Transcription Activation by Ultrabithorax Ib Protein Requires a Predicted α-Helical Region†

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ABSTRACT: Characterization of their transcription activation domains is critical to understanding functional specificity within the Hox family of proteins. However, few Hox activation domains have been identified and none characterized in detail. In this study, promotor−reporter assays in yeast and *Drosophila* S2 cell culture were used to refine the boundaries of the activation domain of the *Drosophila* Hox protein Ultrabithorax (Ubx) and to identify critical elements within this domain. We found that residues 159−242 were sufficient for ∼50% function, and full transactivation capacity was achieved with inclusion of additional N-terminal sequences. Activation domain sequence and placement relative to the homeodomain differ between Ubx and other Hox proteins, consistent with the possibility that diverse activation mechanisms contribute to functional distinctions in vivo. The essential residues 159−242 in the UbxIb activation domain are predicted to contain a β-sheet segment followed by an α-helix. This putative α-helical region was established to be necessary, but not sufficient, for transcriptional activation. Disruption of the helix by proline substitutions abolished activation function, while alteration of side chains presented on the surface of this putative helix with alanine or lysine mutations had no significant effect on activity. Collectively, these data indicate that this secondary structural element is a key component in forming an effective activation domain in the UbxIb protein. Interestingly, the α-helix critical for transcriptional activation is found only for Ubx orthologs from flies and not other species. The mutant Ubx proteins generated in this study have potential applications in deciphering Hox functions in vivo.

The Hox genes encode transcription factors with key roles in patterning the anterio-posterior axis. Altering expression of these genes in *Drosophila* causes homeotic transformations, in which one body part is partially transformed into another (*1*, *2*). On the molecular level, Hox proteins activate or repress transcription through binding short and partially redundant DNA target sequences via the highly conserved homeodomain (*2*, *3*). Specific Hox proteins can tolerate significant DNA sequence variation, and in vivo target sequences can be recognized by multiple members of the Hox protein family (*3*−*8*). Thus, a major discordance exists between the necessity for unique function in vivo and the overlap of DNA binding specificity.

Solutions to the Hox specificity problem may encompass complex and varied combinations of the following properties: small differences in DNA binding, interactions between individual Hox proteins as well as with general Hox cofactors such as Extradenticle and Homothorax, interactions with heterologous transcription factors acting on the same promoter/enhancer, and transcription activation and repression by the Hox proteins themselves depending on the cellular context (*9*−*15*). Sequences outside the homeodomain differ substantially among these proteins and clearly have the potential to contribute to functional specificity (*5*, *11*, *12*, *15*, *16*). An interesting series of experiments utilized chimeric Hox proteins to determine the minimal protein sequences required for specific functions during *Drosophila* development (*15*, *16*). The results demonstrate that distinct regions of a single Hox protein determine specificity in different developmental contexts (*15*, *16*). To ensure that only directly analogous domains of the proteins were exchanged in these experiments, junctions in chimeras between different Hox proteins were limited to conserved regions in or near the homeodomain. As a result, large portions of these proteins remain unexplored. To better understand functional specificity of Hox proteins, an essential step is to map individual functions to amino acid sequence.

Transcription activation requires sequences outside of the homeodomain that are poorly conserved; as a consequence, differences in the sequence and nature of these activation domains may contribute to the functional distinctions observed within the Hox family (*3*, *14*, *17*−*23*). Indeed, sequences outside the homeodomain are responsible for differences in function between *Drosophila* and onychophora Ultrabithorax (Ubx)[1] (*24*). Furthermore, differences in the strength of activation domains within the Hox family may allow one member to predominate over another at the same enhancer. For example, the Ubx-VP16 chimera contains a strong activation domain and alters the normal effect of Ubx to generate an Antennapedia-like phenotype in *Drosophila* (*5*). Identification and comparison of Hox activation domains

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[1] Abbreviations: ONPG, *o*-nitrophenyl-β,D-galactopyranoside; SD medium, synthetic dropout minimal medium; Ubx, Ultrabithorax protein; X-gal, 5-bromo-4-chloro-3-indolyl-β,D-galactopyranoside.

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is therefore of particular interest. However, genetic approaches suffer from the drawback that mutations may also affect DNA binding, transcription repression, or protein interaction, complicating the use of phenotypic experiments in whole flies to investigate amino acid sequences required specifically for gene activation. We have therefore utilized assays in yeast and in *Drosophila* S2 cells in culture, allowing rapid quantitative analysis in a homogeneous cell population (*7*), to identify sequences necessary for the transcription activation function of the *Drosophila* Hox protein Ubx.

Ubx specifies the identities of regions of the posterior thorax and a portion of the first abdominal segment, contributing to the development of the central and peripheral nervous systems as well as midgut, leg, and haltere (a balancing organ unique to dipterans) (*1*, *25*−*32*). Ubx is expressed prior to pupation in the haltere imaginal disc and regulates expression of many genes in the wing/haltere genetic hierarchy (*33*). The UbxIb isoform, which contains all potential coding regions (Figure 1), is capable of both stimulating and repressing transcription based on results from in vivo and in vitro assays (*3*, *8*, *14*, *34*−*39*). Ubx binds RNA polymerase II, a possible target for Ubx transactivation function (*39*, *40*), and this activation requires regions N-terminal to the Ubx homeodomain (*3*, *8*, *14*). Segments of the protein involved in transactivation have been identified to be within regions 1−260 or 36−226 (*3*, *14*), but the contributions of various segments within these large regions to transactivation have not been well-defined (*3*, *8*, *14*, *16*, *39*, *41*).

In this study, we have identified and characterized in greater detail the key regions for transcription activation by Ubx using a series of deletion and point mutants in regions distant from the homeodomain that are not accessible using chimeric constructs. For the yeast analysis, UbxIb was fused to the LexA DNA binding protein, allowing the effects of mutation to be analyzed independently of effects on DNA binding. In the second analysis, UbxIb was expressed in *Drosophila* S2 cells in which natural Ubx target sequences were used in the promoter−reporter constructs. In this system, activation function can be assayed in the context of the native *Drosophila* transcription apparatus. Together, these in vivo systems can be used to specifically isolate and evaluate transcription activation. These studies reveal that amino acid residues 159−242, located in the central region of UbxIb, support partial activity and that full activation function of UbxIb requires extension to include residues 68−159. Of significant interest, a predicted α-helix encompassing residues 221−234 appears *essential* for transcriptional activation, as deletion of this segment or introduction of amino acid substitutions designed to interrupt the predicted helix completely abolishes the transcriptional activation capacity of UbxIb. Furthermore, the absence of significant effects on transactivation by complete alanine or selected lysine substitution within this predicted helical region suggests a structural role for this region. Comparison of the Ubx activation domain with this functional domain in other Hox/homeodomain proteins reveals significant variation in position and amino acid sequence of this domain. Finally, comparison among Ubx orthologs indicates that only fly species contain sequences that contain the α-helix critical for transcriptional activation by *Drosophila* Ubx protein.

EXPERIMENTAL PROCEDURES

Materials, Media, Bacterial Strains, and Yeast Strains. All chemicals used for yeast transformation and β-galactosidase assays were purchased from Sigma (St. Louis, MO). Restriction enzymes and ligase were obtained from Promega (Madison, WI). Synthetic dropout (SD) minimal medium with various supplements was used for yeast culture, maintenance, and selection of transformants. *Saccharomyces cerevisiae* EGY48 [MATα *ura*3 *his*3 *trp*1 $LexA_{op(x6)}$ LEU2] is a reporter host strain that carries a wild-type LEU2 gene under the control of a series of LexA operators (*42*). EGY48 was transformed with the reporter plasmid p8op-*lacZ* that carries the reporter gene *lac*Z also under the control of LexA operators to establish stable transformation prior to introduction of plasmids to test for activation. *E. coli* strain AR120 was used as a host for all plasmid amplification.

Mutagenesis and LexA Fusion Plasmid Constructions. Various UbxIb truncation mutations and amino acid substitutions were produced from pETC6-UbxIb (gift from Dr. P. A. Beachy, Johns Hopkins University) by PCR with primers that introduce an *Eco*RI site at the 5′ end and a *Bam*HI site at the 3′ end following a stop codon. The correct reading frame was ensured by precise location of the *Eco*RI site in each 5′-primer. PCR was performed under various temperature conditions ranging from 48 to 55 °C depending on primer-template stabilities. The vector pLexA (Clontech Laboratories, Inc.) containing the *E. coli* LexA DNA binding protein sequence was used to generate a fusion protein of LexA with various portions of UbxIb. The PCR products were cloned into the vector pGEM-T. After amplification, the pGEM-T plasmids carrying various portions of UbxIb were digested with a combination of *Eco*RI and *Bam*HI, and the resultant fragments were then cloned into the *Eco*RI/*Bam*HI site of pLexA. All constructs were examined by restriction digestion, and the correct reading frames and sequences were confirmed by sequencing.

Yeast Transformations. Plasmids were introduced into yeast according to the one-step transformation method (*43*, *44*). Plasmid DNA (1 μg) encoding a fusion protein was transformed into yeast cells carrying reporter plasmid p8op-*lac*Z. Yeast transformants were plated onto SD plates lacking uracil and histidine and incubated at 30 °C until colonies became visible (~3 days). Growth media were prepared as described by Sherman (*45*) with glucose (2%) as the carbon source. X-Gal (40 mg/L) was added to selection plates to monitor transactivation of the *lac*Z reporter gene. Blue colonies indicate transactivation by the UbxIb fragment in the construct, and the relative shade of blue reflects the level of transactivation. Experiments reported were performed ≥3 times to confirm the results.

β-Galactosidase Assays. Transactivation of the *lac*Z reporter gene, determined by monitoring β-galactosidase production by blue/white colony assessment, was confirmed by direct assay of β-galactosidase activity. These assays were performed as described by Brent and Ptashne (*46*) using whole-cell extracts prepared by the glass bead method. Assays with at least six independent colonies performed on different days were used to generate averaged values. A negative control transformation with a plasmid that did not generate β-galactosidase production was utilized to determine background activity present in the extracts.

Cell Culture and Transfections. Drosophila melanogaster Schneider line 2 (S2) cells (*47*) were grown in 10% heat-

inactivated fetal bovine serum (Life Technologies), 90% Schneider's *Drosophila* medium (Life Technologies) with 10 units/mL penicillin, 10 units/mL streptomycin, and 25 ng/mL antimycotic (Life Technologies). Cells were maintained at a density between 10^6 and 10^8 cells/mL at 26.5 °C in 75 cm^2 flasks and passaged every 3−4 days, including the day before transfection. For transfection, cells at a density of $\sim 7 \times 10^6$ cells/mL were diluted with medium to a final concentration of 1×10^6 cells/mL. Aliquots of 3 mL were plated onto 40 cm diameter plates and grown at 26.5 °C for 9 h. The Invitrogen Calcium Phosphate Transfection kit was used to precipitate 19 μg of DNA per transfection at room temperature for 30−40 min in polypropylene tubes. Cells were incubated with the DNA for 24 h, then centrifuged for 2 min at 1000*g*, and resuspended in growth media. After incubation for 120 h at 26.5 °C following transfection, the cells were centrifuged, resuspended once in PBS, recentrifuged, and resuspended in 250 μL of lysis buffer (Promega, CAT Assay Kit). After vortexing the cells for 8 s, the lysate was heated to 60 °C for 10 min to inactivate endogenous CAT activity. The lysate was centrifuged at 12000*g* for 10 min, and the supernatant was stored at −80 °C.

The pMT/V5-His LacZ reporter plasmid (Invitrogen) was simultaneously transfected in a separate dish to assay transfection efficiency. LacZ expression was induced 48 h after adding DNA by adding 15 μL of 100 mM $CuSO_4$. After an additional 24 h, cells were stained for β-galactosidase using the Invitrogen β-galactosidase staining kit.

Immunoblot Analyses. Yeast protein extracts were prepared as described by Samson et al. (*14*). Samples (50 μL) of protein extracts were electrophoresed through 10% SDS−polyacrylamide gels and transferred to a nitrocellulose membrane. Immunoblotting was carried out with a 1:10000 dilution of LexA murine monoclonal antibody (Clontech) followed by a 1:5000 dilution of peroxidase-labeled secondary anti-mouse antibody (Amersham Life Science). Proteins were visualized with enhanced chemiluminescence (ECL) detection reagents (Amersham Life Science) and quantified with GelExpert software using the NucleoVision system (NucleoTech Corp.).

To test expression in S2 cells, 3 mL volumes of cells transfected with 19 μg of pPAC-UbxIb plasmid expressing wild-type or mutant Ubx were utilized. Cell extracts (30 μL) were mixed with 4× sample buffer (10 μL) and heated to 90 °C for 10 min prior to separation on a 10% 29:1 polyacrylamide gel. Proteins were transferred at 150 V for 20 min onto a nitrocellulose membrane (Schleicher & Schuell). The antibodies FP3.38 (1:200 dilution, gift from Dr. P. A. Beachy, Johns Hopkins University) and 10H.7 (1:100 dilution) (*48*) were used as primary antibodies. Horseradish peroxidase conjugated goat anti-mouse (Calbiochem) was used as secondary antibody at a 1:2000 dilution.

CAT Assays. CAT assays were performed using [^{14}C]-chloramphenicol according to the instructions in the CAT Assay Kit from Promega. Reaction products were detected by scintillation counting. The resulting activities were corrected for protein concentration in the cell lysate and normalized to the wild-type transfection from the same experiment.

RESULTS

Confirming the Transactivation Potential of the UbxIb Protein. UbxIb was expressed as a fusion protein with the LexA DNA binding protein in yeast cells in which a reporter plasmid, p8op-*lac*Z (*42*), had been established (Figure 1A). This plasmid contains *lac*Z as a reporter gene with eight copies of the LexA operator binding site upstream. Blue colonies were generated when the yeast cells EGY 48 carrying p8op-*lac*Z were transformed with pLexA-UbxIb. Thus, as observed previously, the LexA-UbxIb fusion protein is able to transactivate the reporter gene, confirming that the UbxIb protein has intrinsic transcription activating properties (*3*, *8*, *14*, *39*, *41*). Likewise, UbxIb was expressed under the actin 5C promoter in S2 cells using the pPAC-UbxIb plasmid (*3*). The pPAC-UbxIb plasmid was cotransfected with the $pCAT_{UbxIb}$ reporter plasmid, which contains the chloramphenicol acetyltransferase (CAT) gene under the control of a region of the *Ubx* gene promoter activated by Ubx in S2 cells (*3*, Figure 1B). As observed previously, Ubx was able to activate transcription of the CAT gene above background levels (see below) (*3*).

Defining the Transactivation Domain of UbxIb Protein. Analysis of the transactivation activity of fusion proteins in yeast transformation assays has been used to establish the transcription activation function of UbxIb (*8*, *14*) and to define the transcription activation domain(s) in other transactivators using deletion constructs (*49*, *50*). The advantage of this approach is that the LexA component of the hybrid protein supplies the sequence-specific DNA binding domain required to position the protein to be tested for transactivation (Figure 1A). Thus, only that portion of the protein that functions as a transcription activation domain is required to observe activation. To more closely delineate the transcription activation domain of UbxIb, a series of plasmids encoding LexA-UbxIb and its truncated derivatives was constructed and transformed into yeast cells carrying the reporter plasmid p8op-*lac*Z (Figure 1C, Table 1). Each of the effector plasmids produced the designed LexA-UbxIb-(derivative) fusion protein in yeast, and expression levels of the fusion proteins were comparable based on immunoblot analysis of transformed cell extracts using antibody to LexA (Figure 2). This similarity in expression indicates that observed differences in activation cannot be ascribed to different levels of fusion protein produced in the cells.

Two LexA-UbxIb fusion derivatives lacking segments of the C-terminus of the protein were generated. LexA-UbxIb-(1−370) lacks the poly-alanine region between residues 372 and 382, and LexA-UbxIb(1−354) lacks both the poly-alanine region and the predicted coiled-coil region near the homeodomain. Both constructs efficiently activate the transcription of the *lac*Z gene to produce blue colonies (Figure 1C); thus, the deleted C-terminal regions are dispensable for this function. A series of LexA-UbxIb fusion derivatives was generated in which sequential deletions were made from the N-terminus of UbxIb. LexA-UbxIb(42−389), lacking residues 1−41 of UbxIb, exhibited efficient transactivation function with blue colonies. LexA-UbxIb(103−389) and LexA-UbxIb(159−389) showed relatively weaker transactivation function by generating pale blue colonies. In contrast, LexA-UbxIb(216−389), LexA-UbxIb(242−389), and LexA-UbxIb(265−389) were unable to activate transcription of the reporter gene *lac*Z and produced only white colonies, consistent with earlier studies that showed that residues 1−260 were required for activity (*14*) and deletion of 36−226 abolished activity (*3*). These results indicated that UbxIb

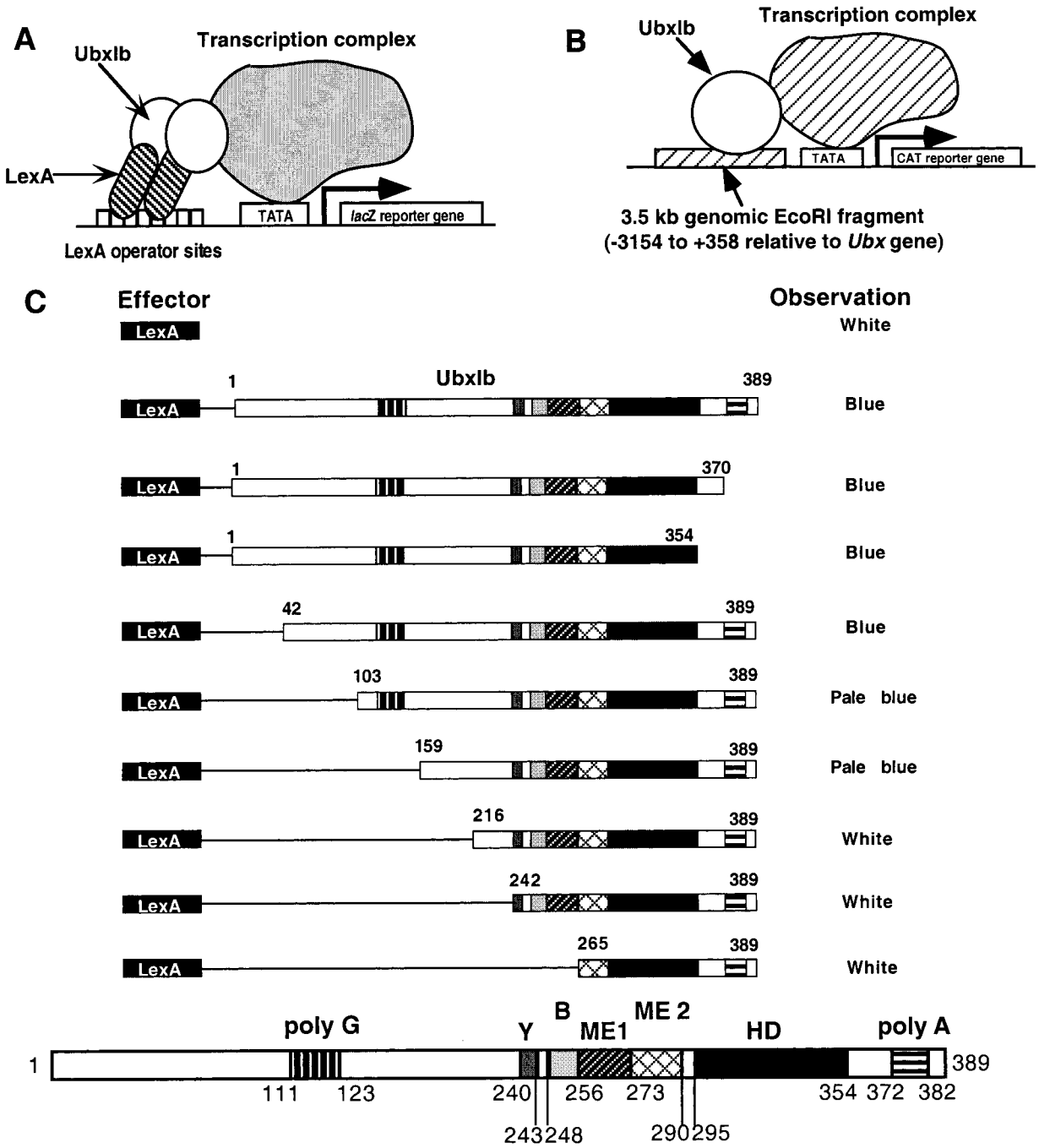

FIGURE 1: Schematic diagrams for assessment of UbxIb transactivation function in yeast and in S2 cells and results of transcription activation for deletion mutants. (A) The yeast one-hybrid fusion protein encompasses the entire LexA protein, including the DNA binding and dimerization domains. The transcription activation domain is provided by segments of UbxIb fused to LexA. Eight copies of the LexA operator binding sites are placed upstream of the *lac*Z reporter gene. Plasmids encoding the fusion protein and reporter gene are coexpressed in yeast cells. LexA alone can bind to the LexA operators but is unable to activate transcription of the reporter gene *lac*Z unless fused to a transcriptional activation domain. When the fusion protein activates transcription, colonies are blue, and the extent of activation can be measured by assays of β-galactosidase activity. (B) The extent of activation by UbxIb and its variants was measured in *Drosophila* S2 cells by assays of CAT activities in extracts. The $pCAT_{UbxIb}$ reporter plasmid contains the 3.5 kb genomic *Eco*RI fragment (−3154 to +358 relative to *Ubx* gene) shown placed upstream of the CAT reporter gene, as a target sequence to which UbxIb binds and activates transcription in *Drosophila*. This region contains the *Ubx U-A* and *U-B* enhancers, each of which has multiple Hox binding sites (*3*). (C) In these schematic diagrams of LexA-UbxIb fusion proteins, the filled box to the left represents the DNA binding protein LexA. The remainder of the regions is shown in the full-length UbxIb at the bottom of the figure. Poly G is a glycine-rich motif located in the amino-terminal half of UbxIb; Y, the YPWM region which is involved in direct interaction with the homeodomain-containing cofactor extradenticle (Exd) (*61*, *83*−*86*); B, b element microexon; ME1 and ME2, sequences encoded by microexons I and II, respectively; HD, the homeodomain capable of sequence-specific DNA recognition and binding; and polyA is an alanine-rich motif located in the carboxyl terminus of UbxIb. Plasmid DNA encoding each of the fusion proteins was transformed into yeast cells carrying reporter plasmid p8op-*lac*Z as described under Experimental Procedures. Yeast transformants were plated onto X-Gal (40 mg/L)-containing SD plates lacking uracil and histidine, and incubated at 30 °C until colonies became visible (~3 days). Blue colonies indicate transactivation function by the UbxIb fragment in the construct, while white colonies indicate lack of transactivation function. The relative shade of blue reflects the level of transactivation. Results shown are the composite of at least three independent experiments. Expression levels for fusion proteins in yeast cells are shown in Figure 2, and quantitation of β-galactosidase expression is provided in Table 1.

Table 1: Transcription Activation by UbxIb Regions

LexA-UbxIb derivatives	normalized β-galactosidase activity (%)[a]	LexA-UbxIb derivatives	normalized β-galactosidase activity (%)[a]
LexA	<1	LexA-UbxIb(103−242)	62 ± 10
LexA-UbxIb	100	LexA-UbxIb(108−242)	51 ± 11
LexA-UbxIb(103−389)	55 ± 5	LexA-UbxIb(148−242)	49 ± 7
LexA-UbxIb(148−389)	51 ± 5	LexA-UbxIb(159−242)	45 ± 5
LexA-UbxIb(159−389)	43 ± 4	LexA-UbxIb(216−242)	<1
LexA-UbxIb(216−389)	<1	LexA-UbxIb(Pro mutant 1)	<1
LexA-UbxIb(42−216)	<1	LexA-UbxIb(Pro mutant 2)	<1
LexA-UbxIb(68−216)	<1	LexA-UbxIb(Pro mutant 3)	<1
LexA-UbxIb(103-216)	<1	LexA-UbxIb(Pro mutant 4)	<1
LexA-UbxIb(159−216)	<1	LexA-UbxIb(Ala mutant 1)	102 ± 6
LexA-UbxIb(42−242)	91 ± 5	LexA-UbxIb(Ala mutant 2)	99 ± 6
LexA-UbxIb(68−242)	94 ± 8	LexA-UbxIb(Ala mutant 3)	102 ± 7

[a] The liquid β-galactosidase assay was performed with the transformants of yeast strain EGY48 producing fusion proteins of LexA and full-length UbxIb, its derivatives with deletion or point mutations. The numbers shown reflect the average percent values relative to LexA-UbxIb assays with measurements on at least six independent colonies performed on different days. Each set of experiments included a negative control (EGY48) that was performed simultaneously to determine background.

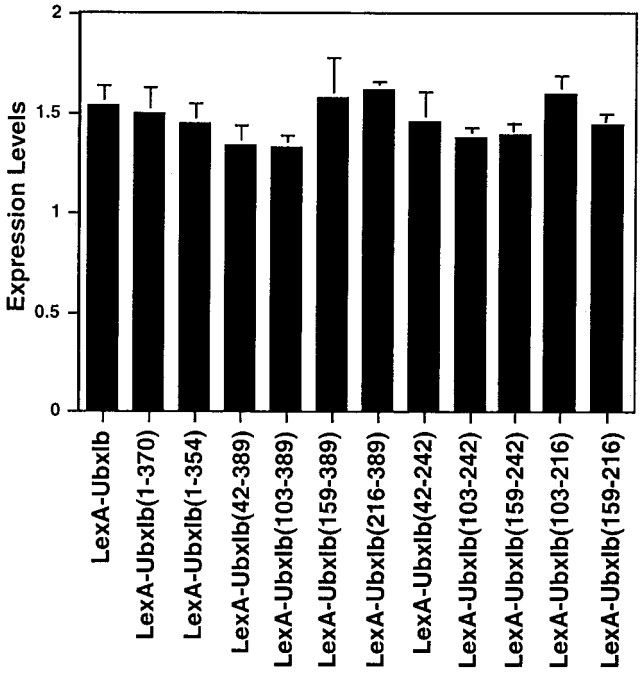

FIGURE 2: Antibody analysis of fusion protein production in yeast. Protein extracts were prepared from yeast culture in late exponential growth phase, and samples were electrophoresed and transferred to nitrocellulose for Western blotting. The bands corresponding to fusion proteins were visualized by antibody to LexA and quantified as described under Experimental Procedures. The level of each of the fusion proteins was determined based on band intensities, and the data were normalized to the protein content of the extracts. Error bars correspond to standard deviation for at least three independent experiments.

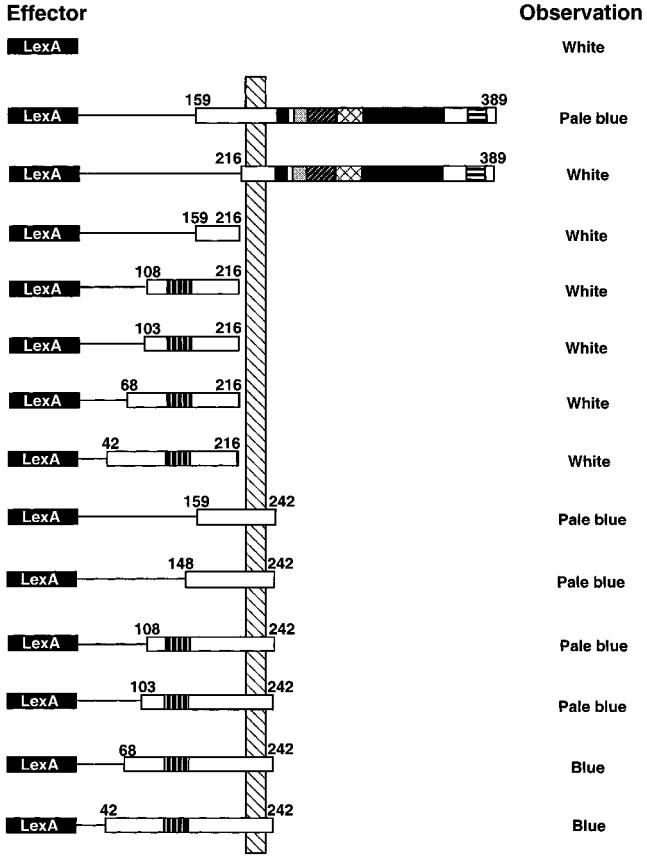

FIGURE 3: Definition of the region of UbxIb required to transactivate the *lac*Z reporter gene in yeast. Yeast cells with reporter plasmid p8op-lacZ were transformed with plasmids encoding the indicated fusion proteins, cultured, and observed for positive colonies as described in the legend to Figure 1. The vertical cross-hatched bar indicates amino acids 221−234 predicted to form an α-helix (see Figure 4 and text). The symbols for different segments are as defined in Figure 1. Results shown are the composite of at least three experiments.

residues 159−216, distant in sequence from the homeodomain (amino acids 295−354), are critical for the transactivation function of UbxIb.

To determine whether the 159−216 region of UbxIb alone has transactivation function, we generated LexA-UbxIb-(159−216) and determined its ability to activate the transcription of the reporter gene (Figure 3, Table 1). LexA-UbxIb(159−216) exhibited no transactivation function, resulting in white colonies. Thus, the fragment of UbxIb encompassing residues 159−216 is necessary but not sufficient for transactivation. On the basis of these results, we extended the length of UbxIb(159−216) in both N- and C-terminal directions and tested the ability of these fragments to activate reporter gene transcription (Figure 3, Table 1). LexA-UbxIb(103−216), LexA-UbxIb(68−216), and LexA-UbxIb(42−216) extend the N-terminal flanking sequence. None of these constructs resulted in transcription activation (Figure 3, Table 1). Therefore, residues 42−159, which contribute to full transactivation in LexA-UbxIb(42−389),

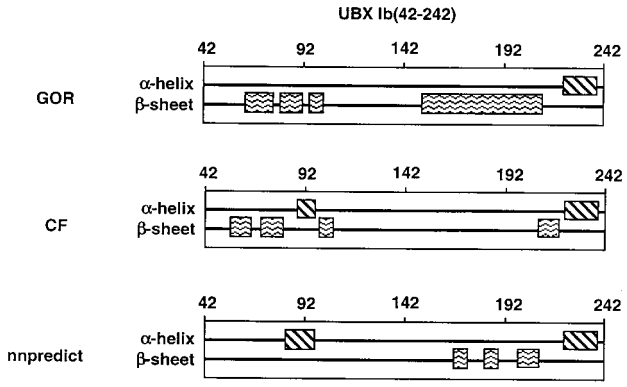

FIGURE 4: Secondary structure predictions of the region corresponding to the activation domain of UbxIb. The secondary structure predictions are based on Garnier−Osguthorpe−Robson (GOR) (*58*), Chou−Fasman (CF) (*59*), and nnpredict (*60*) algorithms. The homeodomain (amino acids 295−354) is C-terminal to the region shown.

cannot activate transcription by themselves or supplement residues 159−216 to activate transcription. LexA-UbxIb-(159−242) extends the C-terminal flanking sequence, and this construct yielded pale blue colonies that gave ∼50% transactivation based on β-galactosidase assays (Figure 3, Table 1). These results revealed that the region encompassing UbxIb residues 159−242 is sufficient for partial function as a transactivation domain and that UbxIb residues 216−242 are indispensable for this function.

Because residues 159−242 can only partially activate transcription, the full transactivation domain of UbxIb was not yet established. Considering LexA-UbxIb(159−389), which includes the C-terminus, only partially activates transcription, the missing region required for full activation must lie to the N-terminus of residue 159. To search for the missing region, the length of the UbxIb fragment fused to LexA was extended toward the N-terminus to generate five new fusion derivatives: LexA-UbxIb(148−242), LexA-UbxIb(108−242), LexA-UbxIb(103−242), LexA-UbxIb-(68−242), and LexA-UbxIb(42−242). While the LexA-UbxIb(103−242) fusion still produced pale blue colonies, the LexA-UbxIb(68−242) protein produced blue colonies and activity comparable to the full-length protein (Figure 3, Table 1). Thus, this segment extending to residue 68 is deduced to contain the missing region required for full activation function in concert with the essential region 159−242.

Correlation of Secondary Structural Elements with the Transactivation Function of UbxIb. Because multiple structural domains may be important for activation by transcription factors (*51*−*57*), we examined the secondary structure of UbxIb protein within the entire activation domain. Using GOR, CF, and nnpredict algorithms (*58*−*60*) to predict the secondary structure of UbxIb, qualitatively similar results were obtained, although there were differences in detail. Specifically, the GOR method indicates that UbxIb has more β-sheet structure, while the CF and nnpredict algorithms suggest that UbxIb has an additional α-helical segment in the region of amino acids 89−98 (Figure 4). The three predictions agree completely, however, on α-helical structure encompassing amino acid residues 221−234, which is within the region essential for transactivation. Further, all these methods also predict the α-helical structure for amino acid sequences in the homeodomain that correspond to the helices identified in the crystallographic structure for Ubx homeodomain complexed with Extradenticle homeodomain (*61*).

Effect of Mutations Designed To Interrupt the Predicted α-Helical Segment on UbxIb Transactivation Function. To determine whether the predicted α-helix is required for transcription activation, mutations were introduced in full-length UbxIb protein within the region predicted by the GOR, CF, and nnpredict algorithms to contain the crucial α-helical segment. These mutations were designed to disrupt the putative helix, and effects of mutation on transcription activation in the full-length protein were assayed in both yeast and S2 cells. Interestingly, no single amino acid replacement in this sequence resulted in loss of the entire predicted helix; therefore, multiple substitutions were required to completely eliminate predicted helical structure in this region. To ensure that the mutations were not in residues for which the side chains (vs helical structure) are specifically required for function, several combinations of substitutions predicted to disrupt the helix were generated in full-length UbxIb: LexA-UbxIb(A223P/A226P/Q233D), LexA-UbxIb-(A223P/Q224P/T225P/A228P), LexA-UbxIb(A227P/Q233D), and LexA-UbxIb(A226P/Q233P) (Figure 5). All mutant proteins were expressed effectively in yeast (data not shown) and in S2 cells (Figure 6A). None of these Ubx derivatives activated transcription of either the LacZ reporter gene in yeast (Table 1) or the CAT reporter gene in S2 cells (Figure 6B). The CAT gene is under the control of a 3 kb segment from the *Ubx* promoter which is activated by Ubx protein in S2 cells (*3*). Within this region, the *Ubx U-A* and *Ubx U-B* sites have been shown to bind Ubx in vitro (*62*). These results confirm the importance of helical structure for residues 221−234 in transcription activation in yeast and for expression from an in vivo target of Ubx in *Drosophila* S2 cells. Even in the context of the full-length protein, alteration of only two amino acids in this region is sufficient to completely abrogate transcription activation. Indeed, the mutant proteins repress transcription. Because different combinations of amino acids were mutated and all four proteins are incapable of transcription activation, it is unlikely that a single mutated amino acid would provide specific contacts critical for activation function.

Effect of Mutations That Alter the Surface of the Predicted α-Helical Segment. To eliminate the possibility that specific contacts were affected by mutations that interrupt helix formation, a series of mutations to alanine were designed within the 221−234 region. If side chains in this region are involved in specific interactions, conversion to alanine would likely disrupt function; however, if only the helical structure is required, conversion to alanine should not affect transactivation. Given the nature of the side chains that occur within this region (many are alanine in the wild-type protein), the residues most likely to participate in specific interactions are on the two faces encompassing Q224/L231 or T225/H232 (Figure 7). To establish the effects of modifying these surfaces, mutations were generated that converted all residues on separate faces of the presumed helix to alanine or changed all of the residues in this region to alanine. Conversion of a subset or even *all* of the residues to alanine did not disrupt (and perhaps enhanced) the ability to activate transcription in yeast or S2 cells (Table 1, Figures 6B and 7). Therefore, either the side chains of this putative α-helix do not

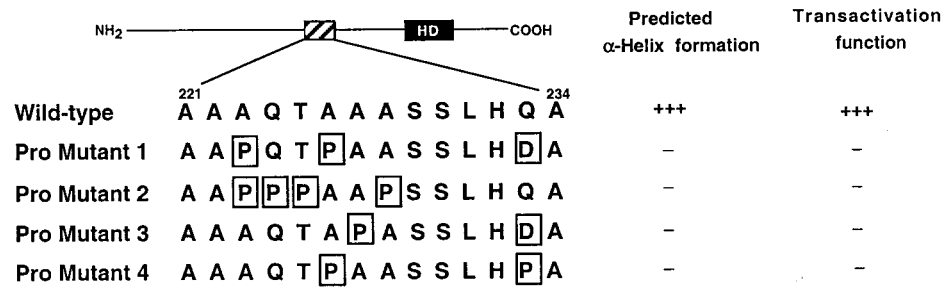

FIGURE 5: Transactivation activity for site-specific mutants that are predicted to preclude α-helix formation for residues 221−234. Mutations were introduced in full-length UbxIb protein within the region between residues 221 and 234. No single mutation in this region abolished the predicted α-helix. As a consequence, combinations of mutations were generated, using different regions of the sequence, that resulted in abolition of predicted secondary structure for this segment. These changes are shown, and the *β*-galactosidase activity in yeast (Table 1) and CAT activity in S2 cells (see Figure 6) were quantified to assess transactivation function.

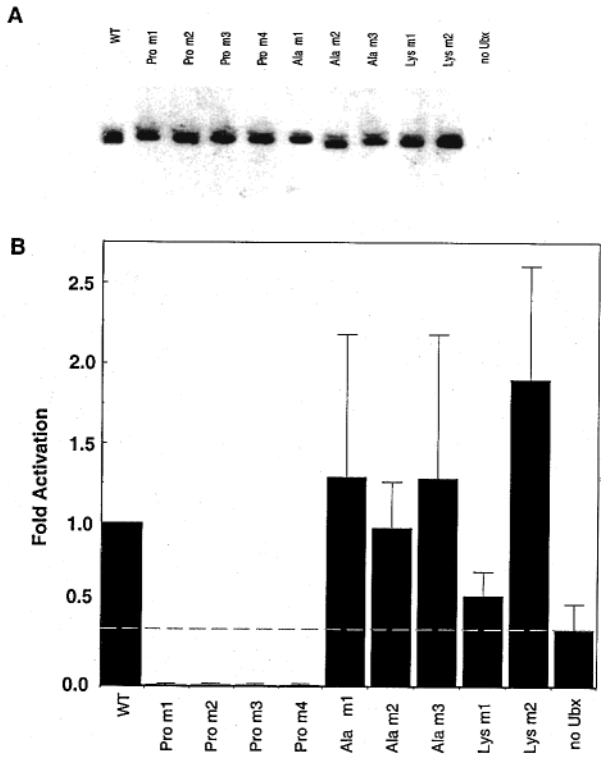

FIGURE 6: Transactivation activity for the site-specific mutants in S2 cells. The expression levels and transactivation abilities of mutants shown in Figures 5, 7, and 8 were measured in *Drosophila melanogaster* Schneider line 2 cells. Cell maintenance and transfections were performed as described under Experimental Procedures. Typical expression levels for mutant proteins in S2 cells are shown in panel A. CAT activities in S2 cells were quantified to assess transactivation activity of these mutants and are shown relative to activation levels by wild-type Ubx, defined as 1.0, in panel B. The dashed gray line marks the level of CAT activity in cells with no Ubx. Each bar represents the average of at least three experiments, each an independent transfection, and the error bars indicate the sample standard deviation.

participate in crucial protein·protein interactions or the methyl group of alanine suffices for these contacts. These results are especially striking when compared with the results from the proline mutations. Mutation of 7 residues to alanine, generating a polyalanine region 14 amino acids long, does not appear to diminish function (Figures 6B and 7). However, mutation of two residues to disrupt the helical structure completely abrogates transactivation (Figures 5 and 6B). We conclude that the ability to form helical structure in this region is likely the key factor influencing the transactivation capacity of UbxIb.

Effect of Alanine to Lysine Mutations. Because alanine may be enriched in activation domains (*63*), one possibility is that alanine itself is required to mediate hydrophobic interactions required for transcription activation. To test whether alanine is required, the mutants UbxIb(A227K) and UbxIb(A227K/A228K) were constructed in the full-length protein (Figure 8). Both mutants retain the predicted helix but place a lysine on a face of the helix that formerly held two alanines, thus inserting a charge on a hydrophobic surface. If these alanines constitute part of a hydrophobic surface required for transactivation, the charged lysine side chain should disrupt these contacts. Both proteins with lysine substitutions are capable of activating transcription, though at different levels than wild-type (Figures 6B and 8). Unlike observations for the proline mutants, these results do not support substantial difference between these mutants and wild-type protein. We conclude that the alanine surfaces interrupted are unlikely to be required for hydrophobic contacts in transcription activation, but the differences observed may reflect functional distinctions that require further study.

DISCUSSION

Activation domains in Hox proteins influence the nature and degree of transactivation, and differences in these domains may provide a source of functional specificity for this family with highly homologous DNA binding domains. Despite this potential regulatory role, few Hox activation domains have been identified and none characterized. Here, we have detailed the components of the Ubx activation domain and identified a critical region for which secondary structure appears to be the central requirement. The core element required for transactivation is located in the central portion of UbxIb spanning residues 159−242. The level of *β*-galactosidase activity observed for transformants of pLexA-UbxIb(159−389) is identical to that detected for transformants of pLexA-UbxIb(159−242), and corresponds to ∼50% of the reporter gene activation observed in the full-length protein (Figure 3, Table 1). Extension of the N-terminus to residue 68 results in full activation function. These observations, in concert with the lack of influence of successive deletions from the carboxyl-terminal portion of UbxIb (Figure 1), demonstrate that the carboxyl-terminal portion of UbxIb from residues 242 to 389 that contains the DNA binding domain is not essential for transactivation function. These data are consistent with previous studies indicating that residues required for transactivation activity were within amino acids 1−260 or 36−226 (*3*, *8*, *14*).

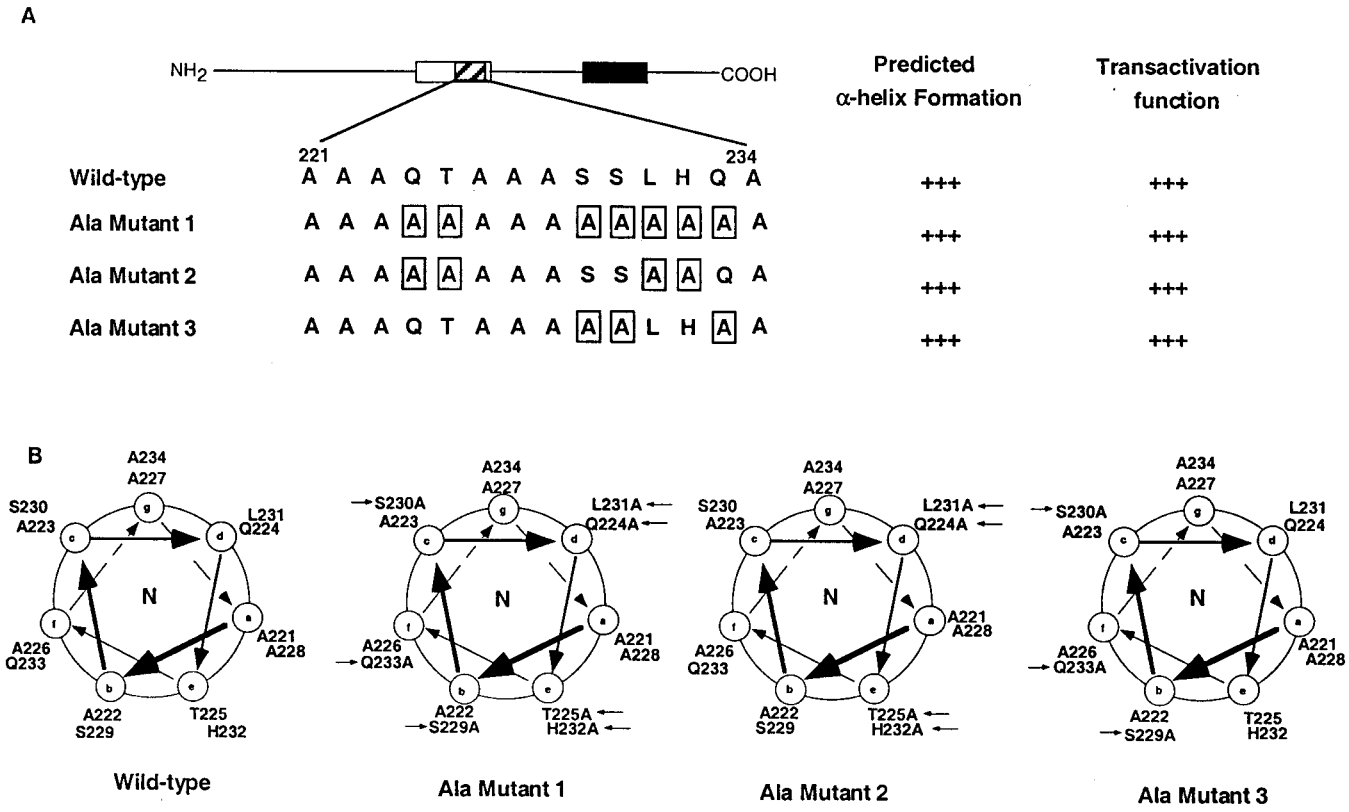

FIGURE 7: Transactivation activity for site-specific mutants to alter the surface of the putative α-helix by alanine substitution. As in Figure 5, mutations were introduced in full-length UbxIb protein. Sequences for mutant proteins that alter the surfaces presented by a helical structure are indicated. These sequence alterations did not affect the predicted formation of helix for this region. The helical wheels show the surface residues for the wild-type and mutant proteins. β-Galactosidase activity in yeast was quantified to assess transactivation activity (Table 1), and results of CAT assay in S2 cells are shown in Figure 6.

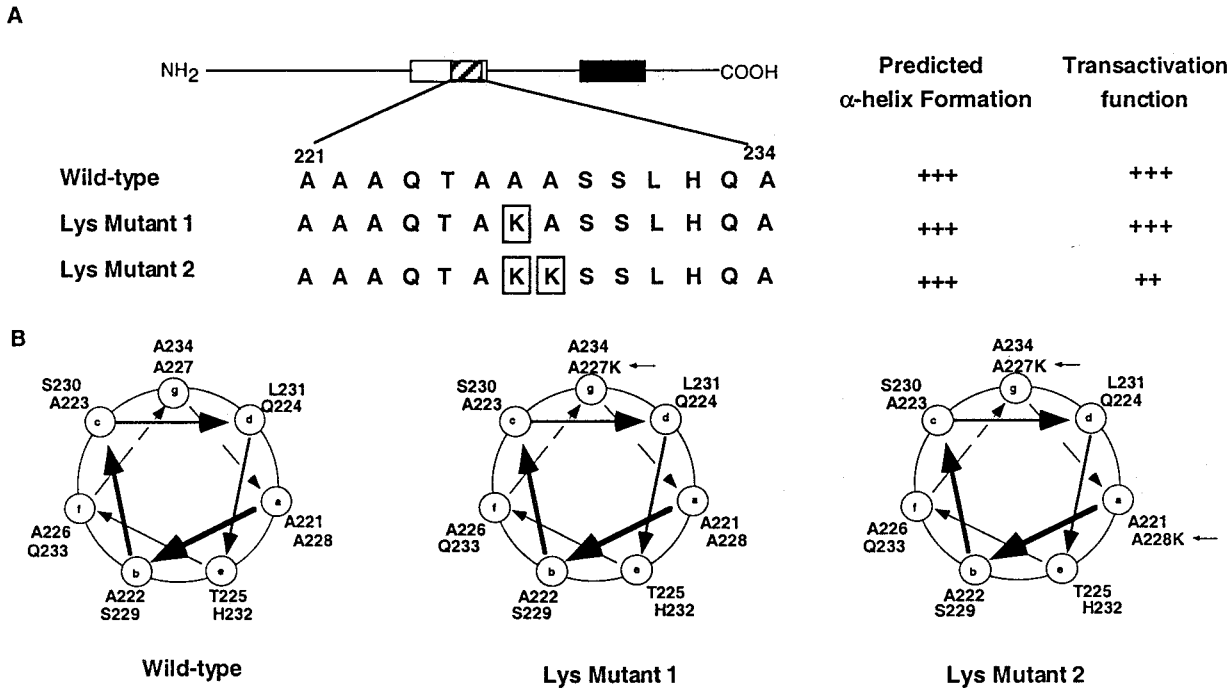

FIGURE 8: Transactivation activity for site-specific mutants to alter the surface of the putative α-helix by lysine substitution. As in Figure 5, mutations were introduced within the α-helix region in the full-length UbxIb protein. The sequence alterations and helical wheels that locate these alterations are shown. The transactivation activities indicated were derived from the color of yeast colonies, and results of CAT assay in S2 cells are shown in Figure 6.

Deletion mutant data demonstrate that amino acids 159−242 in isolation can partially activate transcription. This observation is analogous to other proteins in which successive deletions sequentially reduce activation function, a result traditionally interpreted as evidence for unstructured activation domains (*63*−*65*). The NH_2-terminal portion of UbxIb

(residues 68−242) exhibited activity comparable to full-length UbxIb, and UbxIb(159−242) was 50% as active, while UbxIb(68−216), UbxIb(159−216), and UbxIb(216−389) were unable to activate transcription (Figure 3 and Table 1). Thus, amino acid residues 216−242 are necessary, but not sufficient alone, for activation. Within this region, amino acids 221−234 are predicted to form an α-helix by several algorithms (Figure 4). The GOR-IV algorithm, which scores the strength of the prediction, assigns a higher probability to the α-helix in this region than even the known helices in the homeodomain (*61*). Helix capping motifs are necessary to satisfy unused hydrogen bonds at either end of the α-helix. Consistent with the results of secondary structure prediction, residues near the ends of this region are compatible with Ia (h−xpxhx) N-terminal and Va (hxpx−nxh) C-terminal helix capping motifs, where h is a hydrophobic residue, x is any residue, p is a polar residue, n is a nonbranched residue, and − is the boundary between the rest of the protein and the helix (*66*). Residues 221−234 are rich in alanine and glutamine, very similar to alanine/glutamine-rich sequences that form helices in solution as isolated peptides (*67*−*69*). Furthermore, a single amino acid change only shortened the predicted helix; multiple mutations were required to disrupt the entire predicted α-helix. In composite, these results strongly support the presence of an α-helix for amino acids 221−234.

Because the predicted α-helix falls within the core activation domain, it may contribute directly to activation function either in a structural role or by producing a surface required for specific interactions. Proline substitutions, designed to destroy the predicted helical structure, were introduced into the full-length protein; activation by these mutant proteins was tested in both yeast and S2 cells (Table 1, Figures 5 and 6). All these mutations obliterated transcription activation by UbxIb, despite the fact that protein expression was not compromised. Indeed, the proline mutations resulted in repression relative to the absence of Ubx. Because loss of activation is seen even in the yeast one-hybrid assay, the effects caused by these mutations are not the result of altered DNA binding. Collectively, these results demonstrate that the predicted α-helical region is critical for transcription activation by UbxIb. This requirement for secondary structure is surprising in light of evidence suggesting that secondary structure is not generally required in acidic activation domains (*51*−*53*, *55*, *65*, *70*, *71*). However, some activation domains have "loose" secondary structure (*72*), and binding to general transcription factors may induce secondary structure in activation domains (*51*−*53*, *57*, *73*). Different classes of activation domains are likely to function by different mechanisms. Other activation domains will have to be characterized to determine if α-helices are a requisite part of activation domains for related proteins or if this structure is a unique attribute of UbxIb.

To distinguish structural from interactive roles for the predicted α-helix, multiple residues were altered to create alanine surfaces on the predicted α-helix (Figures 6B and 7). The most extreme mutant consisted of only alanine residues within the predicted helical region between amino acids 221 and 234. Because polyalanine is known to form α-helices (*67*−*69*), the ability of this alanine mutant to activate transcription further supports our hypothesis that residues 221−234 are helical in the wild-type protein. All of the alanine mutants activate transcription at least as well as wild-type UbxIb, and perhaps better, demonstrating that unique side chains in this region are not required for transactivation. Selected alanines in the wild-type sequence were replaced with lysines in a third set of mutants to determine whether the hydrophobic surface presented by the alanine side chains was in some way essential for function (Figures 6B and 8). While Ubx(A227K) has lower activation capacity than wild-type Ubx, the level is above background. The A227K/A228K double mutant has activity similar to wild-type. The transactivation capacity of these derivatives indicates that the alanine surface is not a critical component of a specific interface. Together, these results strongly suggest that the presence of helical secondary structure between amino acids 221 and 234, and not specific amino acid side chains, is requisite for transcription activation by UbxIb.

Transcriptional regulation by Ubx is determined by *both* activation and repression functions. For many transcription factors, the balance between activation and repression is regulated by a discrete "switch" such as dimerization, interaction with a co-repressor or activator, or ligand binding, or can be defined by the spacing of DNA binding sites relative to the promoter (*74*). The source of this switch, if any, for Ubx is unknown. However, because Ubx responds differently to the *Antp* and the *Ubx* enhancers in vivo (*3*), the DNA clearly has some role in determining transcriptional outcome, either by directly influencing Ubx or by recruiting co-repressors or activators. Intriguingly, the Ubx proline mutants used in this study actually repress transcription (i.e., reporter gene expression levels are significantly lower with these mutants than in the absence of the protein, see Figure 6), even though the target is a sequence that normally results in activation by Ubx in S2 cells (*3*). Furthermore, this repression is stronger than wild-type Ubx repression observed for the repression reporter plasmid pAntp-CAT (data not shown, *3*). While DNA sequences bound may contribute to selection of activation or repression, these data indicate that variant Ubx is capable of repressing transcription even from DNA sequences that normally mediate activation. Thus, regulation at a specific site by Ubx may rely on both instruction provided by the DNA (such as binding site spacing or binding by other transcription factors) and specific properties of the Ubx protein.

Mapping individual functions such as transcription activation to Ubx sequence permits analysis of which specific aspects of Ubx function are required for different morphologies in vivo. For instance, Ubx ectopically expressed from the heat shock promoter can induce thoracic to parasegment 6 transformations of first instar larval cuticles (*15*, *16*). Ubx deletion mutants missing most of the activation domain including part of the requisite α-helix can still effect this transformation (*16*). Therefore, parasegment 6 cuticle patterning by Ubx most likely relies on transcription repression. As more data become available delineating activation and repression domains for other Hox proteins, the role of transcription activation/repression in generating tissue-specific identities may be ascertained from experiments with chimeric proteins.

Domain organization of Ubx was compared to several other Hox proteins for which the general locale of the activation domain has been defined (Figure 9) (*14*, *17*−*23*, *63*, *75*, *76*). The position of the activation domain relative

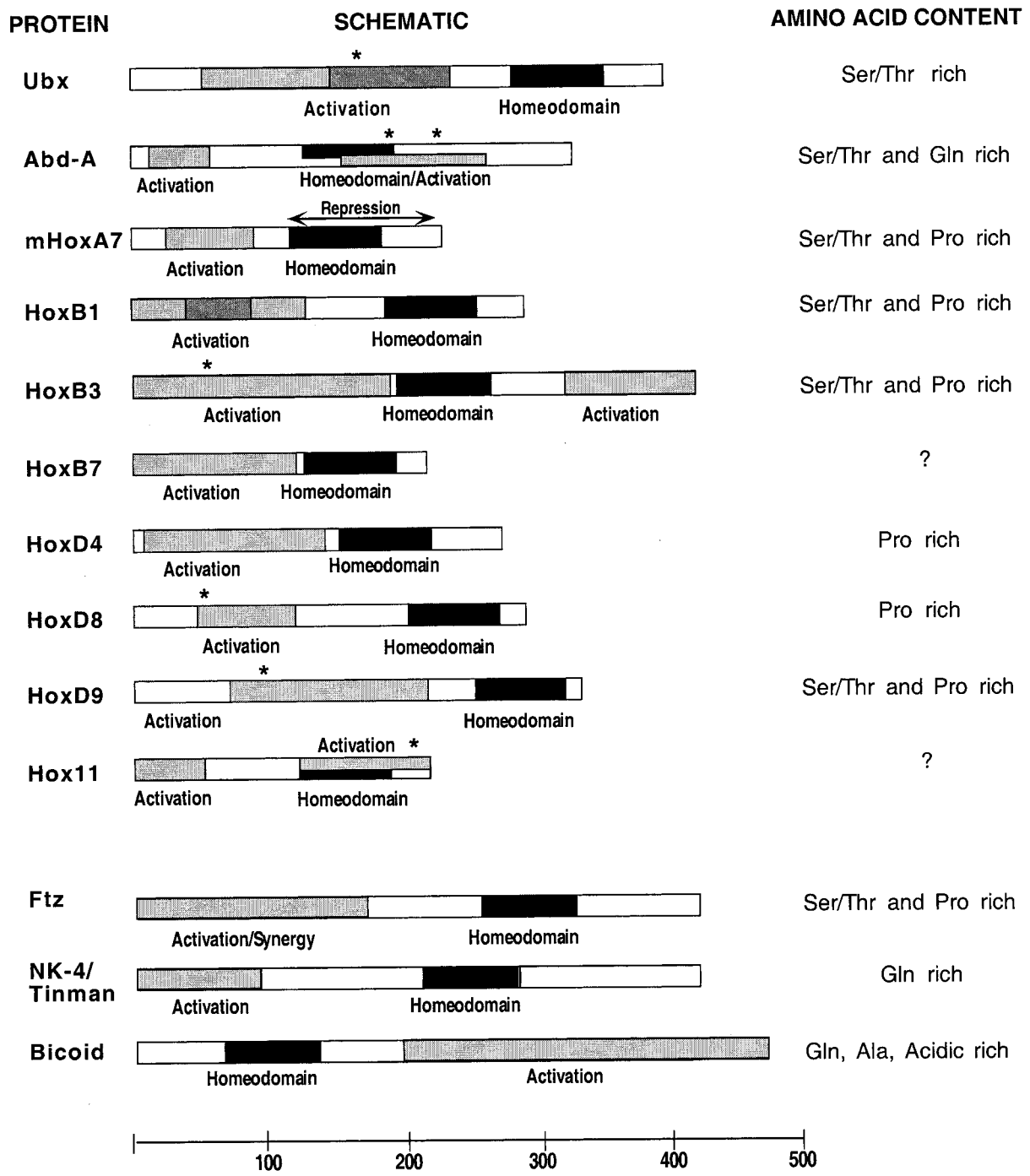

FIGURE 9: Comparison of activation domains in different homeodomain proteins. The arrangement of the regions involved in transcriptional activation for several homeoproteins is depicted (*14*, *17*−*23*, *63*, *75*, *76*). Although the mapping resolution varies between these proteins, the differences in placement of homeodomain and activation domains are nonetheless apparent. The solid bars indicate the homeodomain, and the gray regions correspond to regions that contribute to transcription activation. Where shades of stippling are present, darker regions indicate sequences that are required for transactivation activity, while lighter sequences indicate regions that enhance activity. A ruler for sequence length in amino acid residues is provided at the bottom of the figure. The dominant amino acid composition of the transactivation regions is also indicated. The locations of predicted helices in activation domains are indicated by an (*). The predicted α-helices in HoxB3, HoxD8, and HoxD9 activation domains are polyalanine sequences four to six amino acids in length. Hox11 and Abd-A have long predicted helices C-terminal to the homeodomain, each containing greater than 50% charged residues. Neither pattern matches the predicted α-helix in the Ubx activation domain.

to the homeodomain is clearly not conserved among these proteins, although the activation domain is most frequently found N-terminal to the homeodomain. Interestingly, the distance between the activation domain and homeodomain varies considerably among these proteins. In Hox11 and Abd-A, the DNA binding domain itself is required for transactivation (*14*, *17*, *18*), and in HoxB3, portions of the activation domain are found on either side of the homeodomain (*23*). These differences in the domain organization of Hox proteins may determine which protein·protein contacts are sterically feasible and thereby influence the nature of the transcription complex that can form. Indeed, Hox proteins differentially interact with general transcriptional factors (*77*−*79*). Differences in the ability to form specific protein complexes

ORGANISM	SEQUENCE	ACCESSION NUMBER
D. melanogaster	TISGAAA·QTAAASSLHQASNHTFYPWM	P02834
D. virilis	··SGAAAAQTAAASSLHQASNHTFYPWM	U03180
D. hydei	··SGAAAAQTAAASSLHQASNHTFYPWM	U03178
D. pseudoobscura	TISGAAAAQTAAASSLHQASNHTFYPWM	X05179
D. funebris	TISGAAAAQTAAASSLHQPGNHTFYPWM	X05177
Musca domestica	TISGAAAAQTA··SSLHQASNHTFYPWM	X05178
Anopheles gamiae	SLTGSTGGQAAPSTGLHQ·SNHTFYPWM	AF080562
Acanthokara kaputensis	TTSQVNP··SSETTP·····FYPWM	AF011282

FIGURE 10: Comparison of protein sequences corresponding to the predicted α-helical region for Ubx orthologs. Available sequences for Ubx orthologs in insects were collected from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The sequences N-terminal to the YPWM motif were aligned. Secondary structure prediction was performed as described in the legend to Figure 4, and the regions that were predicted to form α-helical regions are indicated by an underline.

required for transcription may contribute, at least in part, to distinctions in the functional roles of these proteins (*5*).

The amino acid content of activation domains also varies among Hox proteins. According to the relative abundance of particular amino acids, activation domains have been divided into four classes: glutamine-rich, proline-rich, serine/threonine-rich, and acid-rich (*51*, *54*, *74*). Examination of UbxIb residues 159−242 reveals only two acidic residues, three prolines, and two glutamines. However, the proportion of serine and threonine in this region of the UbxIb core is ∼20%, while in the rest of UbxIb, the proportion of serine and threonine is only ∼10%. The high serine and threonine content of the UbxIb activation domain indicates that posttranslational modification may affect transactivation by UbxIb (*41*).

Phosphorylation within the 159−242 region has been demonstrated in vivo, and previous data on transcriptional activation in vivo suggested the potential importance of this region in activation (*41*). Furthermore, all predicted glycosylation sites (at residues Asn186, Asn215, and Asn236) are found in the region of UbxIb identified in this study as the activation domain. However, none of the phosphorylation and glycosylation sites fall within the α-helix, so the mutations introduced are unlikely to affect posttranslational modifications. The precise role of phosphorylation and/or glycosylation in the transactivation function of UbxIb, if any, will not be clearly understood until both the structure of this portion of UbxIb and its cellular target(s) have been defined.

Known activation domains for other Hox proteins are either glutamine-rich, proline-rich, acid-rich, or *both* serine/threonine- and proline-rich, making the composition of the Ubx activation domain unique thus far within the Hox family. Each of these classes of activation domains appears to interact with different targets within the general transcription machinery (*63*, *77*). For instance, the alanine-rich activation domain of bicoid interacts with $TAF_{II}60$, while the glutamine-rich activation domain of bicoid interacts with $TAF_{II}110$ (*63*). Conversely, both Abd-B and Antp have extremely glutamine-rich domains in their N terminus that interact with TFIIEβ (*77*). Interestingly, not all Hox activation domains contain predicted α-helices. For those activation domains that do contain an α-helix, the position within the domain and relative to the homeodomain and YPWM motifs varies (Figure 9). Thus, position, sequence, and predicted secondary structure vary for activation domains in Hox proteins. Presumably, these differences extend to the mechanism of transcription activation and may provide one mechanism for functional specificity in vivo within this family of proteins. The detailed view of the Ubx activation domain presented here opens the possibility for pursuing structural studies that may expand our understanding of transcription activation in this important family of regulatory proteins. Further, the identification of point mutations that abrogate transcriptional activation capacity may be useful in examining Ubx regulatory function in vivo.

Because Hox genes control segment identity and morphology, they are key factors responsible for insect diversity (*80*). For instance, changes in the pattern of *Dll* regulation by Ubx correlate with differences in wing morphology between insects (*80*, *81*). Because the homeodomain, YPWM motif, and C-terminus are important for segment identity and are highly conserved between insect species, diversity seems likely to have arisen from differences in which genes are regulated, distinctions in Hox functional properties, or via subtle changes in Hox expression patterns (*81*, *82*). Indeed, functional distinctions between *Drosophila* and onychophora Ubx arise from sequences outside the homeodomain (*24*), indicating that function other than DNA binding may be key to Ubx evolution. We have shown that a specific region, the putative α-helix N-terminal to the YPWM motif, is critical for transcription activation by Ubx. Interestingly, this helix is conserved in *Drosophila* and *Musca*, whereas prolines and/or glycines disrupt this predicted helix in *Anopheles* (mosquito) and onychophora (Figure 10). This result suggests that differences in Ubx function may be important for diversification of body structures. Further experiments are required to determine whether more divergent Ubx proteins activate transcription in a pattern similar to that for *Drosophila* Ubx and whether distinctions in this function may be tied to differences in body form between species.

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REFERENCES

1. Lewis, E. B. (1978) *Nature 276*, 565−570.
2. Gellon, G., and McGinnis, W. (1998) *BioEssays 20*, 116−125.
3. Krasnow, M. A., Saffman, E. E., Kornfeld, K., and Hogness, D. S. (1989) *Cell 57*, 1031−1043.
4. Ekker, S. C., Young, K. E., von Kessler, D. P., and Beachy, P. A. (1991) *EMBO J. 10*, 1179−1186.
5. Li, X., and McGinnis, W. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 6802−6807.
6. Capovilla, M., Brandt, M., and Botas, J. (1994) *Cell 76*, 461−475.
7. Winslow, G. M., Hayashi, S., Krasnow, M., Hogness, D. S., and Scott, M. P. (1989) *Cell 57*, 1017−1030.
8. Saffman, E. E., and Krasnow, M. A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 7420−7424.
9. Dessain, S., Gross, C. T., Kuziora, M. A., and McGinnis, W. (1992) *EMBO J. 11*, 991−1002.
10. Li, X., Veraksa, A., and McGinnis, W. (1999) *Development 126*, 5581−5589.

11. Mann, R. S., and Affolter, M. (1998) *Curr. Opin. Genet. Dev. 8*, 423−429.
12. Beachy, P. A., Varkey, J., Young, K. E., von Kessler, D. P., Sun, B. I., and Ekker, S. C. (1993) *Mol. Cell. Biol. 13*, 6941−6956.
13. Bondos, S. E., and Tan, X.-X. (2001) *Crit. Rev. Eukaryotic Gene Expression 11*, 157−183.
14. Samson, M.-L., Jackson-Grusby, L., and Brent, R. (1989) *Cell 57*, 1045−1052.
15. Chauvet, S., Merabet, S., Bilder, D., Scott, M. P., Pradel, J., and Graba, Y. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 4064−4069.
16. Mann, R. S., and Hogness, D. S. (1990) *Cell 60*, 597−610.
17. Masson, N., Greene, W. K., and Rabbitts, T. H. (1998) *Mol. Cell. Biol. 18*, 3502−3508.
18. Zhang, N., Shen, W.-F., Ho, A. D., and Lu, M. (1996) *Oncogene 13*, 1781−1787.
19. Schnabel, C. A., and Abate-Shen, C. (1996) *Mol. Cell. Biol. 16*, 2678−2688.
20. Chariot, A., Princen, F., Gielen, J., Merville, M.-P., Franzoso, G., Brown, K., Siebenlist, U., and Bours, V. (1999) *J. Biol. Chem. 274*, 5318−5325.
21. Saleh, M., Rambaldi, I., Yang, X.-J., and Featherstone, M. S. (2000) *Mol. Cell. Biol. 20*, 8623−8633.
22. Zappavigna, V., Sartori, D., and Mavilio, F. (1994) *Genes Dev. 8*, 732−744.
23. Vigano, M. A., Di Rocco, G., Zappavigna, V., and Mavilio, F. (1998) *Mol. Cell. Biol. 18*, 6201−6212.
24. Grenier, J. K., and Carroll, S. B. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 704−709.
25. Gehring, W. J., Affolter, M., and Bürglin, T. (1994) *Annu. Rev. Biochem. 63*, 487−526.
26. Lewis, E. B. (1963) *Am. Zool. 3*, 33−56.
27. Hooper, J. E. (1986) *EMBO J. 5*, 2321−2329.
28. Teugels, E., and Ghysen, A. (1985) *Nature 314*, 558−561.
29. Bienz, M., and Tremml, G. (1988) *Nature 333*, 576−578.
30. Castelli-Gair, J., Müller, J., and Bienz, M. (1992) *Development 114*, 877−886.
31. McGinnis, W., and Krumlauf, R. (1992) *Cell 68*, 283−302.
32. Roch, F., and Akam, M. (2000) *Development 127*, 97−107.
33. Weatherbee, S. D., Halder, G., Kim, J., Hudson, A., and Carroll, S. (1998) *Genes Dev. 12*, 1474−1482.
34. Subramaniam, V., Bomze, H. M., and López, A. J. (1994) *Genetics 136*, 979−991.
35. Bomze, H. M., and López, A. J. (1994) *Genetics 136*, 965−977.
36. Busturia, A., Vernos, I., Macias, A., Casanova, J., and Morata, G. (1990) *EMBO J. 9*, 3551−3555.
37. O'Connor, M. B., Binari, R., Perkins, L. A., and Bender, W. (1988) *EMBO J. 7*, 435−445.
38. Kornfeld, K., Saint, R. B., Beachy, P. A., Harte, P. J., Peattie, D. A., and Hogness, D. S. (1989) *Genes Dev. 3*, 243−258.
39. Johnson, F. B., and Krasnow, M. A. (1990) *Genes Dev. 4*, 1044−1052.
40. Mortin, M. A., Zuerner, R., Berger, S., and Hamilton, B. J. (1992) *Genetics 131*, 895−903.
41. Gavis, E. R., and Hogness, D. S. (1991) *Development 112*, 1077−1093.
42. Estojak, J., Brent, R., and Golemis, E. A. (1995) *Mol. Cell. Biol. 15*, 5820−5829.
43. Chen, D.-C., Yang, B.-C., and Kuo, T.-T. (1992) *Curr. Genet. 21*, 83−84.
44. Gietz, R. D., and Schiestl, R. H. (1991) *Yeast 7*, 253−263.
45. Sherman, F. (1991) *Methods Enzymol. 194*, 3-21.
46. Brent, R., and Ptashne, M. (1985) *Cell 43*, 729−736.
47. Schneider, I. (1972) *J. Embryol. Exp. Morphol. 27*, 353−365.
48. López, A. J., and Hogness, D. S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9924−9928.
49. Hanes, S. D., and Brent, R. (1989) *Cell 57*, 1275−1283.
50. Chae, S.-K., Lee, N.-S., Lee, K.-J., and Kim, E. (1998) *FEBS Lett. 423*, 235−238.
51. Triezenberg, S. J. (1995) *Curr. Opin. Genet. Dev. 5*, 190−196.
52. Tjian, R., and Maniatis, T. (1994) *Cell 77*, 5−8.
53. Hahn, S. (1993) *Cell 72*, 481−483.
54. Mitchell, P. J., and Tjian, R. (1989) *Science 245*, 371−378.
55. Donaldson, L., and Capone, J. P. (1992) *J. Biol. Chem. 267*, 1411−1414.
56. Leuther, K. K., Salmeron, J. M., and Johnston, S. A. (1993) *Cell 72*, 575−585.
57. Van Hoy, M., Leuther, K. K., Kodadek, T., and Johnston, S. A. (1993) *Cell 72*, 587−594.
58. Garnier, J., Osguthorpe, D. J., and Robson, B. (1978) *J. Mol. Biol. 120*, 97−120.
59. Chou, P. Y., and Fasman, G. D. (1978) *Annu. Rev. Biochem. 47*, 251−276.
60. Kneller, D. G., Cohen, F. E., and Langridge, R. (1990) *J. Mol. Biol. 214*, 171−182.
61. Passner, J. M., Ryoo, H. D., Shen, L., Mann R. S., and Aggarwal, A. K. (1999) *Nature 397*, 714−719.
62. Beachy, P. A., Krasnow, M. A., Gavis, E. R., and Hogness, D. S. (1988) *Cell 55*, 1069−1081.
63. Sauer, F., Hansen, S. K., and Tjian, R. (1995) *Science 270*, 1825−1828.
64. Hope, I. A., Mahadevan, S., and Struhl, K. (1988) *Nature 333*, 635−640.
65. Melcher, K. (2000) *J. Mol. Biol. 301*, 1097−1112.
66. Aurora, R., and Rose, G. D. (1998) *Protein Sci. 7*, 21−38.
67. Shalongo, W., Dugad, L., and Stellwagen, E. (1994) *J. Am. Chem. Soc. 116*, 8288−8293.
68. Vila, J. A., Ripoll, D. R., and Scheraga, H. A. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 13075−13079.
69. Scholtz, J. M., Qian, H., Robbins, V. H., and Baldwin, R. L. (1993) *Biochemistry 32*, 9668−9676.
70. O'Hare, P., and Williams, G. (1992) *Biochemistry 31*, 4150−4156.
71. Shen, F., Triezenberg, S. J., Hensley, P., Porter, D., and Knutson, J. R. (1996) *J. Biol. Chem. 271*, 4819−4826.
72. Lee, H., Mok, K. H., Muhandiram, R., Park, K.-H., Suk, J.-E., Kim, D.-H., Chang, J., Sung, Y. C., Choi, K. Y., and Han, K.-H. (2000) *J. Biol. Chem. 275*, 29426−29432.
73. Wright, P. E., and Dyson, H. J. (1999) *J. Mol. Biol. 293*, 321−331.
74. Roberts, S. G. E. (2000) *Cell. Mol. Life Sci. 57*, 1149−1160.
75. Ananthan, J., Baler, R., Morrissey, D., Zuo, J., Lan, Y., Weir, M., and Voellmy, R. (1993) *Mol. Cell. Biol. 13*, 1599−1609.
76. Choi, C. Y., Lee, Y. M., Kim, Y. H., Park, T., Jeon, B. H., Schulz, R. A., and Kim, Y. (1999) *J. Biol. Chem. 274*, 31543−31552.
77. Zhu, A., and Kuziora, M. A. (1996) *J. Biol. Chem. 271*, 20993−20996.
78. Um, M., Li, C., and Manley, J. L. (1995) *Mol. Cell. Biol. 15*, 5007−5016.
79. Zhang, H., Catron, K. M., and Abate-Shen, C. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1764−1769.
80. Weatherbee, S. D., Nijhout, H. F., Grunert, L. W., Halder, G., Galant, R., Selegue, J., and Carroll, S. (1999) *Curr. Biol. 9*, 109−115.
81. Bennett, R. L., Brown, S. J., and Denell, R. E. (1999) *Dev. Genes Evol. 209*, 608−619.
82. Chan, S.-K., and Mann, R. S. (1993) *Genes Dev. 7*, 796−811.
83. Johnson, F. B., Parker, E., and Krasnow, M. A. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 739−743.
84. Peifer, M., and Wieschaus, E. (1990) *Genes Dev. 4*, 1209−1223.
85. Rauskolb, C., Peifer, M., and Wieschaus, E. (1993) *Cell 74*, 1101−1112.
86. Rauskolb, C., and Wieschaus, E. (1994) *EMBO J. 13*, 3561−3569.

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