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Identification of Novel Functional Regions Important for the Activity of HOXB7 in Mammalian Cells

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Members of the HOX family of homeobox transcription factors play a role in pattern formation in diverse developmental systems. The clearly documented role of HOX genes in the proliferation and differentiation of primary hematopoietic cells and cell lines provides a convenient system to pursue a biochemical analysis of HOX gene function in mammalian cells. To explore the role of HOXB7 in myeloid hematopoiesis, a number of mutations and deletions in the gene were constructed that targeted sequences with known functions or in regions that had not been examined previously. The wild-type and mutant B7 constructs were introduced into the murine myelomonocytic cell line, 32D, and assayed for their effects on G-CSF-induced myeloid differentiation. Wild-type HOXB7 inhibited the differentiation of 32D cells, whereas mutations in the Pbx-binding pentapeptide motif or the DNA-binding homeodomain, as well as internal deletions of the N-terminal unique region, blocked this effect. Interestingly, mutations eliminating two target sites for casein kinase II, the glutamate-rich C terminus, or the first 14 amino acids of HOXB7, led to enhanced 32D differentiation. A model proposing a role for these regions of HOXB7 is presented. The Journal of Immunology, 2001, 166: 5058–5067.

Homeobox genes encode transcription factors that are known to be important in development. These genes were originally identified in the Drosophila HOM-C locus and are best known for their role in patterning the anterior-posterior axis during Drosophila embryogenesis. Their name is derived from the 183-bp sequence that encodes the homeodomain, a helix-loop-helix DNA-binding motif.

The HOX genes are vertebrate homologues of the Drosophila HOM-C genes. Like their Drosophila counterparts, HOX genes play a role in axis formation during embryogenesis and also specify additional differentiation events in both embryonic and adult tissues. Thirty-nine HOX genes reside in four chromosomal complexes (A–D) that are analogous to the HOM-C complex and are thought to be the result of an early gene duplication event. Homology is greatest between genes that occupy the same linear position in the respective complexes. Based on this homology, HOX genes are subdivided into 13 groups termed paralogues. HOX genes are designated by the complex in which they reside (A–D), followed by their parologue number (1–13).

A number of studies have implicated HOX genes as important regulators of hematopoiesis, an ongoing developmental process in which the full array of blood cells is continually derived from a pool of pluripotent hematopoietic stem cells. Gene expression surveys have revealed that these genes are differentially expressed in subpopulations of hematopoietic progenitors according to both lineage and stage of maturation (1–3). Furthermore, aberrant expression of individual HOX genes results in distinct perturbations of hematopoiesis in a HOX gene-specific manner (4–7). The compiled data portray the complexity of functions displayed by this gene family, indicating roles in both lineage determination and the maturation of blood cells (8).

The complexity of HOX gene function is illustrated by the effects of manipulated expression of individual HOX genes in normal hematopoietic progenitors. Although deregulated expression of specific HOX genes has distinct effects on hematopoietic differentiation, these effects often involve multiple lineages. For example, overexpression of HOXA10 in murine bone marrow induces expansion of multipotent myeloid progenitors, increases the frequency of progenitors with megakaryocytic potential, and inhibits B cell development (7). Overexpression of HOXB3 inhibits B and T cell development and causes expansion of bilineage granulocyte/monocyte progenitors (6). Mice bearing targeted disruptions in HOXA9 display a reduction in peripheral granulocytes and lymphocytes as well as in committed progenitors of the myeloid, erythroid, and B cell lineages (4).

Thus, a given HOX gene may be involved in multiple, distinct aspects of hematopoiesis. Such observations are consistent with the concept that HOX genes function combinatorially to regulate developmental processes. It is likely that there are specific subsets of HOX genes, the coordinated expression of which is required for proper hematopoiesis, but the precise molecular mechanisms by which they exert these effects are currently unknown.

An understanding of the biochemical basis of HOX gene function would provide an important complement to existing descriptive data. One approach to dissecting these mechanisms would be to identify sequences within these proteins that are required for their various biological activities. Previously described regions of sequence conservation between HOX proteins are the DNA-binding homeodomain (9–11), the pentapeptide motif that has been shown to mediate HOX-Pbx interactions (12, 13), and an amino-terminal octapeptide motif with an as-yet-unknown function. Sequences outside of these conserved segments diverge among HOX proteins and are intriguing for their potential as regions that may interact with different cofactors, regulate DNA binding specificity, or provide targets for posttranslational regulation of protein activity.
HOXB7 was selected as a model gene for further investigation into the mechanisms underlying HOX function. Expression of this gene has been detected in multiple subpopulations of hematopoietic progenitors, including populations enriched for multipotent, erythroid (3), myeloid (1, 3, 14), and megakaryocytic (Y.Y., unpublished results) progenitors. Furthermore, HOXB7 expression is induced after activation of B and T lymphocytes (15, 16). Hence, this gene is potentially important at various stages in virtually all hematopoietic lineages. An inhibitory effect of HOXB7 on granulocytic differentiation has been suggested, based on the observed inhibition resulting from enforced HOXB7 expression on the granulocytic differentiation of the human myelomonocytic cell line HL-60 (14). This effect has recently been confirmed in primary human hematopoietic cells in a study showing that hematopoietic progenitors retrovirally transduced with HOXB7 displayed enhanced proliferation in unilineage granulocytic and monocytic cultures associated with a delay in differentiation (17). This effect was most pronounced in granulocytic cultures and was not observed in unilineage erythroid or megakaryocytic cultures.

To perform a detailed functional analysis of HOXB7, a number of mutations and deletions were introduced into the cDNA and assayed for their effects on the G-CSF-induced differentiation of the murine cell line, 32DClone3 (32D). Wild-type HOXB7 inhibited this differentiation, and the mutant phenotypes led to the identification of regions of the protein that were important for this activity. Importantly, it was demonstrated that HOXB7 could be phosphorylated by the ubiquitous serine/threonine kinase, casein kinase II (CKII), and that this phosphorylation was critical for the function of HOXB7 in cell lines.

Materials and Methods

Generation of FLAG-tagged HOXB7 and HOXB7 mutants in pCDNA3

The coding sequence of HOXB7 was PCR-amplified from HOXB7LXSN, which had been constructed previously in our laboratory (14) with the following primers: 5′-GATTCCATGGTTATGCG-3′ and 3′-TTTCTCCATCCCTCCTCT-3′. The PCR product was subcloned into the TA vector (Invitrogen, Carlsbad, CA), excised with EcoRI and ligated into pCMV-thy-1.2 (kindly provided by the laboratory of Dr. Irvin Chen, University of California, Los Angeles, CA). Electroporation was conducted at 250 V, 960 μF, with a Gene Pulser (Bio-Rad, Richmond, CA). Transfected 293T cells (a human embryonic kidney line) were transiently transfected with HOXB7 coding sequence and subcloned back into pCDNA3, which had been constructed previously in our laboratory (14) with the following primers: 5′-CTGGCAAGAGCTCGAGGAAAAGC-3′ and 3′-GTCGACTCCGAGGAGCTCACTC-3′. In addition, the putative CKII sites was created by digesting FB7S132,133ApCDNA3 with SstII and HindIII, releasing an insert containing the alanine substitutions and ligation of the HOXB7 coding sequence in frame to an upstream FLAG coding sequence to generate FLAG-HOXB7-pBSK. FLAG-HOXB7-pBSK was digested with HindIII and XhoI and the insert ligated into HindIII/XhoI-digested pCDNA3 (Invitrogen), creating FLAG-HOXB7-pCDNA3 (FB7pCDNA3). The various mutants of HOXB7 are summarized in Table I and Fig. 3. All mutations were generated by using standard PCR mutagenesis, introducing the desired mutations into primers used to amplify fragments of HOXB7, and subcloning back into FB7pCDNA3. In each case, the FLAG epitope remained on the 5′ end of each construct to facilitate detection of the expressed protein by immunoblotting. All constructs were sequenced to verify that the only desired mutations were introduced during the PCR and subcloning procedures.

Transient transfection of K562 and 293T cells

The human erythroleukemia cell line, K562 (102 cells), was electroporated with 30 μg of pCDNA3, FB7pCDNA3, or FB7pCDNA3 mutants, and 3 μg of the Thy expression vector, pCMV-thy-1.2 (kindly provided by the laboratory of Dr. Irvin Chen, University of California, Los Angeles, CA). Electroporation was conducted at 250 V, 960 μF, with a Gene Pulser (Bio-Rad, Richmond, CA). Cotransfected cells expressing cell-surface Thy1.2 were purified after 16–24 h by using the Miltenyi MiniMacs columns and magnetic beads conjugated to anti-murine Thy1.2 according to the manufacturer’s instructions (Miltenyi, Sunnyvale, CA). 293T cells (a human embryonic kidney line) were transiently transfected with pCDNA3, FB7pCDNA3, and FB7Δ(1–14)pCDNA3, by using the CalPhos Maximizer transfection kit (Clontech, Palo Alto, CA) according to the manufacturer’s instructions. After transfection (16–24 h), cells were harvested for analysis by Western blotting.

Western blot analysis

32D cells (3.5 × 105, see below), 293T cells (105), or K562 cells (105) were resuspended in reducing SDS sample buffer, boiled for 5 min, and centrifuged 1–2 min to pellet debris. Cell lysates were separated on 12% or 15% polyacrylamide gels and electroblotted to Hybond ECL nitrocellulose (Amersham, Arlington Heights, IL). Blots were blocked with 5% nonfat dry milk and probed with anti-FLAG M2 or M5 Ab (Sigma, St. Louis, MO), followed by a secondary sheep Ab against mouse IgG, coupled to biotin (Amersham). Protein bands were visualized with HRP-conjugated streptavidin (Amersham), followed by detection by ECL (Amersham-Pharmacia Biotech, Piscataway, NJ). For some of the 32D Western blots, a secondary Ab directly conjugated to HRP was used in an attempt to reduce background bands.

Subcellular localization of FLAG-HOXB7 wild-type and mutant proteins

Wild-type and mutant constructs of FB7pCDNA3 were digested with EcoRI, and the released HOXB7 inserts ligated into the EcoRI sites of pEGFP-C2 (Clontech) to create a fusion of the enhanced green fluorescent protein (EGFP) upstream of the HOXB7 coding sequence. NIH3T3 cells were transfected with pEGFP alone or with pEGFP containing wild-type or mutant HOXB7, with the CalPhos Maximizer transfection kit (Clontech) according to the manufacturer’s instructions. After overnight incubation, expression and cellular localization of the EGFP fusion proteins were visualized by fluorescence microscopy.

Generation of 32D stable cell lines

The myeloid murine factor-dependent 32DClone3 cell line (32D; generously provided by Joel Greenberger, University of Pittsburgh, Pittsburgh, PA) was maintained in culture medium (1× IMDM supplemented with 10% FBS (Omega Scientific, Tarzana, CA), 0.4 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin). The medium was supplemented with 200 μM murine GM-CSF (generously provided by Amgen, Thousand Oaks, CA). Cells (107) were electroporated with 30 μg of pCDNA3, FB7pCDNA3, or FB7pCDNA3 mutant constructs at 250 V, 960 μF, by using a Gene Pulser (Bio-Rad). After a 24-h incubation, transfected cells were selected on 1.2 mg/ml G418 (Genetec; Life Technologies, Grand Island, NY). Expression of the FLAG-tagged HOXB7 constructs was confirmed by Western blotting with the anti-FLAG M5 Ab (Sigma).

Granulocytic differentiation of 32D cells

32D cells stably expressing pCDNA3, FB7pCDNA3, or the FB7pCDNA3 mutants were washed three times in 1× PBS to remove the GM-CSF and plated in triplicate wells at a density of 104 cells/ml in culture medium supplemented with 50 ng/ml human G-CSF (kindly provided by Amgen) to induce granulocytic differentiation. After 1, 2, or 3 days plating in G-CSF, 1–2.5 × 104 cells from each well were used to prepare cytopreps slides (Cytospin; Shandon, Pittsburgh, PA). The slides were dried at room temperature and stained with the Diff Quick stain set (Baxter, McGraw Park, IL). Approximately 300 cells per slide were evaluated for nuclear morphology in a blinded fashion. Each cell was scored as either undifferentiated (blast-like morphology, with a rounded, centrally located nucleus) or differentiated (nuclei possessing kidney-shaped, banded, or segmented nuclei). The percentage of differentiated cells for each slide was calculated, and the average value of the triplicates for each construct was determined. A minimum of three experiments was conducted in triplicate for stable cell lines of each construct.

Production of GST-HOXB7 fusion proteins and in vitro CKII assays

FB7(S132, 133A)/(T203, 204A)pCDNA3 containing mutants at both of the putative CKII sites was created by digesting FB7S132,133ApCDNA3 with XhoI and SrlII, releasing an insert containing the alanine substitutions at S132 and S133, and ligating this insert into XhoI/SrlII-digested FB7T203,204ApCDNA3, which released the same insert containing the wild-type sequence. The FB7, FB7S(132, 133A), FB7T(203, 204A), and FB7S(132, 133A)/AT(203, 204A) inserts were released from pCDNA3 by digestion with BstIII and ligated into the BamHI sites of pGEX4T-2 (Amersham-Pharmacia). The four resulting constructs and pGEX4T-2 alone were transfected into the DH5α bacterial strain, and single colonies containing the plasmids inoculated into 2× YT (16 g/L tryptone, 10 g/L...
The inhibitory effect that HOXB7 exerted on the granulocytic differentiation of 32D cl3 cells began to decline, possibly because of a combination of the death of terminally differentiated cells. The inhibitory effect of HOXB7 began to decline by the third day, and the number of differentiated cells in the control population and delayed differentiated cells. This activity, observed in both cultured and primary human hematopoietic cells (14, 17), was further characterized by investigating the effects of HOXB7 in the murine cell line 32D. This myeloblastic cell line is dependent on GM-CSF or IL-3 for maintenance in culture, and undergoes granulocytic differentiation in response to G-CSF treatment (Fig. 1).

Stable 32D cell lines were generated by using the pCDNA3 vector alone, as well as a construct in which pCDNA3 directs the expression of wild-type HOXB7. When the GM-CSF in the medium was replaced with G-CSF, the cells transfected with vector alone differentiated into granulocytes, whereas the cells constitutively expressing HOXB7 consistently showed a lower percentage of differentiated cells (Fig. 2). This inhibition was highly reproducible and most pronounced on the second day after G-CSF treatment (Figs. 2 and 6). By the third day, the inhibitory effect of HOXB7 began to decline, possibly because of a combination of the death of terminally differentiated cells in the control population and delayed differentiation of cells constitutively expressing HOXB7.

The inhibitory effect that HOXB7 exerted on the granulocytic differentiation of 32D cells was consistent with previous observations (14, 17) and provided a convenient method by which to study the importance of different regions of this gene in mediating its activity. A series of HOXB7 mutations was created, targeting regions of known function as well as previously uncharacterized sequences (Fig. 3). A mutant incapable of binding DNA, FB7Δ3h, was generated by deletion of the third α helix of the homeodomain (18). Another mutant was designed to destroy cooperative binding between HOXB7 and isoforms of Pbx (12) by mutating two residues in the pentapeptide, W129 and M130, to phenylalanine and isoleucine, respectively (FB7WM(129, 130)FI).

Previously uncharacterized regions of HOXB7 were targeted for mutagenesis, based on a comparison of the HOXB7 sequence with that of one of its paralogues, HOXA7 (Fig. 3A). Regions conserved...
between these proteins include a glutamate-rich region at the carboxy terminus and a sequence located between the pentapeptide motif and the homeodomain that matches the consensus target sequence ((S/T)XX(D/E)) for CKII. A second potential CKII target site, found between the homeodomain and the glutamate-rich region, exists in both human and murine HOXB7 and in murine HOXA7, although it is absent from human HOXA7. Outside of these regions and the ubiquitous homeodomain, pentapeptide motif, and octapeptide motif, sequence similarity between the two paralogues is limited to the first 14 amino acids, which can be considered as an extension of the octapeptide motif.

Both of the putative CKII target sites in HOXB7, S133 and T204, contain adjacent serine or threonine residues, S132 and T203, respectively. To eliminate the possibility of phosphorylation at these sites, both residues at each site were mutated to alanines, creating FB7(S132, 133)A and FB7(T203, 204)A. A nonsense mutation at glutamate 210 resulted in a construct, FB7ΔGlu, encoding a truncated protein that lacks the glutamate-rich region at the carboxy terminus. Other deletions were designed to remove the first 14 amino acids (FB7Δ(1–14)), as well as to delete portions of the unique N-terminal region corresponding to amino acids 38–79 (FB7Δ(38–79)) and 81–120 (FB7Δ(81–120)). All of the deletions and substitutions are diagrammed in Fig. 3B.

The integrity of each construct was confirmed by transient expression in the human erythroleukemic cell line, K562 (Fig. 4A). Western blots from these lysates with an anti-FLAG Ab confirmed that proteins of the predicted molecular weight were expressed.

Stable 32D cell lines were generated with each of these constructs for the purpose of assessing the extent of granulocytic differentiation upon treatment with G-CSF in parallel with stable cell lines containing vector alone or wild-type HOXB7. Western blotting verified expression of the HOXB7 wild-type and mutant constructs (Fig. 4B). Expression levels of these exogenous proteins were low, probably because of the low copy number of integrated plasmids in stable cell lines.

The Western blots revealed a novel property associated with the first 14 amino acids of HOXB7. When these amino acids were deleted, cellular accumulation of the protein was greatly increased in both of the hematopoietic cell lines examined (Fig. 4, A and B). This phenomenon also was observed in transient transfections of the epithelial cell line, 293T (Fig. 4C), indicating a possible function for this sequence in regulating protein turnover.

Subcellular localization of the HOXB7 constructs was investigated to ensure that any observed functional differences were not the result of defective nuclear targeting. The wild-type and mutant HOXB7 sequences were subcloned into the EGFp vector, pEGFP, and transiently expressed in NIH3T3 cells. When viewed by fluorescence microscopy, all of the resulting fusion proteins localized to the nucleus, as would be expected of a transcription factor, whereas the unconjugated EGFp protein was evenly distributed throughout the cell (Fig. 5). Therefore, any change in activity resulting from the mutations would not be attributed to mislocalization of the proteins but would likely reflect the functional importance of the mutated sequences.

Fig. 6, A–C shows representative experiments examining the effects of HOXB7 and its mutants on the granulocytic differentiation of 32D cells. Differentiated cells were enumerated in a blinded fashion on day 2 after G-CSF treatment. A minimum of three experiments was performed in triplicate for each cell line.
and triplicates were averaged to determine the percent of granulocytic differentiation. In all cases, the stable cell lines remained resistant to G418, and continued protein expression was documented by Western analysis.

As described above, enforced expression of wild-type HOXB7 resulted in inhibition of granulocytic differentiation. Deletion of helix 3 of the homeodomain abolished this inhibitory activity, restoring the level of differentiation to that seen in the vector control (Fig. 6A). This result is consistent with the requirement for DNA binding to elicit the biological activity of HOXB7, as demonstrated previously for other HOX family members. Similarly, mutation of the pentapeptide sequences restored levels of differentiation to that of the control, indicating that dimerization of HOXB7 with Pbx or one of its homologues is also required for this biological effect (Fig. 6A). A similar loss of HOXB7 inhibitory activity was observed in constructs lacking previously uncharacterized regions localized within amino acids 38–79 (FB7D(38–79)) and 81–120 (FB7D(81–120); Fig. 6A).

One class of HOXB7 mutants consistently produced an effect opposite to that observed with the wild-type protein (Fig. 6B and C). In cells overexpressing these mutants, the level of granulocytic differentiation exceeded the level seen in the vector control population. Cells transfected with HOXB7 containing point mutations at either of the putative CKII sites (FB7S(132, 133)A or FB7T(203, 204)A) or lacking the glutamate-rich region (FB7D(Glu)) exhibited a reproducible and dramatic enhancement of differentiation on day 2, whereas a more modest enhancement of differentiation on day 2 was observed in cells stably expressing HOXB7 that contained a deletion of the first 14 amino acids (FB7Δ(1–14)).

A 3-day time course was performed to expand on these findings. The results showed that the dramatic enhancement of differentiation was apparent on the first day after G-CSF treatment for all four constructs (Fig. 7). Deletion of the glutamate-rich region (Fig. 7A) or substitution of the CKII sites (Fig. 7B) reproducibly enhanced differentiation of 32D cells on days 1–3 after treatment. The effect of FB7Δ(1–14) was most pronounced on day 1 compared with the effects seen on days 2 and 3 (Fig. 7A). These trends were observed in three of three experiments for all four constructs.

Growth curves in the presence of G-CSF were performed to evaluate the effects of the various HOXB7 constructs on cell proliferation. In seven of a total of eight experiments, stable cell lines expressing wild-type HOXB7 showed a reduction in total cell numbers on days 1–3 of G-CSF treatment as compared with vector controls (Fig. 8). Thus, it appeared that the inhibitory effect of HOXB7 on granulocytic differentiation of 32D cells was not associated with increased proliferation of these cells. Interestingly, the higher percentage of differentiation seen with FB7Δ(1–14) correlated with a trend toward increasing cell numbers (Fig. 8). This effect was dramatic, and was observed in three of three experiments. Cell populations stably expressing the remainder of the HOXB7 mutant constructs did not reproducibly exhibit cell numbers that differed significantly from plasmid control values (data not shown).

The effect of the mutations at either of the putative CKII target sites on the activity of HOXB7 suggested that HOXB7 is a substrate for this kinase. To further investigate this possibility, we expressed GST-fusion proteins consisting of wild-type HOXB7 or
proteins mutated at either or both of the putative CKII target sites and assayed the ability of CKII to phosphorylate these proteins in an in vitro assay. As shown in Fig. 9, wild-type HOXB7, both of the single site mutants, and a standard substrate (β-casein) all became phosphorylated when incubated with CKII. No phosphorylation was evident when both of the putative CKII sites were mutated together, nor was CKII able to phosphorylate GST alone. Autophosphorylation of CKII served as a convenient internal control, and an immunoblot confirmed that proteins were loaded equivalently (data not shown). Thus, we concluded that CKII can phosphorylate HOXB7 in vitro and that this phosphorylation occurs at both of the CKII target sites, S133 and T204.

Discussion

In this study, we have extended a previously reported inhibitory effect of HOXB7 on granulocytic differentiation to the murine cell line, 32D, and have used this effect in an assay to identify regions in the protein that are important for its activity. The differentiation assay in 32D cells provided a physiologically relevant system in which to further characterize the activity of HOXB7 through structure-function studies, as these cells differentiate in response to a growth factor (G-CSF) that is important for granulocytic differentiation in vivo. The effects of various mutations on the activity of HOXB7 were assessed and could be categorized by functional groups that lend insight into the underlying mechanisms of HOXB7 function.

HOX proteins are known to function as transcriptional regulators, activating or repressing the transcription of target genes, thereby establishing patterns of gene expression that guide cells toward their specific developmental fates. In vitro, these proteins have been shown to bind very similar DNA sequences, a property that contrasts greatly with their distinct and complex biological roles (10, 19). Thus, it has been postulated that mechanisms exist in vivo to direct HOX proteins to relevant target DNA sequences and to affect their activity once they have bound those sequences.

The Pbx family of homeodomain proteins has been identified in this capacity as cofactors that enhance the specificity and affinity of HOX proteins for DNA (20, 21). This family includes three known members, Pbx1, Pbx2, and Pbx3. Pbx1 and Pbx3 also exist as alternatively spliced forms (22). Many of the biological effects of HOX proteins are mediated through interaction with Pbx (23, 24). This interaction requires the pentapeptide motif of HOX proteins, as demonstrated by a number of groups (12, 13, 25). Furthermore, it can be imagined that different effects can be achieved, depending on the Pbx isoform involved in the HOX-Pbx interaction.

In this study, the inhibitory effect of HOXB7 on granulocytic differentiation was specifically mediated via the transcriptional regulation of target genes, as demonstrated by the requirement for the intact HOXB7 DNA binding domain (FB7Dh3). Furthermore, this effect required interactions with Pbx or a related molecule because a mutation of the pentapeptide motif resulted in loss of this activity.

The observed effects of the remaining HOXB7 mutants could be classified into two groups. One group resulted in loss of the inhibitory activity of HOXB7 on granulocytic differentiation, and the other group reversed the effect of HOXB7 overexpression in that cells transfected with these mutant constructs displayed a greater rate and extent of differentiation upon treatment with G-CSF than control cells transfected with the empty vector.

FIGURE 5. Cellular localization of HOXB7 and HOXB7 mutants. HOXB7 and HOXB7 mutants conjugated to EGFP were transiently expressed in NIH3T3 cells. Cells are shown in bright field (left) and corresponding dark field (right). A, EGFP; B, EGFP-HOXB7; C, EGFP-HOXB7Δh3; D, EGFP-HOXB7WM(129,130)FI; E, EGFP-HOXB7ΔGLU; F, EGFP-HOXB7Δ(1–14); G, EGFP-HOXB7Δ(38–79); H, EGFP-HOXB7Δ(81–120); I, EGFP-HOXB7S(132,133)A; J, EGFP-HOXB7T(203,204)A.
dominant negative effect, in which the mutants of HOXB7 interfere with the endogenous pool of HOXB7 proteins, or alternatively, a change in the specificity of the transfected HOXB7 gene for its targets. An RT-PCR protocol specifically designed to amplify HOXB7 mRNA demonstrated no expression of endogenous HOXB7 in 32D cells, either before or after G-CSF-induced granulocytic differentiation (data not shown). These results differ from previous reports demonstrating expression of HOXB7 in the 32D cell line, also by a RT-PCR strategy (26). This discrepancy may be explained by the accumulation of subtle genetic differences after prolonged passage of the same cell line in different laboratories.

Based on these results, because we cannot demonstrate expression of HOXB7 in these 32D cells, a dominant negative effect by the differentiation-enhancing mutants is unlikely. Therefore, we hypothesize that these mutations have altered the specificity of action of HOXB7, possibly by modifying the protein’s DNA binding specificity and/or its transcriptional activity.

The possible functions performed by the different regions of HOXB7 that were investigated in this study are presented in the model shown in Fig. 10. This diagram integrates our data with other studies that have indicated functional significance of some of these same regions in other contexts.

Based on the result that mutations in either of the two putative CKII target sequences led to enhanced differentiation of 32D cells, it was postulated that HOXB7 might be a target for phosphorylation by CKII and that phosphorylation was occurring at both sites in the wild-type protein. Indeed, when CKII was assayed for its ability to phosphorylate HOXB7 GST fusion proteins in vitro, mutations at both CKII target sites were required to completely prevent phosphorylation.

There is growing evidence to support a role for CKII as a regulator of HOX proteins. This serine/threonine kinase is found in both the cytoplasm and the nucleus and is known to phosphorylate a wide variety of transcription factors, including other homeodomain
proteins (27–30). A number of HOX and HOM-C proteins have been shown to exist as phosphoproteins, and in some cases, exhibit different properties, depending on their phosphorylation state (31–33). Recently, the Drosophila HOX homologue, Antennapedia (34), and the vertebrate protein, HOXB6 (35), have been shown to be phosphorylated by CKII, providing additional support for the involvement of CKII in regulating HOXB7 in vivo.

The glutamate-rich region at the carboxy terminus of HOXB7 is found in varying lengths in a subset of other HOX proteins, including HOXA7. Other laboratories have reported the effect of deletions of the C terminus on transcriptional activity in HOXA7 and HOXB7. For example, Schnabel and Abate-Shen (36) deleted the glutamate-rich region and an additional 25 amino acids upstream of it from HOXA7, and observed a reduction in transcriptional repression of a reporter gene in transient transfection assays. More recently, a carboxyl-terminal deletion of HOXB7 was reported to cause reduced transcriptional activation of a reporter gene (37). This deletion spanned the glutamate-rich region, but also eliminated the CKII consensus target sequence at T204. It cannot be determined in either case whether the effects of these respective deletions are attributable solely to the loss of the glutamate-rich regions of these proteins or to loss of other nearby residues that may be important for protein function. In this study, only the glutamate-rich region was deleted, leaving all upstream residues intact, resulting in the reversal of HOXB7 activity. Whether the glutamate-rich region provides either a direct transcriptional activation or repression function is yet to be determined.

The data presented here suggest an alternative hypothesis, which is that the glutamate-rich region is functionally linked to the potential CKII site at T204. CKII requires the presence of an acidic amino acid C-terminal to the phosphoacceptor serine or threonine, preferably located three residues away ((S/T)XX(D/E); Ref. 38). This site becomes an even more potent CKII target if it is followed by a string of acidic amino acids further downstream (34, 38). The similarity of the effects on HOXB7 activity resulting from the respective mutations at T204 and the glutamate-rich region supports the possibility that the glutamate stretch is important for the phosphorylation of the downstream CKII site.

Indeed, mutations at both of the putative CKII sites had effects similar to those seen after deletion of the glutamate-rich region. An attractive hypothesis is that these three regions, by virtue of their proximity to the pentapeptide motif and the homeodomain, help to determine which DNA sites are bound by HOXB7. For example, amino acid C-terminal to the phosphoacceptor serine or threonine, preferably located three residues away ((S/T)XX(D/E); Ref. 38). This site becomes an even more potent CKII target if it is followed by a string of acidic amino acids further downstream (34, 38). The similarity of the effects on HOXB7 activity resulting from the respective mutations at T204 and the glutamate-rich region supports the possibility that the glutamate stretch is important for the phosphorylation of the downstream CKII site.

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FIGURE 10. Possible functions of HOXB7 domains in inhibition of granulocytic differentiation. Known functions are designated by solid lines. Speculated functions are designated by dashed or curved lines. O, Octapeptide; P, pentapeptide; H, homeodomain; h, homeodomain helix 3. See text for discussion.

phosphorylation at S133 may determine which Pbx isoform or splice variant interacts with HOXB7. Such combinatorial interactions may direct HOXB7 to promoters of different target genes. Likewise, phosphorylation at T204 and/or a contribution of the glutamate-rich region may regulate the structure or accessibility of the homeodomain. This regulation could in turn influence interactions with other nuclear factors, or with the DNA itself, to alter the target sequences bound by HOXB7. In this model, mutations in each of these regions could change the set of target genes regulated by HOXB7 and manifest themselves as a change in activity from that of the wild-type protein.

Various deletions at the amino termini of HOX proteins have demonstrated the importance of these residues in mediating transcriptional activity. In results presented here, the deletion of amino acids 1–14 reversed the biological activity of HOXB7 in 32D cells. If this region interacts with a cofactor that facilitates the transcriptional activity of HOXB7, its deletion from the protein could possibly unmask an opposing transcriptional activity and thus reverse the effect of the overexpressed gene, as was observed here. Recently, an interaction between HOXB7 and IκBα has been reported, resulting in enhanced transcriptional activation from a HOXB7 reporter construct (39). This interaction requires the presence of the first 18 amino acids of HOXB7, a sequence comparable to the first 14 amino acids deleted in the present study. Whether this type of interaction occurs in 32D cells is unknown.

Another intriguing function associated with this sequence was its apparent effect on protein accumulation. Based on Western blots from two hematopoietic cell lines, K562 and 32D, and an epithelial cell line, 293T, this region may be involved in the regulation of protein turnover. As this region contains the conserved octapeptide motif found in many HOX proteins, it may represent a global mechanism for controlling HOX protein levels. This is an intriguing property in light of indications that relative levels of HOX gene products are important in establishing certain biological outcomes. This regulation may occur at the RNA or protein level, and may involve synthesis or degradation at these levels.

Loss of HOXB7 activity resulting from large deletions of unique sequences in its amino-terminal half (residues 38–79 and 81–120) may indicate that additional interactions are required to inhibit granulocytic differentiation. The possibility that these large deletions had a deleterious effect on global protein integrity was contradicted by the demonstration that these gene products could be stably expressed, and that they were recognized by the nuclear import machinery. However, it is possible that the effect on protein structure was more subtle, affecting the structure of the DNA-bound protein so that its affinity for the DNA or its ability to interact with cofactors was compromised. Whether these deletions disrupted the activity of the protein because of loss of specific functional regions or because of modification of protein structure remains to be determined.

Functional studies of HOXB7 have been conducted in various settings, including other hematopoietic models (Refs. 14 and 17; and unpublished observations), cancer cell lines (40, 41), and transient transfection assays using a variety of promoters (39, 42). It will be interesting to examine the effects of the HOXB7 mutations presented here in these other biological systems. A perplexing characteristic of HOX genes is their specification of distinct phenotypes in diverse systems. A better understanding of the regions required for the different activities of HOXB7 may help us to understand the complexity, specificity, and versatility that characterize HOX genes in general.

References


12. Knoepfler, P. S., and M. P. Kamps. 1995. The pentapeptide motif of Hox proteins functions are designated by solid lines. Speculated functions are designated by dashed or curved lines. O, Octapeptide; P, pentapeptide; H, homeodomain; h, homeodomain helix 3. See text for discussion.


