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Transcription Regulation and Protein Subcellular Localization of the Truncated Basic Hair Keratin hHb1-ΔN in Human Breast Cancer Cells*

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An aberrant truncated hHb1 hair keratin transcript, named hHb1-ΔN, was previously identified in breast carcinomas. No normal tissue tested so far, including hairy skin, expressed hHb1-ΔN, indicating that hHb1-ΔN is related to carcinogenesis. In the present study, we investigated the mechanism by which such truncated transcript was generated in breast cancer cell lines. We found that hHb1-ΔN transcription is initiated at an unusual cryptic promoter within the fourth intron of the *hHb1* gene and is dependent on two proximal Sp1 binding sites for its baseline activity. Moreover, hHb1-ΔN transcription is increased in response to DNA demethylation by the 5-aza-2'-deoxycytidine drug. This induction is dependent on protein neosynthesis, indicating that an additional factor is required. In addition, we showed that the hHb1-ΔN transcript is translated *in vivo* as a truncated hHb1 protein that is missing the 270 amino-terminal residues. The hHb1-ΔN protein exhibits a filament pattern throughout the cytoplasm and partially co-localizes with cytokeratin filaments, indicating its participation in the cytoskeleton network. hHb1-ΔN might alter the adhesive properties of cancer cells.

Keratin intermediate filaments are expressed in various epithelial tissues. Two types of keratin, the acidic type I and the basic type II, combine in a stoichiometric fashion to form the fundamental unit. Keratin heterodimers further associate to form the 10-nm intermediate filament network (1). Simple or stratified epithelia differentially express pairs of “soft” or cytokeratins, whereas hard epithelia such as hair or nails express specific pairs of “hard” or hair keratins. It has been assumed from biochemical data that hair follicles express four different pairs of keratins. However, nine human acidic hair keratin genes have been identified and classified into three subgroups according to gene structure and sequence conservation (2). From the nine likely basic heterodimeric partners, six genes have been cloned (3).

By differential screening of a cDNA library established from metastatic lymph nodes derived from a breast cancer, we previously identified the MLN137 cDNA that proved to be identical to the 3'-half of the human hair keratin basic 1 (hHb1)¹ cDNA (4). Using 5'- and 3'-probes specific for the hHb1 hair keratin, we established that breast carcinomas specifically express a 5'-truncated form of the hHb1 mRNA, including the second α-helical subdomain and the specific carboxyl-terminal tail domain. MLN137 was therefore called hHb1-ΔN, because the putative protein corresponded to an hHb1 keratin truncated at its amino terminus. *In situ* hybridization showed that the hHb1-ΔN mRNA is ectopically expressed in malignant epithelial cells of primary breast carcinomas and metastases (5). This truncated transcript has thus far never been detected in hairy skin samples.

Aberrant forms of soft keratins have been shown to be responsible for several epidermal genetic diseases (6). Moreover, abnormal cytokeratin expression patterns have been widely used as tumor markers, because they correlate with different types of epithelial differentiation and function (7). Some recent data have revealed point mutations in the hHb1 and hHb6 hair keratins in monilethrix, a rare inherited hair disorder (8–10). In addition, hHb1 expression has been observed in pilomatrixomas, epidermal tumors exhibiting follicular differentiation (11, 12). hHb1-ΔN is, however, the first hair keratin whose expression is detected in carcinomas derived from a tissue different from the epidermis.

Several studies have demonstrated that amino- or carboxyl-terminal-truncated cytokeratins can be incorporated into the intermediate filament network. Keratin proteins bearing small deletions are incorporated without evident modification of the cytoskeleton, whereas larger deletions can lead to the complete collapse of the intermediate filament network (13, 14). Intermediate filaments are likewise physiologically composed of obligatory heterodimers of specific cytokeratins or hair keratins. Forced expression of a foreign keratin through transient transfection showed that it can still efficiently incorporate into the pre-existing network as newly formed intermediate filaments (13, 14). The expression of a truncated keratin could thus have significant relevance for cancer progression, because it could lead to severe disturbances of the intermediate filament network itself and of its interaction with desmosomal or hemidesmosomal proteins, which are essential for the maintenance of cell integrity (6).

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¹ The abbreviations used are: hHb1, human hair keratin basic 1; 5-Aza-2'dC, 5-aza-2'-deoxycytidine; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; DTT, dithiothreitol; GFP, green fluorescence protein; bp, base pair(s); kb, kilobase(s).

Hair is a complex specialized epithelial structure, and hair keratin gene expression patterns are precisely controlled. However, the mechanisms by which hair keratin genes are regulated are poorly understood. Few human hair keratin gene promoters have been studied so far. Comparison of gene promoters of hair keratin and hair keratin-associated proteins of human, mouse, and sheep origin have allowed for the identification of conserved putative regulatory elements (15). Several promoters share consensus binding sites for the Sp1, AP1, AP2, and NF-1 transcription factors. Nevertheless, a prominent motif is recognized as a binding site for lymphoid enhancer factor 1, originally identified as a pre-B and T cell-specific protein (16). A central role for this factor in hair keratin gene transcription and hair follicle patterning has been demonstrated by various mouse transgenesis experiments (17, 18).

In the present study, we characterized the mechanisms leading to the ectopic expression of the truncated *hHb1* hair keratin in breast carcinomas. *hHb1-ΔN* expression does not result from a translocation or rearrangement of the *hHb1* locus. We actually demonstrated that its transcription is controlled by a cryptic promoter present in the 4th intron of the *hHb1* gene. In addition, we studied the subcellular localization of the *hHb1-ΔN* protein in cancer cells.

EXPERIMENTAL PROCEDURES

Cell Cultures—The HBL100 breast-immortalized cell line, the MCF7, SKBR3, and T47D breast cancer cell lines, and the HeLa cervix cancer cell line were maintained in culture with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 0.6 μg/ml insulin (for MCF7 and T47D). When the cells were treated with 5-aza-2'-deoxycytidine (5-Aza-2'dC, Sigma Chemical Co., St. Louis, MO), 4 × 10⁵ MCF7, 2 × 10⁶ SKBR3, and 8 × 10⁵ T47D cells were seeded in 10-cm dishes (day 0). 5-Aza-2'dC was added to the growth medium (at 0.5, 2, or 10 μg/ml) in two (or three, as indicated) 24-h pulses on days 2 and 5 (and day 8). Cells were used 24 h after treatment for RNA isolation. In experiments using cycloheximide (Sigma), 2 μg/ml 5-Aza-2'dC was first added to the medium for 5 days and cells were additionally treated with 2, 10, or 50 μg/ml cycloheximide for the last 72 or 24 h.

cDNA Probes—The *hHb1* 5'- and 3'-specific cDNA probes and the 36B4 (acidic ribosomal protein, GenBank[®] accession number M17885) cDNA probe have already been described (5). Exon 9 of *hHb6* gene was amplified by PCR from SKBR3 genomic DNA using the following primers: 5'-GAGAGAATTCCTACTGCCCCCTGTGTCTC and 5'-GAGAGAATTCCTACAGAGAGAGACCATGGCG (*EcoRI* sites are underlined), cloned into pBluescript and used as a specific probe. All probes were labeled by random priming with [^α-³²P]dCTP using the Klenow enzyme (Amersham Pharmacia Biotech).

Genomic DNA Isolation and Southern Blot Analysis—HBL100, MCF7, SKBR3, and T47D cells were plated in 15-cm dishes. At confluency, cells were washed twice with PBS and lysed in (10 mM Tris, pH 8, 0.1 M EDTA, 20 μg/ml boiled pancreatic RNase, 0.5% SDS, 100 μg/ml proteinase K (Roche Diagnostic Corp., Berkeley, CA)) for 16 h at 42 °C. Genomic DNA was ethanol-precipitated and resuspended in 1 mM Tris, pH 7.5, 0.1 mM EDTA.

20 μg of MCF7, SKBR3, and T47D genomic DNA was digested with the appropriate restriction enzymes under conditions recommended by the supplier (New England BioLabs), separated by 0.8% agarose gel electrophoresis, and transferred onto a Hybond N⁺ nylon membrane according to the manufacturer (Amersham Pharmacia Biotech). Hybridization with the *hHb1* 3'-cDNA probe was performed in the same conditions except for the Northern blot (see below).

RNA Isolation and Northern Blot Analysis—Cell cultures were washed with phosphate-buffered saline (PBS), and RNA extraction was carried out by the guanidinium thiocyanate phenol/chloroform procedure as previously described (19). Northern blot was processed as previously described (4). Briefly, 10 μg of total RNA was denatured at 65 °C for 5 min and electrophoresed on a 1% agarose gel in the presence of 2.2 M formaldehyde, prior to transfer onto a Hybond-N nylon membrane (Amersham Pharmacia Biotech). The Northern blot was hybridized with the appropriate probes for 24 h at 42 °C.

Primer Extension—Primer extension was performed according to the protocol described in a previous study (20). Two picomoles of antisense oligonucleotides complementary to *hHb1-ΔN* cDNA (either 5'-CG-GCAATGATGCAGTCCATGT or 5'-GACAATGTCGTCACTGTGC)

were labeled at the 5' terminus with [^γ-³²P]ATP using the T4 DNA polynucleotide kinase (New England BioLabs). The probe (10⁶ cpm) was co-precipitated with 40 μg of SKBR3 total RNA, or liver total RNA as a negative control, and resuspended in 30 μl of hybridization buffer (80% formamide, 40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA, pH 8). After heat denaturation, hybridization was performed overnight at 37 °C. After ethanol precipitation, cDNA was elongated by 40 units of avian myeloblastosis virus reverse transcriptase (Amersham Pharmacia Biotech, Uppsala, Sweden) in 25 μl of elongation buffer containing 3.5 μl of dNTP (4 mM each), 5 μl of reverse transcriptase buffer, 1.25 μl of RNasin for 90 min at 42 °C. The reaction was stopped by addition of 1 μl of EDTA 0.5 M, and RNA was digested 30 min at 37 °C with 1 μg of RNase A. The elongated cDNA was extracted with phenol/chloroform, ethanol-precipitated, and resuspended in 6 μl of loading buffer (98% formamide, 10 mM EDTA, 0.025% xylene cyanol blue, 0.025% bromophenol blue). The single-stranded cDNA was resolved by electrophoresis on a 6% polyacrylamide denaturing gel containing 8 M urea. *hHb1* cDNA sequencing reactions were performed using the same antisense oligonucleotides according to the manufacturer (Amersham Pharmacia Biotech, Cleveland, OH) and run in parallel to localize the initiation sites of transcription. The gel was vacuum-dried and exposed 24–36 h for autoradiography.

RACE-PCR—RACE-PCR was performed according to the manufacturer (Marathon cDNA Amplification Kit, CLONTECH Laboratories Inc., Palo Alto, CA). Briefly, 2 μg of SKBR3 poly(A)⁺ RNA (5) were reverse-transcribed by 20 units of avian myeloblastosis virus reverse transcriptase. Double-stranded cDNA was ligated to the Marathon cDNA Adaptor by 1 unit of T4 DNA ligase. The 5'-end of the *hHb1-ΔN* cDNA was first amplified by PCR using 5 units of *Taq* polymerase (Sigma Chemical Co.) in the presence of the sense adaptor primer (AP1) and the antisense primer 5'-GACTGAGCCACCGGGCTCCAGCTTG (designed in exon 6) specific of the *hHb1-ΔN* cDNA. The *hHb1-ΔN* cDNA was further amplified using internal primer 5'-GATCTCCTCT-TGGTGC GGCG (designed in the exon 5) and cloned into the pTAdv vector (CLONTECH Laboratories). Thirty *hHb1-ΔN* clones were sequenced.

Plasmid Constructs—The *hHb1* gene was PCR-amplified from SKBR3 and HBL100 genomic DNA using standard conditions with 5 units of Deep Vent polymerase (New England BioLabs) in the presence of 200 μM of each dNTP and 50 pmol of each of the following primers: 5'-primer, 5'-GAGAGAATTCACCCAAACGTCAGGAGGATCATCATG and 3'-primer, 5'-GAGAGAATTCGTAGCTGAGCACTGTCTCCAGGGCG (*EcoRI* restriction sites are underlined). The *hHb1* gene was cloned into the *EcoRI*-digested pBluescript plasmid (Stratagene Inc., La Jolla, CA). The nucleotide sequence was verified from independently amplified genes.

Luciferase constructs containing varying lengths of the *hHb1-ΔN* promoter were generated by PCR and inserted upstream of the firefly luciferase reporter gene into *SacI/XhoI*-digested pGL3basic vector (Promega Corp., Madison, WI). The following primers were used, their positions are referred relative to the most upstream transcription initiation site of *hHb1-ΔN*: the sense primers are 5'-ATTTCTCTATCGATAGGTACCGACTCACGATCAGGATTT (-331/+22), 5'-ATTTCTCTATCGATAGGTACCGACTCGATGTGGACTGCGCCTCA (-305/+22), 5'-ATTTCTCTATCGATAGGTACCGACTGCTCCTGAGGGCGGCTGT (B; -235/+22), 5'-ATTTCTCTATCGATAGGTACCGACTCCTCGGGAGTGTGGACAGGAT (C; -150/+22), 5'-ATTTCTCTATCGATAGGTACCGACTCGGACCAGGAAAGCCTGA (D; -85/+22), 5'-ATTTCTCTATCGATAGGTACCGACTCCTCTGTGTTCTCAACTAAA (E; -38/+22), and the antisense primer is 5'-AAGCTTACTTAGATCGCAGATCTCGAGTTGACAACACCGAGGTTG for all the constructs (*SacI* and *XhoI* restriction sites are, respectively, underlined).

Potential positive regulatory fragments of the *hHb1-ΔN* promoter were PCR-amplified and inserted upstream of the SV40 promoter into the pGL3promoter vector using the sense primers 5'-GAGACTCGAGACTTCTCTGAGGCGGCTGTATG (position B; -235/-214), 5'-GAGACTCGAGGGGAGTGTGGACAGGATGTG (position C; -150/-129), and the antisense primers 5'-GAGACTCGAGACCCCAACTCTCTCTCTG (position C; -172/-151), 5'-GAGACTCGAGCCAAACCTGCCCTCACACA (position D; -107/-86), 5'-GAGACTCGAGCCAAACCCACTGTCCTGACT (position E; -60/-39). A double-stranded oligonucleotide (DE; -85/-39) corresponding to the annealed oligonucleotides 5'-TCGAGGAATTCGGACCAGAGAAAGCCTGAGGAGGTGGAGTCA-GGACATGGTGGGTTGGC, and 5'-TCGAGCCAAACCCACCATGTCTGACTCCACTCTCAGGCTTTCTCTGGTCCGAATTCC, was inserted into the pGL3promoter plasmid.

Mutation of Sp1 and GT box binding sites was performed by site-directed mutagenesis (21) in the -85/+22 construct using the primers

5'-TGTCCTGACTGGAATTCTCAGGCT and 5'-GAGAACACAGC-CCCATATGCCATGTCTGACTC (mutated nucleotides (underlined) generate *EcoRI* and *NdeI* restriction sites (italics)).

All the constructs were verified by sequencing.

Promoter Activity Analysis—MCF7 cells were transiently transfected in 10-cm dishes using the calcium phosphate procedure with 5 μg of luciferase reporter plasmid, 5 μg of pCH110 plasmid (Amersham Pharmacia Biotech) as an internal control for normalization and 5 μg of pBluescribe plasmid. After an overnight incubation, cells were washed and further incubated for 24–36 h. The cells were washed and scrapped in PBS, transferred in an Eppendorf tube, and lysed with 150 μl of passive lysis buffer (Promega Corp., Madison, WI). Cell debris were removed by centrifugation. 50 μl of cell extracts were assayed for luciferase activity in an EG&G Berthold luminometer as outlined by the manufacturer (Promega Corp.). The results were normalized for β-galactosidase activity. All transfections were performed in duplicates or triplicates and repeated three times.

Nuclear Protein Extraction—Nuclear extracts were performed as previously described (20). Briefly, subconfluent cell cultures were washed twice with PBS and resuspended in 0.8 ml of low salt buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (including pepstatin, antipain, aprotinin, leupeptin, and chymostatin at 2.5 μg/ml each, Roche Molecular Biochemicals, Indianapolis, IN)). After 10 min on ice, the lysate was centrifuged and the pellet was resuspended in high salt buffer C (20 mM HEPES-KOH, pH 7.9, 26% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture). Nuclei were incubated for 30 min on ice and centrifuged to remove cell debris. Protein concentration was estimated by Bradford assay.

In Vitro DNase I Footprinting—Footprinting was performed as previously described (20). The -305/+22 promoter deletion fragment was cloned into pBluecript SK+ and released by *EcoRI* and *EagI* digestion. The resulting fragment was isolated and purified to label the coding and non-coding strands by incorporating either [α -³²P]dCTP or [α -³²P]dATP (filling in the *EagI* and *EcoRI* overhangs, respectively) with the Klenow enzyme. The purified labeled probes (20,000 cpm) were mixed with 1 μg of poly[d(I-C)], 50 μg of nuclear extract, and 50 μl of binding buffer (20 mM HEPES-KOH, pH 7.9, 20% glycerol, 1 mM CaCl₂, 10 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT), in the presence of 60 mM KCl in a final volume of 100 μl. Binding was allowed to proceed for 15 min at room temperature. DNA was digested for 2 min by DNase I (grade I, Roche Molecular Biochemicals, Indianapolis, IN) at 15 milliunits/μl for naked DNA, and 1–4 units/μl DNase I in the presence of protein extract. The reaction was stopped by addition of 100 μl of stop solution (1% SDS, 200 mM NaCl, 20 mM EDTA, pH 8, 40 μg/ml tRNA, 100 μg/ml proteinase K) 15 min at 37 °C. DNA was extracted with phenol/chloroform, precipitated and resuspended in 5 μl of denaturing loading buffer. Digested DNA was resolved by electrophoresis on a 6% polyacrylamide denaturing sequencing gel containing 8 M urea. Sequencing reactions of known sequences were run in parallel to define the protected nucleotides. The gel was vacuum-dried and exposed 24–36 h for autoradiography.

Electrophoretic Mobility Shift Assay—Double-stranded oligonucleotides with 5'-overhangs (20): Sp1, 5'-GTCTGAGGAGGTGGAGTCA and 5'-GACTCCTCCACCTCAGTTG; mutated Sp1, 5'-GTCTGATGAGGTTGAGTCA and 5'-GACTACTCCA~~ACT~~CAGTTG; GT box, 5'-GTTTCAGGACATGGTGGGTGGGGCTGTGTTCTCA and 5'-AGTCCTGTACCACCACCCCGACACAAGAGTTG; mutated GT box, 5'-GTTTCAGGACATGGCATA~~CGGG~~CTGTGTTCTCA and 5'-AGTCCTGTACCGTATGCCCGACACAAGAGTTG (core binding site or the mutated nucleotides are underlined) were labeled with [α -³²P]dCTP and [α -³²P]dATP using Klenow polymerase. Labeled probes (20,000 cpm) were added to 20 μl of binding reaction containing 10 μl of binding buffer (20 mM HEPES-KOH, pH 7.9, 20% glycerol, 1 mM CaCl₂, 10 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT), 0.8 μg of poly[d(I-C)] and 60 mM KCl and incubated with 6 μg of nuclear extract for 15 min at room temperature. Competition and supershift assays were performed by adding to the binding reaction a 100× excess of cold oligonucleotide and 0.4 μg of anti-Sp1 and/or anti-Sp3 polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA), respectively. The protein-DNA and protein-DNA-antibody complexes were separated from the free probe by electrophoresis through a 5% non-denaturing polyacrylamide gel. The gels were vacuum-dried and exposed for autoradiography.

Protein Extraction and Western Blot Analysis—The rabbit polyclonal antiserum 1622 was obtained by immunization with the synthetic peptide GISSLVGSCGSSCRKC corresponding to the last carboxyl-terminal residues of hHb1 coupled to ovalbumin using the

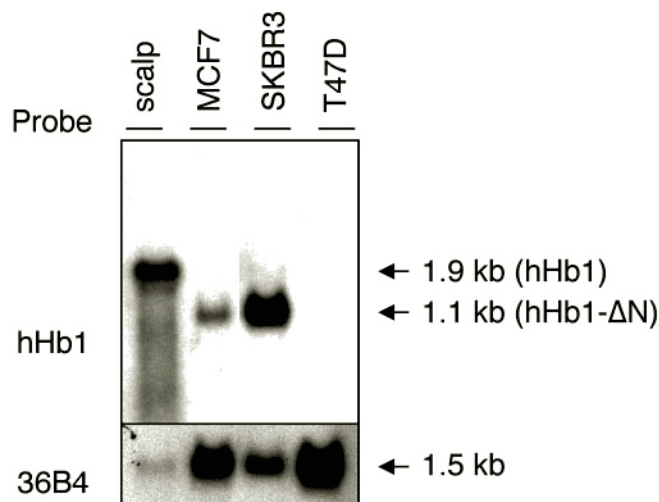


FIG. 1. hHb1 basic hair keratin is expressed as a truncated isoform (hHb1-ΔN) in human breast cancer cell lines. 10 μg of total RNA of scalp, MCF7, SKBR3, and T47D breast cancer cell lines were separated on a 1% agarose gel, transferred onto a nitrocellulose membrane, and hybridized with an hHb1 3'-specific probe. The loading was controlled by 36B4 hybridization.

m-maleimidobenzoyl-*N*-hydroxysuccinimide ester cross-linking reagent (Pierce, Rockford, IL), as previously described (22).

Polytronized mouse back skin and cell lines were twice sonicated in (20 mM Tris, pH 7.5, 0.6 M KCl, 0.1% Triton X-100) to prepare soluble and insoluble protein-enriched fractions. The protein extracts were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred onto a nitrocellulose membrane. The hHb1-ΔN protein was revealed with the rabbit anti-hHb1 antiserum purified against the synthetic peptide. The pan-cytokeratin monoclonal antibody C-11 (Sigma Chemical Co.) recognizes simple- and stratified-epithelial cytokeratins. hHb1-ΔN in fusion with the GFP was recognized by the 1622 antiserum directed against hHb1.

Immunofluorescence—hHb1-ΔN cDNA (GenBank[®] accession number X80197) was fused with the green fluorescence protein (GFP) tag in the *EcoRI*-digested pEGFP-N2 vector (CLONTECH Laboratories Inc.), and hHb1-ΔN protein was localized using GFP fluorescence. Immunofluorescence detection of the intermediate filaments was performed using the pan-cytokeratins C-11 antibody. Isolated HeLa cells were seeded on 12-mm coverslips in 24-well dishes and transfected with the pEGFP-N2-hHb1-ΔN vector using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN). Cells were fixed with 4% paraformaldehyde, washed, and permeabilized by two washes of 10 min with 0.1% Triton X-100. Nonspecific binding sites were blocked with 1% bovine serum albumin. Cells were incubated with the pan-cytokeratin C-11 antibody for 1 h, washed, and then incubated for 30 min with a mouse-specific antibody coupled to the Cy-3 fluorochrome. Nuclei were stained with Hoechst. Labeled cells were analyzed by fluorescence microscopy.

RESULTS

hHb1-ΔN Expression in Human Breast Cancer Cell Lines

Having previously identified the ectopic expression of the truncated hHb1 mRNA, hHb1-ΔN, in breast cancers (4, 5) we extended the characterization of its expression using human breast cancer cell lines. The truncated mRNA is expressed at various levels in the different cell lines tested. hHb1-ΔN is expressed in the MCF7 cell line at basal levels, whereas high mRNA expression was found in the SKBR3 cell line (Fig. 1). The T47D cell line did not synthesize hHb1-ΔN at a detectable level. According to previous data (5), hHb1-ΔN expression was also not detected in normal hairy skin (Fig. 1). So far, hHb1-ΔN was only detected in mammary cancer tissues and cell lines.

Absence of Genomic Alteration in the hHb1 Gene—We decided to evaluate the mechanism responsible for the expression of hHb1-ΔN in breast cancer cells. Expression of tumor cell-specific truncated mRNA can result from gene alterations occurring during cell transformation such as gene deletion, rear-

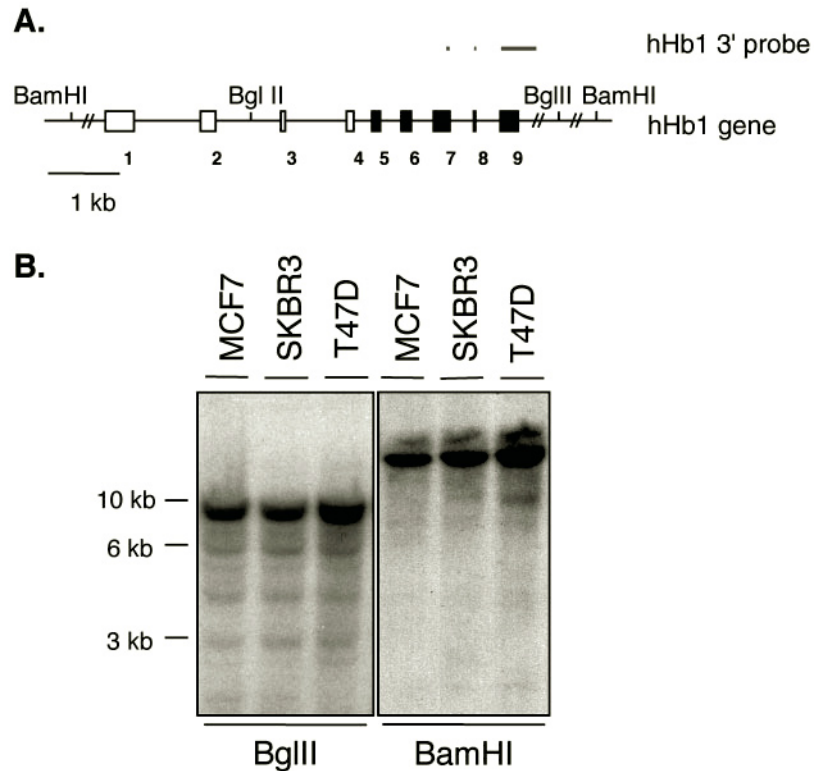


FIG. 2. hHb1-ΔN does not result from genomic alteration at the *hHb1* locus. **A**, schematic representation of the *hHb1* gene. The exons are depicted by boxes; the closed boxes correspond to the hHb1-ΔN coding region. The 3'-specific probe is represented as a broken horizontal line above the *hHb1* gene. BglII restriction sites are indicated by vertical bars. **B**, genomic DNA isolated from MCF7, SKBR3, and T47D cells was digested with BglII or BamHI restriction enzymes. Southern blot was hybridized with the 3'-specific probe of *hHb1*. The size of the restriction fragments containing the hHb1-ΔN coding region is identical in the different cell lines.

rearrangement, and chromosomal translocation. In addition, the *hHb1* gene maps to 12q11-13 (23, 24), a chromosomal region known to present several breakpoints in solid tumors (25). To find out whether hHb1-ΔN results from a genomic deletion or rearrangement, we digested genomic DNA isolated from breast cancer cell lines with five different restriction enzymes and analyzed the restriction patterns of the *hHb1* gene locus by Southern blotting (Fig. 2 and data not shown). The restriction pattern was identical between DNA from cell lines that transcribed the hHb1-ΔN (MCF7 and SKBR3) and the one that did not (T47D). Thus, its expression does not arise from a translocation or an obvious rearrangement event occurring in the *hHb1* locus. We next cloned the *hHb1* gene by PCR from genomic DNA of the SKBR3 cells and of the HBL100 immortalized breast cancer cells that expresses hHb1-ΔN at a low level (data not shown). Up to 500 bp upstream of exon 1 of independently amplified genes from each cell line was sequenced and showed the absence of a minor rearrangement event over the promoter and gene sequences (GenBank[®] accession numbers Y19206 and Y13621) (23). Taken together, these data show that hHb1-ΔN expression is not due to a genomic alteration occurring in the *hHb1* locus.

Mapping of the *hHb1*-ΔN Transcription Initiation Sites—Another mechanism leading to the expression of truncated mRNAs is the use of alternative promoters and/or alternative splicing. To define a putative transcriptional regulatory region, we first mapped the hHb1-ΔN transcriptional start site by primer extension (Fig. 3). An oligonucleotide complementary to the hHb1-ΔN mRNA was hybridized to total RNA isolated from SKBR3 cells, which express high level of hHb1-ΔN, or control liver RNA. Extension of the cDNA resulted in six intense SKBR3-specific cDNA bands, each differing in length by only one nucleotide (92–97 bp), indicating that six adjacent sites located around the *hHb1* intron 4-exon 5 junction (nucleotides 3314–3319, starting from the regular translation initiation site of the *hHb1* gene) (23) are used for hHb1-ΔN transcription initiation. Two additional minor initiation sites were also mapped in exon 5 (nucleotides 3324 and 3345). These sites were

confirmed using different SKBR3 RNA preparations and a second specific oligonucleotide (Fig. 3). We subsequently confirmed the initiator nucleotides by sequencing cDNA obtained by rapid amplification of cDNA 5'-ends (RACE-PCR) of the hHb1-ΔN at the positions 3314–3319 (data not shown). Therefore, hHb1-ΔN transcription is initiated at six adjacent nucleotides located around the intron 4-exon 5 junction of the *hHb1* gene.

Identification of Promoter Activity in Intron 4 of the *hHb1* Gene—To determine the nature of the alternative promoter leading to the hHb1-ΔN expression, we analyzed the promoter activity of the region 5' to the transcription initiation sites. We fused 5'-deleted fragments to the firefly luciferase reporter gene in the promoterless pGL3basic vector and defined their promoter activity by transient transfection of MCF7 cells. The largest construct encompassing exon 1 to part of exon 5 of the *hHb1* gene (–3311/+22) showed a 50-fold induction of the luciferase activity relative to the pGL3basic vector background (Fig. 4A). Shorter 5'-deleted constructs beginning at exon 4 (A; –305/+22) and mid-exon 4 (B; –235/+22) stimulated luciferase activity at a higher extent (140- and 220-fold, respectively). Therefore, the first 235 bp encompassing intron 4 contain a promoter activity responsible for hHb1-ΔN expression. In contrast, the upstream 3-kb region (–3311/–236) possesses a weak silencer activity. We further studied the hHb1-ΔN promoter located in intron 4 of the *hHb1* gene. The 5' deletions of the positive regulatory region (C; –150/+22 and D; –85/+22) progressively decreased luciferase activity (Fig. 4A). The shortest construct (E; –38/+22) retained a 4- to 5-fold stimulation of luciferase activity, indicating the presence of a minimal promoter in the first 38 bp. Analyses of the transcriptional potential of fragments encompassing the 3'-half of the *hHb1* gene did not allow the identification of additional positive or negative regulatory activities (data not shown). Taken together, we conclude that the 235 bp immediately upstream of the initiation sites account for the hHb1-ΔN transcription. hHb1-ΔN expression results from the activation of an alternative cryptic promoter.

FIG. 3. hHb1-ΔN transcription is initiated around the intron 4-exon 5 junction. Specific antisense oligonucleotides 5'-GACAATGTCGTCATACGTGC (*left panel*) or 5'-CGGCAATGATGCAGTCCATGT (*right panel*) were labeled and hybridized at 37 °C to 40 μg of SKBR3 or liver total RNAs. The elongated single-stranded cDNAs were resolved on a denaturing polyacrylamide gel. hHb1-ΔN transcription initiation sites were mapped relative to the full-length hHb1 cDNA (*on the left*) and gene (*on the right*) sequences. Initiator nucleotides are indicated in **boldface capital letters**.

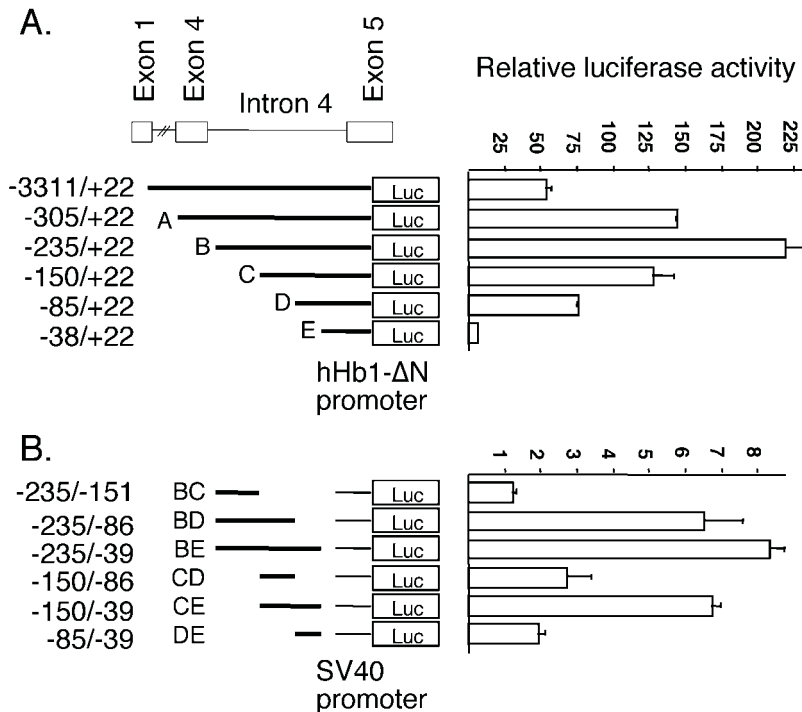
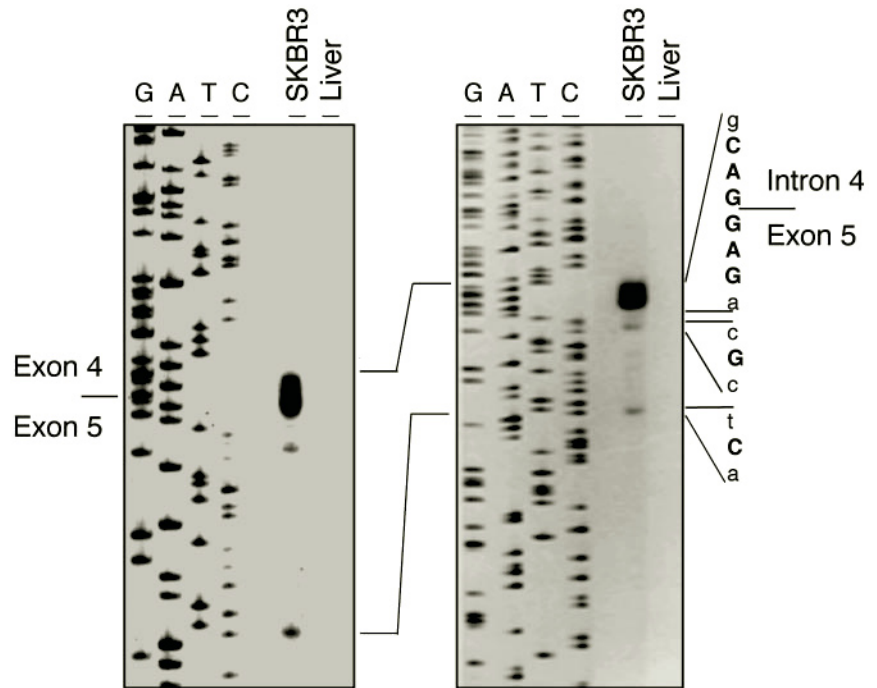


FIG. 4. Intron 4 of hHb1 gene contains hHb1-ΔN promoter activity. In the schematic representation of the hHb1 gene, exons are depicted by open boxes. Luciferase reporter gene (*Luc*) constructs are described under "Experimental Procedures." Transcriptional activity was estimated in transiently transfected MCF7 cells by luciferase assay and normalized for transfection efficiency. Transfections were performed in triplicates and repeated at least three times (mean ± S.D.). *A*, serial 5'-deleted fragments of the hHb1 gene (*thick line*) were fused to the luciferase reporter gene in the pGL3basic vector to estimate promoter activity. *B*, overlapping constructs encompassing hHb1-ΔN regulatory sequences were fused to the strong SV40 promoter in pGL3promoter vector to test their regulatory activity.

To further characterize these *cis*-acting regulatory DNA sequences, we examined whether they could modulate the activity of the heterologous SV40-promoter present in the pGL3-promoter vector (Fig. 4*B*). The highest level of luciferase expression relative to the SV40-promoter activity was found for the largest construct (BE, -235/-39), consistent with the presence of positive regulatory elements. Its activity was only slightly reduced when BC or DE regions were removed (CE, -150/-39 and BD, -235/-86, respectively). The upstream region BC (-235/-151) did not modify by itself the SV40-promoter activity. In contrast, CD (-150/-86) or DE fragments (-85/-39) stimulated 2.5- and 2-fold the luciferase expression, respectively. Positive regulatory elements are thus mainly located in these two CD and DE fragments. In the context of the

hHb1-ΔN proximal promoter, deletion experiments showed that the DE fragment is more active than the CD fragment (Fig. 4*A*). However, in the context of an heterologous promoter, isolated CD and DE fragments have similar positive regulatory activities (Fig. 4*B*). Therefore, our results indicate that CD and DE fragments cooperate together and with the minimal promoter region to induce hHb1-ΔN expression.

Localization and Characterization of hHb1-ΔN Regulatory Elements—To localize more precisely the regulatory elements that account for the hHb1-ΔN promoter activity, we mapped transcription factor-protected regions in the AE fragment by *in vitro* DNase I footprinting experiments (Fig. 5*A*). The antisense strand of the -305/+22 promoter construct (A) was labeled and incubated with or without nuclear extracts from MCF7,

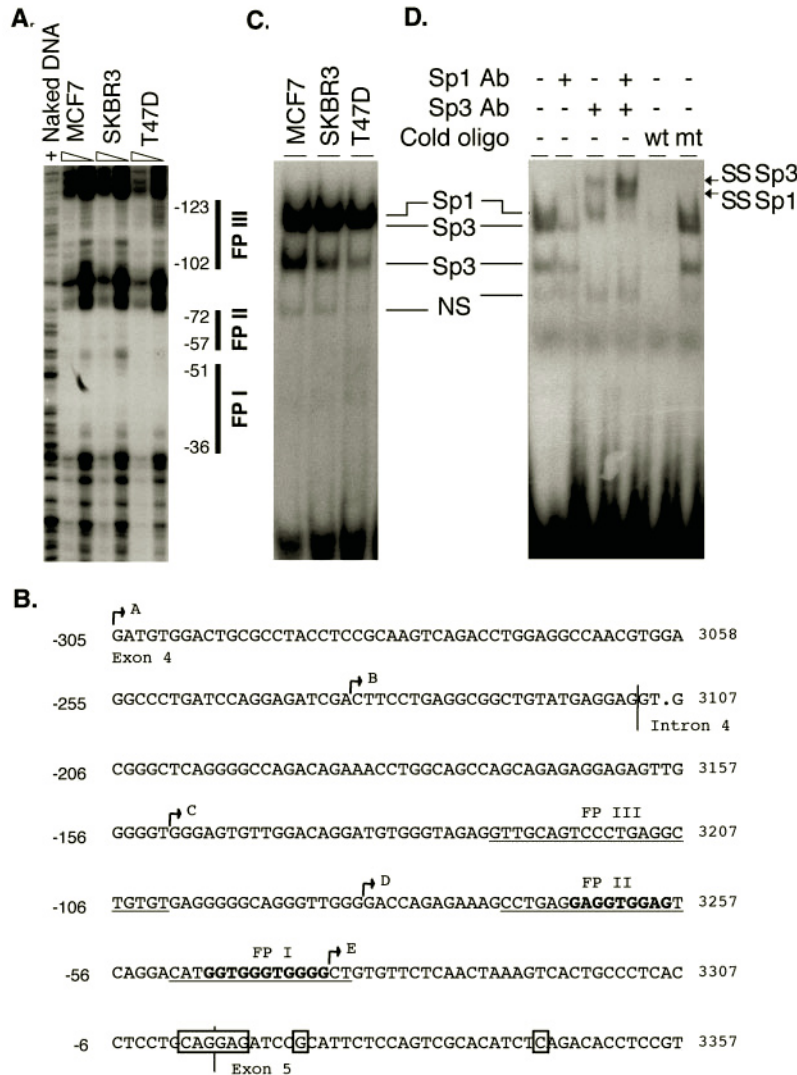
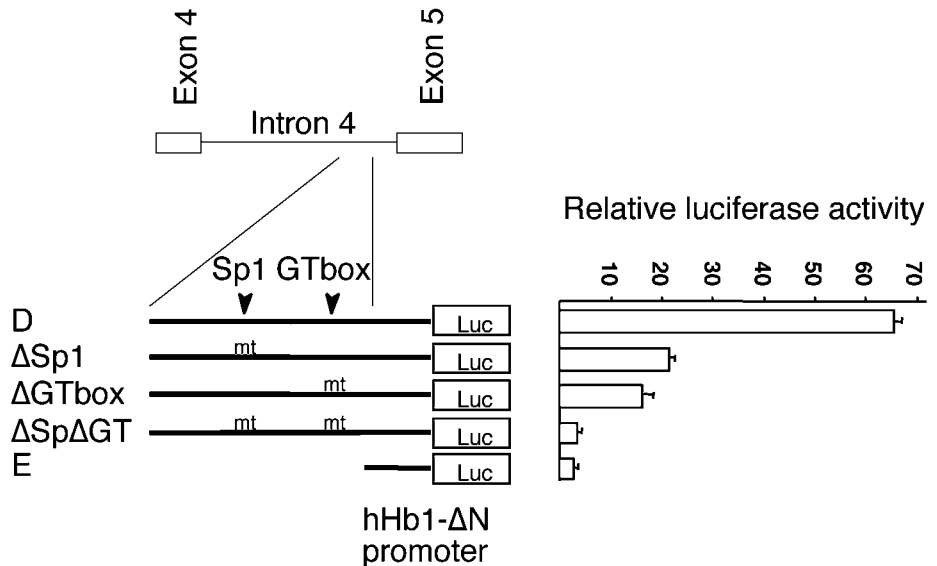


FIG. 5. Analyses of the regulatory elements present in the hHb1-ΔN promoter. A, the $-305/+22$ promoter fragment was analyzed by DNase I footprinting in the absence or the presence of $50 \mu\text{g}$ of nuclear extract from breast cancer cell lines as indicated. Naked and protected DNA were partially digested with decreasing concentrations of DNase I and electrophoresed in denaturing conditions. The three protected regions FP I to III were mapped by means of a sequencing ladder. B, nucleotide sequence of the exon 4 to mid-exon 5 region of the *hHb1* gene is represented, and the positions are indicated on the right in respect to the relative translation initiation site of the hHb1 protein. *hHb1* gene sequence was identical to the data bank gene sequence except two nucleotides (nucleotides 3273 and 3275 are G instead of T and C, respectively). hHb1-ΔN promoter regulatory elements are positioned within the *hHb1* gene. hHb1-ΔN transcription initiation sites are boxed. Promoter sequence positions are indicated on the left relative to the first transcription initiator nucleotide. The 5'-ends of the serial deleted promoter fragments are depicted by arrows and their relative letters. DNase I footprint-protected sequences are underlined and are referred to as FP I to III. Putative Sp1 binding sites have been identified in the protected regions and marked in boldface letters. C, a double-stranded oligonucleotide that encompasses the putative Sp1 binding element in the FP II protected region was labeled and incubated with $6 \mu\text{g}$ of nuclear extract from MCF7, SKBR3, or T47D cells. The complexes were separated from the free probe using a 5% polyacrylamide gel. D, competition and supershift analyses were done using MCF7 nuclear extracts. Competition experiments were performed using a 100 molar excess of the wild-type (wt) or a mutated (mt) oligonucleotide. Addition of Sp1 or Sp3 polyclonal antibodies (Ab) led to the disappearance of the slower and the two faster migrating complexes, respectively, and to the formation of a slower migrating ternary complex (SS, supershift). Co-addition of Sp1 and Sp3 antibodies resulted in the supershift of all three specific complexes. NS indicates nonspecific binding.

SKBR3, and T47D cells. Partial DNase I digestions were electrophoresed and analyzed on a denaturing polyacrylamide gel. In the presence of nuclear extracts, three protected regions were detected between nucleotides -36 and -51 (FP I), nucleotides -57 and -72 (FP II), and nucleotides -102 and -123 (FP III) relative to the most 5'-transcription start site. Thus, FP I- and FP II-protected regions are located within the DE fragment that has a strong positive regulatory activity, whereas the third one, FP III, is located in the CD fragment. These regions were protected with a similar efficiency whether the nuclear extracts were isolated from cells that express the hHb1-ΔN mRNA (MCF7, SKBR3) or the one that did not (T47D).

We next wanted to identify the transcription factors that are able to bind to these protected elements in the hHb1-ΔN promoter. Analysis of their sequences revealed the presence of two putative binding elements for transcription factors of the Sp1 family. We identified a GT box element (5'-GGTGGGTGGGG) in the FP I region and a Sp1 binding element (5'-GAGGTG-GAG) in the FP II region (Fig. 5B). However, no known binding element could be found in the FP III region present in the CD fragment. We next designed double-stranded oligonucleotides that encompass the GT box element or the Sp1 binding element, and carried out their ability to bind to transcription factors by electromobility shift assays. Incubation of the GT box element (data not shown) or the Sp1 binding element probes

FIG. 6. The Sp1 binding element and the GTbox element are responsible for the *in vitro* positive regulatory activity. -85/+22 fragments with single (Δ Sp1 or Δ GTbox) or double (Δ Sp Δ GT) mutated Sp1/Sp3 binding sites were cloned in the pGL3basic vector. Luciferase activity was assayed in MCF7 cells. Transfections were done in duplicates and repeated three times (mean \pm S.D.). Sp1, Sp1 binding element; GTbox, GT box element; *mt*, mutated.



(Fig. 5C) with nuclear extracts from MCF7, SKBR3, or T47D cells revealed three similar protein-DNA complexes. The specificity of these complexes was demonstrated by their efficient competition by an excess of cold wild-type but not of mutated oligonucleotide (Fig. 5D). Addition of polyclonal antibodies raised against Sp1 or Sp3 resulted in the complete elimination of the slower migrating or the two faster migrating complexes, respectively, and allowed for the formation of supershifted complexes (Fig. 5D). Finally, simultaneous incubation with both antibodies supershifted all three specific complexes. Taken together, these data demonstrate that the Sp1 and Sp3 transcription factors are the proteins involved in the specific complexes formed with both Sp1 binding element and GT box element probes.

To ensure that the Sp1 binding sites are effectively responsible for the transcriptional activity, mutated -85/+22 promoter fragments (D) were generated (Fig. 6). Mutation of the Sp1 binding element (Δ Sp1) or GT box element (Δ GTbox) resulted in a significant decrease of the transcriptional activity. Moreover, the double-mutated construct (Δ Sp Δ GT) dramatically inhibited transcriptional activity. This activity is equivalent to that of the -38/+22 proximal promoter (E), showing therefore that mutation of the two elements completely abolished the activity of the DE region. These assays confirm that the two Sp1 binding sites are responsible for the positive activity of the DE fragment.

Epigenetic Regulation of *hHb1-ΔN* Expression—The promoter analyses indicated that the positive regulatory *cis*-elements that drive *hHb1-ΔN* expression are restricted to intron 4 of *hHb1* gene. Moreover, all the regulatory factors we have identified (*i.e.* Sp1 and Sp3 transcription factors) are present and active in cells, independently of their capacity to transcribe *hHb1-ΔN* (Fig. 5, A and C). In addition, six basic hair keratin genes have been identified (3). *hHb1* hair keratin is highly related to *hHb6* (GenBank[™] accession number AJ000263) (23). In particular, intron 4 sequences of *hHb1* and *hHb6* are 98% conserved, and the three protected regions identified by footprinting are strictly conserved. Moreover, using *in vitro* reporter assays in MCF7 cells, the intron 4 of *hHb6* is able to drive the expression of the luciferase reporter gene at a similar extent to that of the corresponding *hHb1* region (data not shown). We studied, therefore, whether *hHb6* hair keratin is expressed in breast cancer cell lines. Neither full-length nor truncated *hHb6* is detected in breast cancer cell lines that express high or detectable level of *hHb1-ΔN*, whereas the 2-kb

hHb6 full-length transcript is detected in the hairy skin sample (Fig. 7). Thus, the specificity of *in vivo* expression of *hHb1-ΔN* in breast cancer does not solely lie in the promoter sequence but might also be dependent on an epigenetic process.

Gene methylation/demethylation events are involved in cancer progression-dependent gene expression (26). To examine whether methylation might regulate *hHb1-ΔN* expression, we treated MCF7, SKBR3, and T47D cells with increasing doses of 5-aza-2'-deoxycytidine (5-Aza-2'dC), a DNA methyltransferase inhibitor (Fig. 8A). 5-Aza-2'dC is incorporated into the newly synthesized DNA strand during replication and depletes the cell of methyltransferase activity by forming a covalent complex with the enzyme. Cells were treated with two 24-h pulses on days 2 and 5 with 0.5, 2, or 10 μ g/ml 5-Aza-2'dC, and RNA were prepared on day 7. At 0.5 and 2 μ g/ml, the cells still grew and no significant changes in their morphology were noticed. However, at 10 μ g/ml, the cells stopped growing and cell death was significant, especially for SKBR3 cells (Fig. 8A, lane 8). The cell lines displayed different responses to 5-Aza-2'dC treatment. The basal level of *hHb1-ΔN* expression in MCF7 cells was increased in a dose-dependent manner. However, the high level of *hHb1-ΔN* expression was not modified in SKBR3 cells, and 5-Aza-2'dC treatment did not induce *hHb1-ΔN* expression in the T47D cell line even at the highest dose. The effect of consecutive 5-Aza-2'dC pulses was also tested (Fig. 8B). MCF7 cells were treated with one, two, or three 24-h pulses with 2 μ g/ml 5-Aza-2'dC dose, and total RNA was prepared 24 h after the end of the last treatment. Northern blot analysis showed that *hHb1-ΔN* expression increased with the number of pulses. We conclude that *hHb1-ΔN* expression is dependent on genomic DNA demethylation.

We analyzed whether *hHb1-ΔN* expression could be induced through an indirect mechanism requiring protein neosynthesis. 2 μ g/ml 5-Aza-2'dC was added in the culture medium of MCF7 cells for 5 days. During the last 72 or 24 h of 5-Aza-2'dC treatment, cells were additionally treated with 2, 10, or 50 μ g/ml cycloheximide, an inhibitor of protein translation. Cycloheximide was not deleterious for MCF7 cells, because the growth rate decreased only slightly at the highest concentration. However, the cycloheximide treatment resulted in the reduction of *hHb1-ΔN* expression in a dose- and time-dependent manner (Fig. 8C). Therefore, these data show that *hHb1-ΔN* induction through DNA demethylation is indirect and requires protein neosynthesis.

hHb1-ΔN* Protein Expression *in Vivo—*hHb1-ΔN* protein

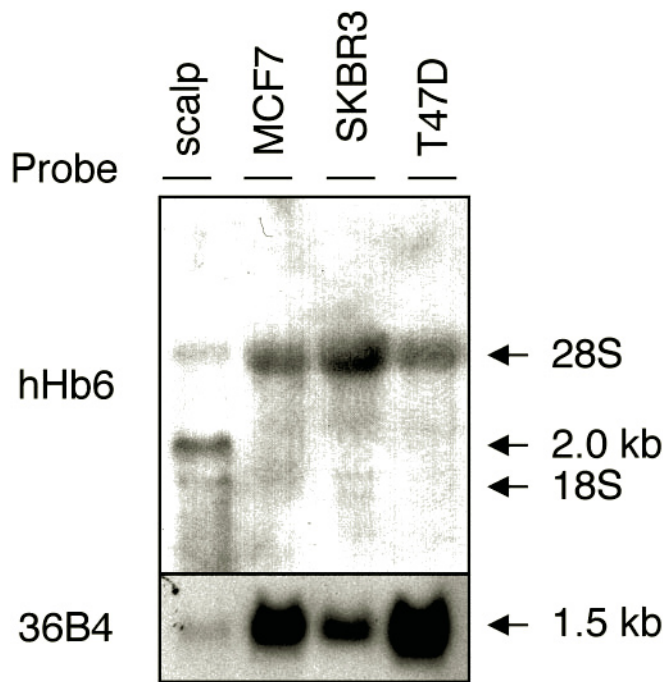


FIG. 7. hHb6 basic hair keratin is not expressed in breast cancer cell lines. The Northern blot of Fig. 1 was rehybridized with a 3'-specific probe of hHb6. Full-length hHb6 is expressed as a 2-kb mRNA in human scalp. No truncated form can be seen in breast cancer cells. The probe slightly hybridized to 28 S ribosomal RNA. The lower band observed in scalp is due to the presence of 18 S RNA.

(Fig. 9A) translation was assessed with a purified rabbit polyclonal antibody raised against the carboxyl-terminal peptide of the tail domain of hHb1 hair keratin. By Western blot, this antibody recognized several proteins from the insoluble fraction of a mouse back skin extract (Fig. 9B). This antibody does not recognize cytokeratins, because no bands were detected in cancer cell line extracts, indicating that it is specific of hair keratins. Accordingly, the pan-cytokeratin C-11 antibody, which recognizes simple and stratified epithelial cytokeratins, detected several bands of about 30–75 kDa in the insoluble fraction of all breast cancer cell line extracts (Fig. 9C). The 30-kDa hHb1- ΔN protein was specifically detected in both soluble and insoluble fractions of SKBR3 cells (Fig. 9B) that exhibited the highest levels of hHb1- ΔN mRNA (Fig. 1). Our results indicate that the hHb1- ΔN protein is translated *in vivo* in breast cancer cells.

Subcellular Localization of the hHb1- ΔN Protein—The expression of such a truncated hair keratin in breast cancer cells prompted us to analyze whether it could participate to the cytoskeleton network. Subcellular localization of hHb1- ΔN protein was studied by fluorescence microscopy (Fig. 9E, middle panel). We could not study the localization of the endogenous hHb1- ΔN keratin in SKBR3 cells, because these cells are poorly adhesive rounded cells and display a very high nucleus-to-cytoplasm ratio. Thus, a GFP tag was fused to the hHb1- ΔN cDNA at the carboxyl-terminal end and transiently transfected in HeLa cells that did not express hHb1- ΔN (Fig. 9B). We verified by Western blot that the fusion protein was expressed and not degraded in HeLa cells (Fig. 9D). A carboxyl-terminal GFP tag has been shown not to interfere with the incorporation of a keratin into intermediate filaments (27). Cells transfected with the GFP alone displayed a faint homogeneous staining in the cytoplasm (data not shown). HeLa cell morphology was not significantly modified upon hHb1- ΔN transfection. The hHb1- ΔN protein was detected in the cytoplasm in a non-homogeneous distribution, illustrative of a filament network.

The hHb1- ΔN protein accumulated at the cell periphery and in large cytoplasmic filaments that became thinner near the cell membrane (Fig. 9E, middle panel).

To determine whether hHb1- ΔN protein co-localizes with cytokeratin filaments, double immunofluorescence was performed with the pan-cytokeratin monoclonal antibody C-11. Intermediate filaments form a network in the cytoplasm that is denser in the perinuclear region (Fig. 9E, upper panel). Double detection of the hHb1- ΔN and cytokeratin proteins showed that hHb1- ΔN partially co-localizes with the endogenous intermediate filament network, notably in some large bundles (Fig. 9E, lower panel). Furthermore, the hHb1- ΔN filament network extends further toward the cell membrane than does the cytokeratin network.

DISCUSSION

The hHb1 hair keratin has been reported to be expressed in pilomatricoma skin tumors, which are characterized by a follicular differentiation (11, 12). Surprisingly, although the normal *hHb1* promoter is silent in normal and malignant breast cells, because no full-length hHb1 mRNA was detected in these cells, a truncated hHb1 mRNA isoform (hHb1- ΔN) was observed in cancerous epithelial cells of breast carcinomas (4, 5). Thus, hHb1 is the first hair keratin whose expression was documented in carcinomas other than skin carcinomas. In addition, this expression of hHb1 is particularly intriguing, because it corresponds to a 5'-truncated isoform never documented so far. In this study, we provide insights into the transcriptional mechanism responsible for this expression and into the presence of the corresponding protein in cancer cells.

We looked for the ectopic expression of other basic hair keratins, namely hHb3, hHb5 and hHb6, as well as the hHb1 acidic heterodimeric partner hHa1 in breast cancer cell lines. None of these keratins were detected in the cell lines. Due to the high conservation of basic hair keratin gene structures and sequences (23, 24), the regulatory mechanism responsible for the ectopic expression of the hHb1- ΔN mRNA must be tightly controlled. Basic hair keratin genes are clustered on chromosome 12q13, a common locale for chromosomal breakpoints in several cancer types, including breast cancers (25). However, we did not detect any chromosomal rearrangement or modification of the *hHb1* gene. hHb1- ΔN expression is therefore not the result of gene or chromosomal alteration. Moreover, no sequence differences were found in the gene locus between breast cancer cell lines that express hHb1- ΔN and those that do not.

hHb1- ΔN expression is never detected in the hair follicle and, consequently, is unlikely to involve the same transcriptional elements as the full-length hHb1 basic hair keratin. Alternative promoter is a frequent mean through which diversity in the complex physiological patterns of gene expression are created (28). Moreover, cancer-specific expression has also been described to be achieved through specific cryptic promoters silent in physiological conditions (29). Thus, because hHb1- ΔN transcription is initiated at the intron 4-exon 5 junction of the *hHb1* gene, its expression may be due to the specific activation of a cryptic promoter. We defined an hHb1- ΔN promoter located in the fourth intron of the *hHb1* gene. A short 38-bp promoter fragment is sufficient to induce transcription of the luciferase reporter gene. Its activity is stimulated by two functional Sp1 binding sites that are located close to the minimal promoter. Site-directed mutagenesis of these elements inhibited the stimulation of the expression of the minimal hHb1- ΔN promoter. Furthermore, electromobility shift assays allowed for the identification of Sp1- and Sp3-containing complexes. These transcription factors are ubiquitously expressed and are involved in the regulation of numerous genes (30).

FIG. 8. hHb1-ΔN is induced in response to 5-Aza-2'-deoxycytidine treatment. **A**, MCF7, SKBR3, and T47D cells were treated with two 24-h pulses using either 0.5 (lanes 2, 6, and 10), 2 (lanes 3, 7, and 11), or 10 μg/ml (lanes 4, 8, and 12) 5-Aza-2'-dC. Total RNA was prepared 24 h after the end of the second treatment. Northern blot (10 μg of RNA) hybridization was performed using 3'-hHb1 and 36B4 probes for loading control. **B**, MCF7 cells were treated with 2 μg/ml 5-Aza-2'-dC during one, two, or three 24-h pulses. RNA were isolated 24 h after the end of the last treatment. hHb1-ΔN expression was assayed using the 3'-hHb1 probe. **C**, MCF7 cells were treated with 2 μg/ml 5-Aza-2'-dC for 5 days, then with 2, 10, or 50 μg/ml cycloheximide for the last 72 or 24 h. hHb1-ΔN expression was assayed by Northern blot using the 3'-hHb1 probe.

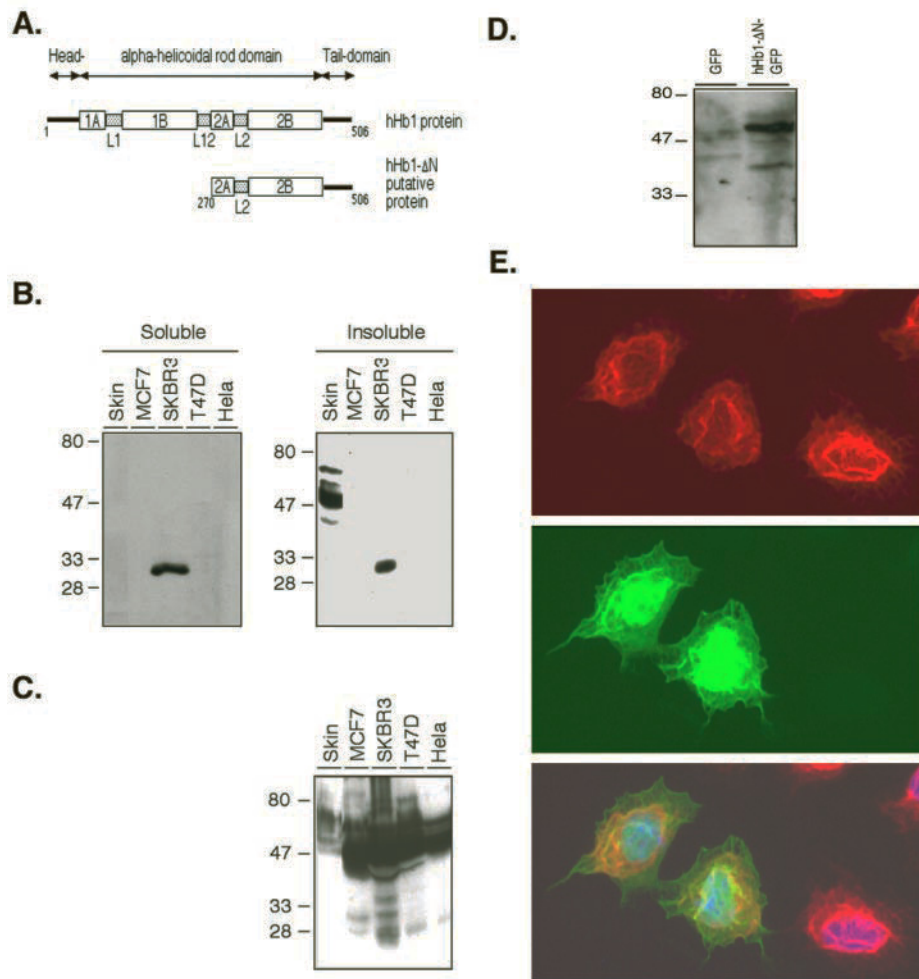
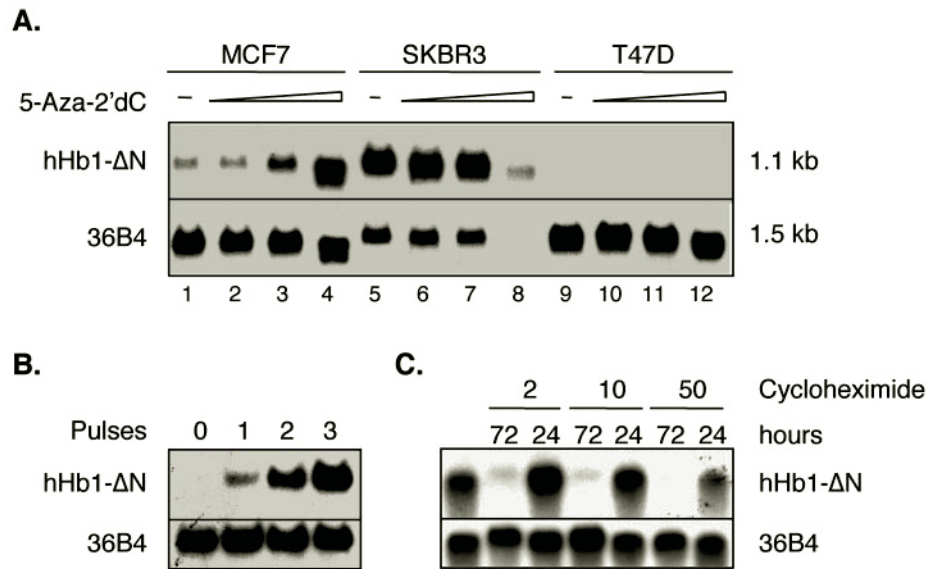


FIG. 9. hHb1-ΔN protein is partially co-localized within cytokeratin intermediate filaments. **A**, schematic representation of the hHb1 (upper) and hHb1-ΔN (lower) proteins. The rod domain of hHb1 contains conserved α -helical structures (1A, 1B, 2A, and 2B) interrupted by linker sequences (L1, L12, and L2). It is flanked by the terminal head- and tail-domains. The hHb1-ΔN protein (amino acids 270–506) contains the second α -helical subdomain and the tail-domain. **B**, soluble (left panel) and insoluble (right panel) protein extracts of mouse back skin or cell lines were resolved by SDS-polyacrylamide gel electrophoresis on a 10% acrylamide gel. hHb1-ΔN protein was detected with the 1622 rabbit polyclonal antibody raised against the carboxyl-terminal peptide of the tail domain of hHb1. The size of the protein detected in SKBR3 cells is consistent with the hHb1-ΔN size. Note that full-length hair keratins were only detected in the insoluble fraction of hairy skin. There is no cross-reaction with cytokeratins. **C**, the blot corresponding to the insoluble extracts was re-probed with the monoclonal antibody C-11 recognizing several cytokeratins. **D**, Western blot analysis of cell extracts (30 μg) from HeLa cells transfected with GFP or hHb1-ΔN-GFP expression vectors. Revelation was performed using the 1622 antiserum directed against hHb1-ΔN. **E**, transfected hHb1-ΔN-GFP was localized in HeLa cells by GFP fluorescence (green, middle panel). The cytokeratin intermediate filament network was detected by indirect immunofluorescence using the pan-cytokeratins antibody C-11 and Cy3-coupled anti-mouse antibodies (red, upper panel). Co-localization of hHb1-ΔN-GFP and intermediate filaments is shown in yellow (lower panel).

Therefore, hHb1-ΔN expression results from the specific activation of a cryptic promoter containing two Sp1 binding sites located next to a minimal promoter.

However, several observations suggest the need for a more complex transcriptional regulation of hHb1-ΔN expression. Thus, the intronic sequence containing the hHb1-ΔN promoter activity is 98% conserved in the *hHb6* basic hair keratin gene. Nevertheless, there is no *in vivo* expression of truncated hHb6,

although this intronic hHb6 region is able to *in vitro* induce transcription. These data suggest that an additional regulatory mechanism should be responsible for hHb1 *in vivo* expression in breast cancer cells. Subsequently, the hHb1-ΔN promoter is composed of an initiator element and two functional Sp1 binding elements. Analysis by DNase I-hypersensitive sites mapping and transient reporter gene assays did not reveal additional *cis*-acting positive regulatory regions within an 8-kb

genomic region encompassing the hHb1-ΔN coding region and the 5'- and 3'-flanking sequences.² Interestingly, transcription from the TATA-less promoter of the *leukosialin* gene is also mediated by a single GTbox element 40 bp upstream of the transcription start site, but no other *cis*-acting regulatory activity responsible for the cell type-specific expression could be identified (31). However, DNA methylation around the 5'-flanking region of the *leukosialin* gene is required to shut off the high level of expression, showing that tissue-specific expression is achieved by alteration of DNA methylation (32). We thus wondered whether the epigenetic processes of methylation could be involved in the breast cancer cell-specific expression of hHb1-ΔN. We showed that the endogenous hHb1-ΔN expression is increased in response to 5-Aza-2'dC, a demethylating reagent, in MCF7 breast cancer cells, and this effect is dose-dependent. DNA methylation is, therefore, clearly involved in hHb1-ΔN transcriptional regulation.

However, because hHb1-ΔN expression is not induced in T47D cells in response to 5-Aza-2'dC, we also propose that another protein should participate to the breast cancer cell specificity of hHb1-ΔN expression. Consistently, because hHb1-ΔN is never expressed under normal physiological conditions but only in transformed cells, its induction in response to DNA demethylation should involve an indirect mechanism. Indeed, blocking protein expression by cycloheximide led to the abolition of hHb1-ΔN transcription in response to 5-Aza-2'dC treatment. Thus, the induction of hHb1-ΔN does not result from the direct demethylation of the hHb1-ΔN genomic region. This result raises the possibility of the involvement of the induction or the increased activation of a transactivator. Sp1 activity was previously shown to be increased in response to demethylating reagents at the level of protein stability (33) or DNA binding activity (34). However, in the hHb1-ΔN promoter, neither Sp1 expression nor its binding activity were notably altered in response to 5-Aza-2'dC.² We thus exclude the possibility that the effect of 5-Aza-2'dC occurs at the Sp1 gene locus or at a gene locus whose product modifies Sp1 activity. hHb1-ΔN induction through demethylation is thus dependent on the synthesis and/or activation of an unknown protein. Therefore, the expression of an undefined factor in response to 5-Aza-2'dC could be sufficient to induce the expression of hHb1-ΔN and presumably other target genes, whose products might be essential in promoting cancer progression.

We have also investigated the translation of hHb1-ΔN mRNA. We raised a polyclonal antibody against the carboxyl-terminal end of the hHb1 protein. This antibody allowed for the specific detection of hair basic keratins in the insoluble fraction of mouse skin extracts. Endogenous hHb1-ΔN protein was detected in both soluble and insoluble fractions of the SKBR3 breast cancer cell line extracts, showing that the truncated hHb1-ΔN mRNA is translated *in vivo*. Because insoluble fractions contain the structural cytoskeleton proteins, this also suggested that hHb1-ΔN might be at least partially associated with the cytoskeleton. Consistently, fluorescence-mediated localization of the hHb1-ΔN protein in HeLa-transfected cells depicts a filament network covering the entire cytoplasm of the cell, indicating that hHb1-ΔN either participates in the cytoskeleton or homo-polymerizes to constitute a particular network.

Keratins form obligatory heterodimers *in vivo* and have specific functions that are fulfilled by the amino- and carboxyl-terminal domains (35–38). In epidermal keratin replacement experiments in mouse, the carboxyl-terminal domain clearly dictates the function of a chimeric keratin (38). Forced expres-

sion of some foreign keratins enables their integration into the endogenous intermediate filament network both in cell culture and in *in vivo* mouse models (35, 38–40). Moreover, forced expression of an amino-terminal-truncated epidermal cyto-keratin beginning at the 1B subdomain was shown to lead to the collapse of the intermediate filament network, resulting in very thin bundles of jagged fibers (39). Altogether, these results suggested that the hHb1-ΔN protein might fulfill a dominant negative function by inducing the collapse of the intermediate filament network. However, the cyto-keratin network, partially overlapping that of hHb1, was not obviously modified upon hHb1-ΔN expression, showing that hHb1-ΔN does not induce its collapse, and suggesting that hHb1-ΔN protein is unlikely to be incorporated during neo-synthesis of intermediate filaments. Finally, the intermediate filaments are a dynamic structure in continuous motion (27), and replacement of incorporated keratins with soluble subunits is a constant mechanism to ensure filament dynamism (41). Thus, hHb1-ΔN may be involved in similar process and alters the function of the pre-existing keratin filaments through its specific carboxyl-terminal domain. In this context, it has been shown that mHa1 or mHb4 hair keratins transiently expressed in HeLa cancer cells can be incorporated in cyto-keratin intermediate filaments without evident alteration of the network (42).

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