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HOXA5 Regulates Expression of the Progesterone Receptor*

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The majority of breast carcinomas show reduced or no expression of the transcription factor, HOXA5. Recently, we have shown that HOXA5 is a potent transactivator of p53 in breast cells and thus may affect the response of breast cancer cells to DNA damage. To determine whether HOXA5 played a role in growth and homeostasis in breast cells, we studied its interaction with the progesterone receptor. The progesterone receptor (PR) belongs to the superfamily of nuclear receptors whose members co-ordinate morphogenesis of the mammary gland in response to binding to their cognate ligands. An increased expression of the endogenous *PR* gene was seen in MCF-7 cells following induced expression of an exogenously transfected *HOXA5* gene. HOXA5, but not HOXB4, -B5, or -B7 activated the *PR* promoter in two breast cancer cell lines, MCF-7 and Hs578T. Deletion and mutation analysis of the promoter identified a single HOXA5-binding site required for transactivation of the *PR* gene by HOXA5. HOXA5 binds directly to this site in the *PR* promoter. Thus, HOXA5 may behave as a transcriptional regulator of multiple target genes, two among which are *p53* and the progesterone receptor.

The proper development of the embryonic body plan depends, in large part, on a family of genes called the *HOX* genes (1, 2). Recently, it was reported that the loss of function of several genes belonging to *HOX* group 9 impaired proper development of mammary glands in mice during and after pregnancy, thereby leading to a strong deficit in milk production and, hence, an abnormal lactation capacity (3). Our own studies have shown that the expression of one member of this family, *HOXA5*, is undetectable in nearly 60% of breast cancers (4). One target of HOXA5 action could be the progesterone receptor. The progesterone receptor belongs to a superfamily of nuclear hormone receptors (5, 6). Through its binding to progesterone, PR[1] is implicated in the control of proliferation, differentiation, and development of the breast and uterine tissues (5, 6). In the breast, while estrogen transmits a proliferative signal, progesterone through its interaction with *PR*, functions as a modulator of estrogen action, leading to pathways of differentiation (5, 6). The most direct evidence for PR function in the mammary gland comes from studies with mice lacking the *PR* gene (7). The mammary glands of these mice show incomplete ductal branching and failure of lobulo-alveolar development (7). Few upstream regulators of *PR* gene expression have been identified (8, 9). In this paper, we provide cellular and biochemical evidence that *PR* is directly regulated by HOXA5.

MATERIALS AND METHODS

Human HOX Recombinant Plasmids—The four *HOX* cDNAs from *pB*-SHOXA5 (John. F. Fuller, UCLA), pBSHOXB4 (C.-P. Chang, Stanford University), pBSHOXB5, and pBSHOXB7 (Corey Largman, UCSF), were subcloned into the *Kpn*I and *Xba*I sites of the mammalian expression vector, pCDM8 (Invitrogen). The nature of the cloned fragments was confirmed by nucleotide sequencing.

Deletion Constructs of Progesterone Receptor Reporter Plasmids—The *PR* promoter construct was prepared by PCR amplification using the following primers (GenBank™ accession number X69068) PR forward primer 5′-ACCTTTCCTCTATCTGCCT-3′ (nt 15–33) and PR reverse primer 5′-GCTTTTCTAACAACGCCTCC-3′ (nt 1135–1114), cloned into TA cloning vector (Invitrogen, CA). The 1121-bp insert was excised from the TA cloning vector by *Xho*I-*Hin*dIII restriction enzymes and cloned into pGL2 basic luciferase reporter vector (Promega Corp., Madison, WI). The resulting clone was sequenced and is referred to as −1121-bp PRPLuc. To make deletion constructs, −1121-bp PRPLuc was digested with *Kpn*I and *Xho*I to create a 3′ and 5′ overhang, respectively, at the multiple cloning site and subjected to exonuclease III nuclease digestion at 30 °C. At 30-s intervals, a sample was transferred into the S1 nuclease mixture as per the manufacturer's instructions (Erase a base kit, Promega Corp.). Following ligation and transformation, the clones containing inserts of varying sizes of the promoter region were selected. The clones were designated as follows: −792-bp PRPLuc, −460-bp PRPLuc, −294-bp PRPLuc, −143-bp PRPLuc, −67-bp PRPLuc, and −48-bp PRPLuc. In addition, we generated a clone lacking the region with a canonical HOXA5-binding site located close to the transcription start site. This was done by digesting −1121-bp PRPLuc with AgeI and *Hin*dIII, followed by recessed end filling with Klenow enzyme followed by self-ligation. The resulting clone is referred to as Δ-68 to −1-bp PRPLuc.

Site-directed Mutagenesis—The core HOXA5-binding site (nt −62 to −59) in the −1121-bp PRPLuc and −67-bp PRPLuc construct was mutated using the Altered Sites™ *in vitro* Mutagenesis System (Promega Corp.) according to the manufacturer's instructions. Mutations were confirmed by nucleotide sequencing.

DNA Transfection and Reporter Plasmid Assay—MCF7 and Hs578T breast cancer cell (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Cells were plated at 5×10^5/60-mm dish. 24 h later, cells were transfected (PanVera Corp.) with 2 μg of the indicated reporter plasmid and/or 1 μg of the expression plasmid pCMVHOXA5, -B4, -B5, or -B7. Transfection efficiency for each assay was assessed by cotransfection of 5 ng of SV40 Renilla luciferase plasmid DNA (Promega Corp.). Luciferase activities were assayed 24 h after transfection using the Dual Luciferase Assay kit (Promega Corp.). The *PR* promoter-firefly luciferase generated light output was normalized to the light output obtained with Renilla luciferase in each cell line.

The Inducible HOXA5 System—The two plasmid, ecdysone-inducible mammalian expression kit (Invitrogen), was used to generate the clones of MCF-7 cells containing an inducible *HOXA5* gene. Briefly, the *HOXA5* gene was cloned into the *Eco*RI site of the vector, pIND, generating the plasmid, pINDHOXA5. MCF-7 cells were transfected with 2 μg each of recombinant pINDHOXA5 and pVgRXR (expresses

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[1] The abbreviations used are: PR, progesterone receptor; ER, estrogen receptor; CMV, cytomegalovirus; Luc, luciferase; bp, base pair(s); Pon A, Ponasterone A.

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a heterodimeric receptor which is derived from *Drosophila* and modified to contain the VP16 transactivation domain and the retinoid X receptor) or with 2 μg each of pIND and pVgRXR using LipofectAMINE (PanVera). Six stable clones from each culture (designated MCF7-HOXA5–1 to 6 and MCF7-VGX-1 to 6) were selected for G418 (Life Technologies, Inc.) and zeocin resistance. The inducibility of HOXA5 upon addition of the ecdysone analog, Ponasterone A (5 μM, Invitrogen, Carlsbad, CA), was determined by Western analysis using the polyclonal antibody, anti-HOXA5–2 (Babco, Richmond, CA). Of the six MCF7-HOXA5 clones, only two (MCF7-HOX-1 and MCF7-HOX-2) survived passage longer than 2 months.

Preparation of Cell Extracts and Immunoblot Analysis—MCF-7 cells expressing HOXA5 under the control of the ecdysone-inducible expression system, MCF7-HOXA5 (4), were rinsed gently, twice in phosphate-buffered saline (20 mM Tris, pH 7.5, and 137 mM NaCl) and lysed in lysis buffer (0.5% Nonidet P-40, 20 mM Hepes, pH 7.5, 120 mM KCl, usb1 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). Blots were incubated with either 1 μg/ml rabbit polyclonal anti-HOXA5 (Babco) antibody, anti-β-actin (AC-15, Sigma), or affinity-purified rabbit polyclonal anti-PR (C-19, Santa Cruz) in 1 × phosphate-buffered saline containing 5% nonfat powdered dry milk and 0.25% Tween 20 for 2 h at room temperature. Immunoreactive proteins were visualized by chemiluminescence and autoradiography.

Gel-shift Assays—Nuclear extracts from SAOS2 cells transiently transfected with pCMV-HOXA5 were prepared, and gel-shift assays were performed as described by Raman *et al.* (10). The reaction was carried out in a 20-μl final volume. Nonspecific binding was eliminated by incubating 2–5 μg of extract in 20 mM HEPES-HCl, pH 7.9, 50 mM KCl, 1 mM EDTA, 10 mM$MgCl_2$, 1 μg of sonicated salmon sperm DNA (200–300 bp), 10 μg of bovine serum albumin, and 6% glycerol for 10 min at 4 °C. [γ-^{32}P]ATP-labeled oligonucleotide probe (approximately 0.3 ng per reaction) was added and incubation continued for a further 15 min, loading buffer was added, and the DNA-protein complexes were separated from the unbound probe in nondenaturing 5% polyacrylamide gels by electrophoresis at 100 V for 3–4 h in 0.25 × TBE (1×: 0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA). The gels were dried and exposed to Kodak-RP film. The 30-mer oligonucleotides (nt −70 to nt −45) containing the canonical HOXA5-binding site within the PR promoter (5′-AGATCCTACCGGTAATTGG GGTAGGGAGGG-3′), as well as the mutated form (5′-AGATCCTACCGGTGGTTGGGG-TAGGGAGGG-3′), were synthesized and high performance liquid chromatography-purified. Double-stranded oligonucleotides were end-labeled with [γ-^{32}P]ATP. To ascertain specificity of binding, unlabeled competitor oligonucleotides were incubated with the protein extract prior to the addition of the labeled oligonucleotide. A supershift assay was also performed by incubating the DNA-protein complex with 2 μg of rabbit polyclonal HOXA5 antiserum, AB2 (Babco) for 10 min on ice.

RESULTS AND DISCUSSION

In this paper, we investigated the hypothesis that HOXA5 plays a role in the development and homeostasis of the breast by regulating the levels of the hormone receptor, PR, which is crucial in its growth and differentiation (5). Seven putative HOX-binding sequences were found in the 1.1-kilobase pairs upstream regulatory sequence of the human progesterone receptor gene. The sequence similarity for the HOX-binding sites (Fig. 1, *A* and *B*) was determined by using the "sequence find" option within the DNA Strider program.

To investigate a possible regulation of PR by HOX proteins, a transient transfection assay was performed using the ER+/PR+ human breast cancer cell line, MCF-7. Cells were cotransfected with the reporter plasmid, −1121 bp PRLuc, together with expression plasmids encoding full-length human HOXA5. Strikingly, we found a 60–66-fold increase in luciferase activity in MCF-7 cells. No such effect was observed when the reporter plasmid was cotransfected with plasmids encoding the paralogous gene, *HOXB5*, or an upstream effector of HOXA5 function, *HOXB4*, or another member of the HOX gene family, *HOXB7* (Fig. 1*C*). None of the HOX proteins affected the renilla luciferase activity of the pRL-SV40-Luc control plasmid (data not shown). To extend these observations, we performed similar experiments in Hs578T cells, a human breast cancer cell line that is ER−/PR−. Transactivation of the PR promoter by

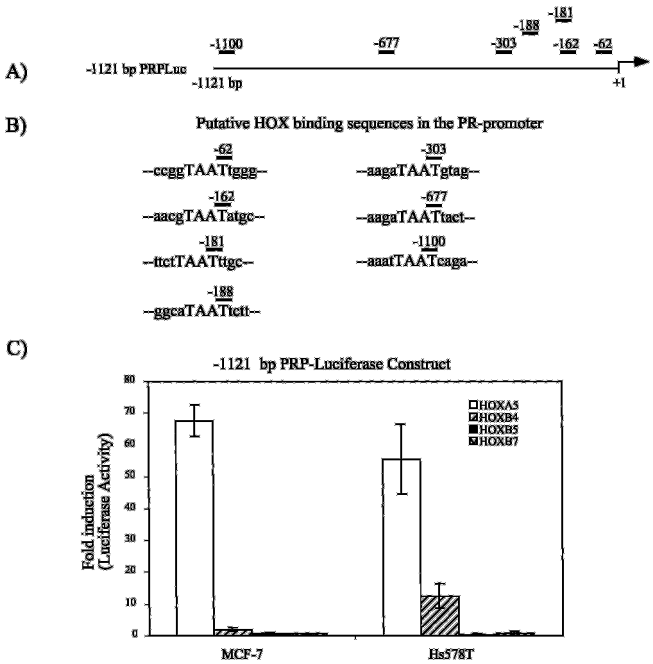

FIG. 1. **The human −1121-bp PRPLuc reporter gene is activated by HOXA5 in MCF-7 and Hs578T cells.** *A*, schematic representation of the *PR* promoter sequence, showing the location of the TAAT putative HOX-binding sites. *B*, putative HOX-binding sequence within the *PR* promoter. *C*, the −1121-bp PRP-Luc reporter reporter plasmid (2 μg) was transiently cotransfected with 1 μg of the *HOXA5*, *-B4*, *-B5*, or *-B7* expression vector. Transfection efficiency for each assay was assessed by cotransfection of 5 ng of renilla luciferase expression plasmid (Promega). Luciferase activities were assayed 36 h after transfection using the Dual Luciferase Assay kit (Promega Corp.). The relative fold activity compared with vector-transfected cells and renilla luciferase plasmid was determined for each experiment. The results are an average of six independent experiments ± S.D.

HOXA5 was also observed here, and the level of activation was comparable with that of MCF-7 cells (Fig. 1*C*). In these cells, HOXB4 showed a low (up to 10-fold), but consistent, level of activation, but HOXB5 and HOXB7 were inactive. These results suggest that in two separate breast cancer cell lines, expression from the PR promoter is highly stimulated specifically by HOXA5, but not by three other members of the *HOX* gene family. Also, the ER/PR status of the cells did not influence the ability of HOXA5 to transactivate the cells, suggesting that factors responsible for the transactivation effect are present in both cell types.

We also varied the dose of *HOXA5* plasmid DNA in experiments in MCF-7 cells. We found that the activation effect increased as the dose was increased from 0.25 to 1 μg and then declined when 2 μg was used (data not shown). 1 μg of expression plasmid was therefore used in all subsequent experiments. A mutant of *HOXA5* that introduced a stop codon leading to a C-terminal truncation was also tested in these experiments. This mutant, which lacked the HOXA5 DNA-binding site, was unable to activate expression from the PR promoter (data not shown). These results indicate that HOXA5 can activate transcription of the PR promoter in this system, and the transactivation is due to an interaction of the DNA binding domain of HOXA5 protein with the PR promoter.

To further define the sequence requirements for the transactivation function, deletion constructs of the PR promoter luciferase construct were tested in cotransfection assays with the full-length *HOXA5* expression plasmids. Deletion of the −1121-bp PRPLuc construct to −792-bp PRPLuc construct, which removes one putative HOX-binding site, resulted in a 27% reduction in luciferase activity in response to HOXA5 in

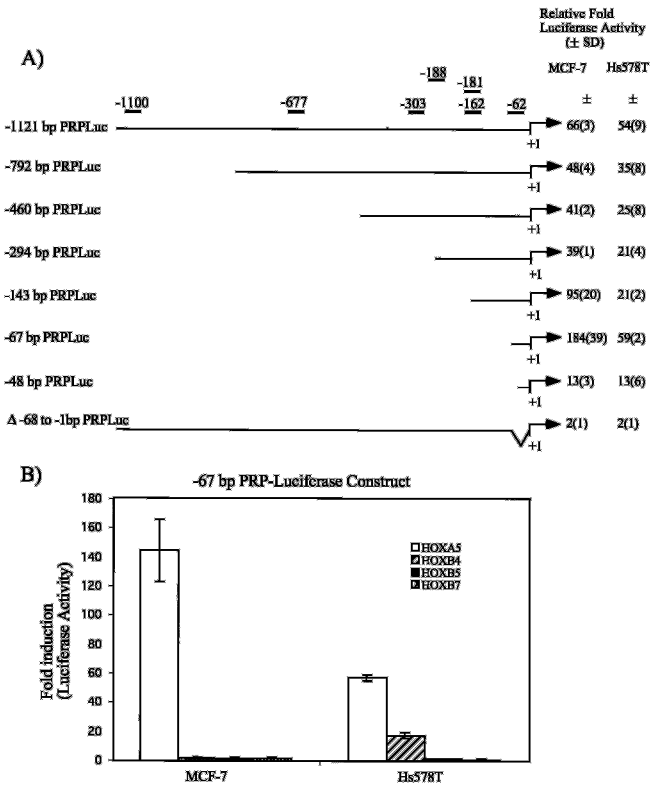

FIG. 2. **Deletion analysis localizes the HOXA5-binding site in the −1121-bp PRPLuc construct.** *A*, schematic representation of the −1121-bp PRPLuc and its deletion constructs, −792-bp PRPLuc, −460-bp PRPLuc, −294-bp PRPLuc, −143-bp PRPLuc, −67-bp PRPLuc, −48-bp PRPLuc or Δ-68 to −1-bp PRPLuc. Cells were transiently transfected with the deletion constructs along with the expression vector, pCMVHOXA5. The results of transcription assays performed with the promoter deletion in MCF-7 and Hs578T cells are shown. Luciferase activities were assayed 36 h after transfection using the Dual Luciferase Assay kit (Promega Corp.). The results are an average of six independent experiments ± S.D. *B*, the −67-bp PRPLuc reporter plasmid was cotransfected into MCF-7 and Hs578T cells with the *HOXA5*, *-B4*, *-B5*, or *-B7* expression vector. The results of the luciferase assay are an average of six independent determinations ± S.D.

MCF-7 cells (Fig. 2*A*) and a 35% reduction in Luc activity in Hs578T cells (Fig. 2*A*). A systematic deletion of the next two HOX-binding sites (−460-bp PRPLuc, −294-bp PRPLuc) resulted in only a slight decrease in luciferase activity in both the cell lines. A further deletion to −143 bp (−143-bp PRPLuc) stimulated luciferase activity 40–45% more than the full-length PR-Luc construct in MCF-7 cells but not in Hs578T cells. This increase in Luc activity in MCF-7 cells, but not in Hs578T, suggests the presence of a negative regulatory molecule in Hs578T cells, which is absent in MCF-7 cells, reflecting the genetic heterogeneity between the two cell lines. However, a further deletion to −67 bp resulted in a 2-fold increase in luciferase activity compared with the −143-bp PRPLuc construct in both MCF-7 and Hs578T cells. This stepwise analysis suggested that the site important for transactivation by HOXA5 is located between −67 and −1. In addition, it is quite likely that the removal of a binding site for negative regulatory molecules between −67 and −143 bp within the PR promoter enhanced the positive transcriptional ability of HOXA5. Cotransfection of *HOXB4*, *-B5*, or *-B7* along with the −67-bp PRPLuc yielded results similar to those shown in Fig. 1*A* in MCF-7 and Hs578T cells (Fig. 2*B*). Further deletion of the last HOXA5-binding site (located at −62 bp), in constructing the −48-bp PRPLuc, resulted in a drastic decrease of luciferase activity in both cell lines. However, luciferase activity was not completely abolished, suggesting the presence of a putative transactivator binding sequence in this region. From the results obtained by the deletion analysis, it appeared that the 3′-most 67 bp, containing only one HOXA5-binding site and the putative transactivator binding sequence, was sufficient for the transactivation. Finally, the specific deletion of these sequences from the full-length PR-Luc construct completely abolished the ability of HOXA5 to transactivate PR-linked reporter activity, providing confirmation that the 3′-most HOXA5-binding site is essential for the activity of PR.

The HOX-binding site that resides at the 3′ end of promoter region contains the core HOX motif, TAAT. To determine whether the "TAAT" core-containing site in the PR promoter is a *bona fide* HOXA5-binding site, electrophoretic mobility shift assays were performed. We tested the binding of oligonucleotides corresponding to this site to HOXA5 protein present in nuclear extracts of SAOS2 cells transfected with pCMVHOXA5. Western analysis of cell extracts using a HOXA5-specific antibody showed the overexpression of the 40–42-kDa HOX protein in *HOXA5*-transfected MCF-7 cells (data not shown). Equal amounts of protein from cells transfected with vector DNA and pCMVHOXA5 were used. Nuclear extracts from vector-transfected and HOXA5-transfected MCF-7 cells were incubated with a ^{32}P-end-labeled 30-mer oligonucleotide probe containing the HOXA5-binding site (Fig. 3*A*). HOXA5 produced a protein-DNA complex efficiently with the probe containing the wild type sequence (TAAT) (*lane 2*). Addition of 50-fold molar excess of unlabeled probe resulted in inhibition of this binding (*lane 3*), while a heterologous probe of a random sequence did not mediate competitive inhibition of protein/DNA binding (*lane 4*). Furthermore, when cell extracts were mixed with an oligonucleotide probe that carries two mutations (TGGT) in the core binding site, no protein/DNA complex formation was observed (*lane 5*). Finally, HOXA5 antibodies (*lane 6*) caused a supershift of the bound HOXA5 protein-oligonucleotide complex. These results, combined with detailed mutational analysis, indicate that the core sequence TAAT is necessary for this binding. These results clearly show that the TAAT-containing sequence present in the −67-bp PR promoter is indeed a HOX-binding sequence.

In a final test to confirm that the site lost in the −48-bp promoter construct is the HOXA5-binding site, we tested the *in vivo* effects of the same mutations in the core binding site that had abolished DNA-protein complex formation in cell extracts. Transient transfection assays were performed using two −67-bp PRPLuc reporter constructs, MUT-1 containing two mutations (TGGT) or MUT-2 carrying one mutation (TAGT, MUT-2), in the core-binding site. The results showed that transactivation of the reporter gene was strikingly reduced with both the mutant promoters (Fig. 3B). In addition, we mutated the 3′-most HOXA5-binding site within the context of the whole PR promoter construct by converting the TAAT to TAGT. The mutated construct had a 6–9-fold lower luciferase activity when cotransfected with *HOXA5* into MCF-7 and Hs578T cells, compared with the wild type 1211-bp PR promoter construct (Fig. 3*C*). Thus, by a number of separate and complementary tests, we have shown that transactivation of the −1121-bp PR promoter-Luc construct is mediated by direct binding of HOXA5 to a single site, at −62 bp, in this promoter.

The above results suggest that HOXA5 is a transactivator of the *PR* gene. To test this possibility, we generated stable MCF-7 cultures that could express the *HOXA5* gene under the control of an ecdysone-inducible system. Within 3 h after induction of HOXA5 expression by the ecdysone analog, Ponasterone A (Pon A), levels of the endogenous 116-kDa PR-B isoform were induced up to 3-fold (Fig. 4*A*), when compared with the control vector. The induction of the PR-B isoform, although

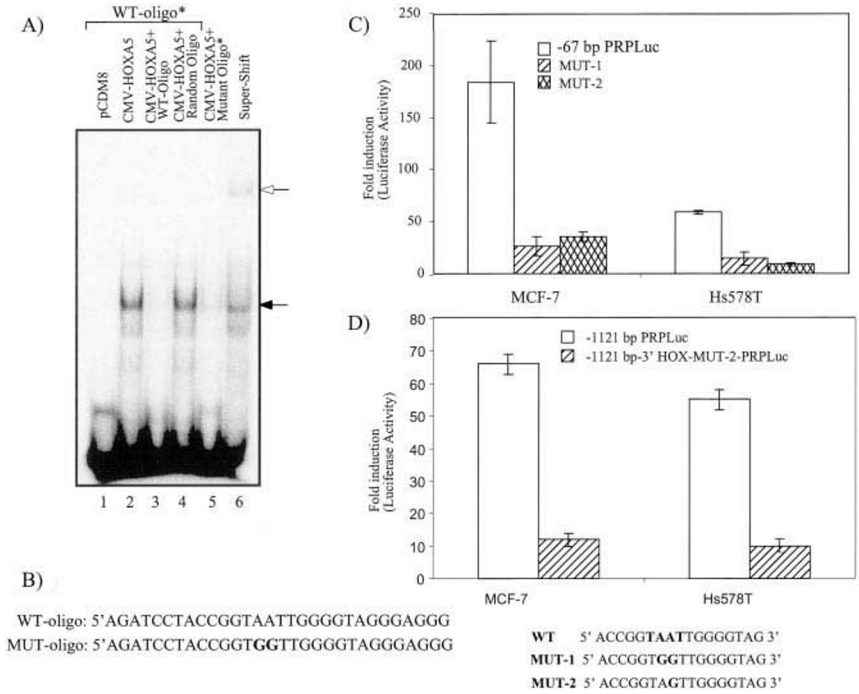

FIG. 3. **Mutations in the HOX-binding core TAAT abolish DNA binding and drastically reduce transactivation of −67-bp PRLuc by HOXA5.** Nuclear extracts of SAOS2 cells transfected with the plasmid vector, pCDM8 or pCMVHOXA5, were prepared and electrophoretic mobility shift assay was performed (described under "Materials and Methods"). For gel-shift analysis (*A*), end-labeled wild type (*WT*) oligonucleotide probe, containing the HOXA5-binding site, was hybridized with extracts from cells transfected with vector alone (*lane 1*) or pCMVHOXA5 alone (*lane 2*), and for competition assays, along with cold, 50-fold excess of the wild type oligonucleotide (*lane 3*) or random oligonucleotide (*lane 4*). *Lane 5* shows binding assay using the mutant oligonucleotide (as described in *B*) with protein extracts from cells transfected with pCMVHOXA5. *Lane 6* shows the supershift caused by binding of HOXA5 protein-oligonucleotide complex to HOXA5 antibodies. The *bold arrow* indicates the bound probe, while the *open arrow* shows the supershifted band. *, double-stranded oligonucleotides end-labeled with [γ-^{32}P]ATP. The oligonucleotide probe used contains the HOX-binding site from the *PR* promoter (*B*). *C*, MCF-7 and Hs578T cells were transiently transfected with the constructs of −67-bp PRPLuc or −67-bp PRPLuc with either a AA to GG or A to G mutation in the core HOX-binding site, along with the expression plasmid pCMVHOXA5. Results are an average of six experiments ± S.D. *D*, MCF-7 and Hs578T cells were transiently transfected with the constructs of −1121-bp PRPLuc or −1121-bp 3′ HOX-MUT-2 PRPLuc (TAAT to TAGT mutation in the core HOX-binding site), along with the expression plasmid pCMVHOXA5. Results are an average of three experiments ± S.D.

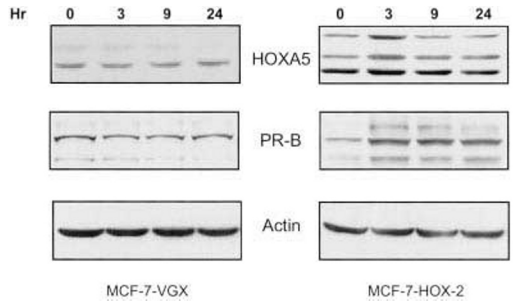

FIG. 4. **HOXA5 up-regulates PR levels in breast cancer cells.** Western blot analysis of cell extracts at different times following addition of Pon A to cultures of MCF7-HOX-2. Antibodies used were HOXA5 (HOXA5–2, Babco), PR (C-19, Santa Cruz), and actin (AC-15, Sigma). HOXA5 is frequently seen as a 40–42-kDa doublet; a third band is seen after Pon A induction of the transfected *HOXA5* gene in MCF-7-HOX2 cells. The multiple bands may be due to post-translational modifications or alternate splicing. The smaller molecular weight bands seen in the blot probed for PR protein may represent degraded PR protein products.

small, was observed in a reproducible manner in duplicate experiments.

In summary, we have described an effect of HOXA5 upon the protein levels of PR-B isoform. HOXA5 is a member of a class of proteins known principally for their role in pattern formation during development. Also, an oncogenic function for both murine and human HOX proteins, by overexpression or by untimely expression, has been well established (11, 12). While the oncogenic activity of overexpressed *Hox* genes has substantial experimental support, these observations and our data suggest additional functions for HOXA5. Our recent studies (4) indicate that HOXA5 is a potent transcriptional activator of *p53* in breast cells. Breast cancer cell lines and primary breast cancer specimens displayed a co-ordinate loss of *p53* and *HOXA5* mRNA and protein expression. We proposed that reduced or lack of expression of HOXA5 could lead to loss of p53 expression, providing a mechanism, other than by mutation, for loss of function of this important tumor suppressor during the development of breast cancer. Based upon the results of the study presented here, we raise the possibility that *HOX* genes play an important role in normal breast development by regulating the expression of the *PR* gene and that its loss in breast cancer could influence the transcription and thereby the expression of several key genes important for normal differentiation.

In our experiments, HOXA5 is a strong positive regulator of PR. We have yet to define the biological significance of the relationship described here. The major modulators of PR concentration are the ovarian hormone estrogen and progesterone itself (13). However, even though estrogen levels are biphasic in the mammary gland during menstrual cycle in normal women, the concentrations of PR are uniform, suggesting that its synthesis is not under the exclusive control of estrogen in breast tissues (14). Similar results were obtained using estrogen receptor-α knock-out mice. In the *ER* null-homozygous mice, *PR* mRNA was nevertheless detected, suggesting the existence of both estrogen-dependent and -independent gene regulation (15). In this study we have identified HOXA5 as a novel upstream regulator of *PR* gene expression in normal breast cells and presented evidence for the presence of an additional factor, other than estrogen, that controls PR expres-

sion in breast cancer. Similar to its well known role in body patterning during embryonic development as an "master regulator of gene action," it is possible that HOXA5 has multiple roles in breast development and that loss of HOXA5 will have a major impact upon the action of multiple genes important in homeostasis.

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