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Advances in Brief

Down-Regulation of Promoter I.3 Activity of the Human Aromatase Gene in Breast Tissue by Zinc-finger Protein, Snail (SnaH)[1]

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Abstract

Aromatase (estrogen synthetase) is expressed in breast cancer tissue, and *in situ* expression of the enzyme stimulates breast cancer growth. Promoter I.3 is one of the major promoters that control the expression of aromatase in breast cancer tissue. Using the yeast one-hybrid approach to screen a human breast tissue hybrid cDNA expression library, we found that the zinc-finger transcriptional factor Snail (SnaH) interacted with a regulatory region near promoter I.3 of the human aromatase gene. DNA mobility shift assays and mutation analyses using recombinant SnaH protein expressed in *Escherichia coli* have revealed that this protein interacts with a segment, 5′-CTGATGAAGT-3′, which is between 66 and 76 bp upstream from the transcriptional start site of promoter I.3. Using mammalian cell transfection experiments, SnaH was found to act as a repressor of promoter I.3 activity. Site-directed mutagenesis experiments have revealed that the NH_2-terminal SNAG domain is important for the repressor activity of SnaH. To demonstrate the inhibitory activity against aromatase expression, a stable SnaH-expressing MDA-MB-231 breast cancer cell line was generated, and the aromatase RNA messages in the SnaH-transfected cell line were found to be 30% of those in the vector-transfected cell line. Reverse transcription-PCR analysis on RNAs isolated from 12 cell lines has confirmed that SnaH is expressed at a higher level in normal breast epithelial cell and stromal fibroblast cell lines than in breast cancer cell lines. In addition, SnaH mRNA was detected in only 16 of 55 breast cancer specimens. On the other hand, aromatase mRNA was detected in 54 of the 55 specimens. Our results indicate that SnaH acts as a repressor that down-regulates the expression of aromatase in normal breast tissue by suppressing the function of promoter I.3. A reduction of the expression of SnaH in breast cancer tissue further suggests a cancer-protective role for this protein in normal breast tissue.

Introduction

Aromatase converts androgen to estrogen. Its expression level in breast cancer tissue has been found to be higher than in normal breast tissue (1). The control of human aromatase gene expression is complex in that several promoters direct aromatase gene expression in a tissue-specific manner (2–5). Although aromatase mRNAs in the noncancerous breast regions are exon I.4-dominant (6), our previous studies (7) on 70 breast tumor specimens using RT-PCR[4] have revealed that exons I.3 and pII are the two major exon Is present in aromatase mRNAs isolated from breast tumors. Our results suggest that promoters I.3 and II are the major promoters directing aromatase expression in breast cancer and surrounding adipose stromal cells. The switching of promoter usage, from promoter I.4 in normal tissue to promoters I.3 and II in breast cancer tissue, was also shown in a study on 49 Japanese breast cancer patients (8) and a study on 18 breast cancer patients (9).

Our laboratory has functionally characterized promoters I.3 (10) and II (11). We have also found a silencer element (S1) that is situated between promoters I.3 and II and that down-regulates the action of these two promoters (12). Several nuclear receptors including ERRα-1 and COUP-TF1 have been shown to be expressed in breast tissue and to bind to S1 (13). A cAMP-responsive element (CREaro) in the region upstream from promoter I.3 was also identified (14). We have proposed that in normal breast stromal cells, aromatase expression is driven by promoter 1.4, which is regulated mainly by glucocorticoid, and the action of promoters 1.3 and II is suppressed by the silencer S1 (14). However, in cancer tissue, cAMP production increases (15), and aromatase promoters are switched to cAMP-dependent promoters, *i.e.*, 1.3 and II. The recently identified CREaro is thought to play an important role enhancing the function of promoter I.3. To better understand the role of CREaro, we applied the yeast one-hybrid approach to screen a human breast tissue hybrid cDNA expression library for proteins binding to the CREaro region. Although we identified cAMP-responsive element binding protein 1 when the library was screened in the presence of cAMP[5], the major proteins identified when the library was screened in the absence of cAMP were the Snail and Slug zinc-finger proteins.

The *snail* gene was identified through the genetic analysis of dorsoventral pattering in *Drosophila*. Snail zinc-finger protein was thought to be required zygotically for mesoderm formation (16, 17). *Drosophila* Snail protein is a transcriptional repressor that acts to maintain the proper germ layers by repressing the expression within the mesoderm of regulatory genes involved in ectodermal development (18). The *snail* genes have also been cloned in mouse (19), *Xenopus laevis* (20), chicken (21), and zebrafish (22). However, Snail had never been reported in humans before our isolation of the cDNA clone. We have named the human snail SnaH. Slug is a member of the snail family of zinc-finger proteins. It is involved in epithelial to mesenchyme cell transition that leads to the maintenance of the progress zone (23) and plays a role in limb bud development (24). The cloning of the human *Slug* gene has been reported previously (25).

We are excited about the Snail and Slug findings for two reasons. First, this was the first time that a human form of Snail cDNA was isolated (the cDNA sequence was submitted to GenBank on February 2, 1999). Second, this was also the first time that SnaH and Slug were found to be expressed in human breast tissue. A series of experiments with our full-length SnaH cDNA clone have been carried out. We performed DNA mobility shift analysis and mutation analysis, which indicated that SnaH binds to a segment in the promoter I.3 region that overlaps with CREaro. cDNA transfection experiments have revealed that SnaH acts as a repressor that reduces promoter I.3 activity. Very importantly, RT-PCR experiments have demonstrated that SnaH (as

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[4] The abbreviations used are: RT-PCR, reverse transcription-PCR; cAMP, cyclic AMP; CAT, chloramphenicol acetyltransferase; ORF, open reading frame.

[5] K. Wu, D. Zhou, and S. Chen, unpublished observations.

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well as Slug) is expressed at a high level in normal breast epithelial cells and stromal fibroblasts but at a very low level in breast cancer cell lines and cancer tissue specimens. Our results support a specific action of SnaH on the activity of promoter I.3 of the human aromatase gene. A hypothesis for possible roles of SnaH and Slug in human breast tissue is presented.

Materials and Methods

Materials. Restriction endonucleases, T4 kinase and T4 DNA ligase were purchased from New England Biolabs, Inc. (Beverly, MA), Roche Molecular Biochemical, Inc. (Indianapolis, IN), or Promega (Madison, WI). α-^{35}S-labeled ATP and [γ-^{32}P]ATP were from New England Nuclear (Boston, MA). [^{14}C]chloramphenicol (D-threo-[dichloroacetyl-l-^{14}C]chloramphenicol; specific activity, 55 mCi/mmol) was from Amersham Life Science, Inc. (Arlington Heights, IL). The CAT expression vector, pUMSVOCAT, was a gift from Dr. K. Kurachi at the University of Michigan (Ann Arbor, MI).

Cell Culture. Heat-inactivated FCS (10%, v/v; Gemini, Calabasas, CA) and 1× antibiotics (Gemini) including 100 units of penicillin/ml, 100 μg of streptomycin sulfate/ml, and 0.25 μg of fungizone/ml were routinely added to media for all of the cell lines examined in this study. Hep-G2 cells, human hepatoblastoma cells, were grown in DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 1 mM sodium pyruvate. SK-BR-3 cells, human breast adenocarcinoma cells, were maintained in McCoy's 5a medium containing 2 mM L-glutamine. WS3PF, WS3TF, GI33PF, GI33TF, and W88TF, human breast fibroblast cell lines, were grown in Weymouth's MB 752/1 medium and were the gifts of Dr. R. J. Santen at the University of Virginia Health Science Center, (Charlottesville, VA). MCF-7 cells, human breast adenocarcinoma cells, were grown in Eagle's MEM with nonessential amino acids and sodium pyruvate. MDA-MB-231, a human breast adenocarcinoma cell line, was maintained in Leibovitz's L-15 medium. MCF-10A and HBL-100 are noncancerous breast epithelial cell lines. MCF-10A was grown in DMEM and Ham's nutrient mixture F-12 containing 5% equine serum, 2 μM insulin, 20 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, and 0.1 μg/ml chorela toxin. HBL-100 was grown in McCoy's 5a medium containing 2 mM L-glutamine.

Library Screening. A hybrid cDNA mammary gland library containing 3.5×10^6 independent clones was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). The library was titered and amplified according to Appendices B and C of MATCHMAKER GAL4 Two-Hybrid User Manual (Clontech Laboratories, Inc.). The library screening followed the MATCHMAKER One-Hybrid User Manual (Clontech Laboratories, Inc.). Complementary strands of a 27-bp aromatase genomic fragment (designated CREaroCF), which included a stretch of eight nucleotides of CREaro sequences, were synthesized to contain three tandem copies with an *Eco*RI site at the 5′ end and *Xho*I and *Xba*I sites at the 3′ end. The sequence for the sense strand is: 5′-CCCCCCGAATTCAACCTGCTGATGAAGTCACAAAATGA-CAACCTGCTGATGAAGTCACAAAATGACAACCTGCTGATGAAGTCACAAAATGACTCTAGACTCGAGCCCC-3′. The complementary strands were annealed and cloned into pHISi and pLacZi vectors. The resulting constructs were linearized with *Xho*I and *Nco*I, respectively, to facilitate integration into the yeast host YM4271 strain genome. Transformation of yeast cells was carried out using the Yeast Maker kit (Clontech Laboratories, Inc.). Integrated pHISi construct yeast strain was used as a reporter to screen the mammary gland cDNA library. Integrated pLacZi construct yeast strain was used to reconfirm positive clones using a β-galactosidase assay. Positive clones coding for fusion proteins that interact with CREaroCF were isolated from yeast hosts and retransformed into *E. coli* DH5α. Sequences of positive clones were then subjected to a database search (BLAST[6]) for homology.

DNA Sequencing and Analyses. DNA sequencing for short stretches of DNA for checking orientation of inserts was performed manually in the laboratory using the T7 Sequenase Version 2.0 (Amersham Life Science, Cleveland, OH) kit and α-^{35}S-labeled ATP for labeling. DNA analyses were performed using GCG Wisconsin software packages. A human Snail homologue (designated SnaH; *H* stands for Human or *Homo sapiens*) has been identified, and a 1696-bp SnaH cDNA sequence was submitted to GenBank (accession no. AF125377) on February 2, 1999.

[6] Internet address: http://www.ncbi.nlm.nih.gov/.

Expression of SnaH in *E. coli*. A 795-bp cDNA containing the coding region of SnaH was generated by PCR using a set of primers containing an *Eco*RI restriction site. The DNA fragment was subcloned into the expression vector pET-28a(+) containing the T7 promoter (Novagen, Inc., Madison, WI). The correct orientation, sequence, and in-frame of the fragment in the vector were confirmed by direct DNA sequencing using T7 promoter primer (5′-TAATACGACTCACTATAGGG-3′). The confirmed construct was designated pET-SnaH. A host cell BL21DE3 was transformed with pET-SnaH, cultured in LB containing 15 μg/ml of kanamycin, and induced by the addition of 1.0 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C for 2 h. The induced cells were harvested by centrifugation. The recombinant SnaH protein was solubilized and refolded according to the pET System Manual and Protein Refolding Kit (Novagen, Inc.). Briefly, the cells were resuspended in 0.1 culture volume of 20 mM Tris-HCl (pH 7.5)/10 mM EDTA/1% Triton X-100 and then put on ice. They were then treated with lysozyme (100 μg/μl) at 30°C for 15 min, sonicated five times (each time for 4×10 s) on ice at 40% Output (Branson Sonifier 450), and centrifuged at $10{,}000 \times g$ for 10 min. Most of the SnaH protein was found to be in the resulting pellet, as demonstrated by 10% SDS-PAGE. The pellet was washed several times and then solubilized in 50 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (pH 11.0) containing 0.3% *N*-lauroylsarcosine. The solubilized sample was incubated at room temperature for 15 min and centrifuged at $10{,}000 \times g$ for 10 min at room temperature. The detergent in the supernatant was slowly removed by two dialyses using 50× volumes of 20 mM Tris HCl (pH 8.5) containing 0.1 mM DTT and then dialyzed two more times in the same buffer without DTT (3 h each). The concentration of the renatured protein was determined by the Bradford method (26).

DNA Mobility Shift Analysis. The oligonucleotide with one copy of CREaroCF (5′AACCTGCTGATGAAGTCACAAAATGAC-3′) and its antisense strand oligonucleotide were synthesized by the DNA/RNA synthesis facility at the City of Hope National Medical Center/Beckman Research Institute. These two oligonucleotides were annealed together at 95°C for 10 min and then cooled down slowly to room temperature. The double-stranded oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 kinase and used as probes in DNA mobility shift analysis. DNA mobility shift analysis was conducted as described by Singh *et al.* (27). Briefly, 10 μl of recombinant SnaH protein was incubated with 60,000 cpm of ^{32}P-labeled probe at room temperature for 30 min in a total volume of 20 μl of the DNA mobility shift analysis buffer containing 12 mM HEPES (pH 7.9), 100 mM KCl, 10 μM $ZnCl_2$, 1 mM DTT, 12% (v/v) glycerol, 0.05% NP40, 20 mg/ml BSA, and 0.1 mg/ml of poly(dI-dC). The mixture was then electrophoresed on a 4% acrylamide gel with 0.5 × Tris-borate EDTA. Gels were dried and autoradiographed.

For the competition assays, the conditions for binding of SnaH protein to each probe were the same as those described above except that the appropriate amounts of the unlabeled oligonucleotides were supplemented in the binding reaction mixture 10 min before adding the labeled probe. To evaluate the interaction of SnaH protein with CREaroCF, five nonradioactive mutated double-stranded oligonucleotides were used as competitors in the DNA mobility shift analysis. The sequence of these oligo-mutants and the wild-type CREaroCF are as follows (only sense strands are shown): wild-type CREaroCF, 5′-AAC CTG CTG A**TG AAG TCA** CAA AAT GAC-3′; Mu 1, 5′-*TTG* CTG CTG A**TG AAG TCA** CAA AAT GAC-3′; Mu 2, 5′-AAC *GAC* CTG A**TG AAG TCA** CAA AAT GAC-3′; Mu 3, 5′-AAC CTG *CAC T***TG AAG TCA** CAA AAT GAC-3′; Mu 4, 5′-AAC CTG CTG A**TG *TT*G TCA** CAA AAT GAC-3′; Mu 5, 5′-AAC CTG CTG A**TG AAG T*TG*** CAA AAT GAC-3′. The CREaro sequence is in bold, *Drosophila* Snail-binding site-like sequence is underlined, and the mutated bases are in italic.

CAT Expression Plasmids. A low background promotorless CAT expression vector, pUMSVOCAT (28), with modification at its cloning sites (29) was used for preparation of the CAT expression constructs. The 1.2-kb aromatase genomic fragment containing 836 bp of 5′ flanking sequences of promoter I.3, promoter I.3 region, and the entire sequence of exon I.3 was subcloned into pBluescript vector, termed pBS-1.2-kb aro. This plasmid was used as a template DNA in the PCR reactions to generate a set of 5′ or 3′ deletion constructs with sets of 5′ and 3′ primers flanking the designated regions with an artificial *Hin*dIII site at the 5′ end and a *Xba*I site at the 3′ end. The fragments, −144/+5 bp, −76/+5 bp, and −32/+5 bp, were restricted with *Hin*dII/*Xba*I, purified on the agarose gel, and subcloned into the *Hin*dII/*Xba*I

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site of the modified pUMSVOCAT vector. These constructs were designated pUMS−144/+5CAT, pUMS−76/+5CAT, and pUMS−32/+5CAT, respectively.

Transfection and CAT Assays. Hepatoblastoma cell line Hep-G2 cells and breast adenocarcinoma SK-BR-3 cells were cotransfected with pSG5-SnaH, pSV-β-Gal, and pUMS−144/+5CAT, pUMS−76/+5CAT, or pUMS−32/+5CAT. Transfection was carried out using lipofectin according to the provided protocol (Life Technologies, Inc.). Briefly, 1 μg of each of the aforementioned constructs in the lipofectin reagent was added to 4×10^5 cells seeded in 60-mm tissue culture dishes. After an overnight incubation, the transfected cells were washed twice with appropriate volume of the regular growth medium and cultured for 24 h. The cells were then harvested by removal from the plates with a scraper, pelleted by centrifugation, and resuspended in 200 μl of 0.25 M Tris-HCl (pH 8.0). The cells were disrupted to release proteins by three quick freeze and thaw cycles. Aliquots of the lysates were used to determine protein concentration in each sample. An equal amount of protein from each sample was used for the CAT assays. Briefly, the appropriate amounts of proteins were incubated overnight at 37°C in a mixture containing [^{14}C]chloramphenicol and *n*-Butyryl-CoA. ^{14}C-labeled CoA was extracted with xylene and subjected to scintillation readings. An equal amount of total protein was also used in the β-galactosidase assay for transfection efficiency normalization.

Site-directed Mutagenesis. The mutants were generated by using a PCR-based mutagenesis method described by Nelson and Long (30). Briefly, the PCR mutagenesis used three forward primers, the nucleotide sequences of which were as follows with the mutated bases and amino acids in bold:

SnaH-P2A: 5′-*ATG GCC TCG AAT TCT ATG* **GCG** *CGC TCT TTC CTC GTC*-3′
M **A** *R S F L V*

SnaH-F5A: 5′-*ATG GCC TCG AAT TCT ATG CCG CGC TCT* **GCC** *CTC GTC*-3′
M P R S **A** *L V*

SnaH-V7A: 5′-*ATG GCC TCG AAT TCT ATG CCG CGC TCT TTC CTC* **GCC** 3′
M P R S F L **A**

The nucleotide sequence of the reverse primer is: 5′-GAA TTC TCA GCG GGG ACA TCC TGA GC-3′.

PCR was carried out with a DNA Thermal Cycler 480 (Perkin-Elmer Corp., Norwalk, CT). The reaction mixture (in 100 μl) contained five units of Taq DNA polymerase, 20 ng of pSG5-SnaH as template, and 200 nmol each of forward and reverse primers. The PCR cycles were 1 min at 94°C to denature the template DNA, 1 min at 50°C to allow the primers to anneal, and 1 min at 72°C for DNA extension, and they were cycled 30 times. The PCR products were resolved over 1.5% agarose gel, purified by using QIAGEN Gel Extraction Kit (Qiagen, Inc., Chatsworth, CA), and cloned by using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). After blue-white selection, transformed competent cells were grown in LB media, and plasmids were extracted by using QIAprep Spin Miniprep Kit (Qiagen, Inc.) followed by *Eco*RI digestion. SnaH fragments were purified from agarose gel and were inserted into pSG5 expression vector (Stratagene, La Jolla, CA) digested with *Eco*RI. The orientation of SnaH-fragments was confirmed by restriction enzyme digestion and sequencing.

Constructs of wild-type SnaH or its mutants (SnaH-P2A, -F5A, and -V7A) were transiently cotransfected with pUMS−76/+5CAT and pSV-β-Gal into HepG2 cells growing on 6-well plates as described above. After washing cells with PBS, cells were lysed in Reporter Lysis Buffer (Promega, Madison, WI), and cell extracts were collected by scraping. The lysates were centrifuged, and the supernatants were used for β-galactosidase assay to define transfection efficiency. Remaining extracts were heated for 10 min at 60°C followed by centrifugation, and the supernatants were used for CAT assay as described above.

RT-PCR Analysis.

RNA Isolation and RT-PCR. Fifty five primary breast carcinoma samples were collected immediately after surgery and quickly frozen by dropping them in liquid nitrogen. The tissue specimens were stored at −70°C until they were analyzed. Total RNA were isolated with the single step method described by Chomczymski (31). All of the RNAs were treated with RNase-free DNase I to get rid of the minimum contamination of genomic DNA.

The semiquantitative analyses were performed using the Titan one tube RT-PCR system (Roche Molecular Biochemical). All of the samples were first analyzed using β-actin primers, and the RNA levels for the analyses of both SnaH and human Slug were normalized based on the results from analyses with a set of β-actin primers. RT-PCR conditions were as indicated below. After 1-h incubation at 50°C, 25 cycles of PCR were run at 94°C for 30 s, 50°C for 1 min, and 68°C for 2 min. The reaction was ended with a 7-min elongation period at 68°C. We performed PCR at a nonsaturating condition, *i.e.*, 25 cycles. In this way, at least we could compare the relative expression levels of nuclear receptors in our samples. It has been shown that with 25 cycles, product accumulation is exponential (7). Furthermore, the quantity of PCR products generated with 25 cycles of PCR increases in a mRNA concentration-dependent manner (7). In addition, a proper control such as PCR analysis with RNA, without treating with reverse transcriptase, was performed to assure us that the PCR products were derived from mRNA. The sequences for the primers and hybridization probes are shown below. SnaH: forward, 5′-AAT CGG AAG CCT AAC TAC AGC GAG-3′; reverse, 5′-CCT TGG CCT CAG AGA GCT GG-3′; hybridization probe, 5′-TGG GCC TCC CTT CGG CTC CAG GAG AGT CCC AGG GTG G-3′; Slug: forward, 5′-GCG CTC CTT CCT GGT CAA GAA GCA TTT CAA CG-3′; reverse, 5′-CTC ACA TAT TCC TTG TCA CAG TAT TTA CAG-3′; hybridization probe, 5′-CCC AGC TAC CCA ATG GCC TCT CTC CTC TTT CCG-3′; Aromatase: forward primer, 5′-GAC TCT AAA TTG CCC CCT CTG; reverse primer, 5′-GTG CCC TCA TAA TTC CAC AC; hybridization probe, 5′ ATG GTT TTG GAA ATG CTG AA; β-actin: forward, 5′-AGG AGC ACC CCG TGC TGC TGA-3′; reverse, 5′-CTA GAA GCA TTT GCG GTG GAC-3′; hybridization probe, 5′-CAT CAC CAT TGG CAA TGA GCG GTT CCG CTG -3′.

Hybridization and Chemiluminescent Detection. The RT-PCR products (10 μl each) were run on 1.5% agarose gel, transferred to the positively charged Zeta membrane (Bio-Rad Laboratories, Hercules, CA) in 20 × SSC, and UV cross-linked. All of the probes were labeled with DIG-11-ddUTP using the 3′-end oligonucleotide labeling kit (Roche Molecular Biochemical). The blots were prehybridized in DIG-Easy-Hyb (Roche Molecular Biochemical) for 1 h and hybridized overnight at each hybridization temperature with each probe. The hybridization temperature was generally 5 to 10°C below the melting temperature of the probe. After hybridization, the blots were washed two times for 1 min in 2 × SSC, 0.1% SDS, and two times for 15 min in 0.1 × SSC, 0.1% SDS.

The RT-PCR products were detected according to the manufacturer's instructions. After hybridization and washes, membranes were rinsed briefly in washing buffer (maleic acid buffer). The membranes were incubated for 30 min in blocking solution, then in anti-DIG-AP (1:10,000) solution for 30 min, and then two times for 15 min in washing buffer and equilibrated for 2 min in detection buffer. CSPD read-to-use solution (Roche Molecular Biochemical) was applied on the membranes that were incubated for 5 min at room temperature. After incubation for 15 min at 37°C, the membranes were exposed to Lumi-film Chemiluminescent detection film (Roche Molecular Biochemical) for 15–30 min at room temperature. Image density was quantified with an Imaging Densitometer (Model GS-670; Bio-Rad Laboratories).

Overexpression of SnaH in MDA-MB-231 Breast Cancer Cell Line by Stable cDNA Transfection. The SnaH cDNA was generated by PCR with the *Eco*RI restriction site at both ends and then ligated into the mammalian expression vector, pHβ-Apr-1-Neo, after a published procedure (32). The orientation and sequence of the cDNA was confirmed by dideoxy sequencing.

MDA-MB-231 cells were cultured in RPMI medium 1640, containing 10% fetal bovine serum (Omega Scientific, Inc., Tarzana, CA), 5 ml of (10,000 units/ml) antibiotic-antimyotic (Life Technologies, Inc., Grand Island, NY), 2 mM L-glutamine, and 1 mM sodium pyruvate (IS; Irvine Scientific, Santa Ana, CA) in 500 ml medium for each cell culture. When cells had grown about 70% confluent, Phβ-Apr-1-Neo vector and plasmid pHβ-SnaH were introduced into the cells with lipofectin. The transfection experiments were performed using lipofectin, following the manufacturer's protocol (Bethesda Research Laboratories). For the selection of transfected cells, the beginning concentration of G418 was 0.6 mg/ml in 5-ml culture volume. The G418 concentration was increased at an increment of 0.1 mg/ml G418/week, and the final concentration of G418 was 1 mg/ml.

Semi-quantitative Analysis of Aromatase and SnaH in Transfected MDA-MB-231 Cell Lines. The SnaH-stable-transfected MDA-MB-231 and the vector-transfected cells were grown in culture media containing 100 μg/ml G418. For RT-PCR, total RNAs were extracted from cultured cells using TRIzol (Life Technologies, Inc.). PCR was carried out under the conditions indicated below. β-actin (15), SnaH (25), aromatase (35) cycles of PCR were run at 94°C for 1 min, 50°C for 1 min, and 68°C for 2 min. The reaction was

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ended with a 10-min elongation period at 68°C. The PCR products were run on 1.5% agarose gel, transferred to the positively charged Zeta membrane GT (Bio-Rad) in 20 × SSC, and UV cross-linked. All of the probes were labeled with ^{32}P using T4 DNA kinase. The blots were prehybridized in 20 mM sodium phosphate buffer (pH 7.2) containing 7% SDS for 1 h and hybridized overnight at 41°C (aromatase) or 65°C (SnaH and β-actin). After hybridization, the blots were washed at hybridization temperature two times for 30 min in 20 mM sodium phosphate buffer (pH 7.2) containing 5% SDS and two times for 30 min in 20 mM sodium phosphate buffer (pH 7.2) containing 1% SDS. The membranes were exposed to X-ray films for 6 h-3 days at −80°C. Image density was quantified with an Imaging Densitometer (Model GS-710; Bio-Rad).

Statistical Analysis. The results were analyzed by the Student *t* test. Results were considered significant when $P < 0.05$.

Results

Identification of Snail and Slug Zinc-finger Proteins in Human Breast Tissue That Bind to the CREaro. Screening of a cDNA mammary gland library containing 1×10^6 clones using a yeast-one hybrid system generated 14 positive clones. Five of the positive clones were homologues of *Drosophila* Snail, six were identical with human Slug, and three shared homology with rRNAs.

Sequence Analysis and Characterization of Human Snail Homologue (SnaH). Sequence analysis of the 1696-bp *Eco*RI/*Xho*I insert from positive clone 6A, which contains a sequence that is homologous with known Snail sequences from other species, revealed an ORF. The 795-bp ORF shares highest homology with mouse Snail zinc-finger protein Sna (83% identity; Fig. 1). The encoding protein was named SnaH, where *H* stands for human or *Homo sapiens*. There are 61-bp upstream and 840-bp, including a poly(A) sequence, downstream from the ORF region (Fig. 2). The translated SnaH contains 264 amino acids and four zinc fingers (Fig. 2). The length of the SnaH is identical to mouse Sna but significantly shorter than *Drosophila* Snail, which is 390 amino acids in length. We have performed DNA mobility shift analyses and transcriptional function studies using expression constructs containing the coding region of SnaH cDNA.

Demonstration of Interaction of SnaH with CREaro by DNA Mobility Shift Analyses and DNA Mutation Experiments. The nuclear extracts from SK-BR-3 cells and breast fibroblasts have been shown to bind to the CREaro/snail regulatory site near the aromatase promoter I.3 (14). Although these cell lines express SnaH (discussed below), we feel that it is necessary to use a recombinant SnaH protein to determine, in a direct fashion, whether SnaH indeed interacts with CREaro and whether a specific nucleotide sequence present in CREaro is responsible for the binding of SnaH. The nucleotide sequence of the genomic region containing the CREaro element is shown in Fig. 3. We generated a series of five double-stranded oligonucleotide competitors that contain di- or tri-nucleotide mutations in the CREaroCF (as indicated in "Materials and Methods"). These oligomutants were independently tested in a DNA mobility shift analysis to determine whether they could compete with a radiolabeled wild-type CREaroCF probe for the binding of SnaH protein. If the mutated competitors are able to compete with the wild-type probe for SnaH protein binding, then the nucleotides that were mutated are not involved in the binding. Conversely, if the oligomutants cannot compete with the wild-type probe for binding, then the regions containing mutated nucleotides are probably involved in the interaction with the DNA-binding proteins. We have demonstrated that the recombinant SnaH interacts with CREaroCF in a dose-dependent manner and that nonspecific DNA, poly(dI-dC) cannot compete with the binding in DNA mobility shift analysis (data not shown). We added 30-, 60-, 120-fold molar excess of the unlabeled wild-type CREaroCF or five oligomutants in the reaction mixture in the competition experiments. Whereas Mu 1, Mu 2, and Mu 5 competed as well as the wild type, Mu 3 and Mu 4 oligonucleotides did not compete as well as the wild type (Fig. 4). The results of the DNA mobility shift analysis lead us to propose that the region at −70 bp to −61 bp, -CTG ATG AAG T-, is involved in the interaction with SnaH.

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   1 cagcgagtggttcttctgcgctactgctgcgcgaatcggcgaccccagtgcctcgaccac  60
  61 tatgccgcgctctttcctcgtcaggaagccctccgaccccaatcggaagcctaactacag 120
       M  P  R  S  F  L  V  R  K  P  S  D  P  N  R  K  P  N  Y  S

 121 cgagctgcaggactctaatccagagtttaccttccagcagccctacgaccaggcccacct 180
       E  L  Q  D  S  N  P  E  F  T  F  Q  Q  P  Y  D  Q  A  H  L

 181 gctggcagccatcccacctccggagatcctcaaccccaccgcctcgctgccaatgctcat 240
       L  A  A  I  P  P  P  E  I  L  N  P  T  A  S  L  P  M  L  I

 241 ctgggactctgtcctggcgccccaagcccagccaattgcctgggcctcccttcggctcca 300
       W  D  S  V  L  A  P  Q  A  Q  P  I  A  W  A  S  L  R  L  Q

 301 ggagagtcccagggtggcagagctgacctccctgtcagacgaggacagtgggaaaggctc 360
       E  S  P  R  V  A  E  L  T  S  L  S  D  E  D  S  G  K  G  S

 361 ccagccccccagcccaccctcaccggctccttcgtccttctcctctacttcagcctcttc 420
       Q  P  P  S  P  P  S  P  A  P  S  S  F  S  S  T  S  A  S  S

 421 cttggaggccgaggcctatgctgccttcccaggcttgggccaagtgcccaagcagctggc 480
       L  E  A  E  A  Y  A  A  F  P  G  L  G  Q  V  P  K  Q  L  A

 481 ccagctctctgaggccaaggatctccaggctcgaaaggccttcaactgcaaatactgcaa 540
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 541 caaggaatacctcagcctgggtgccctcaagatgcacatccgaagccacacactgccctg 600
       K  E  Y  L  S  L  G  A  L  K  M  H  I  R  S  H  T  L  P  C

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       V  C  G  T  C  G  K  A  F  S  R  P  W  L  L  Q  G  H  V  R

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       T  H  T  G  E  K  P  F  S  C  P  H  C  S  R  A  F  A  D  R

 721 ctccaacctgcgggcccacctccagacccactcagatgtcaagaagtaccagtgccaggc 780
       S  N  L  R  A  H  L  Q  T  H  S  D  V  K  K  Y  Q  C  Q  A

 781 gtgtgctcggaccttctcccgaatgtccctgctccacaagcaccaagagtccggctgctc 840
       C  A  R  T  F  S  R  M  S  L  L  H  K  H  Q  E  S  G  C  S

 841 aggatgtccccgctgaccctcgaggctccctcttcctctccatacctgcccctgcctgac 900
       G  C  P  R  *

 901 agccttccccagctccagcaggaaggaccccacatccttctcactgccatggaattccct 960
 961 cctgagtgccccacttctggccacatcagccccacaggactttgatgaagaccatttttct 1020
1021 ggttctgtgtcctctgcctgggctctggaagaggccttcccgtggccatttctgtggagg 1080
1081 gagggcagctggccccagccctgggggattcctgagctggcctgtctgcgtgggttttt 1140
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1201 ctgggaagctcccaccccactcagggggaccccactccctcacacacccccccccacaag 1260
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1441 gaaaagggactgtgagtaatggctgtcacttgtcgggggcccaagtggggtgctctggtc 1500
1501 tgaccgatgtgtctcccagaactattctgggggcccgacaggtgggcctgggaggaaaat 1560
1561 gtttacatttttaaaggcacactggtatttatatttcaaacattttgtatcaaggaaacg 1620
1621 ttttgtatagttatatgtacagtttattgatattcaataaagcagttaatttatatatta 1680
1681 aaaaaaaaaaaaaaaa 1696
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Fig. 1. Complete DNA sequences of 1696-bp *Eco*RI/*Xho*I fragment of positive clone 6A from screening the cDNA mammary gland library and translated SnaH (62-856) amino acid sequences. Four regions of amino acid sequence containing zinc-finger motif are *underlined, italicized*, and *bold-faced*. The internal *Eco*RI cutting site is *underlined* and *bold-faced* (953-958).

Functional Studies Revealed Repressive Role of SnaH on Promoter I.3 of the Human Aromatase Gene. The biological significance of SnaH on aromatase expression was examined by mammalian cell transfection experiments. Human breast adenocarcinoma SK-BR-3 and hepatoblastoma Hep-G2 cells were cotransfected with pSG5-SnaH, CAT reporter vectors with two different lengths of promoter I.3 regions (*i.e.*, −144/+5 and −76/+5 constructs that contain CREaro) and pSV-β-Gal for normalization of data according to transfection efficiency. Expression of SnaH reduces CAT activity significantly in both SK-BR-3 and Hep-G2 cells in comparison with the controls (Fig. 5, *A* and *B*). As a control experiment, SnaH did not have an effect when the analysis was performed using a reporter construct that did not contain the SnaH-binding site (*i.e.*, −32/+5; see Fig. 5*B*). In addition, the inhibitory activity of SnaH was not modified by incubating cells in the presence of forskolin (data not shown), indicating that cAMP is not needed for the interaction of SnaH with its binding element. Our results indicate that SnaH protein binds to the

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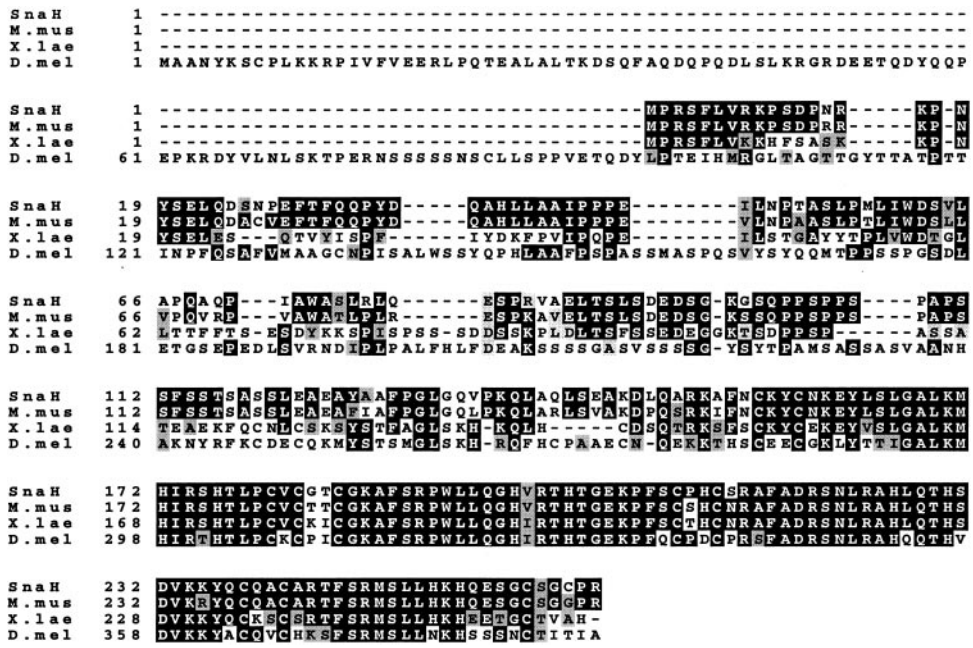

Fig. 2. Alignment of amino acid sequences of SnaH, Sna of *Mus musculus* (*M. mus*), snails of *Xenopus laevis* (*X. lae*), and *Drosophila melanogaster* (*D. mel*). Identical residues are shaded in *black*. Similar residues are shaded in *gray*.

region upstream from promoter I.3 in the human aromatase gene and reduces the promoter I.3 activity.

Grimes *et al.* (33, 34) have suggested that Snail and Slug belong to a protein family containing a SNAG domain, *i.e.*, a 20-amino acid NH_2-terminal repressor domain. Nakayama *et al.* (35) have demonstrated that the maximal repressive effect of mSna, the murine homologue, is dependent on both the zinc-finger DNA-binding domain and the NH_2-terminal, seven-amino acid SNAG domain. To determine the role of the NH_2-terminal seven-amino acid SNAG domain of SnaH for its repressor activity, we prepared three mutants, SnaH-P2A, SnaH-F5A, and SnaH-V7A. The Pro-2 to Ala mutation totally eliminated the ability of SnaH to suppress promoter I.3 activity (Fig. 5*C*). These results indicate that the NH_2-terminal SNAG domain of SnaH is important for its repressor activity.

Expression of SnaH and Slug in Breast Cell Lines and Tissue Specimens. We examined the levels of SnaH and Slug mRNAs through RT-PCR Southern analysis with RNA isolated from 55 breast cancer tissues, 5 breast fibroblast primary cell lines, 3 breast cancer cell lines, 1 hepatoblastoma Hep-G2 cell line, and 2 non-cancer breast epithelial cell lines. As can be seen in Fig. 6, the two breast normal epithelial cell lines (*i.e.*, MCF-10A and HBL-100) and five breast stromal fibroblast cell lines have relatively high levels of SnaH and Slug RNA messages, but aromatase mRNA was not detected in these cell lines. On the other hand, aromatase mRNA can be detected in breast cancer cell lines, and the levels of

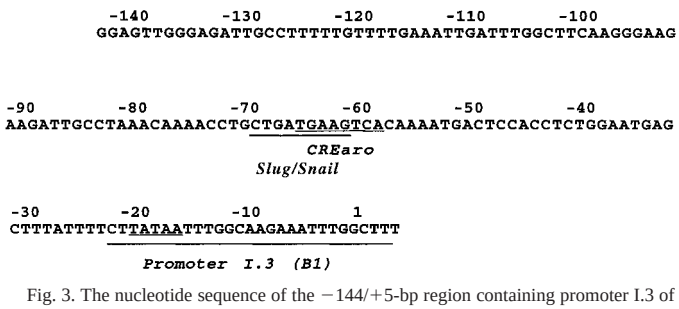

Fig. 3. The nucleotide sequence of the −144/+5-bp region containing promoter I.3 of the human aromatase gene. The important regulatory segments, CREaro, promoter I.3, and Snail/Slug-binding site (identified in this study), are *underlined*.

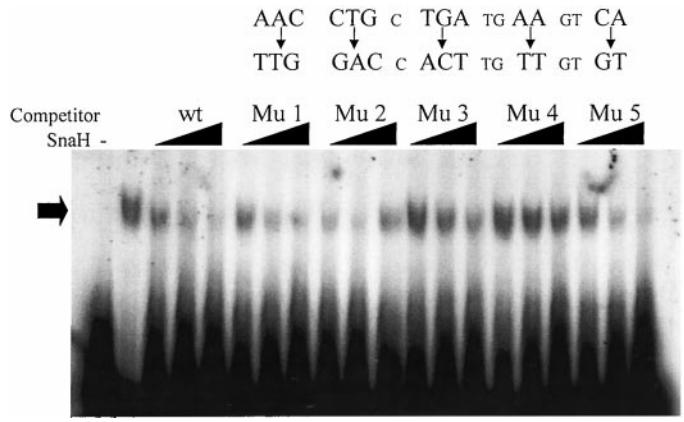

Fig. 4. DNA mobility shift analysis of CREaro containing oligonucleotide in the presence of recombinant SnaH protein generated in *E. coli*. SnaH protein was incubated with a ^{32}P-labeled CREaroCF probe in the absence (−) or presence of an excess of nonradiolabeled competitor. Six different competitors were used, (*wt*) the wild-type CREaroCF and (*Mu1-Mu5*) sequential di- or tri-nucleotide mutations made in the context of CREaroCF sequence (mutated di- or tri-nucleotide shown below the wild-type sequence). The *crescendo triangles* represent increasing excess of nonradiolabeled competitor (30-, 60-, and 120-fold) added to the reaction.

SnaH and Slug mRNAs in these cell lines are low. SnaH expression was not detected in MCF-7 and MDA-MB-231 cell lines, and Slug expression was not detected in the MCF-7 cell line. Most interestingly, SnaH mRNA was detected in only 16 of 55 breast cancer specimens, and Slug mRNA was absent in all of the 55 specimens. On the other hand, aromatase mRNA was detected in 54 of the 55 specimens. Although additional analyses on normal breast tissues are needed, our results indicate that these two zinc-finger proteins are regularly expressed in normal breast tissue, and that their expressions are reduced in breast cancer tissue.

Characterization of SnaH Overexpressing MDA-MB-231 Cells. To confirm the results that SnaH down-regulated the aromatase promoter activity, we have prepared a SnaH-overexpressing MDA-MB-231 breast cancer cell line. As indicated above, SnaH expression was not detected in the MDA-MB-231 cell line. The expression of SnaH in the transfected cell line was confirmed by RT-PCR

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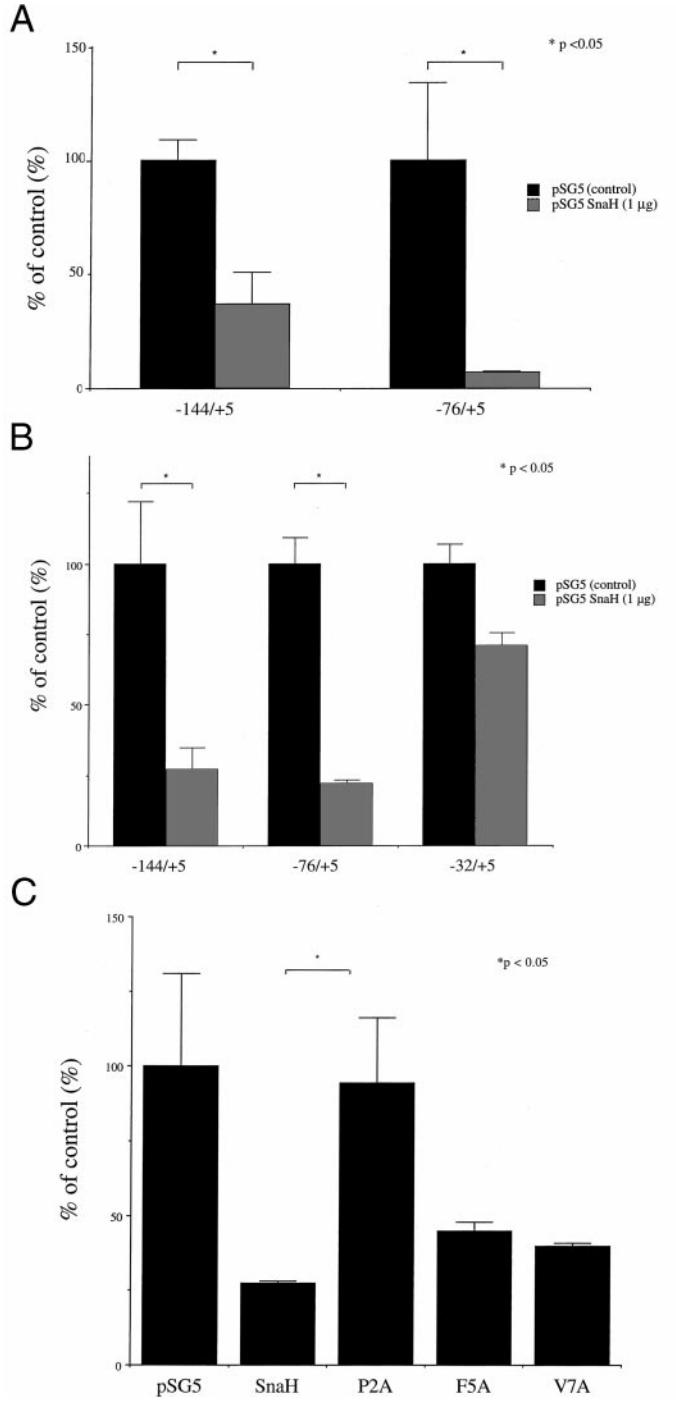

Fig. 5. Functional analysis of SnaH on the promoter I.3 activity of human aromatase gene. SK-BR-3 (*A*) and Hep-G2 (*B*) cells were cotransfected with a CAT deletion construct containing different lengths of the 5′ flanking region of promoter I.3 and SnaH-expressing construct, pSG5-SnaH (1 μg), or just an empty vector, pSG5. pUMS −144/+5 CAT and pUMS −76/+5 CAT contain CREaro region, but pUMS −32/+5 CAT does not contain CREaro. The CAT activities are expressed as the mean ± SE of three independent experiments, and activities in each cell transfected with SnaH-expressing constructs are expressed as a percentage of each control. *C*, functional analysis of SnaH by site-directed mutagenesis. Hep-G2 cells were cotransfected with pUMS −76/+5 CAT and pSG5-SnaH (1 μg), its mutants or an empty vector, pSG5. The CAT activities in cells transfected with the pSG5 vector and pUMS −76/+5 CAT were taken as 100%. ∗, $P < 0.05$.

analysis (data not shown). The aromatase RNA messages in the SnaH-transfected cell line were found to be 30% of those in the vector transfected cell line (Fig. 7). These results indicate that overexpression of SnaH in MDA-MB-231 cells suppresses aromatase expression.

Discussion

Our laboratory previously identified and characterized CREaro in the region upstream from promoter I.3 of the human aromatase gene and demonstrated that cAMP-responsive element-binding protein 1 and some other proteins may bind to this region to control the promoter activity (14). In the present study, we used yeast one-hybrid screening to identify proteins that bind to this region. In the absence of cAMP, the proteins identified were SnaH and Slug zinc-finger proteins. SnaH has 83% homology with mouse Snail protein (Sna). Because we submitted the SnaH cDNA sequence data to the GenBank Data Libraries under accession no. AF125377 on February 2, 1999, two papers describing the characterization of human *snail* genes have been published (36, 37).

The nucleotide and amino acid sequence analyses revealed that the similarity of human, mouse, *Xenopus*, and *Drosophila* Snail extends throughout the coding region, and the human and mouse Snail proteins are shorter than the others (Fig. 2). Both the coding region and amino acid sequences of SnaH share the highest homology with those of mouse Snail (Sna, 83%; xsna, 54.5%; snail, 39.0% homology, respectively). The zinc-finger regions are highly conserved among them. Structural and functional conservation suggests that these proteins may have originated in the extremely ancient common ancestors of insects and vertebrates. However, SnaH has only four zinc fingers and does not encode for the first zinc finger found in *Xenopus* and *Drosophila*. Mouse and zebrafish Snail proteins also have only four zinc fingers. These observations suggest that the first zinc finger in *Drosophila* and *Xenopus* snail may not be critical for the protein functions or may have a different function. In addition, the translated SnaH contains 264 amino acids (Fig. 2). The length of the SnaH is identical to mouse Sna but significantly shorter than *Drosophila* Snail, which is 390 amino acids in length.

By DNase I footprinting analysis, Kasai *et al.* (38) and Ip *et al.* (39) identified *Drosophila* Snail-binding sites in the promoter regions of the gene *single-minded* (*sim*), ANCACCTGTTNNCA, and *rhomboid* (*rho*), C(A/C)ACTTGC, respectively. Mauhin *et al.* (40) defined six Snail-binding sequences (CAGGTG, CAAGTG, CAGGTT, CAGATG, CACGTG, and CATGTG) using a pool of oligonucleotides with random sequences, and the base frequency analysis suggested that each motif has its own binding capacity. On the basis of our DNA mobility shift analysis, it was found that SnaH protein binds to the CREaroCF region and probably interacts with the region between −70 bp to −61 bp, CTGATGAAGT, relative to the human aromatase transcriptional start site of promoter I.3. Because SnaH is a four-finger protein instead of a five-finger protein, as is the *Drosophila* Snail, it is not surprising that, of the two species, Snail recognizes different DNA sequences. Furthermore, the findings from our laboratories and others suggest that SnaH binds to DNA with a broad specificity.

The functional analysis revealed that SnaH is a repressor that reduces the activity of promoter I.3 of the human aromatase gene. We used two reporter constructs containing CREaro (*i.e.*, −144/+5 and −76/+5) and performed transfection experiments in two mammalian cell lines, SK-BR-3 and HepG2. SK-BR-3 is a breast cancer cell line. The HepG2 cell line (human hepatoblastoma cell line) was chosen because aromatase is expressed in this cell line. We have also performed the control experiment using a reporter construct that does not contain the SnaH-binding site (*i.e.*, −32/+5). SnaH did not have an effect when the analysis was performed using the latter construct, supporting the hypothesis that SnaH suppresses the promoter activity by interacting with the proposed SnaH-binding site. As indicated in "Results," the SnaH expression was not detected in the human breast cancer MDA-MB-231 and MCF-7 cell lines. To further analyze the effect of SnaH on aromatase expression, we have generated a SnaH-

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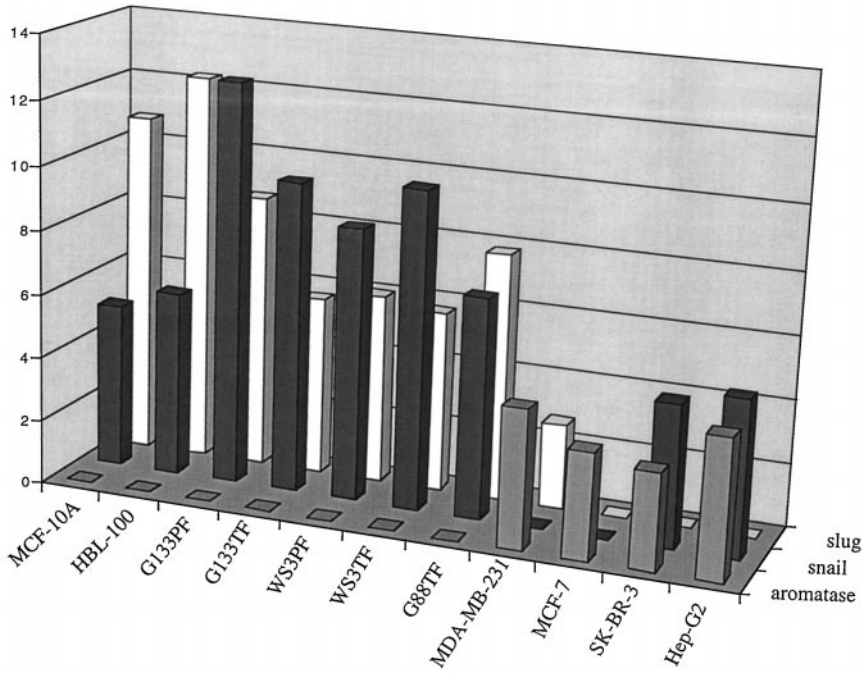

Fig. 6. RT-PCR Southern analysis of mRNA expression levels of aromatase, SnaH, and Slug in two noncancerous breast epithelial cell lines (MCF-10A and HBL-100), five breast fibroblast primary cells (WS3PF, WS3TF, GI33PF, GI33TF, and W88TF), three breast cancer cell lines (MDA-MB-231, MCF-7, and SK-BR-3), and a hepatoblastoma cell line (Hep-G2). Each *bar* represents a relative mRNA expression level normalized by that of β-actin.

overexpressing MDA-MB-231 cell line. The aromatase mRNA level in the SnaH-expressing cell line was found to be one third that of the vector-transfected cell line, confirming that SnaH suppresses aromatase expression (Fig. 7). Attempts were also made to generate a SnaH-expressing MCF-7 cell line. However, we have not yet been able to obtain any live cells after the transfection process.

As shown in Fig. 2, SnaH, mSna, and *Xenopus* snail have the identical NH_2-terminal seven-amino acid SNAG domain. Our results from site-directed mutagenesis experiments confirm that this domain is important for the repressor activity of SnaH (see Fig. 5*C*).

The proposed SnaH-binding element overlaps six nucleotides with CREaro, an enhancer element situated upstream from promoter I.3 of the human aromatase gene (see Fig. 3). It is thought that SnaH can quench the CREaro activity. "Quenching" is a form of gene regulation whereby activators and repressors cooccupy neighboring sites in a target promoter, but the repressor blocks the ability of the activator to contact the transcription complex (41). Gray *et al.* (41) have reported that Snail is not a dedicated repressor but, instead, appears to block disparate activators. We identified SnaH (and Slug) in a human mammary cDNA library in the absence of cAMP. In addition, these zinc-finger proteins are expressed at a relatively high level in normal breast epithelial cells and fibroblasts, but their expression levels in breast cancer tissue are very low. The present observation and our previous work lead us to propose that in normal breast epithelial cells and stromal fibroblasts, SnaH is expressed and prevents proteins from binding to CREaro. This results in a suppression of promoter I.3 activity. On the other hand, in cancer tissue, human aromatase promoter usage switches to promoter I.3, which is in response to cAMP activation of cAMP-responsive element-binding proteins and to a decrease in SnaH (and Slug) expression.

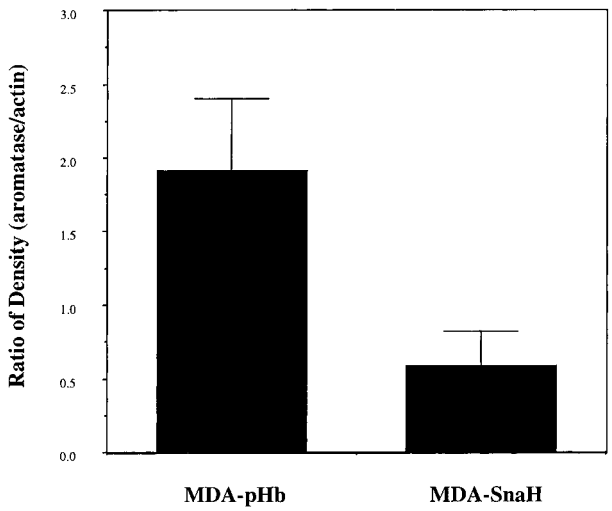

Fig. 7. Aromatase expression in the SnaH-expressing MDA-MB-231 cell line. The aromatase mRNA PCR products in the aromatase-expressing cell line (*MDA-SnaH*) and in the vector-transfected cell line (*MDA-pHb*) were generated and analyzed as described in "Materials and Methods." The results are shown as the mean ± SE of three independent experiments.

The *snail* gene was originally identified in *Drosophila* (16). It acts to restrict neuroectoderm and neural fate in the invaginating mesoderm (17) as a regulator of neurogenesis (42) and is necessary for the maintenance of vestigial expression in the wing disc (43). *Xsna*, a *Xenopus* gene, is expressed zygotically in all of early mesoderm (20). A mouse gene (*Sna*) encodes a 264-amino acid protein that contains four zinc fingers, like the SnaH. *Sna* transcripts are expressed throughout postimplantation development (44). *CSnR* is expressed in the right-hand lateral mesoderm during normal chick development (21). Our study represents the first direct functional analysis of human Snail, SnaH. Although we do not yet know the role of this protein in early development, we have demonstrated a specific role for this protein in modulating estrogen formation in breast tissue. A recent report by Batlle *et al.* (45) has suggested that Snail is a repressor of E-cadherin gene expression in epithelial tumor cells. The study was mainly performed using mouse Snail, *i.e.*, Sna. As shown in our RT-PCR studies and in Northern analysis by Batlle *et al.* (45), SnaH is expressed at a higher level in noncancer cell lines and fibroblasts than in breast cancer cell lines and breast tumor specimens. The role of SnaH may not be as simple as that proposed by Batlle *et al.* (45). The *Drosophila* Snail contains a P-DLS-K sequence and was thought to interfere with the interaction between CtBP and adenovirus E1A protein, which is involved in transcriptional activation and tumori-

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genesis (46). It is important to point out that SnaH is shorter than the *Drosophila* Snail and that the P-DLS-K sequence is not present in SnaH (see Fig. 1).

The *Slug* gene is a second member of the *snail* family. *Slug* expression is correlated with areas of undifferentiated mesenchyme at various stages of tissue differentiation, suggesting a role in maintaining the mesenchymal phenotype and repressing the differentiation processes in early development (24). The mouse slug (Slugh) is not required for mesoderm formation or for neural crest generation, migration, or development in mice (47). These results indicate that neither the expression pattern nor the biological function of the *Slug* gene is conserved among all of vertebrates. The findings that Slug is expressed in noncancer cell lines and fibroblasts and expressed at very low levels in breast cancer cell lines and tumor tissue are very interesting. Because the Slug cDNA clones that we isolated from yeast one-hybrid screening were not full-length clones, we did not include the Slug functional studies in this investigation. We have recently generated the full-length Slug clone by RT-PCR, and experiments are being designed to study the role of this protein in breast cancer development.

The *Escargot* is a third member of the *Snail* family. The *Drosophila Escargot* has a 72% nucleotide identity with *Snail* and a 76% nucleotide identity with *xsnail* (48). The protein has a similar zinc-finger domain. Escargot protein regulates tracheal branch fusion in *Drosophila*. During development of tubular networks, epithelial tubes must fuse to each other to form a continuous network. Little is known about the cellular mechanisms or molecular control of epithelial tube fusion. Escargot is an early fusion marker. Its ectopic expression activates the fusion process and suppresses branching throughout the tracheal system, leading to ectopic tracheal connections that resemble certain arteriovenous malformations in humans (49).

In summary, using the yeast one-hybrid approach to screen a human breast tissue hybrid cDNA expression library, we found that a zinc-finger transcriptional factor Snail (SnaH) and another member of the Snail family, Slug, interacted with a regulatory region near promoter I.3 of the human aromatase gene. In addition, these proteins are expressed in noncancerous cell lines and stromal fibroblasts, but they are expressed at significantly lower levels in breast cancer cell lines and tumor tissue. Our functional analysis revealed that SnaH suppresses the activity of promoter I.3 of the human aromatase gene. These results explain, in part, our previous findings that promoter I.3 is down-regulated in normal breast tissue by SnaH and a previously characterized silencer element (12). On the other hand, promoter I.3 activity is up-regulated in breast cancer tissue, possibly by a decrease of the expression of SnaH and Slug. It is not unexpected that these zinc-finger proteins have additional functions in human breast tissues. Considering the regulatory action of Escargot, a member of the Snail family, on tracheal branch fusion in *Drosophila*, we propose that SnaH/Slug may be involved in the development of ductal networks in the breast. This hypothesis is presently being examined in our laboratory.

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