The Oncogenic Potential of Deregulated Homeobox Genes

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Abstract
Homeobox genes encode DNA-binding proteins that regulate gene expression during embryonic development. Deregulation of homeobox gene expression has been invoked as the molecular basis of a number of leukemias. We now demonstrate, using in vitro and in vivo transformation assays, that the overexpression of homeobox genes is the basis for transformation and tumorigenesis. Thus, clustered homeobox genes related to Drosophila Hom genes (Hoxa-7, Hoxa-5, Hoxa-1, Hoxb-7, and Hoxc-8) as well as the homeobox genes Evx-1 and Cdx-1 constitute a new family of nuclear protooncogenes. This finding suggests that many homeobox genes regulating embryogenesis can—if deregulated—also contribute to tumorigenesis.

Introduction
Chromosomal translocations are one of the major causes of oncogene activation in neoplastic transformations. The activation of the c-abl gene in chronic myelogenous and other acute leukemias and the activation of the c-myc gene in Burkitt's lymphoma are examples of this phenomenon in human neoplasia. Protooncogenes were identified because of their abnormal expression in such neoplastic transformations and are often members of pathways controlling development and differentiation, in addition to cell proliferation (see Ref. 1 for a review). In particular, many nuclear oncogenes that are thought or known to act as transcription factors appear to fulfill these dual roles. Prominent examples include the c-rel oncogene, which is the homologue of the Drosophila dorsal gene (2), and the N-myc and Drosophila hairy homology (3). The concept of protooncogenes as control elements of normal development may help to explain the molecular mechanisms that govern embryogenesis and differentiation.

Recently, genes that contain sequence elements first identified in developmental control genes were shown to be deregulated in neoplastic transformations. The homeobox gene PBX-1 (Phl) is thought to be involved in pre-B-cell leukemias (4, 5). Elevated expression due to chromosomal translocation of the protooncogene tcl-3 is thought to be the molecular cause of 5 to 10% of T-cell acute lymphoblastic leukemias. The homeobox of tcl-3 (HOX-11) is very similar to the homeobox of Hoxa-1 (6).

Deregulated homeobox gene expression by the insertion of a proviral LTR3 upstream of the Hoxb-8 gene, in combination with the overexpression of IL-3, resulted in a mouse myeloid leukemia (7). The LTR Hoxb-8 construct alone was able to transform NIH3T3 cells (8–10).

These reports suggest that deregulated homeobox gene expression leads to aberrant cell proliferation. This identifies the homebox gene as a new type of nuclear protooncogene. A second role of homeobox genes, in addition to the control of embryogenesis and differentiation, may thus be the control of cell proliferation. All homeobox genes carry the 183-base pair homeobox, which encodes a trihelical motif of 61 amino acids. Homeobox proteins were shown to bind DNA via this motif. They are thought to directly interact with transcription factors to regulate transcription from target promoters (11). The control of cell proliferation by homebox-containing genes may thus be executed by transcriptional regulation of growth control genes.

Most of the more than 40 murine homeobox genes identified are related to the Drosophila Antp gene (12). Fig. 1 shows the four clusters to which many of these genes belong. Some members of these clusters might play a role in the hematopoietic system, and in some cases, they have been linked to aberrant growth in various leukemias (5). Apart from the transformation assays with Hoxb-8 and IL-3, no molecular study has thus far systematically tested the oncogenic potential of homeobox genes, as suggested by the correlation of their expression with certain leukemias. To determine whether overexpressed homeobox genes can, in general, act as oncogenes, we decided to test members of the Hox family (Hoxa-7, Hoxa-5, Hoxa-1, Hoxb-7, Hoxc-8) (13, 14) as well as Evx-1 and Cdx-1 (15, 16) homeobox genes for their transforming potential using in vitro and in vivo transformation assays.

Results
Experimental Strategy and Transfection Assays
A number of murine homeobox genes have been isolated and shown to be differentially expressed throughout embryonic development (see Ref. 13 for a review). The homeobox genes used were chosen such that they represent members of different Hox clusters (Fig. 1). Hoxa-7, Hoxa-5, and Hoxa-1 are members of the Hoxa cluster located on chromosome 6 of the mouse (Fig. 1); Hoxb-7 is a member of the Hoxb cluster located on chromosome 6 of the mouse.
11; and Hoxc-8 is a member of the Hoxc cluster located on chromosome 15. Evx-1 is also located on chromosome 6 (16) but is not closely related to genes of the Hoxa cluster. Hoxa-1 represents the most anterior gene of the clusters tested, whereas Hoxc-8 represents the most posterior Hox gene tested. In addition, Hoxa-1 belongs to a different subfamily of Hox genes (labial), whereas the paralogous genes Hoxa-7, Hoxb-7, and the Hoxc-8 gene are genes of the Antp/UbxB subfamily. Hoxa-5 lies between these two gene families and belongs to the Scr subfamily (17). Evx-1 and Cdx-1 genes were chosen since they do not contain the conserved hexapeptide that is found in all homeobox genes in anterior and medium positions of the cluster; additionally, they represent homeobox genes only distantly related to the Antp prototype gene (13).

The cDNAs for Hoxa-7, Hoxa-5, Hoxa-1, Hoxb-7, Hoxc-8, and Evx-1 were inserted into the pCMV5 expression plasmid (Fig. 2). pCMV5 contains the human cytomegalovirus promoter/enhancer, a polylinker into which the cDNAs were inserted, the human growth hormone transcript polyadenylation and termination signals, as well as the SV40 origin of replication. pCMV5 was a gift from M. Stinski and is based on the pCMV expression plasmid series (18). The pCMV Cdx-1 expression plasmid was a gift from B. Meyer. Fig. 2 describes the experimental scheme to select and test for cell clones that overexpress the homeobox genes to be assayed. The pCMV homeobox gene expression constructs were cotransfected with pGKneo (19) into nontransfected mouse NIH3T3 cells or 208-F cells (Fig. 2). 208 cells are a subclone of Rat-1 cells that have been isolated for their flat growth (provided by R. Müller, Marburg, Germany). From each transfection, two-thirds of the cells were used for in vitro transformation assays (focus assay and soft agar assay). One-third of the cells were exposed to G418, and resistant clones and cell pools were isolated (Fig. 2), tested for gene expression and for in vitro transformation characteristics, and subsequently used "in vivo" in the tumorigenicity assay.

Expression Analysis

The G418-resistant cell colonies were expanded and tested for homeobox gene expression in gel retardation assays. In general, homeodomain proteins are thought to bind to DNA as monomers, which may explain their relatively low binding affinities (20). We preferred gel retardation analysis of the extracts from isolated cell clones to standard mRNA expression studies, because the gel retardation assays test directly whether a functional homeobox protein is overexpressed in these cells. Hoxa-7, Hoxa-5, Hoxa-1, Hoxb-7, Hoxc-8, and pCMV (vector only) transfectant cell extracts were analyzed, using an end-labeled oligonucleotide containing a trimer of the Antennapedia binding site B52 (Ref. 21; with modifications by M. Gross). Each homeobox gene transfectant extract formed a new DNA-protein complex of a specific mobility, not seen in extracts from cell clones that only contained the pCMV5 and the pGKneo plasmids (Fig. 3A). Extracts from SV40 T-antigen-transfected NIH3T3 cells and 208 cells did not form specific complexes with the 3 Antp oligonucleotide (data not shown). This is an indication that the specific complexes observed are not due to secondary proteins induced by neoplastic transformation, but rather represent Hox protein binding. The 3 Antp oligomer is able to bind more than one of the produced Hox proteins, visible in Fig. 3A as additional, more slowly migrating complexes.

The Drosophila eve gene product binds sequences (e5) both in its own promoter as well as in the promoter of the engrailed gene (22). In vitro translated mouse Evx-1 protein also recognizes the consensus eve binding site

* M. Gross, in preparation.
Neo-expression Plasmid

Fig. 2. Experimental scheme for the transformation assay. Homeobox protein cDNAs were inserted into the multiple cloning site of pCMV5 (18). Plasmid expression plasmid. Each of these constructs, together with a neomycin expression plasmid, were transfected into recipient NIH3T3 and 208 cells. Cells were seeded into three 100-mm plates 24 h after the glycerol shock. One-third of the transfection was incubated until foci of transformed cells appeared. Cells were then fixed and stained with methylene blue to facilitate the counting and scoring of foci. One-third of the cells were seeded into 0.3% agar to assay for anchorage-independent growth. Cell colonies were counted 2 to 4 weeks after incubation. One-third of the cells were seeded into soft agar to test for anchorage-dependent growth properties. The last third of the cells were selected for DNA uptake by the addition of G418 (GIBCO) 24 h after shock of the cells. Morphologically transformed foci were picked and propagated thereafter. In some cases, pools of G418-resistant cell clones were used as well. These cell isolates were expanded by continuous incubation in the selection medium and used for expression analysis and transformation assays.

e5, suggesting that the binding is mediated through the homeodomain, the only region conserved between the two proteins. Evx-1 expression was assayed by the gel retardation of the e5-oligomer by Evx-1 transfectant extracts. In vitro translated Evx-1 protein produced a shifted complex of the same mobility as specific complexes formed with cell extracts from both NIH3T3 and 208 Evx-1 transfectants (Fig. 3B).

The Cdx-1 transfectants were analyzed by Western blot analysis (Fig. 3C), since the DNA target of the Cdx-1 protein is not known, but an antibody is available (23).

Transfectants which carried the respective pCMV homeobox gene expression construct and expressed a functional protein were expanded and used in three types of transformation assays: focus assay, soft agar assay, and tumorigenicity assay (Fig. 2).

Transformation Assays

Focus Assay. Two-thirds of the cells of each transfection assay were not exposed to G418. One-third of each transfection assay was maintained as a monolayer of cells in order to monitor for cells that had lost contact inhibition. These cells gave rise to foci, detectable after staining with methylene blue (Fig. 2). Transfected cells that constituted a focus were able to overcome contact inhibition and overgrow the surrounding monolayer of cells that had not taken up the expression construct and pGKneo (Fig. 4). This outgrowth or focus formation is a property of a transformed cell. The cell lines used vary in their permissivity for outgrowth. The NIH3T3 cells are susceptible to transformation and are capable of low levels of spontaneous focus formation (24). The 208 cells show a flatter growth pattern, do not spontaneously form foci, and are less likely to form foci even upon transfection of an oncogene-expressing construct.5 Fig. 4 shows foci obtained after transfection of NIH3T3 cells with the Hoxa-7 CMV expression construct (Fig. 4A) and 208 cells (Fig. 4B). Fewer foci were obtained in 208 cells in repeated transfections when compared to NIH3T3 cells, and the foci were smaller and appeared 2 to 5 days later than NIH3T3 cell foci (Table 1; Fig. 5). The "pretransformed" status of NIH3T3 cells means that a single additional oncogene will strongly transform the recipient cells. Pretransformation is thought to be induced by prior activation of an endogenous protooncogene or deactivation of an antioncogene (24). 208 cells more closely resemble untransformed cells and may provide a more stringent test for the transforming capacity of the potential oncogene.5 By analogy to the multiple steps in malignancy that lead to tumor formation, NIH3T3 cells would represent the initiated cells, whereas 208 cells would represent more normal cells. In agreement with this hypothesis, 208 cells exhibited slower growth in the focus assay.

The in vitro transformation assays were repeated with isolated homeobox gene-expressing cell clones. Fig. 4C shows Hoxa-7-transformed 208 cells expanded from an isolated focus. The cells acquired the ability to overgrow the cell monolayer, and they exhibited a more rounded and diverse morphology. The transformed 208 cell morphology differs considerably from untransformed 208 cells shown in Fig. 4D. Fig. 4E shows a monolayer of Hoxa-7-transformed 208 cells grown from an isolated

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5 R. Müller, personal communication.
focus, indicating that not only the individual cell morphology, but also the overall appearance of the cell "monolayer," had changed. Clumps of cells, as well as large, often multinucleated cells, can be observed. Fig. 4f shows that the Hoxa-7-transformed 208 cells are able to grow suspended in semisolid medium.

All homeobox-containing genes tested (Hoxa-7, Hoxa-5, Hoxa-1, Hoxb-7, Hoxc-8, Evx-1, and Cdx-1) were able to induce focus formation in both NIH3T3 cells and 208 cells (Table 1; Fig. 5). Transfections with either the pCMV expression plasmid alone, a zinc finger protein-CMV expression construct pCMV Zfp-2 (Ref. 25; and data not shown), or Oct protein expression constructs [pCMV Oct-2, pCMV Oct-4 (26)] did not result in focus formation in 208 cells. Foci formed in NIH3T3 cells did not exceed background frequencies seen in untransformed NIH3T3 cells. An SV40 large T-antigen expression construct, which served as a positive control in the transformation assays, induced foci 3 to 7 days faster than any of the homeobox gene constructs.

We also picked two independent foci of both Hoxa-7-transfected 208 and NIH3T3 cells that were not selected with G418, expanded them, and tested for homeobox gene expression and in vitro transformation abili-
Fig. 2. Morphology and growth pattern of cells transformed with homeobox proteins. Only Hoxa-7-transfected cells are shown, as morphological and growth pattern changes observed were similar for all of the homeobox gene-transfected cells. A, focus obtained upon transfection of NIH3T3 cells with the pCMV Hoxa-7 expression plasmid. The cells lost the contact inhibition and had a rounded appearance characteristic of the transformed phenotype. B, the foci of 208 cells were smaller, but the Hoxa-7-transformed cells were still able to overgrow. Noteworthy is the flatter appearance of the 208 cell monolayer when compared to the NIH3T3 monolayer in A, C, and D. Phase contrast photomicrographs of the Hoxa-7-transformed 208 cells (C) and untransformed 208 cells (D). The disordered multilayer growth (C) is typical of malignant cells. At this magnification, the cross-over growth and the more rounded cell morphology can be seen clearly (C) when compared to the flat and regular morphology of the untransformed 208 cells in D. E, this photograph shows the cell layer of the cells seen in C. The cells tend to aggregate and form three-dimensional bodies, whereas other cells remain large and flat and are often multinucleated. F, Hoxa-7-transformed 208 cell colony growing suspended in 0.6% soft agar. Cells that are not transformed cannot grow in suspension and can be seen as small cell clumps next to the large colony of transformed cells.
Table 1  Transformation of 208 and NIH 3T3 cells by homeobox expression plasmids

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>Soft agar assay (No. of colonies/150 ng DNA)</th>
<th>Focus assay (No. of colonies/100 ng DNA)</th>
<th>Tumorigenicity (No. of tumors in nude mice/ No. of injections): 208</th>
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<tbody>
<tr>
<td></td>
<td>0.6%</td>
<td>0.9%</td>
<td>1.2%</td>
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<tr>
<td>pCMV</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>pSV</td>
<td>&gt;100</td>
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<tr>
<td>Hoxa-7</td>
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<td>Hoxa-5</td>
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<td>Hoxb-1</td>
<td>&gt;100</td>
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<td>Hoxb-7</td>
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<td>&gt;100</td>
<td>&gt;100</td>
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<td>Hoxc-8</td>
<td>&gt;100</td>
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<td>Cdx-1</td>
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At left are listed the DNAs that were introduced into the cells to give rise to the cell lines tested in the transformation experiment. pCMV, cells which harbor only the pCMV construct as the negative control; pSV, the T-antigen expression construct used as a positive control. The various pCMV homeobox expression constructs are listed by the name of the homeobox protein they express.

Growth of the cells was tested in 0.3%, 0.6%, 0.9%, and 1.2% soft agar. Cell colonies were counted 2 to 3 weeks after plating. At 0.3% soft agar, even the pCMV-harboring cells were able to grow; therefore, the results for this soft agar concentration are not listed. The experiments were performed at least twice for each cell type. The first column lists the average number of colonies observed in two 208 cell transfections. The second column lists the average observed in two NIH3T3 cell transfections, each representing approximately 1 x 10^6 cells transfected with 150 ng expression plasmid.

The last column gives the number of colonies obtained from newly transfected cells. The number is an average of two transfections, each representing approximately 1 x 10^6 cells transfected with 300 ng DNA/transfection. Qualitatively, these numbers agree with focus induction upon mixing of normal cells with transformed cells.

The last column shows the number of injections that resulted in tumor formation per number of nude mice given injections. Tumors appeared as early as 2 weeks after injection for T-antigen-transformed 208 cells and as late as 9.5 weeks after injection for Hoxa-5-transformed cells.

As described above, expression levels and transformed cell properties were similar to those observed in the expanded G418 colonies (Fig. 3; and data not shown), excluding unspecific effects induced by the selection procedure.

**Anchorage-independent Growth.** The observations of the focus assay were supported when transfected cell growth was monitored in a suspension of semisolid medium. The ability to grow in high concentrations of soft agar is correlated to the lesser or greater degree of transformation, manifested in the ability to form tumors and metastasize in vivo (27). In general, transformed 208 cells grew more slowly and formed smaller cell colonies in suspension than did transformed NIH3T3 cells (Table 1). Homeobox protein-expressing cells were able to grow in high concentrations of 0.6 to 1.2% soft agar, as were cells transformed by the SV40 T-antigen. The Hoxa-5 gene transfecants were unable to grow in 1.2% soft agar, an indication of a weaker transformed phenotype. Control cells transfected with the pCMV expression plasmid or Zip-2, Oct-2, or Oct-4 expression constructs were unable to grow in agar concentrations higher then 0.3%. Growth in 0.3% agar was observed for both the untransformed NIH3T3 and, a few times, for 208 cells. The soft agar experiment was repeated twice for every transfected cell type, both after transfection and with G418-selected isolated cell clones (Fig. 2). Table 1 presents the data for both NIH3T3 and 208 cells transfected with the respective homeobox gene. When compared to T-antigen-transformed NIH3T3 and 208 cells, homeobox protein-transformed cell colonies were fewer (between one-half and one-quarter, depending upon different experiments and different genes) and grew more slowly. This indicates that the transforming potential of the viral oncogene is greater than that of the homeobox gene, provided that both proteins were produced at similar levels.

**Tumorigenicity Assays.** Because the oncogenic capacity of the homeobox genes appeared to be strong enough to transform the more resistant 208 cells in two types of in vitro assays, we decided to test only the 208 cells in in vivo transformation assays.

Athymic 4-week-old male nude mice (NMRI) were given injections of 1 to 5 x 10^5 cells administered s.c. in the flank. Two mice received injections in parallel of the same transformed cell type at one time. Three such independent injections were performed. The animals receiving injections were observed weekly for tumor formation. The animals were sacrificed once the tumor had grown to a 0.5- to 1-cm maximal diameter. Animals that were tumor free at the end of 3 months were scored as negative for tumor formation.

Tumors were observed as early as 12 days postinjection for Hoxa-1-expressing cells. Mice that were given injections of Hoxa-7, Hoxa-5, Hoxa-1, Hoxb-7, Hoxc-8, Evx-1, and Cdx-1-expressing cells grew tumors close to the site of injection. In two of six injections of the Hoxa-5-transformed cells, no tumors grew, which is consistent with the results of the soft agar assay indicating that this homeobox gene has a weaker transforming potential.

**Tumor Morphology**

Histological sections from the tumors were prepared. Generally, Hox-induced tumors were classified as poorly differentiated spindle cell fibrosarcomas. The tumors had cell-rich centers, sometimes with necrotic regions, interspersed with fat and smooth muscle tissue from their hosts. Infiltration into nerves, blood vessels, and adipose tissues was observed in some tumors (Fig. 6, Hoxa-7 (A), Hoxc-8, Hoxc-1). Systematic studies to compare the appearance or absence of infiltration in the various Hox tumors are currently being pursued. The histological appearance of the homeobox gene-induced tumor was distinct from the T-antigen-induced tumor, which was a round cell sarcoma. All homeobox protein-induced tumors were well vascularized, with extensive capillary proliferation from within (Fig. 6). The Hoxa-5 tumors were smaller and not as solid, and they required 2
Fig. 5. Focus assay for the ability to overcome contact inhibition. This assay was performed twice in nonselected cells following transfection, as shown in this figure, and twice with selected cell clones pooled and mixed at a ratio of 1:1000 with nontransformed cells (data not shown). Cells that have taken up the transforming DNA are able to overgrow nontransformed cells, forming multilayered cell mounts which are visible as dark-staining foci of cells. Depending upon transfection efficiencies, the number of foci obtained varied from plate to plate. The average number of foci in two transfections is shown in Table 1. At bottom, control cells which harbor only the pCMV expression vector construct are shown. No foci are visible in the 208 cells (right), whereas the NIH3T3 cells (left) gave a significant background of spontaneously transformed cell foci. SV40 T-antigen-transformed cells exhibited strong focus formation, the onset of which occurred a few days earlier than for the homeobox protein-transformed cells.

additional weeks to display visible growth. In contrast to the growth observed from the injection of $1 \times 10^7$ 208 cells that contained only the CMV vector, the Hoxa-5 tumor had a more reddish color, displayed small blood islands indicative of some vascularization, and was more solid; for that reason, it was scored as a tumor. The capacity to actively recruit blood vessels enables the transformed cells to expand and to form solid tumors. Without this blood supply, tumors are limited in their growth by the diffusion of oxygen and nutrients to the cells, as seen in microtumors induced by injection of $1 \times 10^7$ of the control 208 CMV cells (Fig. 6). In this case, the microtumor is merely a small, pale body of cells that is not vascularized and thus unable to expand. After prolonged growth (longer than 3 months), these cells did not grow further, suggesting that the potential tumor cells are probably at a semistable equilibrium, in which cell growth will occur only upon cell death, and further outgrowth is restricted. A secondary transforming event, as often observed in progressing malignancies, is needed to activate angiogenesis and enable the outgrowth of the tumor. It has been reported that, upon vascularization, a
tumor can expand to many times its original size within 2 weeks (28). We observed that 2 weeks after the first tumor growth was detected, growth was extremely rapid, with a doubling of tumor size within 2 to 4 days.

We did not observe metastasis. In theory, blood vessels provide a route for tumor cells to travel and colonize. Metastasis requires that these cells migrate, which is one of the characteristics that tumor cells and embryonic cells seem to share. We did not observe tumor formation removed from the site of injection, although some of the histological tumor sections indicated that the homeobox-transformed cells were infiltrating striated muscle and adipose tissues, as well as nerves and blood vessel walls (Fig. 6). A longer incubation within the animals might enable metastasis to occur, considering that the in vitro growth properties strongly suggest that cell transformation has progressed far enough to cause full malignancy.

**Discussion**

This study has shown that various homebox-containing genes are able to fully transform recipient fibroblast cells. Homebox genes localized at the anterior end of the cluster belonging to the labial subfamily of homeobox genes (Hoxa-1), genes belonging to the Antp/Ubx homeobox subfamilies (Hoxa-7, Hoxa-5) within one cluster (Hoxa), homeobox genes in different clusters (Hoxc-8) and of the same paralogue group (Hoxb-7) (17) were capable of transformation, in both in vitro and in vivo transformation assays (Fig. 1). All of these genes contain the conserved hexapeptide sequence element, whereas the homeobox-containing genes Evx-1 and Cdx-1 do not, but they are equally competent in transforming recipient fibroblast cells. Thus, the hexapeptide element is not required for transformation.

This study supports the idea that the homeobox genes are a new family of transcriptional activator-type pro-
to oncogenes. We also tested other developmental control genes that are thought to act as transcriptional activators for their transforming potential. Proteins that bind DNA via a different DNA-binding motif, such as the zinc finger protein Zif-2 (25), or several octamer proteins that contain a homeobox subdomain (29), were tested in the in vitro transformation assays. Neither were positive in these assays, which is consistent with the notion that all homeobox-containing genes have transforming potential and the notion that all putative developmental control genes that can act as transcription factors will also be capable of transformation. Some of these genes provide housekeeping functions and are expressed ubiquitously (Oct-1), whereas others may activate genes that are not involved in proliferation, in which case an overexpression will not necessarily lead to transformation.

A number of homeobox genes were shown to cause leukemias upon deregulation (5). HOX-11 is overexpressed, and PBX-1 is an activated hybrid protein. Both are found in acute leukemias and are nonclustered homeobox genes like Cdx-1, which was used in this study. The up-regulation of the Hoxb-8 gene through an adjacent viral LTR isolated from a mouse myeloid leukemia cell line provided the first step in transforming NIH3T3 cells, although another transforming event was necessary to obtain full metastasis. These were the first findings and detailed studies suggesting that a deregulated homeobox gene could lead to neoplastic transformation. Hoxb-8 is thought to maintain the ability to self-renew or to directly or indirectly impede terminal differentiation in hematopoietic cells. This function would be shared with nuclear oncogenes such as myc or myb (1). As in the case of these nuclear oncogenes, which require the ras oncogene for full transformation, Hoxb-8 needs IL-3 or the pretransformed NIH3T3 cells to allow full transformation (7, 10).

It seems likely that the deregulation of a group of homeobox genes is the molecular basis of many other leukemias. Interestingly, most cases of leukemia in which a homeobox gene is thought to be involved are acute leukemias that occur early in childhood. Chronic leukemias that appear mostly in older patients are thought to be caused by nonnuclear oncogenes, such as c-ABL. The deregulation of developmental control genes may preferentially result in childhood malignancies. This notion is supported by an oncogene, Pem, first isolated from a T-lymphoma and then found to contain a homeobox. It is designated as oncelfetal, because its expression is temporally and spatially restricted in embryogenesis and is otherwise confined to immortalized and malignant cell lines (30).

The term "oncelfetal" emphasizes the close relationship of tumorigenesis and embryogenesis. Common tumor and embryonic cell characteristics, such as the capacities for cell migration, invasion, and enhanced mitotic rates support this notion. Gene products that normally regulate the proliferation, mitotic arrest, and differentiation of cells throughout embryonic development may well, upon altered regulation, induce processes leading to tumorigenesis.

This study has shown that the overexpression of homeobox genes results in many cases in transformation under controlled experimental conditions. Our results support earlier observations and confirm that the up-regulation of a homeobox gene may indeed be the defect induced by chromosomal translocations in certain leukemias. We demonstrated, however, that only a subgroup of homeobox-containing genes carry the transforming potential, whereas others, such as the octamer genes, do not. It should prove useful to screen leukemias that involve chromosomal translocations for the transcriptional activation of this class of homeobox-containing genes.

We propose that in embryogenesis the down-regulation of a homeobox gene arrests growth and leads to differentiation. This is supported by the correlation of in situ hybridization signals from homeobox gene expression in developing embryos with areas of strong mitosis (14, 31, 32). Hoxa-1 expression was identified in the adult intestine, testis, brain, kidney, and liver (33). Hoxa-5 expression was identified in the adult liver, kidney, ovary, testis, and spinal cord (34), whereas Hoxb-7 expression in the adult was restricted to kidney, testis, and spinal cord (35). It appears that the expression of homeobox-containing genes is often confined to proliferating cells in the adult organism, such as blood stem cells (expression in the adult spinal cord) or germ cells in adult ovary and testis. Homeobox genes are expressed widely throughout embryonic development, yet preferentially in restricted (mitotically active) tissues in the adult organism. Consequently, one function of the homeobox proteins appears to be the control of cell proliferation. This notion was suggested by earlier work with the homeobox gene Hoxb-8 (7, 9, 36) and is now strongly supported by the systematic transformation studies presented here for seven homeobox-containing genes. Alternatively, the down-regulation of the homeobox gene in different tissues should result in mitotic arrest. The link to mitosis suggests that mitotic regulator proteins are likely candidates for transcriptional trans-activation. This would relate the homeobox genes to other immediate early genes, such as c-rel, c-jun, or c-fos, that are thought to activate targets which in turn promote cell cycling upon mitogen stimulation (1). The identification of the targets of these homeobox genes and the molecular basis of that control may not only further our understanding of embryogenesis, but also of tumorigenesis.

Homeobox-containing genes appear to constitute a new family of nuclear oncogenes that, by altering transcriptional control of cell proliferation, may provide the molecular basis for tumor formation, especially in the hematopoietic system.

Materials and Methods

Cell Lines. The 208-F cells are a subclone of Rat-1 cells isolated by R. Müller, IMT, Marburg, Germany. 208 cells were maintained in Dulbecco's modified Eagle's medium (Biochrome) supplemented with 10% fetal calf serum (Boehringer Mannheim). NIH3T3 cells are a subclone originally isolated by F. Cuzin for their low frequency of spontaneous transformations. The NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum.

Molecular Clones. The cDNAs of the Hoxa-7, Hoxa-5, Hoxa-1, Hoxb-7, Hoxb-8, and Evi-1 genes were subcloned into the pCMV expression plasmid using standard cloning techniques. The Hoxb-7 cDNA was a gift from F. Meijlink. The pCMV Cdx-1 expression plasmid was constructed by B. Meyer. The pSV T-antigen plasmid was
obtained from M. Botchan. pGKn eo was described previously (19).

**DNA Transfection and Selection.** Two μg of the pCMV-homebox protein expression plasmid, together with 1 μg pGKn eo and 7 μg carrier DNA, were transected on a 70% confluent cell monolayer in a 100-mm tissue culture dish using the calcium phosphate method, with modifications (37). The transfected plate was split 1:3 after 24 h. One plate was split again and left for 2 to 4 weeks, depending upon the onset of focus formation. Thereafter, the cells were stained with a few drops of glutaraldehyde (Sigma) and stained with methylene blue (1% in water; Sigma). Finally, the tissue culture plates were rinsed with water, and the foci were counted.

One-third of the cells were seeded into 0.3%, 0.6%, 0.9%, or 1.2% soft agar as described (27). The last third of the cells were selected for DNA uptake by the addition of G418 (GIBCO) 24 h after shock of the cells. The 208 cells received 0.4 mg/ml and the NIH3T3 cells 0.6 mg/ml of G418. Pools of G418-resistant cells and isolated cell clones were expanded by continuous incubation in the selection medium and used for expression analysis and transformation assays. The cells were maintained at subconfluent densities to prevent spontaneous focus formation.

**Expression Analysis.** Total cell extracts were prepared, and the protein concentration was determined as described for the electrophoretic mobility shift assay (29). Reaction conditions were essentially as described for Hoxa-5 binding (34), with modifications. The binding reactions and gel electrophoresis were carried out at 4°C, as the homebox protein-DNA complexes seemed very labile at room temperature. Binding reactions contained 6 μg total cell extract, 25 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.6), 1 mm EDTA, 0.5 mm dithiothreitol, 10% glycerol, 50 mm NaCl, as well as 1 μg polydeoxyminosinic-deoxycytidylic acid. Reactions were incubated at 4°C for 60 min and resolved in the cold on an 8% polyacrylamide gel.

The in vitro transcription for Evx-1 was performed with 1 μg of linearized Evx-1 cDNA cloned into Bluescript (Stratagene) (16), 2 μl 5 mm capping reagent (Boehringer), 40 mm Tris-HCl (pH 8), 10 mm MgCl₂, 2 mm spermidine, 50 mm NaCl, 1 mm each ATP, CTP, GTP, and UTP, 10 mm dithiothreitol, 2 μl T3 polymerase (Boehringer), 1 μl RNasin (Boehringer) in 20 μl for 60 min at 37°C. Evx-1 protein was translated in vitro with a rabbit reticulocyte extract (BRL), using a mix of 10 μl extract, 3 μl BRL reaction buffer without methionine, 3 μl BRL reaction buffer without leucine, 0.65 μl potassium acetate (20 mm, pH 7.2), 0.5 μl magnesium acetate (20 mm, pH 7.2), and 1 μl of the transcription reaction in a volume of 30 μl. Reactions were incubated at 30°C for 60 min. Five μl of this reaction mix were used in the DNA binding reactions. Binding reactions were performed as described for octamer proteins (29).

For a Western blot analysis, 50 μg of cell extract were prepared as described (38). The extracts were separated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel that was subsequently transferred onto an Immobilion-P membrane by semidry electric transfer. The membrane was blocked in 5% dry milk powder-PBS, incubated overnight with a 1:200 dilution of the Cdx-1-peptide antibody, and developed with the peroxidase-diama-benzidine reaction.

**Tumorigenicity Assay.** The transformed cells were trypsinized and washed twice with PBS to exclude stimulating effects of the serum. Approximately 1 to 5 × 10⁶ cells were injected in a volume of 200 μl PBS s.c. into the flank of a 4-week-old athymic (nu)de male mouse. The animals were monitored twice weekly for tumor occurrence and size. At a tumor size of 1 cm, the animal was sacrificed, and the tumor was photographed. Cells of the tumor were explanted into tissue culture, expanded, and frozen. A part of the tumor was fixed in 4% paraformaldehyde and embedded in Paraplast following standard in situ hybridization protocols. Sections (8 μm) were cut and stained for histological analysis. Staining with hematoxylin and eosin was performed as described (39). Animals that showed no tumor growth after 3 months were sacrificed and recorded as tumor free.

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**References.**


