Journal of Cellular Biochemistry 71:46–54 (1998)

Molecular Cloning of a Mutated *HOXB7* cDNA Encoding a Truncated Transactivating Homeodomain-Containing Protein

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Abstract Homeodomain-containing proteins regulate, as transcription factors, the coordinated expression of genes involved in development, differentiation, and malignant transformation. We report here the molecular cloning of a mutated *HOXB7* transcript encoding a truncated homeodomain-containing protein in MCF7 cells. This is a new example of mutation affecting the coding region of a *HOX* gene. In addition, we detected two *HOXB7* transcripts in several breast cell lines and demonstrated that both normal and mutated alleles were expressed at the RNA level in MCF7 cells as well as in a variety of breast tissues and lymphocytes, suggesting that a truncated HOXB7 protein might be expressed in vivo. Using transient co-transfection experiments, we demonstrated that both HOXB7 proteins can activate transcription from a consensus HOX binding sequence in breast cancer cells. Our results provide evidence that HOXB7 protein has transcription factor activity in vivo and that the two last amino acids do not contribute to this property. J. Cell. Biochem. 71:46–54. © 1998 Wiley-Liss, Inc.

Key words: homeobox; breast; ligase chain reaction; transcription

Homeobox-containing genes have been identified as transcriptional regulators controlling the coordinated expression of genes involved in development and differentiation [Gehring, 1987; Levine and Hoey, 1988; Favier and Dollé, 1997]. They share a highly conserved 180 bp region that codes for a 60 amino acid DNA binding domain ("homeodomain") [Gehring et al., 1994]. Initially discovered in Drosophila where they control segment identity and segment position [Lewis, 1978; McGinnis et al., 1984; Scott and Weiner, 1984], homeobox genes now have been isolated from multiple species [Levine et al., 1984; Akam, 1989]. To date, 39 human class I homeobox genes have been discovered. They are organized in four clusters (HOX loci A, B, C, and D), located on chromosomes 7, 17, 12, and 2 [Acampora et al., 1989], respectively. The physical organization of the homeobox genes may play a role in their spatiotemporal expression [Graham et al., 1989].

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Received 3 November 1997; Accepted 14 April 1997

HOXB7, one member of the *HOXB* cluster, was initially isolated from an SV40-transformed human fibroblast cDNA library [Simeone et al., 1987]. The clone encoded a protein of 217 amino acids. Its carboxyterminus is highly acidic, containing seven glutamic acids out of the final eight amino acids. Because of its tissue selective and stage-related pattern of expression in embryogenesis, HOXB7 was proposed to be involved in a variety of developmental processes. HOXB7 has also been implicated as a regulatory protein in hematopoietic differentiation as well as in lymphoid development [Shen et al., 1989; Deguchi et al., 1991; Lill et al., 1995]. The pattern of *HOXB7* expression was investigated in human tumors [Deguchi et al., 1991; Cillo et al., 1992; Celetti et al., 1993; De Vita et al., 1993; Tiberio et al., 1994]. Whereas most known tissues express two *HOXB7* mRNAs of 1.6 and 1.4 kb, additional longer transcripts were detected in some colon cancer biopsies [De Vita et al., 1993]. In contrast, no difference was detected between renal carcinomas and normal kidney tissues [Cillo et al., 1992]. Because of its expression in lymphoid as well as nonlymphoid cells, the *HOXB7* gene

product has been implicated in the regulation of a common transcriptional event rather than in the determination of lineage-specific gene expression [Baier et al., 1991].

The DNA binding properties of HOXB7 recombinant protein have been previously reported [Corsetti et al., 1992]. These studies demonstrated that HOXB7 protein displays a low affinity for the regulatory sequences of upstream homeobox genes and a high affinity for HOXB7 promoter and downstream *HOX* genes. However, little is known about transcriptional properties of HOXB7 protein in vivo.

In this report, we describe the identification of a mutated *HOXB7* transcript encoding a truncated protein with a shortened acidic tail. Furthermore, we provide evidence that both HOXB7 proteins activate transcription in human breast cancer-derived cells.

MATERIALS AND METHODS

Cell Lines and Tissue Specimens

MCF7, MDA-MB231, T47D, Hs578T, Hs578Bst, and MCF10F cell lines were obtained from the American Type Tissue Collection (Rockville, MD). Cloned subpopulations of MCF7 (MCF7-2B3, -10B5, -5H7, -4C10, -2E5) were the generous gift of P. Horan Hand [Greiner et al., 1985; Wewer et al., 1986] and J. Greiner (National Cancer Institute, Bethesda, MD) and have been described. Specimens of breast cancer, normal adjacent tissues as well as blood samples from healthy patients and patients treated for leukemias were collected.

Nucleic Acid Isolation

Total cellular RNA was extracted by the guanidium isothiocyanate extraction followed by a gradient centrifugation [Glisin et al., 1974]. Poly (A) RNA was selected by two passages on an oligo (dT) cellulose column (Collaborative Research, Bedford, MA). Genomic DNA from the various cell lines was prepared as described [Fernandez et al., 1985]. Genomic DNA from blood samples was extracted using the QIamp Blood kit (Qiagen, Chatsworth, CA).

Isolation of MCF7 *HOXB7* cDNA Clones

An MCF7 cDNA library into UniZap XR phage DNA (Stratagene, La Jolla, CA) was constructed and screened with a nick translated ^{32}P-labeled EcoRI-XhoI cDNA fragment encoding a partial homeodomain region of *HOXB6* obtained as described [Castronovo et al., 1994]. Hybridization and washing conditions for phage filters were as recommended by Stratagene for Uni-Zap XR phage. Positive purified phage were subcloned into the EcoRI and XhoI sites of pBluescript vector and analyzed by DNA sequencing using the dideoxy method. Both strands were systematically sequenced.

Northern Blot Analysis

Equal amounts (5 µg) of poly (A) RNA were separated by size through a 1.2% (w/v) agarose-formaldehyde gel, transferred to nytran membrane (Schleicher and Schuell, Keene, NH), and hybridized at 42°C as described [Wewer et al., 1986]. The *HOXB7*-specific 504 bp probe was generated from the 3′ untranslated region of the mRNA by RT-PCR and was ^{32}P-labeled by nick-translation. The RNA blot was washed under stringent conditions (0.2XSSC, 0.5% SDS at 55°C) and exposed. A housekeeping probe from glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was used for normalization.

RT-PCR Experiments

cDNAs were synthesized for 1 h using 5 µg template total cellular RNA and 50 pmol of primer containing the sequence 5′-GAGGGCTTACAAAACTGCAAGAT-3′. The reverse transcriptase was then inactivated at 95°C for 10 min. To amplify a 793 bp *HOXB7*-specific fragment, PCR was performed using 50 pmoles of *HOXB7* specific upstream primer containing the sequence 5′-ATGGGCTCGAGCCGAGTTCC-3′; 35 cycles were performed for this PCR, each cycle including denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 90 seconds. The final cycle was followed by a final extension at 72°C for 3 min. 1% of the reaction products was used for LCR experiments.

Amplification of *HOXB7* Genomic DNA Fragments for LCR Experiments

Genomic DNA (1 µg) was used as template to perform the PCR, and 50 pmoles of *HOXB7* specific primers were added to the reaction mixture. The downstream primer was identical to the one used for the RT-PCR experiments; however, to avoid amplification of an intronic sequence, the upstream primer was located downstream of the splicing site and contained the sequence 5′-AACTGACCGCAAACGAG-

GCC-3′. 35 cycles were performed as described above. The 623 bp expected fragments were visualized on an ethidium bromide stained 5% polyacrylamide gel. 1% of the reaction products was used for LCR experiments.

Ligase Chain Reaction

Parameters for the LCR experiments were adapted from the procedure of Barany [1991]. Allele specific oligonucleotides (ASOs) were designed for two possible templates as diagrammed in Figure 1.

ASO-3 and ASO-4 were common for both templates and were labeled with ^{32}P at their 5′ ends. Diagnostic ASOs for the T allele (203ASO-1 and 203ASO-2) and for the G allele (209ASO-1 and 209ASO-2) were designed for specificity at their 3′ ends. To help discriminate between specific ligation products on diagnostic acrylamide gels, varying amounts of T tails were added to the 5′ ends of the diagnostic ASO as shown in Figure 1.

Thus, reaction products for the T and G allele products should be 53 bp and 49 bp, respectively. In some experiments, a third set of ASOs was used as a negative control for a template representing a possible A allele. In all cases, all the LCR experiments have been confirmed at least three times.

Expression Constructs

The coding sequences of clone 203 (T allele) and clone 209 (G allele) were subcloned by PCR amplification into the expression vector pcDNA3 (Invitrogen, San Diego, CA). Primers, containing artificial sites, were 5′-TATAGGATCCATGAGTTCATTGTATTATGC-3′ and 5′-ATATTCTAGATTCTCCATCCCTCACTCTT-3′. The amplified fragments were cloned in 5′-3′ orientation into the BamHI-XbaI sites within the polylinker of expression vector pcDNA3. Integrity of the amplified coding regions was confirmed by sequencing representative clones on both strands, as described above. The pD9 expression vector was kindly provided by V. Zappavigna (Laboratory of Gene Expression, Department of Biology and Technology, Istituto Scientifico H.S. Raffaele, Milan, Italy) and represents the complete open reading frame of HOXD9 cDNA cloned into the BamHI site of the pSG5 expression vector [Zappavigna et al., 1991]. The pT109 and the pTCBS reporter plasmids were also kindly provided by V. Zappavigna. The pTCBS plasmid contains an eightfold multimerized form of a homeodomain consensus-binding sequence and a luciferase reporter gene under the control of a HSV-TK promoter, whereas the pT109 construct does

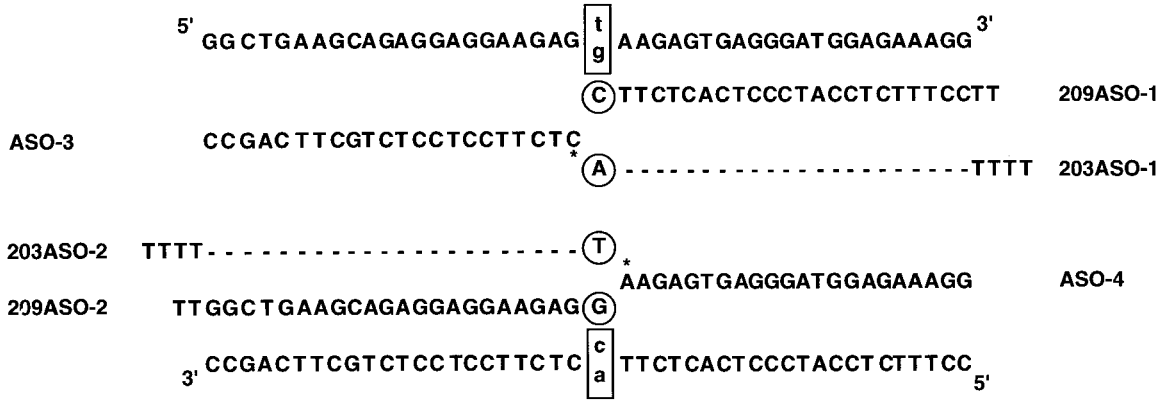

Fig. 1. Allelic specific oligomers for ligase chain reaction (LCR). On the top and bottom lines, in bold, the template sense and antisense strands, respectively, are shown, as derived from the sequences surrounding the G to T transversion. The boxed lower case bases designate suspected polymorphic or mutated nucleotides. Allelic specific oligomers (ASO) are positioned between the template strands. 203ASO-1 and 203ASO-2 are complementary to the T allele template strands and contain a diagnostic A and T, respectively, at their 3′ ends (circled bases). At their 5′ ends, a tail of 4 Ts was added. 209ASO-1 and 209ASO-2 are complementary to the G allele and contain 5′ tails of 2 Ts as well as circled 3′ diagnostic nucleotides C and G. ASO-3 and ASO-4, which are complementary to both possible templates, are labeled with 32P at their 5′ ends (*) and will be ligated to the appropriate adjacent diagnostic ASOs if the latter's diagnostic (circled) nucleotide is exactly complementary to an available template. Since 203ASO-1 and -2 contain longer T tails than 209ASO-1 and -2, ligated ASOs can be easily discriminated on autoradiographs of a denaturing polyacrylamide gel. Ligated ASOs for the T and G allele will be 53 bp and 49 bp, respectively.

not contain the CBS sequence and is used as a control [Zappavigna et al., 1994].

In Vitro Translation

Both 1 µg of the 209 pcDNA3 or the 203 pcDNA3 contructs were used as template to perform the *in vitro* translation with the T7 RNA polymerase and the wheat germ extracts, according to the protocol provided by the manufacturer.

Transient Transfections and Luciferase Assays

Transfections in MDA-MB 231 cells were performed using the cationic liposome reagent DOTAP (Boehringer Mannheim, Mannheim, Germany), 1 µg of reporter plasmid, and 1 µg of HOX209, HOX203, or 4 µg of HOXD9 expression vectors per 35 mm dish. Total concentrations of DNA and expression vectors were kept constant throughout by adding appropriate amounts of pcDNA3 without insert. Cells were harvested 48 h after transfection, using the lysis buffer provided by the Luciferase Reporter Gene Assay kit (Boehringer Mannheim). Luciferase assays were carried out as recommended by the manufacturer. Luciferase activities were normalized to the protein concentration of the extracts.

RESULTS AND DISCUSSION

Cloning and Expression of *HOXB7* Transcripts

To isolate full-length homeobox cDNAs from human breast cancer cells, an MCF7 cDNA library was screened using a well-conserved partial HOXB6 homeodomain cDNA as a cross-hybridizing probe [Castronovo et al., 1994]. The phage isolates included 3 *HOXC6* clones, 10 *HOXA10* clones, 1 *HOXA4* clone, and 12 *HOXB7* clones. We further studied the levels of *HOXB7* mRNA in several human breast cancer-derived cell lines by RT-PCR experiments as illustrated in Figure 2A. Hs578Bst (right panel, lane 1) as well as T47D (right panel, lane 3) mRNA generated a reverse transcriptase-dependent amplified product, whereas there was no detectable amplification from MCF10F (right panel, lane 5) and MDA-MB 231 (right panel, lane 7) RNA. The identity of this HOXB7 fragment was further confirmed by restriction analysis (data not shown). This pattern of expression was in agreement with a Northern blot as illustrated in Figure 2B. Two mRNA transcripts of 1.6 and 1.4 kb were detected in the *HOXB7*-expressing cell lines (Fig. 2B, lanes 1,3, and 5), as has been described in other model systems [Deguchi et al., 1991; Cillo et al., 1992; Celetti et al., 1993; De Vita et al., 1993; Tiberio et al., 1994].

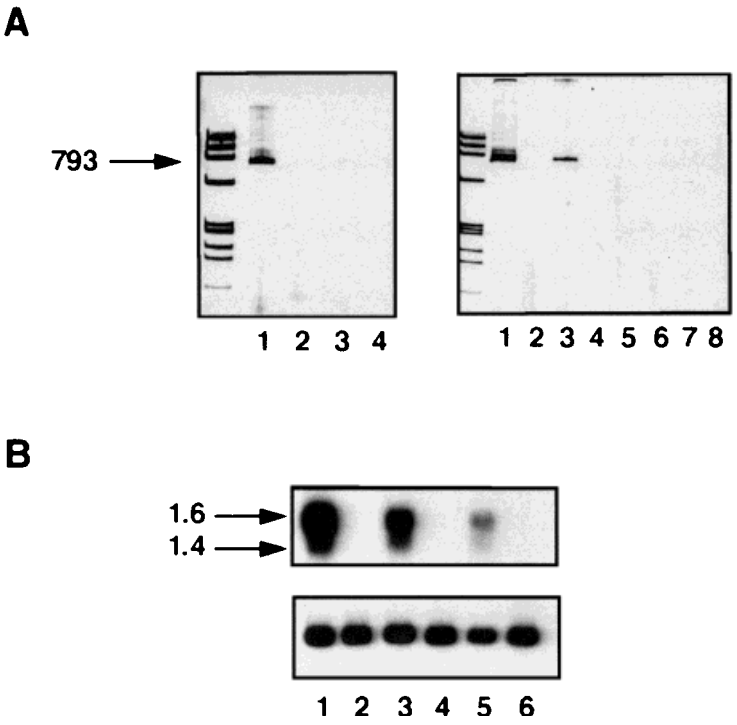

Fig. 2. Detection of HOXB7 expression in breast cancer. **A.** Amplification of a 793 bp *HOXB7* fragment (arrow) from mRNA sequences in human breast-derived cell lines by RT-PCR experiments. Left panel: Amplification of MCF7 *HOXB7* mRNA. **Lane 1:** MCF7 RNA; **Lane 2:** without reverse transcriptase; **Lane 3**: RNA pretreated with RNAse; **Lane 4:** no RNA template. Right panel: Amplification of HOXB7 mRNA from various cell lines. Lanes 1–2: Hs578Bst; Lanes 3–4: T47D; **Lanes 5–6:** MCF10F; **Lanes 7–8:** MDA-MB 231. Lanes 2, 4, 6, 8: without reverse transcriptase. **B.** Northern blot experiment: using a HOXB7 specific probe (top panel) and a control GAPDH probe (bottom panel). **Lane 1:** Hs578Bst; **Lane 2:** Hs578T; **Lane 3:** MCF7; **Lane 4:** MCF10F; **Lane 5:** T47D; **Lane 6:** MDA-MB 231.

Identification of a Base Change Resulting in an Alternate Stop Codon of HOXB7

The 12 independent isolates of MCF7 *HOXB7* cDNA clones were completely sequenced to determine possible polymorphisms or mutations that might be present. Unexpectedly, 9 of the 12 MCF7 *HOXB7* cDNA clones displayed a G to U transversion in the first base of codon 216, resulting in an alternate translational stop codon (UAA) instead of a glutamic acid (GAA) and thus leading to a protein that is two amino acids shorter than expected.

At this point, we could not rule out the fact that this mutation was generated by a mistake of the polymerase. We then decided to perform another approach in order to confirm the existence of this mutation in MCF7 cells. An adaptation of the ligase chain reaction (LCR) technique was then developed. LCR has been described as a reliable DNA diagnostic method for the detection of point mutations [Barany, 1991]. To use this method for the detection of single base changes at the RNA level, LCR experiments were performed on the RT-PCR products obtained from the breast cell lines, using two sets of allele specific oligonucleotides (ASOs) as illustrated in Figure 1.

Plasmids representing T and G alleles were linearized and used as templates for a control LCR experiment to assist in the identification of LCR reaction products (Fig. 3A top, lanes 1–4). Signals were detected when the template was matched with the correct ASO (lanes 1,3) but not with a noncorresponding ASO (lanes 2,4). When the 793 bp RT-PCR generated fragment from MCF7 was subjected to LCR, evidence for the existence of both "U" and "G" mRNA transcripts was confirmed (Fig. 3A top, lanes 5–8). The LCR was dependent upon the presence of reverse transcriptase and MCF7 mRNA in the original RT-PCR experiments (Fig. 3A top, lanes 5 and 7). No LCR signal was generated in the absence of reverse transcriptase (Fig. 3A top, lanes 6 and 8), in the absence of template MCF7 mRNA (lanes 11 and 12), or when MCF7 mRNA was treated with Rnase (lanes 9 and 10). All these experiments were performed at least four times: identical results were obtained, thus demonstrating the reproducibility of this approach. Among the in vitro grown cell lines tested, the "U" transcript appears to be unique to the MCF7 cell line. T47D, Hs578Bst, MCF10F, and MDA-MB 231 cells contain only "G" transcripts (Fig. 3A bottom). Although the Northern blot and nonradioactive RT-PCR experiments failed to detect HOXB7 transcript in MCF10F and MDA-MB 231 cells, long radioautographic exposures of the LCR reaction products permitted detection of *HOXB7* transcripts, demonstrating the high sensitivity of this technique.

In MCF7 cells, both alleles appear to be expressed, potentially resulting in the presence of two HOXB7 proteins that differ in length by two amino acids. In the other cell lines tested, the T allele was not detected either because it was not transcribed at detectable levels or because it is not present in the genome. To address this issue, an LCR experiment was performed on genomic DNA from these cell lines. Again, a preliminary amplification experiment (PCR) was performed using two *HOXB7* specific primers in order to increase the sensitivity of the reaction (data not shown). PCR products were used as templates for the LCR experiments (Fig. 3B). Both T and G alleles were detected in the MCF7 genome (Fig. 3B, left panel, lanes 1 and 2), whereas only the G allele was detected in the other cell lines tested (Fig. 3B, right panel, lanes 2 and 5). The reliability and specificity of the LCR technique were further tested by performing LCR using another set of ASOs for a hypothetical allele containing an A instead of a G (Fig. 3B, left panel, lane 3, right panel, lanes 3 and 6).

It was not clear if the presence of both G and U HOXB7 mRNAs in MCF7 cells reflects the presence of two different expressed alleles in individual cells or a subpopulation of cells that express the T allele instead of the G allele. Genomic DNA was extracted from subcloned populations of MCF7 cells and assayed by LCR for the presence of the T and G alleles: as illustrated in Figure 3C, 4 of the 5 subclones tested contained both alleles. The fifth subclone contained only the G allele. A similar experiment performed in the absence of any DNA template did not lead to any amplified fragment (data not shown). Therefore, individual MCF7 cells appear to have both T and G alleles, or are homozygous for the G allele. We found no evidence for homozygous T allele. To determine whether the G to U transversion in some *HOXB7* transcripts can be detected in vivo, we performed our assay using total cellular RNA extracted from breast cancer and adjacent tissues. Preliminary RT-PCR experiments al-

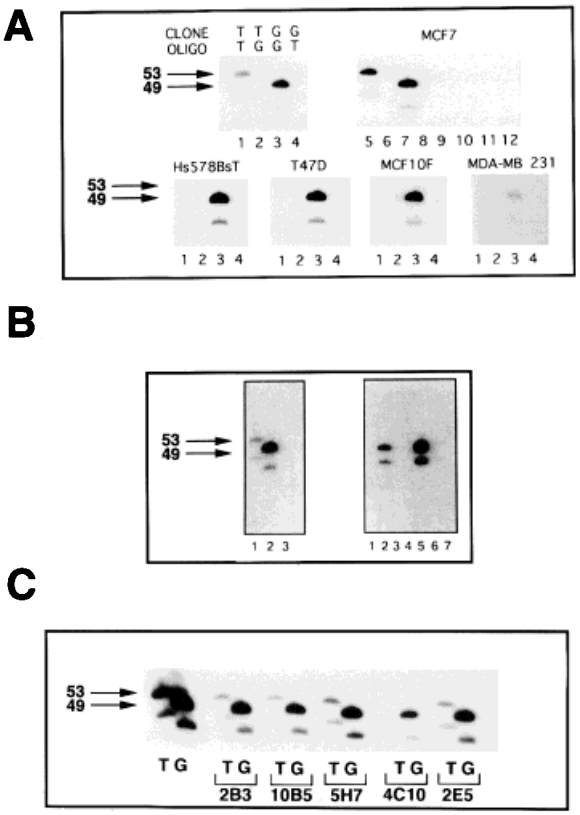

Fig. 3. LCR experiments performed on breast cell lines. **A.** Top left: Positive and negative controls to determine specificity of LCR. Clone T (= clone 203) and clone G (= clone 209) were used as template with appropriate ASOs (see Fig. 1). Top right: LCR of MCF7 HOXB7 mRNA: demonstration of T and G alleles. 1% of the reaction products shown in Figure 2A (**lanes 1–4**) were subjected to LCR using both sets of ASOs. **Lanes 5 and 7** used MCF7 RT-PCR fragment as shown in Figure 2A (lane 1), **lane 6 and 8** (no reverse transcriptase), **lane 9 and 10** (RNase-treated RNA), **lane 11 and 12** (no RNA template). Lanes 5,6,9,11 used "OLIGO T" conditions (oligos 203ASO-1, 2 and ASO-3, 4) and lanes 7,8,10,12 used "OLIGO G" conditions (oligos 209ASO-1, 2 and ASO-3, 4) as described above. Bottom panels: LCR of *HOXB7* mRNA from various cell lines. For each cell line, lanes 1 and 2 used "OLIGO T" conditions and lanes 3 and 4 used "OLIGO G" conditions. For each cell line, lanes 1 and 3 show the LCR products using the odd numbered lane from Figure 2B, and lanes 2 and 4 show the corresponding negative control using the even numbered lane (no reverse transcriptase) from Figure 2B. **B.** LCR of genomic DNA from various cell lines. Left panel: LCR of MCF7 *HOXB7* DNA. **Lane 1:** "OLIGO T" conditions; **Lane 2:** "OLIGO G" conditions; **Lane 3:** "OLIGO A" conditions as described in the text (negative control for specificity). Right panel: LCR of HOXB7 DNA from various cell lines. 1% of the PCR-generated HOXB7 DNA products were used as template for LCR reactions. T47D: lanes 1–3; MCF10F: **lanes 4–6; lane 7:** no template. Lanes 1,4,7 used "OLIGO T" conditions; lanes 2,5 used "OLIGO G" conditions; lanes 3,6: "OLIGO A" conditions. **C:** LCR of genomic DNA from subcloned populations of MCF7 cells. For each sample, a pair of lanes representing LCR products using ASOs corresponding to the OLIGO T allele (53 bp, **left lane**) and to the OLIGO G allele (49 bp, **right lane**) are shown. The T and G lanes to the left of the figure demonstrate the positive OLIGO T allele and OLIGO G alleles, respectively.

lowed us to select the samples that contained amplifiable *HOXB7* mRNAs. We identified both transcripts in 5 out of 6 cancerous tissues as well as in some adjacent tissues as illustrated in Figure 4A. These results suggest then that alternate alleles are expressed not just in a specific cell line but also in a variety of cancerous and adjacent breast tissues. We further extracted genomic DNA from blood obtained from healthy patients as well as patients treated for leukemia and subjected them to LCR experiments. As illustrated in Figure 4B, the mutated allele was systematically detected in the leukemia. Moreover, as illustrated in Figure 4C, the transversion was detected in eight healthy patients, whereas the signals for the T allele were barely detectable in two other samples suggesting that this mutation was not specific to breast tissues. No amplified fragment was obtained in the absence of any DNA template (data not shown). Interestingly, no homozygotes for this transversion were observed. Taken together, our results suggest that two distinct HOXB7 gene products that differ by their C-terminal tail are expressed in a variety of tissues. Since the mutated transcript is expressed in breast cancer cells as well as in normal tissue, no oncogenic abilities can be attributed to this product. This is the first example of a truncated HOX protein generated from an alternate allele. HOX truncated polypeptides that are encoded by distinct transcripts generated by alternate splicing events have been previously described. Such truncated proteins can lack either the entire homeodomain [Shen et al., 1991; Chariot et al., 1995], or a putative transcriptional regulatory region [Cho et al., 1988]. They can also display an alternative regulatory domain [Fitzpatrick et al., 1992]. Some truncated proteins have been proposed as competitors with full-length proteins for binding to other cellular factors [Fitzpatrick et al., 1992].

Our report is a new example of mutation affecting the coding sequence of a *HOX* gene. Recently, two other studies have established a link between alterations of *HOXA13* and *HOXD13* coding sequences and inherited abnormality of limb development [Muragaki et al., 1996; Boncinelli, 1997; Innis, 1997; Mortlock and Innis, 1997]. These results, combined with our study, strongly suggest that multiple *HOX* genes might be altered by mutations that could, in some cases, lead to developmental or other kind of defects yet to be characterized.

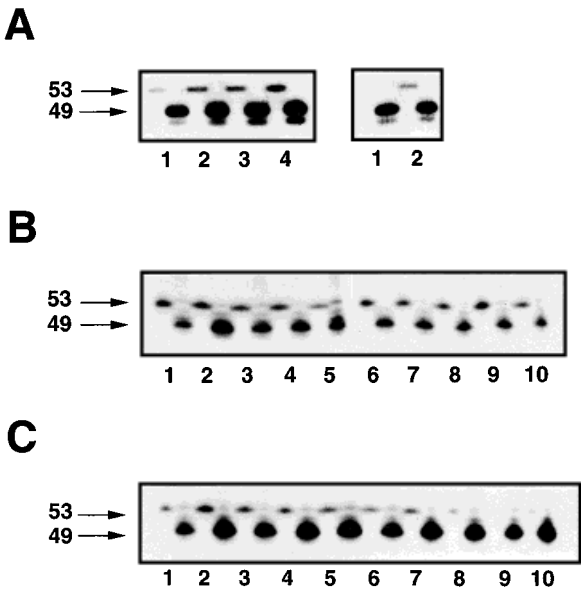

Fig. 4. Detection of the mutated *HOXB7* allele *in vivo.* For each tissue sample, a pair of lanes representing LCR products using ASOs corresponding to the "OLIGO T" allele (53 bp, **left lane**) and to the "OLIGO G" allele (49 bp, **right lane**), are shown. **A.** LCR of mRNA from breast cancer samples (left panel) and from normal adjacent breast tissues (right panel). **B.** LCR of genomic DNA from patients treated for leukemia. **C.** LCR of genomic DNA from healthy patients.

Both HOXB7 Proteins Can Activate Transcription in MDA-MB 231 Cells

To investigate HOXB7 transcription factor activity, we performed transient transfection experiments in MDA-MB 231 cells that contain barely detectable levels of *HOXB7* mRNA (see Fig. 2) and using both pTCBS and pT109 as reporter plasmids. CBS sequence is recognized by most HOX proteins in transient transfections [Zappavigna et al., 1994] and displays low level of basal activity in MDA-MB 231 cells. Both HOXB7 gene products were equally generated from their respective expression vectors as demonstrated by in vitro translation experiments (Fig. 5A, lanes 1 and 2). As shown in Figure 5B, the luciferase activity of pTCBS in MDA-MB 231 cells was increased fourfold when cotransfected with HOXD9 expressing vector, a gene product acting as an activator in NIH3T3 cells [Zappavigna et al., 1994] as well. When co-transfected with pcDNA3–209, the luciferase activity of pTCBS was increased 3.5–3.7 fold. A similar induction (3.4–3.6 fold) was observed when pTCBS was co-transfected with pcDNA3–203. The observed transactivation effects are mediated by the binding of the HOXB7 proteins on the CBS since no effect were obtained with the pT109 construct used as reporter plasmid. We cannot, however, rule out the hypothesis that other HOX proteins whose expression are induced by both HOXB7 products may contribute to the observed effect. Inductions observed in our experiments were weak and dose dependent, which could be explained by an imperfect recognition of the CBS sequence in vivo. Precise identification of HOXB7 target DNA sequence should address this issue. In this context, a potential HOXB7-binding site has been identified by Electrophoretic Mobility Shift Assay experiments within the HOX3D promoter [Corsetti et al., 1992]. It would then be of interest to determine whether both HOXB7 proteins can recognize that sequence in vivo and still act as transactivator when bound to such sequence in transfected cells.

The transcriptional properties of homeodomain-containing proteins remain largely uncharacterized. Indeed, despite in vivo specificity for each member of this family, all these transcription factors share very similar DNA binding affinities. The protein-protein interaction are likely to play a major role in the regulation of their function. In this context, structural analysis have revealed that two homeodomains can bind to one 10 bp DNA sequence in a tandem fashion [Hirsch and Aggarwal, 1995], thus suggesting that these transcription factors can physically interact on their target DNA sequences. Depending on the proteins investigated, specific domains have been proposed for these protein-protein interactions: the amino terminus and helix 1 of the homeodomain of HOX proteins belonging to the D loci [Zappavigna et al., 1994], the homeodomain and carboxy terminal sequences of Ultrabithorax [Chan et al., 1993] and the C-terminal tail of yeast $\alpha 2$ [Mak and Johnson, 1993; Stark and Johnson, 1994]. Moreover, the conserved pentapeptide as well as the N-terminal domain of HOX proteins are required for cooperative DNA binding activity with cofactors such as Pbx proteins and Meis products, respectively [Chang et al., 1995; Shen et al., 1997]. Taken together, these studies suggest that a combination of several domains that can contact DNA and/or define protein interaction interface are required for the transcriptional activity of homeodomain-containing proteins. This hypothesis is supported by a recent study illustrating that HOXA7 protein harbors repressing abilities that are mediated by an unique combination of several do-

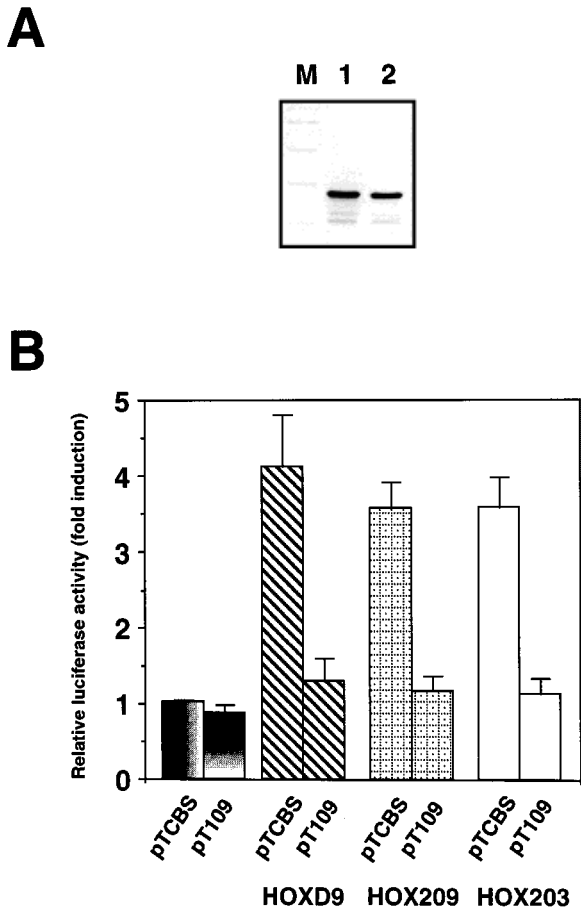

Fig. 5. **A.** In vitro translation of both the wild type and the mutated HOXB7 proteins. M: marker; **Lane 1:** HOXB7 wild type; **Lane 2:** mutated HOXB7 product. 1 µl of the resulting products has been subjected to eletrophoresis on a denaturing 10% polyacrylamide gel and exposed by autoradiography. **B.** Analysis of HOXD9 and HOXB7 gene products on the CBS target sequence by transient cotransfections in MDA-MB 231 cells. The pT109 does not contains any HOX-binding sequence and is used as a negative control. The figure shows the relative luciferase activity over the activity observed with the pTCBS or the pT109 reporter plasmids alone. Each value represents the mean of at least three independent experiments after normalization to the protein concentration of the extracts. Bars, mean + S.D.

mains including both activator and repressor regions [Schnabel and Abate-Shen, 1996]. Interestingly, the acidic C-terminal tail contributes to the repressing activity of this transcription factor. Although the entire acidic C-terminal tail is not deleted in the identified truncated product, it seems that the two last amino acids of this domain are not involved in the transcriptional properties of HOXB7. However, multiple studies have demonstrated the involvement of similar acidic region in the transcriptional activity of other DNA binding proteins such as p53 [Unger et al., 1992]. Additional mutagenesis experiments should lead to the identification of the domain(s) that mediate HOXB7 transactivation effect. Interestingly, we demonstrate that both HOXB7 gene products can activate transcription from the CBS sequence in MDA-MB231 cells, whereas the paralogue HOXA7 displays repressing abilities in NIH3T3 cells. The transcriptional activity of HOX proteins may then potentially depend on the cell type, the target DNA, the cofactors as well as on subtle amino acid changes within homeodomain sequences and less conserved regions.

ACKNOWLEDGMENTS

The authors are grateful to Vincenzo Zappavigna for many helpful discussions and for the generous gift of the pTCBS and pT109 reporter plasmids and the pD9 expression construct. We are grateful to Muriel Chapelier, Giusy Bonizzi, and Jacques Gielen for their support. A.C. and V.C. are, respectively, Research Assistant and Senior Research Associate of the National Fund for Scientific Research (Belgium). This work was also funded by the Intramural National Cancer Institute budget.

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