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HOXB6 Protein Is Bound to CREB-binding Protein and Represses Globin Expression in a DNA Binding-dependent, PBX Interaction-independent Process*[S]

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Although *HOXB6* and other *HOX* genes have previously been associated with hematopoiesis and leukemias, the precise mechanism of action of their protein products remains unclear. Here we use a biological model in which HOXB6 represses α- and γ-globin mRNA levels to perform a structure/function analysis for this homeodomain protein. HOXB6 protein represses globin transcript levels in stably transfected K562 cells in a DNA-binding dependent fashion. However, the capacity to form cooperative DNA-binding complexes with the PBX co-factor protein is not required for HOXB6 biological activity. Neither the conserved extreme N-terminal region, a polyglutamic acid region at the protein C terminus, nor the Ser^{214} CKII phosphorylation site was required for DNA binding or activity in this model. We have previously reported that HOX proteins can inhibit CREB-binding protein (CBP)-histone acetyltransferase-mediated potentiation of reporter gene transcription. We now show that endogenous CBP is co-precipitated with exogenous HOXB6 from nuclear and cytoplasmic compartments of transfected K562 cells. Furthermore, endogenous CBP co-precipitates with endogenous HOXB6 in day 14.5 murine fetal liver cells during active globin gene expression in this tissue. The CBP interaction motif was localized to the homeodomain but does not require the highly conserved helix 3. Our data suggest that the homeodomain contains most or all of the important structures required for HOXB6 activity in blood cells.

The HOX homeodomain (HD)[1] proteins function as master regulators of the body plan (1). In addition, *HOX* genes are expressed in blood cells in lineage-specific patterns (2, 3), and HOX proteins are important in the growth and differentiation of normal bone marrow cells and in leukemias (4). We cloned two alternatively spliced *HOXB6* cDNAs from HEL cells, a human leukemic cell line with erythroid/myeloid bipotential differentiation capacity (3, 5). In a previous study, enforced expression of one of these cDNAs that encoded a 224-amino acid, HD-containing HOXB6 protein repressed the erythroid phenotype in human leukemic cells, as reflected by the loss of α-, β-, and γ-globin gene expression; loss of erythroid surface markers; and down-regulation of heme synthesis (6). However, it has proven difficult to describe a precise biochemical mechanism of action to account for these effects. Similarly, despite genetic studies showing that *HOX* genes influence the expression of downstream targets, the efforts of numerous investigators have yielded few mechanistic details to explain the action of HOX proteins.

Although they are thought to function as transcription factors, most full-length HOX proteins, including HOXB6, bind only weakly by themselves to DNA targets containing a TAAT sequence (7–9). Several laboratories demonstrated that HOX proteins exhibited weak activation or repression on reporter genes containing either TAAT multimers (reviewed in Ref. 10) or *HOX* gene auto-regulatory elements containing TAAT sequences (11, 12). However, in our own studies, HOXB6 and other HOX proteins did not produce changes in transient reporter gene assays using either synthetic TAAT multimers or putative gene regulatory regions (13). We and others demonstrated that HOX proteins gain both DNA binding avidity (9) and site specificity by forming DNA-binding complexes with the PBX HD proteins (reviewed in Ref. 14). For the HOX proteins from paralog groups 1–8, binding as heterodimers with PBX was far stronger than binding as monomers to TAAT sites (9, 15). Transgenic reporter studies demonstrated the importance of consensus PBX-HOX sites within auto- or cross-regulatory regions of *HOX* genes (16, 17). However, except for the auto-regulatory sites, few mammalian gene targets have been identified that contain PBX-HOX sites, and no direct gene targets of any type have been reported for HOXB6. Indeed, reports that other HOX proteins activate important genes, including p21 (18), β-fibroblast growth factor (19), the progesterone receptor (20), and p53 (21) all relied on computerized identification of TAAT sequences as putative binding sites. Biochemical and genetic evidence suggests that the *Drosophila* DFD protein (HOXB4 homolog) directly activates the apoptosis protein *reaper* (22). However, the putative DFD-binding sites do not contain PBX/EXD consensus motif, adding further confusion regarding whether PBX-HOX sequences are the biologically relevant regulatory binding sites. These studies, showing HOX proteins as activators, contrast to a large body of data suggesting that these proteins function as genetic repressors (8, 23, 24). It has been proposed that HOX proteins function

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental tables.

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[1] The abbreviations used are: HD, homeodomain; CREB, cyclic AMP response element binding protein; CBP, CREB-binding protein; HAT, histone acetyltransferase; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; EMSA, electrophoretic mobility shift assay; PIM, PBX interaction motif; PKA, cAMP-dependent protein kinase; GST, glutathione *S*-transferase; IP, immunoprecipitation; PKC, protein kinase C; CK II, casein kinase II.

alone as repressors and are converted to activators by forming cooperative DNA-binding complexes with PBX/EXD (25). Thus, despite the efforts of many laboratories, little progress has been made in defining biologic targets and the biochemical mechanism of action of the HOX proteins.

The CBP/p300 proteins have been a focus of interest because they appear to link transcription factors to chromatin remodeling mechanisms, thus facilitating eukaryotic gene transcription (26, 27). CBP/p300 are thought to increase general transcription *via* their histone acetyltransferase (HAT) activity (26, 27). One model is that CBP/p300 function by mediating the acetylation of histones within the nucleosome core, thereby reducing DNA interactions and facilitating and/or stabilizing steric changes that permit increased access of the general transcriptional machinery to target genes (28). An alternative mechanism by which CBP-HAT may regulate transcription is through the direct acetylation of transcription factors, thus modulating their activity. The GATA1 transcription factor plays a critical role in red cell development (29). CBP interacts with the GATA1 protein and is required for red cell differentiation (30). Consistent with the second model, CBP directly acetylates the GATA1 protein, which increases its transcriptional activity (31, 32). In addition, a recent study suggested that the Pu.1 protein blocks erythroid differentiation by blocking the CBP-HAT-catalyzed acetylation of GATA1 and other proteins (33).

We previously demonstrated that HOXB6 and other HOX proteins bind to CBP/p300 and inhibit their HAT activity (13). HOXB6 blocked CBP-HAT-dependent, transient *in vivo* reporter gene transcriptional activity. Conversely, CBP prevented DNA binding by HOXB6 and other HOX proteins. To gain insights into the mechanism of action of HOXB6, we have performed a structure/function analysis, using a modified version of our previously described system, in which the biological activity of HOXB6 can be readily observed as readout. In addition, our data on CBP-HOX protein interactions stimulated us to explore the importance of HOXB6-CBP interactions in this model system.

MATERIALS AND METHODS

Establishment of HOXB6-expressing Cell Lines—A full-length human *HOXB6* cDNA (5), engineered to encode a full-length protein fused to an N-terminal FLAG epitope, was cloned into a bicistronic murine stem cell virus retroviral vector in which an internal ribosomal entry site allows GFP expression (gift from K. Humphries). Standard techniques were used with the ExSite mutagenesis kit (Stratagene) to produce a series of mutant HOXB6 proteins. These included proteins in which asparagine 196 was changed to alanine (N196A) to disrupt DNA binding. Tryptophan 130 was changed to glycine (W130G) to disrupt PBX interactions. Serine 214 was changed to glutamic acid (S214E) or to alanine (S214A) to mimic a constitutively phosphorylated molecule or one incapable of phosphorylation on Ser^{214}. A set of deletion mutants were constructed in which ΔNterm is missing the first 12 amino acids; ΔCterm is missing 9 amino acids; and Δ119, Δ127, and Δ134 are missing amino acids 1–119, 1–127, and 1–134, respectively. ΔHD is missing amino acids 135–224, whereas HDΔhelix 3 represents a protein extending from 135–224 but missing amino acids 187–203. The correct mutations were confirmed by DNA sequencing. Each of the mutant proteins was checked by expression as a T7 epitope-tagged fusion protein in the TnT system (Promega). Viral supernatants prepared in 293T cells using helper plasmids (34) were used to infect K562 cells in two rounds of spinoculation. The cells were sorted for GFP expression and expanded to produce lines. Most experiments were performed within a 20-day period after sorting when HOX protein levels were relatively high. A few experiments were performed using cells in which exogenous HOXB6 expression levels were similar to those of the endogenous protein.

Immunochemistry Studies—Immunoblotting, immunohistochemical localization, co-immunoprecipitation, and Western blotting were all performed following standard procedures with appropriate secondary antibodies as outlined (35). Antisera used for chromatin IP were: rabbit α-acetyl lysine histone 3 (Upstate Biotechnology Inc.), murine α-FLAG (Sigma), and rabbit α-HOXB6 against the N-terminal 128 amino acids (antiserum 1), as described previously (36). Antibodies used for HOXB6 immunoprecipitation were either the α-HOXB6 antiserum 1 or a mixture of two rabbit antisera (antiserum 2) against peptides RKSDCAQDKSVFGET and ESKLLSASQLSAEE, respectively. These peptides are from regions of the HOXB6 protein N- and C-terminal to the HD, which are conserved between human and mouse. Antibody 1 was also used for Western blotting and for immunohistochemistry experiments in combination with the TSA Cy3 amplification kit (704A; PerkinElmer Life Sciences). For immunohistochemical staining, α-HOXB6 antiserum 1 or a third antibody to HOXB6 (antiserum 3) (S20, Santa Cruz) was used. All three antisera to HOXB6 were shown to recognize the full-length protein by Western blotting and immunoprecipitation of *in vitro* translated protein (data not shown). Mouse α-T7 (Novagen) was used for immunoprecipitation of *in vitro* translated T7 epitope-tagged HOXB6 associated with DNA. Rabbit α-CBP (A-22, Santa Cruz) was used for immunoprecipitation, Western blotting, and histochemical detection, whereas a murine α-CBP (C-1; Santa Cruz) was used to confirm CBP co-precipitation with HOXB6 by Western blotting. Goat α-tubulin (C20) was used to confirm protein loading.

Determination of Globin mRNA Levels—Sixteen separate transfection experiments were performed for the HOXB6-GFP and the corresponding MIG control vector (Supplemental Tables I and II). Supplemental Tables III and IV show experiments in which the different mutant HOXB6 proteins were studied in addition to the parental HOXB6 and the MIG control vector. Following FACS separation of GFP+ cells, total RNA was isolated from each population using an RNAeasy kit (Qiagen) and quantitated by A_{260}. Approximately 15 μg of RNA from each cell pool was analyzed for human α-globin and γ-globin on separate Northern gels, using specific cDNA probes as previously described (6). For each experiment all of the samples to be compared were run on a single gel and Northern blotted, and the resulting filters were probed for either α-globin or γ-globin. Filters were stripped and reprobed for human β-actin to allow correction for loading differences. The blots were scanned using a Lacie Silver Scanner, and the bands were quantitated using National Institutes of Health Image software. The ratios of the γ-globin or α-globin signals to the actin signal, in the HOXB6-transfected cells and in the MIG vector-transfected control cells, are shown in Supplemental Tables I and II. The globin/actin signals vary between experiments because of efficiency of probe labeling, autoradiography times, and film development conditions. To better compare data between experiments, the globin/actin ratio for the MIG control in each experiment was arbitrarily set to 1.0 (see Supplemental Tables III and IV). The other globin/actin ratios within each experiment were then normalized to its MIG control. Changes in globin gene levels for each of the mutant HOXB6 proteins relative to the wild type HOXB6 protein were then compared using the mean values shown in Supplemental Tables III and IV.

Analysis of heme synthesis, EMSA analysis, CBP interaction studies, and phosphorylation of HOXB6 protein were performed as described (6, 9, 13, 37). HOXB6 proteins were measured by fractionating 15 μg of total cell extract by PAGE, followed by Western blotting with α-FLAG sera, hybridization with goat α-mouse IgG, and subsequent development for 30 min with ECL Plus (Amersham Biosciences).

RESULTS

A Cell Model for Analyzing HOXB6 Activity in K562 Cells—The purpose of the current study was to perform a structure/function analysis of the HOXB6 protein using a convenient cell culture model as a readout. Previously studies employed a replicon-based transfection assay that utilized a long drug selection protocol to show that HOXB6 down-regulated erythroid markers when expressed in erythroid cell lines (6). In the current study we utilized a bicistronic murine stem cell virus-derived retroviral vector coupled with FACS sorting of GFP-positive cells to rapidly obtain K562 lines expressing exogenous HOXB6 protein without prolonged drug selection (Fig. 1*A*). Using this protocol it is likely that multiple different viral integration sites are present in each pool of FACS-sorted cells. Endogenous *HOXB6* mRNA expression can be detected in K562 cells (3), but Western blotting revealed only weak signals for endogenous HOXB6 protein in vector-infected controls (not shown). In contrast, HOXB6 or a HOXB6 DNA-binding mutant

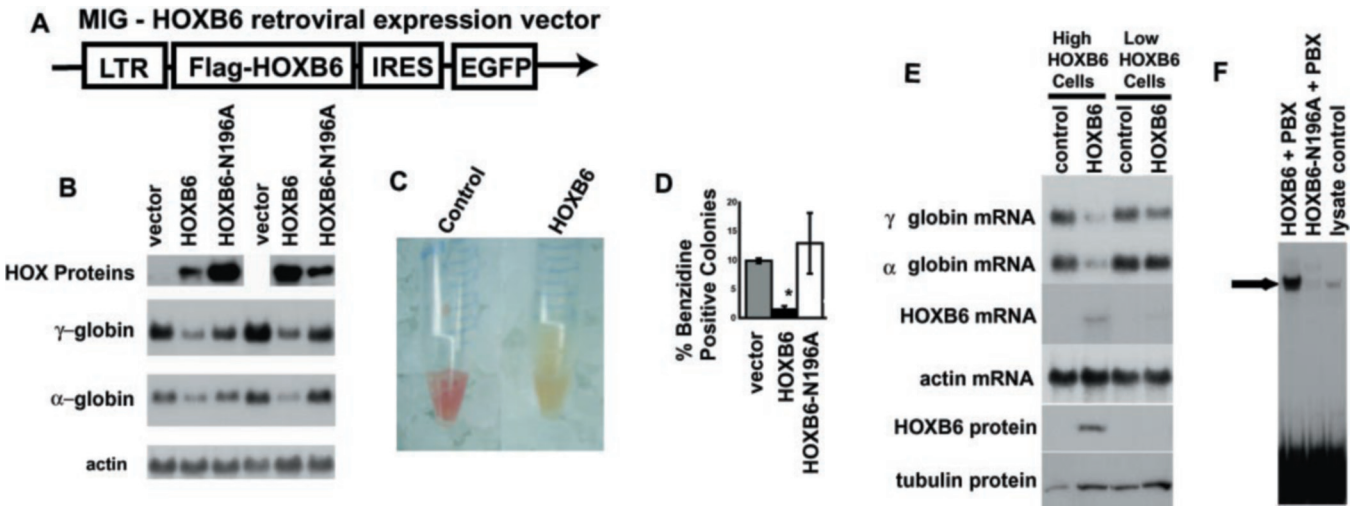

FIG. 1. **HOXB6 down-regulates globin mRNA levels in a DNA binding-dependent manner.** *A*, MIG-HOXB6 expression vector. IRES, internal ribosomal-binding protein; EGFP, enhanced green fluorescent protein. *B*, HOXB6 down-regulates γ- and α-globin mRNAs, but a DNA-binding mutant (HOXB6-N196A) protein does not. Globin mRNAs were measured by Northern analysis, and the HOX proteins were measured by Western blotting. The mean values for 16 separate transfection experiments for γ- and 15 experiments for α-globin expression in MIG and HOXB6-transfected cells are reported in Table I. *C*, HOXB6-expressing cells lose red color, reflecting low globin. *D*, HOXB6 down-regulates heme synthesis (benzidine staining). *E*, low HOXB6 expressing clone exhibits reduction in globin mRNA. *F*, EMSA showing HOXB6-N196A mutant protein cannot form DNA-binding complexes with PBX. *LTR*, long terminal repeat.

(HOXB6-N196A) protein was readily detected in representative stably transduced cell lines (Fig. 1*B*). The relative levels of the HOX proteins varied to some degree between infections, possibly reflecting differences in viral integration sites within chromatin. A qualitative estimate of the relative HOXB6 protein expression for the 16 separate transfection experiments is shown in Supplemental Table I. For most of the different infection experiments, the levels of HOXB6 protein were lower than or approximately equal to those detected for the HOXB6-N196A or other HOXB6 mutant proteins (Figs. 1, 3, and 4 and data not shown).

HOXB6 Represses Globin mRNA Levels—We first confirmed our earlier findings that the HOXB6 protein repressed globin mRNA levels. HOXB6 overexpression resulted in a statistically significant ($p < 0.0001$) 3–4-fold decrease in both γ- and α-gamma globin gene expression (Fig. 1*B* and Table I). Northern gel analysis showed that although there was variation in the absolute degree of globin mRNA repression among the 16 experiments (ranging from 1.6- to 50-fold for γ-globin and 1.7- to 12.5-fold for α-globin, respectively), there was a decrease in both transcripts for each experiment (see Supplemental Tables I–IV). The repression of globin mRNA levels roughly paralleled the estimated HOXB6 protein levels in the 16 experiments.

The production of mature hemoglobin, consisting of globin protein chains and the heme group, is a defining feature of terminal erythroid differentiation. K562 cells have been shown to express low levels of hemoglobin, reflecting their myeloid/erythroid progenitor bipotential phenotype (38). Cells in which HOXB6 was expressed were markedly white compared with the red parental K562 cells containing a control vector (Fig. 1*C*). To further characterize HOXB6-induced differentiation changes, we measured heme production using benzidine staining of colonies grown in soft agar, as performed previously (6). There was a marked decrease in heme synthesis in the HOXB6-expressing cells (Fig. 1*D*). One possible explanation for low globin synthesis was that high levels of HOXB6 were squelching general transcription. Although the relatively uniform actin expression argued against this phenomenon, we repeated the globin analysis on cells in which HOXB6 expression was estimated to be less than 5-fold elevated over the low endogenous levels. In these cells, α- and γ-globin expression was still reduced, albeit to a lesser extent than observed in cells in which higher levels of HOXB6 protein were detected (Fig. 1*E*).

TABLE I
Down-regulation of α- and γ-globin mRNA levels by HOXB6 proteins

HOXB6 construct	N	α-Globin[a]	N	γ-Globin*
MIG control	15	1.0	16	1.0
HOXB6	15	0.28 ± 0.18[b]	16	0.37 ± 0.23[b]
HOXB6-N196A	5	1.04 ± 0.35[c]	5	0.83 ± 0.17[c]
HOXB6-W130G	5	0.25 ± 0.19[d]	5	0.26 ± 0.15[d]
HOXB6-S214A	3	0.31 ± 0.12[d]	5	0.43 ± 0.28[d]
HOXB6-S214E	3	0.42 ± 0.24[d]	3	0.32 ± 0.09[d]
HOXB6-ΔNterm	3	0.40 ± 0.05[d]	3	0.48 ± 0.19[d]
HOXB6-ΔCterm	2	0.32 ± 0[d]	2	0.55 ± 0.04[d]

[a] The relative expression values shown are the means and standard deviation of globin mRNA levels normalized to actin to control for mRNA loading and then normalized to the MIG control, run in parallel for each experiment, which was set to 1.0. Raw MIG vesus HOXB6 globin/actin ratios are reported in Supplemental Tables I and II, and data for each experiment, normalized to MIG are shown in Supplemental Tables III and IV.
[b] $p < 0.0001$, compared to MIG control.
[c] Not satistically different from MIG control.
[d] Not statistically different from the HOXB6 value.

DNA Binding Is Required for HOXB6 Activity—In a previous study HOXB6 did not display robust transcriptional activity on a range of reporter genes in transient assays (13). To test whether DNA binding is a prerequisite for HOXB6 activity in the K562 cell system, we first designed a mutant that lacked DNA binding capability. In HOX proteins, the absolutely conserved Asn at position 51, within the 60-amino acid HD directly interacts with an adenine base in the x-ray crystal structure of the HD bound to an oligonucleotide target (39). We have previously shown that mutation of the corresponding asparagine in HOXB4 renders the protein incapable of binding DNA (40). In the HOXB6-N196A mutant protein this asparagine was changed to alanine. Because HOXB6 binds only weakly to DNA in the absence of PBX (see below), we used an oligonucleotide containing a PBX-HOX-binding site in the presence of PBX (41) to demonstrate that the N196A protein does not bind to DNA under the conditions of the EMSA (Fig. 1*F*). Despite being

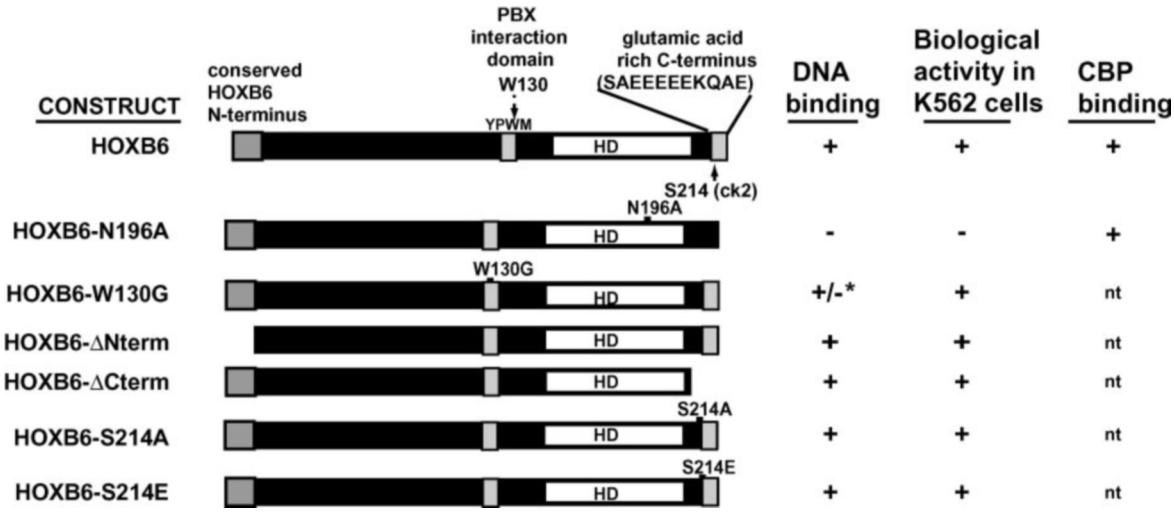

FIG. 2. **Summary of HOXB6 mutant protein activity.** Mutant HOXB6 proteins were tested in three systems: 1) capacity to form DNA-binding complexes in EMSAs; 2) biological activity when expressed in K562 cells; and 3) capacity to bind to CBP-HAT as measured by co-IP. All of the HOX proteins were expressed as N-terminal FLAG epitope fusion proteins. *, cooperative DNA binding with PBX is abolished. *nt*, not tested.

expressed at 2–5-fold higher levels than wild type protein, the N196A mutant HOXB6 protein exhibited only minimal or no repression of the γ- or α-gamma globin gene expression levels ($p < 0.001$ for both γ- or α-gamma globin *versus* HOXB6 and not significant *versus* MIG control) or heme synthesis, respectively (Fig. 1, *B* and *D*, and Table I). The activities of the HOXB6-N196A and subsequent mutant HOXB6 proteins for DNA binding, inhibition of globin expression, and CBP binding (see below) are summarized in Fig. 2.

HOXB6 Does Not Require PBX Interaction for Globin Repression—Although the HD has long been defined as a DNA-binding domain, we have previously noted that full-length HOXB6 does not avidly bind DNA (see below) (9). We and others demonstrated that HOXB6 and other HOX proteins from paralog groups 1–10 gain both DNA binding avidity and specificity through forming cooperative DNA-binding complexes with the PBX proteins (9, 41–46). We thus next investigated the requirement for PBX interaction for HOXB6 activity in the K562 cell model. The HOXB6-W130G protein was made, in which the conserved tryptophan within the critical YPWM that comprises the PBX interaction motif (PIM), which is required for PBX interaction, was mutated to glycine. We have previously shown that mutation of this tryptophan prevents HOXB4 from forming cooperative DNA-binding complexes (9). The mutant HOXB6-W130G protein did not form a cooperative DNA-binding complex with PBX1a (Fig. 3*A*). When the HOXB6-W130G mutant was tested in the K562 cell system, it repressed both α- and γ-globin mRNA levels to a similar degree as wild type HOXB6 protein (Fig. 3*B* and Table I). The HOXB6-W130G transfected cells were also white, further indicating that the mutant protein had globin repressing activity (data not shown).

Conserved Regions Outside the HD Are Not Required for HOXB6 Activity—The HOX HD protein family is defined on the basis of relatively high sequence homology within the HD and conservation with the ancestral *Antennapedia* HD (47). Comparison of the 39 HOX proteins shows that with the exception of the HD and the PIM, the only relatively conserved region is the extreme N terminus consisting of the first 10–12 amino acids. This region was removed in the HOXB6-ΔNterm mutant protein. This protein displayed wild type DNA binding and biological activity in K562 cells (Fig. 4 and Table I). The only other remarkable structural feature within the HOXB6 sequence is a stretch of polyglutamic acids at the extreme C terminus, a domain that is shared with other HOX6 paralog proteins and the HOXB5 protein. A HOXB6-ΔCterm protein that lacks the polyglutamic acid region exhibited full biological activity in the K562 cell assay and DNA binding by EMSA (Fig. 4 and Table I). Both of these HOXB6 deletion mutants were also white, indicating a loss of mature hemoglobin synthesis. Because none of the regions outside the HD affected HOXB6 activity, a mutant HOXB6 protein consisting of only the HD and short N- and C-terminal flanking regions (HOXB6Δ134) was constructed to test for biological activity in K562 cells. However, although this polypeptide was stable when synthesized in the TNT system, the FLAG epitope fused HD protein could not be detected in transfected K562 cells, despite the presence of the corresponding mRNA, suggesting that the truncated HOXB6-HD protein was unstable. In an attempt to produce a stabilized HD polypeptide, the YPWM PIM interaction motif (HOXB6-Δ127) or the YPWM plus nine additional N-terminal amino acids were fused to the HD (HOXB6-Δ119). However, neither of these proteins could be detected by Western blotting following transfection into K562 cells, despite the presence of the respective mRNAs.

The HOXB6 Protein Is Phosphorylated by Several Kinases, but Phosphorylation Does Not Alter DNA Binding—Phosphorylation of HOXB6 has been described previously (48). These authors identified a major CKII phosphorylation site at Ser^{214}, which is located in the short C-terminal tail between the HD and the polyglutamic acid region, and also reported that PKA was capable of phosphorylating the protein. We were able to confirm CKII and PKA phosphorylation of bacterially expressed HOXB6 (Fig. 5*A*). To test the importance of the known CKII phosphorylation site, a mutant HOXB6 protein in which Ser^{214} was changed to alanine to make a protein incapable of phosphorylation (HOXB6-S214A) was tested in the K562 cell assay. HOXB6-S214A showed full activity in repressing globin gene expression (Fig. 4*A* and Table I). In a similar manner, HOXB6-S214E, in which Ser^{214} was changed to glutamic acid to make a protein that models constitutive phosphorylation, was also active in the K562 cell assay (Fig. 4*A* and Table I). The finding that neither amino acid substitution at Ser^{214} altered activity was consistent with the observation that both mutant HOXB6 proteins bound DNA (Fig. 4*B*) and that CKII treatment of HOXB6 did not alter DNA binding in EMSAs (Fig. 5*B*). In addition to phosphorylation by CKII and PKA, we detected very strong labeling of HOXB6 by PKC and lower amounts of phosphorylation by CKI (Fig. 5*A*). These kinases were not examined in the previous analysis of HOXB6 phosphorylation (48). Although PKC appeared to be the most active kinase

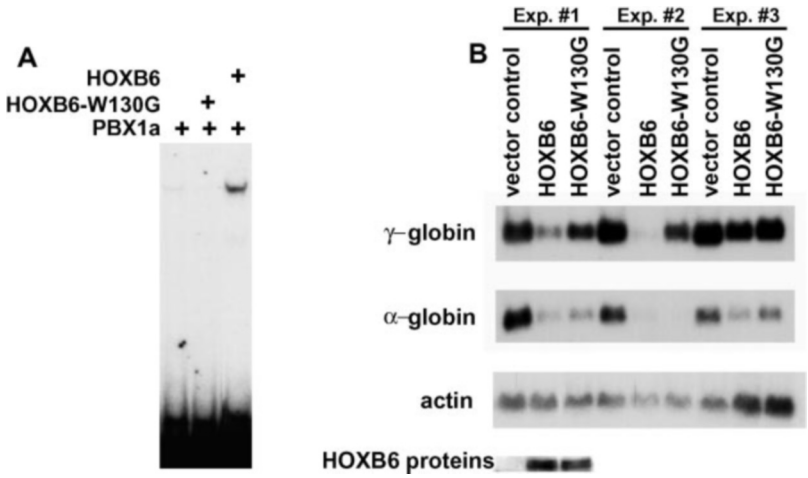

FIG. 3. **Interaction with PBX is not required for HOXB6-mediated down-regulation of globin expression.** *A*, the HOXB6-W130G protein does not form cooperative DNA-binding complexes with PBX on a PBX-HOXB6 consensus oligonucleotide target by EMSA. *B*, the HOXB6-W130G PBX interaction mutant protein down-regulates α- and β-globin to an extent similar to the wild type HOXB6 protein.

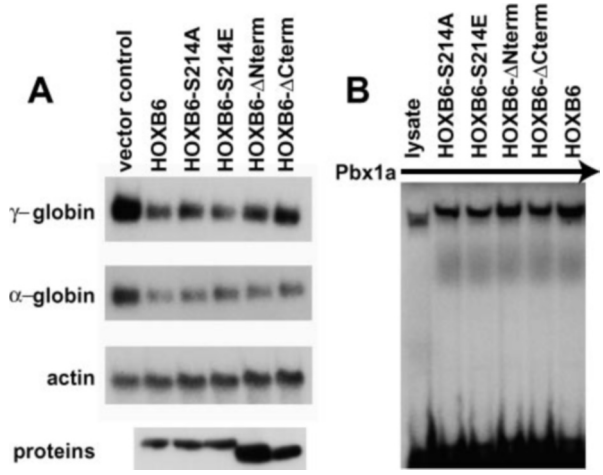

FIG. 4. **A series of non-HD mutations do not affect HOX6 biological activity in K562 cells or DNA binding with PBX.** *A*, mutant proteins down-regulate globins. HOXB6 proteins containing mutations deleting the conserved extreme N-terminal region (Δ*Nterm*), the polyglutamic acid extreme C-terminal region (Δ*Cterm*), or altering a previously described ckII phosphorylation site to either a nonphosphorylated residue (*S214A*), or mimicking constitutive phosphorylation (*S214E*) all repressed globin expression to a similar degree as the wild type protein. *B*, all of the mutant proteins form cooperative DNA-binding complexes with PBX1a in EMSA analysis. The TNT lysate used to synthesize the various HOXB6 and PBX1a proteins contains endogenous protein(s) capable of shifting the target oligonucleotide to form a nonspecific complex that migrates faster than the HOXB6-PBX band and exhibits variable intensity (compare for instance with Fig. 3*A* or 5*B*).

toward HOXB6, among those tested, none of these enzymes modified HOXB6 in a manner that altered DNA binding (Fig. 5*B*).

Modification of Ser^{214} Does Not Alter HOXB6 Subcellular Distribution—HOXB6 was predominantly localized to the cytoplasm in epidermal keratinocytes (36). Western blotting of retrovirally transfected K562 cells showed that ~75% of the HOXB6 or HOXB6-N196A protein was localized to the cytoplasm (Fig. 6*A*). These data were confirmed using immunohistochemical localization. Two different antisera to HOXB6 detected the majority of immunoreactive material in the cytoplasm in HOXB6 transfected cells (Fig. 6*B*). Endogenous HOXB6 protein was also predominantly cytoplasmic by this assay. Because HOX proteins are thought to function as nuclear transcription factors and because phosphorylation by CKII as well as other kinases has been reported to regulate nuclear localization, we asked whether the previously identified CKII phosphorylation might regulate HOXB6 cytoplasmic/nuclear translocation. Salt fractionation was used to examine the subcellular distribution of the Ser^{214} mutant proteins. Both the S214A and S214E proteins were detected in the same predominantly cytoplasmic distribution as wild type HOXB6 (Fig. 6*C*), suggesting that CKII-mediated phosphorylation of Ser^{214} does not change HOXB6 localization in the cell.

HOXB6 Blocks CBP-HAT Activity and CBP Blocks HOXB6 DNA Binding—We have previously reported that CBP binds to a large number of HOX proteins, including HOXB6 (13). As part of these studies, we showed that the HOXB6 protein could block CBP-HAT-mediated activation of a reporter gene in transient transfection studies and that this repression required the HOXB6 HD. To extend these studies to the K562 cell model, we first asked whether the HOXB6 protein could block *in vitro* CBP-HAT activity using purified proteins. A GST fusion protein containing the CBP-HAT domain exhibited robust acetylation of histone H3 (Fig. 7*A*). A HOXB6-maltose-binding protein fusion blocked HAT activity, whereas the maltose-binding protein control did not (Fig. 7*A*). To confirm and extend our previous data showing that binding to CBP prevented DNA binding, two different assays were utilized. EMSA was used to show that a full-length FLAG epitope-tagged CBP protein competitively reduced the capacity of HOXB6 to form DNA-binding complexes. There was only a minimal effect of addition of CBP on the cooperative DNA-binding complex with PBX (Fig. 7*B*, *upper band*). We have previously noted that PBX appears to stabilize HOX protein binding to DNA such that CBP poorly competes these complexes (13). However, there was a profound repressive effect on the capacity of HOXB6 alone to bind DNA in the absence of PBX (Fig. 7*B*, *lower band*). Because the capacity of CBP to block HOXB6-PBX binding to DNA as measured by EMSA was modest and the EMSA band for HOXB6 alone is weak, a DNA precipitation assay was developed to further demonstrate the effect of CBP on HOXB6 DNA interactions. In this assay, ^{32}P-labeled DNA was incubated with a T7 epitope-tagged HOXB6 protein with and without PBX cofactor (Fig. 7*C*). An antibody to the T7 epitope was used to precipitate the labeled DNA, indicating complex formation with HOXB6 (*lane 4*) and stronger HOXB6 binding to DNA in the presence of PBX (*lane 2*). The addition of a FLAG epitope-tagged CBP-HAT protein greatly reduced the amount of DNA brought down by either HOXB6 alone (*lane 5*) or together with PBX (*lane 3*). Taken together, these assays demonstrate that binding to CBP by the HOXB6 protein is not compatible with DNA binding.

HOXB6 and CBP Are Co-immunoprecipitated from K562 and Fetal Murine Liver Cells—Because HOXB6 can block CBP-HAT activity, one possible mechanism for HOXB6 repression of globin gene expression would be by blocking CBP-HAT-medi-

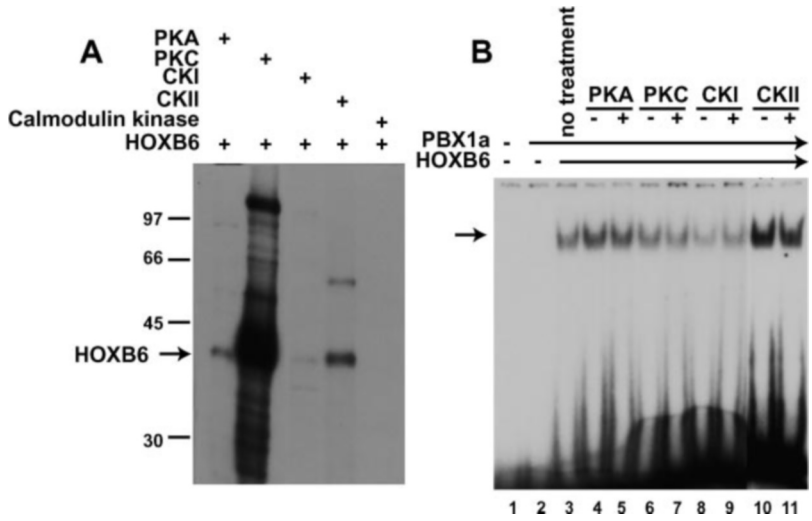

FIG. 5. **Phosphorylation of HOXB6 does not alter DNA binding.** *A*, several commercially available kinases phosphorylate bacterially expressed HOXB6 *in vitro*. All of the phosphorylation experiments were performed simultaneously, so that the relative levels of isotope incorporation reflect a combination of relative kinase activity of the various preparations and selectivity of the enzyme for HOXB6 target sites. *B*, phosphorylation with these kinases does not alter DNA binding measured by EMSA in the presence of PBX1a. For each assay pair, the control lane was incubated under the buffer conditions used for the respective phosphorylation assay. The intensity differences in EMSA band ascribed to HOXB6 among the untreated lanes (*lanes 3*, *4*, *6*, *8*, and *10*) result from differences in the salt concentrations used for each of the kinase assays.

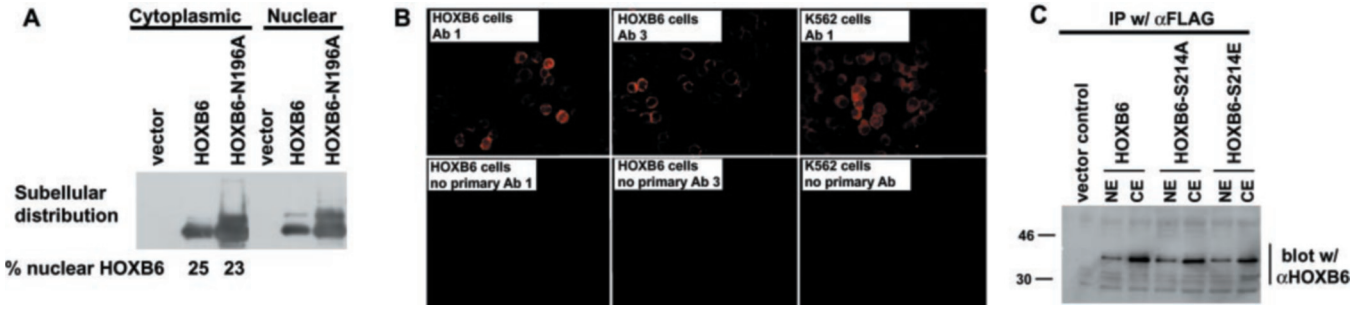

FIG. 6. **HOXB6 is predominantly cytoplasmic in K562 cells.** *A*, the majority of HOXB6 and HOXB5 proteins are cytoplasmic by subcellular fractionation. Subcellular fractions of transfected K562 cells were analyzed by Western blotting using a FLAG antibody. The fraction of immunoreactive protein in the nucleus, calculated by normalizing for a 3-fold cytoplasmic to nuclear extract volume ratio, is shown below the blot. *B*, exogenous and endogenous HOXB6 protein is predominantly cytoplasmic by immunohistochemical analysis. Affinity purified antibodies against HOXB6 protein are described under "Materials and Methods." The signals for endogenous HOXB6 in the *right-hand panel* has been electronically contrast enhanced to better visualize the weak signals that are predominantly cytoplasmic. *C*, the S214E mutant HOXB6 protein, designed to mimic constitutive phosphorylation, does not exhibit an altered nuclear localization. Western blotting of nuclear and cytoplasmic fractions showed similar distribution of wild type HOXB6, HOXB6-N196A, and HOXB6-S214E.

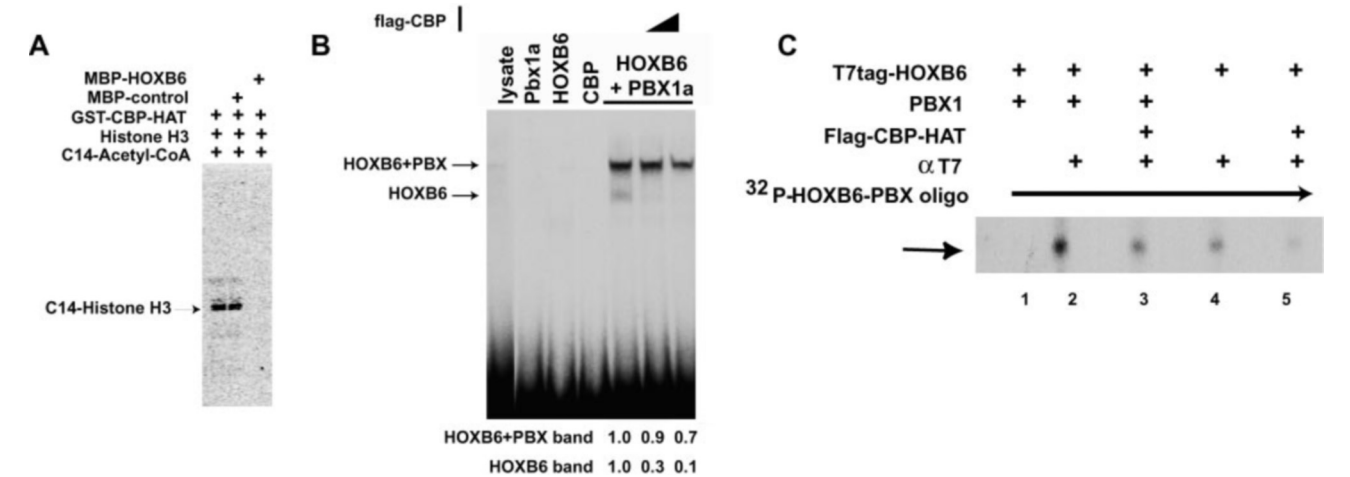

FIG. 7. **CBP and HOXB6 inhibit each other's activities.** *A*, HOXB6 blocks CBP-HAT mediated acetylation of histone H3. A maltose-binding protein-HOXB6 fusion protein blocked CBP-HAT activity, whereas the maltose-binding protein alone did not. *B*, CBP inhibits HOXB6-DNA interactions as measured by EMSA. The addition of increasing amounts of a full-length CBP protein containing an N-terminal FLAG epitope fusion competed away the EMSA complex formed by HOXB6 with PBX1a with an oligonucleotide containing a PBX-HOXB6 binding site. *C*, the CBP-HAT domain inhibits HOXB6 binding to DNA by co-IP. A labeled oligonucleotide containing a PBX-HOXB6-binding site was preincubated *in vitro* with HOXB6 and PBX1a (*lanes 1–3*) or with HOXB6 alone (*lanes 4* and *5*). DNA bound to HOXB6 was precipitated in the absence of CBP-HAT using antisera to a T7 epitope fused to the HOXB6 protein (*lanes 2* and *4*). The addition of CBP-HAT protein reduced the binding of HOX protein complexes to the labeled oligonucleotide (*lanes 3* and *5*).

ated acetylation. Given that HOXB6 and CBP functionally disrupt each other's activity, we asked whether the two proteins are associated *in vivo*. We first established that ^{35}S-labeled HOXB6, synthesized *in vitro*, could be precipitated by GST-CBP protein (Fig. 8*A*). To test whether the HOXB6 protein was bound to CBP *in vivo*, co-immunoprecipitation experiments were performed on nuclear and cytoplasmic extracts from K562 cells transfected with HOXB6, HOXB6-N196A, or

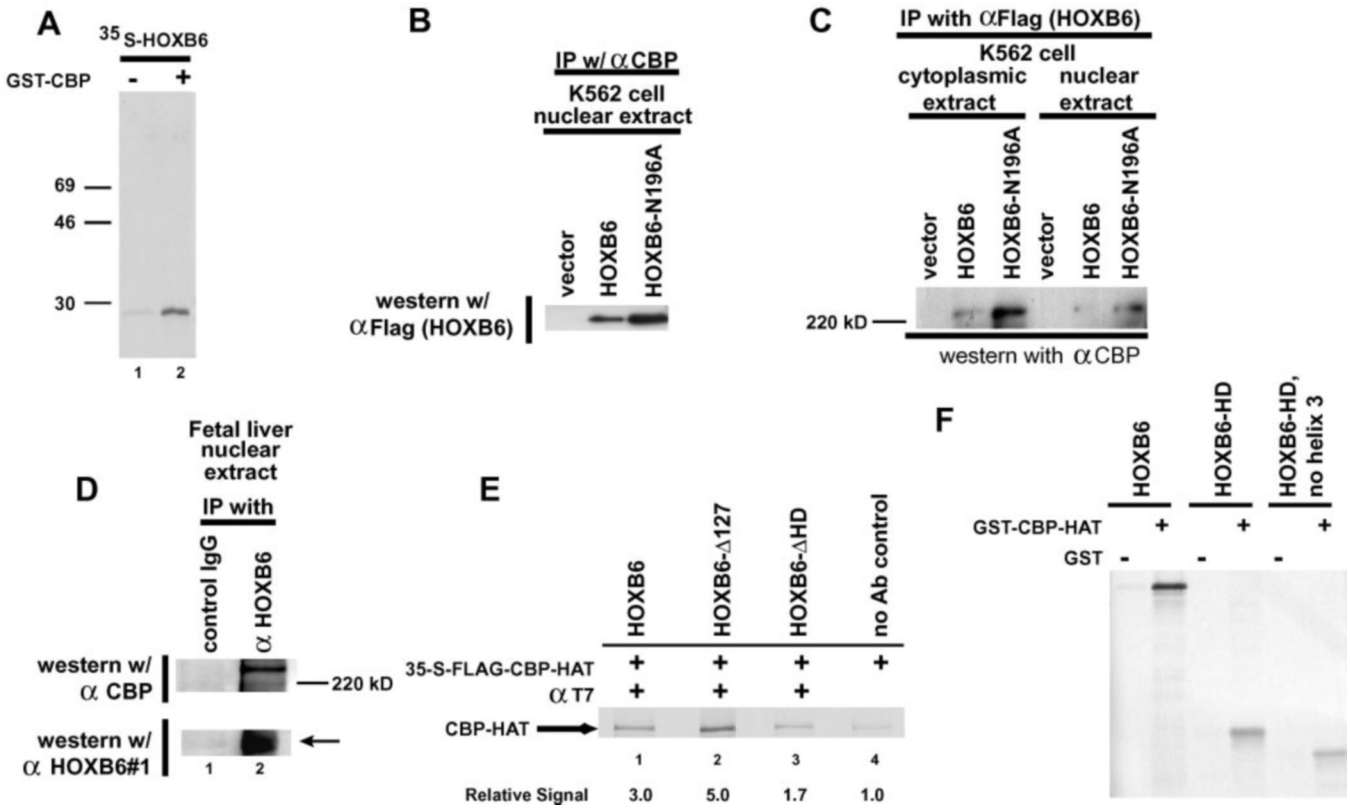

FIG. 8. **HOXB6 binds CBP *in* vitro and *in vivo*, and the HD mediates the interaction.** *A*, ^{35}S-labeled HOXB6, synthesized *in vitro*, was precipitated by purified GST-CBP-HAT domain but not by GST control protein. *B*, exogenous HOXB6 is co-precipitated by antisera to endogenous CBP protein *in vivo*. *C*, endogenous CBP protein is co-precipitated with antisera to the FLAG epitope tag on exogenous HOXB6 *in vivo*. In these experiments, detection of endogenous CBP was difficult because of the inefficient Western blot transfer of the very large CBP protein. Longer exposures show full-length CBP in the HOXB6 nuclear fraction more clearly. A second antisera to CBP confirmed co-immunoprecipitated bands by Western gel (not shown). *D*, endogenous CBP is precipitated by antisera against endogenous HOXB6 in 14-day fetal liver cells. The proteins were precipitated from nuclear and cytoplasmic extracts using a mixture of two antisera against peptides from the HOXB6 sequence (antiserum 2; see "Materials and Methods") (*lane 2*) or control IgG (*lane 1*). Following SDS gel resolution of the precipitated proteins and Western blotting, the blot was cut in half, and proteins migrating above 55,000 kDa were probed with antisera to CBP, whereas the lower molecular mass protein portion of the blot was probed with antisera (antiserum 1) to HOXB6. The *arrow* points to the band that co-migrates with HOXB6 protein expressed *in vitro* from the cloned cDNA (5). The cytoplasmic fraction yielded a similar but weaker set of co-precipitated CBP and HOXB6 immunoreactive bands (not shown). *E*, the HD is required, but the N-terminal half of HOXB6 is not required for CBP binding. Full-length HOXB6 and the HOXB6 HD (HOXB6-Δ127) co-precipitate CBP-HAT, but the N-terminal portion of HOXB6 lacking the HD (HOXB6-ΔHD) cannot bring down CBP-HAT. ^{35}S-Labeled *in vitro* synthesized CBP-HAT was subjected to co-immunoprecipitation with HOXB6 polypeptides, using an antibody to the T7 epitope tag fused to the HOXB6 proteins. *F*, the HOXB6 HD binds CBP, and conserved helix 3 is not required. ^{35}S-Labeled full-length HOXB6, the FLAG-HD fragment, or a FLAG-HD polypeptide lacking helix 3 all were bound to immobilized GST-CBP-HAT domain, whereas minimal background binding was observed for the GST control beads.

control vector. When antibodies to CBP were used to precipitate the endogenous protein, immunoreactive FLAG-HOXB6 protein was detected by Western analysis of the co-precipitated proteins in the nuclear fraction (Fig. 8*B*). Confirming these interactions, when antisera directed against the FLAG epitope tag fused to the HOX proteins was used for immunoprecipitation, bands for immunoreactive endogenous CBP were detected by Western blotting, using two different CBP antisera (Fig. 8*C*). In this experiment, co-precipitated bands of endogenous immunoreactive CBP were detected in both the cytoplasm and nuclear fractions, probably reflecting the large amount of HOXB6 localized to the cytoplasm. Because substantially more HOXB6-N196A than HOXB6 was present in these extracts (Fig. 1), the co-IP data suggest that both forms of HOXB6 bind to CBP equally well.

To investigate the association of the endogenous HOXB6 and CBP proteins, nuclear and cytoplasmic proteins were isolated from 14-day fetal murine liver cells, as a relatively rich source of HOXB6 protein. Previous studies have shown that the *Hoxb6* gene is maximally expressed in the developing fetal liver, during which time hematopoiesis is localized to this tissue (49). A combination of antisera directed against peptides from the N- and C-terminal regions, excluding the HD (antibody 2) was used to precipitate HOXB6 protein from liver cell nuclear extracts. As shown in Fig. 8*D*, a strong immunoreactive band was observed for the endogenous CBP protein co-precipitated by the specific antisera to the HOXB6 protein but not by control IgG. Two different antisera to the HOXB6 protein were used to confirm its immunoprecipitation (Fig. 8*D*, *bottom panel*, and data not shown).

HOXB6 Interacts with CBP through Helix 3 of the HD—We next wished to define the region of HOXB6 that interacts with CBP. Our previously published experiments showed that a HOXB6 protein lacking the HD was incapable of blocking *in vivo* CBP-HAT-mediated gene transcription (13), suggesting that the HD was required for binding. To confirm that the HD was the site of HOXB6 interaction with CBP, we used FLAG-tagged polypeptide fragments to pull down labeled CBP-HAT protein (amino acids 1099–1877, containing the Cys, ZZ, and TAZ zinc finger domains and the HAT domain (26)) in an *in vitro* co-IP assay. In this assay, the HD exhibited robust binding (Fig. 8*E*, *lane 2*), whereas the full-length HOXB6 protein also exhibited binding to CBP-HAT (*lane 1*). In contrast, the N-terminal 134 amino acids of HOXB6 lacking the HD did not appear to bind to CBP-HAT (*lane 3*).

Because previous studies showed that all of the HOX pro-

teins examined bound to CBP-HAT, we next asked whether helix 3, the most highly conserved region among the HOX HDs, was required for HOXB6 interaction with CBP-HAT. A HOXB6 mutant protein in which helix 3 of the HD was deleted was still co-precipitated with the CBP-HAT protein (Fig. 8*F*), indicating that the CBP-binding site in HOXB6 resides within the first two-thirds of the HD. Taken together, these data appear to explain why CBP-HAT binding is incompatible with DNA binding for the HOXB6 protein.

DISCUSSION

Among the 39 mammalian HOX proteins, the HD of HOXB6 shares the highest sequence identity to the canonical ANTP HD (47). The fact that the ANTP protein was shown to be a DNA-binding protein that altered transcriptional activity in reporter gene assays (50) has long suggested that HOXB6 and the other mammalian HOX proteins function as DNA-binding transcription factors. However, despite intense interest in the role of HOX proteins in a broad range of developmental and disease processes, little is known about their precise biochemical mechanism of action, either as transcriptional activators or repressors. As a first step in determining the portions of the molecule that contribute to function, we have performed a structure/function analysis for the HOXB6 protein, utilizing a biological readout of repression of globin expression in K562 cells. Given that the HOXB6 protein is expressed during skeletal formation, neurogenesis, and kidney development, as well as in hematopoiesis, it is unclear whether results from any one model can be generalized to other tissues. Our data demonstrate that none of the features examined, including the conserved N terminus, the polyglutamic acid C terminus, or the PIM motif are required for HOXB6 activity. In addition, the CKII site at Ser^{214} was not required for HOXB6 activity in this particular model system. A study of the ANTP protein suggested that the HD is the major effector of the observed phenotype during a specific phase of *Drosophila* development but that both the N- and C-terminal flanking regions potentiate the effect of the HD (51). The dominance of the HD was further illustrated in a series of studies in which various fly HD-containing gene mutational effects on early development were complemented or phenocopied by the mammalian homologs HOXB6, HOXD4, and HOXB1, respectively (52–54), despite the fact that there are no regions outside of the HD and PIM domains that are conserved between the respective protein pairs.

These data demonstrating a pre-eminent role for the HD thus strongly support our finding that the HOXB6 HD is the dominant motif in controlling the phenotypic change in our model system. However, in contrast to the results for ANTP in flies, no other region of HOXB6 was important for repression of globin mRNA levels in transfected K562 cells. Our results differ from those obtained for the other structure/function analysis performed on a mammalian HOX protein. Yaron *et al.* (55) analyzed the HOXB7 protein, using the differentiation of 32D hematopoietic cells as a readout. HOXB6 and HOXB7 share several features, namely the PIM domain, a polyglutamic acid C-terminal, a CKII site between the HD and polyglutamic acid tail, in addition to a relatively poorly conserved extreme N-terminal region. Although both studies found that DNA binding was required for activity, these authors found that for HOXB7, the extreme N terminus and glutamate-rich C terminus were important, as was the CKII site located C-terminal to the HD. These authors also found that HOXB7 required PBX interaction through the conserved YPWM PIM domain.

The PIM domain is not required for HOXB6 activity in our blood cell differentiation model. The discovery of cooperative HOX protein DNA binding with PBX/EXD proteins was anticipated to be a mechanism by which the HOX would gain specificity and thus explain the differential phenotypic output of these proteins that bind alone to very similar sequences (56). PBX interactions were reported to be important for HOXB1 and HOXB4 regulation of rhombomere development (16, 17) and in a HOXB4-induced fibroblast transformation model (40). In contrast, although a few studies in blood cells indicate an important role for interaction with PBX (55, 57), most studies in hematopoietic cells show HOX proteins functioning by PBX-independent mechanisms. Thus, the immortalizing and the transforming activities of HOXA9 have been reported to be PBX-independent (58, 59). In addition to our current studies, we have recently demonstrated that HOXB6-mediated immortalization of bone marrow progenitor cells occurs by a PBX-independent pathway.[2] Removal of the PIM domain did not change the stem cell expanding properties of HOXB4 (60). Paradoxically, PBX may play a role in this system, because RNAi-mediated PBX knockdown enhanced HOXB4-induced stem cell expansion.

Our data suggest that the weak interactions of the HOXB6 protein with DNA in the absence of PBX are sufficient for biological activity. Because we have shown that HOXB6 is bound to CBP and that CBP binding is incompatible with HOXB6 binding to DNA, we posit that weak DNA binding facilitates increased localized concentration of HOXB6 protein at specific target sites within chromatin. In this model, the reversible weak DNA interactions would allow localized HOXB6 to bind to and block the HAT activity of local CBP molecules, leading to repression of CBP-mediated gene transcription. The finding that the HOXB6 HD mediates binding to CBP is consistent with our data showing that biological activity requires this motif. Our proposed model for HOXB6 inhibition of CBP-HAT activity would join a growing list of DNA-binding proteins that block CBP/p300-HAT activity. The Pu.1 protein appears to mediate erythroid differentiation by blocking CBP-mediated acetylation of GATA1 (33), and the Twist transcription factor has been shown to block p300-HAT activity (61). Zhao *et al.* (62) recently proposed a mechanism for the early B cell transcription factor that was similar to what we propose for HOXB6. These authors found that early B cell factor bound CBP/p300 and blocked HAT activity but that such interaction was incompatible with the DNA binding function of the protein. Our data support a proposed model in which HOX proteins function as repressors when binding weakly to TAAT sites and function as transcriptional activators when binding more tightly to DNA together with PBX proteins (25). Although we show that CBP can compete a HOXB6-PBX EMSA complex, the competition with HOXB6 alone appeared more efficient. Because simple TAAT sites occur frequently, our model is also consistent with the report that two *Drosophila* non-HOX HD proteins are detected by chromatin IP analysis to be bound at numerous sites along the chromosomes (63).

Because HOX proteins are thought to function as transcription factors, our current and previous (36, 64–66) findings that much of the HOXB6 and other HOX proteins are cytoplasmic has been somewhat perplexing. Active export of the non-HOX HD protein, EN (Engrailed), from the nucleus has been reported (67). A Leu/Ile-rich nuclear export signal spanning the turn between helices 2 and 3 and part of helix 3 of the HD, which was described for EN, is shared by HOXB6. EN nuclear export appears to be regulated by CKII-mediated phosphorylation (68). Although HOXB6 contains a serine-rich region preceding the HD, this sequence does not contain a consensus

[2] N. Fischbach, S. Rozenfeld, W. Shen, S. Fong, D. Chrobak, D. Ginzinger, S. Kogan, A. Radhakrishnan, M. M. Le Beau, C. Largman, and H. J. Lawrence, manuscript submitted.

CKII site, and HOXB6 is phosphorylated by CKII on Ser^{214}, which is C-terminal to the HD (48). We now show that phosphorylation of Ser^{214} does not appear to alter the HOXB6 subcellular localization. Although the regulation of HOXB6 localization remains to be elucidated, our results showing strong PKC activity and weaker CKI-mediated phosphorylation of HOXB6 suggest that PKC or CKI phosphorylation events may alter HOXB6 subcellular distribution.

Previous data suggest a role for HOXB6 in the regulation of red blood cell differentiation. Most data show *HOXB6* gene expression restricted to myeloid/erythroid leukemic cell lines (3, 69–71) and primary acute myeloid leukemias (72, 73). *HOXB6* expression correlated with erythropoietin production sites and erythropoiesis throughout murine fetal development but was not detected in hematopoietic stem cell populations (49). Disruption of the *HOXB6* gene resulted in a selective increase in early murine bone marrow erythroid progenitor cells (74), whereas treatment of adult human hematopoietic progenitor cells with an antisense *HOXB6* oligonucleotide results in selective decrease in granulomonocytic (75) or myeloid and erythroid progenitor cells (76). Our data present an apparent paradox in that HOXB6 appears to be a marker of erythroid tissues and yet acts to block markers of terminal erythroid differentiation. The complex series of interactions that we observe between HOXB6, CBP, and DNA suggest a speculative model for the molecular mechanisms that underlie cellular commitment. In biologic terms, an undifferentiated cell is considered to be committed if subsequent developmental events show that the cell can only differentiate into one or only a few lineages. We propose that cellular commitment is mediated by the "marking" of sets of lineage-specific genes, such as globins, for future activation in response to appropriate differentiation signals. HOXB6 may accomplish this by reversibly and competitively binding cognate DNA-binding sites and CBP, thereby creating and maintaining high local concentrations of CBP at specific genomic sites and yet inhibiting CBP-enhanced activation of these genes at the same time. In this model, the differentiation-dependent decline of HOXB6 protein would alleviate the HOXB6-mediated inhibition of CBP-HAT activity and permit full gene activation as cells are induced to mature.

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