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The scs' Boundary Element: Characterization of Boundary Element-Associated Factors

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Boundary elements are thought to define the peripheries of chromatin domains and to restrict enhancer-promoter interactions to their target genes within their domains. We previously characterized a cDNA encoding the BEAF-32A protein (32A), which binds with high affinity to the scs' boundary element from the *Drosophila melanogaster* 87A7 *hsp70* locus. Here, we report a second protein, BEAF-32B, that differs from 32A only in its amino terminus. Unlike 32A, it has the same DNA binding specificity as the complete BEAF activity affinity purified from *Drosophila*. We characterize three domains in these proteins. Heterocomplex formation is mediated by their identical carboxy-terminal domains, and DNA binding is mediated by their unique amino-terminal domains. The identical middle domains of 32A and 32B are dispensable for the functions described here, although they may be important for boundary element function. 32A and 32B apparently form trimers, and the ratio of 32A to 32B varies at different loci on polytene chromosomes as judged by immunofluorescence. The scs' element contains a high- and low-affinity binding site for BEAF. We observed that interaction with the low-affinity site is facilitated by binding to the high-affinity site some 200 bp distant.

Much evidence suggests that chromatin is organized into independent functional units called domains (reviewed in reference 37). Functional independence requires restricting the activity of DNA elements such as enhancers to the domain in which they reside. For instance, proper temporal, developmental, and tissue-specific gene expression requires that enhancers activate only their target promoters, which might be tens of kilobases away, despite their ability to activate diverse promoters (22). Domains are presumably separated by special nucleoprotein structures, or boundary elements, that restrict interactions between DNA elements located in different domains (reviewed in references 7 and 38). Boundary elements, also called insulators, are likely to accomplish this through effects on higher-order chromatin structure, nuclear organization, or both. Boundary elements should be identifiable by two *in vivo* assays. First, when bracketing a transgene, they should shield the transgene from position effects caused by integration into different chromosomal environments (position-independent expression); and second, activation of a promoter by an enhancer should be blocked by an intervening boundary element since the enhancer and promoter should then be in separate domains (enhancer blocking). In addition, they should not act as enhancers and they should localize to domain edges.

The best-characterized putative domain boundary elements are from *Drosophila melanogaster*. These are the scs and scs' special chromatin structures found at the proximal and distal boundaries of the 87A7 *hsp70* puff of polytene chromosomes (8, 35) and a 340-bp fragment from the gypsy retrotransposon (12). Bracketing a gene by scs and scs' leads to position-independent expression of that gene, while enhancer blocking is observed when they are located between an enhancer and promoter (20, 21, 36). Enhancer blocking is not observed when scs or scs' is located immediately upstream of the enhancer and promoter. Consistent with the model in which boundary ele-

ments separate domains of genetic function, enhancer blocking does not inactivate either the promoter or the enhancer. The promoter can still respond to other, unblocked enhancers (21), and the blocked enhancer can still mediate the activation of a divergently transcribed promoter lacking an intervening scs element (3).

Like scs and scs', the gypsy-derived element confers position-independent expression on a bracketed reporter gene (31) and blocks enhancer action when it is located between the enhancer and promoter without inactivating the promoter (12) or enhancer (3, 33). It has been additionally shown that when located in an intron, this element insulates the promoter from downstream enhancers without interfering with transcription (12). Binding of the zinc finger protein su(Hw) to the 12 binding sites in the gypsy fragment mediates boundary activity (31). Interestingly, mutations in the enhancer of position-effect variegation protein mod(mdg4) lead to bidirectional silencing by su(Hw) (i.e., rather than blocking, the gypsy-derived element leads to inactivation of the enhancer, promoter, or both, even when it is not located between the enhancer and promoter [11]), as well as allowing repression of enhancer-promoter interactions in the paired gene of the homologous chromosome (10). Since mod(mdg4) and su(Hw) interact *in vitro* (11), mod(mdg4) might directly influence the association of su(Hw) with itself or other components. This might localize su(Hw) action by limiting spreading of the complex and preventing su(Hw)-mediated transvective pairing. It is of interest to know if mod(mdg4) mutations affect scs or scs' function.

Despite the similarities between the gypsy-derived element and scs and scs', there are some important differences. In particular, su(Hw) blocks enhancers in transient-expression assays (16) while scs' does not (39). Thus, it is likely that scs' requires a special chromatin structure or nuclear compartmentalization not necessary for su(Hw) function. Another difference is that scs and scs' localize to the edges of the 87A7 heat shock domain and presumably limit the heat shock response there to the *hsp70* genes; thus, they might represent prototype elements used to define boundaries of many normal domains. On the other hand, su(Hw)-mediated boundary activity has

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been shown to occur only in conjunction with the clustered binding sites from near the 5' long terminal repeat of the gypsy retrotransposon and thus may be a special situation. Also, evidence argues against mediation of *scs* or *scs'* boundary activity by clustered binding sites for a single protein. Indeed, as described below, an *scs'*-specific binding activity has been identified, but reiterated binding sites for this protein are not sufficient for full boundary activity. How these elements physically accomplish their function is unknown.

We identified a DNA binding activity in *Drosophila* nuclear extracts with a high affinity for the *scs'* boundary element (boundary element-associated factor [BEAF]), which led to the cloning of a cDNA encoding BEAF-32A (BEAF with a molecular mass of 32 kDa [39]). Consistent with a role in boundary element function, the distribution of BEAF binding sites coincided with the previously mapped chromatin structure of *scs'* (35). Also consistent with a role in boundary element function, either a 200-bp *scs'* fragment containing a high-affinity BEAF binding site or seven tandem repeats of a 48-bp oligonucleotide with this high-affinity binding site partially blocked an enhancer in stably transfected cells, suggesting that BEAF partially accounts for boundary activity. BEAF was immunolocalized to numerous interbands and puff boundaries on polytene chromosomes, suggesting that a chromosomal domain is represented on polytene chromosomes by a band plus part of the adjacent interbands and that BEAF-associated boundaries are commonly used.

Our subsequent finding that BEAF and BEAF-32A have different DNA binding specificities despite being antigenically related led us to screen for another cDNA encoding a related protein. Here we report the cloning of a cDNA encoding a protein, BEAF-32B, that has a similar DNA binding specificity to that of BEAF. We characterize domains in BEAF-32A and BEAF-32B, which differ only in their amino termini, demonstrate that they associate, and show they mostly colocalize on polytene chromosomes. We additionally show that binding to a high-affinity site facilitates the interaction of BEAF with a low-affinity binding site located 200 bp away.

MATERIALS AND METHODS

Band shift and DNase I footprinting assays. Band shift assays were performed as previously described, with empirically determined amounts of protein (39). Binding reactions for DNase I footprinting were similar, except that the probe DNA was labeled on only one end. After treatment with approximately 20 ng of DNase I (Sigma) for 90 s at room temperature, the products were resolved on 6 or 8% sequencing gels, dried, and exposed to an X-ray film at -70°C .

Isolation of the BEAF-32B cDNA. A PCR-generated 0.8-kb fragment of the BEAF-32A cDNA (39) was used to screen a cDNA library from *Drosophila* embryos (provided by M. Goldschmidt-Clermont). Seventeen positive clones, all of which encoded BEAF-32B, were isolated from 10^5 plaques. The cDNA was subcloned into the *EcoRI* site of pBSKS(−) to generate pBS-32B, and both strands were sequenced with the T7 sequencing kit from Pharmacia. Southern blot analysis of *Drosophila* genomic DNA isolated from KC cells with probes specific for the unique N-terminal sequence of 32A (200-bp *NsiI*-*Bam*HI restriction fragment) or 32B (PCR-amplified fragment of the coding sequences for the first 80 amino acids) was done by standard methods.

Proteins and expression vectors. BEAF was affinity purified from *Drosophila* tissue culture cell line KC 161 nuclear extracts as described previously (39).

Oligonucleotide 5'CAGCATATGCCCAAGGGTCGTGT and the T7 primer were used to amplify the coding sequence of BEAF-32B cDNA. The fragment was digested with *NdeI* and *EcoRI* and cloned into the corresponding sites of the pET-3b(NSEB) T7 expression vector (39), and the protein was expressed as described by Studier et al. (34). BEAF-32B was partially purified by phosphocellulose chromatography before use.

Plasmids expressing N-terminally or C-terminally truncated 32A and 32B were constructed by amplifying the corresponding cDNA fragments by PCR and inserting them into the proper sites of pET-3b(NSEB). pET-32AΔ82/204 and pET-32BΔ81/203 were constructed by inserting the corresponding N-terminal domain into pET-3b(NSEB) to generate pET-32A81E and pET-32B80E, respectively, followed by insertion of the C-terminal domain into these constructs. Expressed proteins were partially purified by phosphocellulose chromatography before use.

Antibodies. Rabbit antibodies were raised against 32A(1–67) or 32B(1–75) as described previously (1). Mouse antibodies were raised against the same proteins essentially as described by Harlow and Lane (15). Anti-32B antibodies were affinity purified by a filter method (1). Anti-32A antibodies were purified with 32A143 coupled to Affi-Gel 10 (Bio-Rad).

Immunoprecipitation. Protein A-Sepharose beads (5 mg) were swollen in phosphate-buffered saline (PBS). A 20- μl volume of affinity-purified anti-32A or anti-32B antibodies was incubated with the beads in 100 μl of PBS for 1 h at room temperature with rotation. After being washed three times for 5 min each with 100 μl of PBS followed by 5 min with 100 μl of NEB200 (10 mM HEPES [pH 7.6], 0.2 M KCl, 3 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 0.2% Trasyolol, 10% glycerol), the beads were incubated with 30 μl of KC nuclear extract for 2 h at 4°C with rotation. The beads were washed three times for 5 min each with 100 μl of NEB200, and the bound proteins were eluted with 30 μl of 2 $\times$ sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. The samples were split into two parts, resolved by SDS-PAGE, blotted onto nitrocellulose membranes, and detected with anti-32A and anti-32B antibodies by using an enhanced chemiluminescence kit (Amersham).

Immunostaining of polytene chromosomes. Polytene chromosomes from late-third-instar larvae were prepared as described by Zhao et al. (39). As the primary antibody, affinity-purified rabbit anti-32A antibodies were used at a 1:4 dilution and mouse anti-32B antibodies were used at a 1:2 dilution. Dilutions (1:200) of rhodamine-conjugated goat anti-rabbit or fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibodies were used. The chromosome preparations were viewed and photographed through a Bio-Rad MRC 600 confocal microscope.

In vitro translation. In vitro translation was performed with the Promega TNT coupled reticulocyte lysate system as specified by the manufacturer. After translation, 1 μl of the solution was used for gel mobility shift assays and 5 μl of the ³⁵S-labeled solution was used for immunoprecipitations.

Chemical cross-linking and glycerol gradient ultracentrifugation. A 7.5- μg portion of KC nuclear extract or 1 optical density unit of KC nuclei was incubated at room temperature in 100 μl of NEB100 with 10 μg of dithio-bis(succinimidyl propionate) (Pierce) per ml for various times up to 30 min. Reactions were stopped by addition of trichloroacetic acid to 25%, and the precipitated samples were analyzed by SDS-PAGE and Western blotting as for immunoprecipitations.

KC nuclear extract (100 μl), together with molecular weight markers, was dialyzed into NEB200 (without glycerol) and loaded onto 5-ml gradients of 5 to 20% glycerol in NEB200. The samples were centrifuged for 14 h at $22,000 \times g$ at 4°C . Fractions (0.2 ml) were collected from the gradient bottom and analyzed by SDS-PAGE and Western blotting as for immunoprecipitations.

Two-hybrid assay. The two-hybrid assay was performed with plasmids, yeast strains, and protocols derived from the Clontech Matchmaker kit. Plasmids were created by inserting PCR-amplified fragments into the appropriate vector. Bait constructs utilizing the GAL4 DNA binding domain were derivatives of the 2 μm TRP1 plasmid pGBT9 (2). Target constructs utilizing the GAL4 activation domain were derivatives of the 2 μm LEU2 plasmid pGAD424 (2).

Plasmids were transformed into yeast strain Y190 by the lithium acetate method (13). Bait fusion proteins containing an N-terminal GAL4 DNA binding domain and target fusion proteins containing an N-terminal GAL4 activation domain were produced constitutively under the control of the *ADHI* promoter. Cotransformants were selected on minimal medium lacking tryptophan and leucine. After incubation for 3 days at 30°C , a single colony was picked and streaked onto a plate of minimal medium additionally lacking histidine to select for colonies in which interactions between the bait and target proteins activated *HIS3*. To assay for colonies in which interactions between the bait and target proteins activated *lacZ*, a single colony was streaked onto a plate of minimal medium lacking tryptophan and leucine and incubated for 2 days. The color reaction was then performed by the recommended filter-lifting assay.

Nucleotide sequence accession number. The EMBL accession number for the complete BEAF-32B cDNA sequence is Y09475.

RESULTS

Isolation of the BEAF-32B cDNA. We previously reported the purification from *Drosophila* nuclear extracts of a protein activity called BEAF, which binds with high affinity to a palindromic sequence of the *scs'* boundary element, and we presented evidence that this activity accounts, at least in part, for the *scs'* boundary function (39). A cDNA was cloned encoding a novel protein, BEAF-32, that was shown by biochemical and immunological criteria to correspond to one of the *Drosophila* BEAF proteins. Although our studies indicated a close relationship between the *Drosophila* BEAF activity (called BEAF) and the bacterially expressed BEAF-32 protein, we also noted important differences. In particular, unlike BEAF, cloned BEAF-32 was observed to interact with a mutant D probe

FIG. 1. DNase I footprints show that *Drosophila* BEAF and 32B have similar interactions that differ from those of 32A. After treatment with DNase I, radiolabelled scs' D fragment DNA was separated on denaturing polyacrylamide gels and visualized by autoradiography. The same strand is labelled in all panels, and the CGATA motifs (vertical arrows) and DNase I-hypersensitive sites (HS) are indicated. (A) DNase I footprints in the presence of increasing amounts of affinity-purified, bacterially expressed 32A (lanes 3 to 5) or affinity-purified *Drosophila* BEAF (lanes 7 to 9). Lane 6, no protein. Lanes 1 and 2 show G and G+A sequencing reaction products, respectively. (B) DNase I footprints in the presence of increasing amounts of bacterially expressed 32B partially purified by phosphocellulose chromatography (lanes 3 to 5). Lane 2, no protein. Lane 1, G+A sequencing reaction products. (C) DNase I footprints in the presence of increasing amounts of the 80 N-terminal amino acids of 32B (lanes 2 and 3). The bacterially expressed truncated protein was partially purified by phosphocellulose chromatography. Lane 4, no protein. Lane 1, G+A reaction products.

containing point mutations in the palindrome (see below). This difference in DNA binding specificity was confirmed by DNase I footprinting (Fig. 1A). BEAF gives a major footprint over the CGATA palindrome and over the single CGATA sequence located 18 bp upstream. This pattern includes major hypersensitive sites bracketing the palindrome; the one between the single and palindromic CGATA sequences is particularly noteworthy for its strength. In contrast, the footprint of bacterially expressed BEAF-32 includes the single CGATA and a sequence downstream of the palindrome but does not encompass the palindrome and lacks hypersensitive sites. A number of footprinting experiments suggest that the main recognition motif of BEAF-32 is a direct repeat of TCACG (15a), with 52 bp between repeats in the footprint shown in Fig. 1. The region with which both BEAF and BEAF-32 interact has the two recognition motifs merged into the sequence TCACGATA.

The difference in binding specificities of BEAF and BEAF-32 prompted us to search for a cDNA encoding further BEAF-related proteins. For this purpose, a *Drosophila* cDNA embryo library was screened with the same PCR-amplified probe described previously (39). A number of identical clones were isolated which were partially homologous to BEAF-32.

Sequencing revealed an open reading frame encoding a protein of 282 amino acids whose calculated molecular mass is 31.8 kDa (Fig. 2). Comparison with the original cDNA revealed that the 80 N-terminal amino acids encoded by the new cDNA differ from the 81 N-terminal amino acids encoded by the original cDNA, while the remainder of the predicted proteins are identical. We will refer to the protein encoded by the original cDNA as BEAF-32A (39) and that encoded by the new cDNA as BEAF-32B; we also use the shorter terms 32A and 32B. Southern blot analysis with probes specific for BEAF-32A or BEAF-32B revealed that the two cDNAs are derived from the same single-copy gene (data not shown). The unique 5' sequences are presumably encoded by two independent exons, with alternative transcription initiation or alternative splicing resulting in either one or the other being joined to the common 3' sequences of the mature mRNAs.

In contrast to the 32A protein encoded by the previously cloned cDNA, the DNase I footprint of 32B (Fig. 1B) is nearly identical to that of BEAF, covering both the single and palindromic CGATA sequences. However, the hypersensitive sites induced by 32B appear to be less prominent.

1 MPKGRVSNVWQHYDINEECENFAICRYCGN
 31 NISRGGMASNLKGNNTTNLWTHLRHKHRDE
 61 VLVSPVQQRFPQARMIFIKKDKTVRITKAK
 91 TLREPLRVAKPKIESNVANYLGEAGALPQK
 121 WEYEQNDIEISEDIKDIIYSEDPLCYSPIH
 151 VMDDEGLDQFEKQVTVLTHSTSPAGGSSRP
 181 IGVGSGVQATTVASTSSSSSSAKQLKNNLE
 211 TSIERLTAVSEQLSYIIQQNHEELTKDDDY
 241 YFALSVPAMRHLSSLRKMVVRSKIQDILF
 271 KESEDSTLAKDE

FIG. 2. Amino acid sequence of BEAF-32B. The amino acid sequence of 32B deduced from the cDNA sequence is shown. Amino acids are numbered on the left from the first methionine. Unique sequences are underlined (amino acids 1 to 80); the rest are identical to 32A amino acids 82 to 283. Of relevance to the present work, amino acids 200 to 230 are predicted to have a high propensity to form a coiled-coil structure containing an atypical leucine zipper (27). See reference 39 for a description of other sequence features.

BEAF-32A and BEAF-32B interact. To aid the study of the two BEAF proteins, we raised antibodies directed against their unique N-terminal regions. These antibodies are specific for the BEAF protein against which they were raised and do not cross-react (Fig. 3A and 4A). With these antibodies, we demonstrated the presence of heterocomplexes of 32A and 32B in KC nuclear extracts, because both BEAF-specific antibodies immunoprecipitated complexes consisting of 32A and 32B (Fig. 3A). Both anti-BEAF antibodies also recognize the BEAF doublet described previously (39). This doublet arises by post-translational phosphorylation of 32A and 32B, since treatment with alkaline phosphatase eliminated the upper protein band (data not shown).

As mentioned above, 32A interacts with the mutant D fragment despite the mutated palindrome which abolishes binding by *Drosophila* BEAF. This is illustrated by a band shift experiment in which a complex of 32A with the wild-type D fragment, unlike that of BEAF, is dissociated by excess mutant D DNA (Fig. 3B, compare lanes 1 and 2 with lanes 10 and 11). We do not observe 32A binding activity in KC nuclear extracts or purified *Drosophila* BEAF, possibly because its DNA binding behavior is altered by being predominantly complexed to 32B (see below). This view is supported by semiquantitative Western blot analyses that indicate a fourfold excess of 32B over 32A in KC nuclear extracts (e.g., Fig. 3A).

We could not generate functional heterocomplexes of 32A and 32B by using purified bacterially expressed proteins, but we achieved this goal by cotranslation in reticulocyte lysates as shown by DNA binding (Fig. 3B) and immunoprecipitation (Fig. 4A). Complexes formed on the D fragment by in vitro-cotranslated 32A plus 32B and by affinity-purified BEAF had indistinguishable mobilities and were insensitive to dissociation by excess mutant D DNA (Fig. 3B, compare lanes 7 to 9 with lanes 10 to 12). In contrast, the complex with in vitro-translated 32B is distinguishable by a higher mobility (Fig. 3B, lanes 4 and 5). 32B is also distinguishable by a roughly twofold-weaker affinity for the D fragment than that of BEAF or 32A, which have similar affinities despite binding to different sequences on the D fragment (data not shown). Thus, 32A-32B heterocomplexes form a more stable complex on the palindromic target sequence than do 32B homocomplexes.

FIG. 3. BEAF-32A and BEAF-32B form heterocomplexes. (A) Coimmunoprecipitation of 32A and 32B from KC nuclear extracts with protein-specific antibodies. Antibodies directed against the unique N-terminal sequences of 32A or 32B were affinity purified before use. Protein A-Sepharose beads bound with these or the control anti-p23 (an unrelated *Xenopus* protein) antibodies were incubated with KC nuclear extracts. Bound proteins were washed, eluted, separated by SDS-PAGE together with 2, 4, and 8 ng of 32A (lanes 5 to 7) and 10, 20, and 40 ng of 32B (lanes 8 to 10), transferred to nitrocellulose, and detected with either anti-32A (upper panel) or anti-32B (lower panel) antibodies. Lanes 5 to 10 demonstrate the specificity of the antibodies, indicating that 32A and 32B are both immunoprecipitated (IPP) by anti-32A (lane 3) or anti-32B (lane 4) but not by anti-p23 (lane 2) antibodies. Lane 1, input KC nuclear extract. From blots such as these, we estimate a ratio of 4:1 for 32B to 32A. (B) Heterocomplexes of 32A and 32B have different DNA binding properties than homocomplexes. After 32A and 32B were translated separately or together in a reticulocyte extract, the proteins were used in DNA binding assays with the radiolabelled D probe in the presence of a 200-fold molar excess of unlabelled competitor DNA, as indicated. Like *Drosophila* BEAF (lanes 10 to 12), the cotranslated proteins (lanes 7 to 9) exhibit a binding activity that migrates more slowly than that of 32B homocomplexes (lanes 4 to 6) and, unlike 32A homocomplexes (lanes 1 to 3), is resistant to mutant D (mD) fragment competition.

BEAF-32A and BEAF-32B share a C-terminal protein interaction domain. As described below, we have identified three functionally distinguishable domains in the BEAF proteins, which, for convenience, we call the N, M, and C domains (corresponding to the N-terminal, middle, and C-terminal regions, respectively). The C domain contains a region (amino acids 200 to 230) predicted by computer to have an atypical leucine zipper and a high propensity for a coiled-coil structure (27). We demonstrated, by cotranslation of full-length 32A together with truncated 32B proteins in reticulocyte lysates, that the C domain is responsible for interactions between BEAF molecules. Specific immunoprecipitation with either anti-32A or anti-32B antibodies demonstrated no interaction of full-length 32A with mutant protein B142, which deletes the C-terminal amino acids 143 to 282 (Fig. 4A, lanes 5 and 10). In contrast, complex formation occurred between 32A and the mutant protein BΔ81/203, which deletes the internal M domain (amino acids 81 to 203) to fuse the N and C domains (Fig. 4A, lanes 3 and 8). Taking all these results together, we con-

FIG. 4. BEAF-32A and BEAF-32B interact through the C-terminal C domain. (A) Heterocomplex formation detected by coimmunoprecipitation requires the C domain. 32A and B142 or BΔ81/203 (truncated 32B proteins of amino acids 1 to 142 containing the N-terminal N domain, or lacking amino acids 81 to 203 to delete the M domain and fuse the N and C domains, respectively) were translated separately or together in a reticulocyte extract in the presence of [³⁵S]methionine. After immunoprecipitation with anti-32A-specific (lanes 1 to 5) or anti-32B-specific (lanes 6 to 10) antibodies prebound to protein A-Sepharose beads, the precipitated proteins (IPP) were eluted, separated by SDS-PAGE, and detected by autoradiography. Coimmunoprecipitation occurred only if both proteins had the C domain (lanes 3 and 8). Lanes 11 to 15, input proteins used for immunoprecipitation. I, translation products derived by initiation at an internal AUG codon. (B) Summary of the yeast two-hybrid results showing that the C domain is necessary and sufficient for interactions between 32A and 32B. N, M, and C represent the N-terminal, middle, and C-terminal domains, respectively, of 32A and 32B (see the text and Fig. 5A for details). These domains were fused to the GAL4 DNA binding domain (bait fusions) or the GAL4 activation domain (target fusions) and cotransformed into the yeast strain HF7c as indicated. Only bait and target cotransformants that both contained at least the C domain exhibited β-galactosidase activity and histidine autotrophy (indicated by +), both of which required interaction between the bait and target constructs, whereas absence of the C domain from either construct eliminated these activities (indicated by -). Numbers in parentheses correspond to the numbers in panel C. (C) Growth of the yeast strain HF7c on minimal medium lacking histidine requires the BEAF C domain on both the bait and target plasmids. The numbers correspond to those in panel B; see the legend to panel B for details.

clude that BEAF interactions are mediated by the C domain from amino acids 204 to 282. Note that initiation at an internal initiation codon in the 32B constructs presumably gives rise to the lower-molecular-weight protein band observed in Fig. 4 for both mutant B constructs.

The yeast two-hybrid system was used to confirm and extend these results to an in vivo situation (Fig. 4B and C). "Bait" proteins consisted of an N-terminal GAL4 DNA binding domain fused to full-length or truncated forms of 32A and 32B. Similarly, target proteins consisted of an N-terminal GAL4 activation domain fused to full-length or truncated forms of 32A and 32B (details are given in Fig. 4B). Transformed yeast cells were plated onto medium lacking histidine so that growth required activation of the *HIS3* gene driven by a GAL1 promoter. No growth occurred after transformation with single constructs, indicating that the BEAF proteins do not activate transcription (data not shown). Cell growth and hence gene activation occurred only after pairwise cotransformation with combinations where the C domain is present in both the bait and target proteins (Fig. 4C no. 1, 3, 5, 7, 9, and 11 [summarized in Fig. 4B]). The C domain was found to be not only necessary but also sufficient for complex formation, as robust growth was observed when only the C domain was fused to the activation domain (Fig. 4B and C, no. 9 and 11). Clearly, the C domain is capable of mediating protein-protein interactions in vivo, possibly via the putative leucine zipper.

BEAF-32A and BEAF-32B have N-terminal DNA binding domains. The difference in the protein sequence of BEAF 32A and 32B is restricted to their N-terminal domains (amino acids 1 to 80), which consequently must be mediating their different DNA binding specificities. We established that the N domains harbor the DNA binding domains by testing the DNA binding behavior of a series of deletion mutants of either protein (summarized in Fig. 5A; representative band shifts are shown in Fig. 5B). The results with 32B are discussed first. Deletion of the internal M domain (amino acids 81 to 203) does not alter the DNA binding behavior, and truncation of 32B following amino acid 142 or 204 does not interfere with the formation of specific DNA complexes (Fig. 5B). However, unlike with the full-length protein, a ladder of bands is observed with the C-terminal deletions. Supposedly, these truncated 32B proteins bind individually rather than cooperatively due to the missing C domain, which is necessary for protein-protein interactions (see above). Based on these results, the lack of DNA binding by the two 32B proteins truncated in the C domain (truncated after amino acid 234 or 252) is thought to be due to misfolding of these proteins. This view is supported by the finding that the 80 N-terminal amino acids (protein B80) suffice to bind and protect the CGATA palindrome of the D probe from DNase I digestion (Fig. 1C), demonstrating a similar DNA binding specificity to that of intact 32B or the *Drosophila* activity.

A similar N-terminal DNA binding domain could not be detected for 32A. As expected, an N-terminal deletion to amino acid 66, AΔ1/66, abolishes DNA binding (Fig. 5A). Also, C-terminal deletion proteins, obtained by either bacterial expression or in vitro translation, had no detectable DNA binding activity (summarized in Fig. 5A). By analogy with 32B, we assumed that the DNA binding domain is in the N-terminal 80 amino acids but that DNA binding might require stabilization by C-terminally mediated interactions and folding. Consistent with this reasoning, DNA binding could be detected when the N and C domains of 32A were fused by deleting the internal M domain (Fig. 5B, AΔ82/204). The single shifted band is similar in mobility to that observed with the equivalent mutant 32B protein, suggesting the presence of cooperative binding by two or more subunits. Although direct involvement

FIG. 5. BEAF-32A and BEAF-32B have N-terminal DNA binding domains. (A) Summary of DNA band shift results showing that the N domain is the DNA binding domain and that inclusion of the C-terminal protein interaction domain leads to cooperative DNA binding. The indicated bacterially expressed deletion mutants were partially purified by PC chromatography, and their DNA binding activities were tested in band shift and DNase I footprinting experiments with the scs' D subfragment probe. Cooperative binding was deduced from band shift experiments by the position of the shifted band and the absence of multiple shifts (B). The N-terminal, middle and C-terminal domains are represented by solid, open, and hatched bars, respectively. Numbers above the bars represent positions of truncation, with those for 32B in parentheses. nd, not determined. (B) Band shift experiment with radiolabelled D probe and increasing concentrations of B142 (lanes 2 to 5), AΔ82/204 (lanes 7 to 10), BΔ81/203 (lanes 12 to 15), B204 (lanes 17 to 19), or full-length 32B (lanes 21 to 23). Lanes 1, 6, 11, 16, and 20 contain no protein. See panel A for details.

of the 32A C domain in DNA binding cannot be rigorously excluded, the C domain does not contribute to or alter the 32B N domain DNA binding specificity. Thus, we believe that the C domain indirectly stabilizes DNA binding by the 32A N domain by promoting protein-protein interactions, proper folding of the N domain, or both, as suggested above. We conclude that the N domains of both proteins bind DNA.

Drosophila BEAF can form a trimer. The suppression of 32A binding in nuclear extracts combined with the large DNase I footprint on the D probe led us to examine the size of the BEAF complex. The DNase I footprint of BEAF on the D probe suggests that BEAF could bind as a trimer to protect the three CGATA sequences present. This is supported by results of an experiment involving the B142 protein that should bind CGATA motifs individually rather than cooperatively since it lacks the C domain (Fig. 6A). A single complex is formed with a mutant 48-bp BEAF target site oligonucleotide (39) containing only the single CGATA motif, while two complexes form on a mutant 48-bp BEAF target site oligonucleotide containing only the CGATA palindrome. A protein concentration-dependent ladder of three complexes forms on the wild-type 48-bp BEAF target site oligonucleotide containing all three CGATA motifs.

We determined the number of subunits in a BEAF complex

by cross-linking proteins with dithio-bis(succinimidyl propionate). The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and detected with anti-BEAF antibodies. Complexes with mobilities consistent with BEAF dimers (about 75 kDa) and trimers (about 105 kDa) were detected by using either KC nuclei (Fig. 6B) or KC nuclear extracts (data not shown). While more 105-kDa species than 75-kDa species was detected in nuclei, the opposite was found when nuclear extracts were used. In another approach, glycerol gradient analysis of KC nuclear extracts yielded a molecular mass of about 100 kDa for the BEAF complex (data not shown). These results suggest the presence of a trimer of BEAF in solution or on DNA. Based on DNase I footprints (Fig. 1), the inability of BEAF or 32B to bind the mutant D fragment (Fig. 3B), and evidence that the BEAF binding activity is composed of a heterocomplex of 32A and 32B (Fig. 3), we presume that the activity in nuclear extracts is composed mainly of two 32B subunits and one 32A subunit. However, as shown below, the composition of BEAF complexes in vivo varies.

BEAF-32A and BEAF-32B partially colocalize on polytene chromosomes. If BEAF is present exclusively as heterocomplexes in vivo, as it possibly is in nuclear extracts, 32A and 32B should colocalize on polytene chromosomes. To test this, we used rabbit and mouse antibodies specific for the unique amino

FIG. 6. *Drosophila* BEAF can form a trimer. (A) Band shift experiment showing that it takes three 32B molecules to bind the scs' D-site CGATA motifs protected by the *Drosophila* BEAF activity. Increasing concentrations of B142 were incubated with the 48-bp D-site BTS oligonucleotide containing three CGATA motifs (39) (lanes 1 to 5), the m1-BTS oligonucleotide containing two CGATA motifs (lanes 6 to 10), or the m2-BTS oligonucleotide containing one CGATA motif (lanes 11 to 15). The single CGATA motif was changed to CTCGA in m1-BTS, while the palindromic CGATA motifs had this change in m2-BTS. (B) Protein cross-linking with KC nuclei results in a BEAF species that migrates as a trimer during SDS-PAGE. KC nuclei (0.5 optical density unit) were treated with dithio-bis(succinimidyl propionate) to cross-link proteins, separated by SDS-PAGE in the absence of reducing agents, transferred to nitrocellulose, and detected with anti-BEAF antibodies by enhanced chemiluminescence (Amersham). The positions of the molecular weight standards are indicated on the right (in thousands).

terminus of either 32A or 32B, as tested by Western blotting (e.g., Fig. 3). No staining of polytene chromosomes was observed when preimmune sera were used. We previously immunolocalized *Drosophila* BEAF to interbands and puff boundaries of polytene chromosomes of third-instar larvae with an antibody that recognized both 32A and 32B (39) and confirmed this staining pattern with the protein-specific antibodies. Also as previously noted (39), no staining of the centric heterochromatin was detectable. Double staining with anti-32A (red) plus anti-32B (green) antibodies revealed that 32A and 32B predominantly colocalize (yellow) on polytene chromosomes. However, there are many interbands that stain mainly for either 32A or 32B (Fig. 7), indicating that the relative ratio of these proteins varies. It should be noted that the fluorescence data cannot be used to calculate the actual ratios of 32A to 32B.

Facilitated binding of BEAF-32B to the B and D sites of the scs' core element. Although 32A, 32B, and BEAF all bind with

high affinity to the scs' D fragment, this fragment had significantly less ability than the entire scs' core element to block an ecdysone response element in an enhancer-blocking assay with stably transformed *Drosophila* D1 cells (39). The scs' core element has two inverted CGATA repeats (Fig. 8A), one on the D fragment with 1 bp between repeats (D site) and one on the B fragment with 3 bp between repeats (B site). Although 32A does not bind the B site and BEAF has a much lower affinity for the B site than for the D site (K_d , 600 and 25 pM, respectively [data not shown]), the enhancer-blocking assay results suggested that both sites might be important for boundary function.

Using both a band shift assay (Fig. 8B) and a footprinting assay (Fig. 8C), we found that binding of 32B to the B site was facilitated by the presence of the D site in the scs' core element. When the D-site inverted repeat was mutated, there was a weak shift of the scs' fragment at the concentrations of 32B tested, consistent with binding to the low-affinity B site. Under the same conditions, there was a much stronger shift of the wild-type scs' fragment, and the majority of the shift had a position consistent with simultaneous binding to the high-affinity D site and the low-affinity B site. Footprinting of scs' confirmed that both the B and D sites were protected by 32B to the same extent from DNase I digestion. Mutation of the D site inverted repeat eliminated protection of both sites, consistent with the partial shifting of the mutant scs' probe under these conditions. We conclude that the binding of 32B to the B site is stabilized by binding to the D site.

DISCUSSION

BEAF-32A and BEAF-32B are related proteins that form heterocomplexes with properties distinct from homocomplexes. *Drosophila* BEAF is a DNA affinity-purified activity derived from nuclear extracts that specifically binds the scs' boundary element of the 87A7 *hsp70* domain as well as hundreds of interbands and puff peripheries on polytene chromosomes (39). Our efforts to elucidate the mechanism by which boundary elements work led to the cloning of the *BEAF-32A* cDNA (39). Here we report the cloning of a cDNA encoding the highly related protein BEAF-32B, which, in contrast to 32A, essentially reproduces the footprint of the *Drosophila* BEAF activity on the scs' D site. The major difference is that BEAF appears to induce a much stronger hypersensitive site downstream of the palindrome.

The cDNAs encoding 32A and 32B are derived from the same gene, presumably by alternative splicing or alternative transcription initiation. The 32A and 32B proteins differ only in their N-terminal 81 (or 80) amino acids, which harbor the DNA binding domains. The remaining 202 amino acids are identical and can be experimentally partitioned into two further domains. The middle domain, M (amino acids 81 to 203), is dispensable for the interactions reported here, although it may be important for boundary function. The C-terminal part (amino acids 203 to 282) harbors the protein interaction domain that results in homo- and heterocomplex formation by 32A and 32B. Our DNA binding and physical data are most consistent with a trimer of (32A)(32B)₂ comprising the binding activity detectable in nuclear extracts. The predominance of this activity may be due to the predominance of 32B over 32A (about fourfold) in our nuclear extracts. However, our immunofluorescence data suggest that BEAF complexes have different compositions at different genomic sites in vivo. Although we cannot calculate actual ratios of 32A to 32B from the relative fluorescence signals, it appears 32A and 32B mostly colocalize while some locations are richer in either 32A

FIG. 7. BEAF-32A and BEAF-32B predominantly colocalize on polytene chromosome interbands and puff borders. Polytene chromosomes from third-instar larvae were immunostained with rabbit anti-32A (red) and mouse anti-32B (green) antibodies. The staining localizes to interbands and puff borders. Although the signals predominantly overlap (yellow regions), many interbands stain mainly for 32A or 32B. Note that different colors indicate different ratios of 32A to 32B but cannot be used to calculate actual ratios. Scale bar, 5 μ m.

or 32B. Since their DNA binding domains are different, it is reasonable to suggest that compositional variation is allowing specific interaction with BEAF sites of different sequence contexts. It is also possible that interactions with other, presently unknown DNA binding partners also contribute to compositional heterogeneity. It will be of interest to characterize other BEAF target sequences and identify common sequence features and assay for boundary function.

Efficient boundary function might require interactions between the B and D sites of the *scs'* element. While it is not yet known what is necessary for efficient boundary element function, it is clear that neither a single copy of the 200-bp D subfragment derived from *scs'* nor seven tandem copies of the 48 bp BEAF target site oligonucleotide, which contains the D-site palindrome, block an enhancer as efficiently as the 515-bp *scs'* core element does (39). Thus multimerized D sites do not act synergistically in this context, in contrast to the case for many transcription factors (4, 28). The second palindrome in *scs'*, the B site, is located about 200 bp away from the D site. Although the affinity of BEAF for the B site is about 25 times lower than for the D site, the presence of the D site facilitates binding of 32B to the B site. The B and D sites should lie in the two nuclease-hypersensitive regions, with the resulting loop between them corresponding to the DNase I-resistant region mapped by Udvardy et al. (35). While it is possible that other *scs'* sequences are necessary for boundary activity, for instance to recruit other proteins, another non-mutually exclusive possibility is that proper spacing between BEAF binding sites is important. Perhaps some protein complex such as a nucleosome occupies the loop between BEAF binding sites and collaboratively forms a special structure capable of interrupting the propagation of open and closed chromatin states.

Other putative boundary elements are also associated with nuclease-hypersensitive sites. The vertebrate β -globin domain is controlled by a 5' locus control region consisting of a series of tissue-specific nuclease-hypersensitive sites bounded on the 5' side by a constitutive hypersensitive site. The constitutive hypersensitive site from humans (5' HS5 [26]) and chickens

(5'HS4 [5]) has boundary activity, and the chicken 5' HS4 also has this activity in transgenic *Drosophila*, suggesting a broad conservation of the activity. It should be pointed out that these elements are different than those found at the edges of certain nuclease-sensitive domains encompassing active genes such as the chicken lysozyme domain (29), the human beta interferon domain (23), and the human apolipoprotein B domain (18). The latter elements have been reported to contain scaffold attachment regions that confer position-independent expression to transgenes after integration into the genome. Although their native localization implies that they or an adjacent sequence is responsible for the transition from nuclease-sensitive to -insensitive chromatin, the latter elements also lead to elevated expression levels. It is not clear if these elements reduce position effects by acting as boundary elements or by stimulating transcription via facilitation of chromatin opening (25, 30).

The *Mcp* and *Fab-7* regions of the *Drosophila* bithorax complex have been proposed to contain boundary elements that separate parasegment-specific regulatory units from each other, with *Mcp* separating the *iab-4* regulatory unit from *iab-5* and *Fab-7* separating *iab-6* from *iab-7* (9, 19, 37). Since the inactive regulatory units are located between the active units and the target promoter, these boundary elements are special because they do not interfere with interactions between active units and the target promoter despite preventing interactions between adjacent active and inactive regulatory units. A combination of chromatin mapping and analysis of deletion mutants in these regions provides a correlation between the location of the boundary activity and nuclease-hypersensitive sites. The putative *Mcp* boundary element localizes to a 400-bp region encompassing a 300-bp nuclease-hypersensitive site (19), while the *Fab-7* element localizes to a 1-kb region encompassing three nuclease-hypersensitive sites. A partial loss of boundary activity is observed when some of the *Fab-7* sites are deleted (9).

Deletion analysis of *scs* implicates its hypersensitive sites in boundary activity while showing that the central nuclease-resistant region is dispensable (36). As for *Fab-7*, partial deletion

FIG. 8. The scs' D site facilitates binding of BEAF-32B to the scs' B site. (A) Map of the 515-bp scs' fragment showing the locations of the high-affinity D site and the low-affinity B site and their dissociation constants for BEAF. Also shown are the mutations introduced into the high-affinity site of the mutant scs' fragment (lowercase letters). (B) Band shift experiment showing the affinity of 32B for the wild-type and mutant scs' fragment. Increasing amounts of bacterially expressed 32B, partially purified by PC chromatography, were incubated with wild-type (lanes 1 to 6) or mutant (lanes 7 to 12) scs' and subjected to electrophoresis through a 4% polyacrylamide gel in 0.25× TBE (lanes 1 and 7 contain no protein). The highest concentration of 32B used completely shifts the scs' probe but only partially shifts the mutant scs' probe. In addition, the scs' probe gives two shifts consistent with binding to one or both binding sites present. For a given amount of 32B protein, there is a higher occupancy of the B site in the presence than in the absence of the D site (compare the scs' probe upper shift to the mutant scs' probe shift). (C) DNase I footprints in the presence of increasing amounts of 32B. Binding conditions were as in panel B. Lanes: 1 to 5, wild-type scs' element; 6 to 10, mutant scs' element. Elimination of the high-affinity D site in the mutant scs' element leads to loss of protection of the low-affinity B site under conditions that result in full protection on the wild-type scs' element. Arrowheads indicate the positions and orientations of the CGATA motifs in the wild-type B and D sites. Lanes 1 and 6, G+A sequencing reaction products. Lanes 2 and 7, no protein.

of the hypersensitive sites impaired boundary activity. Multimerization of sequences encompassing a subset of the hypersensitive sites restored activity, implying a redundancy of relevant features. Since the smallest multimerized sequence was roughly 200 bp, it may have worked better than seven tandem copies of the 48-bp BEAF target site oligonucleotide because it had a longer, more optimal spacing between protein binding sites. In summary, boundary activity appears to be associated with certain nuclease-hypersensitive sites. Disturbing these regions interferes with boundary activity, although the relevant features of these regions might be redundant. Finally, it may be that the spacing between hypersensitive sites is more important than the sequences found between them. Further analysis of scs' variants will shed light on the importance of the BEAF binding sites and the spacing between them.

Interbands and boundary function. BEAF localizes to hundreds of interbands on polytene chromosomes, suggesting that BEAF-utilizing boundary elements are common in *Drosophila*.

As mentioned above, 32A and 32B do not completely colocalize on polytene chromosomes. Because BEAF apparently binds as a trimer, this suggests that different combinations of the four possible trimers are present in different boundary elements that utilize BEAF. Another nonexclusive possibility is that 32A and 32B can be directed to different sites through interactions with other DNA-binding proteins. Further studies are necessary to determine the functional differences between the putative elements detected by immunofluorescence. Because 32A and 32B can form homo- and heterocomplexes and these complexes can interact to facilitate binding to multiple sites (e.g., the B and D sites of scs'), one interesting possibility is that there are extensive interactions between boundary elements in diploid cells that help organize chromatin in the nucleus. Proteins involved in another aspect of chromatin organization, heterochromatin formation, form such extensive interactions. These interactions can lead to the physical association of a euchromatic gene containing a heterochromatic

insertion, together with its wild-type homologous copy, with heterochromatic compartments in interphase nuclei (6). Such interactions would not be apparent from immunostaining of polytene chromosomes, because of the special configuration of chromatin in polytene chromosomes.

RNA polymerase II has been immunolocalized to most interbands and puffs of *Drosophila* polytene chromosomes (17, 24), suggesting an interband location of 5' gene-regulatory sequences. This is supported by high-resolution mapping of the *Notch* locus in third-instar salivary glands, where it is not expressed. It was found that the transcribed sequences are in polytene chromosome band 3C7 while sequences 5' of the start of transcription are in the interband between bands 3C6 and 3C7 (32). The apparently similar localization of BEAF-related boundary elements and gene-regulatory sequences to interbands is consistent with a role for boundary elements in transcriptional regulation.

Consideration of the structure of the *scs'* element provides some insight into how a boundary element might block enhancer activity. Although reported to have no enhancer activity in transformed flies (20), the *scs'* core element fused 5' of the *hsp27* basal promoter increased CAT activity about fourfold in transiently transfected tissue culture cells (38a). Two divergent promoters are located in *scs'* (14), suggesting that the stimulation of reporter gene activity could be due to these endogenous promoters. As proposed above, this colocalization of BEAF binding sites to *Drosophila* promoter regions is not unique (6a). While the relationship between the promoters, special chromatin structure, and BEAF binding sites of *scs'* is presently unclear, they could all be important for boundary function. What is clear is that BEAF is not a typical transcription factor. Perhaps a boundary element limits enhancer activity by limiting the spread of enhancer-induced changes in chromatin structure, and this is often coupled with a neighboring promoter that can act as a sink for the enhancer. The result could be either the induction of a boundary element-associated transcript or interactions with a defective initiation complex that merely prevent the enhancer from activating another promoter further downstream.

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