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HOXB7 Constitutively Activates Basic Fibroblast Growth Factor in Melanomas

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Homeobox (*HOX*) genes control axial specification during mammalian development and also regulate skin morphogenesis. Although selected *HOX* genes are variably expressed in leukemias and kidney and colon cancer cell lines, their relationship with the neoplastic phenotype remains unclear. In both normal development and neoplastic transformation, *HOX* target genes are largely unknown. We investigated the expression and function of *HOXB* cluster genes in human melanoma. The *HOXB7* gene was constitutively expressed in all 25 melanoma cell lines and analyzed under both normal and serum-starved conditions, as well as in in vivo primary and metastatic melanoma cells; conversely, *HOXB7* was expressed in proliferating but not quiescent normal melanocytes. Treatment of melanoma cell lines with antisense oligomers targeting *HOXB7* mRNA markedly inhibited cell proliferation and specifically abolished expression of basic fibroblast growth factor (bFGF) mRNA. Band shift and cotransfection experiments showed that HOXB7 directly transactivates the *bFGF* gene through one out of five putative homeodomain binding sites present in its promoter. These novel findings indicate a key role for constitutive *HOXB7* expression in melanoma cell proliferation via bFGF. The results also raise the possibility that growth factor genes are critical *HOX* target genes in other developmental and/or neoplastic cell systems.

The homeobox (HOX) is a 183-bp DNA sequence, originally discovered in *Drosophila melanogaster* and highly conserved during evolution from very simple organisms to vertebrates, including mice and humans (18, 43).

Homeoproteins are localized in the nucleus and represent one of the major classes of transcription factors that control cellular differentiation during development. The homeodomain is a four-α-helix helix-loop-helix DNA binding motif, and its flanking sequences provide either activating or repressing functions for target gene transcription (18, 43).

Homeogenes are organized in multigene clusters in all species analyzed. In the human genome, four clusters are present, named *A*, *B*, *C*, and *D* and mapped on chromosomes 7, 17, 12, and 2, respectively (54). Several studies of *HOX* genes during ontogenetic development have indicated that their expression is stage related and tissue or region specific and that their relative position along the chromosome is connected to their expression domains along the anterior-posterior axis of the central nervous system (18, 37, 43). Functional data have been derived from gain- or loss-of-function mutations in mouse embryos, indicating that *Hox* genes play a fundamental role in the determination of positional identity by embryonic precursor cells (4, 14, 48).

Recent data suggest a role for *Hox* genes in murine skin morphogenesis (41, 53). Analysis of cultured fetal melanocytes shows the expression of several genes of the *A* and *C* clusters (7, 53). Interestingly, the epidermal differentiation process seems to involve different growth factors, including retinoic acid (3), which is known to be a strong inducer of *HOX* genes in embryonic stem cells (43).

Current studies indicate an important role for HOX-containing genes in normal and neoplastic adult cells (11, 17, 23, 38, 47, 50). Selected *HOX* genes are expressed in early hematopoietic cells from bone marrow and peripheral blood (23, 38, 50), as well as activated NK (47) and T- and B-lymphocytic cells (11, 17). Moreover, *HOX* genes may be involved in different neoplastic processes, as suggested by their expression in several leukemic primary cells and in the cell lines of both lymphoid and myeloid origin (11, 17, 39, 47). Evidence for involvement of a homeogene in leukemia is represented by *Hoxb8*, which is constitutively activated in the mouse myeloid cell line WEHI-3B, because of the insertion of a retrovirus-like intracisternal A particle in its first exon (45, 46). Gene transfer of both *Hoxb8* and interleukin 3 into mouse bone marrow cells induces acute myeloid leukemia, inhibiting the normal differentiation program (8). The oncogenic potential of *Hoxb8* and other *Hox* genes, i.e., *Hoxa7*, *-a5*, *-a1*, *-b7*, and *-c8*, has been demonstrated in both in vitro and in vivo transformation assays (42). Several divergent *HOX* genes, such as *Pbx-1*, *tcl-3*, and *HB24*, are involved in some specific chromosomal translocations associated with different leukemias (15, 16, 27). Finally, several *HOX* genes are associated with various human carcinomas (12, 19). Although selected *HOX* genes are variably expressed in leukemias (38, 39, 47), and kidney (12) and colon (19) cancer cells and cell lines, their relationship with the neoplastic phenotype remains unclear. In both normal development and neoplasia, *HOX* target genes are largely unknown (20).

In the present study, we have investigated the expression pattern and the functional role of *HOXB* cluster genes in human normal and neoplastic melanocytic cells.

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MATERIALS AND METHODS

Cell cultures. All melanoma cell lines were cultured in RPMI 1640 medium supplemented with 5 or 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere until confluent. HeLa cells and NT2/D1 teratocarcinoma cells were kept in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Human embryonal melanocytes were purchased from Promo Cell (Heidelberg, Germany) or were obtained as cell lines from M. Herlyn (The Wistar Institute, Philadelphia, Pa.). Melanocytes were cultured in the melanocyte growth medium formulated and sold by Promo Cell.

Synthetic oligomers. (i) PCR amplification. Unmodified 18-mer deoxynucleotides were synthesized and purified by ethanol precipitation according to standard procedures.

(ii) Antisense oligomers. Phosphorothioate oligomers (NBI, Plymouth, Mass.), which are characterized by increased cell uptake and stability, were used for antisense inhibition experiments.

RT-PCR. Reverse transcription (RT)-PCR analysis was performed according to standard procedures. Briefly, total RNA was extracted by the standard guanidium thiocyanate-CsCl technique. Two micrograms of total RNA was reverse transcribed with oligo(dT) as the primer. After glyceroldehyde-3-phosphate dehydrogenase (GAPDH) normalization (20 PCR cycles), an aliquot of RT-RNA (corresponding to 0.02 to 0.05 μg of input RNA) was amplified. *HOX* RT-RNA amplification within the linear range was obtained by 30 PCR cycles (i.e., this cycle number allowed a linear RT-RNA dose response). PCR products were analyzed by Southern blotting with an internal oligomer as a probe. The sequences of the primers and probes, as well as PCR conditions, are reported in references 5 and 11.

RNase protection. RNase protection analysis was performed according to standard procedures (49). Hybridization was carried out overnight at 60°C, RNase digestion was carried out for 1 h at 37°C, and urea-acrylamide electrophoresis was run at 50°C. DNA fragments to be transcribed consisted of specific sequences 3′ to the box: *HOXB2*, a 198-bp *Sma*I-*Pst*I fragment; *HOXB3*, a 219-bp *Ava*I-*Ava*II fragment; *HOXB5*, a 240-bp *Hin*fI fragment; and *HOXB7*, a 139-bp *Sma*I-*Taq*I fragment. Basic fibroblast growth factor (bFGF) and GROα were a 280-bp *Hin*cII fragment and a 250-bp *Bam*HI fragment, respectively. The β-actin insert, a 93-bp *Rsa*I fragment, was always labeled in the presence of cold GTP to obtain a low specific activity.

In situ hybridization and immunohistochemistry. Specimens obtained from surgical biopsies were cryopreserved until use. Sections (6 μm thick) were mounted on gelatin-coated slides. In situ hybridization was performed as described previously (10), including hybridization at 50°C in 50% formamide, RNase treatment, and high-stringency washes at 65°C in formamide. Antisense and sense (control) riboprobes for *HOXB7* (139-bp *Sma*I-*Taq*I fragment), *GAPDH* (281-bp *Ava*I fragment), and *bFGF* (280-bp *Hin*cII fragment) were labeled by ^{35}S-UTP incorporation; 10^6 dpm was applied to each section. In all experiments, the sense probe was routinely included and gave no signal above background. Slides were counterstained with hematoxylin. Immunohistochemistry was carried out according to instructions for the Dako labeled streptavidin biotin peroxidase kit (Dako Corporation, Carpinteria, Calif.). Sections were incubated with 5 μg of HMB45 monoclonal antibody (Dakopatts) per ml in phosphate-buffered saline for 1 h.

Antisense experiments. Cells (5×10^3) of each sample were incubated in triplicate in 96-well plates in 0.2 ml of culture medium supplemented with 10% fetal bovine serum (heat inactivated) for 30 min at 65°C to destroy the nucleases. The cells were cultured in the presence or absence of antisense, sense, or randomly scrambled phosphorothioate oligomers. The sense and scrambled sequences used to demonstrate the absence of toxicity and the specificity of inhibition were first tested against sequences from the EMBL database. The antisense oligomers were complementary to the translation start site. Dose-response curves were determined with oligomers at concentrations of 15, 20, and 30 μM. Oligomers were added 24 h and 3 days after cell plating. Cells were tested for proliferation on day 5 and, in some experiments, on day 7 by addition of [^{3}H]thymidine to duplicate wells 8 h before the cells were harvested. Antisense inhibition was calculated relative to that of sense- and scrambled-oligomer-treated cells, whose inhibition never exceeded 15% of the proliferation rate shown by their untreated counterparts. Growth inhibition was calculated as $[1 - ([^3\text{H}]$thymidine incorporation of treated cells/$[^3\text{H}]$thymidine incorporation of control cells$)] \times 100$.

On the last day of culture, cells from the third well of each sample were lysed for total RNA extraction by a modification of the guanidium thiocyanate-CsCl gradient made in a microultracentrifuge (TL-100; Beckman Instruments, Inc., Palo Alto, Calif.) in the presence of rRNA as the carrier. *HOXB2* and *HOXB4* oligomer sequences are given in reference 11. The *HOXB7* antisense sequence was 5′ CGCATAATACACTGAACT 3′, and the *HOXB7* scrambled sequence was 5′ AGATCAACCTAGTAACTA 3′.

Electrophoretic mobility shift assay. Proteins were produced with a TnT in vitro transcription-translation kit (Promega, Madison, Wis.).

For the mobility shift binding assay, an end-labeled oligomer (2×10^4 cpm per sample [0.2 to 0.5 ng]) containing the homeodomain binding sequence (CCAG ATTAGCG) was bound to nuclear extracts obtained from different melanoma cell lines (i.e., A375, WM983A, WM35, and Me 665/1) and from the NT2/D1 teratocarcinoma cell line as the negative control. Binding reaction mixtures (containing 5 μg of nuclear proteins) were incubated for 90 min at 4°C in a reaction buffer containing 12% glycerol, 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.9]), 5 mM $MgCl_2$, 100 mM KCl, 1 mM EDTA, 4 mM Tris-HCl (pH 7.9), 1 mM dithiothreitol, 300 μg of bovine serum albumin per ml, and 1 μg of poly(dI-dC). Controls included competition with oligonucleotides (i) identical to the hot probe, (ii) mutated by two nucleotide substitutions within the binding site, and (iii) containing a totally unrelated sequence. The reactions were run through a 5% polyacrylamide gel which was then dried and autoradiographed overnight.

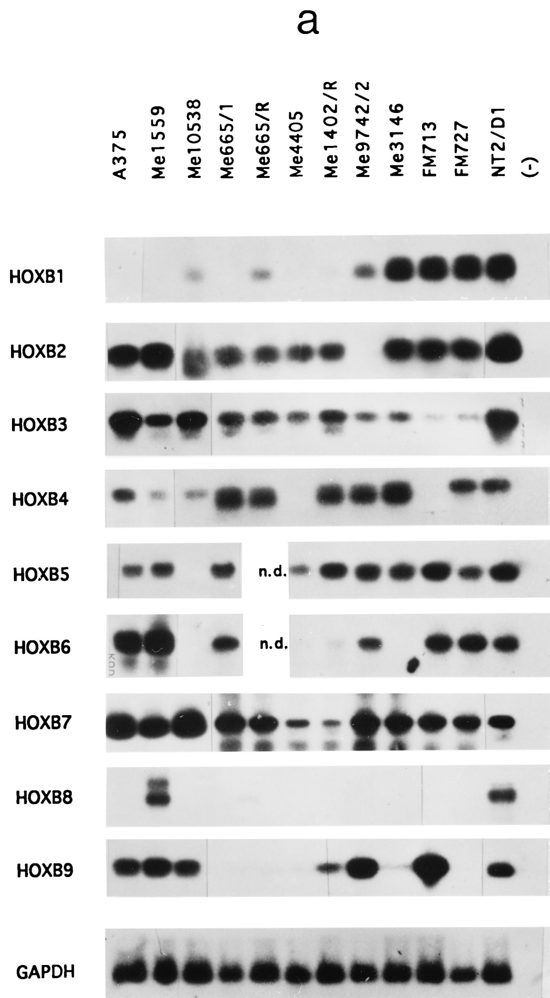
Transfections and CAT assays. For the transactivation experiments, five different bFGF promoter fragments (22) were used: (i) pbFGFproL-CAT (chloramphenicol acetyltransferase), consisting of the sequence from nucleotide −1001 to +314; (ii) pbFGFproS-CAT (−455 to +314); (iii) pbFGFproD-CAT (−1001 to −455 joined to +175 to +314); (iv) pbFGFproSS-CAT (+175 to +314); and (v) pbFGF proM-CAT, corresponding to the whole promoter (−1001 to +314) but containing two point mutations in the core ATTA sequence of the binding site around position +142 (ATTA to ATCT). All of these constructs, produced by PCR amplification, were sequenced and cloned in the pKT-CAT vector. Site-specific mutagenesis was carried out according to the in vitro mutagenesis system Altered Sites II (Promega). Ten micrograms of the bFGFpro-CAT reporter plasmids was transfected together with 8 μg of pSG5/HOXB7 or other pSG5/HOX gene expression vectors into HeLa cells by calcium phosphate precipitation. Two micrograms of pSVβgal plasmid (44) was cotransfected as an internal control. Cells were harvested 48 h after transfection. CAT activity, given as picograms per 100 μg of total protein, was quantitated with an enzyme-linked immunosorbent assay detection kit (Boehringer, Mannheim, Germany). Normalized amounts of CAT activity are given as fold increase of activation (mean of at least three separate experiments for each construct) over that of the promoterless pKT vector.

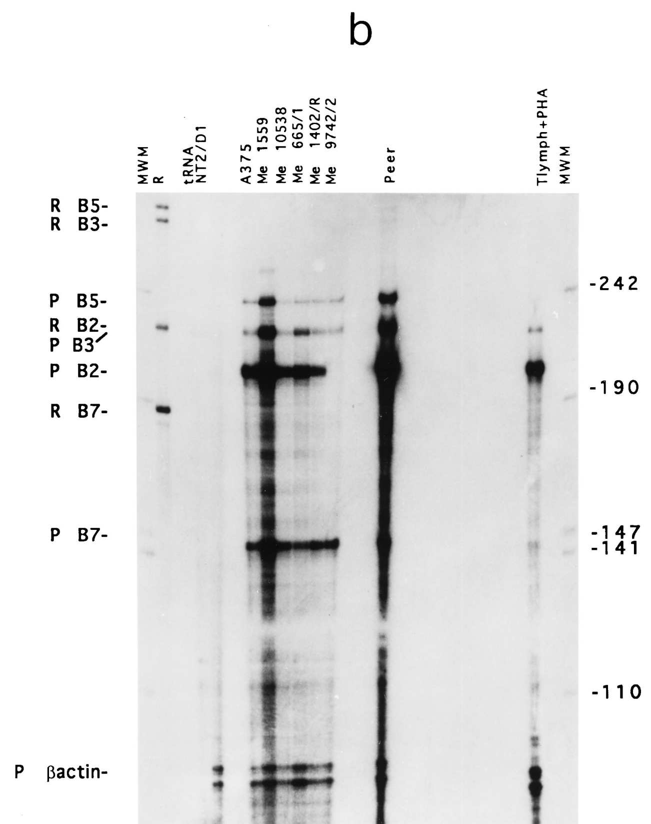
Analysis of DNA content by propidium iodide staining. Cells (5×10^5) were fixed with 2% paraformaldehyde (Sigma) (10 min on ice), washed twice in 50 mM Tris-buffered saline (pH 7.5), and then incubated for 30 min at 37°C in 0.4 ml of 0.1% saponin (Sigma) in Tris-buffered saline in the presence of 1 mg of RNase A (Sigma) per ml; 25 μg of propidium iodide (Sigma) per ml was then added, and incubation was continued for 20 min on ice in the dark. Samples were analyzed with a FACScan (Becton Dickinson) fluorescence-activated cell sorter (FACS).

RESULTS

***HOXB* gene expression in normal and neoplastic melanocyte cells.** We evaluated *HOXB* cluster gene expression in a panel of nine melanoma cell lines. As determined by RT-PCR (Fig. 1a) and confirmed by RNase protection for *HOXB2*, *-B3*, *-B5*, and *-B7* (Fig. 1b), the *HOXB* cluster showed a variable pattern of expression with different genes turned on or off in each sample. Interestingly, *HOXB7* was consistently expressed in all nine melanoma cell lines (Fig. 1a and b). In view of these observations, we examined 16 other melanoma cell lines derived from tumors at different stages of progression, including radial growth phase and vertical growth phase primary melanomas as well as subcutaneous and lymph-node metastases (Table 1). Although the level of expression varied as much as sixfold, *HOXB7* was the only *HOXB* gene expressed in all samples (Fig. 1c and data not shown). In order to demonstrate that *HOXB7* expression in melanoma cell lines was not a culture artifact, in situ hybridization analysis was performed. *HOXB7* expression was present in all melanoma samples from a total of five different patients, including a bone metastasis, confirming in vivo the results obtained in vitro. Representative results with a primary melanoma and its bone metastasis show *HOXB7* expression restricted to neoplastic cells and to some infiltrating lymphocytes (Fig. 2m to p).

To test whether *HOXB7* expression was associated with the melanocyte lineage, five melanocyte cell cultures were also analyzed. Results show consistent *HOXB7* expression in all cultures tested (Fig. 1a and c and data not shown). However, further studies indicated that *HOXB7* expression was differentially regulated in normal or neoplastic melanocytes. In normal melanocytes, *HOXB7* expression was linked to cell proliferation, whereas in melanomas, the expression was constitutive. For the analysis, melanoma cells were cultured in 0.1% serum for 24 to 48 h, inducing accumulation of cells in the G_0/G_1 phase as indicated by FACS analysis on propidium iodide-





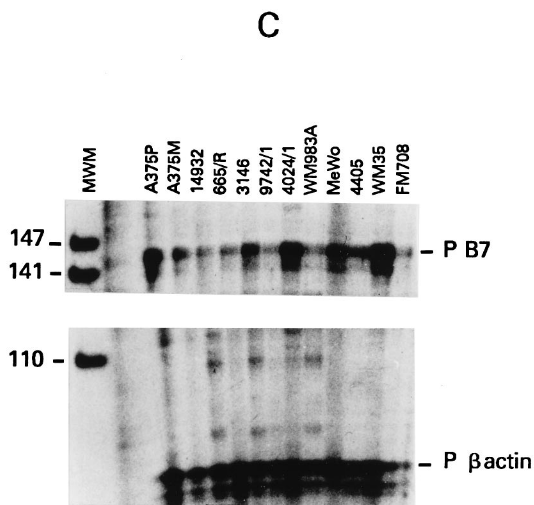


FIG. 1. Expression of *HOXB* cluster genes in melanocyte and melanoma cell lines. (a) A375 (36) and eight other melanomas of the Me series (2), as well as FM713 and FM727 melanocyte cell lines, were analyzed for *HOXB* gene expression by RT-PCR, as previously described (11). *HOXB* genes were amplified for 30 cycles, while GAPDH remained in the exponential phase at 20 cycles. NT2/D1 teratocarcinoma cells activated for 14 days with retinoic acid were the positive control. GAPDH expression is shown as a housekeeping gene internal control. (−) is the negative control, including all reaction reagents except RT-RNA. n.d., not done. (b) RNase protection analysis of the *HOXB2*, *HOXB3*, *HOXB5*, and *HOXB7* genes. Ten micrograms of total RNA from six melanoma cell lines was analyzed. β-Actin expression is shown as the internal control. The Peer T acute lymphocytic leukemia line and 48-h phytohemagglutinin-activated T lymphocytes (Tlymph + PHA) were the positive controls. Ten micrograms of tRNA and 10 μg of noninduced NT2/D1 teratocarcinoma cells were used as the negative control. Riboprobes (R) and protected fragments (P) are shown on the left, and molecular size (base pairs) markers (MWM) (pGEM4Z Hpa II) are shown on the right. (c) RNase protection analysis of *HOXB7* in 11 melanoma cultures and 1 melanocytic cell culture. Eight micrograms of total RNA was used. β-Actin was used to normalize; protected fragments (P) are shown on the right, and molecular size (base pairs) markers (MWM) are shown on the left.

stained cultures of the A375 cell line (Fig. 3a, right panel). This deprived culture condition gradually increased the number of cells accumulating in G_0/G_1 up to 75% after 48 h, with a parallel decrease in the number of cells in the S and G_2 plus M phases. RNase protection analysis performed with total RNA obtained from aliquots of the same starved cells (Fig. 3a, left panel) showed a constitutive expression of *HOXB7*. In contrast, the following results were obtained. (i) In cultured primary melanocytes that require multiple growth factors and a tumor promoter, such as tetradecanoyl phorbol acetate, for proliferation (29), *HOXB7* expression was abolished when cells shifted to basal medium without any supplement were growth arrested (Fig. 3b). (ii) *HOXB7*, as demonstrated by in situ hybridization experiments, was not expressed in quiescent melanocytes from four different normal skin samples (Fig. 2a to d). (iii) In compound nevi, melanocytes proliferate in the epidermal layer but are growth arrested when entering the underlying dermis, and epidermal but not dermal melanocytes show *HOXB7* expression by in situ hybridization and are stained by monoclonal antibody HMB45, which has been indicated to be associated with the melanocyte-specific silver protein involved in pigment synthesis, thus confirming the

TABLE 1. Melanocyte and melanoma cell lines analyzed in this study

Cell line	Type	Source or reference
FM707[a]	Melanocyte cell culture	M. Herlyn (28)
FM708	Melanocyte cell culture	M. Herlyn
FM713	Melanocyte cell culture	M. Herlyn
FM723	Melanocyte cell culture	M. Herlyn
FM727	Melanocyte cell culture	M. Herlyn
WM35	RGP[b] lesion	M. Herlyn
WM1552C	RGP and VGP[c]	M. Herlyn
WM3211	Primary tumor VGP	M. Herlyn
WM983A	Primary tumor VGP	M. Herlyn
MeWo	Lymph node metastasis	35
A375P	Metastatic melanoma	24
A375M	Melanoma variant metastatic in *nu/nu* mice	36
Me 665/1	Lymph node metastasis	2, 13
Me 665/2	Subcutaneous metastasis	2, 13
Me 665/R	Recurrence of subcutaneous metastasis	2, 13
Me 4405	Primary tumor VGP	13
Me 1402	Primary tumor VGP	13
Me 1402/R	Recurrence of primary tumor	13
Me 9742/1	Lymph node metastasis	13
Me 9742/2	Subcutaneous metastasis	13
Me 3146	Lymph node metastasis	13
Me 1007	Primary tumor VGP	13
Me 1340	Subcutaneous metastasis	13
Me 4024/1	Lymph node metastasis	13
Me 4024/2	Lymph node metastasis	13
Me 1559	Lymph node metastasis	13
Me 1811	Lymph node metastasis	13
Me 10221	Lymph node metastasis	13
Me 14932	Lymph node metastasis	13
Me 10538	Primary tumor VGP	13

[a] FM, melanocytes isolated from newborn foreskin.
[b] RGP, radial growth phase.
[c] VGP, vertical growth phase.

melanocytic origin of the hybridizing cells (1) (Fig. 2g to l [also described below]).

Functional role of the *HOXB7* gene: antisense oligonucleotides specific to the *HOXB7* gene, but not to the *HOXB2* and *HOXB4* genes, inhibit proliferation of melanoma cell lines. On the basis of our previous findings that both T lymphocytes stimulated by phytohemagglutinin and hematopoietic progenitors triggered by growth factors require expression of selected *HOXB* genes to proliferate (11, 23), we treated melanoma cell lines with different doses of phosphorothioate oligomers complementary to *HOXB2*, *-B4*, and *-B7* mRNA sequences, including the translation start sites, and analyzed their proliferation (representative results with the A375 cell line are shown in Fig. 4). Anti-*HOXB2* and anti-*HOXB4* oligomers, previously shown to block T-lymphocyte proliferation (11), did not change the proliferative rate of A375 (Fig. 4c), Me 665/1, and Me 4405 melanomas (not shown). In all of the antisense experiments, RT-PCR analysis was used to verify the abrogation of the specific transcripts (i.e., *HOXB2* and *HOXB4*) in the antisense-treated cells; the expected bands were present in the control and in the sense or scrambled-oligomer-treated groups. RT-RNA amounts were always normalized for the GAPDH expression level (Fig. 4b and d). In contrast, A375, Me 665/1, and Me 4405 melanoma cell lines treated with 20 μM *HOXB7* antisense oligomer showed 60 to 80% growth inhibition, as indicated by [^{3}H]thymidine uptake (results of a representative experiment with the A375 cell line are shown in Fig. 4a). A significant difference was shown between the proliferative rate of *HOXB7* scrambled-oligomer- and antisense-treated cells ($P < 0.001$; Student's *t* test). The specificity of this inhibitory effect was further ensured by the following series of controls. (i) The same antisense oligomer treatment did not inhibit the proliferation of the NT2/D1 teratocarcinoma cell line, which does not express *HOXB7* (not shown). (ii) RT-PCR analysis of *HOXB7* RNA from antisense or scrambled-oligomer-treated and untreated cells showed that the specific *HOXB7* 445-bp fragment (55) was detected in all but the antisense oligomer-treated cells (Fig. 4b). (iii) In all experimental groups, unrelated control mRNAs were not modified, including GAPDH and manganese superoxide dismutase (Fig. 4b).

The expression analysis of genes known to be involved in melanoma proliferation showed that the mRNA for *bFGF* was specifically abrogated in the *HOXB7* antisense-treated cells (Fig. 4b). These data indicated a coexpression of *HOXB7* and *bFGF* in melanoma cell lines: both genes were constitutively expressed under normal and serum-deprived culture conditions (Fig. 3a). This correlation was confirmed in normal melanocytes, since *HOXB7* and *bFGF* mRNAs were negative in quiescent cells from four different normal skin samples (Fig. 2a, c, and d) and were sharply downmodulated in primary melanocytes that were growth arrested in culture (Fig. 3b). Moreover, as demonstrated by in situ analysis, in compound nevi, proliferating epidermal melanocytes express both *HOXB7* and *bFGF* mRNAs while quiescent dermal melanocytes were negative for both genes (Fig. 2g to l show corresponding melanocytes positive for *HOXB7* and *bFGF*).

The *HOXB7* product binds to and transactivates the *bFGF* promoter. In a final series of studies, we investigated whether *HOXB7* directly activates the *bFGF* promoter. Analysis of the promoter region of *bFGF* for putative homeoprotein binding sites identified five homeodomains with at least 70% homology to canonical sequences (see Fig. 6a). The mobility shift assay revealed a protein-DNA specific complex between nuclear extracts from some melanoma cell lines (i.e., A375, Me 665/1, WM983A, and WM35) expressing the *HOXB7* gene and a DNA fragment encompassing nucleotides +130 to +159, but there was no protein-DNA complex formed with the other four putative HOX protein-binding sequences of the *bFGF* promoter (22) (Fig. 5 shows experiments with the A375 cell line), confirming the presence of a functional binding site for at least one nuclear protein in this promoter region. A comigrating band was also obtained when an in vitro-translated HOXB7 protein instead of melanoma nuclear extract was used (Fig. 5a, lane 3). Addition of unlabeled oligomer inhibited this interaction (Fig. 5a, lanes 2 and 4). The nearly complete abrogation of the DNA-protein binding was also obtained in similar experiments by labeling a mutated oligomer containing two base substitutions in the core ATTA sequence (A→G and T→C) (Fig. 5b). Furthermore, this interaction was not inhibited by unlabeled oligomer containing either a two-base substitution or totally unrelated sequences (Fig. 5c, lanes 4 and 5). The specific complex was not detected in nuclear extracts from a noninduced teratocarcinoma cell line, which does not express any of the *HOX* genes (Fig. 5a, lane 6), nor was it detected with the HOXB2 or -D3 in vitro-translated proteins (Fig. 5d).

Altogether, the results shown in Fig. 5 indicated a direct and selective interaction of the HOXB7 protein with the sequence encompassing nucleotides +130 to +159 of the *bFGF* promoter. Although the ATTA box is shared among several homeoproteins (33), unique flanking sequences may account for their high-affinity binding.

To further confirm an actual role for the HOXB7 protein in *bFGF* promoter activity, we cotransfected HeLa cells with the

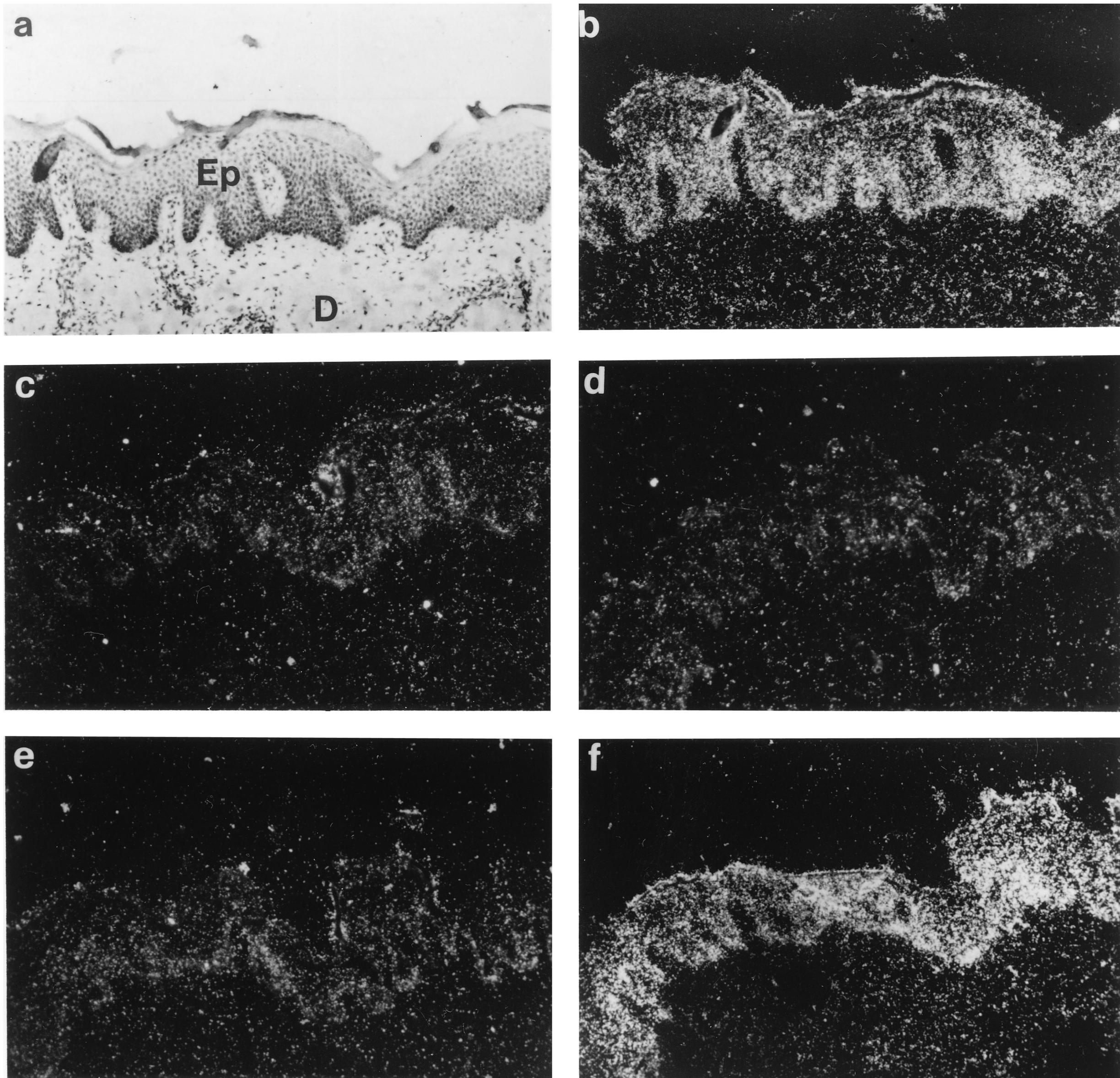


FIG. 2. Expression pattern analysis of the *HOXB7* gene in normal melanocytes of the skin, in compound nevi, and in melanomas as detected by in situ hybridization of sections from surgical biopsies (10). (a to f) Hybridization of normal skin. (a and b) GAPDH under bright-field (a) and dark-field (b) illumination; (c and d) *HOXB7* sense (c) and antisense (d) riboprobes under dark-field illumination; (e and f) bFGF sense (e) and antisense (f) riboprobes under dark-field illumination. GAPDH was used as a positive control. No signal was present on the adjacent section hybridized to the *HOXB7* antisense riboprobe. mRNA for the *bFGF* gene was detected on keratinocytes in the epidermal layer. Magnification, ×200. Ep, epidermis; D, dermis. (g to l) Compound nevus. (g) Immunostaining with HMB45 monoclonal antibody, which is associated with the melanocyte-specific silver protein (arrows); no signal was present on intradermal quiescent melanocytic cells (arrowheads). Biotin-streptavidin peroxidase staining was used. Magnification, ×200. (h) Bright field of an adjacent section. (i and l) *HOXB7* (i) and *bFGF* (l) in situ hybridization under dark-field illumination. Magnification, ×200. Note corresponding cells positive for *HMB45*, *HOXB7*, and bFGF (long arrows) in the junctional components of the nevus. Pl, pilus. (m to p) Melanomas. (m and n) Bright (m) and dark (n) fields of primary melanoma in vertical growth phase. Tumor cells infiltrating the dermal region show a higher level of *HOXB7* mRNA expression. Magnification, ×100. Me, melanoma cells. (o) Higher magnification of panel m (×500). The arrows show positive areas of melanotic and amelanotic tumor cells infiltrated with positive T lymphocytes (arrowheads). (p) Biopsy of a melanoma metastasis localized in the bone showing expression of *HOXB7*. Magnification, ×600. Is, intraneoplastic stroma.

pSG5/HOXB7 expression plasmid and the human *bFGF* promoter linked to a CAT reporter gene. Five different promoters containing or not containing the ATTA sequence around position +142 as well as its mutagenized variant were tested (Fig. 6a). They included a long *bFGF* promoter, proL-CAT (nucleotides −1001 to +314), and its shorter version, proS-CAT (nucleotides −455 to +314), since the region between nucleotides −854 and −521 seems to contain a negative regulatory domain in a human medulloblastoma cell line (22). The proD-CAT construct had a deletion encompassing nucleotides −455 to +175, thus lacking the putative homeodomain binding site around position +142; proSS-CAT conserved as little as the portion between nucleotides +175 and +314; and proM-CAT was identical to proL-CAT, except for the site-directed mutagenesis in the ATTA around position +142 (ATCT). Cotransfection of 10 μg of bFGF-CAT proL and proS plasmids in

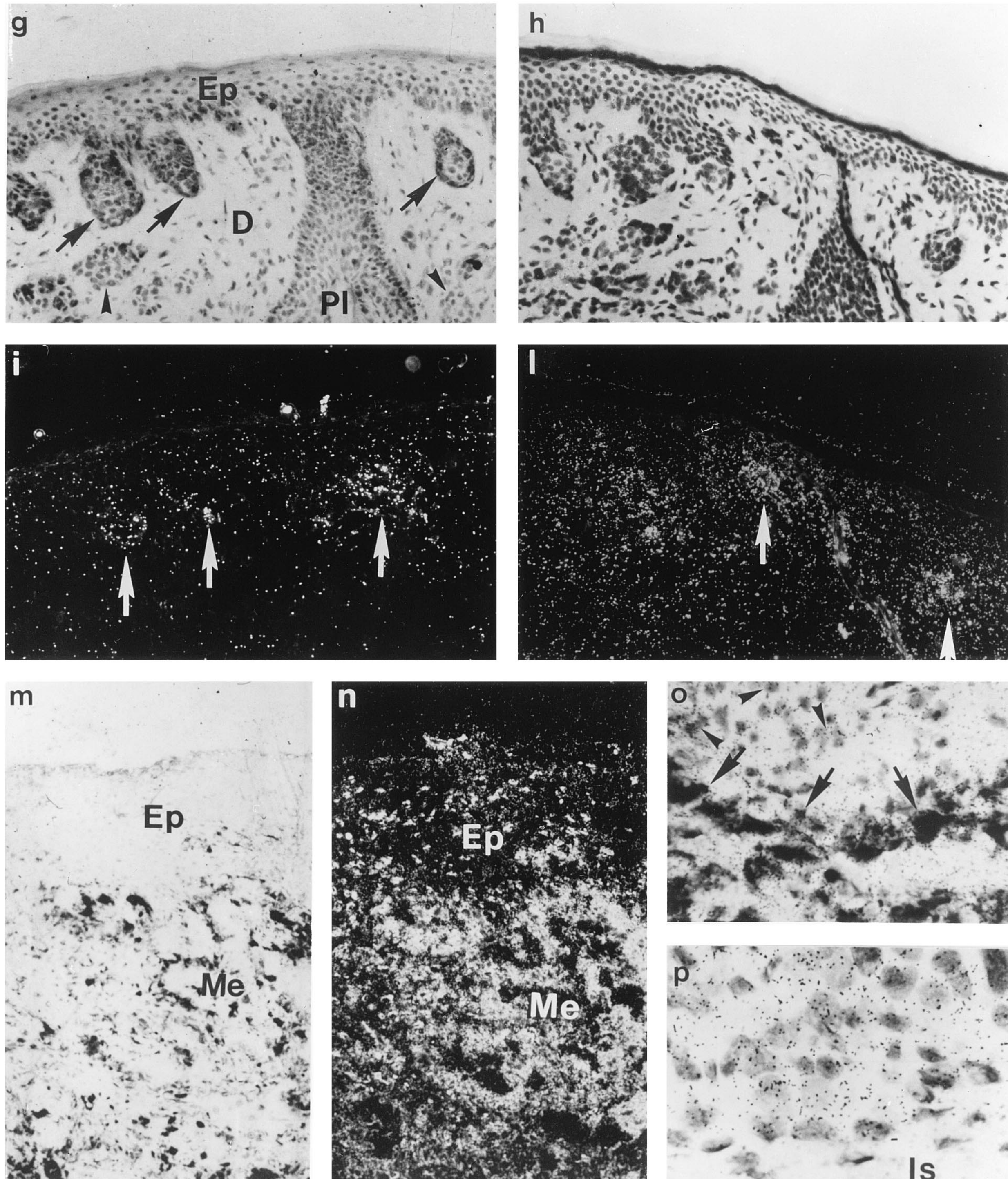


FIG. 2—*Continued.*

the presence of pSG5/HOXB7 increased CAT activity 40- and 30-fold over that of the promoterless pKT vector, respectively, while the proD, proSS, and proM constructs, which lacked the +142 binding site, did not increase CAT activity (Fig. 6a). Moreover, CAT activity dose-response curves were obtained by transfection of increasing amounts of the *HOXB7* construct in the presence of proL and proS constructs (not shown). As controls, pSG5/B3 and pSG5/D3 constructs were transiently transfected in HeLa cells together with the *bFGF* promoter fragment linked to a CAT reporter gene. No significant increase in CAT activity was observed in either case (Fig. 6b).

DISCUSSION

Homeoproteins represent transactivating factors that regulate gene expression (18, 43). The homeodomain can bind to

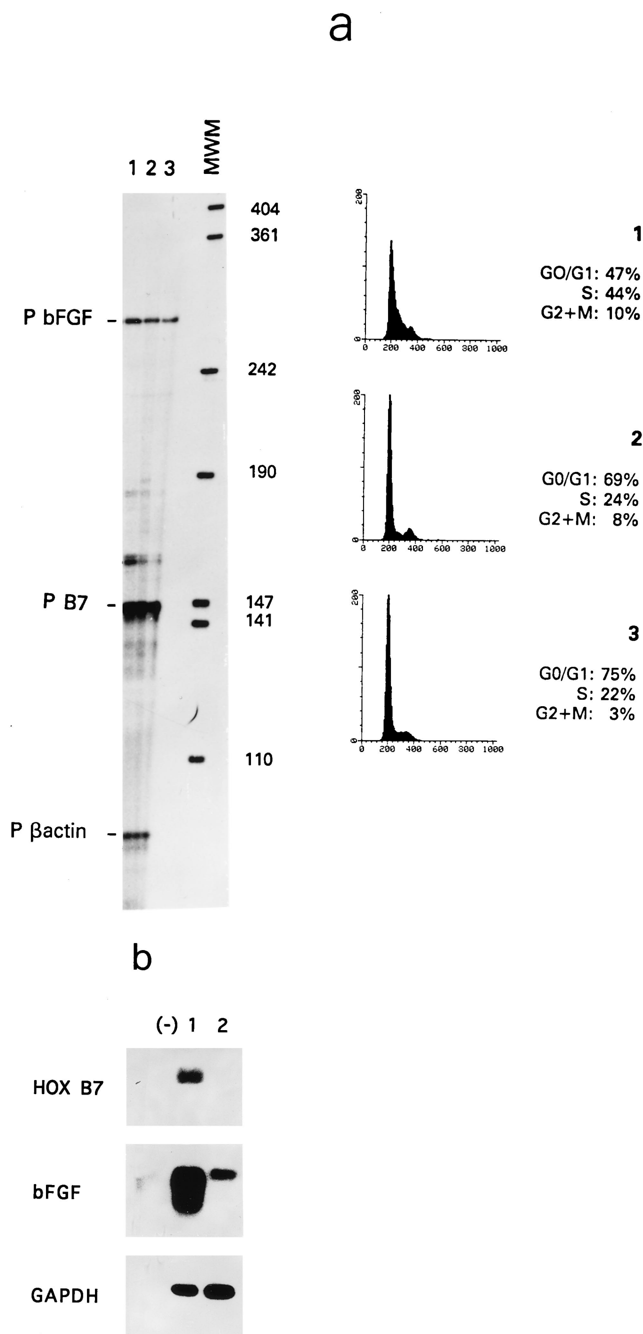


FIG. 3. *HOXB7* and *bFGF* expression in melanoma cells starved by low-concentration serum. (a) (left) RNase protection (P) analysis of the A375 melanoma cell line under standard (10% serum, lane 1) or starved (0.1% serum) growth culture conditions for 24 h (lane 2) and 48 h (lane 3). (Right) FACS profile of DNA content of the same cells used for RNA extractions. MWM, molecular size (base pairs) markers. (b) RT-PCR analysis of *HOXB7* and *bFGF* in primary melanocytes grown in supplemented medium (lane 1) and shifted to a medium deprived of growth factors for 24 h (lane 2). The normalized ratio between *bFGF* expression in sample 1 and that in sample 2 was approximately 15:1. *GAPDH* was the internal control.

specific DNA sequences, including promoters of other *HOX* genes, to enhance or inhibit transcription. Alteration of this complex network controlling cell proliferation may contribute to the multistep process underlying the onset of neoplasia. *HOX* genes are expressed in several primary leukemic cells and cell lines of both lymphoid and myeloid origin (11, 17, 39, 47), and several divergent *HOX* genes, such as *Pbx-1*, *tcl-3*, and *HB24*, are involved in some specific chromosomal translocations associated with different leukemias (15, 16, 27). Furthermore, in rodents, the oncogenic potential of *Hoxb8* and other *Hox* genes (i.e., *Hoxa7*, *-a5*, *-a1*, *-b7*, and *-c8*) has been demonstrated by both in vitro and in vivo transformation assays (42).

Despite a coordinated 3′→5′ expression pattern found in NT2/D1 teratocarcinoma cells after differentiation induced by retinoic acid (43) and in normal T lymphocytes and hematopoietic progenitors after a proliferative stimulus (11, 23), complex noncoordinated *HOXB* expression has been described for both normal and neoplastic adult cells of ectodermal origin (12, 19).

In melanomas, *HOXB7* was the only *HOXB* gene always expressed in a total of 25 cell lines analyzed by both RNase protection and RT-PCR. *HOXB7* expression was also confirmed in five surgical specimens analyzed by in situ hybridiza-

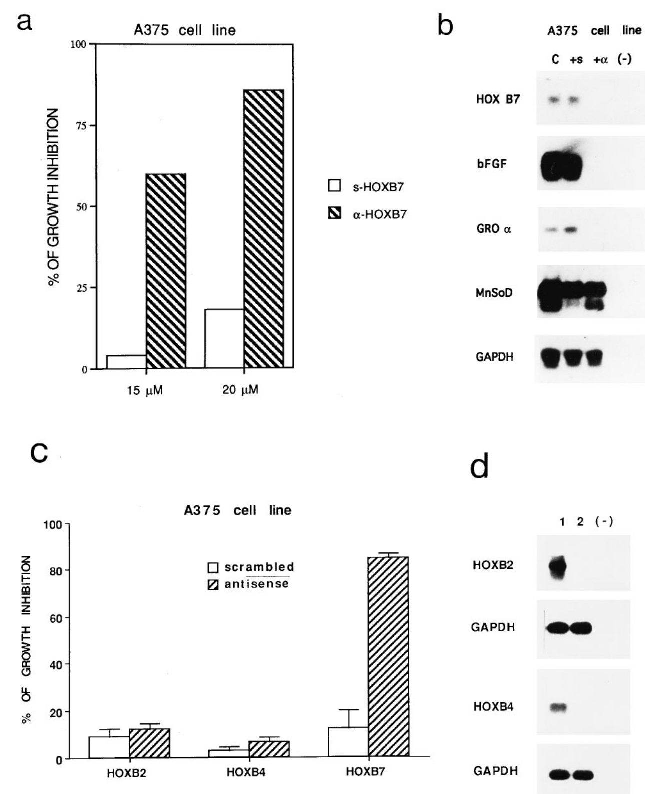


FIG. 4. Inhibition of melanoma cell proliferation by *HOXB7* antisense oligodeoxynucleotides results in *bFGF* and *GROα* mRNA downregulation. We show representative experiments with the A375 melanoma cell line: similar inhibitory results were obtained with diverse melanoma cell lines (e.g., Me 665/1 and Me 4405). (a) Inhibition of A375 melanoma cell proliferation upon treatment with 15 and 20 μM anti-*HOXB7* (α-HOXB7) oligomer compared with that after scrambled-oligomer (S-HOXB7) addition. Results from a representative experiment out of four separate experiments with identical results are shown. Inhibition after scrambled versus antisense oligomer treatments differed significantly ($P < 0.001$ [Student's t test]). (b) RT-PCR analysis of *HOXB7*, *bFGF*, *GROα*, manganese superoxide dismutase (MnSoD), and *GAPDH* gene expression with total RNA derived from cells treated with 20 μM antisense reagents shown in panel a. C, untreated control. (c) Inhibition of A375 melanoma cell proliferation upon treatment with 20 μM anti-*HOXB7*, *-B2*, and *-B4* oligomers compared with that after scrambled-oligomer addition. (d) RT-PCR analysis of *HOXB2*, *-B4*, and *GAPDH* genes from cells treated in panel c. Lanes: 1, cells treated with scrambled oligomer; 2, cells treated with antisense oligomer.

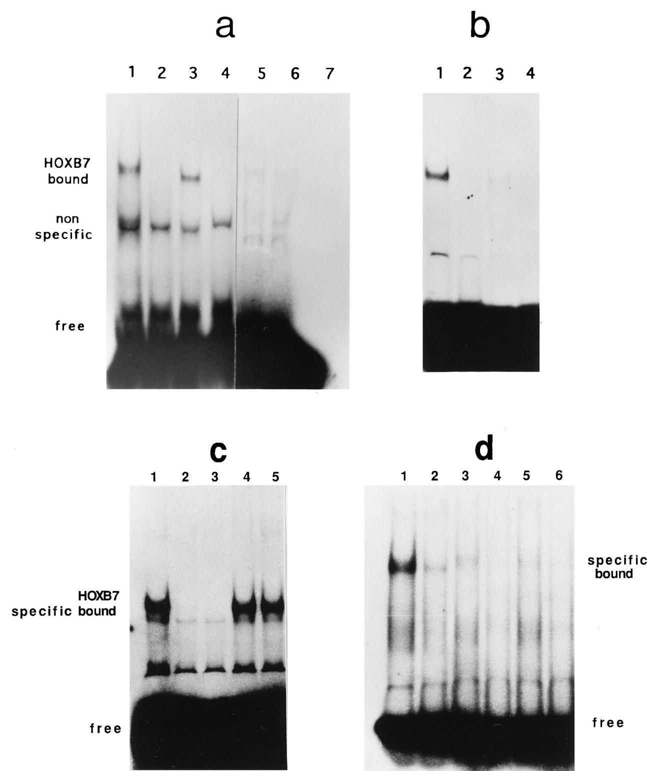


FIG. 5. HOXB7 protein binds and activates the bFGF promoter. (a) Electrophoretic mobility shift assay of the oligonucleotide encompassing bases +130 to +159 of the bFGF promoter sequence (22) after binding as follows: lane 1, nuclear extracts from the A375 melanoma cell line; lane 2, in the presence of a 200× molar excess of cold competitor oligonucleotide; lane 3, in vitro-translated HOXB7 protein; lane 4, in the presence of 200× cold competitor oligomer; lane 5, noninduced NT2/D1 teratocarcinoma cells; lane 6, in the presence of 200× cold competitor oligomer; and lane 7, unbound oligonucleotide. (b) Mobility shift assay carried out with the oligonucleotide encompassing bases +130 to +159 mutated (lanes 3 and 4) or not mutated (lanes 1 and 2) in the ATTA sequence (ATTA to GTCA). Lanes 1 and 3, in vitro-translated HOXB7 protein; lanes 2 and 4, HOXB7 protein plus 200× cold competitor oligomer. (c) Mobility shift assay performed with the labeled +130/+159 oligonucleotide without cold competition (lane 1) or in the presence of a cold competitor identical to the hot probe (lanes 2 and 3, 200× and 400×, respectively); a mutated oligonucleotide containing two base substitutions in the binding site, ATTA to GTCA (lane 4, 400×); and a totally unrelated sequence (lane 5, 400×). (d) Mobility shift assay of the nucleotide +130/+159 and in vitro-translated proteins HOXB7 (lanes 1 and 2), HOXB2 (lanes 3 and 4), and HOXD3 (lanes 5 and 6) in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of cold competitor.

tion. Its expression in melanomas is constitutive and is not lineage specific, since in melanocyte cell cultures, *HOXB7* is expressed only in proliferating cells, and in compound nevi, it is expressed only by the proliferating melanocytes of the epidermal layer. *HOXB7* has a functional role in that its inhibition by antisense oligonucleotide impairs melanoma proliferation.

Other *HOX* genes have been described as necessary for cell proliferation; studies with antisense oligomers to *HOXB* genes established a correlation between expression of diverse *HOXB* genes and proliferation of hematopoietic progenitors (23) and T lymphocytes (11) but shed no light on the underlying mechanism(s). It has been shown that bFGF (6), GROα (9), and interleukin 8 (51) sustain melanoma cell growth in an autocrine manner. Although other autocrine loops might be involved, in view of the diverse cytokines, growth factors, and receptors expressed by melanoma cell lines (40), only the autocrine production of bFGF and GROα is indicated as an early event in melanoma progression (34). If *HOXB7* acts through these factors, *bFGF* and *GROα* expression should be inhibited after *HOXB7* downregulation. Indeed, *bFGF* and *GROα* mRNAs were inhibited in melanoma cells treated with antisense oligomers specific to *HOXB7*. Furthermore, *bFGF*, like *HOXB7*, was constitutively expressed in melanomas maintained under both normal and serum-starved conditions, whereas *GROα* expression was not constitutive, in that it was downregulated in serum-starved, quiescent cells (not shown).

In normal melanocytes, the relationship between proliferation and *HOXB7* or *bFGF* expression was shown by (i) the absence of transcripts for *HOXB7* and *bFGF* in quiescent melanocytes from normal skin obtained from four different donors, (ii) the sharp downmodulation of both *HOXB7* and *bFGF* mRNAs in primary melanocytes growth arrested in culture, and (iii) the in situ analysis of compound nevi, whereby proliferating epidermal melanocytes, but not quiescent dermal melanocytes, express both *HOXB7* and *bFGF* mRNAs. Moreover, we found combined expression of *HOXB7* and *bFGF* in other neoplasias, including carcinoma and leukemia cell lines. In all cases examined (i.e., four glioblastoma, one lung, one renal carcinoma, and seven hematopoietic cell lines), expression of *HOXB7* was correlated with that of *bFGF*; moreover, two cell lines derived from breast and ovary carcinomas were negative for both genes (not shown).

Therefore, the regulated expression of *HOX* genes, as found in teratocarcinomas, activated T lymphocytes, and progenitor hematopoietic cells, appears to be lost in neoplasias of different origin. *HOXB7* constitutive expression may simply reflect the higher rate of proliferation of these tumors or may represent a step in the transformation process. It is likely that deregulated expression of *HOXB7* may contribute to neoplastic

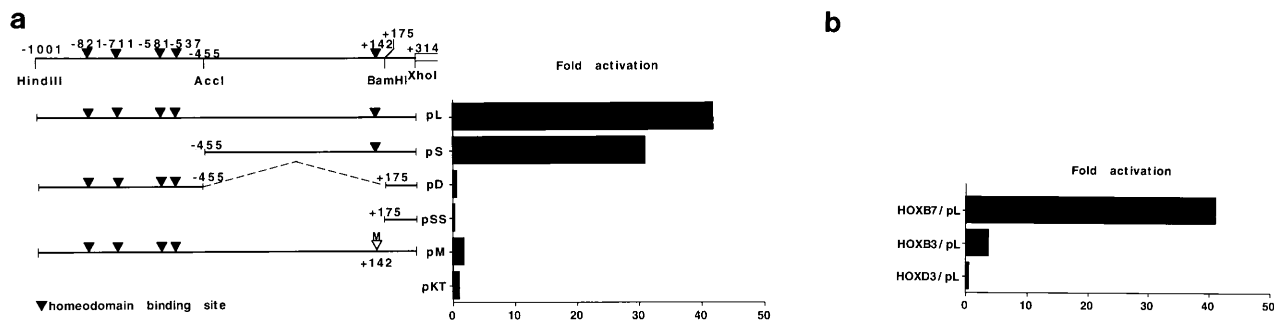


FIG. 6. Deletion constructs of the *bFGF* promoter and their relative transactivation, calculated as CAT activity, by the HOXB7 protein (a) and *bFGF* promoter transactivation by HOXB7, HOXB3, and HOXD3 homeoproteins (b). pL, pS, pD, pSS, and pM, proL-CAT, proS-CAT, proD-CAT, proSS-CAT, and proM-CAT constructs, respectively.

transformation and progression via bFGF, which has been described as promoting an autocrine loop in melanomas (6, 26), and it is also one of the major angiogenetic factors in both cancer (52) and embryogenesis (21). The functional association between *HOXB7* and bFGF was confirmed by cotransfection experiments showing a direct transactivation of the bFGF promoter by *HOXB7*. Despite the numerous controls performed here, we cannot exclude the possibility that other *HOX* gene-encoded proteins as well as other transcription factors can bind to the bFGF promoter. However, both gel shift analysis and cotransfection experiments suggest the specificity of *HOXB7*, since no other *HOX* gene tested (*HOXB2*, *-B3*, *-B4*, or *-D3*) was active and other *HOXB* genes (*HOXB1*, *-B8*, and *-B9*) were actually not expressed in A375 and Me 665/1 melanomas.

In conclusion, the present study raises the possibility that the regulatory function of *HOX* genes could be mediated, at least in some cell systems, via crucial growth factor genes. After the identification of the genes encoding several adhesion molecules, such as cytotactin (30), N-CAM (31, 32), and L-CAM (25), as target genes for homeoproteins (33), our results indicate that bFGF is the first growth factor involved in the complex network underlying *HOX* gene effects on cell proliferation and differentiation.

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