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HOMEOBOX GENES: POTENTIAL CANDIDATES FOR THE TRANSCRIPTIONAL CONTROL OF THE TRANSFORMED AND INVASIVE PHENOTYPE

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Abstract—The transformation of a cell and the acquisition of the invasive and metastatic phenotype result from the activation of a group of complex cellular processes rather than from the effect of a single gene product. It is likely that the coordination of the multiple genes involved in malignancy is under the control of a few genes that act as master genes or orchestrator genes. The latter probably code for transcription factors that control the genetic program for tumor invasion and metastasis. Homeobox genes are a family of transcription factors that contain a 183 bp highly conserved nucleotide sequence coding for a 61 amino acid domain that binds specifically to DNA. First discovered in *Drosphila* as genes controlling segmentation and segment identity, homeobox genes have since been identified in many other species including nematodes, frog, mouse and human. There is strong support for the suggestion that homeobox genes play a key role in development and differentiation. In humans, there are 38 homeobox genes organized in four clusters that are localized on chromosomes 2, 7, 12 and 17. The specific functions of each of these genes are generally unknown. Alterations in expression of several homeobox genes have been reported in a variety of malignant lesions, suggesting that they could play a role in the development of cancer. Using reverse transcriptase reaction coupled with polymerase chain reaction and degenerate oligonucleotides corresponding to the 5′ and 3′ ends of the highly conserved homeodomain, we amplified 130 bp cDNA fragments from the human breast cancer cell line MCF7 that were subsequently cloned into pBluescript vector. Sequencing of the clones, resulted in the identification of the homeodomains of four different human homeobox genes: HOXB6, HOXA1, HOXA10 and HOXC6. Further studies should determine the specific role of these four homeobox genes in the development and progression of human breast cancer and potentially determine if they might be good targets for gene therapy.

Tumor invasion and metastasis: a complex biological process

The most life-threatening aspect of cancer is certainly tumor invasion and metastasis [1, 2]. The genetic changes responsible for the imbalance of growth regulation lead to uncontrolled proliferation. These mutations are necessary for both primary tumor and metastatic colony expansion, but are not sufficient to initiate tumor invasion and metastasis. Progression to metastasis probably requires additional genetic modifications that will enable the cells to leave the tissue of origin and to disseminate locally and distally [3, 4]. Metastases result from a cascade of linked sequential steps involving complex interactions between cancer cells and the host [3]. In order to successfully form a metastatic colony, a cell or a group of malignant cells have to leave the primary tumor, invade the adjacent tissue and the wall of local blood or lymphatic vessels, enter the circulation, attach to the endothelium at a distant vascular bed, intravasate into the target organ or tissue and proliferate as a secondary tumor (Fig. 1). Angiogenesis induced by cancer cells is required for the expansion of the primary lesion as well as the metastatic colony [5]. At all stages of the metastatic cascade, the tumor cells must overcome both passive and active host defence mechanisms to reject tumors.

Only a very small percentage of circulating malignant cells (<0.01%) succeed in initiating a metastatic colony [6]. It is most likely that only the cells that express the right combination of genes, that allow them to pass each step of the metastatic cascade, will end up forming a secondary colony. The successful metastatic cells must express the correct genes at the right time and place. Our hypothesis is that this extraordinary complex coordination of the gene expression throughout the metastatic cascade is the result of the application of genetic programs that control in a spatiotemporal fashion the expression of the genes needed for invasion and metastasis. Such genetic programs may be similar to those used in the early stages of embryogenesis and fetal development since organ formation involves invasion and dissemination of cells. For example, trophoblastic invasion of the endometrium is a physiological, biological event that requires the expression of genes that lead to the penetration of the endometrium and the degradation of uterine blood vessel walls [7, 8]. According to our hypothesis, tumor invasion and metastasis results from the activation of specific genetic programs contained in the genome of each cell. Interestingly,

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several studies have pointed out the similarity of the molecular mechanisms involved in trophoblast invasion and in cancer invasion. These invasion genetic programs are very probably under the control of master genes that orchestrate the expression of the batteries of genes needed for the correct execution of the program. Such orchestrator genes are probably transcription factors that can control multiple genes, leading to the expression of a specific phenotype. The strategic importance of identifying the master genes controlling invasion and metastasis is obvious since it will dramatically reduce the target for anticancer therapies. One can indeed postulate that the inactivation of the specific invasion genetic program would stop cancer invasion.

The extreme complexity of development as well as the large number of differentiated cell types in higher eukaryotic organisms suggest that a large number of proteins are needed to regulate specific developmental processes. However, as postulated by Dressler [9], if the phenotype of a particular cell is specified by a combination of factors rather than by a single protein, significantly fewer regulatory elements will be needed to obtain a unique genetic program. Furthermore, if the expression level of a single regulatory protein, in combination with others, has *per se* a regulatory value, an even smaller number of controlling factors would be required. In recent years, there has been an accumulation of evidence to show that several developmental and cell specific factors contain homeoboxes. The homeobox genes are a family of genes that play key roles during development and differentiation [10–13]. Several examples exist in which homeobox genes appear to be the master genes that command a specific genetic program, particularly in development. The evidence that homeobox genes encode transcription factors during vertebrate development is mostly circumstantial, but several tissue-specific transcription factors contain homeobox domains. Based on their role as orchestrators in several developmental biological processes, the homeobox genes could be potential candidates for the control of the invasive genetic program.

Homeobox genes

Homeotic transformations or homeosis correspond to developmental anomalies in which one part of the body develops in the likeness of another [10, 11]. This phenomenon, observed in most of the higher eukariotic organisms, has been mostly studied in the fruit fly, *Drosophila*. The most commonly studied homeotic mutations are those that result in transformation of one body segment to another. Mutations in the gene Antennapedia (Antp), for example, result in the formation of leg structures on a segment where the antennae belongs [10, 11]. Antp was the first homeobox gene identified out of a large family of at least 25 *Drosophila* developmental genes that control segmental identity [14]. Comparison of the nucleotide sequence of these genes showed that they shared a highly conserved short stretch of DNA

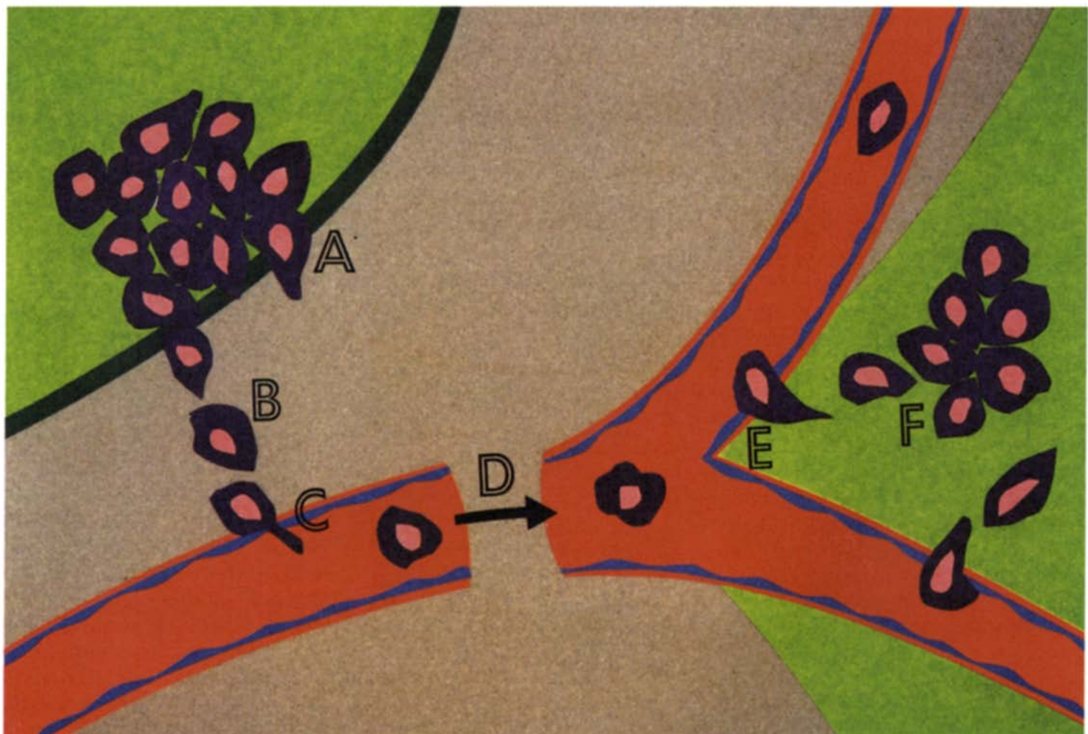

Fig. 1. The metastatic cascade. In order to form a secondary colony, a cancer cell must (A) leave the primary tumor, (B) invade the adjacent stroma, (C) penetrate the wall of blood or lymphatic vessels, (D) circulate in the bloodstream, (E) attach to the endothelium and invade the vessel wall during extravasation, (F) invade the parenchyma of the target organ and proliferate.

(183 nucleotides) which was called "homeobox" or "homeodomain". Most of the homeobox genes in *Drosophila* are arranged in two clusters, the Antennapedia Complex (ANT-C) and the bithorax complex (BX-C) [10, 11]. During embryonic development of the fruit fly, these genes have very precise but overlapping zones of expression from head to tail, with the anterior limit of somatic expression of each gene related to its position in the gene cluster. Because these genes appear to have similar functions, but at different places, it has been proposed that homeobox genes might all have evolved from an ancestral gene by duplication and divergence. Homeobox genes with striking nucleotide sequence homologies in the homeodomain regions have been identified in many other species including, *Xenopus laevis, nematoda*, mouse and human. Based on their presence in fungi, plants and animals it has been estimated that the homeodomain structure is over a billion years old [15]. In addition to the homeodomain, homeobox proteins contain other highly conserved structures that are represented in a prototype homeobox protein presented in Fig. 2 [12]. There is a conserved amino-terminal region that extends in some homeobox genes for up to 45 amino acids followed by a variable region often rich in alanine, serine, glycine, proline or glutamine. A highly conserved pentapeptide called homeopeptide (IYPWM) is present just upstream from the homeodomain. Three-dimensional NMR of the 61 amino acid homeodomain has proven directly that it contains a helix-turn-helix, which is a DNA binding motif. It ends with an acidic tail whose function is not yet known, but which could act as a transcriptional activation domain like in certain other transcription factors described previously [12].

Several studies have shown that homeobox proteins exhibit a transcription factor function in *Drosophila* as reviewed in Refs. 10, 11 and 16. In mammals, evidence that homeobox proteins act as transcription factors has been provided by biochemical experiments showing that many mammalian promoters and enhancers contain copies of an octanucleotide sequence (ATTTGCAT) that is required for transcriptional activity [12]. The transcription factors that bind to this sequence contain a homeodomain. Furthermore, Pit-1, a factor required for the transcription of prolactin also contains a homeodomain [17].

In humans, as well as in mice, there are 38 class I homeobox genes (designated as HOX genes) that are organized in four clusters designated as A, B, C and D, located on chromosomes 7, 17, 12 and 2, respectively (Fig. 3) [15, 17–19]. These homeobox genes are designated class I genes or HOX genes because they are related to the homeobox sequence of the Antennapedia complex. Recently, a new nomenclature has been proposed to avoid the confusion caused by the existence of different names given to the same homeobox gene and by the use of different letter and decimal designations to individual homeobox genes [15]. The genes of the HOX clusters can be aligned with the *Drosophila* homeobox genes. Interestingly there is a conservation of the spacial localization of genes within each cluster and between mammals and *Drosophila* indicating that the four clusters arose from the reduplication of a single ancestral homeobox complex [15]. A number of genes coding for proteins containing the homeodomain are found outside the cluster complexes and are called diverged homeobox genes. For practical reasons, these genes do not bear the name "HOX". Homeobox genes in mammals appear to be master genes controlling genes for body development [16]. They also seem to be implicated in the phenotypic commitment of a variety of cells, e.g. the hematopoietic cells [20].

An interesting aspect of homeobox genes relates to the fact that they are expressed as alternatively spliced transcripts [20, 21]. The multiple transcripts encode either full-length homeobox proteins, or proteins lacking the homeodomain. The latter would be without putative transcriptional regulatory domains or would contain alternative putative regulatory regions. The role of this truncated homeobox protein is still unknown, but, it has been suggested that partial homeobox proteins could modulate transcription through competition with complete molecules for binding to other cellular factors [22].

Homeobox genes and cancer

The concept that homeobox genes might be involved in cancer is recent. A putative role of

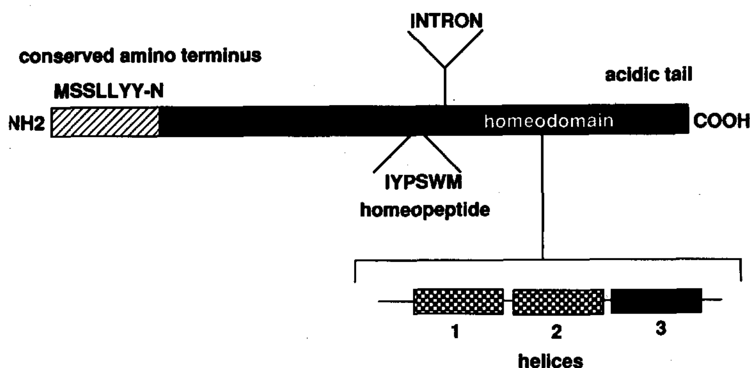

Fig. 2. Schematic prototype homeobox genes. The homeodomain is a 61 amino acid highly conserved domain which bears a helix-turn-helix motif. Other conserved domains include the conserved amino terminus, the homeopeptide and an acidic tail.

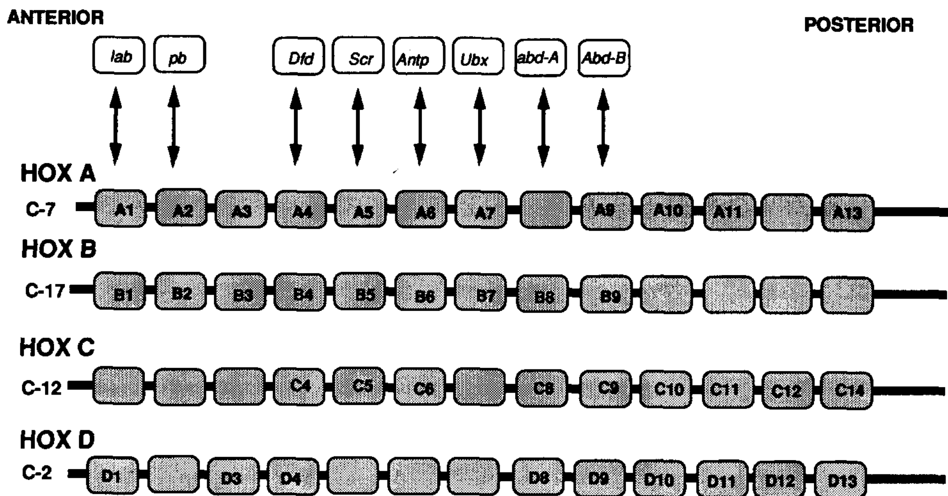

Fig. 3. The four mammalian HOX complexes (adapted from Scott). Four clusters of HOX genes and their new names are presented. Genes expressed more anteriorly, compared to the corresponding *Drosophila* genes shown at the top, have the lowest number. Each column of HOX genes indicates corresponding genes based only on homeodomain sequences. Empty boxes indicate that no gene has been found in human. The four clusters, HOX A, HOX B, HOX C and HOX D are located on chromosomes 7, 17, 12 and 2, respectively.

homeobox genes in malignant processes was first documented in leukemia, as reviewed recently [20]. The first evidence of the involvement of a homeobox gene in leukemia was for the HOXB8 (previously, Hox-2.4) gene which is transcriptionally activated in the mouse myeloid leukemia cell line WEH1-3B. The constitutive over-expression of this homeobox gene is due to the insertion of its first exon of a retrovirus-like intracisternal A particle (IAP) [23, 24]. It was shown that the over-expression of HOXB8 inhibited specific pathways of the myeloid differentiation program [24]. Interestingly, the oncogenic potential of HOXB8 was demonstrated in NIH3T3 fibroblasts. NIH3T3 transfectants bearing an activated HOXB8 gene exhibited a transformed phenotype and produced fibrosarcomas in nude mice [25].

The t(10;14)(q24,q11) chromosomal translocation found in 5–7% of T-cell acute lymphoblastic leukemias involves the juxtaposition of the T-cell receptor delta chain with a novel homeobox containing gene called tcl3 proto-oncogene [26]. This translocation results in the expression in T-cells of a homeobox gene which is not normally expressed. Additionally, a variant translocation t(7;10)(q35;q24) involves the juxtaposition of the tcl3 gene and the TCR beta gene on chromosome 7, resulting in over-expression of the homeobox protein tcl3. These data strongly suggest a role for tcl3 in the genesis of T-cell leukemia. The transforming properties of this homeodomain containing protein are further documented by experiments showing that over-expression of tcl3 in NIH3T3 cells led to transformation of the cells [27].

Pbx1 gene is a novel divergent homeobox gene located on chromosome 1. Thirty per cent of cases of pre-B-cell acute lymphocytic leukemia have t(1;19) translocation [28]. Most of these involve a fusion of the Pbx1 gene with the E2A gene, coding for the enhancer-binding protein E12:E47. The chimeric proteins contain the amino two-thirds of the E2A protein with a homeodomain replacing its normal DNA-binding region [29]. Several studies have shown that this fusion protein exhibits transforming properties [30]. For example, the introduction of the chimeric protein into normal bone marrow cells yields myeloid leukemia in the animals. Very recently, it was demonstrated that the chimera gene E2A-Pbx1 induced proliferation, apoptosis and malignant lymphoma in transgenic mice [31].

HB24 is another diverged human homeobox gene expressed in hematopoietic progenitor cells [32]. It has been involved as a transcription factor that plays a key role during hematopoietic progenitor proliferation [33]. Specific cell type differentiation required down-regulation of this homeobox gene. High expression of HB24 was shown to confer to a human T cell line the ability to form metastasis in mouse [34].

The pattern of expression of homeobox genes contained in the four human clusters has been studied in different types of human leukemia [35]. The results suggest that hematopoietic malignant cells express a repertoire of HOX genes characteristic of a particular cell lineage at a specific stage of differentiation.

Several homeobox genes are also involved in solid cancers. Preliminary studies pointed out that homeobox proteins were augmented in various human carcinomas, such as breast, colonic, rectal, gastric, lung, renal and testicular cancers [36]. Alterations in the expression of homeobox genes were studied in N-ras transformed PA1 human teratocarcinoma cells [37]. It was shown that enhanced proliferation of these transformed cells

compromised cell differentiation by a mechanism that suppresses homeobox gene induction.

Hox 7.1 is homeobox gene involved in the terminal differentiation of myogenic cells [38]. It plays an important role in the proliferation of the myoblast cells and differentiation, if the cells required a down-regulation of the expression of Hox 7.1. Transfection of Hox 7.1 into myoblasts inhibited terminal differentiation and induced cell transformation [38].

Alterations in expression of homeobox genes have been reported in kidney and colorectal cancer [35, 39]. It appears that while some homeobox genes have the same expression in normal and malignant tissues, others exhibit altered expression in the cancer lesions suggesting an association with cancer progression.

In general the specific role of specific homeobox genes in the genesis of cancers has not been completely elucidated. Future study should determine their specific participation in the oncogenic process and in the control of the invasive and metastatic phenotype.

Identification and cloning of homeobox genes expressed in human breast cancer

To explore the possibility that human breast

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5' PRIMER      TCG   GAR   YTN   GAR   AAR   GAR   TT

                .                 .                 .                       .                 .
M2           1 TCG   GAG   TTG   GAG   AAA   GAG   TTT   CAC   TAC   AAT   CGC   TAC   CTG   ACG  42
HOX B6         CT-   ---   C--   ---   --G   ---   ---   ---   ---   ---   ---   ---   ---   ---
                                              .                 .                       .
M3                                          1 GAA   TTC   CAC   TTC   AAC   AAG   TAC   CTG   ACG  27
HOX A1                                        ---   ---   ---   ---   ---   ---   ---   ---   ---
                                              .                 .                       .
M13                                         1 GAA   TTC   CAC   TTC   AAC   CGC   TAC   CTG   ACA  27
HOX C6                                        ---   ---   ---   ---   --T   ---   ---   --A   --G
                .                 .                 .                       .                 .
M20          1 TCG   GAG   TTT   GAG   AAA   GAA   TTT   CTG   TTC   AAT   ATG   TAC   CTT   ACT  42
HOX A10        CT-   ---   C-G   ---   --G   --G   ---   ---   ---   ---   ---   ---   ---   ---
-----------------------------------------------------------------------------------------------
                            .                       .                 .                 .
M2          43 CGG   CGG   CGG   CGC   ATC   GAG   ATC   GCG   CAC   GCC   CTG   TGC   CTG   ACG  84
HOX B6         ---   ---   ---   ---   ---   ---   ---   ---   ---   ---   ---   ---   ---   ---
                      .                 .                       .                 .
M3          28 CGC   GCC   CGC   AGG   *TG   GAG   ATC   GCT   GCA   TCC   CTG   CAG   CTC   AAC  68
HOX A1         ---   ---   ---   ---   G--   ---   ---   ---   ---   ---   ---   ---   ---   ---
                      .                 .                 .                       .
M13         28 CGG   CGC   CGC   CGC   ATC   GAG   ATC   GCC   AAC   GCG   CTT   TGC   CTG   ACC  69
HOX C6         ---   ---   --G   ---   ---   ---   ---   ---   ---   ---   ---   ---   ---   ---
                            .                       .                 .                 .
M20         43 CGA   GAG   CGG   CGC   CTA   GAG   ATT   AGC   CGC   AGC   GTC   CAC   CTC   ACG  84
HOX A10        ---   ---   ---   ---   ---   ---   ---   ---   ---   ---   ---   ---   ---   ---
-----------------------------------------------------------------------------------------------
                            .                 .                 .
M2          85 GAG   AGG   CAG   AGG   AAA   ATA   TGG   TTC   CAA   AAC   CCC  117
HOX B6         ---   ---   ---   -TC   --G   ---   ---   ---   --G   ---   -GA
                .                 .                       .                 .
M3          69 GAG   ACC   CAA   GTGG  AAG   ATT   TGG   TTC   CAA   AAC   CCC  102
HOX A1         ---   ---   ---   ---   ---   --C   ---   ---   --G   ---   -G-
                .                 .                       .                 .
M13         70 GAG   CGA   CAG   AGC   AAG   ATA   TGG   TTC   CAA   AAC   CCC  102
HOX C6         ---   ---   ---   -T-   --A   --C   ---   ---   --G   ---   -G-
                            .                 .                 .
M20         85 GAC   AGA   CAA   GTG   AAA   ATC   TGG   TTC   CAG   AAC   CCC  117
HOX A10        ---   ---   ---   ---   ---   ---   ---   --T   ---   ---   -G-

3' PRIMER                          N   AAR   ATH   TGG   TTY   CAR   AAY   CCC
```

Fig. 4. Identification of cloned homeodomain fragments. Four of the cloned homeodomain fragments had sequences that were homologous with members of the HOX clusters. Underneath the sequence of the cloned amplified MCF7 homeodomain fragments M2, M3, M13 and M20 are the actual sequences of the HOX genes B6, A1, C6 and A10, respectively. Identity between the cloned fragment and the published sequence are noted with –, while any mismatches are provided. The * between positions 39 and 40 of M3 designates a deletion in M3 compared to HOX A1. Note that there is an internal EcoR1 site within HOX A1 and HOX C6, so when we restricted the PCR amplified fragment, we lost upstream sequences. For probes M3 and amplified fragment, we lost upstream sequences. For probes M3 and M13, number 1 is the first base of the internal EcoR1 site in HOX that matches up. For probes M2 and M20, number 1 is equivalent to base 10 of the 5′ degenerate primer (which is the second T of EcoRI), as described in the text. The 3′ degenerate primer is described in the text. Its complementary reverse sequence was: NAARATHTGGTTYCARAAYCCCCTCGAGCGCGC, with the XhoI site underlined. The complementary reverse sequence of the 3′ primer is shown at the bottom of the figure, however the last 11 bases were deleted to exclude the XhoI site and the tail of the primer.

cancer cells express homeobox genes and to identify any genes that may be involved in the progression of breast cancer, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) experiments on the human breast cancer-derived cell line MCF7. To amplify homeobox gene mRNA transcripts in MCF7 cells, cDNAs were synthesized using 5 μg of total cellular MCF7 RNA and a degenerate primer that was derived from a well conserved homeodomain and that included a 5 base tail and XhoI recognition sequence at the 5′ end: GCGCTCGAGGGRT-TYTGRAACCADATYTTN (IUPAC codes) [40]. The reverse transcriptase was then inactivated at 95° for 10 min. The PCR was performed in a Perkin Elmer (Norwalk, CT, U.S.A.) thermal cycler 480 in the same buffer, by adding 5 U of native Taq DNA polymerase (Perkin Elmer) and 50 pmol of an upstream degenerate primer form the well conserved homeodomain region and that included at the 5′ end a 5 base tail and EcoR1 recognition sequence: AGCCGGAATTCGGARYTNGARAAGARTT. The expected size of the PCR products was 130 bp. PCR steps included an initial denaturation at 95° for 5 min followed by 5 cycles of denaturation (95° for 90 sec), annealing (45° for 30 sec) and extension (ramp from 45° to 72° for 2 min and 72° for 2 min), and 25 more cycles of denaturation (92° for 90 sec), annealing (55° for 30 sec), extension (ramp from 55° to 72° for 2 min and 72° for 3 min) followed by a final extension at 72° for 2 min. This PCR protocol led to the amplification of multiple 130 bp DNA fragments (data not shown). The RT-PCR products were purified from acrylamide gels and were restricted with EcoR1 and Xhol. The cDNA fragments were then cloned into the EcoR1 and Xhol sites of p-Bluescript vector (Stratagene, La Jolla, CA, U.S.A.). Due to internal Xhol and EcoR1 sites within some of the amplified fragments, some clones contained smaller than 130 bp cDNA inserts. Sequencing of the subcloned RT-PCR products led to the identification of some of the 38 human clustered homeobox family. Indeed, as shown in Fig. 4, partial homeodomains of HOX A1, HOX A10, HOX B6 and HOX C6 were isolated using this procedure. These observations provide the first evidence that specific homeobox genes are expressed in a human breast derived cell line. These preliminary data provide us with evidence that specific homeobox genes might be involved in breast cancer. Further study, including exhaustive screening of cDNA libraries and full length identification of the homeobox genes expressed in breast cancer cells, should lead to the specification of homeobox genes involved in mammary carcinoma. Studies of the expression of such candidate genes in a variety of human breast lesions using techniques such as *in situ* hybridization or RNase protection assays, and determination of the molecular mechanisms involved in their expression should help elucidate the exact role of the master genes in transformation and control of the invasive and metastatic phenotype.

Perspectives and speculations

Homeobox genes are probably the best candidates to be master genes playing a key role in the control of embryogenesis and in the phenotypic commitment of specific cells. The study of the exact functions of these transcription factors is complex because they are usually expressed at low levels. The existence of multiple alternatively spliced transcripts further complicates the task of understanding the mechanism of action of homeobox genes. Furthermore the observation that several different homeobox genes are expressed in the same cell suggests that it could be the specific combination of homeobox proteins being expressed rather than the expression of a single specific homeobox gene that determines the phenotype of this cell. The target genes regulated by homeodomain proteins have not been identified to date. It is highly likely that these genes will code for proteins involved in differentiation. The involvement of homeobox genes in cancer supports the concept that these genes play a central role in the control of the differentiation status of a cell. Alteration in the qualitative and quantitative expression of homeobox genes could result in the expression of a particular combination of homeobox proteins that will commit the cells to activate the genetic program of invasion and metastasis. Studies including transfection of sense and anti-sense specific homeobox genes, alone or in combination, might elucidate the role played by these transcription factors in the control of the invasive and metastatic phenotype and should determine whether they are a good target for gene therapy.

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