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Cooperative DNA Binding of the Human HoxB5 (Hox-2.1) Protein Is under Redox Regulation In Vitro

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The human HoxB5 (Hox-2.1) gene product is a sequence-specific DNA binding protein. Cooperative interactions stabilize in vitro DNA binding of the HoxB5 protein to tandem binding sites by at least 100-fold relative to binding to a single site. The HoxB5 homeodomain is sufficient for sequence-specific DNA binding but not for cooperative DNA binding. Here we report that the additional protein sequence required for cooperativity is a small domain adjacent to the homeodomain on the amino-terminal side. We further show that cooperative DNA binding is under redox regulation. The HoxB5 protein binds to DNA in vitro both when oxidized or reduced but binds cooperatively only when oxidized. Mutational analysis has revealed that the cysteine residue in the turn between homeodomain helices 2 and 3 is necessary for cooperative binding and redox regulation. The enhanced DNA binding of oxidized HoxB5 protein is the opposite of the redox regulation reported for other mammalian transcription factors such as Fos, Jun, USF, NF-κB, c-Myb, and v-Rel, in which oxidation of cysteine residues inhibits DNA binding. Thus, specific oxidation of nuclear proteins is a potential regulatory mechanism that can act to either decrease or increase their DNA binding activity.

The homeodomain (HD) is a highly conserved, 60-amino-acid DNA binding domain encoded by the homeobox. The HD was originally discovered in several genes that control *Drosophila* pattern formation, and subsequently, a large number of vertebrate genes containing HDs have been identified (43). The mammalian HD genes most closely related to the *Antennapedia* (*Antp*)-type HDs of *Drosophila melanogaster* are the *Hox* gene family. The evolutionary conservation of *Hox* genes with *Antp*-type HDs in vertebrates, along with their similarity to *Drosophila* HD-containing genes in genomic organization and pattern of expression (12, 16), has led to the idea that *Hox* genes play a similar role in the specification of embryonic development. The postulated role of *Hox* genes as developmental regulators is further supported by a number of studies in which ectopic expression or loss of expression of *Hox* genes leads to defects in embryonic development (31).

The initial hypothesis that Hox proteins are transcription factors was based on the facts that *Drosophila* HD proteins are transcription factors, that a family of diverged (POU domain) mammalian HD proteins are transcription factors, and that Hox proteins are sequence-specific DNA binding proteins (27, 56). Recently, some Hox proteins have indeed been shown to be transcription factors. Hox proteins found to transactivate reporter genes include Hox-4.4 and -4.5 (17), CHOX-1.7 (41), Hox-3.3 (2), Hox-2.5 (19), Hox-4.2 (38), and the related Evx-1 (18). However, in similar assays, transactivation was not seen with many other *Hox* gene products, such as Hox-4.3 (17), CHOX-1.1, -1.4, -2.6, and -4.2 (41), Hox-3.4 and -3.5 (2), Hox-1.3 (18), Hox-2.4 (19), Hox-1.6 (38), or Hox-3.1 (53) (for the proposed new names of these Hox proteins, see reference 42). Some of these proteins (Hox-4.3, CHOX-1.4, Hox-2.4, and Hox-3.1) can suppress activation by other HD proteins in cotransfection assays. Together, these studies suggest that Hox proteins, which possess similar DNA binding specificities, can have widely different transcriptional effects on target genes.

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Several mechanisms have been proposed for how *Antp*-type HD proteins, with their very similar HDs, can achieve functional specificity (15, 25). Studies of chimeric *Drosophila* HD proteins and chimeric *Drosophila*-mammalian HD proteins suggest that much of their regulatory specificity resides in or adjacent to the HD (31). It is thought that the HD region achieves this specific regulation by a combination of DNA sequence specificity and interactions with other proteins (15, 31). One type of protein-protein interaction that can influence DNA binding is cooperative binding. Clustered HD protein binding sites have been found in the promoters of most *Drosophila* HD-containing genes and *HOX* genes. Cooperative DNA binding to clustered sites has been observed for the *Drosophila* engrailed and fushi tarazu (Ftz) proteins (7) and a hybrid Deformed/Ultrabithorax protein (8). In a mixed binding reaction to multiple binding sites, the engrailed and Ftz proteins appear to bind homocooperatively but not heterocooperatively (35). The sequences required for cooperative binding of *Antp*-type HD proteins have not previously been defined. Cooperative DNA binding has been reported for other classes of evolutionarily diverged HD proteins, and in some cases, the domains necessary for cooperativity have been determined. The POU-specific domain, which is a short distance towards the N terminus from the POU HD, is required both for homocooperative and heterocooperative DNA binding of mammalian POU HD proteins (26, 51). A short, unstructured sequence adjacent to the N-terminal side of the HD has also been found to mediate the heterocooperative DNA binding of the yeast α2 HD protein with the MCM1 protein (52). A human protein related to MCM1, the serum response factor, also exhibits heterocooperative DNA binding with a human HD protein, pHox1. In this case, the HD of pHox1 is sufficient for cooperative DNA binding (13). Another example of homocooperative DNA binding of a highly diverged HD protein is the yeast STE12 protein (59), but the required sequences have not been reported.

We have been studying the DNA binding of the human HoxB5 protein, which was formerly called Hox-2.1, but for this work, we have adopted the recently proposed new

vertebrate *Hox* gene nomenclature (42). We previously showed that the human HoxB5 protein binds cooperatively to tandem consensus sites, and that HoxA5 (Hox-1.3) exhibits similar cooperative DNA binding (10). It was found that the HoxB5 protein can bind to a single 12-bp consensus site (LP site [7]) but that, when it binds to each of two or more tandem LP sites, the half-life of HoxB5-DNA complex is increased from seconds for a single site to hours for tandem sites. We further showed that the HoxB5 HD is sufficient for sequence-specific DNA binding but not the cooperative stabilization of DNA binding (10). Therefore, the interactions that stabilize HoxB5 binding to tandem sites require protein sequences outside of the HD. We have now mapped the protein sequences required for cooperative binding and have also shown that there is cooperative DNA binding to sites in the *HoxB5* promoter. In the course of mapping the HoxB5 protein sequences necessary for cooperative binding, we found that cooperative interactions are quite sensitive to the redox state of the assay buffer. It has recently been reported that the in vitro DNA binding of a number of mammalian transcription factors is regulated by oxidation. Spontaneous oxidation of cysteine residues that occurs during protein purification decreases or abolishes the DNA binding of Jun and Fos (1), BZLF-1 (4), NF-κB (32, 49), v-Rel (24), c-Myb (14), bovine papillomavirus (BPV) E2 (30), and USF (39). The cysteine residue required for redox regulation of these proteins is found within the DNA binding domain and is often part of a small conserved motif (24, 30, 58). A cellular redox protein, Ref-1 (57), has been found to activate the DNA binding of several proteins whose in vitro DNA binding is inhibited by oxidation (58). In contrast to these DNA binding proteins, oxidation of HoxB5 actually enhances DNA binding in vitro. We show here that oxidation of HoxB5 protein does not affect the stability of binding to a single site but is required for cooperative stabilization of binding to tandem sites. We further show that this redox regulation requires the presence of the cysteine residue in the HD. Although the HoxB5 response to oxidation is opposite that seen for previously described mammalian regulatory proteins, it is quite similar to that of the bacterial transcriptional regulator OxyR, which binds to DNA both when oxidized or reduced but forms a transcriptional activation complex only when oxidized (47, 48). The redox regulation of HoxB5 cooperative DNA binding illustrates that oxidation may be a mechanism by which mammalian cells can positively or negatively modulate the DNA binding activity of regulatory proteins.

MATERIALS AND METHODS

Expression and purification of truncated HoxB5 proteins. Polymerase chain reaction (PCR)-mediated mutagenesis of the previously described full-length human *HoxB5* T7 expression construct (10) was employed to generate truncated *HoxB5* coding sequences. These truncated coding sequences contained an *Nde*I site with an in-frame methionine codon at the 5′ end and a termination codon (UGA) followed by a *Bcl*I site at the 3′ end. To create expression vectors for the proteins diagrammed in Fig. 1A, the PCR-generated coding sequences were digested with *Nde*I and *Bcl*I and inserted into *Nde*I- and *Bam*HI-cut pET11A, an *Escherichia coli* T7 expression vector (9). Substitution mutants were also made by PCR mutagenesis, in which one primer was the same as the truncated mutants and the other contained the desired substitution and extended to a unique restriction site. The PCR product generated with these primers was cleaved and inserted in place of the same fragment in the appropriate wild-type T7 expression construct. Each mutant construct was verified by DNA sequencing. All of the T7 expression constructs produce essentially nonfusion proteins, with the exception of the initiating methionine. Protein expression in *E. coli* BL21(DE3) (9) containing the expression plasmids was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 30°C. The truncated HoxB5 proteins are much less toxic to the bacteria than full-length HoxB5 protein, and this allowed a longer IPTG induction, resulting in a higher yield (10 μg of truncated HoxB5 protein per liter of culture) than previously described for the full-length protein (10). The bacteria were harvested, and the expressed HoxB5 proteins were purified in buffers without reducing agents by the previously described methods (10). Briefly, a nondenaturing lysis procedure was used to make soluble protein extract from the bacterial cells. The extract, in buffer containing 0.2 M KCl, was passed over DEAE-Sepharose CL6B (Pharmacia). The HoxB5 proteins were further purified by sequence-specific DNA affinity chromatography (20). The DNA affinity resin contained the self-annealed, ligated oligonucleotide LP_2: GATCAATTAATTGATCAATTAATT. The purification procedure resulted in truncated HoxB5 proteins that were estimated on silver-stained gels to be 40 to 80% pure.

Footprint challenge assay. The footprint challenge assays were performed with purified, bacterially expressed HoxB5 proteins, and no footprinting activity was seen after purification of extract from bacteria containing only the pET11A expression vector. The plasmids for LP_1, LP_2, and LP_4 probes contain one, two, or four tandem copies, respectively, of the palindromic LP site (7) and were prepared by ligating the self-annealed oligonucleotide GATCAATTAATT into the *Bam*HI site of pBLCAT2 (28). The LP probes were prepared by end labeling these plasmids with polynucleotide kinase at the *Hin*dIII site and then by a second cutting at the *Xho*I site, as previously described (10). The *HoxB5* genomic sequence probe gS3, containing the genomic sequences from a *Bss*HII site at bp −355 to a *Sac*I site at bp +7 (numbered from the predicted translational start site, as in reference 10), was labeled at a *Bam*HI polylinker site adjacent to the *Sac*I site. We have found that the HoxB5 protein forms dimers (see below) and other multimers in solution (11), and an advantage of footprint analysis relative to gel mobility shift assays is that the occupancy of each DNA binding site can be determined independently of protein multimer formation. Prior to footprint analysis, binding reactions took place for 30 min in a 50-μl volume at 4°C. The binding and competition conditions used for all experiments were 50 mM KCl, 25 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, 2% polyvinyl alcohol, and 400 ng of poly(dI-dC) competitor DNA per ml. Binding reactions for footprint challenge assays were performed by a 30-min incubation of the probe with approximately twofold-more purified protein than is required to completely protect the binding site(s) from DNase digestion. Stability of binding was assessed by the addition of 200 ng of unlabeled self-annealed LP_2 competitor oligonucleotide (250-fold molar excess over the labeled probe) for various amounts of time. After the indicated time of competition at 4°C, the samples were subjected to DNase I digestion as previously described (10). The samples in the footprint challenge lanes labeled 0 had competitor added, were mixed for several seconds, and then were immediately subjected to the typical 1-min DNase I digestion. Therefore, this assay can determine that a protein-DNA half-life is less

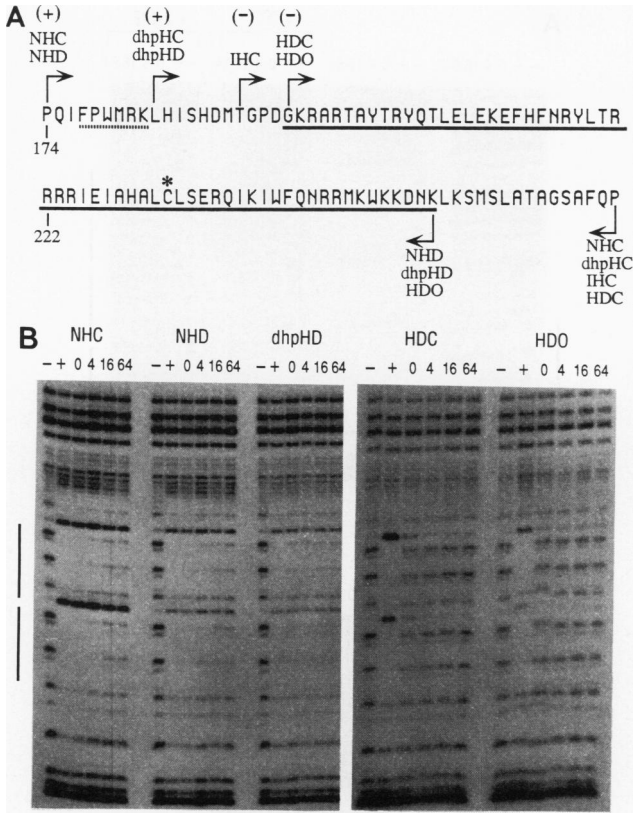

FIG. 1. Mapping of the sequences required for HoxB5 cooperative binding. (A) Amino acid sequence of the human HoxB5 HD and surrounding regions. The HD is underlined, and the conserved upstream hexapeptide is indicated by a dotted underline. The single cysteine residue, Cys-232, is marked with an asterisk. The numbering is relative to the predicted translational start of the protein, and the proline residue shown at the carboxy terminus (residue 269) is the C-terminal residue of the HoxB5 protein (10). The amino terminus of each indicated truncated HoxB5 protein is a methionine residue followed by the sequence marked by the rightward-facing arrow. The carboxyl terminus of each indicated protein is marked by the leftward-facing arrow. Proteins that can bind cooperatively are indicated by (+) above their N termini, and loss of cooperative binding is indicated by (−). (B) Footprint challenge assay for cooperative binding of truncated HoxB5 proteins to LP_2 sites. Saturating amounts of each indicated protein were allowed to bind to the LP_2 probe (except for no protein control lanes), and then a 250-fold molar excess of unlabeled LP_2 sites was added for the indicated time and was followed by standard DNase I digestion and footprint analysis. The footprints of the LP sites are shown by bars on the left side. Lanes: −, no protein added; +, no competitor added; 0, 4, 16, and 64, minutes of competition prior to DNase I digestion.

than 30 s but cannot further quantitate highly unstable binding. Concentrated aqueous stock solutions were used to add dithiothreitol (DTT), 2-mercaptoethanol (2-ME), 1,1′-azobis[*N*,*N*-dimethylformamide] (diamide), and *N*-ethylmaleimide (NEM) as described below. Quantitative analysis of footprint challenge experiments was performed by scanning gels with a radioanalytic imaging system (AMBIS Systems, San Diego, Calif.) and quantitating the normalized counts per minute in the footprint areas as previously described (10). Calculations of the K_d and the protein-DNA binding half-life were also performed as previously described (10).

Western blot analysis. Protein samples were boiled in sample buffer (60 mM Tris-HCl [pH 6.8], 20% glycerol, 2% sodium dodecyl sulfate [SDS], 0.005% bromphenol blue) with or without 10% 2-ME and then resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 17% polyacrylamide gels. The proteins were then electroblotted in 20 mM sodium phosphate (pH 6.8) on to nitrocellulose membrane. The membrane was blocked with 2% bovine serum albumin. The primary antibody used was a 1/1000 dilution of rabbit serum containing polyclonal antibodies raised against a full-length human HoxB5-TrpE fusion protein (11). The specificity of this antibody has been demonstrated by Western blotting (immunoblotting), by immunoprecipitation, and by immunofluorescence (11). Primary antibody binding was visualized with an Immunoselect kit (Bethesda Research Laboratories, Gaithersburg, Md.) per the manufacturer's recommendations. This procedure uses a biotinylated secondary goat anti-rabbit immunoglobulin G followed by streptavidin-alkaline phosphatase and then color development with 5-bromo-4-chloro-3-indolylphosphate toluidinium (BCIP) and nitroblue tetrazolium (NBT).

RESULTS

The non-HD sequences required for cooperative binding. Because the HoxB5 HD is sufficient for sequence-specific DNA binding but not for cooperative binding to tandem sites seen for full-length HoxB5 protein (10), deletion mutants were constructed to map the domain required for cooperativity. Loss of cooperative binding of mutant HoxB5 protein was previously shown to reduce the half-life of the protein-DNA complex from hours to seconds (10). Cooperative stabilization of protein binding to tandem LP DNA binding sites was measured by a footprint challenge assay. The LP binding site is the double-stranded 12-bp palindromic sequence (TCAATTAATTGA) found to bind many Antp-type HD proteins (7). For this assay, a saturating amount of purified protein was bound to an end-labeled probe containing either one, two, or four tandem copies of the LP binding site (LP_1, LP_2, or LP_4, respectively). The stability of binding was then determined by addition of a large molar excess of unlabeled LP_2 sites and subsequently measuring the amount of time necessary for the loss of the DNase I protection of the labeled binding sites. As a starting point for defining the sequences required for cooperative DNA binding, we examined the binding of a truncated HoxB5 protein that contains the HD and surrounding regions that are conserved in the HoxA5 (Hox1.3) protein (10, 50). This protein, $HoxB5_{NHC}$ (the amino acid sequence of which is shown in Fig. 1A), exhibits the same cooperative stabilization of binding as full-length HoxB5 protein (Fig. 1B) (10). In fact, quantitative analysis (see Materials and Methods) of the $HoxB5_{NHC}$ binding shown in Fig. 3A revealed that this truncated HoxB5 protein binds to an LP_4 site with a half-life of 237 min (K_d = 2.92×10^{-3} min^{-1}), which is very similar to the 224-min DNA binding half-life we previously found for full-length HoxB5 (10). The very short half-life (<30 s) of the $HoxB5_{NHC}$ protein specifically bound to a single LP site (see Fig. 3A) shows that the relatively stable binding of this truncated protein to each of multiple tandem LP sites is due to cooperative interactions. Thus, the HD, with an additional 20 amino acids on the N-terminal side and with the 15 C-terminal amino acids, is sufficient for cooperative binding. Further analysis of mutants revealed that only the N-terminal extension to the HD is necessary for the magnitude of cooperative stabilization seen with $HoxB5_{NHC}$ (Fig. 1, $HoxB5_{NHD}$). A very small but reproducible stabilization of

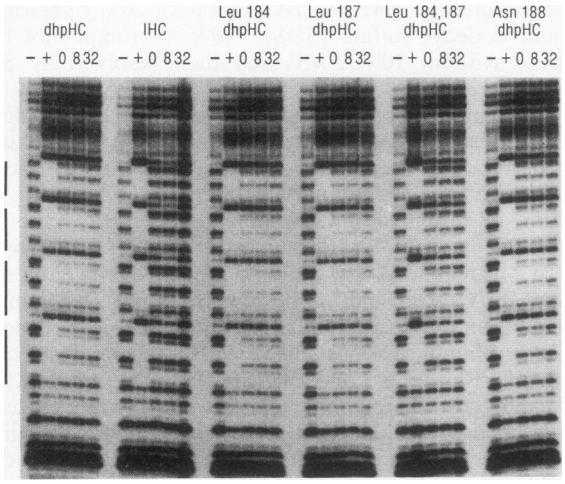

FIG. 2. Defining the HoxB5 cooperative binding domain adjacent to the HD. Footprint challenge assays are as described in the legend to Fig. 1B, with the indicated proteins and LP_4 probe. The footprints of the LP sites are shown by bars on the left side. Leu-184 and Leu-187 are substitution mutants for His residues, Leu-184,187 is a double-substitution mutant, and Asn-188 is a substitution mutant for an Asp residue in $HoxB5_{dhpHC}$. Lanes: −, no protein added; +, no competitor added; 0, 8, and 32, minutes of competition prior to DNase I digestion.

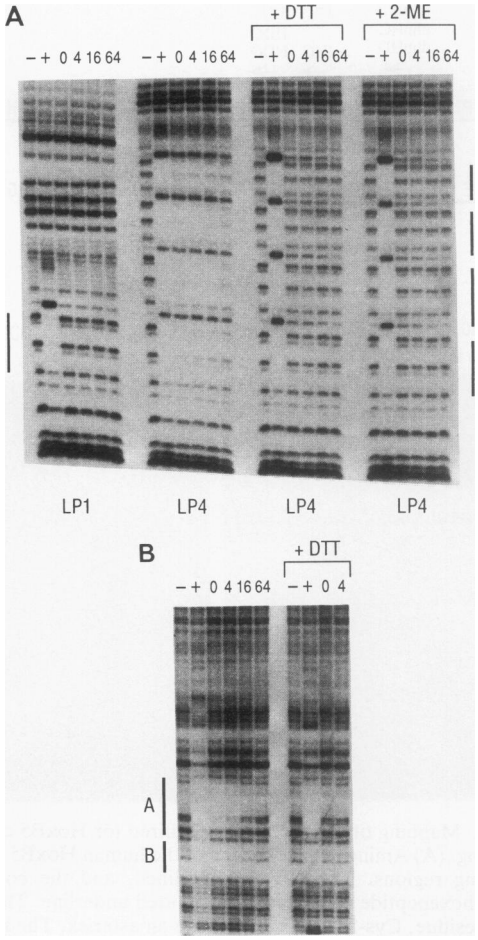

FIG. 3. Cooperative DNA binding by HoxB5 is inactivated by reducing agents. (A) Footprint challenge assays with $HoxB5_{NHC}$ protein on LP_1 or LP_4 probes, as indicated beneath the footprint assays. The single LP site in the LP_1 probe is shown by a bar on the left, and the four LP sites of the LP_4 probe are shown by bars on the right. The lanes are labeled as described in the legend to Fig. 1B. Where indicated, 5 mM DTT or 20 mM 2-ME was present during the binding and competition reactions. (B) Footprint challenge assays with full-length HoxB5 protein on the *HoxB5* promoter probe gS3. The lanes are labeled as for Fig. 1B. Sites A (−86 to −73) and B (−68 to −57), numbered as in reference 10, are indicated by bars. Where indicated, 5 mM DTT was present during the binding and competition reactions.

binding is conferred by the C-terminal domain (Fig. 1B, $HoxB5_{HDC}$). As was previously found (10), the HD by itself binds to LP sites but shows no cooperative binding, as the footprint is lost immediately upon competitor addition (Fig. 1B, $HoxB5_{HDO}$, time 0). All the proteins shown binding to LP_2 probe in Fig. 1B can specifically bind to a single LP site, but like the HDO binding to LP_2 shown in Fig. 1B, the footprint is lost immediately after competitor addition (data not shown). The region N terminal to the HD contains a conserved hexapeptide sequence (indicated with a dotted underline in Fig. 1A) which is found a short distance from the HD in a number of *Hox* and *Drosophila* HD-containing genes (29). However, analysis of a mutant with a deletion of the hexapeptide sequence (Fig. 1, $HoxB5_{dhpHD}$) demonstrated that the hexapeptide and the sequences C terminal to the HD are not required for cooperative binding. Therefore, a small N-terminal domain, (M)LHISHDMTGPD, is sufficient to confer cooperative stabilization of DNA binding by the HoxB5 HD.

To more closely analyze the N-terminal cooperativity domain, a further deletion mutant, $HoxB5_{IHC}$, was constructed (Fig. 2). This deletion removed the (M)LHISHD sequence, leaving only the sequence MTGPD followed by the HD and the C-terminal domain. This construct, $HoxB5_{IHC}$, contains the entire coding sequence 3′ to the single intron in the *HoxB5* gene (10) and thus might represent an evolutionary domain boundary. Figure 2 shows that the $HoxB5_{IHC}$ protein does not bind cooperatively to tandem sites, as the footprint is lost immediately upon competitor addition. To identify important residues in the essential MLHISHD amino-terminal cooperativity domain, substitution mutants in each of the three charged amino acids were prepared. A footprint challenge assay with these mutant $HoxB5_{dhpHC}$ proteins is shown in Fig. 2. Mutation of either histidine (His-184 or His-187) to leucine or of the aspartate (Asp-188) to asparagine has little effect on cooperative stabilization of binding (Fig. 2). However, a double mutant of $HoxB5_{dhpHC}$, with both His-184 and His-187 changed to leucine, does exhibit significantly diminished cooperative binding (Fig. 2). All of the proteins shown in Fig. 2 can also specifically bind to a single LP site, but the footprint is lost immediately after competitor addition (data not shown).

Cooperative DNA binding, but not sequence-specific binding, is inactivated by reducing agents. Because we found that preparations of HoxB5 protein treated with DTT no longer showed enhanced stability of binding to tandem DNA sites, we used the footprint challenge assay to examine the effect of reducing agents on cooperative binding. As previously reported for full-length HoxB5 protein (10), and as Fig. 3A shows for the truncated $HoxB5_{NHC}$ protein, the stability of HoxB5 protein binding to DNA is greatly enhanced when the protein binds to multiple tandem sites (compare LP_1 probe to LP_4 probe [Fig. 3A]). Figure 3A shows that $HoxB5_{NHC}$

protein treated with DTT or 2-ME still binds to each of the four identical LP sites of LP_4 probe and exhibits increased enhanced bands between the LP sites (see no competitor lanes [labeled +]). However, treatment of the $HoxB5_{NHC}$ protein with reducing agents completely abolished cooperative stabilization of DNA binding (Fig. 3A). A total loss of cooperative binding was also seen with an LP_2 probe or with full-length HoxB5 protein (data not shown). Thus, after treatment with reducing agents, the half-life of protein binding to each of the four tandem DNA binding sites in LP_4 probe is diminished to the stability of binding to the single site in LP_1 probe (Fig. 3A). This is a profound alteration of the stability of HoxB5 binding to tandem sites, as reducing agents decrease the in vitro half-life of HoxB5 protein bound to each of the LP_4 sites from about 4 h to less than 30 s (Fig. 3A). Titration experiments revealed that addition of as little as 0.5 mM DTT or 2 mM 2-ME significantly diminishes cooperative binding and that cooperative binding is equally sensitive to DTT if the protein is bound to the probe prior to addition of the DTT (data not shown).

To test cooperative binding to naturally occurring sites, two adjacent HoxB5 binding sites found in the *HoxB5* promoter were examined. We previously showed that sites A and B are near the *HoxB5* transcriptional start site and bind the HoxB5 protein (10). Figure 3B shows that HoxB5 protein binds cooperatively to these sites, as the footprint is still present 16 min after specific competitor addition. The loss of the HoxB5 footprints on this promoter sequence after 64 min of competition indicates that binding to these sites is not as stable as that to tandem LP sites. As with the synthetic LP sites, the addition of 5 mM DTT to the binding reaction does not prevent specific DNA binding to the *HoxB5* promoter sites, but the stabile binding of HoxB5 to these sites is abolished. Figure 3B shows that the footprints of DTT-treated HoxB5 protein on the *HoxB5* promoter probe (lane +) are immediately lost after competitor addition (lane 0). Thus, the HoxB5 protein binds cooperatively to naturally occurring promoter sites, and this cooperative DNA binding is also under redox regulation.

DTT inactivation of cooperative DNA binding is reversible. To determine whether reducing agents irreversibly inhibit the cooperative binding of HoxB5 protein, DTT-treated protein was reoxidized with diamide and tested for cooperative binding (Fig. 4A). $HoxB5_{NHC}$ protein was incubated at 0°C for 20 min in binding buffer containing 5 mM DTT, and then a portion of the reduced protein was then incubated for 20 min at 0°C in the presence of 10 mM diamide. The cooperative binding of the $HoxB5_{NHC}$ protein treated with DTT only or with DTT and then with diamide was compared with that in the untreated protein in a footprint challenge assay. All three protein samples were incubated on ice for the same total time prior to the footprint challenge assay. Figure 4A shows that the DTT-treated $HoxB5_{NHC}$ protein lost the ability to cooperatively bind DNA but that diamide treatment of the reduced protein partially restores the cooperative stabilization of binding. In contrast to the DTT-only-treated protein, for which the footprint and enhanced bands are lost immediately after competitor addition (Fig. 4A), a partial footprint still visible in the DTT-plus-diamide experiment at 0 and 8 min, and the strong enhanced bands persist even at 32 min after competitor addition. Therefore, DTT inactivates cooperative HoxB5 binding in a reversible manner.

Treatment with oxidizing agents or NEM does not affect cooperative binding. Because oxidation inhibits the DNA binding of other mammalian redox-regulated transcription

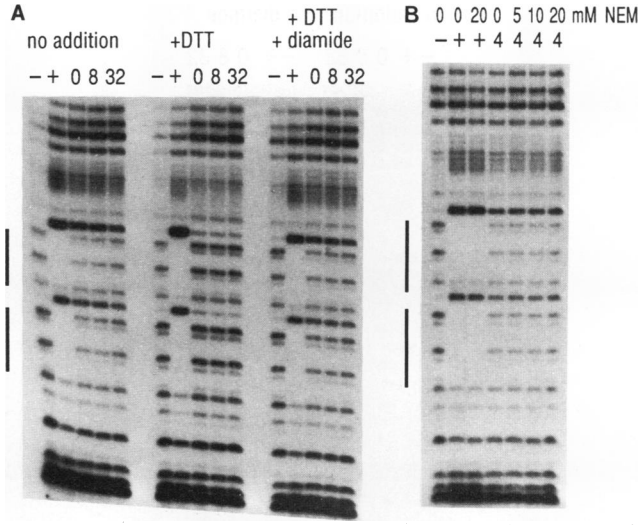

FIG. 4. (A) DTT inhibition of HoxB5 cooperative binding is reversed by diamide. Footprint challenge assay was with $HoxB5_{NHC}$ and LP_2 probe, with the lanes labeled as described in the legend to Fig. 1B. The $HoxB5_{NHC}$ protein used in this assay was untreated, incubated with 5 mM DTT, or incubated with 5 mM DTT and then with 10 mM diamide, as described in the text. (B) NEM treatment does not affect binding or cooperativity of HoxB5. Footprint challenge assay was with $HoxB5_{NHC}$ and LP_2 probe. Lanes: −, no protein added; +, no competitor added; 4, 4 min of competition prior to DNase I treatment. Prior to the binding reaction, the protein was incubated at 20°C for 30 min with the concentration of NEM shown above the lanes.

factors (1, 4, 14, 30, 32, 39, 49), we tested the effect of oxidizing agents on the DNA binding and cooperativity of HoxB5. Treatment of HoxB5 protein with 5 mM sodium tetrathionate, 10 mM diamide, or 5 mM H_2O_2 had no effect on binding or cooperativity (data not shown). Another common feature of mammalian DNA binding proteins, whose in vitro binding requires a reducing environment, is that DNA binding is inhibited by 5 mM NEM, which alkylates free sulfhydryl groups (1, 14, 30, 49). To test whether the purified $HoxB5_{NHC}$ protein contains a reduced cysteine residue sensitive to alkylation, $HoxB5_{NHC}$ protein was treated with NEM at 20°C for 30 min prior to the standard DNA binding reaction. Figure 4B shows that there is no effect of NEM on DNA binding or cooperativity. Even after treatment with 20 mM NEM, the $HoxB5_{NHC}$ protein still binds to LP sites and shows no detectable loss of cooperative binding (Fig. 4B).

Identification of the cysteine required for cooperative binding. Although there are three cysteines in full-length HoxB5 protein, the truncated HoxB5 protein, $HoxB5_{NHC}$, which binds cooperatively to tandem DNA sites, contains only a single cysteine residue (Cys-232 [Fig. 1A]). By analogy to the nuclear magnetic resonance structure of the Antp HD (37) and the crystal structure of the engrailed HD (22), this cysteine (HD residue 39, by the numbering system of references 22 and 37) is predicted to reside in the turn between helices 2 and 3 of the HD. This region is the helix-turn-helix structure conserved in the DNA binding domain of a variety of proteins (22). To determine whether Cys-232 is required for redox regulation, it was replaced in the $HoxB5_{NHC}$ protein by a serine residue. This altered protein, $HoxB5_{NHCser232}$, still specifically binds to LP sites (Fig. 5, no competitor lane) but has lost the ability to cooperatively

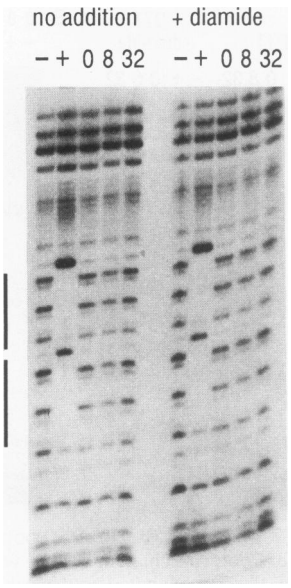

FIG. 5. Cys-232 is required for cooperative binding and redox regulation. Footprint challenge assay was with $HoxB5_{NHCser232}$ and LP_2 probe, with the lanes labeled as described in the legend to Fig. 1B. The Ser-232 mutant has a serine residue in place of the single cysteine residue in the $HoxB5_{NHC}$ protein. Where indicated, 10 mM diamide was present during the binding and competition reactions.

bind to tandem sites, as the footprint was lost as soon as competitor was added (Fig. 5, lane 0). To test whether loss of cooperative stabilization of DNA binding was due specifically to replacing Cys-232 with a serine residue, Cys-232 was also replaced with glycine, histidine, or asparagine residues. As does $HoxB5_{NHCser232}$, these mutant proteins all specifically bind to single LP sites with similar affinity but show no cooperative binding to tandem sites (data not shown). Because cooperative binding of DTT-treated $HoxB5_{NHC}$ protein can be restored by oxidation with diamide (Fig. 4A), we tested whether treatment of $HoxB5_{NHCser232}$ with 10 mM diamide during binding and competition causes this mutant protein to cooperatively bind to tandem sites. Figure 5 shows that diamide treatment does not lead to cooperative binding of the $HoxB5_{NHCser232}$ mutant protein.

HoxB5 forms intermolecular disulfide bonds. Cys-232 is the only cysteine residue in the entire $HoxB5_{NHC}$ protein, and thus intramolecular disulfide bonds are not possible. Because it appears that the cysteine residue in the HD needs to be oxidized for cooperative DNA binding, we examined whether Cys-232 participates in intermolecular disulfide bond formation. Purified HoxB5 proteins were analyzed by nonreducing or reducing SDS-PAGE followed by immunoblotting with an antibody directed against full-length HoxB5 protein. Western blot analysis of a nonreducing gel (Fig. 6) shows that even after being boiled in SDS-containing buffer, a significant portion of the purified $HoxB5_{NHC}$ runs at about twice the predicted size. The higher-molecular-weight forms are not present when the protein was reduced by treatment with 2-ME prior to electrophoresis (Fig. 6). This result, along with the absence of a higher-molecular-weight forms in unreduced mutant proteins in which Cys-232 was replaced by a serine residue (Fig. 6), reveals that the higher-molecular-weight form is a disulfide-linked dimer. Furthermore, the fact that the higher-molecular-weight form changes size proportionally to the different truncated HoxB5 monomers

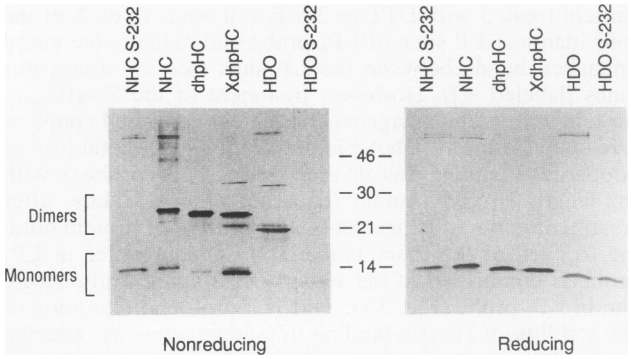

FIG. 6. Truncated HoxB5 proteins from disulfide-linked homodimers. Western blot analysis of reduced or unreduced purified HoxB5 proteins was performed as follows. The purified HoxB5 proteins indicated above the lanes were boiled in sample buffer without (nonreducing) or with (reducing) 10% 2-ME and fractionated by SDS-PAGE on separate gels. The proteins were transferred to nitrocellulose, and the immunoblots were probed with anti-HoxB5 polyclonal antibodies. The expected sizes of monomers and dimers are shown on the left. The positions of molecular mass markers (in kilodaltons) are indicated between the two gels.

and the absence of sufficient amounts of small bacterial proteins in the purified preparations indicate that the higher-molecular-weight forms are disulfide-linked HoxB5 homodimers. Surprisingly, all of the truncated proteins containing wild-type Cys-232 form disulfide-linked dimers (Fig. 6). These dimers are present even in purified $HoxB5_{HDO}$ protein, which is only the HoxB5 HD, a protein that does not bind cooperatively (Fig. 1). Thus, while an oxidized cysteine residue may be necessary for cooperative binding, disulfide bond formation between $HoxB5_{HDO}$ monomers capable of sequence-specific DNA binding is not sufficient for cooperative binding. In an initial attempt to examine whether disulfide bond formation is necessary for cooperative binding, purified $HoxB5_{NHC}$ protein that cooperatively bound DNA was enriched by extensively washing the protein bound to DNA affinity resin with LP_2 competitor. The expectation was that in the presence of a large amount of specific competitor DNA, only the cooperatively stabilized HoxB5 protein would remain bound to the tandem sites on the DNA affinity resin. The stably bound protein was then eluted with high salt and analyzed by SDS-PAGE and immunoblotting; it was found that there was no change from the starting material in the ratio of monomers to covalent dimers (data not shown). This result is consistent with the idea that not all the HoxB5 proteins that participate in cooperative DNA binding are disulfide-linked dimers.

DISCUSSION

In this work, we have defined the non-HD sequences necessary for cooperative DNA binding of human HoxB5 protein in vitro. We have also shown that the cooperative stabilization of HoxB5 DNA binding, but not sequence-specific DNA binding, is under redox regulation. Cooperative binding and redox regulation were found to require the presence of the cysteine residue in the turn between HD helices 2 and 3. Although the in vitro DNA binding of a number of mammalian proteins has been shown to be inhibited by oxidation, the HoxB5 protein is an example of protein oxidation stabilizing DNA binding.

Multiple clustered HD protein binding sites are found in

the promoters of *Drosophila* and vertebrate genes whose expression is regulated by Antp-type HD proteins. Thus, cooperative interactions could have a large influence on the DNA binding of HD proteins to these sites. Furthermore, the specificity of cooperative protein-protein interactions may be influenced both by the identity of the HD proteins and the sites to which they are bound. The homocooperative binding described here, as well as possible heterocooperative interactions, represents a mechanism beyond simple sequence-specific DNA binding that could allow specific transcriptional regulation by HD proteins with similar in vitro DNA binding specificities. Cooperative HoxB5 binding to *HoxB5* promoter sites A and B (shown in Fig. 3B) may have an important function. These sites are in the region of the TATA box, are conserved in the *HoxB5* paralogs *HoxA5* (*Hox1.3*) and *HoxC5* (*Hox3.4*), and were found essential for *HoxC5* promoter activity (2, 34). Although the transcriptional activity of HoxB5 has not yet been defined, the overlap of the TATA box and the HoxB5 protein binding sequences may be significant, because in vitro analysis has revealed that the *Drosophila* engrailed protein competes with TFIID (36), and both the *Drosophila* Ubx and eve HD proteins act to regulate transcription preinitiation complex formation (23). A rather short (12-amino-acid) sequence adjacent to the N-terminal side of the HoxB5 HD is sufficient to confer cooperative DNA binding to the HD. Without this sequence, as with other HD-only polypeptides that have been examined, the HoxB5 HD binds to DNA in a sequence-specific manner but not cooperatively to tandem sites. The region just N terminal to the HD is most conserved among Hox paralogs and thus may confer specific regulatory functions to each paralog group. A recent nuclear magnetic resonance analysis of the Antp protein in the region adjacent to the N-terminal side of the HD found this sequence to be flexibly disordered (40). However, these findings are not directly applicable to HoxB5 structure, because with the exception of the hexapeptide sequence, which we have shown here is not necessary for cooperative DNA binding, there is little sequence similarity between these regions of Antp and HoxB5. The role of the evolutionarily conserved hexapeptide sequence in HD proteins remains unclear, as no function has been revealed by studies of transactivation (3), DNA binding (40), or the putative protein-protein interactions of homocooperative DNA binding (this work).

Because reducing agents block the cooperative DNA binding of HoxB5 to tandem sites but not sequence-specific binding to single or multiple sites, it is reasonable to conclude that the target for reducing agents is involved in protein-protein interactions rather than protein-DNA interactions. There is only one cysteine residue in the entire $HoxB5_{NHC}$ protein (Cys-232), and replacement of this cysteine residue in the HD with various other amino acids abolishes cooperative binding. Thus, replacement of Cys-232 has the same detrimental effect on cooperative binding of $HoxB5_{NHC}$ as reducing agents, except that cooperativity of a cysteine replacement mutant protein cannot be rescued by oxidizing agents (Fig. 5). Taken together, these data suggest that redox regulation is mediated by reduction and oxidation of Cys-232. The mechanisms by which oxidized cysteine residues act to alter the DNA binding activity of redox-regulated proteins are not clear. In preliminary experiments with mammalian cells, we have found no disulfide-linked HoxB5 dimers in transfected human cells expressing high levels of HoxB5 protein. We have shown here that an intermolecular disulfide bond is formed in a high proportion of the HoxB5 protein purified after expression in *E. coli*. However, the formation of these covalent dimers is not sufficient for cooperative DNA binding, as the purified $HoxB5_{HDO}$ protein (just the HD) forms disulfide-linked dimers but does not bind cooperatively to tandem sites. Thus, cooperative DNA binding to tandem sites is not simply a consequence of covalently linking two DNA binding domains together with a disulfide bond. Nonetheless, while our data show that disulfide-linked dimerization is not sufficient for cooperative binding, they do not yet distinguish whether disulfide bond formation is necessary for cooperativity. Gel filtration analysis and cross-linking studies have revealed that $HoxB5_{NHC}$ forms noncovalent dimers in the absence of DNA and that dimerization is not prevented by replacement of Cys-232 with a serine residue (11). These results also suggest that cooperative binding requires more than simple dimerization. It is possible that the disulfide-linked homodimers are a consequence of reactive cysteine residues brought together by noncovalent dimerization, similar to what is seen with C/EBP-related proteins (54). This view is supported by the finding that a significant portion of the HoxB5 proteins exists as disulfide-linked dimers, even in unpurified extracts of bacteria expressing these proteins (11). Spontaneous intermolecular disulfide bonds were also found to form in vitro between the corresponding cysteine residues of the bacterially expressed Antp HD, but noncovalent dimers were not detected (33).

The *E. coli* OxyR protein, which exhibits the most similar regulation by oxidation to HoxB5, binds to DNA both when oxidized or reduced but activates transcription only when oxidized (47). On the basis of differences seen in the enhanced bands of DNase I footprints of oxidized and reduced OxyR, it was postulated that oxidation causes a conformational change in OxyR (47). Oxidation-induced changes in the DNase I footprint patterns were also consistently seen with HoxB5, as reducing agents cause a large increase in the intensity of the enhanced band between each LP site (Fig. 3A). Prior to competitor addition, the four LP sites are completely protected by HoxB5 protein in the presence or absence of DTT or 2-ME, indicating that there is no change in binding site occupancy. Thus, the reducing agent-mediated increase in DNase I cleavage between the LP sites (Fig. 3A, lanes +) reveals that reducing agents alter the conformation of the HoxB5-DNA complex.

For proteins whose DNA binding is inhibited by oxidation, the alternative inhibition of DNA binding by NEM suggests that they contain reactive and exposed sulfhydryl groups. In contrast, NEM has no effect on HoxB5 DNA binding or cooperativity (Fig. 4B). We cannot distinguish from this result whether the HoxB5 Cys-232 is sterically inaccessible to NEM, is in an unreactive form, or is alkylated without any effect on DNA binding or cooperativity. It has been proposed that under some conditions, cysteine residue oxidation can lead to formation of sulfenic acid (1, 47). Analysis of the crystal structure of both papain (21) and NADPH peroxidase (46) has revealed that they contain oxidized cysteine residues in their active sites, with histidine residues nearby. The tertiary structure of cocrystals of the engrailed HD (22) or the MATα2 HD (55) bound to DNA suggests that the N terminus of the HD is close to the turn between helices 2 and 3. Thus, the small domain required for cooperativity that is just N terminal to the HD may be in a position to interact with Cys-232. On the bases of this potential interaction, the data suggesting oxidized cysteine residues can be stabilized by histidines, and the loss of cooperative DNA binding of $HoxB5_{dhpHC}$ after substitution of leucine residues for His-184 and His-187, a possible model

is that tertiary structure allows these histidine residues to participate in stabilizing Cys-232 in an oxidized form. This oxidized cysteine residue could play an essential role in cooperative interactions. However, none of our mutant or redox data exclude the possibilities that oxidation of Cys-232 leads to disulfide-linked dimers and that the disulfide linkage, along with the small domain N terminal to the HD, leads to the proper HoxB5 conformation for cooperative stabilization of binding to adjacent DNA sites.

While redox regulation is a mechanism by which bacteria alter transcription factor activity to cope with oxidative stress (6, 47), it may be a more general eukaryotic mechanism for rapid, reversible, posttranslational modulation of DNA binding activity. It has been established for a number of proteins that in vitro DNA binding is inhibited by oxidation of cysteine residues, and we have shown here that cysteine oxidation can enhance the DNA binding of HoxB5. We are now engaged in an analysis of the transcriptional activity of the HoxB5 protein, and the influence of cooperative DNA binding and redox regulation on target gene expression. Redox regulation may be a way to fine-tune the spatial and temporal expression of genes regulated by Hox proteins, perhaps even to respond to gradients of oxygen. Enhanced DNA binding of oxidized proteins may easily be overlooked, because reducing agents are routinely added to most buffers used in protein purifications and DNA binding assays. Studies of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase suggest that under redox conditions similar to those found inside mammalian cells, oxidized cysteine residues can be stably formed (5). Many cysteine residues that have been identified as targets of redox regulation are in a local basic environment, which greatly enhances their potential for oxidation (45). Redox regulation may be carried out by specific redox proteins in the cell, as sequence similarities have been observed in the region adjacent to the target cysteines of redox-regulated proteins (24, 30, 58), and a nuclear protein (Ref-1) has been shown to restore DNA binding in vitro to several of these proteins (58). The region around Cys-232 of HoxB5 does not conform to the sequence similarities seen around oxidation-inhibited DNA binding proteins, and while HD is rich in basic amino acids, Cys-232 is flanked on either side by leucine residues. Thus, the susceptibility Cys-232 to oxidation may be due to interactions with basic residues brought about by the tertiary structure of the protein. Sequence comparison of Hox proteins reveals that the cysteine residue is not one of the highly conserved HD residues, but the presence or absence of the cysteine is conserved within all of the Hox cognate groups. With the exception of the deformed protein, this conservation extends to the corresponding *Drosophila* homologs and to the more highly diverged families of mammalian HD-containing genes (44). Because the engrailed and Ftz proteins cooperatively bind DNA (7, 35) but do not contain a cysteine residue in their HDs, it appears some cooperative DNA binding by Antp-type HD proteins is either not subject to redox regulation or involves other protein domains. Overall, to understand the function of Hox proteins, it will be important to determine the effects and generality of cooperative DNA binding and the role of redox regulation in modulating this cooperative binding.

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