Cloning and expression of a new HOXC6 transcript encoding a repressing protein

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Homeodomain-containing proteins are transcription factors that regulate the co-ordinated expression of multiple genes involved in development, differentiation and malignant transformation. In an attempt to characterize expressed homeobox (*HOX*) genes in breast cancer cells, we cloned two distinct HOXC6 transcripts from an MCF7 cDNA library. Interestingly, one of them represents a new HOXC6 mRNA encoding a homeodomain-containing protein harbouring a unique N-terminal sequence. Moreover we demonstrate that this HOXC6 transcript is less abundant in human breast cancer cells than in non-tumorigenic cell lines, is detected in breast carcinomas and adjacent tissues and is expressed in a variety of human tumours. In addition, transient co-transfection experiments illustrated that both HOXC6 transcripts encode gene products that repress transcription from a *HOX* binding sequence in MDA-MB231 cells and co-operate with other *HOX* gene products such as HOXB7 on their target genes. Taken together, our results suggest that HOXC6 proteins might contribute to the breast cell phenotype through co-operative interactions with other *HOX*-derived proteins and repression of their target genes.

INTRODUCTION

The identification of genes differentially expressed in human cancer remains a key step in improving our understanding of the molecular mechanisms underlying neoplasia. Cellular transformation involves the expression of intact or altered proteins that are co-ordinately regulated by multiple transcription factors including homeodomain-containing proteins. These latter polypeptides are encoded by homeobox (*HOX*) genes described as 'master genes' for the crucial role that they play in the development of many species [1,2]. *HOX* genes share a highly conserved 183 bp DNA-binding region (the homeobox), conferring on the resulting proteins the ability to modulate the expression of a variety of target genes [3]. Initially discovered in *Drosophila*, where they control segment identity [4], *HOX* genes have since been cloned from many species, including human [5]. They are organized in four distinct clusters (loci A, B, C and D) located on chromosomes 7, 17, 12 and 2 respectively [6]. Their chromosomal localization is associated with their spatio-temporal pattern of expression in the developing embryo [7].

Most of the *HOX* cDNAs have yet to be completely characterized. A HOXC6 clone, previously named HOX c8.5111, has been isolated from SV-40-transformed human fibroblasts [8]. Additional HOXC6 cDNAs have been subsequently cloned from a human placenta library [9]. Among these clones, a cDNA named HOX cp25 has been further characterized. It displayed a 3′ untranslated region 82 bp longer than clone HOX c8.5111 because of the use of an alternative polyadenylation signal [9]. Interestingly, all isolated HOXC4, C5 and C6 cDNA clones that belong to cluster C shared an identical 5′ end sequence, suggesting that a common primary transcript was transcribed from one single promoter and subjected to alternative splicing events to generate distinct *HOX* transcripts belonging to this cluster [9].

Expression studies have illustrated the detection of HOXC6 transcripts in a variety of adult tissues, including normal kidney [10], normal and neoplastic colon [11] and small-cell lung cancers [12], whereas its expression in human breast tissues remain unknown. Interestingly, some kidney and colon biopsies expressed a 2.2 kb mRNA [10,11] whereas some lung biopsies expressed both a 2.2 kb and a second 1.8 kb mRNA whose sequence remain uncharacterized [12]. These observations suggested a role for HOXC6 in cellular differentiation. Such a hypothesis is supported by functional studies illustrating a down-regulation of the *Xenopus* homologue XIHbox-1 during muscle differentiation [13] as well as an involvement of this *HOX* gene in an early step in the proliferation of the erythroid colony-forming unit subset of progenitor cells [14].

There is growing evidence to suggest a potential role of homeodomain-containing proteins in neoplasia [15]. Translocations as well as viral integrations affecting *HOX* genes have been described and lead to proteins harbouring oncogenic properties [16–23] whereas ectopic expression of intact *HOX* genes causes cellular transformation [24]. Moreover, altered expression of multiple *HOX* genes has been detected in a variety of human cancer lesions [10–12]. Such phenomena have also been attributed to other homeodomain-containing proteins that do not belong to the HOX class I family (homeoproteins sharing an Antennapedia-like homeodomain) [25–28].

Apart from a number of studies performed at the RNA level, little information is available about the transcriptional properties of the human HOXC6 protein *in vivo*. A recent report describes the localization of HOXC6 protein in the nucleolus [29]. Despite this precise localization, the ability of HOXC6 protein to activate or repress transcription and to interact with other transcriptional factors remains unknown.

Here we describe the molecular cloning of one 1.8 kb cDNA (clone 211) as well as a 2.2 kb cDNA (clone 173) from MCF7 cells. The new 1.8 kb transcript encodes a homeodomain-containing protein harbouring a unique N-terminal sequence. Moreover we demonstrate that the 2.2 kb HOXC6 transcript is down-

Abbreviations used: CBS, consensus binding sequence; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *HOX*, homeobox gene; RT–PCR, reverse transcriptase PCR.

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regulated in human breast cancer cells whereas the 1.8 kb mRNA is expressed in many human tumours, including breast and ovarian carcinomas. Furthermore we show that both HOXC6 gene products act as repressor of transcription in MDA-MB 231 cells and can co-operate with other *HOX* proteins such as HOXB7 on their target genes. Taken together, our results suggest that both HOXC6 proteins might contribute to breast cell phenotype through repression of their target genes.

MATERIALS AND METHODS

Cell cultures

All cell lines were obtained from the American Type Tissue Collection (Rockville, MD, U.S.A.). MCF7, MCF7D, MDA-MB 231 and T47D cell lines were derived from the pleural effusions of patients with breast adenocarcinoma. MCF7D is a variant of the MCF7 cell line that was kept in culture for 50 more passages than the parent line. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal calf serum (Life Technologies, Grand Island, NY, U.S.A.) and antibiotics. The Hs578T cell line was derived from a breast adenocarcinoma and the Hs578Bst cell line from peripheral normal fibroblasts from the same patient. Hs578T cells were maintained in DMEM supplemented with 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. Hs578Bst cells were maintained in DMEM supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 % (v/v) NCTC 109 (Biofluids, Rockville, MD, U.S.A.), 30 ng/ml epidermal growth factor (Gibco), 10 mM Hepes, pH 7.3, and 8 ng/ml insulin. The MCF10F cell line was established from the mammary tissue of a patient with fibrocystic breast disease. The cells were maintained in DMEM/F12 (Life Technologies) supplemented with 10 % (v/v) fetal calf serum and antibiotics.

Tissue specimens

Breast cancer samples as well as adjacent tissues were collected from patients receiving surgery in local institutions. The tissues were immediately frozen in liquid nitrogen for further extraction of RNA.

Screening of the MCF7 cDNA library

A MCF7 cDNA library in Unizap XR phage DNA [30] (Stratagene, La Jolla, CA, U.S.A.) was screened with cDNA fragments encoding partial HOXB6 and HOXC6 homeodomains in accordance with a protocol described previously [30]. Various HOXC6 cDNA clones were sequenced in both strands by the dideoxy method with the Sequenase Version 2.0 kit (United States Biochemicals, Cleveland, OH, U.S.A.). Sequence ambiguities were resolved by running the reactions on a 40 % (v/v) formamide gel. The resulting sequences were compared with the GenBank and EMBL Nucleotide Sequence Databases with software provided by the Genetics Computer Group (Madison, WI, U.S.A.).

RNA isolation and Northern blots

Total RNA extracted from ovarian cancer was provided by A. Berchuck (Duke University, Durham, NC, U.S.A.), total RNA isolated from human placenta was provided by F. Van Den Brule (Metastasis Research Laboratory, University of Liège, Liège, Belgium), and samples isolated from colon cancers and adjacent tissues were provided by R. Gol (Molecular Oncology, University of Liège). Total RNA extracted from leukaemic patient leucocytes was provided by V. Bours (Laboratory of Clinical Chemistry and Clinical Oncology, University of Liège). Total cellular RNA from breast cancer cells, cancerous and adjacent breast tissues was extracted by the guanidinium isothiocyanate extraction procedure and caesium chloride gradient centrifugation [31]. Poly(A)$^+$ RNA was purified by two passages on an oligo(dT)–cellulose column (Pharmacia Biotech, Uppsala, Sweden). Poly(A)$^+$ RNA was separated by electrophoresis on a 1.2 % (w/v) agarose/formaldehyde gel, transferred to Nytran membrane (Schleicher and Schuell, Keene, NH, U.S.A.) and hybridized at 42 °C as described previously [32]. HOXC6-specific probes (probes A, B and C; see Figure 1) were generated by PCR with clones 173 and 211 respectively as templates and were ^{32}P-labelled by nick-translation. The RNA blot was washed under stringent conditions (0.2 × SSC, 0.5 % SDS at 55 °C) and exposed for 2 days at −80 °C to Kodak XAR-2 films in an intensifying screen cassette.

Reverse transcriptase PCR (RT–PCR)

cDNA synthesis was performed at 42 °C for 30 min with 1 μg of total cellular RNA and 20 units of Superscript reverse transcriptase (Life Technologies, Gaithersburg, MD, U.S.A.) and a 3′-specific HOXC6 primer 3-RT (5′-TCCTCTTCTGTCTCT-TCCCGC-3′) common to both HOXC6 transcripts (see Figure 1). After RNA template degradation by RNase H (Life Technologies), the cDNAs were then purified through a GlassMAX Spin Cartridge (Life Technologies). As a control, all reactions were performed in parallel in the absence of reverse transcriptase. HOXC6 PCR amplifications were performed with primer 3-PCR (5′-TTCTCCAGTTCCAGGGT-3′), which is common to both HOXC6 transcripts, and primers 211-PCR (5′-TCTGTCCTG-GATTGGAGCCGT-3′) or 173-PCR (5′-AATGAGGGAAG-ACGAGA-3′) located on different exons (see Figure 1). As a control for total RNA integrity, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT–PCR experiments were performed systematically on each sample. GAPDH cDNA synthesis was performed with 1 μg of total RNA and primer 5′-AGGCAG-GGATGATGTTCT-3′ whereas the subsequent PCR amplification was performed with 5′ primer 5′-ATGGGGAAGGT-GAAGGTC-3′ and 3′ primer 5′-TGATGGCATGGACTG-TGG-3′ [33]. For both HOXC6 and GAPDH amplification, PCR steps included 35 cycles of denaturation (95 °C for 60 s), annealing (55 °C for 60 s) and extension (72 °C for 2 min) followed by a final extension at 72 °C for 5 min. The resulting amplified fragments were analysed on a 1 % ethidium bromide-stained agarose gel.

Transient transfections and luciferase assays

The *Bam*HI–*Xho*I fragments containing the coding sequence of clone 173 and 211 were subcloned into the expression vector pcDNA3 and driven by a CMV promoter (Invitrogen, San Diego, CA, U.S.A.). The pD9 expression vector was provided by V. Zappavigna (Laboratory of Gene Expression, Department of Biology and Technology, Istituto Scientifico H. S. Raffaele, Milan, Italy) and contains the complete open reading frame of HOXD9 cDNA cloned into the *Bam*HI site of the pSG5 expression vector and driven by a SV-40 promoter [34]. The HOXB7-expressing vector in pcDNA3 was constructed from a cDNA clone isolated from MCF7 cells (A. Chariot and M. E. Sobel, unpublished work). The pTCBS reporter plasmid provided by V. Zappavigna contains an eight-fold multimerized form of a homeodomain consensus-binding sequence and a luciferase reporter gene under the control of an HSV-TK promoter [35].

MDA-MB 231 cells were maintained in culture as described above. Transfections were performed with the cationic liposome reagent *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulphate (DOTAP) (Boehringer Mannheim, Mannheim, Germany), 1 μg of reporter plasmid pTCBS and 0.5–2 μg of 173-pcDNA3, 0.5–2 μg of 211-pcDNA3, 0.5 μg of HOXB7 or 4 μg of HOXD9 expression vectors per 35 mm dish. Total concentrations of transfected DNAs were kept constant throughout by adding appropriate amounts of pcDNA3 vector without insert. Cells were harvested 48 h after transfection, by 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

Cloning of HOXC6 cDNAs from an MCF7 cDNA library

To investigate whether *HOX* genes are expressed in breast cancer, we had previously amplified several partial homeodomains from MCF7 total RNA by RT–PCR [36]. To characterize these *HOX* transcripts further, we screened an MCF7 cDNA library by using partial HOXB6 and HOXC6 homeodomains as probes. Among the various *HOX* cDNA clones isolated, ten clones were identified as representing full-length HOXC6 cDNAs. These clones displayed the expected 183 bp highly conserved DNA-binding HOXC6 homeodomain as well as the homeopeptide. The sequence of these representative clones was compared with the previously published human HOXC6 sequence named HOX c8.5111 [8]. Our representative clone 173 displays a longer 5′ untranslated region (Figure 1). Our coding sequence harbours a 153-residue open reading frame and is identical with the previously published HOXC6 sequence except for the codon 136, which encoded a leucine residue (CTG) in our sequence as opposed to a methionine residue (ATG) in the HOX C8.5111 clone. To determine whether this single base change was specific to MCF7 cells, we sequenced an RT–PCR fragment obtained from human fibroblast D551 total RNA, again obtaining a CTG (results not shown).

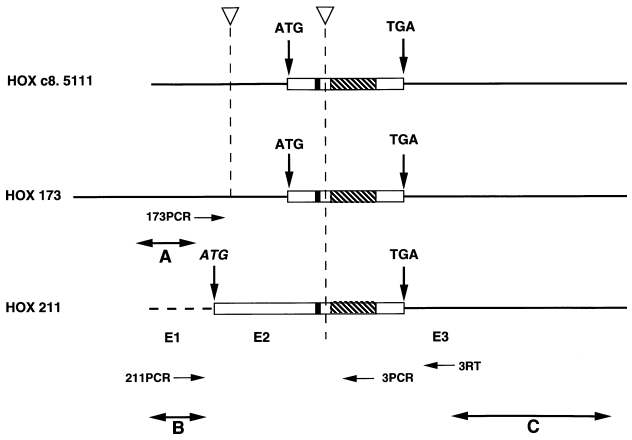

Figure 1 Schematic representation of both HOXC6 transcripts isolated from MCF7 cells

Coding sequences are illustrated by rectangles, and untranslated regions are represented by straight lines. E1, E2 and E3 designate exon sequences. Homeodomains are represented by hatched rectangles and homeopeptides by black rectangles. Splicing signals are delimited by vertical broken lines. HOX c8.5111 is a previously described HOXC6 cDNA clone isolated from SV-40-transformed human fibroblasts [8]. Clone 173, whose coding sequence is identical with that of clone HOX c8.5111, displays a longer 5′ untranslated sequence. Clone 211 represents the newly cloned HOXC6 transcript and encodes a homeodomain-containing protein 82 residues longer. Its distinct 5′ terminal sequence is represented by a horizontal broken line. Specific HOXC6 primers used to perform RT–PCR experiments (3-RT, 173-PCR, 211-PCR and 3-PCR) are indicated by arrows below the clones. Probe A derived from the 5′ untranslated region of clone 173, probe B derived from the 5′ untranslated region of clone 211, and probe C derived from the common 3′ untranslated region of both clones are indicated below the HOXC6 primers and are used in Northern blot experiments.

Two HOXC6 transcripts differ at their 5′ ends but share the same homeodomain

Among the HOXC6 cDNAs isolated, six clones shared a distinct 5′ end region and represented a new cDNA sequence. The sequence of the first exon E1 of the representative clone 211 (Figure 2) differed markedly from that of clones HOX c8.5111 and 173 (Figure 1). Interestingly, the E1 sequence was identical with the 3′ end of the 11 kb intron of the primary transcript that generates clone cp25, an additional HOXC6 cDNA isolated from a human placenta cDNA library [9]. Moreover, as this HOXC6 transcript represented by clone 211 contained a distinct initial ATG, the corresponding mRNA would produce a 235-residue protein, 82 residues longer than the polypeptide encoded by the HOXC6 transcript represented by clone 173. Both human clone 211 and the mouse Hoxc6 homologue gene products [37] are highly conserved throughout evolution because they are 99 % similar. Indeed, only two amino acids located downstream from the homeodomain differ among the 235 amino acids whereas both homeodomains are identical (results not shown).

To confirm the existence of this new HOXC6 transcript in MCF7 cells, we performed an RT–PCR experiment using, as 5′ primer, an oligonucleotide located within the sequence of exon E1 of our clone 211 (Figure 1). As illustrated in Figure 3(A), a single 580 bp amplified fragment was obtained from these cells. The existence of both HOXC6 transcripts was further supported by Northern blot analysis performed on poly(A)$^+$ RNA extracted from several breast cancer-derived cell lines. A 1.8 kb HOXC6 transcript was detected by probe B in MCF7 cells (Figure 1 and Figure 3B, lane 1). Interestingly, this transcript is not expressed in MCF10F and Hs578T cells. However, a 2.2 kb mRNA corresponding to clone 173 was detected by probe A in these cells (Figure 1 and Figure 3B, lanes 2 and 3). Because of the low sensitivity of this approach, we could not detect any signal in the MCF7 cells despite the presence of this HOXC6 transcript in MCF7 RNA as confirmed by RT–PCR experiments (results not shown). As expected, probe C, which derives from the common 3′ untranslated region of both clones (Figure 1) hybridized to both HOXC6 transcripts in the three cell lines investigated (Figure 3B). Our cloning results suggest that both HOXC6 transcripts can be generated either from two distinct promoters or by an alternative splicing event affecting their 5′ ends.

Expression of the 2.2 kb transcript in human breast cell lines

To explore HOXC6 expression in other breast cells, we performed similar Northern blot experiments with poly(A)$^+$ RNA extracted from a variety of human breast cell lines and probe A (Figure 1). As illustrated in Figure 4, the 2.2 kb HOXC6 transcript was detected in Hs578Bst cells (lane 1) as well as in MCF10F cells (lane 5), whereas its expression was decreased in breast cancer-derived cell lines such as Hs578T (lane 2), MCF7 (lane 3), MCF7D (lane 4) and T47D (lane 6) and undetectable in other breast cancer cells such as the MDA-MB231 cell line (lane 7).

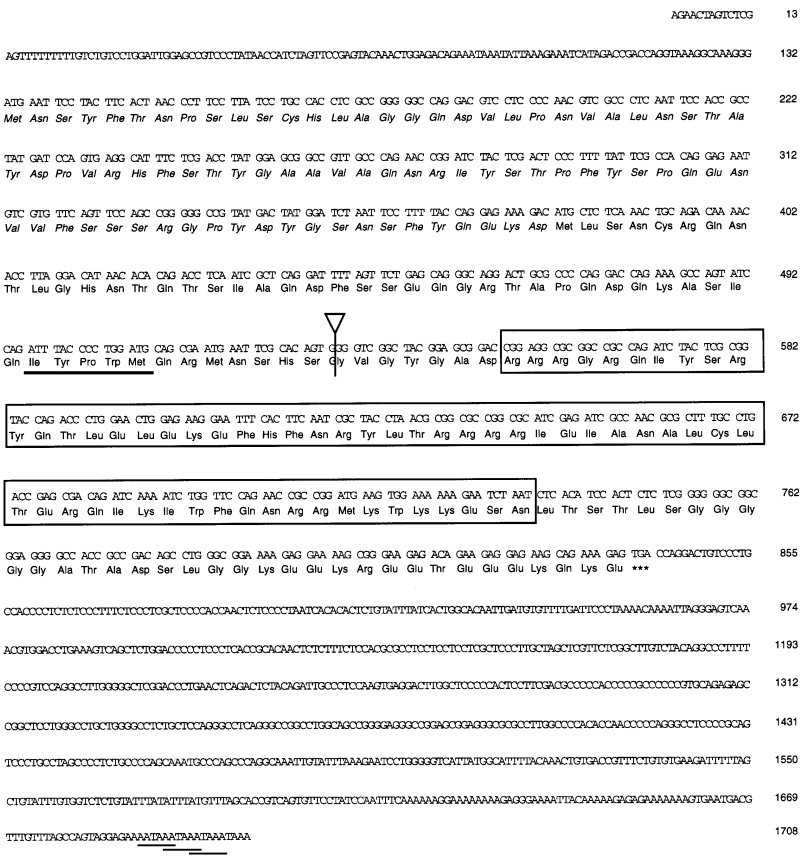

Figure 2 Complete cDNA sequence of the new HOXC6 transcript (clone 211) and its predicted amino acid sequence

The conserved homeobox domain is represented by a white rectangle, and the homeopeptide is underlined. The numbers at the right represent bases starting at the cloned 5′ untranslated region of HOXC6 mRNA. The polyadenylation signals are underlined.

HOXC6 is expressed in human breast tissues

To explore the expression of this *HOX* gene in mammary glands, we performed RT–PCR experiments with, as templates, total RNA extracted from a variety of human breast carcinomas and adjacent tissues. Both HOXC6 1.8 and 2.2 kb transcripts were detected in some breast carcinomas as well as in some adjacent tissues. Indeed, some biopsies do not express any HOXC6 transcript (Figure 5A, lanes 2 and 4; Figure 5B, lane 3) whereas other samples contained either both HOXC6 transcripts (Figure 5A, lane 5; Figure 5B, lanes 1, 4, 5, 6 and 8) or one of the two mRNA species (Figure 5A, lanes 1, 3 and 6; Figure 5B, lanes 2 and 7). We further investigated HOXC6 gene expression in three breast carcinomas and in their corresponding adjacent tissues (Figure 5C). Both HOXC6 transcripts can display a distinct pattern of expression in some breast tissues. These experiments illustrate that positive HOXC6 expression in the breast carcinoma is not necessarily associated with HOXC6 expression in the corresponding adjacent tissue.

The new HOXC6 transcript is expressed in a variety of human tumours

To investigate the expression of this HOXC6 gene in other human tumours, we performed RT–PCR experiments with total RNA extracted from ovarian cell lines and ovarian tissues (Figure 6A), colon cell lines and colon tissues (Figure 6B) and leukaemic patients' leucocytes and three human placentas. The

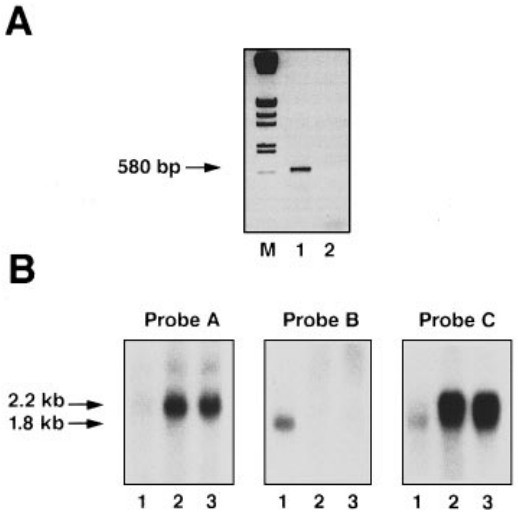

Figure 3 Detection of the new HOXC6 transcript in several breast cancer-derived cell lines

(**A**) RT–PCR experiment on MCF7 cells: a 580 bp amplified fragment was obtained by using primers 3-RT, 211-PCR and 3-PCR, as described in the Materials and methods section and in Figure 1. Lane M, marker; lane 1, 1 μg of MCF7 total RNA; lane 2, RT–PCR experiment performed without any reverse transcriptase as a negative control. (**B**) Northern blots with probes A, B and C (see Figure 1): the 2.2 and 1.8 kb transcripts corresponding to clone 173 and clone 211 respectively are indicated by arrows. Lane 1, MCF7; lane 2, MCF10F; lane 3, Hs578T.

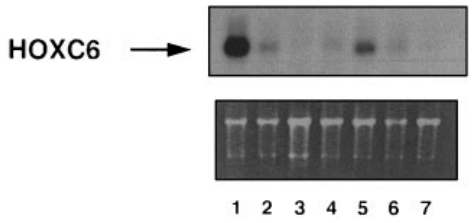

Figure 4 Northern blot performed on 5 μg of poly(A)$^+$ RNAs extracted from a variety of human breast cell lines, with probe A (see Figure 1)

The ethidium bromide-stained agarose gel is illustrated below the HOXC6 blot, in which the position of HOXC6 is arrowed. Lane 1, Hs578Bst; lane 2, Hs578T; lane 3, MCF7; lane 4, MCF7D; lane 5, MCF10F; lane 6, T47D; lane 7, MDA-MB231.

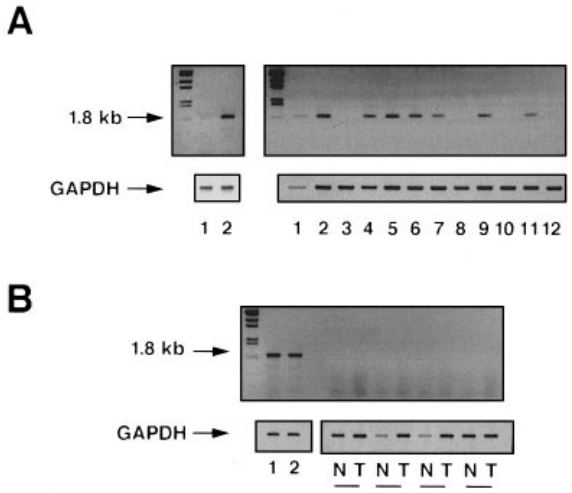

Figure 6 Detection of the new HOXC6 transcript in a variety of human tumours

RT–PCR experiments were performed on 1 μg of total RNA as template with primers 3-RT, 3-PCR and 211-PCR (see Figure 1). GAPDH RT–PCR experiments were performed on each sample: the amplified fragments are shown below each HOXC6 RT–PCR experiment. (**A**) Left panel, ovarian cell lines SK-OV3 (lane 1) and OVCAR 3 (lane 2); right panel, 12 ovarian carcinomas. (**B**) Left panel, colon cell lines HT29 (lane 1) and HTM29 (lane 2); right panel, colon cancers and adjacent tissues.

new HOXC6 transcript was detected in OVCAR cells (Figure 6A, left panel), in eight out of twelve ovarian carcinomas (Figure 6A, right panel, lanes 1, 2, 4, 5, 6, 7, 9, 11 and 12) and in HT29 and HTM29 cells, two cell lines derived from colon cancer. No transcripts were detected in the colon cancer investigated and in the adjacent tissues (Figure 6B), in the leukaemic leucocytes investigated and in the placenta samples (results not shown).

Both HOXC6 gene products repress transcription in MDA-MB231 cells

To study further the functional properties of both HOXC6 proteins in breast cancer cells, we designed co-transfection experiments with pTCBS, as reporter plasmid. This construct contains a multimerized consensus binding sequence (CBS) that can mediate *trans*-activation of multiple *HOX*-derived proteins [35]. We performed these experiments in MDA-MB231 cells that do not express detectable levels of HOXC6 transcripts (Figure 4). As a positive control for our transfections, we used HOXD9 gene product, which *trans*-activates pTCBS in NIH3T3 cells [35] as well as in MDA-MB231 cells, as illustrated in Table 1. Both HOXC6 gene products repress the transcription from the CBS in a dose-dependent manner (Table 1). Moreover, when co-transfected with HOXB7 gene product, which causes a *trans*-activation by itself, the 173 or 211 pcDNA3 constructs significantly suppressed the induced expression of the luciferase gene in a dose-dependent manner. Our results suggest that both HOXC6 products harbour intrinsic repressing abilities and can co-operate with other *HOX*-derived proteins on the CBS.

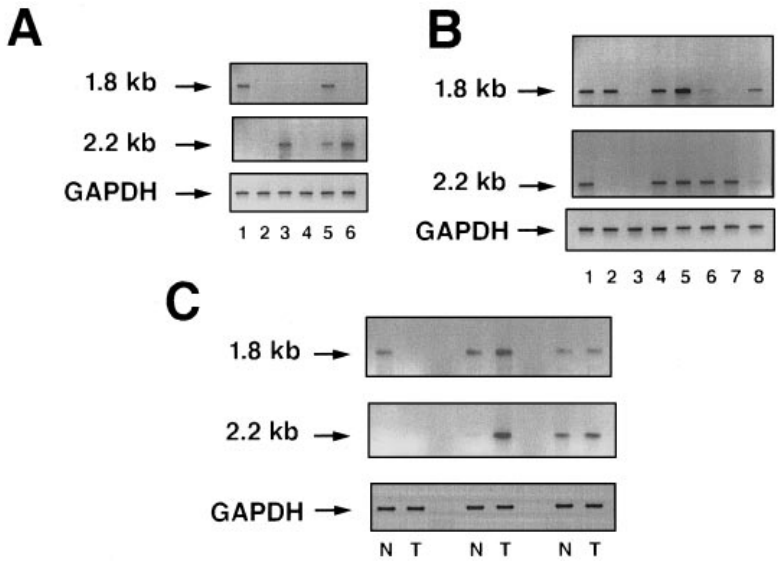

Figure 5 RT–PCR experiments performed with primers 3-RT, 3-PCR, 173-PCR and 211-PCR

The experiments were designed to detect both HOXC6 transcripts in human breast cancers (**B**) and adjacent tissues (**A**). N and T designate adjacent tissues and corresponding breast carcinomas respectively in (**C**). GAPDH RT–PCR experiments performed on each sample are shown in each bottom panel. The resulting amplified fragments were detected after electrophoresis on a 1% ethidium bromide-stained agarose gel.

Table 1 Analysis of HOXD9, HOXB7 and both HOXC6 gene products (HOX 173 and HOX 211) on the CBS target sequence by transient co-transfections in MDA-MB 231 cells

The luciferase activities compared with the value obtained with 1 μg of reporter plasmid alone are shown. Each value represents the mean ± S.D. for three independent experiments after normalization to the protein concentration of the extracts. For each experiment, 1 μg of reporter plasmid was added to the cells. The identities of the transfected HOX gene products are underlined.

Constructs (μg)	Luciferase activity (fold induction)
HOXD9 (4)	4.120 ± 0.680
HOXB7 (0.5)	2.904 ± 0.337
HOX 173 (0.5)	0.803 ± 0.108
HOX 173 (2)	0.353 ± 0.043
HOX 211 (0.5)	0.713 ± 0.041
HOX 211 (2)	0.198 ± 0.056
HOXB7 (0.5) + HOX 173 (0.5)	2.038 ± 0.158
HOXB7 (0.5) + HOX 173 (2)	0.322 ± 0.004
HOXB7 (0.5) + HOX 211 (0.5)	1.274 ± 0.027
HOXB7 (0.5) + HOX 211 (2)	0.405 ± 0.049

DISCUSSION

This report presents the molecular cloning and characterization of two HOXC6 transcripts that differ in their 5′ ends and encode distinct homeodomain-containing proteins in MCF7, a cell line derived from human breast cancer. Interestingly, Simeone et al. [9] had previously reported the isolation of multiple *HOX* transcripts belonging to cluster C and sharing a common 5′ end sequence. This observation suggested that these mRNAs were transcribed from a common promoter and generated by alternative splicing of a common primary transcript [9]. Because our HOXC6 transcripts display distinct 5′ ends, we speculate that they are either generated by an alternative splicing event or transcribed from two distinct promoters. Despite the lack of experimental evidence, we believe that the transcription from two distinct promoters is the most likely hypothesis because such a phenomenon has been experimentally demonstrated for mouse [37] as well as for *Xenopus* Hoxc6 homologues [38]. Indeed, in those species, two promoters located approx. 9 kb apart have been identified [37,38]. Because of the high conservation of similarity in sequences and in genomic organization of *HOX* genes throughout evolution, our results illustrate the potential existence of a second promoter in the human cluster C that controls the synthesis of the newly cloned HOXC6 transcript. When these results are compared with those described by Simeone et al. [9], we conclude that transcription of *HOX* genes belonging to cluster C might then imply two distinct mechanisms that would account for a complex network of transcription regulatory mechanisms.

We illustrate the detection of the 2.2 kb HOXC6 transcript in human breast cell lines. Interestingly, the abundance of this mRNA was lower in several cell lines derived from breast cancers than in two cell lines derived from non-tumorigenic breast tissue. Our observation is in agreement with a previous report illustrating a similar phenomenon in the mouse mammary gland [39]. Moreover we have detected the new HOXC6 transcript as well as the 2.2 kb mRNA in a variety of breast cancer lesions displaying distinct histological grades as well as in the adjacent tissues. Quantitative analysis with a large panel of breast tissues should determine whether the particular differential pattern of expression of this transcript in breast cancer cells grown *in vitro* also occurs *in vivo*. This is the first evidence of the expression of a defined *HOX* gene in human neoplastic mammary glands. In the mouse system, Hoxc6 expression has not been detected in breast cancer lesions. The more sensitive RT–PCR method used in this study might explain this apparent discrepancy. Moreover, the expression of the mouse homologue Hoxc6 is modulated by an unidentified steroid [39]. We have observed that HOXA1 expression was induced by progestins in MCF7 cells [40]. However, this observation cannot at present be extended to other *HOX* genes such as HOXC6.

The existence of both HOXC6 transcripts is not specific to breast tissues but also occurs in other cell types such as ovarian cell lines and ovarian tissues. However, we did not detect the newly cloned HOXC6 transcript in the colon tissues investigated. A previous study had reported the detection of the 2.2 kb HOXC6 transcript corresponding to our clone 173 in normal and neoplastic colon biopsies [11]. However, the 1.8 kb transcript corresponding to our clone 211 was not detected in any of the samples investigated. This observation and our results suggest that neither promoter is systematically turned on in colon cells or in breast tissues, suggesting that they can work independently under the control of distinct transcription factors to regulate their expression. Such a phenomenon might reflect the existence of a specific regulatory network controlling HOXC6 expression.

Both HOXC6 proteins can repress the transcription of a reporter gene under the control of a CBS in MDA-MB231 cells. Their effect can be modulated by co-operative interactions with other *HOX*-derived proteins such as HOXB7 or HOXD9 (results not shown). The existence of such protein–protein interactions is further supported by structural analysis [41]. Two homeodomain-containing proteins can indeed bind to a single 10 bp DNA sequence in a tandem fashion [41] thereby allowing the interaction of both homeodomains and other less conserved regions, leading to the observed modulation of gene expression. Despite distinct N-terminal domains, both HOXC6 gene products, however, share identical homeopeptides as well as homeodomain sequences that have been involved in protein–protein interactions, as suggested by recent studies [20,42]. Their *trans*-activation effect might be mediated by these particular sequences. Mutagenesis analysis should lead to the identification of the precise domains responsible for the biological effects of HOXC6.

The transcriptional properties of human *HOX*-derived proteins have been previously investigated [20,43]. These studies combined with ours suggest that *HOX*-derived proteins can act either as activators or repressors of transcription. Because all *HOX* members bind to similar *cis*-regulating DNA sequences [44–46], it is tempting to speculate that each cell expresses at a given time a set of *HOX*-derived proteins that interact between themselves and presumably with other transcription factors to modulate the expression of their target genes.

The identification of target genes and polypeptides expressed by *HOX* in different types of tissue should lead to a better understanding of the roles played by these critical proteins.

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