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 cytotkeratin 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 stratified epithelia differentially express pairs of "hard" or cytoskeleton filaments. Keratin proteins bearing small deletions can lead to the complete intermediate filament network. Keratin proteins bearing small deletions can be incorporated into the pre-existing network as newly formed intermediate filaments. 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).

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) 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 a-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).
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-N 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-N 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 × 10^5 MCF7, 2 × 10^6 SKBR3, and 8 × 10^6 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 24 h. cDNA Probes—The hHb1 5’- and 3’-specific cDNA probes and the 36B4 (acidic ribosomal protein, GenBank accession number M17885) cDNA probe were already described (5). Exon 9 of hHb6 gene was amplified by PCR from SKBR3 genomic DNA using the following primers: 5'-GAGAGAATTCACCCAAACGTCCAGGAGGATCATC-3'; 5'-GATCTCCTCTGTTGCGGCCG (designated in exon 9) and the antisense primer 5'-GAGAGAATTCACCCAAACGTCCAGGAGGATCATC-3'. The hHb1-N cDNA was further amplified using internal primer 5'-GATCTCCTCTGTTGCGGCCG (designated in the exon 9) and cloned into the pTDvec vector (CLONTECH Laboratories). Thirty hHb1-N 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'-GAGAGAATTCACCCAAACGTCCAGGAGGATCATC-3' and 3’-primer, 5'-GAGAGAATTCACCCAAACGTCCAGGAGGATCATC-3'. The hHb1-N cDNA was further amplified using internal primer 5'-GATCTCCTCTGTTGCGGCCG (designated in the exon 9) and cloned into the EcoRI-digested plBluescript plasmid (Stratagene Inc., La Jolla, CA). Exon nucleotide sequence was verified from independently amplified genes.

Luciferase constructs containing varying lengths of the hHb1-N promoter were generated by PCR and inserted upstream of the firefly luciferase reporter gene in Sacl/Axl-digested pGL3basic vector (Promega Corp., Madison, WI). The following primers were used, their positions are referred to the most upstream transcription initiating nucleotide (MUTP) as 0 (position 1). The following primers were 5'-GAGAGAATTCACCCAAACGTCCAGGAGGATCATC (A; -305/+22), 5'-ATTTCTCTCTGTTGCGGCCG (designated in exon 9) and cloned into the EcoRI-digested plBluescript plasmid (Stratagene Inc., La Jolla, CA). Exon nucleotide sequence was verified from independently amplified genes.

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Potential positive regulatory fragments of the hHb1-N promoter were PCR-amplified and inserted upstream of the SV-40 promoter in the pGL3promoter vector using the sense primers 5'-GAGAGAATTCACCCAAACGTCCAGGAGGATCATC (A; -305/+22), 5'-ATTTCTCTCTGTTGCGGCCG (designated in exon 9) and cloned into the EcoRI-digested plBluescript plasmid (Stratagene Inc., La Jolla, CA). Exon nucleotide sequence was verified from independently amplified genes.

Luciferase constructs containing varying lengths of the hHb1-N promoter were generated by PCR and inserted upstream of the firefly luciferase reporter gene in Sael/Axl-digested pGL3basic vector (Promega Corp., Madison, WI). The following primers were used, their positions are referred to the most upstream transcription initiating nucleotide (MUTP) as 0 (position 1). The following primers were 5'-GAGAGAATTCACCCAAACGTCCAGGAGGATCATC (A; -305/+22), 5'-ATTTCTCTCTGTTGCGGCCG (designated in exon 9) and cloned into the EcoRI-digested plBluescript plasmid (Stratagene Inc., La Jolla, CA). Exon nucleotide sequence was verified from independently amplified genes.

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5'-TGCTCTAGGAGAATTCCTCAGGCT and 5'-GAGAACACAGC-CCCATATTGCCATGCCTGACT (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 pBlueScript 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 nuclear 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 (26). Briefly, subconfluent cell cultures were washed 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, washed twice with PBS and resuspended in 0.8 ml of low salt buffer A lactosidase activity. All transfections were performed in duplicates or triplicates and repeated three times.

In Vitro DNase I Footprinting—Footprinting was performed as previously described (20). The ~305/32 promoter deletion fragment was cloned into pBlueScript SK+ and released by EcoRI and EagI digestion. The resulting fragment was isolated and purified and labeled to 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(dI-dC), 50 μg of nuclear extract, and 50 μl of binding buffer (20 mM HEPES-KOH, pH 7.9, 20% glycerol, 1 mM EDTA, 0.5 mM dithioether, 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.

Polyctonized 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-cytokeratin 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. Non-specific 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 Cy3-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 expressed 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. 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.
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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 luminescence bands (Fig. 4), corresponding to the transcriptional activity at a higher extent (140- and 220-fold, respectively). Therefore, the first 235 bp encompassing exon 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.
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. 4B). The highest level of luciferase expression relative to the SV40-promoter activity was found for the largest construct (BE, -235/+22), consistent with the presence of positive regulatory elements. Its activity was only slightly reduced when BC or DE regions were removed (CE, -150/+22 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/+22) 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. 4A). However, in the context of an heterologous promoter, isolated CD and DE fragments have similar positive regulatory activities (Fig. 4B). 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. 5A). The antisense strand of the -305/+22 promoter construct (A) was labeled and incubated with or without nuclear extracts from MCF7, FIG. 3. hHb1-ΔN transcription is initiated around the intron 4-exon 5 junction. Specific antisense oligonucleotides 5'-GACAATGTCTGATCATCTGTGC (left panel) or 5'-GGGAAATGCTGAGTCCATGTCAT (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.
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 236 and 251 (FP I), nucleotides 257 and 272 (FP II), and nucleotides 2102 and 2123 (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'-GAGGTGGAG) 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...
of elements that drive hHb1-D promoter analyses indicated that the positive regulatory cis-the two Sp1 binding sites are responsible for the positive ac-

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lent to that of the

Moreover, the double-mutated construct (GTbox Sp1 binding element (D)) dramati-

ically inhibited transcriptional activity. This activity is equiv-

alent to that of the ~38/22 proximal promoter (E), showing therefore that mutation of the two elements completely abol-

ished 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’-desoxycytidine (5-Aza-2’-dC), a DNA methyltrans-

ferase 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 μg/ml 5-Aza-2’-dC, and RNA were prepared on day 7. At 0.5 and 2 μg/ml, the cells still grew and no significant changes in their morphology were noticed. However, at 10 μg/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 μg/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 μg/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 μg/ml cycloheximide, an inhibitor of protein translation. Cyclo-

heximide 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-depend-

ent 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
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 pilomatrixoma 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 protein 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).
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 GT-box 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 supposed 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 activity 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 carboxy-terminal end of the hHb1 protein. This antibody allowed for the specific detection of hair basic keratin 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-

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2 A. Boulay, unpublished results.