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Human Homeobox HOXA7 Regulates Keratinocyte *Transglutaminase Type 1* and Inhibits Differentiation*

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Keratinocyte proliferation and differentiation result from expression of specific groups of genes regulated by unique combinations of transcription factors. To better understand these regulatory processes, we studied *HOXA7* expression and its regulation of differentiation-specific keratinocyte genes. We isolated the homeobox transcription factor HOXA7 from keratinocytes through binding to a differentiation-dependent viral enhancer and analyzed its effect on endogenous differentiation-dependent genes, primarily *transglutaminase 1*. HOXA7 overexpression repressed *transglutaminase 1*-reporter activity. HOXA7 message markedly decreased, and transglutaminase RNA increased, upon phorbol ester-induced differentiation, in a protein kinase C-dependent manner. Overexpression of HOXA7 attenuated the *transglutaminase 1* induction by phorbol ester, demonstrating that *HOXA7* expression is inversely related to keratinocyte differentiation, and to *transglutaminase 1* expression. Antisense HOXA7 expression activated transglutaminase 1, involucrin, and keratin 10 message and protein levels, demonstrating that endogenous HOXA7 down-regulates multiple differentiation-specific keratinocyte genes. In keeping with these observations, epidermal growth factor receptor activation stimulated *HOXA7* expression. *HOX* genes function in groups, and we found that *HOXA5* and *HOXB7* were also down-regulated by phorbol ester. These results provide the first example of protein kinase C-mediated homeobox gene regulation in keratinocytes, and new evidence that HOXA7, potentially in conjunction with HOXA5 and HOXAB7, silences differentiation-specific genes during keratinocyte proliferation, that are then released from inhibition in response to differentiation signals.

The epidermis provides a protective barrier that undergoes constant renewal. Keratinocytes of the innermost or basal layer withdraw from the cell cycle and become displaced outwardly, differentiating to form layers of flattened, interconnected envelopes of cross-linked proteins packed with keratin filament bundles. Suprabasal cells inactivate genes expressed in basal cells, such as keratins 5 and 14, and activate differentiation-specific genes such as keratins 1 and 10 (1), cornified envelope proteins such as involucrin, loricrin, and small proline-rich proteins, and the enzyme required for envelope cross-linking, transglutaminase type 1 (reviewed in Refs. 2–4). EGF[1] receptor activation triggers keratinocyte proliferation (5, 6) and inactivates differentiation-specific genes (7). Rising extracellular calcium concentration, thought to be a key physiological mediator, activates differentiation-specific gene expression and morphological changes (8, 9). Calcium-induced differentiation of cultured keratinocytes is protein kinase C (PKC)-dependent, and markers of differentiation can be induced by PKC activators such as TPA (10, 11). Recent studies suggest that several differentiation-specific keratinocyte genes are regulated by the integrated action of DNA-binding factors, including members of the AP-1, AP-2, Sp1, and ets families (reviewed in Ref. 12) and, perhaps least understood, the homeobox family of transcription factors (13, 14).

Homeobox genes encode a family of transcription factors sharing a conserved 60-amino acid homeodomain (15). Duplication of gene clusters first described in *Drosophila* has produced four conserved mammalian *Hox* clusters, A–D (16), with capitalized names indicating the human homologs. Their importance in segment identity, pattern formation, and cell fate determination during development (16, 17) suggests that Hox factors regulate batteries of genes culminating in differentiation (18). Both Hox and non-Hox homeobox factors have also been implicated in regulation of differentiation in adult tissues, such as blood (19) and skin (13, 20).

In mouse skin, numerous non-*Hox* and *Hox* homeodomain genes are differentially expressed during development (21–24). The non-Hox POU (Pit-Oct-Unc) homeodomain gene *Oct-11*, or *Skn-1a*, message is associated with basal mouse epidermal cells by one study (25), but with suprabasal cells in another study (26). The *Drosophila Distal-less*-related non-*Hox* homeodomain gene *Dlx3* is transcribed in differentiating keratinocytes (27). Ectopic expression inhibits growth and induces the expression of differentiation-associated proteins, suggesting a

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF026397.

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[1] The abbreviations used are: EGF, epidermal growth factor; *TGM1*, transglutaminase 1 gene; TGase1, transglutaminase type 1; *K3*, a 1.7-kilobase pair *TGM1* gene upstream regulatory DNA fragment; PKC, protein kinase C; NHK, primary neonatal human keratinocytes; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; HPV-16, human papillomavirus 16; RT-PCR, reverse transcription-polymerase chain reaction; AP-1, activator protein 1; AP-2, activator protein 2; Sp1, transcription factor Sp1; SPRR2A, small proline rich-related peptide 2A; HOX, class 1 homeobox transcription factors related to the *Drosophila* Antennapedia complex and Bithorax complex genes; POU, Pit-Oct-Unc-related transcription factors containing a conserved homeodomain and POU domain; Dlx3, *Drosophila* distal-less-like homeobox transcription factor; CBP, cAMP-regulated enhancer binding-binding protein; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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role in regulation of differentiation (13). In human keratinocytes, Oct-11/Skn-1a activates expression of the differentiation markers K10 (26) and the small proline-rich envelope protein SPRR2A (28), and Oct-6 inhibits expression of the proliferation-associated *K5* and *K14* genes (29), suggesting activation of differentiation. However, others observed Oct-6 RNA in all living layers of normal epidermis (29), and several POU family members, including Skn-1a, are capable of inhibiting the differentiation-dependent involucrin promoter (30). *HOX* cluster genes have also been implicated in the regulation of keratinocyte differentiation. HOXC4 message and HOXB6 protein correlate with differentiation in normal skin, and HOXA4 is absent in proliferative basal cell carcinomas (14, 31). Stelnicki *et al.* (32) identified predominantly HOXA4, HOXA5, and HOXA7 expression in suprabasal fetal human epidermis, with expression persisting in the adult epidermis, but not in the dermis.

HOX transcription factors recognize similar DNA sequences *in vitro*. The diversity of their effects and targets *in vivo* is believed to result from modulation by cofactors that affect binding or function. For example, multiple copies of the *Drosophila* Ultrabithorax (Ubx) bind cooperatively to target gene sites (33), and HOX factors exhibit altered DNA sequence recognition and cooperative DNA binding with either of two homeodomain proteins, PBX1 (34, 35), via a conserved hexapeptide motif, or MEIS-1 (36). Furthermore, HoxB7 exhibits no change in DNA binding, but is transcriptionally coactivated by the histone acetylase CBP (CREB-binding protein) (37).

Another mechanism underlying specificity of function is the combined action of unique sets of *Hox* genes. In development, *Hox* genes act in groups comprising paralogous genes (corresponding genes from different clusters), as well as nearby genes within clusters, to bring about target regulation. This mechanism may also underlie the expression of predominantly HOXA4, HOXA5, HOXA7, but also HOXB6 and HOXB7, in a similar differential manner in human skin (32). In this study we isolated and sequenced the *HOXA7* cDNA from keratinocytes through binding to a differentiation-dependent HPV-16 E6/E7 enhancer fragment. HOXA7 also bound to a regulatory fragment of the differentiation-specific *transglutaminase 1* gene and repressed *transglutaminase 1*-reporter activity. HOXA7, HOXA5, and HOXB7 were down-regulated in keratinocytes induced to differentiate with TPA, and overexpressed HOXA7 inhibited transglutaminase 1 expression during TPA-induced differentiation. Antisense HOXA7 expression up-regulated keratinocyte differentiation markers and slowed growth, and HOXA7 message was up-regulated in growth-activated keratinocytes. These results indicate that HOXA7, potentially in conjunction with related family members, functions in silencing differentiation-specific genes, prior to its own PKC-mediated down-regulation during differentiation. A transient increase in HOXA7 expression observed as cultured cells reach confluence may act as a brake initially to limit the rate at which differentiation progresses, as cells become exposed to differentiation signals.

EXPERIMENTAL PROCEDURES

Isolation of HOXA7 cDNA—An epidermal cDNA expression library (CLONTECH, Palo Alto, CA), prepared in λ*gt11* from human keratinocyte messenger RNA, was screened for expressed proteins that recognize a 232-bp HPV-16 *E6/E7* enhancer *Dra*I fragment (bp 7524–7756) by a standard method (38). Briefly, phage plaques on cultures of infected *Escherichia coli* were overlaid with nitrocellulose filters pretreated with isopropyl-1-thio-β-D-galactopyranoside and dried. Expressed, adsorbed proteins were denatured with 6 M guanidine HCl in Tris-buffered saline and renatured with washes in six decrements to zero denaturing agent. Filters were blocked with 5% nonfat dried milk and 100 μg/ml salmon sperm DNA in Tris-buffered saline with 0.5% Triton X-100, and expressed proteins were probed with a ^{32}P-labeled 232-bp *Dra*I fragment (bp 7524–7756), containing the CK element, a small cytokeratin homology motif, from the HPV-16 *E6/E7* enhancer, or a neighboring 209-bp *Dra*I control fragment (bp 7286–7495). Bound probe was visualized by autoradiography. Positive plaques underwent two further cycles of *E. coli* infection, plating, induction of protein expression, and probing. Clone 124, containing the full-length *HOXA7* cDNA, was subcloned into pCDNAI and sequenced.

Tissue Culture, Vectors, and Cell Transfection—Neonatal human keratinocytes (NHK) were obtained from human foreskin by overnight dispase digestion, followed by trypsinization of the separated epidermis, and plating in keratinocyte serum-free medium (Life Technologies, Inc.), containing recombinant human EGF and bovine pituitary extract. ME180 epidermoid carcinoma cells were obtained from the American Tissue Culture Collection (ATCC) and grown in RPMI 1640 (Biowhitaker, Walkersville, MD) with 8% fetal calf serum (Sigma). HaCaT spontaneously immortalized keratinocytes were a generous gift from Dr. N. E. Fusenig and were cultured in Dulbecco's modified Eagle's medium with 8% fetal calf serum.

The full-length HOXA7 cDNA (forward primer 5′-GTCGCCATGGGTTCTTCGTATTATGTG-3′, generating a Kozak translation start sequence, and vector reverse primer) and a 5′ fragment from the translation start to just 5′ of the hexapeptide domain (Fig. 1) (5′-ATACTCGAGTAGATGCGGAAATTGG-3′ reverse primer) were PCR amplified by *pwo* polymerase (Roche Molecular Biochemicals), subcloned into the vector pCR2.1 (Invitrogen, Carlsbad, CA), then moved to the eukaryotic expression vectors pCS2 (pCS2-HOXA7), pCDNA3 (Invitrogen) (p3-HOXA7, p3-HOXA7frag), and pEF3 (pEF3–124fragRev) in the forward and reverse orientation and sequenced. The expression vector pEF3 was constructed by subcloning the EF-1α promoter, released from the vector pEF6/HisA (Invitrogen) by partial digestion with *Hin*dIII/*Bgl*II, into the pCDNA3 (Invitrogen) backbone in place of the cytomegalovirus promoter. The 1.7-kilobase pair *transglutaminase 1* upstream regulatory region *K3*, isolated previously (39, 40), was subcloned into the enhancerless and promoterless reporter vectors pCAT-Basic (pCAT-K3), and pGL3-B (pGL3-K3) (Promega), for transient transfection. The control reporter pHβA-*LacZ* was constructed by inserting the *lacZ* cDNA from pCH110 (Amersham Pharmacia Biotech) into the human β-actin promoter-driven expression vector pHβAPr-1 (41).

Third passage NHK, and ME180, were transfected with Fugene 6 (Roche Molecular Biochemicals), and HaCaT keratinocytes were transfected with Exgen 500 (Fermentas, Hanover, MD) under the recommended conditions. Cells were transiently cotransfected with expression and reporter vectors (either *HOXA7* or *K3*-containing, respectively, or empty vector) and with the pHβA-*LacZ* control vector. Lysates obtained 2 days after transfection were assayed for chloramphenicol acetyltransferase activity using [^{14}C]chloramphenicol and acetyl-CoA by thin layer chromatography and autoradiography, and by xylene extraction and scintillation counting, presented as counts/min acetyl-[^{14}C]chloramphenicol per milligram of lysate protein. Determinations of luciferase activity (Promega luciferase substrate), β-galactosidase activity (Tropix, Applied Biosystems, Foster City, CA), and total protein concentration (BCA, Pierce) were made according to the reagent supplier's instructions. Results are expressed as relative light units per milligram of protein. Cotransfection of expression vectors with empty reporters resulted in low background level reporter signals in all cases (data not shown). Transfection efficiency was determined as the relative number of β-galactosidase-stained cells. Cells were fixed for 5 min with an ice-cold equivolume mixture of acetone and methanol, washed in phosphate-buffered saline, and stained for 16 h at 37 °C with 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside in 40 mM citrate phosphate buffer, pH 7.5, with 5 mM ferro- and ferricyanide, 2 mM $MgCl_2$, and 150 mM NaCl.

Stably transfected HaCaT were selected starting 48 h after transfection with 500 μg/ml Geneticin (Life Technologies, Inc.) and maintained in 300 μg/ml Geneticin. For analysis of growth rate, 1.5×10^5 antisense HOXA7, or vector control-transfected HaCaT, were plated in 35-mm dishes. Triplicate samples of unattached cells, and attached cells released by trypsinization, were counted at 24-h intervals. Data are reported as cells per well $\times 10^{-4} \pm$ S.D. Statistical differences were determined by analysis of variance and *t* test.

Electrophoretic Mobility Shift Assay—To verify binding to the HPV-16 *E6/E7* enhancer, HOXA7 was expressed in λ*gt11*-transformed 1090 *E. coli* by induction with 2 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h, followed by sonication in the presence of phenylmethylsulfonyl fluoride and centrifugation. For electrophoretic mobility shift assay, 232-bp (bp 7524–7756) and 209-bp (bp 7286–7495) *Dra*I fragments of HPV-16 were released by restriction digestion, purified, and ^{32}P-labeled. Binding reactions containing 4×10^5 cpm probe (about 1 ng of DNA) and 5 μg of HOXA7 bacterial phage expression lysate or

nontransformed bacterial lysate and 2 μg of poly(dI-dC) in 20 mM Hepes, pH 7.5, 50 mM potassium chloride, 1 mM dithiothreitol, 6% glycerol, and 0.2 mM phenylmethylsulfonyl fluoride were incubated at room temperature for 20 min, separated on 5% polyacrylamide gels with 0.5× Tris/borate/EDTA at 4 °C for 4 h, dried, and autoradiographed.

To investigate HOXA7 binding to the *TGM1* upstream regulatory region (K3), recombinant HOXA7 was expressed by *in vitro* transcription/translation using the ATNT-coupled Reticulocyte System® from Promega according to the included protocol, using 1 μg of template DNA per reaction. In control synthesis reactions, [^{35}S]methionine was added, and expression was monitored by SDS-PAGE and autoradiography. For electrophoretic mobility shift assay, a 264-bp K3 fragment (5′ end at −710), containing a CK element similar to that found in the 232-bp *Dra*I HPV-16 fragment, and a 212-bp neighboring *K3* fragment (5′ end at −444), were prepared by PCR (bp −709 to +90), digested with *Bam*HI and *Hpa*II, purified, ^{32}P-end-labeled, and shifted with 5 μg of the HOXA7 *in vitro* translation lysate as described above.

RNase Protection Assay—Total RNA was isolated using TRIzol reagent (Life Technologies, Inc.) from third passage neonatal human keratinocytes treated with 50 ng/ml TPA (Calbiochem). ^{32}P-UTP-labeled RNA probes were synthesized using T7 polymerase (Ambion, Austin, TX) from the linearized plasmid DNA template pCDNA3-HOXA7fragRev, containing a nonhomologous 5′ fragment of the *HOXA7* cDNA (Fig. 1), and pBSII-GAPDH (Stratagene, La Jolla, CA), containing a 360-bp human *glyceraldehyde phosphate dehydrogenase* cDNA fragment, both in the reverse orientation. Following DNase I digestion for 15 min at 37 °C, the probes were electrophoretically purified in a 5% polyacrylamide gel containing 1× TBS and 8 M urea (Sequagel, National Diagnostics, Atlanta, GA), eluted (60 min, 37 °C in 0.5 M ammonium acetate, 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 50 μg/ml yeast RNA (Ambion)), precipitated (1 M ammonium acetate and 3 volumes of 2-propanol), dissolved in H_2O, and scintillation counted. For RNase protection, labeled probe was ethanol/ammonium acetate-precipitated together with 12 μg of normal human keratinocyte or yeast RNA, redissolved in hybridization buffer (Ambion), denatured (90 °C, 4 min), and hybridized overnight at 42 °C. Single-stranded RNA was digested using 1 part in 100 RNase A1/RNase T1 in digestion buffer (Ambion) for 30 min at 37 °C. Protected RNA was separated by denaturing polyacrylamide gel electrophoresis as described for probe purification and visualized by autoradiography.

For detection of antisense HOXA7 RNA in stably transfected HaCaT, total RNA was hybridized as above, without the addition of probe, digested with RNase A/T for various times, and phenol-chloroform-extracted and precipitated, before detection of 5′ HOXA7 RNA by RT-PCR. The PCR product, spanning bases 291–451 (Fig. 1), was produced using the HOXA7 forward primer described below under "RT-PCR" and the reverse primer 5′-CTCGTCCGTCTTGTCGCAGG-3′.

RT-PCR—To determine the range of target DNA concentrations giving rise to a linear relationship between target concentration and product concentration, restriction fragments of *HOXA7*, *TGM1*, and *β-actin* cDNA were purified, and 100–100,000 molecules were PCR amplified using *Taq* DNA polymerase (Promega) and PCR buffer (Sigma), in the presence of 1.8 mM $MgCl_2$, 400 nM primers, 400 μM each dNTP (Roche Molecular Biochemicals), and 5 μCi of [^{32}P]dCTP. Samples were removed after 30 cycles of 94 °C, 20 s; 57 °C, 20 s; 72 °C, 30 s, electrophoresed in 6% acrylamide, 0.5× Tris-borate-EDTA-buffered gels, and autoradiographed. Quantification of product bands, using Scion Image (www.scioncorp.com), indicated a linear relationship between the amount of target cDNA and the amount of PCR product formed across the range of target concentrations tested.

Total RNA (0.75 μg) from control, or 50 ng/ml TPA-treated NHK, denatured at 68 °C for 10 min in the presence of 2 μM oligo(dT) primer (Invitrogen) and 1.25 mM each dNTP served as the template for cDNA synthesis by Superscript II reverse transcriptase (Life Technologies, Inc.) in the supplied buffer, supplemented with 0.5 unit/μl RNase inhibitor (Promega) and 10 mM dithiothreitol, at 42 °C for 60 min. The reaction was stopped with EDTA and heating for 15 min at 70 °C. cDNA diluted 1:10 with H_2O and added at 0.10 volume to a 30-cycle PCR reaction under the above conditions gave rise to a signal intensity corresponding to a target concentration for HOXA7 and TGM1 within the linear range of the PCR assay. PCR using β-actin primers generated a signal equivalent to standards in the linear range of PCR after 18 cycles. HOXA5 and HOXB7 were also amplified for 30 PCR cycles, and involucrin, K5, and K10 were amplified for 25 cycles. Sham reactions in which no reverse transcriptase was added produced no PCR bands. The HOXA7 and transglutaminase 1 PCR primer sequences span intron splice sites to distinguish products arising from amplification of cDNA. Treatment of RNA samples with 2 units of DNase I (Ambion) followed by heat inactivation before cDNA synthesis had no affect on the PCR products formed. Primers of the following sequence (5′ to 3′) were used: HOXA7 forward (CTTATACAATGTCAACAGCC) and reverse (TCCTTATGCTCTTTCTTCC and TCTTCTTCATCATCGTCCTCCTCG), TGM1 forward (TCTGTGGGTCCTGTCCCATCCATCCTGACC) and reverse (CCCCAACGGCCCACATCGGAACGTGGCCCATCCATCATGC), human β-actin forward (CAGGCTGTGCTATCCCTGTAC) and reverse (CACGCACGATTTCCCGCTCGG), human cytokeratin K10 forward (GGCTCTGGAAGAATCAAACTATGAGC) and reverse (GGATGTTGGCATTATCAGTTGTTAGG), involucrin forward (TGTTCCTCCTCCAGTCAATACCC) and reverse (ATTCCTCATGCTGTTCCCAGTGC), keratin K5 forward (CTGTCTCCCGCACCAGCTTCACCTCC) and reverse (CTCCACAAGCACCCGCAAGGCTGACC), HOXA4 forward (GGCGCTGACATGGATCTTCTTCATCC) and reverse (CAACTACATCGAGCCCAAGTTCCCTCC), HOXA5 forward (CCTCTCTGCTGCTGATGTGGGTGC) and reverse (ACGGCTACGGCTACAATGGCATGG), HOXB7 forward (AAGTTCGGTTTTCGCTACCGGAGCC and CGCGCAGTGCATGTTGAAGG).

Transglutaminase Assay—HaCaT keratinocytes stably transfected with pCDNA3 (control) or pCDNA3-HOXA7fragRev (HOXA7 antisense), carrying a 5′ HOXA7 cDNA fragment (Fig. 1) insert in the reverse orientation, grown to 75% confluence in 100-mm dishes, were washed in 10 ml and then scraped up in 0.3 ml of 50 mM Tris, pH 7.4, 150 mM NaCl, 0.2 mg/ml bovine serum albumin, 20 mM EDTA, and complete protease inhibitor mixture (Roche Molecular Biochemicals), microfuged 30 s at 4 °C, forming the cell supernatant, and resuspended in the same buffer with 20 mM EDTA. Cells were lysed by sonication, and centrifuged at 100,000 × *g* for 45 min at 4 °C, forming the cytosolic supernatant and transglutaminase 1-containing particulate pellet (42). TGase I activity was extracted from the disrupted pellet for 30 min on ice in the lysis buffer supplemented with 1% Triton X-100 and centrifuged at 10,000 × *g* for 15 min at 4 °C, with the supernatant designated the particulate fraction. The cell supernatant and cytosolic fractions were made 1% in Triton X-100 corresponding to the detergent level in the particulate fraction. Transglutaminase activity of cellular fractions relative to total protein content was determined essentially as described previously (42), by incorporation of 20 μCi/ml (0.2 μmol/ml) [^{14}C]putrescine into 2 mg/ml dimethylcasein in the presence of 20 mM Tris, pH 7.4, 60 mM NaCl, 20 mM $CaCl_2$, 5 mM dithiothreitol, and 0.4% Triton X-100 for 45 min at 37 °C, followed by trichloroacetic acid precipitation and scintillation counting.

Western Blot—Control and antisense HOXA7-transfected HaCaT cells were scraped up in sodium dodecyl sulfate sample buffer, electrophoresed in 8.5% polyacrylamide gels with a discontinuous Tris-glycine buffer system, transferred to nitrocellulose in Tris-glycine with 20% methanol at 300 mA for 1 h, blocked for 1 h with 4% dry milk or bovine serum albumin in Tris-buffered saline with 0.1% Tween 20, and incubated 1 h at 37 °C with primary antibody, diluted 1:1000 in blocking solution, against human involucrin (BioTechnologies Inc., Staughton, MA), human K1 (1:1000), or mouse K5 (1:200,000, cross-reacts with human) (Babco, Richmond, CA). Bound antibody was detected by incubating filters for 45 min with a 1:5,000 dilution of horseradish peroxidase-conjugated goat-anti-rabbit IgG (Dako, Carpinteria, CA) in Tris-buffered saline with 0.1% Tween 20 and exposing autoradiographic film after treatment with luminescent substrate (Amersham Pharmacia Biotech).

Northern Blot—Northern analysis of antisense HOXA7 RNA in stably transfected HaCaT keratinocytes was performed according to standard methods. Total RNA (25 μg) from 80% confluent vector control and antisense HOXA7-expressing HaCaT cells was isolated using TRIzol following the provided instructions (Life Technologies, Inc.), separated on a 1.2% agarose, MOPS, 1.1% formaldehyde gel, and transferred to a nylon membrane. The blot was hybridized with a ^{32}P-labeled riboprobe prepared as recommended (Maxiscript, Ambion) from the 5′ region of the HOXA7 cDNA inserted in the reverse orientation in the vector pCDNA3, using SP6 phage RNA polymerase. The blot was washed at high stringency (0.1% SSC, 0.1% SDS at 68 °C), and hybridized probe was detected by autoradiography. The blot was stripped and reprobed with a GAPDH control probe, ^{32}P-labeled by random priming (Life Technologies, Inc.), washed as above, and autoradiographed.

RESULTS

Isolation of the HOXA7 cDNA through Binding to the HPV16 E6/E7 Enhancer—Epidermal differentiation results from the coordinated expression of keratinocyte genes, perhaps by the

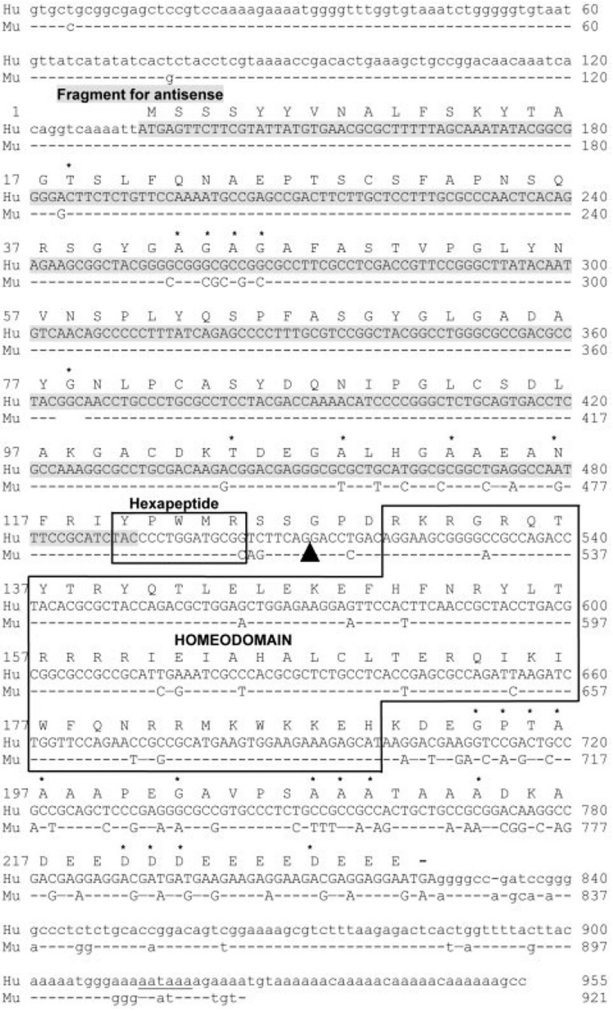

FIG. 1. **The human HOXA7 cDNA sequence exhibits high sequence homology with the murine HoxA7 (Hox1.1).** *Dashes* in the murine sequence indicate nucleotide identity with the human; the predicted human amino acid sequence is shown above, with *asterisks* indicating differences in the amino acid sequences. The nonhomologous 5′ fragment used for expression of antisense RNA in transfected cells, and for RNA probe synthesis, is *shaded*. The conserved hexapeptide, required for certain protein-protein interactions, and the DNA-binding homeodomain are *boxed*, and the polyadenylation signal is *underlined*. The Intron 1 boundary is marked with an *arrowhead*. The human HOXA7 cDNA sequence is listed in the GenBank™ data base under accession number AF026397.

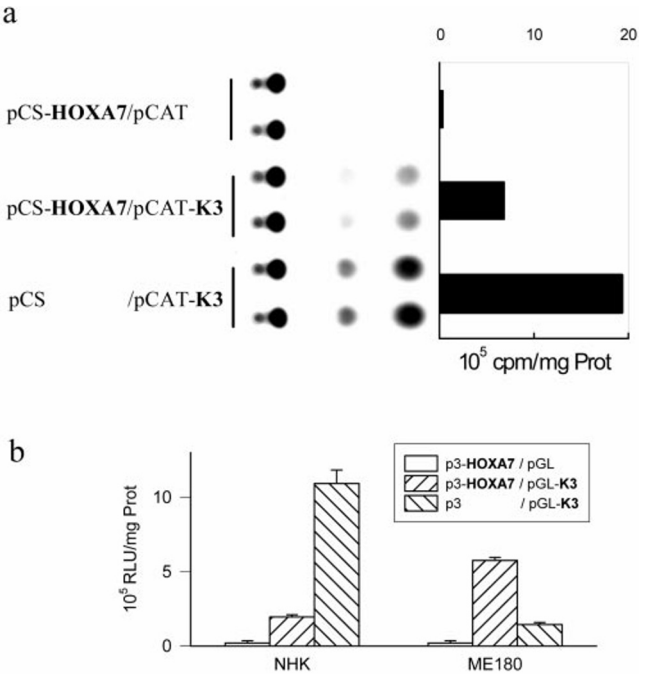

FIG. 2. **HOXA7 represses *TGM1 K3* transcription in NHK, but stimulates *K3* transcription in ME180 carcinoma cells.** *a*, CAT reporter activity of NHK transfected with the empty pCS vector, or pCS2-HOXA7 producing a *c-myc*-HOXA7 fusion peptide, and the reporter pCATB-K3; results are expressed (duplicate lanes) as a thin layer chromatography autoradiograph and as counts/min organically extracted acetyl-[^{14}C]chloramphenicol per milligram of lysate protein. *b*, relative light units (*RLU*) per milligram of protein of NHK and ME180 transfected with pCDNA3 (p3), or pCDNA3-HOXA7 (p3-HOXA7), and pGL3B-K3 (pGL-K3). Results are representative of at least three experiments.

action of a specific set(s) of transcription factors. The human papilloma virus-16 (HPV-16) *E6/E7* enhancer (p91) exhibits keratinocyte- and differentiation-specific activation. To take advantage of any functional *cis*-acting elements pirated by the HPV-16 p91 enhancer from the differentiation-specific keratinocyte gene regulation system, we used a 232-bp *Dra*I fragment of the HPV-16 *E6/E7* enhancer to screen a keratinocyte cDNA expression library. This fragment encompasses an AA-PuCCAAA motif (CK element) also found within the transglutaminase 1 (TGM1) gene upstream regulatory region (K3) (40, 43), keratins K1 and K14 (44, 45), and involucrin upstream regulatory regions (46). A phage clone expressing a binding protein that did not adhere to a neighboring 209-bp enhancer fragment was isolated, as verified by electrophoretic mobility shift assay (data not shown).

Sequence analysis revealed a 954-bp cDNA encoding HOXA7, a class I homeobox transcription factor of 230 amino acids, with a homeodomain extending from amino acid 130 to 189, a conserved six-amino acid (hexapeptide) sequence just upstream of the homeodomain, and an acidic C-terminal domain (Fig. 1). The homeodomain and hexapeptide amino acid sequences of the human HOXA7, and its mouse homolog Hoxa7 (formerly Hox1.1), are identical despite some divergence at the nucleic acid level. The proteins also share overall similarity, including an acidic C-terminal domain. The mammalian hexapeptide and homeobox sequences represent remarkable conservation, varying by only one amino acid from the *Drosophila antennapedia*, but sequences outside the homeodomain exhibit no obvious sequence similarity to *D. antennapedia*. Examination of the genomic sequence indicates a structure similar to the mouse *Hoxa7* gene (47), with a 944-bp intron separating the two translated exons, between the hexapeptide and homeodomain coding sequences (*arrow* in Fig. 1).

HOXA7 Transrepresses TGM1 Gene Upstream Regulatory Region (K3) Reporters in NHK, but Transactivates K3 Reporters in the Epidermoid Carcinoma Line ME180 and Binds Specifically to a K3 Fragment in Vitro—To determine whether the highly conserved HOXA7, recognized by the HPV-16 enhancer, might regulate differentiation-specific keratinocyte genes such as *TGM1*, we examined the affect of transiently overexpressed HOXA7 on *TGM1*-K3 reporter activity. HOXA7 overexpressed as a c-*myc* fusion (pCS-HOXA7) in primary neonatal keratinocytes (NHK) repressed transcriptional activity of *K3* (pCAT-K3) relative to the empty vector (Fig. 2*a*). To rule out any effect of the *c-myc* portion of the fusion peptide, the HOXA7 cDNA was subcloned into the eukaryotic expression vector pCDNA3; the K3 regulatory DNA was subcloned into the promoterless, enhancerless pGL3B. As before, HOXA7 expression inactivated *K3* transcription relative to empty pCDNA3 in NHK. Interestingly, HOXA7 had the opposite effect in the epidermoid carcinoma cell line ME180 and transactivated K3 relative to vector control (Fig. 2*b*).

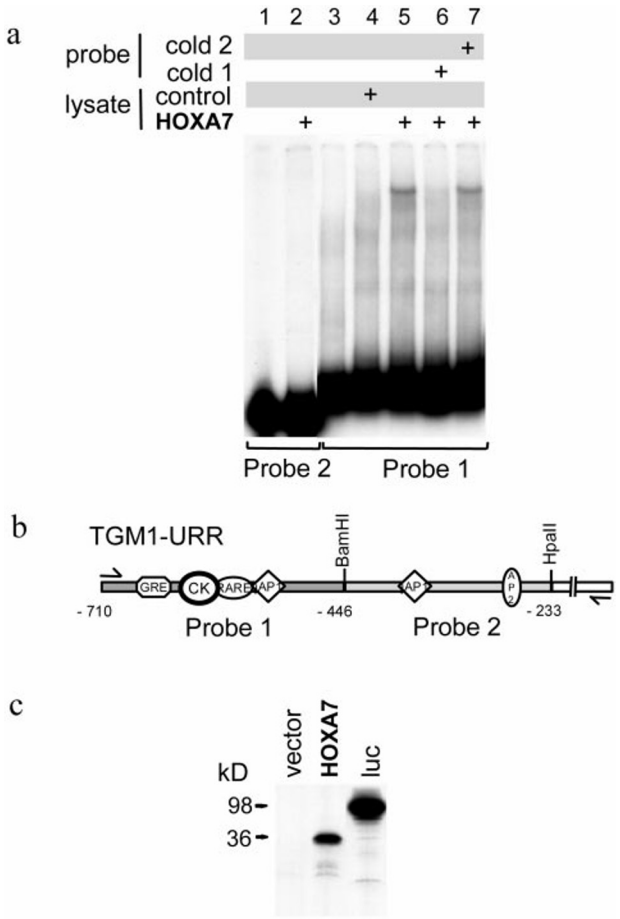

FIG. 3. **HOXA7 binds specifically to a fragment of the *TGM1* upstream regulatory region.** *a*, HOXA7 expressed by *in vitro* transcription/translation formed a complex with a 264-bp *transglutaminase 1* gene fragment containing the CK-8-mer, probe 1 (*lane 5*), but not with the neighboring 212-bp control fragment, probe 2 (*lane 2*). The binding was abrogated by competition with cold probe 1 (*lane 6*), but not cold probe 2 (*lane 7*). *Lanes 1 and 3*, probes 1 and 2 alone. *Lane 4*, probe 1 with control reticulocyte lysate. *b*, delineation of the *TGM1 K3* region comprising probes 1 and 2. *c*, the [^{35}S]methionine-labeled HOXA7 product (*center lane*), control reaction containing the empty vector pCDNA3 (*left lane*), and expression of the luciferase gene product (positive control) (*right lane*). Two additional experiments produced comparable results.

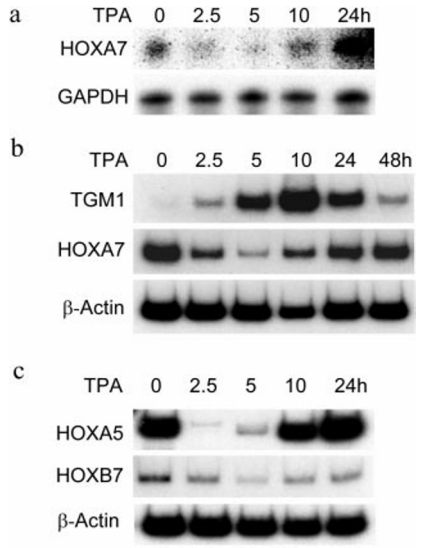

FIG. 4. **HOXA7 message falls in NHK induced to differentiate with TPA** *a*, RNase protection of HOXA7 message in total RNA from NHK treated with 50 ng/ml TPA, immediately after reaching confluence, for the indicated times (*top panel*), relative to GAPDH (*bottom panel*). Data are representative of four independent experiments. *b*, HOXA7 message (*center panel*), transglutaminase 1 message (*top panel*), and β-actin message (*bottom*) in NHK as determined by RT-PCR after treatment with 50 ng/ml TPA and isolation of total RNA, in one of six repeated experiments. *c*, HOXA5 message (*top panel*) and HOXB7 message (*middle panel*) in NHK as determined by RT-PCR after TPA treatment as in *a*. *Bottom panel*, β-actin control. Data reflect results of three experiments.

Since HOXA7 affected *TGM1 K3* reporters by transient transfection, we tested the hypothesis that HOXA7 may recognize a potential binding site in the *K3* regulatory region near the CK element by electrophoretic mobility shift assay. The *in vitro* transcribed and translated HOXA7 protein electrophoretically retarded a 264-bp 5′ *K3* fragment (Fig. 3*a*, *lanes 5* and *7*), but the empty vector control lysate did not (*lane 4*). Binding was abolished by competition with cold probe (*lane 6*), but not by competition with a neighboring 212-bp *K3* fragment (*lane 7*). Protein synthesis was monitored in control reactions with [^{35}S]methionine by SDS-PAGE and autoradiography (Fig. 3*c*). These results indicate that HOXA7 can specifically bind K3 and regulate *K3* transcriptional activity and suggest that HOXA7 regulates *TGM1* gene activity in keratinocytes, via a mechanism altered in ME180 carcinoma cells.

HOXA7, HOXA5, and HOXB7 Expressions Are Repressed, and TGM1 Is Activated, in NHK Stimulated to Differentiate with TPA by a PKC-dependent Mechanism—With the potential to transrepress the differentiation-specific gene TGM1 in keratinocytes, we investigated the level of *HOXA7* expression in keratinocytes induced to differentiate with TPA, which activates *TGM1* gene expression. The concentration of HOXA7 RNA was markedly reduced, as measured by RNase protection assay, by 2.5 h after treatment of NHK with TPA, reaching a minimum at 5 h and remaining in decline for at least 10 h (Fig. 4*a*). The concentration of HOXA7 RNA was also reduced as measured by RT-PCR analysis (Fig. 4*b*, *middle panel*). HOXA7 message was reduced by treatment with as little as 0.5 ng/ml TPA for 10 h (not shown). TPA treatment also resulted in a decline in HOXA5 and HOXB7 message levels (Fig. 4*c*), suggesting coregulation with HOXA7. The HOXA7 autoradiographic bands represent processed mRNA only, as the primer hybridization sites span the intron splice site (Fig. 1, *arrow*). HOXA4 message was detected only at trace levels by RT-PCR (not shown). RNA pretreatment with DNase I had no affect on the bands produced by RT-PCR, and sham RT samples yielded no detectable PCR product. RNA isolated from untreated NHK at each time point exhibited a constant level of HOXA7 message (not shown).

As expected, TPA treatment of normal human keratinocytes caused an increase in TGM1 message level (Fig. 4*b*, *top panel*), which reached a maximum at a time later than the maximum drop in HOXA7 message level. The TPA induction of *TGM1* was transient, as described previously (11), as was the inhibition of *HOXA7*. The β-actin message level remained relatively constant over the time course tested (Fig. 4, *b* and *c*, *bottom panels*). The number of PCR cycles was varied for each primer pair to maintain linearity of the relationship between the number of target molecules and the amount of PCR product formed, as determined using purified linear cDNA.

As expected, we found that TPA modulation of HOXA7, like that of TGM1, occurs downstream of PKC activation. The marked decline in HOXA7 message levels with TPA treatment of cultured NHK was blocked by the PKC inhibitor bisindolylmaleimide (*bIM*) (Fig. 5, *top row*). Treatment with bisindolylmaleimide alone had no effect on the HOXA7 message level in nonconfluent cells and increased the HOXA7 message level in post-confluent cells (not shown), demonstrating that the de-

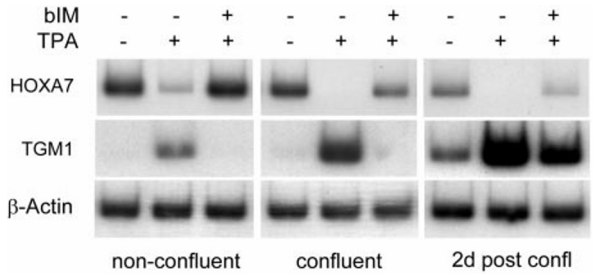

FIG. 5. **Repression of *HOXA7* gene expression with TPA treatment is PKC-dependent.** On successive days after plating, NHK were pretreated for 30 min with 100 mM PKC inhibitor bisindolylmaleimide (*bIM*) (+), or Me_2SO (−), then treated for 10 h with 20 ng/ml TPA (+) or Me_2SO (−). HOXA7 (*top row*), TGM1 (*middle row*), and β-actin (*bottom row*) message levels were determined by RT-PCR. Results from 60–70% confluent (nonconfluent, *left column*), confluent (*middle column*), and 2 days postconfluent cells are shown. Results represent three independent experiments.

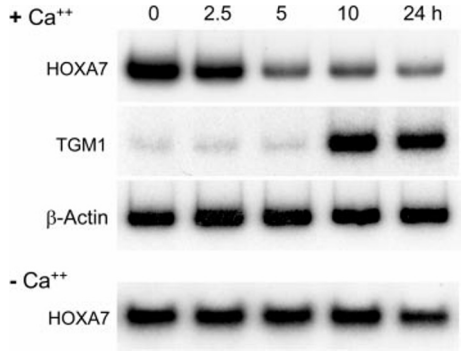

FIG. 6. **Calcium treatment represses *HOXA7* and stimulates *TGM1* expression.** NHK grown to confluence in low calcium medium were treated with 1.8 mM $CaCl_2$ (*top three panels*) or maintained in low calcium medium (*bottom panel*). Total RNA was isolated before and at 2.5, 5, 10, and 24 h after initiation of calcium treatment. *HOXA7*, *TGM1*, and *β-actin* expression were determined by RT-PCR.

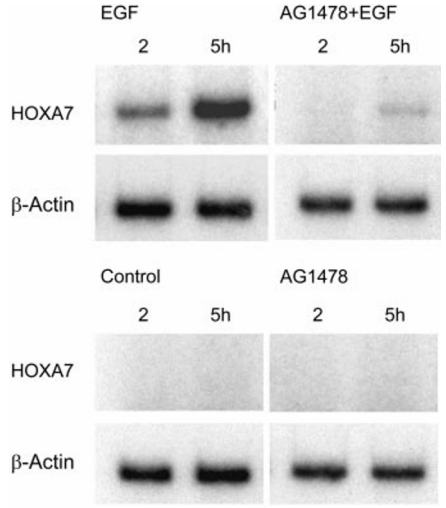

FIG. 7. **EGF activates expression of *HOXA7* in cultured keratinocytes.** NHK were cultured in serum-free medium, and cells at low density were switched to EGF-free serum-free medium for 30 h with one change of medium. Selected groups of wells were pretreated for 30 min with 3 μM AG1478, and some additionally with 5 ng/ml EGF, such that groups of wells received each of the following: EGF alone (*upper block, left column*), AG1478 and EGF (*upper block, right column*), no treatment (*Control, lower block, left column*), or AG1478 alone (*lower block, right column*). Total RNA was isolated from wells in each group at 2 and 5 h after EGF treatment, followed by RT-PCR, PAGE, and autoradiography for HOXA7 (*top rows*) and β-actin (*bottom rows*) message levels.

cline of HOXA7 seen after keratinocytes reach confluence is PKC-mediated. β-Actin message levels remained unaffected by TPA and bisindolylmaleimide treatment.

HOXA7 Expression Is Also Repressed, and TGM1 Is Activated, in NHK Stimulated to Differentiate with Calcium—NHK cultured in low calcium medium remain undifferentiated (8). Raising the calcium concentration above 0.1 mM induces differentiation-associated proteins and morphological changes, and *TGM1* expression rises in proportion to the extracellular calcium concentration (10, 11). We found that NHK demonstrated a drop in HOXA7 message levels by 5 h after raising the extracellular calcium concentration to 1.8 mM (Fig. 6, *top row*). High calcium treatment also stimulated a rise in the TGM1 message level within 10 h (*center row*), while β-actin message was unaffected. The HOXA7 message level in calcium-treated cells continued to fall at 48 h (not shown), but as seen in Fig. 5, HOXA7 message levels decline and TGM1 message levels rise even in untreated cells with the passing of successive days post-confluence, as the cells contact inhibit and begin to differentiate. It is therefore difficult to attribute changes solely to calcium signaling. Together, these data indicate that stimulation of differentiation, either following cell-cell contact, or by TPA or calcium treatment, or by a combination, results in a drop in HOXA7 and an increase in TGM1 message levels.

EGF Stimulation of Keratinocyte Proliferation Activates HOXA7 Gene Expression—*HOXA7* expression is down-regulated in differentiating keratinocytes, and overexpression represses the differentiation marker gene *TGM1*. These observations suggest that *TGM1* expression occurs upon differentiation when *HOXA7* expression is low and that the higher level of HOXA7 functions to block *TGM1* expression during proliferation. We therefore tested whether *HOXA7* expression is activated under conditions promoting keratinocyte proliferation. Adding back EGF to nonconfluent, EGF-starved NHK cultured in serum-free medium stimulated *HOXA7* expression relative to β-actin, as measured in total RNA by RT-PCR (Fig. 7, *upper left panels*). The increase was blocked by pretreatment with the selective EGF receptor tyrosine kinase activity inhibitor AG1478 (48) (Fig. 7, *upper right panels*). Control cells that received no EGF (*lower left panels*), or AG1478 alone (*lower right panels*), exhibited no detectable HOXA7 message.

Overexpression of HOXA7 Attenuates TPA-induced TGM1 Expression, and Antisense HOXA7 Activates Expression of Differentiation-associated Genes at the RNA and Protein Level in Nonconfluent Keratinocytes—Our data indicate that HOXA7 binds to the *TGM1* upstream regulatory region *K3*, transrepresses exogenous *TGM1* reporters in NHK, is turned off prior to *TGM1* gene activation in differentiating cells, and is turned on in proliferating cells at a time when *TGM1* is inactivated. To test whether modulation of *HOXA7* would also affect endogenous *TGM1* gene activity, NHK were transiently transfected with a HOXA7 expression vector and then treated with TPA or with an antisense HOXA7 expression construct. Fig. 8*a* shows that cells overexpressing HOXA7 (*HOXA7* +) exhibited a reduced TPA activation of *TGM1* compared with control vector transfected cells (HOXA7 −). The relatively small attenuation is believed to reflect the transfection efficiency of 20%, as determined by β-galactosidase staining (data not shown).

As seen in Fig. 8*b*, nonconfluent NHK transfected with a vector expressing antisense HOXA7 showed a dose-dependent increase in TGM1 message level. The 5′ fragment of the HOXA7 cDNA chosen for antisense expression (Fig. 1, *shaded sequence*) is not homologous to other human homeobox transcription factor sequences, or open reading frames of other known human genes, affirming that the TGM1 gene activation is the result of HOXA7 message targeting.

Since antisense HOXA7 up-regulated *TGM1* in transiently transfected normal human keratinocytes, the effect of anti-

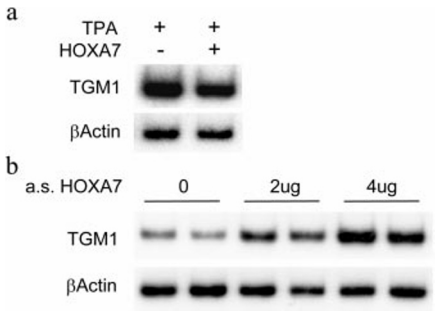

FIG. 8. **Overexpression of HOXA7 attenuates the TPA-induced expression of *TGM1* in NHK, and HOXA7 antisense overexpression causes a dose-dependent increase in *TGM1* gene expression in untreated NHK.** *a*, nonconfluent, proliferating NHK were transfected in 60-mm dishes with 16 μg of pcDNA3 (HOXA7 −, *left column*), or pcDNA3-HOXA7 (*HOXA7* +, *right column*), and 28 μl of LipofectAMINE. Cells were treated with 0.5 ng/ml TPA for 8 h, beginning 16 or 40 h after transfection. Total RNA was analyzed by RT-PCR for TGM1 (*top row*) and β-actin (*bottom row*) message levels. *b*, proliferating NHK in six-well plates were transfected in duplicate with 0 (*left column*), 2 (*middle column*), or 4 μg (*right column*) of the antisense-HOXA7 expression construct pEF3-HOXA7fragRev, plus control vector (pEF3) for a total of 6 μg of DNA and 4 μl of Fugene 6 per well. Relative amounts of TGM1 (*top row*) and β-actin message (*bottom row*) were assayed in total RNA isolated 48 h after transfection, by RT-PCR, PAGE, and autoradiography.

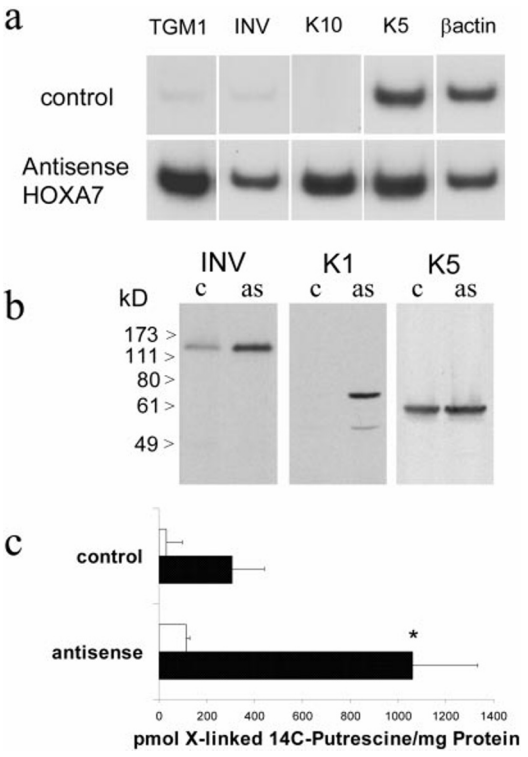

FIG. 9. **Antisense HOXA7-transfected HaCaT keratinocyte cell lines express the differentiation markers TGM1, involucrin, and keratin 10, at both the RNA and protein level.** *a,* stably transfected HaCaT keratinocytes expressing the neomycin selection marker introduced in the empty pCDNA3 vector (*control, top row*), or the HOXA7 antisense-containing vector pCDNA3–124fragRev (*Antisense HOXA7, bottom row*), were cultured at 60–70% confluence. Total RNA was analyzed for TGM1 (*first column*), involucrin (*INV, second column*), keratin 10 (*K10, third column*), keratin 5 (*K5, fourth column*), and β-actin (*sixth column*) message by RT-PCR, PAGE, and autoradiography. *b*, control (*c*) and HOXA7 antisense (*as*) expressing cells were cultured as in *a*, and SDS/antiprotease mixture-containing cell lysates separated by 8.5% polyacrylamide gel electrophoresis were blotted and probed with antibody against involucrin (*INV*, *left panel*), keratin 1 (*K1, center panel*) or keratin 5 (*K5, right panel*). *c*, transglutaminase type 1 enzyme activity was determined in control and antisense HOXA7 cells grown to 60–70% confluence, by [^{14}C]putrescine incorporation into dimethylcasein, followed by trichloroacetic acid precipitation and scintillation counting. Shown is a plot of picomoles cross-linked [^{14}C]putrescine per milligram of protein from three experiments ± S.D. *Open bars*, high speed supernatant after sonication (cytosol), containing mainly the ubiquitous transglutaminase type 2. *Filled bars*, detergent extract of the high speed pellet after sonication (particulate), containing the bulk of the keratinocyte-specific TGase1 activity. * indicates a statistically significant difference ($p < 0.05$) by analysis of variance and *t* test. Similar results were observed in at least three experiments.

sense HOXA7 expression in stably transfected cells was studied. Immortalized HaCaT keratinocytes were transfected with the HOXA7 antisense expression construct, selected, and cell lines were cloned and analyzed for expression of differentiation markers. Proliferating antisense cells showed an increase in the expression of the differentiation-specific genes *TGM1*, *involucrin*, and *keratin K10*, compared with vector control HaCaT (Fig. 9*a*). In contrast, the message level of the proliferating basal cell-associated keratin K5 increased to only a minor degree, and the control β-actin message was not altered, in the antisense cells. As seen in Fig. 9*b*, involucrin and keratin K1 proteins were also greatly up-regulated in the antisense HOXA7-transfected cells, while the keratin 5 protein concentration remained relatively constant. Proliferating antisense-transfected cells also contained an increased amount of TGase 1 enzyme activity compared with vector control cells (Fig. 9*c*), measured as putrescine incorporation into dimethyl casein. The TGase 1 enzyme is membrane-anchored and is found in the pellet after keratinocyte sonication and centrifugation. TGase activity in the cytosolic fraction represents the ubiquitous type 2 enzyme, which is down-regulated in differentiating keratinocytes, plus TGase 1 released from the plasma membrane by proteolytic cleavage. HOXA7 antisense expressing HaCaT also grew at a slower rate, as seen in Fig. 10. Expression of antisense RNA in antisense HOXA7-transfected HaCaT keratinocytes was verified by Northern blot analysis (Fig. 11*a*) and by RNase protection (Fig. 11*b*).

Taken together, these results demonstrate that the *HOXA7*, *HOXA5*, and possibly *HOXB7*, genes are down-regulated during differentiation, and *HOXA7* is up-regulated upon stimulation of proliferation. Furthermore, endogenous HOXA7 regulates differentiation-associated genes and affects cell growth rate in transfected cells, suggesting that these HOX factors represent an important component in the control of proliferation *versus* differentiation in keratinocytes.

DISCUSSION

Keratinocytes differentiate as cells withdraw from the cell cycle and migrate suprabasally in association with expression of differentiation-specific genes. Induction of keratinocyte differentiation *in vitro* is also associated with differentiation marker expression. This report establishes that keratinocyte expression of *HOX* genes *HOXA7*, *HOXA5*, and possibly *HOXB7*, is down-regulated upon TPA-induced differentiation *in vitro*. *HOXA7* is also down-regulated upon differentiation triggered by cell-cell contact or by raising the extracellular calcium concentration. Furthermore, antisense HOXA7 expression up-regulates the differentiation-associated genes *TGM1*, *involucrin*, and *keratins 1* and *10* in nonconfluent cells, and slows growth. HOXA7 protein binds to a *TGM1* regulatory DNA fragment *in vitro* and transrepresses a *TGM1*-reporter construct. These results provide evidence that HOXA7, likely in combination with other HOX transcription factors, functions in down-regulation of differentiation-associated keratinocyte genes prior to the onset of differentiation and that part of the differentiation program involves release of these genes from HOX inhibition.

We analyzed the role of HOXA7 in regulation of differentiation-specific keratinocyte genes. HOXA7 physically interacted

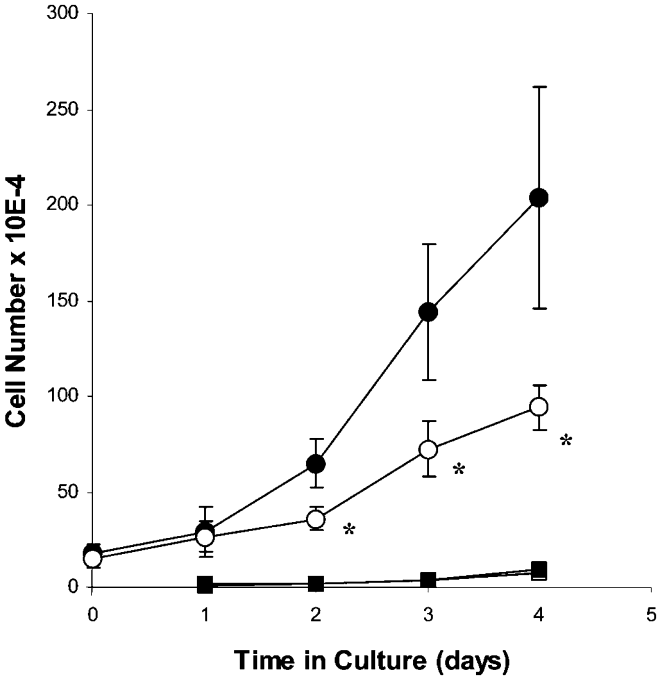

FIG. 10. **Antisense HOXA7 HaCaT cell lines are growth-inhibited compared with vector control cells.** Antisense HOXA7 or vector control HaCaT were plated at 1.5×10^5 cells/well in 35-mm dishes. Detached (HOXA7 antisense, *open squares*; control HaCaT, *filled squares*) and trypsin-released adherent cells (HOXA7 antisense, *open circles*; control HaCaT, *filled circles*) were counted every 24 h. Plotted is cells per well $\times 10^{-4}$ from five experiments ± S.D. * indicates a statistically significant difference ($p < 0.001$) by *t* test.

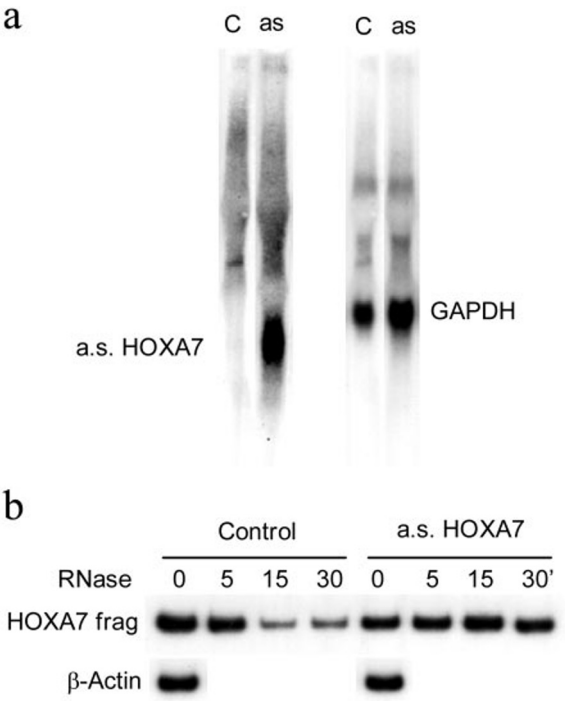

FIG. 11. **Antisense HOXA7 RNA is detected in antisense HOXA7-transfected HaCaT cells by Northern analysis and by RNase protection.** RNA from antisense HOXA7-transfected HaCaT, but not from vector control cells, hybridized a HOXA7 riboprobe (*a*). Total RNA from antisense HOXA7 and control vector-transfected HaCaT was RNase A/T-digested, and a HOXA7 sequence represented in the antisense expression construct was detected by RT-PCR (*b*).

with a fragment of the differentiation-specific TGM1 gene promoter *in vitro* and transrepressed a *TGM1* promoter fragment (40) linked to a reporter in normal human keratinocytes. Basal *TGM1*-reporter activity in the absence of the HOXA7 cDNA (Fig. 2) may represent saturation by the reporter construct of a limiting pool of inhibitory endogenous HOXA7 protein or depletion of a corepressor. Conversely, HOXA7 turned on the *TGM1*-reporter in ME180 carcinoma cells, which exhibit a high level *TGM1* expression (49) in association with abnormal growth. This observation demonstrates the potential of HOXA7 to act as a transcriptional activator. Whether a transcription factor transactivates or represses depends on the nature of its functional domains (DNA binding, protein-protein interaction, transcriptional activation, and repression), on the number, sequence, and relative positions of *cis* elements in the target, and on the presence of cofactors. The murine Hoxa7, over 90% identical to the human HOXA7 (Fig. 1), comprises both transcriptional activating and inhibitory domains, and the whole molecule has demonstrated both transcriptional repression (50) and activation (51). Multiple copies of Hoxa7, like other Hox proteins, exhibit cooperative binding and transactivation of targets with multiple recognition sites (51). Cooperativity involves proteins bound to nearby, as well as to distant sites, presumably through a DNA looping action (33). In addition, HOX proteins physically interact with the homeodomain cofactor PBX or MEIS1, or both, resulting in altered site recognition and cooperative DNA binding (35, 36, 52–54). Other known homeobox cofactors include the acetylase/integrator CREB-binding protein (37, 55), IκB and NF-κB (56), the glucocorticoid receptor (57, 58), and serum response factor (59–61), presaging the existence of more, potentially tissue-specific, cofactors. ME180 may constitutively express a HOXA7 cofactor that yields *TGM1* transcriptional activation, or that masks an inhibitory HOXA7 domain, such as the acidic C-terminal region (50).

In addition to repressing *TGM1* reporter activity, HOXA7 also down-regulated endogenous *TGM1* gene activity. HOXA7 overexpressed in normal keratinocytes attenuated the TPA-induced expression of *TGM1*. The maximum observed attenuation is limited by the transfection efficiency. Even if the overexpressed HOXA7 blocked all *TGM1* expression in transfected cells, the apparent attenuation would not exceed the measured transfection efficiency of 20%. The relatively small observed decrease in *TGM1* induction is therefore to be expected and nevertheless implies a large inhibition of *TGM1* activation in those cells transfected. TPA treatment activates PKC-α, and induces transcriptional activation by AP-1 factors (62), which can regulate differentiation-specific genes such as *TGM1* (63), *involucrin* (64), *filaggrin* (65), *keratins* (reviewed in Refs. 66 and 67)), and HPV-16 and -18 (68, 69). The observed HOX *versus* TPA antagonism could result from mutually exclusive promoter binding and function by HOX and AP-1 factors, as observed in the case of the POU factor Pit-1 gene autoregulation (70). Alternatively, since CBP binding has been observed to be obligatory and limiting in AP-1 transactivation (71, 72), and CBP also binds and enhances HOXB7 transactivation potential (37), HOXA7 and AP-1 antagonism may represent competition for available CBP. Whether or not the mechanism of the observed HOXA7 antagonism of TPA activity is so direct, HOXA7 may function to prevent basal AP-1 levels from activating differentiation markers in proliferating cells, or to delay expression until later stages of differentiation.

Since overexpression of the otherwise rare HOXA7 protein might affect transcription of genes outside its normal sphere of regulation, we determined whether endogenous HOXA7 regulates *TGM1* gene activity. Targeting endogenous HOXA7 by antisense expression resulted in marked *TGM1* gene activation, in both normal and immortalized human keratinocytes, in agreement with our *in vitro* binding and HOXA7 sense trans-

fection data. The antisense sequence is derived from a nonhomologous 5′ portion of the HOXA7 cDNA, excluding the conserved hexapeptide, homeobox, and acidic C-terminal-encoding regions (Fig. 1), so that the results reliably reflect selective HOXA7 transcript targeting. In this case, the observed TGM1 induction, the product of the fold induction within transfected cells, times the fraction of cells transfected, is not limited to the value of the transfection efficiency. That the observed induction (Fig. 8*b*) is severalfold means that the fold induction in transfected cells was large, indicating the presence of a *TGM1* gene activation signal, balanced by *HOXA7* inhibition, in proliferating cultured keratinocytes. In immortalized HaCaT keratinocytes, stable transfection with antisense HOXA7 up-regulated not only *TGM1*, but also *involucrin*, and *keratin 1* and *keratin 10* expression, and resulted in slower growth compared with vector control cells, suggesting that HOXA7 regulates multiple genes associated with differentiation.

Keratinocyte differentiation, induced by TPA treatment (Fig. 4), by raising extracellular calcium (Fig. 6), or by prolonged high density culture (Fig. 5), down-regulates *HOXA7* expression. We therefore investigated whether mitogenic activation stimulates HOXA7 message levels. We found that *HOXA7* expression is induced by EGF receptor activation, supporting the hypothesis that HOXA7 helps silence *TGM1* during proliferation, until the appropriate time during differentiation. Further investigation will be required to define the relevant regulatory pathways in more detail. It will be interesting to determine whether the *HOXA7* and *HOXA5* genes are down-regulated by AP-1 factors activated in differentiating keratinocytes. Also, our results indicate that HOXA7 modulates both the early differentiation markers *K1* and *K10*, and the later marker *TGM1*, and is itself regulated by both calcium and TPA treatment, whereas TPA treatment of mouse keratinocytes activates late differentiation markers but blocks calcium-induced expression of *K1* and *K10* (73).

Overlapping expression and function of *Hox* genes is a common theme in development. In mice, disruption of *Hoxb6*, *Hoxa7*, *Hoxb7*, and *Hoxb9* all contribute to first and second rib defects. *Hoxa7* disruption alone causes no defects, but adding *Hoxa7* mutations markedly increases the rate and severity of rib defects observed in *Hoxb7*−/− mice, suggesting that Hoxa7 has functional roles that were not revealed in the *Hoxa7*−/− mice, and that these two genes act together (74). Hoxa7 may also have a functional role in the epidermis, requiring closer examination of morphology and differentiation marker expression, requiring some additional challenge, or requiring a different double mutant gene partner, such as *Hoxa5*, for manifestation. Synergistic and overlapping functions have also been observed with *Hoxa3*, *Hoxb3*, and *Hoxd3* and with *Hoxa11* and *Hoxd11* disruption in mouse development (reviewed in Ref. 75). Overlapping function of groups of HOX factors regulating sets of target genes may be retained, following cessation of development, where continual proliferation and subsequent differentiation occur. In keeping with this idea, Stelnicki *et al.* (32) detected predominantly HOXA4, HOXA5, and HOXA7, but also HOXB7 and HOXC4 message, in fetal and adult human epidermis, but not in the dermis, by RT-PCR using a set of degenerate *HOX* gene primers. *HOXA7* was consistently the most frequently identified gene upon cloning and sequencing the PCR products, suggesting an important role in epidermal development and homeostasis. These findings are in good agreement with our results from neonatal human keratinocytes, where *HOXA7* and *HOXA5* were expressed much more highly than *HOXB7*, although we detected only a weak *HOXA4* signal. They observed further a suprabasal expression pattern of HOXA4, HOXA5, and HOXA7 in neonatal and adult epidermis. Interestingly, we measured a transient increase in HOXA7 message level as cultured keratinocytes reach confluence (not shown), as well as compelling evidence of HOXA7 down-regulation upon PKC activation and up-regulation with EGF receptor activation. The apparent conflict is resolved if we postulate that HOXA7 functions as an inhibitor of differentiation during proliferation, but also as a brake system during the onset of differentiation. Small amounts of HOXA7 may suffice to inhibit expression of differentiation-specific genes during proliferation, when differentiation signal-transduction pathways are nearly idle. *HOXA7* expression may be activated progressively, in parallel with differentiation signals during early stages of differentiation, to limit the rate and extent of potentially destructive elements of the process, allowing completion of important intermediate steps involving protein synthesis and vesicle transport. This would account for the reported increase in suprabasal expression by *in situ* hybridization. *HOXA7* expression may ultimately be silenced, as mimicked by our TPA treatment of keratinocytes, at a time appropriate for completion of the keratinization process.

We found that, in addition to *HOXA7*, the *HOXA5*, and possibly the *HOXB7* gene (very low expression was observed), is also down-regulated in cultured keratinocytes induced to differentiate with TPA. It would be interesting to ascertain whether combined *HOXA5* and *HOXA7*, and even *HOXB7* disruption, or inducible ectopic epidermal coexpression, would yield a pronounced skin defect. Interestingly, our HOXA5 RT-PCR product comprised two bands (Fig. 4), raising the possibility that like HOXB6 (14), HOXA5 exhibits differential expression of alternatively spliced message, and perhaps protein, although the band of anomalous electrophoretic migration rate may represent an RT-PCR artifact. These results support the contention that regulation of keratinocyte differentiation involves multiple HOX gene family members, including *HOXA7* and *HOXA5*, and perhaps *HOXB7*.

It may be worth noting that expression of all three of these genes has been associated with a nondifferentiated state or with cellular proliferation. Among HOX genes, *HOXA7* and *HOXB7* are highly expressed in chemically induced papillomas (76), suggesting a shared function in the etiology of growth deregulation. Murine Hoxa7 and Hoxa9 cooperativity with Meis-1 has been implicated in murine myeloid leukemia (54). Overexpression of Hoxa5, Hoxa7, or Hoxb7 leads to transformation and tumorigenicity in two fibroblast cell lines. HOXA5 and HOXB7 are associated with hematopoietic progenitor proliferation (77, 78), and *Hoxa4* and *Hoxa5* are up-regulated in association with inhibition of differentiation by retinoic acid in the developing mouse lung (79). Furthermore, *HoxB7* is expressed in proliferating mammary epithelial cells and disappears with matrix-induced differentiation (80). Finally, *HOXB7*, normally expressed in proliferating melanocytes, is up-regulated in, and implicated in the enhanced growth of, metastatic melanomas (81, 82). However, these observations represent potential function, since examples of induction of differentiation can also be cited, and further studies are required to fully elucidate the role of HOX genes in regulation of keratinocyte proliferation and differentiation.

We isolated HOXA7 from a human keratinocyte library, via specific binding to the HPV-16 epithelial-dependent enhancer, which restricts viral oncogene *E6*/*E7* expression to differentiating keratinocytes (44). The HPV-16 epithelial-dependent enhancer may fall within the spectrum of differentiation-dependent genes repressed by HOXA7. Inspection of the enhancer fragment suggests that HOXA7 may bind to a HOX consensus core motif, preventing cooperative Oct-1/NF-1 transactivation (83) at an overlapping site, thus adding to the repression me-

diated by YY1 steric inhibition of AP-1 and Sp1 transcription factors (84). Down-regulation of *HOXA7* may contribute to *E6/E7* activation, in combination with up-regulation of HPV-16 transactivation factors skn-1a (26, 85), AP-1 (86), and Sp1 (84). Although the HOXA7 message appears to be rare (32), the stability and steady-state accumulation of HOXA7 protein in proliferating keratinocytes remains to be determined.

In summary, these results suggest that *HOXA7*, likely in combination with other *HOX* genes, plays an important role in regulating keratinocyte *TGM1* expression and possibly more generally in regulating the expression of differentiation-specific keratinocyte genes. These properties have implications for HOX involvement in keratinocyte oncogenesis, and in keratinocyte-specific viral activation, that warrant continued investigation.

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