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 HOX5 and HOX7 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 HOX5 and HOX8, 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). EGF3 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

The abbreviations used are: EGF, epidermal growth factor; TGM1, transglutaminase 1 gene; TGate1, 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 FOU 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, HOAX5, 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 Droso- sophila 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 homodomain 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, HOAX5, 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 keratinoocytes 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, HOAX5, 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 Agt11 from human keratinocyte messenger RNA, was screened for expressed proteins that recognize a 232-bp HPV-16 E6/E7 enhancer Dral fragment (bp 7524–7756) by a standard method (38). Briefly, phage plaques on cultures of infected Escherichia coli were overlaid with nitrocellulose filters pre-treated 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 six washes in decreasing amounts 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 32P-labeled 252-bp Dral 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 Dral control fragment (bp 7726–7495). 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 pCDNA1 and expressed in COS-7 cells.

**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. More than 50% of the immortalized keratinocytes cells were obtained from the American Tissue Culture Collection (ATCC) and grown in RPMI 1640 (Bioswichtaker, 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′-GTCGGCGATGGG- TCCTCCTGATATTGCG-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′-ATCTGC- GAGTAGATGCCGAATTTGG-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 vector pc92 (p-S2-HOXA7), pCDNA3 (Invitrogen) (p5- HOXA7, p3-HOXA7 frag), 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/HiasA (Invitrogen) by partial digestion with HindIII/BglIII, 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 (Amshamer Pharmacia Biotech) into the human β-actin promoter-driven expression vector pHβA-Pr-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 required 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 (CAT) activity (14, 42) and 124 fragment, based on CoA by thin layer chromatography and autoradiography, and by xylene extraction and scintillation counting, presented as counts/min acetyl-[14C]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 were expressed as relative activity per milligram of protein. Cotransfection of expression vectors with empty reporter vectors 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-galactosidase in 40 ml citrate phosphate buffer, pH 7.5, with 5 mM ferrous- and ferricyanide, 2 mM MgCl2, 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 × 105 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 × 10–4 ± 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 Agt11-transformed 1090 E. coli by induction of transcription and translation 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 7726–7495) Dral fragments of HPV-16 were released by restriction digestion, purified, and 32P-labeled. Binding reactions containing 4 × 104 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 HOX7 binding to the TGM1 upstream regulatory region (K3), recombinant HOX7 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, [35S]methionine was added, and translation 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 DraI HPV-16 fragment, and a 212-bp neighboring K3 fragment (5’ end at −444), were prepared by PCR (bp −709 to −90), digested with BamHI and HaPII, purified, 32P-end-labeled, and shifted with 5 μg of the HOX7 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). 32P-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 HOX7 cDNA (Fig. 1), and pBluescript-GATC (Stratagene, La Jolla, CA). Human recombinant 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% urea (Sequagel, National Diagnostics, Atlanta, GA), eluted (60 min, 37 °C, 0.5 M ammonium acetate, 1 μl EDTA, 0.1% sodium dodecyl sulfate, and 50 μg/ml yeast RNA, precipitated (1× ammonium acetate and 3 volumes of 2-propanol), dissolved in H2O, 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 unit in 100 μl RNAse A/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 HOX7 RNA in stably transfected HaCaT, total RNA was hybridized as above, without the addition of probe, digested with RNase A/F at various annealing temperatures, extracted and precipitated, before detection of 5’ HOX7 RNA by RT-PCR. The PCR product, spanning bases 291–451 (Fig. 1), was produced using the HOX7 forward primer described below under “RT-PCR” and the reverse primer 5’-CTCGTCCGGTCTCTGCCCATCTG-C-3’. RT-PCR—To determine the range of target DNA concentrations giving rise to a linear relationship between target concentration and product signal, restriction was removed from a 264-bp K3 fragment of 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 μM MgCl2, 400 μM primers, 400 μM each dNTP (Roche Molecular Biochemicals), and 5 μC of [32P]dCTP. Samples were removed after 30 cycles of 94 °C, 20 s; 57 °C, 20 s; 72 °C, 30 s, and 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 μl oligo(dT) primer (Invitrogen) and 1.25 μM 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/ml RNAse inhibitor (Promega) and 10 μM 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 H2O and added at 0.1 volume to a 30-cycle PCR reaction under the above conditions gave rise to a signal intensity corresponding to target concentration for HOX7 and TGM1 over 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. HOX5 and HOX7 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 HOX7 and transglutaminase 1 PCR primer sequences span intro

** RESULTS**

Isolation of the HOX7 cDNA through Binding to the HPV16 E6/E7 Enhancer—Epidermal differentiation results from the coordinated expression of keratinocyte genes, perhaps by the

**Homeobox HOX7 Regulates Transglutaminase 1**
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 DraI 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).

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.

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, and its mouse homolog Hoxa7, we examined the affect of transiently overexpressed HOXA7 on TGM1-K3 reporter activity. HOXA7 overexpressed a sac-myc fusion (pCS-HOXA7) in primary neonatal keratinocyte (NHK) repressed transcriptional activity of K3 (pCAT-K3) relative to the empty vector (Fig. 2a). 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 vector (Fig. 2b).
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. 3a, 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 [35S]methionine by SDS-PAGE and autoradiography (Fig. 5). Protein synthesis was monitored in control reactions containing the empty vector pCDNA3 and control reaction containing the empty vector pCDNA3 (left lane), and expression of the luciferase gene product (positive control). Two additional experiments produced comparable results.

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. 4a). The concentration of HOXA7 RNA was also reduced as measured by RT-PCR analysis (Fig. 4b, 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. 4c), 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 effect 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. 4b, 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-
cline of HOXA7 seen after keratinocytes reach confluence is PKC-mediated. β-Actin message levels remained unaffected by TP 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 TP 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. 8a 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. 8b, 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-
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. 9a). 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. 9b, 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 TIGase 1 enzyme activity compared with vector control cells (Fig. 9c), measured as putrescine incorporation into dimethyl casein. The TIGase 1 enzyme is membrane-anchored and is found in the pellet after keratinocyte sonication and centrifugation. TIGase activity in the cytosolic fraction represents the ubiquitous type 2 enzyme, which is down-regulated in differentiating keratinocytes, plus TIGase 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. 11a) and by RNase protection (Fig. 11b).

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 conjunction 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...
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 coexpressor. 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 activation 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 overexpression 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,

![Graph](image)

**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 x 10^5 cells/well in 35-mm dishes. Detached (HOXA7 antisense, open squares; control HaCaT, filled circles) and trypsin-released adherent cells (HOXA7 antisense, open circles; control HaCaT, filled circles) were counted every 24 h. Plotted is cells per well x 10^-4 from five experiments ± S.D. * indicates a statistically significant difference (p < 0.001) by t test.

![Graph](image)

**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).

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. 8b) 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 HOX5 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 Hoxb7−/− 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 Hoxa11 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 HOXB7, 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 HOX5 were expressed much more highly than HOXB7, although we detected only a weak HOX4 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 ideal. 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 HOX5, 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 HOX7, and even HOXB7 disruption, or inducible ectopic epidermal coexpression, would yield a pronounced skin defect. Interestingly, our HOX5 RT-PCR product comprised two bands (Fig. 4), raising the possibility that like HOXB6 (14), HOX5 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 HOX5, 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. HOX5 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 oncoprotein 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-
HOX7, likely in combination with other HOX genes, plays an important role in regulating keratinocyte TG1 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.

In summary, these results suggest that HOX factors, that warrant continued investigation for HOX involvement in keratinocyte oncogenesis, and in keratinocyte-specific viral activation, that warrant continued investigation.
Human Homeobox HOXA7 Regulates Keratinocyte Transglutaminase Type 1 and Inhibits Differentiation

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