VILIP-1 (gene name VSNL1), a member of the neuronal Ca$^{2+}$ sensor protein family, acts as a tumor suppressor gene by inhibiting cell proliferation, adhesion, and invasiveness. VILIP-1 expression is down-regulated in several types of human cancer. In human non-small cell lung cancer, we found that down-regulation was due to epigenetic changes. Consequently, in this study we analyzed the VSNL1 promoter and its regulation. Serial truncation of the proximal 2-kb VSNL1 promoter (VP-1998) from its 5' terminus disclosed that the last 3’ terminal 100-bp promoter fragment maintained similar promoter activity as compared with VP-1998 and therefore was referred to as VSNL1 minimal promoter. When the 5’ terminal 50 bp were deleted from the minimal promoter, the activity was dramatically decreased, suggesting that the deleted 50 bp contained a potential cis-acting element crucial for promoter activity. Deletion and site-directed mutagenesis combined with in silico transcription factor binding analysis of VSNL1 promoter identified nuclear respiratory factor (NRF)-1/α-PAL as a major player in regulating VSNL1 minimal promoter activity. The function of NRF-1 was further confirmed using dominant-negative NRF-1 overexpression and NRF-1 small interfering RNA knockdown. Electrotransfection of the proximal 100-bp promoter fragment into various cell lines confirmed using dominant-negative NRF-1 overexpression and NRF-1 small interfering RNA knockdown. Electrotransfection of the proximal 100-bp promoter fragment into various cell lines.
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EXPERIMENTAL PROCEDURES

Cell Culture—Two non-small cell lung cancer cell lines, NCI-H522 and NCI-H520, which express different levels of VILIP-1, and normal human bronchial epithelial cells were cultured as previously described (11).

Deletion and Mutation Constructs of VSNL1 Promoter—pGL4.10[luc2] vector and pGL4.73 luciferase reporter vectors (Promega, Madison, WI) were used for VSNL1 promoter reporter assays. VP-1998 (VP2kb) was constructed as described (11). All of the deletion constructs of VSNL1 promoter were derived from VP-1998 by PCR using the same reverse primer: 5’-GAAAGATCTGAGATTGGGAAATCCCATAG. The forward primers were as follows: for VP-354, 5’-GGGGTGCCTGGAGAGG; and for VP-100, 5’-GGGGTACCAGTCGGCAGCGCAGCAGG; and for VP-90Sp1m, 5’-GGGGTACCTCGCGC; for VP-60, 5’-GGGGTACCAATTTAGGAAAGGGGAGGG; for VP-70, 5’-GGGGTACCACTGCGCCAGGGGAGGG; for VP-80, 5’-GGGGTACCCGGTTCGGTAACCACTGGG; for VP-85, 5’-GGGGTACCGCGCATCGCCAGGCG; for VP-90, 5’-GGGGTACCCGGTTCGGTAACCACTGGG; for VP-100, 5’-GGGGTACCGCGCATCGCCAGGCG; for VP-174, 5’-GGGGTACCGTGCCAGGGGAGGG; for VP-186, 5’-GGGGTACCTCCGCC. The underlined bases were mutated within enzyme sites. The cDNA fragments were digested with HindIII and XbaI and ligated into pGL4.10[luc2] vector. The constructs and 8 ng of pGL4.73, luciferase activity was measured in 1 or 2 days.

For siRNA transfections, NRF-1 siRNA (SC-38105) and scrambled control siRNA (SC-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Different amounts of NRF-1 siRNA were tested for knocking down NRF-1 protein, and 50 pmol was finally used in the reporter gene assay with 0.8 μg of VP-174 and 8 ng of pGL4.73 in 24-well plate. Luciferase activity was measured in 3 days. For immunoblot analysis of VILIP-1 protein after NRF-1 knockdown, scrambled (D-001810-10-05) and NRF-1 (L-017924-00-0005) siRNAs from Thermo SCIENTIFIC were also used. All of the data were obtained from at least three individual experiments.

Electrophoretic Mobility Shift Assays (EMSA) and Chromatin Immunoprecipitation (ChIP)—Nuclear proteins were extracted from cells using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the protocol provided by the company. The following NRF-1 oligonucleotides were used: forward, 5’-TCGCCGGCGCTGCGCATCCAG, and reverse, 5’-GCTGGATCAGCCAGGGCGCGGG; for methylated NRF-1 M2: forward, 5’-GCTGGATCCAGCCAGGGCGCGGG; and for methylated NRF-1 M2+1: forward, 5’-TCCGGAGCCAGGGCGCGGG; for methylated NRF-1 M1: forward, 5’-TCGCCGGCGCCAGGGCGCGGG; and for methylated NRF-1 M2: forward, 5’-TCGCCGGCGCCAGGGCGCGGG. The equal volumes of the forward and reverse oligonucleotides with the same molar concentration were mixed and boiled for 10 min and were annealed at room temperature overnight. The probe labeling and EMSA were performed using a gel shift assay system (Promega). Briefly, the annealed double-stranded oligonucleotides were labeled using T4 polynucleotide kinase before purification with Microspin G-25 columns (GE Healthcare, Buckinghamshire, UK). The DNA binding was conducted at 4°C for 30 min in a mixture containing 5 μg of nuclear extract, 1× gel shift binding buffer, and 1 μl of 32P-labeled probe. In the competition experiment, 60× unlabeled double-stranded oligonucleotides were used as competitors and incubated with nuclear extract and binding buffer for 30 min before the addition of 32P-labeled probe. The reactions were further incubated at 4°C for 30 min. In supershift assays, the antibodies were also incubated with the reaction mixture at 4°C for 30 min before the addition of the 32P-labeled probe.

gene assay, DNA mixture containing 0.8 μg of VSNL1 promoter constructs and 8 ng of pGL4.73, a transfection efficiency control, was diluted in 50 μl of Opti-Mem 1 medium (Invitrogen) and mixed with 2 μl of Lipofectamine 2000 diluted in 50 μl of Opti-Mem 1 medium. 100 μl of DNA-Lipofectamine 2000 complexes were added to each well of 24-well plates after 20 min of incubation at room temperature, and the transfected cells were left in the incubator for 24 h before the lysis step with 150 μl of passive lysis buffer. Reporter gene activity was measured in 96-well plates according to the protocol from Dual-Luciferase reporter 1000 assay system kit (Promega) by using an Envision plate reader (PerkinElmer Life Sciences). For assessing the effect of DN NRF-1 on VSNL1 promoter activity, different amounts of pCDNA3.1 Myc-DN-NRF-1 or vector were co-transfected with 0.8 μg of pGL4.10 VP-174 and 8 ng of pGL4.73, and luciferase activity was measured in 1 or 2 days.

—The expression plasmid of DN NRF-1 was constructed previously described (11).

—For all experiments, the cells were transfected by Lipofectamine 2000 (Invitrogen) using the manufacturer’s protocol. For reporter
Anti-NRF-1 antibodies were kindly provided by Dr. Claude Piantadosi (13) and Dr. Kimitoshi Kohno (14). The control sc2025 mouse IgG was from Santa Cruz Biotechnology. Finally the reaction mixtures were analyzed on a 6% DNA retardation gel (Invitrogen) at 10 V/cm on ice for 1.5 h. The gel was dried and analyzed by autoradiography.

ChiP was performed as before (11) using a chromatin immunoprecipitation assay kit (Upstate Biotechnology, Lake Placid, NY) following the manufacturer’s protocol. Briefly, the proteins were cross-linked to DNA by incubating 1 × 10⁶ cells with 1% formaldehyde for 2.5 or 10 min at 37 °C. The cell pellets were resuspended in 200 μl of SDS lysis buffer followed by DNA sonication for a total of 16 times (each time for 20 s at 30% of maximal power) by using the Ultrasonic Processor (Cole Parmer, Vernon Hills, IL). Immunocomplexes were captured from the rest of the lysates with 10 μg of antibody. After the cross-linking was reversed by heating the sample at 65 °C for 4 h, DNA was then extracted with phenol/chloroform and precipitated with ethanol. PCR was performed by using 1% of immunoprecipitated material and the same primers as used before (11). The following PCR program was used: 95 °C for 5 min followed by 35-40 cycles of 95 °C for 35 s, 54 °C for 45 s, and finally 72 °C for 10 min. Rabbit anti-NRF-1 serum was kindly provided by D. Reines (15). Antibodies from Santa Cruz Biotechnology include: normal rabbit IgG serum was kindly provided by D. Reines (15). Antibodies from Santa Cruz Biotechnology include: normal rabbit IgG serum was kindly provided by D. Reines (15).

RESULTS

Identification of a Core Promoter Essential for Transcription of VSNL1—In our previous study, we had cloned a 2-kb fragment of VSNL1 promoter (VP-1998) from normal human bronchial epithelial cells and showed that its regulation by epigenetic mechanisms included promoter hypermethylation and histone acetylation (11). To define the boundaries of the minimal promoter and to identify cis elements that govern the transcriptional activity of VSNL1, we prepared a series of truncation constructs (Fig. 1A) and tested them in a transient luciferase reporter system. As shown in Fig. 1 (B and C), VP-354 was observed to have stronger transcriptional activity than VP-1998 in two non-small cell lung cancer cell lines (NCI-H522 and NCI-H520), indicating that putative negative cis-acting regulatory elements are located between the −1998 and −354. A 5’-deletion of 180 bp (VP-174) reduced promoter activity to the level comparable with that of VP-1998 in NCI-H522 cells. However, in the other non-small cell lung cancer cell line (NCI-H520), the reduction of activity was not as remarkable as in NCI-H522 cells. Further deletion of 74 bp from VP-174 caused a 14% decrease in activity when using NCI-H522 cells. In contrast, a 37% increase in activity was noted with NCI-H520 cells. VP-50 containing only the last 50 bp displayed the lowest luciferase activity in both cell lines. Its activity was 4 and 35% of VP-1998 in NCI-H522 and NCI-H520 cells, respectively, strongly indicating that the region from VP-100 to VP-50 is critical to VSNL1 promoter activity.

Identification of cis-Acting Elements in the VSNL1 Core Promoter—To further examine which part within the truncated 50 bp could sustain transcriptional activity, we designed 10-bp stepwise deletion constructs (Fig. 1D) and tested them in the same assay system. The stepwise 10-bp deletion caused a gradual loss of promoter activity, and VP-60 still maintained 26 and 43% of VP-100 activity in NCI-H522 and NCI-H520, respectively (Fig. 1E and F). Nevertheless, removal of 10 bp from VP-60 dramatically decreased the activity to 6 and 14% of VP-100 in NCI-H522 and NCI-H520 cells, respectively.

We searched the sequence of VP-100 for homology to known regulatory elements by using the TESS, AliBaBa2.1, and Mat-
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Inspector bioinformatics programs. There were a number of putative binding sites for transcriptional factors in this region (Fig. 2), among which Sp1 and NRF-1 were frequently identified. Combining this in silico analysis with the result from the 10-bp stepwise deletion experiment, it could be concluded that the first Sp1 site was located between VP-100 and VP-90 and the second Sp1 site was located between VP-90 and VP-80 and that a NRF-1 site spanning between VP-60 and VP-40 could have regulatory effect on \(VSNL1\) promoter activity. To confirm these findings, we introduced point mutations to the sites and tested the mutant constructs in both cell lines (Fig. 3). Comparable with the 10-bp stepwise deletion results, mutation of two Sp1 sites demonstrated that these sites played a positive regulatory role in \(VSNL1\) transcription activity (Fig. 3, B and C). The putative NRF-1 site in the \(VSNL1\) promoter is an 11-base tandem repeat sequence, GCGCCTGCGCA. When the core CCTGCG was replaced by GCATTC, an 80 and 74% drop in promoter activity was observed as compared with intact VP-60 in NCI-H522 and NCI-H520 cells, respectively. The effect of mutation of the NRF-1-binding site was further verified in a longer \(VSNL1\) promoter setting (VP-174). This mutation caused a 69% decrease of VP-174 activity in NCI-H522 cells and a 44% decrease in NCI-H520 cells. These results suggested that the NRF-1-binding sequence is a very important regulatory element in the \(VSNL1\) promoter.

\textbf{NRF-1 Is a Transcription Factor Regulating VSNL1 Promoter Activity—}

The requirement of NRF-1 for effective \(VSNL1\) promoter activity was verified by co-transfection of the Myc-tagged DN NRF-1 expression vector with VP-174 reporter construct. Overexpression of Myc-DN-NRF-1, containing only the N-terminus DNA-binding and nuclear localization domains, should compete with the binding of endogenous NRF-1 to the NRF-1-binding site on the \(VSNL1\) promoter and therefore interrupt the activating function of endogenous NRF-1 because it does not contain the C-terminal transactivation domain. As shown in Fig. 4 (A and B), the VP-174 activity was inhibited upon the expression of Myc-DN-NRF-1, exhibiting \(64\) and \(84\%\) inhibition (0.8 \(\mu\)g) in NCI-H522 and NCI-H520 cells, respectively. The expression of Myc-DN-NRF-1 was confirmed by immunoblotting analysis with anti-Myc antibody (Fig. 4 C). The second evidence for NRF-1 regulation of the \(VSNL1\) promoter was obtained by NRF-1 siRNA treatment. Transfection of 50 pmol of NRF-1 siRNA resulted in a strong inhibition of NRF-1 protein synthesis as compared with that of scrambled siRNA control (Fig. 5 A). When the same amount of NRF-1 siRNA was co-transfected with VP-174, an approximately 90% reduction in promoter activity in both cell lines was observed (Fig. 5 B). In addition, the essential requirement of NRF-1 for \(VILIP-1\) transcription was corroborated by the observation that NRF-1 silencing led to a significant down-regulation of \(VILIP-1\) protein (Fig. 5 C).

To determine whether the NRF-1 sequence is effectively recognized by cellular NRF-1 protein, we performed EMSA with the nuclear proteins extracted from NCI-H520 cells. Two major bands were observed (Fig. 6A, lane 2) that were abolished by cold (unlabeled) NRF-1 oligonucleotides (lane 3), indicating...
that these DNA-protein complexes contain proteins able to recognize and bind to the NRF-1 response element. The upper band represented the major NRF-1-containing DNA-protein complex because it was specifically supershifted by using two different NRF-1 antibodies from Drs. Claude Piantadosi and Kimitoshi Kohno, respectively (lanes 4 and 5), whereas the control antibody was unable to supershift it (lane 6).

To determine the in vivo occupancy of the VSNL1 promoter by NRF-1, we performed a ChIP assay. As negative controls, a normal rabbit IgG and a nonrelated anti-α1-trypsin antiserum were included for immunoprecipitation using the same batch of cell lysates as used in NRF-1 antibody immunoprecipitation. The VSNL1 promoter was co-immunoprecipitated only with NRF-1 antibody (Fig. 6B), indicating the specific binding of VSNL1 promoter by NRF-1 in vivo.

Regulation of VSNL1 Promoter by Methylation of the NRF-1-binding Site—Methylation is one of the major epigenetic mechanisms governing VSNL1 gene expression (11). Methylation of all the CpG islands in VP-1998 with M.SssI treatment nearly abolished VSNL1 promoter activity (decreased to 11%) (11). Within VP-100, the NRF-1-binding site is the only sequence containing two GCGC sequences. To further study the epigenetic regulation of VSNL1 expression and the regulation of VSNL1 promoter activity by methylation of the NRF-1-binding site, we took advantage of HhaI DNA methyltransferase that methylates the cytosine residue in the GCGC sequence. Therefore, the methylating effect observed can be attributable exclusively to methylation of the NRF-1 site. Whole plasmids (pGL4.10 and pGL4.10VP-100) were modified by HhaI, and the complete modification was confirmed by digestion with restriction enzyme HhaI (data not shown). Methylation of the GCGC sequence of pGL4.10VP-100 almost completely inhibited VP-100 activity (leading to 94 and 95% decrease in NCI-H520 and NCI-H520 cells, respectively) (Fig. 7A). Nevertheless, methylation of the vector itself also suppressed its basal activity to 30 and 44% of the unmethylated counterparts because the vector backbone harbors GCGC sequences. To further confirm the methylation effect, a promoter-specific methylation approach was utilized, and the ligated plasmids were introduced into cells. A decrease of 55% in NCI-H520 cells and 65% in NCI-H520 cells was observed because of methylation of the NRF-1-binding site within VP-100 (Fig. 7B).

The mechanisms underlying the regulation of transcription by DNA methylation remain unclear. We first investigated whether the methylation of NRF-1-binding sites could block its binding by performing an EMSA assay. Two oligonucleotides in which two (M2) or three (M1) CpGs within the NRF-1 sites were methylated were used. When the EMSA was performed with the unmethylated NRF-1 probe, we observed two major
because they were supershifted by the NRF-1 antibody (upper bands represented the NRF-1-containing complex formation of these two bands (Fig. 8A, lane 2)). The upper band was supershifted by the NRF-1 antibody (lane 4), as in Fig. 6A. However, methylation of two or three CpGs of NRF-1-binding sites failed to abolish the formation of these two bands (lanes 5 and 7), and similarly, the upper bands represented the NRF-1-containing complex because they were supershifted by the NRF-1 antibody (lanes 6 and 8). Furthermore, the formation of the NRF-1-containing complex (Fig. 8B, lane 2) was not observed when either the unlabeled NRF-1 oligonucleotides (lane 3) or the unlabeled methylated counterparts (lanes 4 and 5) were used, indicating that the methylation of the CpG sites within the NRF-1-binding site has no effect on the binding of NRF-1 to the VSNL1 promoter. Methylation of the CpG sites within gene promoters can recruit HDAC and histone deacetylases, thus leading to the alteration of chromatin structure (16, 17). We next examined the association of all five members of MBP family and class I HDACs with VSNL1 promoter by using ChIP assay. As shown in Fig. 8C, MBD1 and MBD4 were able to bind VSNL1 promoter in NCI-H522 and NCI-H520 cells, whereas MBD3 binding was detected predominantly in NCI-H522 cells. Interaction of MBD2 and MeCP2 with VSNL1 promoter was not observed in any cell line. Binding of HDAC1, HDAC2, and HDAC3 to VSNL1 promoter was found in NCI-H520 cells, nevertheless, among these three HDACs, it appears that the association of HDAC1 was the most intense in NCI-H522 cells, which do not express endogenous VILIP-1 (Fig. 8C).

The Potential Regulation of the Promoter Activity of Other Visinin-like Subfamily Members by NRF-1—The recent data showing that neurocalcin (18), a calcium sensor in Drosophila neurons, was down-regulated in the ewg (NRF-1 homologue in Drosophila) mutant prompted us to examine whether NRF-1 could be implicated in regulating the expression of each member of the visinin-like subfamily. The promoters of the human visinin-like subfamily were poorly studied, and most of the promoter sequences were unavailable; therefore, we first searched and identified over 2.5 kb upstream and downstream sequence of the first exons of HPCAL1, HPCAL4, HPCAL1, and HPCA genes. None of the first exons from these genes is transcribed, and CpG islands were found to exist in all three promoters using the criteria formulated by Gardiner-Garden and Frommer (19), i.e. a 200-bp or greater stretch of DNA with a C/G content of >50% and an observed CpG/expected CpG ratio in excess of 0.6. In silico analysis of these fragments revealed that all of these gene promoters harbor the potential NRF-1-binding sites (Table 1). The analysis of the promoter fragment of NCALD was initially done using a Drosophila genomic DNA sequence (data not shown). A perfect potential ewg binding site was located within the second intron of Drosophila neurocalcin (CG7646). Similarly, we found a NRF-1-binding site with the consensus sequence GCGCATGCGCA within the first intron of the human NICALD gene. After we subcloned the DNA fragments containing the potential NRF-1 site, we studied the effect of DN NRF-1 on the promoter transcriptional activity. Application of DN NRF-1 significantly inhibited the promoter activity of
human **HPCAL4**, **HPCAL1**, **HPCA**, and **NCALD** in both NCI-H522 (Fig. 9A) and NCI-H520 (Fig. 9B) cells, suggesting that NRF-1 could also be the *trans*-acting element regulating these gene promoters. Among the promoters, we selected **NCALD** for the study of the effect of a NRF-1 mutation on promoter transcriptional activity because the promoter of **NCALD** harbors a canonical NRF-1 site, and it showed the most remarkable decrease after DN NRF-1 transfection in our cell systems. A NRF-1 mutant construct in which the core GCA sequence was replaced by TTT demonstrated a decrease of ∼64 and 72% in the promoter activity in NCI-H522 cells and NCI-H520 cells, respectively (Fig. 9C), indicating the direct regulatory effect of NRF-1 on the **NCALD** promoter.

**DISCUSSION**

We characterized the **VSNL1** promoter by serial promoter deletion experiments and identified a 100-bp fragment that sustained most of the **VSNL1** promoter activity. Further deletion of this minimal promoter revealed a **cis**-acting element at -60 to -50 nucleotides upstream of the first **VSNL1** gene exon. Alignment of human, mouse, and rat **VSNL1** promoters revealed a high homology of the core promoter among all three species (Fig. 2). By using three transcription factor searching programs, we identified one potential binding site (GCGCGTCGCG) that is a perfect match for the NRF-1-binding site (20). This putative NRF-1-binding site is well conserved in human, mouse, and rat (Fig. 2), suggesting that it plays an essential role in regulating **VSNL1** promoter activity. As expected, deletion and mutation of this binding site dramatically decreased the promoter activity in human lung cancer cell lines.

NRF-1 is one of the major transcription factors involved in regulating the expression of nuclear genes essential for mitochondrial biogenesis (21, 22). NRF-1 binding is required for the expression of genes encoding subunits of all the five respiratory
complexes (23). Additionally, acting on major regulators of mitochondrial transcription including Tfam and TFB (24, 25), NRF-1 coordinates the respiratory subunit expression with that of the mitochondrial transcriptional machinery. Furthermore, NRF-1 has been implicated indirectly in specific respiratory functions by regulating a number of nuclear genes such as outer membrane transporter TOMM20 (26) and a cytochrome oxidase assembly factor (27). The induction of mitochondrial biogenesis in response to environment signals such as cold exposure and exercise is coordinated by co-factors that interact with NRF-1, including the transcriptional co-activators PGC (peroxisome proliferator-activated receptor γ co-factor)-1α, PGC-1β and PRC (PGC-1-related co-activator) (28–30). These NRF-1 co-activators bind to NRF-1 and trans-activate NRF-1 target genes that are involved in mitochondrial respiration and transcription. The NRF-1-responsive element has been identified to be a GC-rich palindrome with the core sequence YGCGCAY-GCGCR (21). Interestingly, the NRF-1 recognition site is one of the seven transcription factor-binding sites that are most frequently found in the proximal promoters of ubiquitous genes (31), indicating a broader spectrum of target genes for NRF-1. A recent study using CHIP-on-chip has revealed a collection of human promoters that are occupied by NRF-1 in vivo (32). Besides the genes that are expected to mediate mitochondrial biogenesis, NRF-1 target genes overlap significantly with those of E2F, suggesting a role in the regulation of DNA replication and cell cycle progression. It is plausible that deregulation of

**TABLE 1**

*The in silico prediction of the potential NRF-1-binding sites of the gene promoters of the other visinin-like subfamily members compared with VSNL1*

The potential binding sequences are in bold type, and the positions of the promoter fragments are numbered relative to the start of the first exon.

| Gene   | Position | Sequence                        |
|--------|----------|---------------------------------|
| VSNL1  | −60/−41  | TCGCCGCTGCGCAGCTCCAG            |
| HPCAL4 | −4/−23   | GGAAGGCGGCCGCCAGACCTTTGG         |
| HPCAL1 | +1283/+1310 | AGGCCCTCCGCGGCAAGGCGGCCCTGCGGC |
| NCALD  | +43298/+43316 | AGGGCGGCAAGGCGGAAAGCC          |
| HPCA   | −46/−21  | GCCTGTGGCGCGCGCGCAAGGCGCAAGGC |

**FIGURE 9.** A and B, the promoter activity of HPCA, NCALD, HPCAL4, and HPCAL1 was suppressed by the overexpression of DN NRF-1 in NCI-H522 cells (A) and NCI-H520 cells (B). DN NRF-1 transfection and the luciferase assay were performed as described in the legend to Fig. 4. V, vector; DN, DN NRF-1. C, site-directed mutagenesis of the NRF-1-binding site in NCALD promoter inhibited its activity. The promoter constructs containing the wild type (WT) or the mutated (Mutant) NRF-1-binding sites were introduced into the cells, and the luciferase assay was performed.
NRF-1 could contribute to cell proliferation during tumorigenesis. Interestingly, it has been noted that certain gene products related to tumor cell growth, differentiation, migration, and invasion are NRF-1-regulated targets. These genes include GalNac-T3 (14), CAPNS1 (33), E2F6 (34), TOMM34 (35), and VHL (36). Our findings showing that NRF-1 is a key trans-acting factor regulating the transcription of the tumor suppressor VSNL1 further extends this observation. To our knowledge, this is the first characterization of human VSNL1 promoter as well as the first description of NRF-1 as a critical cis-acting element in VSNL1 promoter.

Although targeted disruption of NRF-1 gene in mouse resulted in an embryonic lethal phenotype (37), the data from deletion of NRF-1 homologues in other species implicates NRF-1 in the physiology of the nervous system. Not really finished (nrf), a NRF-1 homologue in zebra fish, is expressed ubiquitously throughout the developing retina and central nervous system and is crucial for the development of the zebra fish outer retina (38). Mutations of the NRF-1 homologue locus in Drosophila, ewg, exhibit severe neural phenotype leading to embryonic death (39). In addition to its prominent function in maintaining the respiratory chain, the extensive EWG protein expression pattern in all neurons of Drosophila suggests its potential role as a transcription factor in the regulation of neuron-specific gene expression. Indeed, recent analysis of ewg mutant (ewgl), in which ewg is inactivated, showed that a spectrum of genes other than mitochondrial function-related genes were either up- or down-regulated by EWG in neurons (18).

VILIP-1 expression was detected in the hippocampus where VILIP-1 might influence signaling pathways, such as cAMP/cGMP pathway and the activity of neurotransmitter receptors and ion channels, which are related to learning and memory processes (40–42). Interestingly, a number of other neuronal genes, including IAP (12), Glur2 (43), and FMR-1 (15), all containing NRF-1-binding sites in their promoter regions, can also regulate the synaptic plasticity and learning and memory processes, indicating that NRF-1 might participate in these complex activities.

VILIP-1 belongs to a subfamily of NCS proteins including VILIP-1, VILIP-2, VILIP-3, hippoculin, and neurocalcin-δ (1, 2). The finding that neurocalcin, a calcium sensor in Drosophila neurons, was down-regulated in ewg mutant suggested that VSNL1 might not be the only NRF-1-regulatable member of this subfamily (18). The in silico analysis of the promoters of the other four genes in the same family predicted the presence of NRF-1 cis-acting regulatory elements in most of the promoters of visinin-like subfamily. Blocking the endogenous NRF-1 function with a dominant-negative form led to a significant decrease in the promoter transcriptional activity of HPCA, NCALD, HPCAL4, and HPCAL1, indicating that all of these promoters can be directly or indirectly regulated by NRF-1. Mutation of the NRF-1-binding site within the NCALD promoter suppressed its activity, further substantiating the importance of NRF-1 site as a cis-acting regulatory element in the gene promoters of this NCS subfamily. The potential regulation by NRF-1 could further extend the function of NRF-1 to a spectrum of physiological and pathological processes that are mediated by this NCS subfamily. Interestingly, hippocalcin, another member of the same subfamily highly expressed in the hippocampal pyramidal cells, has also been implicated in hippocampal long-term depression (44) and in memory processes because the hippocalcell-deficient mice display defects in spatial and associative memory (45).

VSNL1 gene expression can be regulated epigenetically in human lung cancer (11). Global methylation of CpG in VP-1998 by M.Sasl abolished VSNL1 promoter activity, probably through methylation of a group of CG-containing transcription factor binding sites including Sp1, NRF-1 (46), AP2 (47), and NF-κB (48). In this study, we have identified VP-100 as the VSNL1 minimal promoter and NRF-1 site as a key cis-acting regulatory element. The fact that NRF-1 is the only GCGC-containing sequence present within the core promoter allowed us to pinpoint the effect of NRF-1 site methylation on VSNL1 transcription. Using both whole plasmid and promoter-specific methylation approaches, we found that methylation decreased VSNL1 gene expression by 55–65%. Interestingly, the extent of the observed promoter activity suppression is comparable with those found in studies of FMR-1 and hTfam promoters using different methylation methods (49, 50). Comparing the reduced activity induced by NRF-1 site methylation (Fig. 7) with that produced by NRF-1 site mutation (Fig. 3), it appears that both led to similar levels of VSNL1 promoter inhibition, suggesting that methylation of the NRF-1 site in the VSNL1 promoter could block NRF-1 binding. However, failure of the methylated oligonucleotides to block the NRF-1 binding in EMSA (Fig. 8, A and B) excluded the possibility that direct impeding of transcription factor binding by methylation could account for the VSNL1 promoter repression. MBP family proteins can be recruited to hypermethylated promoter and act as important “translators” between DNA methylation and histone-modifier genes, therefore establishing a transcriptionally inactive chromatin environment (16, 51). Except MBD3, all the other MBP proteins, MBD1, MBD2, MBD4, and MeCP2, were able to recognize and bind methylated DNA (52). MBD3 is a bona fide component of a large macromolecular complex, Mi-2-NuRD (nucleosome remodeling and deacetylase), that couples a chromatin-remodeling ATPase and histone deacetylation (53). In addition to MBD3, the Mi-2-NuRD complex consists of other multiple subunits, including at least two Mi-2 proteins, HDAC1 and/or HDAC2, Rbpbp4 (p48), Rbpbp7 (p46), and MTA proteins, the activity of which implicates it in repressing gene transcription. Our analysis of the association between MBP, class I HDACs, and VSNL1 promoter by using chromatin immunoprecipitation suggests that the repression of VSNL1 promoter activity might be mediated through the recruitment of Mi-2-NuRD complex containing MBD3 and HDAC1 and HDAC2 to the VSNL1 promoter.

In summary, we have characterized the human VSNL1 gene promoter and identified the NRF-1-binding site as a major regulatory site for VSNL1 gene transcription. The functional importance of NRF-1 was confirmed by demonstrating VSNL1 promoter repression by DN NRF-1 and NRF-1 siRNA interference and the consequent VILIP-1 protein down-regulation by NRF-1 silencing. EMSA and ChIP assay further demonstrated the direct binding of NRF-1 protein to its binding site. Further-
more, in silico analysis suggested that NRF-1 might be able to regulate the entire visinin-like subfamily of NCS.

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