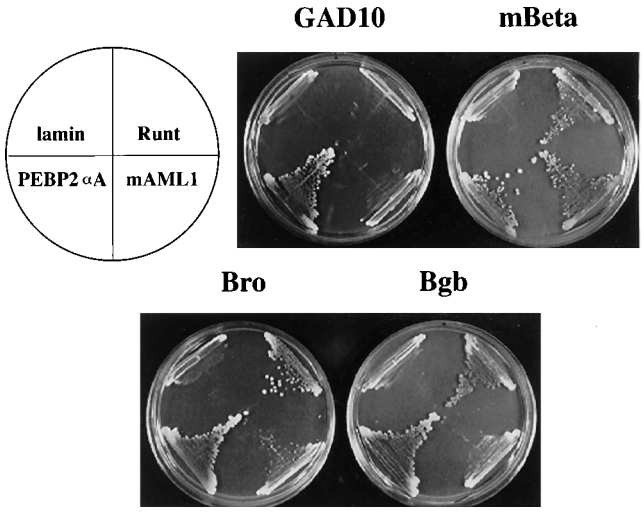

FIG. 1. Interaction of Runt domain proteins with the Bro family of proteins. Plates with streaked-out yeast transformants that contain plasmids expressing different GAL4 DNA-binding domain ($GAL4_D$) and GAL4 activation domain ($GAL4_A$) fusion proteins are shown. The schematic in the top left corner indicates the $GAL4_D$ fusions in the transformants streaked in each quadrant of the four plates. Transformants on each plate contain the same $GAL4_A$ plasmid, as indicated. Transformants were initially plated on medium that selects for the presence of both plasmids (SC −Leu, −Trp). The plates shown here were generated by streaking these cotransformants onto similar plates that detect a two-hybrid protein-protein interaction due to activation of a *HIS3* reporter gene (SC −Leu, −Trp, −His). The growth observed for transformants containing PEBP2αA and GAD10, the activation domain vector, suggests that this Runt domain protein contains an activation domain that functions in yeast cells. The $GAL4_D$/human lamin C fusion is a control for the specificity of the interaction between the Bro-related proteins and Runt domain proteins.

the mBeta fusion and other GAL4 DNA-binding domain fusions (Fig. 1 and data not shown). The interaction between mBeta and PEBP2αA could not be detected in this assay because PEBP2αA gives a positive signal when cotransformed with the activation domain vector pGAD10 (Fig. 1). One interpretation that is consistent with transactivation studies done in mammalian cells (2, 31) is that PEBP2αA contains a transcriptional activation domain. However, from these studies, the failure of mAML1 to activate transcription in yeast cells is somewhat unexpected. Although the relevance of the activities of these proteins in yeast cells to their properties as transcriptional regulators in mammals and flies is unknown, the important practical consequence of these experiments is that *Drosophila* homologs of mBeta could be identified by using the two-hybrid system to screen for Runt-interacting proteins.

The yeast two-hybrid reporter strain was cotransformed with a 0- to 6-h *Drosophila* embryonic cDNA library (courtesy of L. Pick) constructed in the GAL4 activation domain vector pACT (8a) and the plasmid that expresses the GAL4 DNA-binding domain/Runt fusion protein. Among more than 400,000 initial transformants, approximately 400 showed growth above background on selective medium. These colonies were restreaked on plates lacking histidine and then assayed for *lacZ* expression. From this set, 90 independent yeast transformants containing pACT cDNAs that encoded putative Runt-interacting proteins were recovered. Initial sequence analysis indicated that most of these cDNAs were from two related genes that were homologous to mBeta. These genes are herein referred to as *Brother* (Beta for Runt and others, abbreviated *Bro*) and *Big-brother* (*Bgb*). Of the 90 two-hybrid positives, 52 represented five distinct cDNAs from the *Bro* gene and 10 clones represented two distinct cDNAs from *Bgb*. The ability of representative *Bro* and *Bgb* cDNAs to interact with Runt in the two-hybrid selection assay is shown in Fig. 1. As determined by this assay, these two *Drosophila* proteins also interact with the

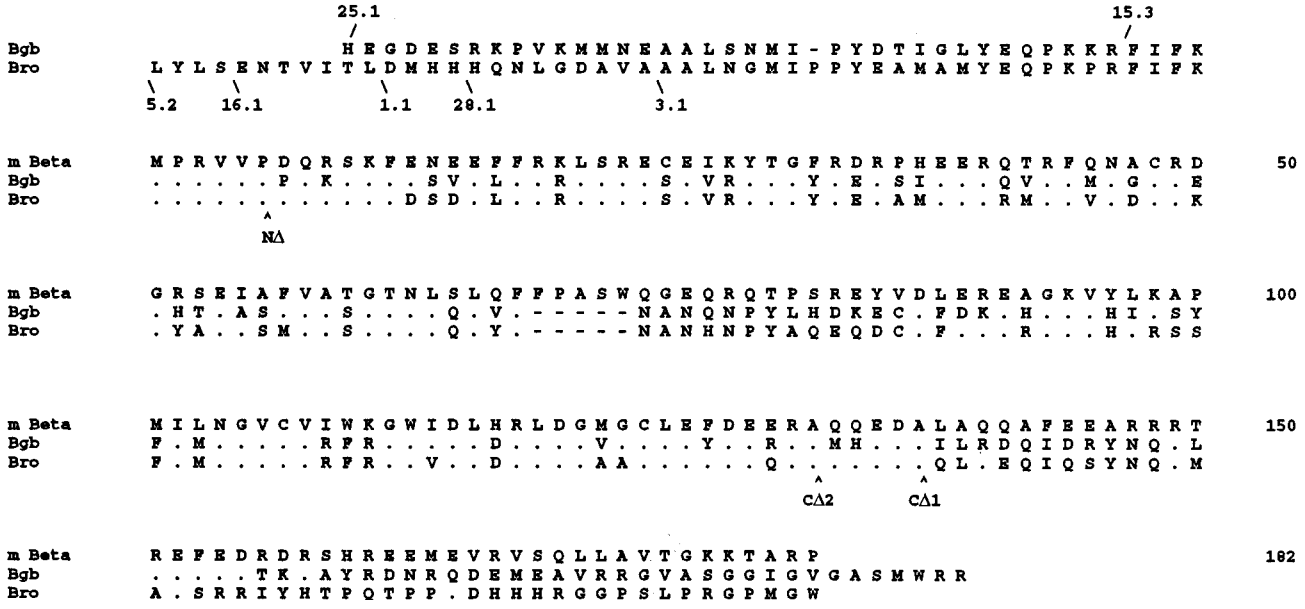

FIG. 2. Sequence comparison of the Bro family of proteins. The deduced sequences of the Bro, Bgb, and mBeta proteins are shown in a three-way alignment. The amino acid numbers shown on the right are for mBeta. Amino acids in Bro and Bgb that are identical to those in mBeta are indicated with a period. In this alignment, mBeta has a five-amino-acid insertion (indicated with dashes) relative to the two *Drosophila* proteins. The starting points of *Drosophila* cDNA isolates are indicated by clone number. Arrows indicate the start and end points of the Bro deletion constructs used for two-hybrid and gel shift assays. Note that the translation initiation site is uncertain for both Bro and Bgb. In both cases, the deduced open reading frames extend upstream of the methionine that aligns with the N terminus of mBeta and there are multiple in-frame methionines.

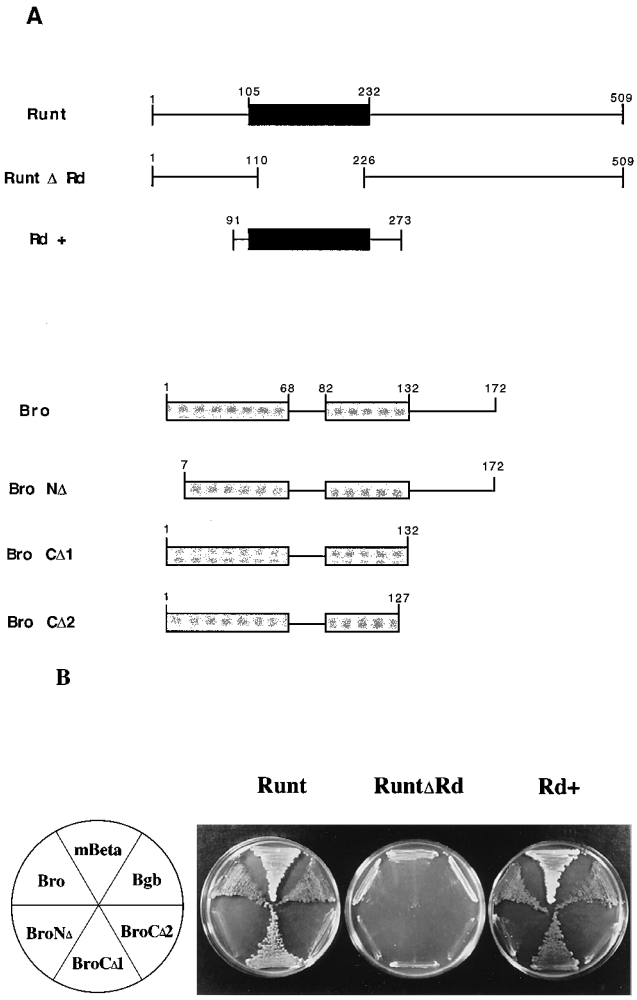

FIG. 3. Regions required for Bro and Runt interaction. (A) Schematic diagram depicting deletion derivatives of Runt and Bro. For Runt, the darkly shaded box indicates the Runt domain. For Bro, the shaded areas denote regions of high homology with both Bgb and mBeta. (B) Two-hybrid assay with Runt and Bro deletion constructs. Bro deletions expressed as $GAL4_A$ fusions were co-transformed into yeast strain Y153 with $GAL4_D$/ Runt constructs. Transformants harboring both plasmids were streaked onto plates that select for activation of a *HIS3* reporter gene. The schematic on the left indicates the location on the plates of transformants containing each of the $GAL4_A$ plasmids. The different $GAL4_D$/ Runt fusion derivatives carried in the yeast transformants are indicated above each plate. Growth was detected for all full-length proteins. The only deletion constructs to show activation of the *HIS3* reporter were Bro(CΔ1) and Rd+.

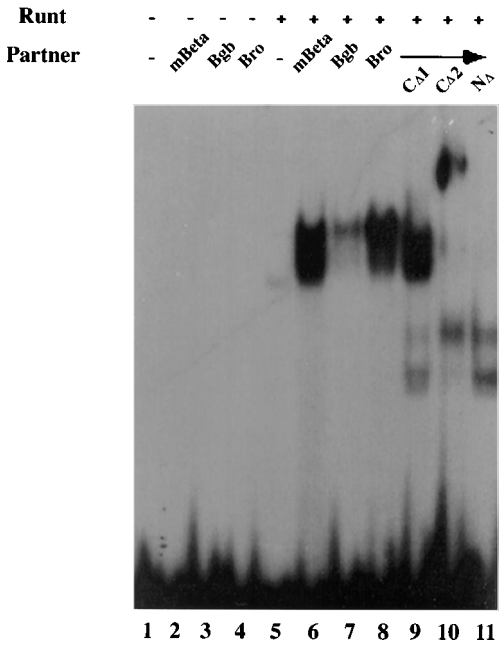

FIG. 4. Bro and Bgb stimulate Runt's ability to bind DNA. Bacterially expressed hexahistidine-tagged proteins were purified by nickel-agarose chromatography and tested for DNA-binding activity in an electrophoretic mobility shift assay under the conditions of Pepling and Gergen (32). The radiolabeled probe was a fragment of the polyomavirus enhancer containing a PEBP2/CBF binding site. Lane 1, on the left, shows free probe. The presence or absence of Runt protein (1 μg) in the binding reaction is indicated above each lane. The inclusion of recombinant mBeta, Bgb, Bro, and Bro deletion derivative proteins (ca. 400 ng each) is also indicated above each lane. The Bro(CΔ2) protein gives a variable and weak stimulation of DNA binding that is not apparent in this experiment. Nonspecific complexes that migrate below the Runt and Runt-Bro complexes are also observed for all three Bro deletion derivatives in this experiment. These background bands, which are not always observed, may be due to contaminating proteins present in these preparations, all three of which contain a relatively low concentration of the Bro protein derivatives.

murine AML1 protein (Fig. 1). This interaction is specific for these Runt domain proteins, as no growth is observed for cells transformed with a control GAL4 DNA-binding domain/lamin fusion protein.

Sequencing of these cDNAs revealed that the *Bro* and *Bgb* transcripts encode open reading frames that are highly homologous to mBeta. Sequence alignment identifies a large block of homology among all three proteins that begins with the initiating methionine of the mBeta protein (Fig. 2). By analogy, it seems likely that this methionine defines the amino terminus of the Bro and Bgb proteins. However, for both genes, the open reading frames extend upstream of this position, and there are additional in-frame methionines. Furthermore, there is significant homology between the deduced protein sequences in this upstream region (18 of 26 identities [see Fig. 2]). These observations strongly suggest that the Bro and Bgb proteins begin upstream of the conserved region. The block of sequence homology between all three proteins extends from amino acids 1 to 137 of the mBeta protein. All three proteins extend C-terminal to this conserved block by approximately 50 amino acids, with some limited homology between Bro and Bgb (nine identities) and mBeta and Bgb (seven identities) but with little to no homology in the three-way alignment. Within the large conserved block, all three proteins are identical at 70 positions (51% identity). There is a single 5-amino-acid insertion in the mBeta sequence that splits the conserved block approximately in half. Much of the variation within the conserved block is adjacent to this insertion, suggesting that the conserved block actually comprises two separable subregions or domains. Database searches revealed no other proteins with obvious homology to Bro and Bgb except for mBeta. Thus, these proteins define a novel family. Although the high degree of homology in the conserved regions of these proteins implies that they will have similar properties, the divergence found at the C terminus and within the center of the conserved block indicates there are also likely to be functional differences.

Dimerization is mediated by conserved regions of the Runt and Brother proteins. The two-hybrid assay was used to localize regions of the Runt and Bro proteins that are required for their interaction. The Runt(ΔRd) protein is an internal deletion mutant that lacks all but the first six and last seven amino acids of the Runt domain but contains the rest of the Runt protein (Fig. 3A). No interaction is detected between this pro-

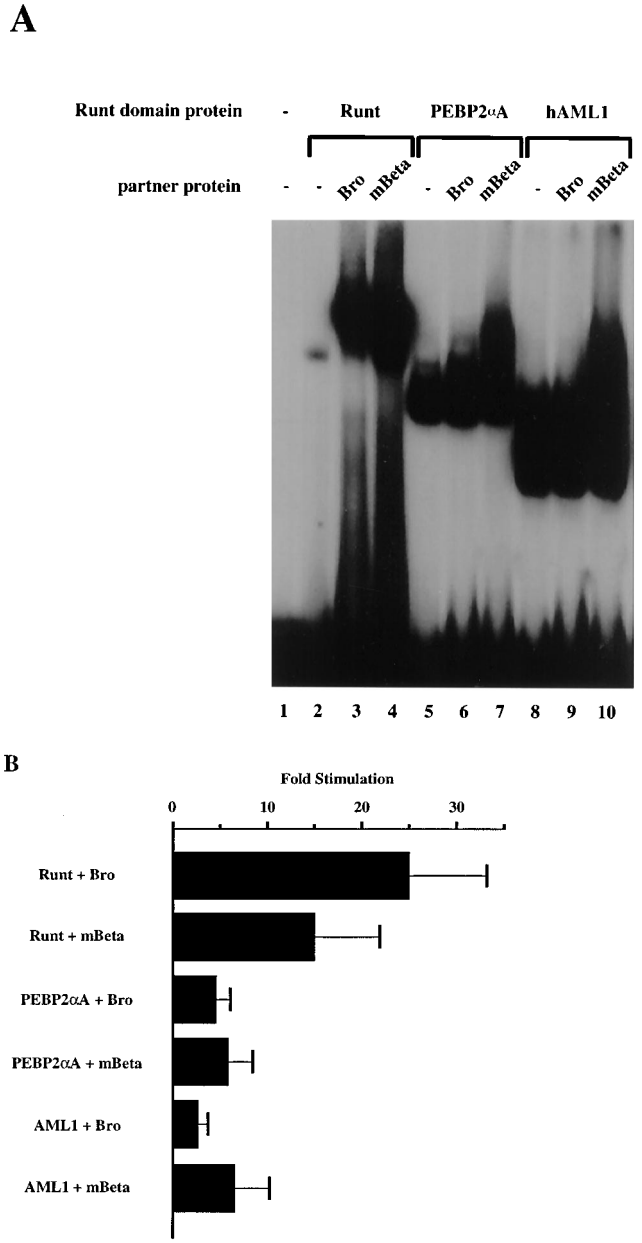

FIG. 5. Conserved stimulation of DNA binding by Bro and mBeta. (A) Assay showing binding of recombinant, full-length Runt domain proteins to the PEBP2/CBF site in the polyomavirus enhancer A element in the absence of a partner (lanes 2, 5, and 8), in the presence of 1 μg of Brother protein (lanes 3, 6, and 9), or in the presence of 1 μg of mBeta (lanes 4, 7, and 10). Lanes 2 to 4, 10 μg of Runt protein; lanes 5 to 7, 10 μg of PEBP2/CBFαA; lanes 8 to 10, 10 μg of human AML1. The mobility of free probe is shown in lane 1. The complexes formed with human AML1 migrate more quickly because the protein isoform used (from cDNA pP6-1 [28]) contains only 250 amino acids. The high concentration of Runt domain proteins used here maximizes the detection of complexes obtained in the absence of a partner protein. This exposure obscures the monomeric Runt complexes obtained in the presence of mBeta but reveals the weak heteromeric PEBP2αA-Bro complex (upper complex in lane 6). (B) Bar graph indicating the fold stimulation of DNA binding of Runt domain proteins by partner proteins. Fold stimulation was measured in three separate experiments, and the average values are shown. Cross bars indicate standard deviation. The binding reactions for these experiments all contained 1 μg of the respective Runt domain proteins and a saturating amount of the respective partner protein (typically 500 ng).

tein and the Bro family in the two-hybrid assay (Fig. 3B). Thus, Runt's interaction with the Bro family of proteins requires an intact Runt domain. Experiments with another deletion derivative, Rd+, strongly suggest that the Runt domain is also sufficient for interaction. This protein, which contains the Runt domain plus 12 N-terminal and 54 C-terminal amino acids, interacts with the Bro proteins in the two-hybrid assay (Fig. 3B). Although this does not exclude the possible involvement of amino acids outside of the Runt domain, the data with Runt(ΔRd) show that these flanking amino acids are not sufficient for interaction. Thus, the Runt domain appears to be both necessary and sufficient for this protein-protein interaction.

Similar experiments reveal that the conserved regions of the Brother protein are required for interaction with the Runt domain. From the lack of sequence conservation, we anticipated that the C-terminal tail of the Bro protein would not be required. This was confirmed by deletion analysis. The deletion derivative Bro(CΔ1), which is truncated precisely at the end of the conserved block (see Fig. 2 and 3A), interacts with the Runt domain in the two-hybrid assay (Fig. 3B). In contrast, no interaction is detected with Bro(CΔ2) (Fig. 3B). This protein lacks the five C-terminal-most amino acids of the conserved block, three of which are identical in mBeta and Bgb (Fig. 2 and 3A). From this and other results shown below, this conserved triplet of amino acids appears to define a functional C-terminal boundary of the Bro protein.

Two observations suggested that the methionine in alignment with the initiator methionine of mBeta would define a functional N-terminal boundary for the Bro protein. First, all of the *Bro* and *Bgb* cDNAs that were isolated in the two-hybrid screen extended upstream of this methionine (Fig. 2). Second, the sequence beginning with this methionine is the most conserved region of these proteins. The first 12 amino acids of mBeta are identical to the corresponding region of Bro, and there are only two substitutions in Bgb, one of which is conservative. Consistent with this, a GAL4 activation domain fusion protein that contains Bro sequences starting at this methionine interacts with Runt (not shown), whereas the construct Bro(NΔ), which lacks the first six amino acids of the conserved region, does not (Fig. 3B). Taken together, these results define a 132-amino-acid segment of the Bro protein that interacts with Runt. The N- and C-terminal boundaries of this segment coincide with boundaries identified by sequence homology with the mBeta and Bgb proteins.

Brother proteins stimulate DNA binding by Runt. The mBeta protein interacts with Runt domain proteins to form a heteromeric DNA-binding complex (20, 32). We used in vitro DNA-binding assays to determine if this is a conserved property of the *Drosophila* Bro and Bgb proteins. Recombinant proteins were expressed as hexahistidine fusions and used in electrophoretic mobility shift assays with a DNA probe that contains a PEBP2/CBF binding site from the polyomavirus enhancer. No binding complexes are detected when the mBeta, Bro, or Bgb protein is used in the absence of Runt (Fig. 4, lanes 2 to 4). As shown previously, the weak binding of Runt to this DNA probe is enhanced by addition of mBeta (Fig. 4, compare lanes 5 and 6). A similar enhancement of DNA binding is also observed when the *Drosophila* Bro and Bgb proteins are added to Runt (Fig. 4, lanes 7 and 8). Thus, these two proteins are functional homologs of mBeta. Truncated derivatives of the Bro protein were tested for their ability to interact with Runt in this assay. The Bro(CΔ1) protein retains the ability to stimulate formation of DNA-binding complexes (Fig. 4, lane 9). Bro(CΔ2), in which an additional five amino acids are deleted from the C terminus, gives lower but detectable

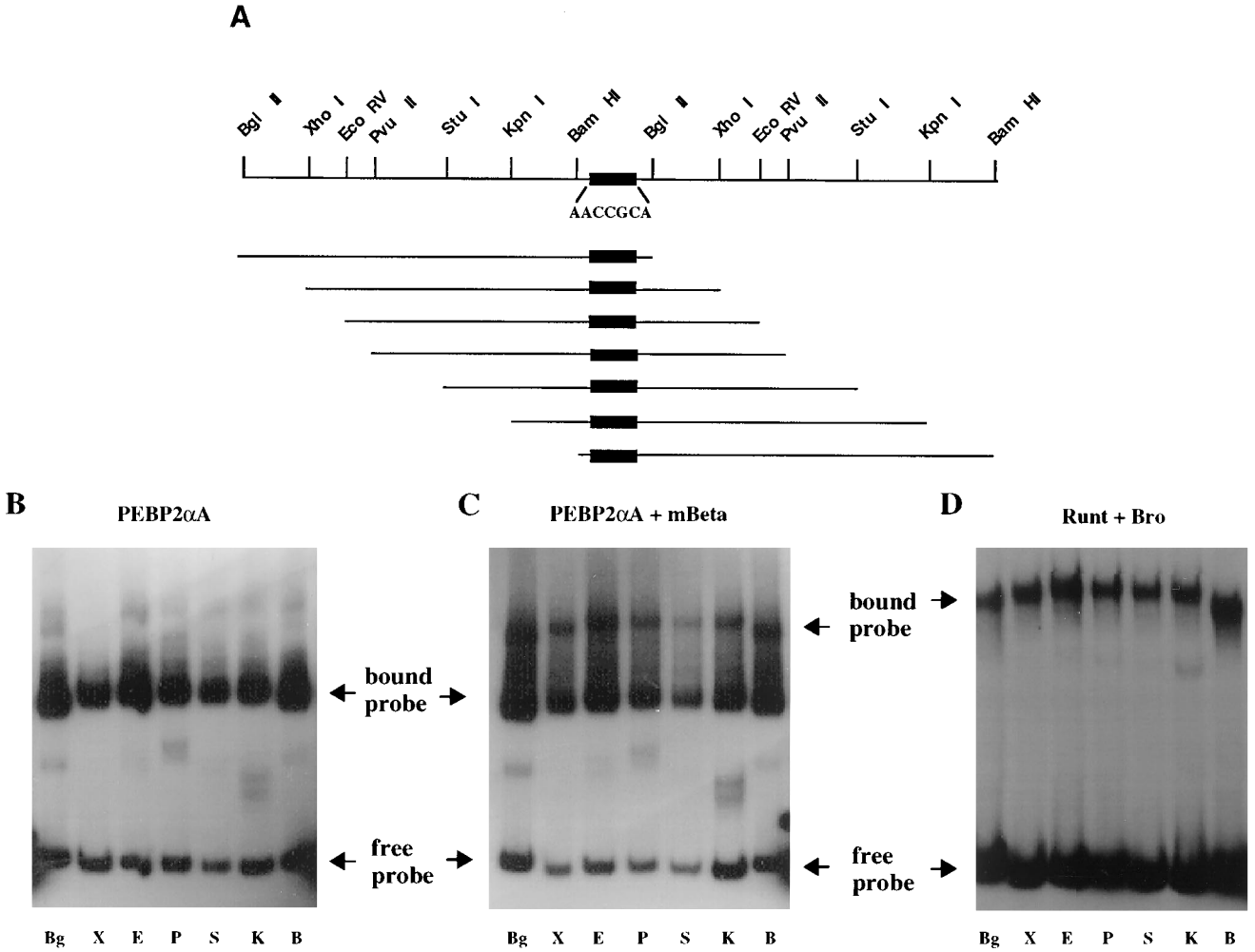

FIG. 6. DNA bending by Runt domain proteins is enhanced by partner proteins. (A) Schematic diagram of the DNA probes used to detect bending. The boxed area indicates the binding site for Runt domain proteins that was cloned into the pBend2 vector. Restriction enzyme cleavage sites are shown. (B, C, and D) DNA-bending assays with PEBP2/CBFαA (αA), αA plus mBeta, and Runt plus Bro proteins, respectively. Restriction enzymes: Bg, *Bgl*II; X, *Xho*I; E, *Eco*RV; P, *Pvu*II; S, *Stu*I; K, *Kpn*I; B, *Bam*HI.

stimulation, and Bro(NΔ) is inactive in this assay (Fig. 4, lanes 10 and 11). These results are in general concordance with the results obtained in the two-hybrid assays and identify a minimal region of Bro that is required for interaction with Runt and stimulation of its DNA-binding activity.

There are several informative differences in the binding complexes obtained with Runt and the different partner proteins. Addition of mBeta leads to stimulation of two complexes. The lower, more rapidly migrating complex is interpreted to contain only Runt, as it comigrates with the complexes formed in the absence of partner protein. Conversely, the upper complex is interpreted to be heteromeric, as it is only observed in the presence of partner protein. Addition of Bro and Bgb leads to the formation of single DNA-binding complexes that approximately comigrate with the putative Runt-mBeta complex. The results obtained with the Bro(CΔ1) protein demonstrate that Runt and Bro form a heteromeric DNA-binding complex. The upper complex formed in the presence of this truncated Bro protein migrates close to but faster than the complex observed with Runt and full-length Bro protein (Fig. 4, compare lanes 8 and 9). This altered mobility is strong evidence that the upper complex contains the Bro protein. Interestingly, Bro(CΔ1) also stimulates formation of the lower Runt monomer complex (similar to the results obtained with mBeta), and the weak stimulation produced by Bro(CΔ2) produces only this lower complex. How do the mBeta, Bro(CΔ1), and Bro(CΔ2) proteins enhance the formation of complexes that contain only Runt protein? One explanation is that dimerization with the partner proteins facilitates the formation of a Runt-DNA complex but that once the complex is formed, the partner protein is free to dissociate. In the context of this explanation, the observation that only the upper, heteromeric complexes are observed with full-length

TABLE 1. DNA bending by Runt domain proteins

Runt domain protein	Partner	Mean bending angle (°) ± SE
PEBP2αA	None	41.6 ± 1.3
	mBeta	55.8 ± 0.9
Runt	Bro	61.0 ± 1.6
	Bgb	63.5 ± 3.0
	mBeta	67.4 ± 0.9

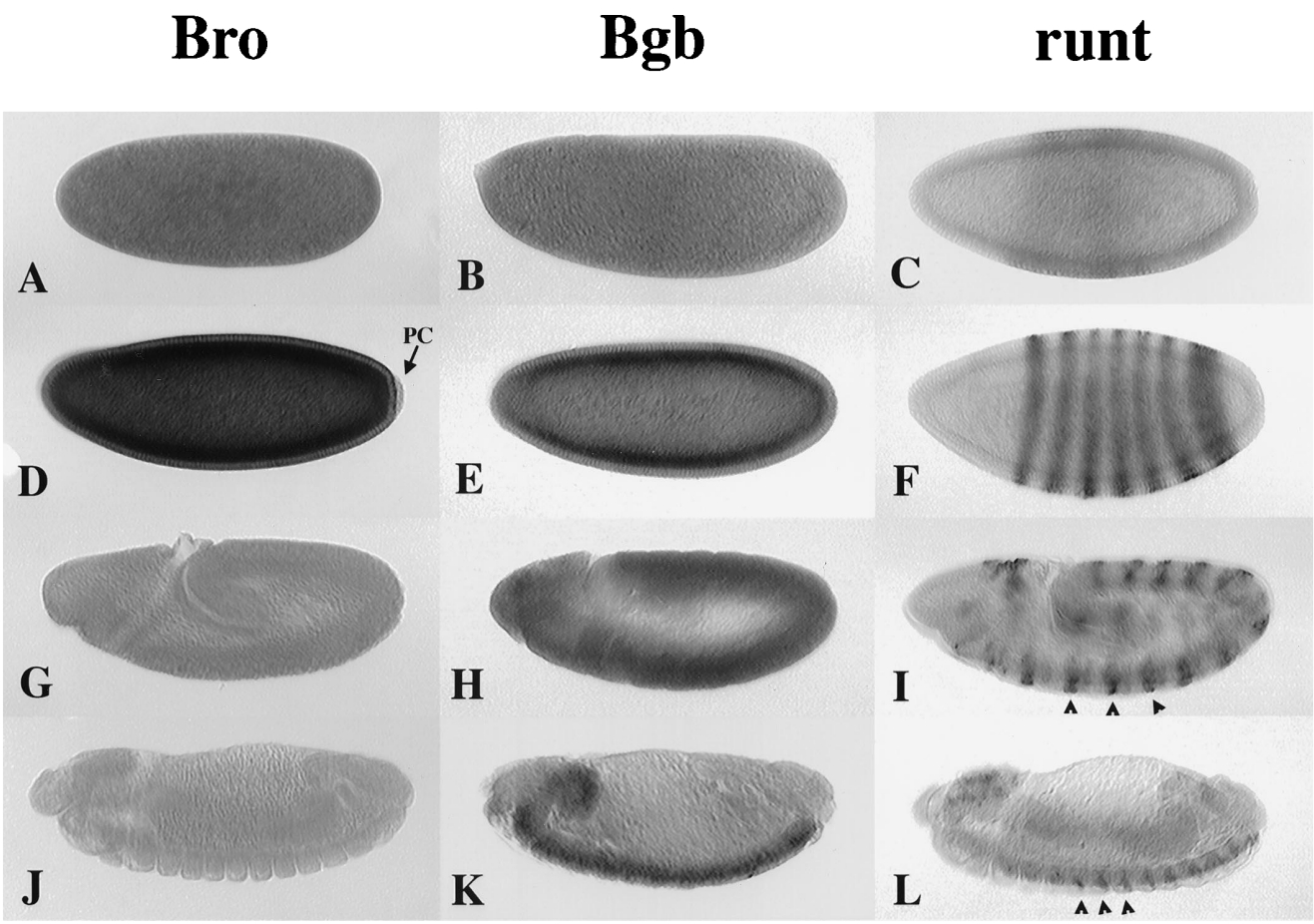

FIG. 7. Localization of Bro and Bgb transcripts. In situ hybridizations were done on embryos with digoxigenin-labeled antisense *Bro*, *Bgb*, and *runt* probes by the method of Klingler and Gergen (24). Embryos are depicted with the anterior to the left and the dorsal side up. The age of the embryos increases from top to bottom. The *Bro* expression pattern (A, D, G, and J) shows a ubiquitous maternal deposition (A) followed by a strong zygotic contribution with transcripts notably absent from the pole cells (PC) (arrow in D). After germband extension, the levels of *Bro* transcript are reduced. *Bgb* expression (B, E, H, and K) is similar up to germband extension (H). At this point, *Bgb* message increases at the periphery and becomes highly expressed in the CNS (K). *runt* expression (C, F, I, and L) does not have a maternal component and is first detected in a broad, central domain in syncitial blastoderm-stage embryos (C). This pattern is later refined to 7 (F) and then 14 stripes with some head expression (I) and at later stages is found in a segmentally repeated pattern in a subset of cells in the CNS (arrowheads in I and L).

Bro and Bgb suggests that Runt associates with these proteins more avidly than with mBeta and the C-terminal Bro deletions. Furthermore, the apparent instability of the Runt-Bro(CΔ1) complex and the absence of a detectable Runt-Bro(CΔ2) complex suggest that the C-terminal tail of Bro contributes to the stability of its interaction with Runt.

The two-hybrid results indicate that mBeta, Bro, and Bgb are all similar enough to interact both with Runt and with mAML1. We used DNA-binding assays to further confirm the functional homology of these interspecific protein-protein interactions. DNA binding by Runt is increased some 20-fold by the Bro and mBeta proteins, with Bro giving a somewhat greater stimulation (Fig. 5). In agreement with previous reports (30, 43), we find that DNA binding by the mammalian Runt domain proteins is enhanced five- to sixfold by addition of mBeta (Fig. 5B). Bro also stimulates DNA binding by mammalian Runt domain proteins but does so less well than mBeta (Fig. 5). This specificity is also revealed by the relative instability of the heteromeric DNA-binding complexes formed between proteins from different species (Fig. 5B). The in vitro assays presented above convincingly demonstrate the functional homology of the Bro and mBeta proteins. These results also emphasize the point that enhanced DNA binding by Runt domain proteins does not require stable association with a Bro-related protein partner.

DNA bending: evidence for a partner protein-dependent conformational change. The mechanism by which the mBeta and Brother partner proteins enhance the DNA-binding activity of Runt domain proteins is not known. For mammalian PEBP2/CBF, addition of partner protein decreases the rate of dissociation from DNA (43). As shown above, the partner proteins do not bind to DNA on their own, suggesting that the change in DNA-binding affinity is not due to interactions between the partner protein and DNA. Stronger evidence for this conclusion comes from the findings that mBeta causes no detectable alteration in the methylation and ethylation interference patterns obtained with mammalian Runt domain proteins (21, 43). Combined, these observations imply that the altered DNA binding observed upon addition of mBeta involves a conformational change in the Runt domain. Such a conformational change in the Runt domain may be associated with a conformational change in its DNA target. To test this idea, we investigated the DNA-bending properties of Runt domain proteins in the presence and absence of protein partners.

The electrophoretic mobility of DNA is reduced by bending, and the effect is greatest when the bend is in the middle of the

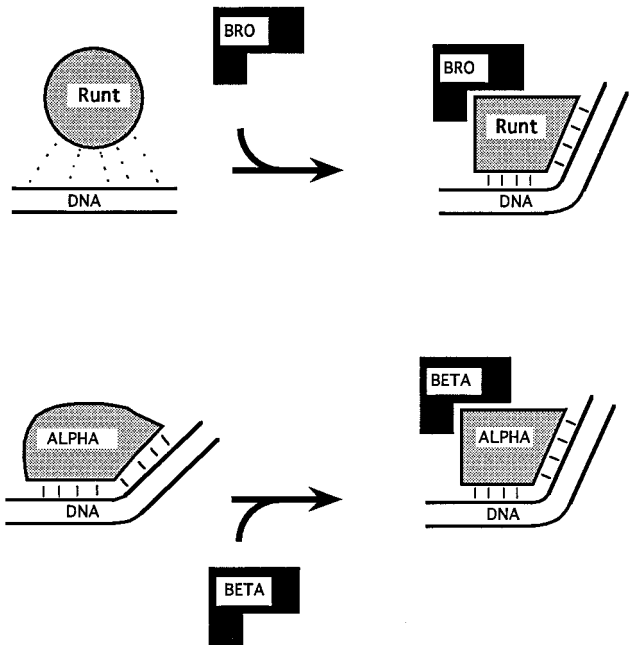

FIG. 8. Conformational model of DNA-Runt-Bro interactions. A hypothesis of the interactions described herein gives a model that stresses induced changes in protein conformation. In this model, Runt protein by itself favors a conformation which binds to DNA poorly. Addition of a Bro family member alters Runt's conformation that is more favorable to DNA binding. This binding is associated with a significant DNA bend. In the case of mammalian Runt domain proteins, their conformation in the absence of a partner protein is permissive for DNA binding with some induced bending. Addition of mBeta is proposed to alter the conformation of mammalian Runt domain proteins so that there is a change in the DNA-bending angle. This alteration is proposed to decrease the rate at which the protein dissociates from the DNA.

DNA fragment (46). Furthermore, the bending angle induced by a specific DNA-binding protein can be calculated by measuring the relative mobility of identically sized DNA fragments that contain the binding site for this protein at different positions. The pBend-2 plasmid vector (23) was used to generate a panel of DNA probes that are the same size and that contain the same but variably positioned PEBP2/CBF binding site. Experiments with this panel of probes indicate that binding of PEBP2/CBFαA is associated with a slight bend in the DNA. Probes with the binding site near the middle of the DNA fragment form complexes that migrate consistently and reproducibly more slowly than probes with the binding site near the end (Fig. 6A). The relative mobilities obtained in several such experiments were used to calculate a bending angle of 41.6° for complexes that contain only PEBP2/CBFαA (Table 1). Similar experiments indicate that the heteromeric complexes formed upon addition of mBeta are associated with a more severe DNA bend (Fig. 6B and Table 1). As there is no evidence that mBeta makes any direct contacts with DNA, these results support the hypothesis that interaction with mBeta causes a conformational change in the Runt domain.

Bending assays were used to investigate interactions between the Runt protein and DNA. The complexes formed with Runt and Bro are associated with a DNA bend of 61° (Fig. 6C and Table 1). This is somewhat greater than that observed for PEBP2/CBFαA and mBeta. Similar if not even slightly greater DNA bends are obtained with Runt-Bgb and Runt-mBeta combinations (Table 1). These results suggest that the three different partner proteins form similar DNA-binding complexes with Runt. Because of Runt's poor ability to bind the PEBP2/CBF site, we were not able to determine a reliable bending angle for Runt in the absence of partner proteins. There are subtle differences in the mobilities of the different DNA probes with Runt. However, unlike the complexes obtained in the presence of partner protein, these relative mobilities do not correlate with the location of the PEBP2 binding site. These results indicate that there are significant differences in the way that Runt interacts with DNA in the presence or absence of Bro and the other related partner proteins.

Brother and Big-brother are widely expressed in embryogenesis. The *runt* gene shows a complex expression pattern during *Drosophila* embryogenesis that correlates well with its regulatory roles in several different developmental pathways. In order to determine which of these regulatory functions might involve *Bro* and *Bgb*, we used in situ hybridization to examine the mRNA expression patterns of these two genes. The first significant accumulation of *runt* transcript is detected as a broad band that spans the presegmental region of syncytial blastoderm-stage embryos (Fig. 7C). This early central domain provides a gap-like activity during segmentation and is also important for the activation of the *Sex-lethal* gene in female embryos (7, 39). There are low, uniform levels of both *Bro* and *Bgb* mRNAs during this stage (Fig. 7A and B). This ubiquitous expression is primarily due to maternal deposition rather than zygotic transcription, because similar levels of accumulation are seen in unfertilized eggs (data not shown). The transcript levels of both genes increase during the blastoderm stage up to the time of cellularization (Fig. 7D and E). This increase, which is presumably due to zygotic transcription, occurs uniformly throughout the embryo with one exception. The pole cells express *Bgb* but contain no detectable levels of *Bro* transcripts. Given the ubiquitous accumulation observed prior to this stage, the generation of this pattern probably involves active elimination of *Bro* transcripts in the pole cells.

The high level of *Bro* and *Bgb* expression during the process of cellularization temporally correlates well with *runt's* peak levels of expression in a pair-rule pattern during segmentation (Fig. 7F). The expression of *Bro* declines following the completion of cellularization and the onset of gastrulation, and only a low level of uniform transcript accumulation is detected during the subsequent stages of embryogenesis. In contrast, *Bgb* transcript levels persist during germband extension (Fig. 7H) and are then increased specifically in the central nervous system (CNS) during germband retraction. During these later stages, *Bgb* is expressed at uniformly high levels throughout the ventral nerve cord and in the procephalic lobes. This expression persists during CNS contraction (Fig. 7K). This aspect of *Bgb* expression pattern overlaps temporally with *runt's* expression in a subset of CNS cells during these embryonic stages (Fig. 7I and L). However, *Bgb* is expressed much more globally than *runt*, which is restricted to a small subset of cells within each hemisegment. The overlaps in the expression patterns of *Bro* and *Bgb* with that of *runt* indicate that Runt's function as a transcriptional regulator is likely to involve interactions with these two partner proteins. Furthermore, the much more widespread expression of these two genes suggests that they also interact with other factors.

DISCUSSION

This work describes two *Drosophila* proteins, Bro and Bgb, that are homologs of mBeta, a subunit of the heteromeric mammalian transcription factor PEBP2/CBF. These three proteins share the ability to interact with and modulate the DNA-binding properties of Runt domain proteins. The regions required for these functions coincide with the conserved areas

identified by aligning the three protein sequences. The homology between the three proteins begins with the methionine that defines the amino terminus of the mBeta protein. Previous findings showed that the first 11 amino acids of mBeta are required for association with PEBP2/CBFα (16). Our results show that removal of the first six amino acids from the corresponding region of Bro renders this protein nonfunctional. Our experiments also provide information on the C-terminal boundary of the region required for Bro protein function. Deletion derivatives of mBeta that extend to position 141 are functional by several criteria (16, 30). Conversely, a CBFβ-myosin heavy-chain fusion protein that contains only amino acids 1 to 133 of mBeta retains only partial activity in vivo and very poor activity in vitro (16). The strong sequence homology of the mammalian and *Drosophila* proteins extends to a position that corresponds to amino acid 137 of mBeta. Consistent with this, we find that Bro(CΔ1), which extends precisely to this position, interacts with Runt in both two-hybrid and DNA-binding assays. The Bro(CΔ2) derivative (which extends to position 132) gives no detectable interaction with Runt in a two-hybrid assay and shows only marginal activity in DNA-binding assays. The Bgb protein contains nonconserved substitutions at positions corresponding to 133 and 134 of mBeta. This suggests that the reduced activity of Bro(CΔ2) is due to removal of the amino acids corresponding to positions 135 to 137, a triplet of amino acids that is identical in all three proteins.

Our results reveal several conserved in vitro properties of the Bro family of proteins. Bro and mBeta both stimulate the DNA-binding activity of Runt approximately 20-fold, although Bro is slightly more effective. Similar 60° to 65° bends in DNA are produced by Runt in the presence of each of the three partner proteins that we tested. The one consistent difference in the activity of these proteins involves the stability of the heteromeric complexes formed with different Runt domain proteins. Not surprisingly, the interspecific complexes formed between mammalian and *Drosophila* proteins are less stable than the complexes formed when the two proteins are from the same species. There are several regions where the Bro and Bgb proteins are similar to each other and different from mBeta that could account for this difference. Within the highly conserved region, there are 26 amino acid identities between Bro and Bgb that are different in mBeta. Also, 6 of the 11 amino acids just C-terminal to the conserved region are identical in Bro and Bgb and different in mBeta. Although further studies are required to define the regions responsible for stabilizing the interaction with Runt, our results indicate that the C-terminal tail is important. Bro protein deletion derivatives that are truncated at the C terminus retain the ability to stimulate Runt's DNA-binding activity but form relatively unstable heteromeric complexes.

Bro proteins influence the DNA-binding properties of the Runt domain. Exactly how the DNA-binding affinity of Runt domain-containing proteins is increased by addition of a partner remains unclear. Like mBeta, the Bro and Bgb proteins do not show appreciable DNA-binding activity on their own. The methylation and ethylation interference patterns seen with mammalian Runt domain proteins are not altered by the addition of mBeta (21, 43). This suggests that the heteromeric complex makes substantially the same DNA contacts as are made by the Runt domain alone. The relatively weak DNA-binding activity of Runt in the absence of a partner protein makes it difficult to determine if the addition of Bro affects the pattern of contacts with DNA. However, other results suggest that the enhanced formation of DNA-protein complexes does not involve an interaction between Bro and DNA. One indication of this is the stimulation of the Runt monomeric complex by the Bro(CΔ1) and Bro(CΔ2) proteins. This indicates that Bro alters the DNA-binding properties of Runt by a mechanism that does not require its continued presence in a DNA-protein complex.

There are several other instances in which interaction between two proteins results in the enhanced formation of DNA-protein complexes. Examples include the effect of the Phox homeodomain protein on DNA binding by SRF (15), the effect of *Drosophila* Extradenticle on Ultrabithorax (5), and a cooperative DNA-binding interaction between PEBP2αA and Ets-1 (45). Significantly, these cases all involve interactions between proteins that have some DNA-binding ability on their own. In contrast, the Bro-related proteins possess no detectable intrinsic DNA-binding activity. There are some similarities between the Bro proteins and the β subunit of the heteromeric transcription factor GABP. This protein does not bind to DNA in the absence of the Ets domain-containing GABPα subunit. However, an important distinction is that GABPβ, when complexed with GABPα, is thought to make contacts with DNA (37). There are also parallels between the Bro proteins and the Tax protein of human T-cell leukemia virus type 1. This viral protein does not bind DNA efficiently but instead appears to regulate transcription by increasing the DNA-binding affinity of several factors (12, 29), including, for example, CREB, SRF, Fos-Jun, and NF-κB (1, 47).

One way in which a non-DNA-binding protein such as Bro could modulate the DNA-binding affinity of another protein would be to alter the conformation of the DNA-binding domain. Our findings that PEBP2αA bends DNA and that the bending angle is increased by addition of mBeta provide strong support for this idea. A model based on conformational changes can account for many of the in vitro properties of different Runt domain proteins (Fig. 8). In this model, the Runt domain, in the absence of a partner protein, favors a conformation that is suboptimal for DNA binding. The addition of mBeta to PEBP2αA is proposed to stabilize an altered conformation that has a moderate increase in affinity for DNA and that is associated with an increase in the bending angle. The effect of the partner protein on the DNA-binding affinity of Runt is more pronounced. The weak binding obtained in the absence of a partner protein is not associated with a measurable bend in the DNA. In contrast, the bending angle obtained with Runt in the presence of any of the three partner proteins is more extreme than that produced by combining PEBP2αA and mBeta. In the context of this model, we predict that the partner protein-induced conformational changes in Runt will be more substantial than for PEBP2αA.

Implications of the Bro proteins for Runt's function as a transcriptional regulator. Runt's activity as a transcriptional regulator is likely to involve the DNA-binding function of the evolutionarily conserved Runt domain. Indeed, a region encompassing the Runt domain is conserved with near identity in *Drosophila* species that diverged from *D. melanogaster* some 40 million years ago (32). Consistent with this, recent studies in this laboratory have identified DNA-binding sites for Runt in *cis*-regulatory elements of the *hairy* and *Sex-lethal* genes that are thought to be direct targets of *runt* (15a). Even on these putative natural targets, DNA binding by Runt is strongly dependent on Bro or a related partner protein. The finding that Runt is a DNA-bending protein has important implications for understanding how Runt and other factors may interact to regulate the activity of these *cis* elements. One well-characterized example of a eukaryotic *cis* element that is regulated by DNA-bending proteins is the minimal enhancer of the TCRα gene. The related HMG domain proteins

TCF-1α and LEF-1 bind to a site in center of this element that is required for transcriptional activation (38, 44). The bend in the DNA helix caused by these proteins appears to facilitate interactions between proteins bound to an ATF/CREB site on one side of the bend and to an Ets-1 site on the other side of the bend (13, 14). These studies have led to the view that the central function of the TCF-1α and LEF-1 proteins is architectural and involves assembling a higher-order enhancer complex that contains other transcriptional regulators. It is intriguing that PEBP2/CBF is also implicated as a regulator of the TCRα gene enhancer. Two adjacent PEBP2/CBF binding sites are located between the TCF-1α/LEF-1 site and the Ets-1 site. Furthermore, cooperative binding to this minimal enhancer is observed with recombinant PEBP2αA and Ets-1 proteins (14). Although the 130° DNA bend associated with LEF-1 binding is more extreme, it seems likely that the bend associated with the binding of PEBP2/CBF to its two sites will contribute to the overall architecture and thus the activity of this enhancer. Further experiments that investigate how the relative spacing of binding sites affects the activity of this enhancer, as well as similar studies on Runt-responsive *cis* elements in *Drosophila* cells, should reveal the functional significance of DNA bending by Runt domain proteins.

The above discussion considers the role of the Bro proteins with respect to Runt's activity as a DNA-binding transcription factor. However, the regulatory effects of *runt* on some genes appear to be mediated by mechanisms that do not involve DNA binding. One example is the antagonistic effect that overexpression of Runt has on activation by the Bicoid (Bcd) protein. This effect is observed in transgenic *Drosophila* embryos that carry a *lacZ* reporter gene with multimerized Bcd binding sites (39). A second example is the activation of a *lacZ* reporter construct containing multimerized binding sites for the orphan nuclear receptor proteins FTZ-F1 and DHR39 (40). There is no evidence that Runt can bind to the regulatory elements that mediate these two opposing regulatory effects. Thus, these effects have been interpreted to be due to protein-protein interactions. In principle, an interaction between Runt and Bro could account for either of these effects. For example, if Bro is an obligatory cofactor for Bcd, then overexpression of Runt would reduce Bcd activity by titrating Bro away. Alternatively, if Bro had a negative effect on transcriptional activation by FTZ-F1, then titration by Runt could lead to activation.

In vivo functions of the Bro proteins. The mammalian Runt domain proteins were copurified with mBeta as subunits of the PEBP2/CBF transcription factor, providing evidence that these proteins interact with each other in vivo (30, 31, 43). In addition, the human Runt domain gene *AML1* and the human homolog of the gene for the mBeta protein are both frequent targets of chromosomal rearrangements associated with acute myeloid leukemia (26, 28), further suggesting a functional connection between the activity of these two genes. A complicating observation is the cytoplasmic localization of the mBeta protein in NIH 3T3 cells transfected with an expression construct. This unexpected subcellular localization is observed even when the cells are cotransfected with a PEBP2αA expression construct (27). If the primary reason for association of these two proteins is to form a heteromeric DNA-binding factor, it would seem imperative that they colocalize to the nucleus. Interestingly, mBeta is found in the nucleus when coexpressed with truncated derivatives of PEBP2αA (27). These observations suggest that the formation and nuclear translocation of PEBP2/CBF heterodimers are regulated. Regulation at this level could provide an important mechanism for coordinating the activity of PEBP2/CBF with other cellular signaling pathways.

It is notable that *D. melanogaster* contains two genes homologous to mBeta, whereas only one gene is known in mammals. While it is possible that Bro and Bgb simply provide redundant functions, their divergent expression patterns at later stages of development suggest that this may not be the case. The divergence in the protein sequences, especially in the C-terminal regions, further suggests that there will be some functional differences in the activity of these proteins. Although only a single gene for mBeta has been found in both mice and humans, there are several different alternatively spliced mRNA transcripts (30, 43). Two of the isoforms characterized to date retain the ability to interact with Runt domain proteins and have differences in their respective nonconserved C-terminal regions. Two other spliced variants have deletions within the conserved region and produce proteins that do not interact with the Runt domain (30, 43). There are also differences in the expression patterns of these isoforms. The two isoforms that contain intact conserved regions seem to be widely, if not ubiquitously, expressed, although at slightly different levels. Conversely, only a subset of cell lines express the variant form of mBeta that lacks exon 5 (corresponding to amino acids 134 to 166 in the mBeta sequence in Fig. 2) (30). It will be interesting to determine the functional relevance of these differences in protein structure and expression.

A final issue regarding the Bro-related proteins is whether their functions are restricted to interactions with Runt domain proteins. This question is raised by another evolutionarily conserved feature of this protein family, namely, that Bro and Bgb (like mBeta) are widely expressed. This pattern contrasts with the highly regulated expression of *runt* during *Drosophila* embryogenesis (22, 24). This difference strongly suggests that the Bro proteins are likely to interact with other factors. The molecular identification and characterization of the *Bro* and *Bgb* genes presented here provide a starting point for the initiation of a genetic analysis of the function of these proteins. Ongoing experiments in this direction are aimed at elucidating the role that these proteins have in *Drosophila* development. These studies should provide important insights on the functional significance of the interactions between the Bro proteins and Runt. From the expression patterns, we believe that they are likely to have an even broader significance. The rich developmental genetic framework available in *D. melanogaster* makes it an attractive system for future studies on the structure and function of the Bro family of proteins.

ACKNOWLEDGMENTS

The clones for PEBP2αA, mAML1, and mBeta that were provided by Yoshiaki Ito were invaluable to the early phases of this work. We are also extremely indebted to Leslie Pick for graciously providing the *Drosophila* embryo cDNA library in the yeast GAL4 activation domain vector. The eager assistance with the yeast work that we received from the Sternglanz and Fields labs, especially from Paul Bartel, is greatly appreciated. Frosoulla Reagan provided excellent technical assistance. Tom Triolo helped to construct the Runt domain deletion construct. J.P.G. thanks Marty Freundlich for encouraging the DNA-bending studies. Wen-Chen Chang assisted with the analysis of the bending data. Comments from Ben Aronson, Bernadette Holdener, Nancy Speck, and Sid Strickland helped to improve the manuscript.

This research was funded by grants from the NIH (GM5322909) and the International Human Frontier Science Program (RG35794M) to J.P.G. G.G. is a recipient of a Hoffmann-La Roche-sponsored Predoctoral Fellowship from the Institute for Cell and Developmental Biology. J.P.G. is a recipient of a Faculty Research Award from the American Cancer Society (FRA428).

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