Chromatin Inactivation Precedes De Novo DNA Methylation during the Progressive Epigenetic Silencing of the RASSF1A Promoter†

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Epigenetic inactivation of the RASSF1A tumor suppressor by CpG island methylation was frequently detected in cancer. However, the mechanisms of this aberrant DNA methylation are unknown. In the RASSF1A promoter, we characterized four Sp1 sites, which are frequently methylated in cancer. We examined the functional relationship between DNA methylation, histone modification, Sp1 binding, and RASSF1A expression in proliferating human mammary epithelial cells. With increasing passages, the transcription of RASSF1A was dramatically silenced. This inactivation was associated with deacetylation of histone H3 and an impaired binding of Sp1 at the RASSF1A promoter. In mammary epithelial cells that had overcome a stress-associated senescence barrier, a spreading of DNA methylation in the CpG island promoter was observed. When the RASSF1A-silenced cells were treated with inhibitors of DNA methyltransferase and histone deacetylase, binding of Sp1 and expression of RASSF1A reoccurred. In summary, we observed that histone H3 deacetylation and H3 lysine 9 trimethylation occur in the same time window as gene inactivation and precede DNA methylation. Our data suggest that in epithelial cells, histone inactivation may trigger de novo DNA methylation of the RASSF1A promoter and this system may serve as a model for CpG island inactivation of tumor suppressor genes.

Epigenetic inactivation of tumor suppressor genes by transcriptional silencing is a hallmark of cancer. DNA methylation is one of the major epigenetic processes involved in gene silencing. In a recent study, we reported that Suv39h-mediated H3-K9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin in Arabidopsis thaliana (28). In vertebrates, it has been shown that a repressive chromatin modification, like histone H3 lysine 9 (H3-K9) methylation, can direct DNA methylation to major satellite repeats at pericentric heterochromatin (28). In a recent report, H3-K9 methylation and DNA methylation were excluded as the cause of silencing of a transgene (30).

Normal human mammary epithelial cells (HMEC) grown in culture exhibit two types of proliferation barriers, a stress-associated senescence barrier (stasis) and a senescence barrier resulting from telomere erosion and dysfunction (36, 42). Prestasis HMEC can grow for several population doublings prior to a proliferative arrest associated with elevated levels of p16 (4). Under certain culture conditions, a small number of cells spontaneously emerge from stasis and continue proliferation with ongoing telomere erosion (17, 42). These poststasis cells show a loss of p16 expression associated with promoter DNA methylation to major satellite repeats at pericentric heterochromatin (28). In a recent report, H3-K9 methylation and DNA methylation were excluded as the cause of silencing of a transgene (30).

In proliferating human mammary epithelial cells, the transcription of RASSF1A was dramatically silenced. This inactivation was associated with deacetylation of histone H3 and an impaired binding of Sp1 at the RASSF1A promoter. In mammary epithelial cells that had overcome a stress-associated senescence barrier, a spreading of DNA methylation in the CpG island promoter was observed. When the RASSF1A-silenced cells were treated with inhibitors of DNA methyltransferase and histone deacetylase, binding of Sp1 and expression of RASSF1A reoccurred. In summary, we observed that histone H3 deacetylation and H3 lysine 9 trimethylation occur in the same time window as gene inactivation and precede DNA methylation. Our data suggest that in epithelial cells, histone inactivation may trigger de novo DNA methylation of the RASSF1A promoter and this system may serve as a model for CpG island inactivation of tumor suppressor genes.

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been located in 3p21.3, a segment which is frequently lost in a variety of human tumors (7). The RASSF1 gene consists of two major transcripts, termed RASSF1A and RASSF1C, which are expressed from two distinct CpG island promoters (9). Both transcripts are present in normal human tissues. However, the RASSF1A message is missing in a variety of human cancer cell lines and primary tumors, including lung and breast carcinomas (7, 9, 10). Silencing of RASSF1A transcript has been correlated with aberrant DNA methylation of the RASSF1A CpG island (7). Methylation profiling of breast tumors suggests that DNA methylation spreads from the first exon into the CpG island area (49). However, a functional promoter sequence has not been mapped, and the exact mechanisms of RASSF1A inactivation have not been investigated at all.

To gain insight into the mechanisms of epigenetic inactivation of RASSF1A, we analyzed the promoter sequence of RASSF1A by a luciferase reporter assay, electrophoretic mobility shift assay (EMSA), in vivo footprinting, and chromatin immunoprecipitation (ChIP). We identified four functional Sp1 sites in the promoter of RASSF1A. In proliferating HMEC and breast cancer cells, we investigated the expression of RASSF1A. DNA methylation, chromatin modification, and Sp1 binding at the RASSF1A promoter. In posttranslational HMEC, drastic silencing of RASSF1A was observed, and a spreading of DNA methylation occurred. Our data indicate that histone inactivation precedes DNA methylation and the repressed chromatin state is associated with occlusion of Sp1 binding at the RASSF1A promoter.

**MATERIALS AND METHODS**

**Cells.** Four breast cancer cell lines (T47D, MDA-MB-231, MCF7, and ZR75-1), HeLa cells, and the A549 lung cancer cell line were obtained from the American Type Culture Collection and cultured in the recommended medium. Human mammary epithelial cells (HMEC-184 and HMEC-48R) were obtained from reduction mammoplasty tissue as described previously (41). Additional mammary epithelial cell (HMEC-219 and HMEC-1001) were purchased from Clonetics (BioWhittaker, Verviers, Belgium) or isolated from mammary epithelium (HMEC-141). Primary HMEC were grown in mammary epithelial cell growth medium and/or serum-containing medium (41). Posttranslational HMEC were cultivated in serum-free mammary epithelial cell growth medium (PromoCell, Heidelberg, Germany) to no more than 80% confluence. Cells were grown at 37°C in 5% CO2 and medium was changed every 3 days. To determine the population doublings, the cells were counted at each passage.

**Luciferase reporter gene analysis.** Regulatory sequences of RASSF1A and RASSF1C were cloned in the pRL-null vector (renilla luciferase; Promega, Mannheim, Germany). Promoter sequences of RASSF1A, RASSF1C, and RASSF1 were amplified from fibroblast DNA and cloned in the pRL-null vector. All primer sequences are available in Table S1 in the supplemental material. To transfect the cells, the A-511 construct at 10 ng of plasmid DNA was treated with 30 U of Ssml methylase (New England Biolabs [NEB], Frankfurt, Germany). Truncation and mutation in the promoter reporter gene were generated by single-site-directed mutagenesis (QuickChange XL kit; Stratagene, Amsterdam, The Netherlands) using specific primers. A-213 was cloned by creation of a novel XhoI site at position 196 of A-511 and deletion of the upstream fragment. A-129 was generated through an Xmal site at position 112 of A-511 and deletion of the downstream fragment. All plasmids were verified by sequencing and cotransfected in HeLa cells with pGL3-SV40 promoter vector control (firefly luciferase; Promega). After 24 h, the expression of luciferase reporter gene was determined and normalized by the Dual-Luciferase reporter assay (Promega).

**EMSA.** Nuclear extracts were prepared as previously described with some modifications (46). Briefly, HeLa cells were washed with and incubated in lysis buffer. The isolated nuclei were resuspended in extraction buffer and dialyzed overnight. Nuclear extracts were stored at −80°C in aliquots. To label the EMSA probe, two complementary single-stranded oligonucleotides (200 pmol) were mixed in equimolar amounts, annealed in a water bath, and labeled with 20 μCi of [γ-32P]ATP and 10 U of T4 polynucleotide kinase (NEB). Labeled probes were generated with 2 U of Ssml methylase. The DNA-binding assays were carried out in 20 μl of binding buffer with 5 μg of nuclear extract and 2 μl of the probe (10 pmol/μl) for 1 h on ice. If necessary, 500 pmol of cold competitor oligonucleotides or 2 μg of anti-Sp1 or anti-XPA (Santa Cruz Biotechnology, Santa Cruz, Calif.) was included. DNA-protein complexes were mixed with loading buffer and resolved on native 6% polyacrylamide gels at 100 V for 4 h in Tris-borate-EDTA.

**LMPCR.** For genomic footprinting experiments, HeLa cells and genomic DNA were treated with 0.2% dimethyl sulfoxide (DMS). Ligation-mediated PCR (LMPCR) of the cleaved DNA was performed as previously described (8). Primer sequences are available in Table S1 in the supplemental material. The amplified fragments were separated on 8% polyacrylamide–7 M urea gels and ethidiumblotted onto nylon membranes, and the sequences were visualized by hybridization with a single-strand gene-specific PCR probe.

**ChIP.** In order to perform chromatin immunoprecipitation (ChIP) analysis, proteins were cross-linked to DNA by adding 1% formaldehyde to the cells for 10 min at 37°C. The cells were lysed in 1% sodium dodecyl sulfate, and the lysates were sonicated to shear DNA to lengths of approximately 300 bp and diluted in 2 ml of ChIP buffer with protease inhibitors. One percent of the diluted DNA supernatant was kept to quantify the amount of DNA present in different samples. This probe is considered to be the input control, and DNA-protein cross-links were reversed. To reduce nonspecific background, the 2 ml of diluted DNA supernatant was preincubated with 75 μl of salmon sperm DNA-protein A agarose (Upstate, Charlottesville, Va.). The immunoprecipitating antibody (0.9 μg of histone H3-trimethyl K9 antibody [ab8898] from Abcam, Cambridge, United Kingdom; anti-acetyl-histone H3 [Ac-K9 and Ac-K14] from Biomol, Hamburg, Germany) or 1 μg of Sp1 antibody from Santa Cruz Biotechnology was added to the supernatant fraction and incubated overnight at 4°C. The antibody-histone complex was isolated by adding 60 μl of salmon sperm DNA-protein A agarose and pelleted. For a negative control, a no-antibody immunoprecipitation was utilized by incubating the supernatant fraction with only salmon sperm DNA-protein A agarose. The protein A agarose–antibody complex was washed, and the DNA was eluted as described in the ChIP protocol of Upstate. Protein-DNA cross-links were reversed in 0.25 M NaCl at 65°C for 4 h. The DNA was purified by proteinase K digestion, phenol–chloroform–isoamyl alcohol extraction, ethanol precipitation. The DNA was resuspended in Tris-EDTA buffer, and the amount of histone modification and Sp1 binding were quantified by real-time PCR with the primers listed in Table S1 in the supplemental material. The input sample and no-antibody probe were used as positive (100%) and negative (0%) controls, and the bound-to-input (B/I) fraction was determined. Real-time analyses were repeated at least three times from two independent experiments.

**Real-time reverse transcription-PCR.** Total cellular RNA was extracted from cells using the Trizol reagent (Invitrogen, Groningen, The Netherlands) and quantified. Total RNA of normal mammary glands was obtained from Clontech (BD Biosciences, Erembodegem, Belgium). cDNA was synthesized from 0.5 μg of RNA with the iScript cDNA synthesis kit (Bio-Rad, Munich, Germany). Real-time PCR was carried out using the Rotor Gene 2000 (Corbett Research, Sydney, Australia), using SYBR Green (Biozym, Germany), and 0.7 μl of each deoxynucleoside triphosphate, 20 pmol of each primer (see Table S1 in the supplemental material), 0.2× SYBR Green (Biozym, Germany), and 0.7 μl of cDNA. After an initial denaturing step at 95°C for 5 min, the cycling conditions were as follows: 95°C for 30 s, annealing temperature for 30 s, 72°C for 30 s, and a fluorescence measurement at the appropriate melting temperature (63°C for RASSF1A) for a total of 30 cycles. cDNA prepared from 1 μg of RNA of human fibroblast was used as an internal standard and diluted 20 times (10 relative units), 4 times (50 relative units), or 2 times (100 relative units). The relative amounts of DNA were determined with the software Rotor-Gene 4.4 in the quantification mode. Real-time analyses were repeated at least three times with independent cDNA preparations.

**Methylation analysis of the RASSF1 locus.** The methylation status of the RASSF1 locus was determined by combined bisulfite restriction analysis (COBRA) (48). For COBRA, 100 ng of bisulfite-treated genomic DNA was amplified for 25 cycles with 20 pmol of each primer and conditions described in Table S1 in the supplemental material. One-fifth portions of the first PCR products were used as templates for a second PCR with internal primers for 35 cycles. Twenty to 30 ng of PCR products was digested with 2 U of restriction enzyme (NEB) as described in Table S1 in the supplemental material. DNA sequencing was carried out from HeLa cells and treated with Ssml methylase. No enzyme was used as a control for complete digestion. The restriction products were resolved on 2% Tris-acetate-EDTA agarose gels, and data were analyzed by ImageJ 1.26v (National Institutes of Health). Amplified bisulfite PCR products were subcloned into pGEM-T vector (Promega) according to the manufacturer’s instructions.
RESULTS

Characterization of regulatory sequences in the RASSF1A gene. RASSF1A is frequently epigenetically inactivated in human tumors; however, transcriptional regulators have not been mapped in its promoter region. To identify functional elements of the RASSF1A promoter, we cloned 511 bp of the RASSF1A promoter region in a luciferase reporter vector and found high transcriptional activity of this construct (A-511) in HeLa cells (Fig. 1). The fragment includes four putative Sp1 binding sites (GGGCGG), the upstream CpG island of RASSF1A, and the translation start site (Fig. 1A). These Sp1 sites were the only significant transcription binding sites revealed by in silico analysis (www.cbil.upenn.edu/tess and http://genome.ucsc.edu). Deletion of an upstream Sp1 site at position −480 (Sp1 A) or mutation of this Sp1 site resulted in a 50% reduction of the promoter activity (Fig. 1B). Deletion of three Sp1 sites located in the CpG island resulted in 30% reduction. In vitro methylation of the RASSF1A promoter containing reporter plasmid reduced the expression completely. In the RASSF1C promoter, five putative Sp1 binding sites were identified (Fig. 1B). A 527-bp fragment (C-527) including these Sp1 sites was analyzed in the luciferase assay (Fig. 1B). Sequential mutation of four Sp1 sites (Sp1-1 to Sp1-4) in C-527 reduced the activity significantly. Mutational inactivation of the Sp1 site located at −330 had only minor effects. Subsequently, we analyzed the Sp1 binding sites of the RASSF1A promoter by EMSA (Fig. 1C). When the Sp1 sites containing oligonucleotides were incubated with nuclear extract, a shift was detected. A similar shift was detected with the methylated oligonucleotide (Fig. 1C). Shifts with unmethylated probes were further supershifted with Sp1 antibody but not with anti-XPA antibody. The cold Sp1 probes competed for the binding. However, competitors that harbor mutations in the Sp1 site (GTTCCG) were not able to compete significantly (Fig. 1). To verify the binding of Sp1 to the RASSF1A promoter, we performed in vivo footprinting and ChIP assays (Fig. 1D and E). Genomic footprinting of the upper Sp1 site revealed a hyperreactive G in the Sp1 consensus sequence (Fig. 1D). In ChIP assays, two fragments of the RASSF1A promoter (A-1 and A-2) and a fragment of RASSF1C were analyzed for the abundance of cross-linked Sp1 factor (Fig. 1E). The A-2 and A-1 regions contain one Sp1 binding site and three Sp1 binding sites, respectively, and the analyzed RASSF1C fragment harbored two Sp1 binding sites (at positions −270 and −190). In HeLa cells with active RASSF1A and RASSF1C genes, Sp1 binding at both RASSF1 promoters was observed (Fig. 1E). In contrast, reduced binding of Sp1 at the RASSF1A promoter was detected in the breast cancer cell line ZR75-1. In these cancer cells, the RASSF1A promoter is silenced by hypermethylation of the CpG island promoter, but RASSF1C is unmethylated (Fig. 2).

Epigenetic status of the RASSF1A locus. To investigate the DNA methylation pattern of the RASSF1A gene, we analyzed 12 regions of a 7-kb segment flanking the RASSF1A CpG island by combined bisulfite restriction analysis (COBRA) (Fig. 2). This analysis was performed in RASSF1A-expressing cells (HeLa cells, human fibroblasts, and blood) and in breast and lung cancer cell lines (T47D, MDA-MB-231, ZR75-1, MCF7, and A549), which lack RASSF1A expression. The RASSF1A (RA and D1) and RASSF1C (RC) CpG island fragments revealed differences in their methylation patterns. In human fibroblasts, blood, and cancer cell lines, the RC fragment is completely unmethylated (Fig. 2B and C). In contrast, the CpG island of RASSF1A is completely methylated in all cancer cell lines but not in fibroblasts, blood, and HeLa cells (Fig. 2B and C). Then, we analyzed the methylation pattern of the sequences flanking the RA fragment in the breast cancer cell lines. All six segments (D1 to D6) located downstream of the RASSF1A promoter were frequently methylated (Fig. 2C).

In the RASSF1A-expressing cells, this downstream region was less methylated, but two fragments, D3 and D5, located in different Alu elements were methylated at 50 and 100%, respectively. Three upstream fragments (U2 to U4) of the RASSF1A CpG island, which are located in the BLU gene, were frequently methylated in cancer and normal cells. Analysis of the U1 fragments, which contain the upstream Sp1 binding site and the BLU termination sequence, showed low-frequency methylation in the RASSF1A-expressing cells (0 to 33%) compared to cancer cells (>90%). In addition to DNA methylation, chromatin modification by histone methylation and acetylation is involved in the inactive and active chromatin state. To investigate the chromatin state of the RASSF1A and RASSF1C promoter, we performed ChIP with antibodies against histone H3 lysine 9 trimethylation (H3-K9) and acetyl-histone H3 (Ac-H3) in HeLa and ZR75-1 breast cancer cells (Fig. 3). In the unmethylated RASSF1A promoter of HeLa cells, Ac-H3 is more frequently detected than in the methylated RASSF1A CpG islands of ZR75-1. In contrast, H3-K9 methylation is exclusively detected in the methylated promoter of ZR75-1. At the RASSF1C promoter, no differences in histone modification between HeLa and ZR75-1 cells were observed (Fig. 3A and B). Furthermore, we treated the ZR75-1 cells with trichostatin A (TSA) and 5-aza-2-deoxycytidine (Aza), which inhibit histone deacetylase and DNA methyltransferase, respectively. This treatment led to acetylation of H3 at the RASSF1A promoter (Fig. 3C). For the region A-1, which is located in the CpG islands, a 3.4-fold increase in Ac-H3 was observed. Additionally, a dramatic 30-fold decrease in H3-K9 methylation was revealed (Fig. 3C). For the upper A-2 region, only minor changes in histone modification were detected. Treatment with TSA and Aza enhanced the binding of Sp1 at both analyzed regions fourfold (Fig. 3C), and after Aza treatment RASSF1A expression increased threefold (Fig. 4).

Silencing of RASSF1A in HMEC during senescence. To analyze the epigenetic inactivation of RASSF1A, we utilized normal HMEC, which were grown for consecutive passages. It has been reported that pretransit HMEC exhibit increasing p16 expression with ongoing proliferation, while in the posttransit cells p16 is silenced (4). When we analyzed the expression of p16 in HMEC, we observed epigenetic silencing of p16 in posttransit HMEC (data not shown). Subsequently, we investigated the expression of RASSF1A in different HMEC isolates by real-time reverse transcription-PCR (Fig. 4A). As the pretransit HMEC approached the stasis barrier, RASSF1A expression was drastically reduced (Fig. 4A and B). At stasis, HMEC-48R (passage 5) showed a 70% reduction of expression, and HMEC-184 showed a 50% reduction compared to

After transformation, DNA of positive clones was prepared with a Qiagen spin kit (QIAGEN, Hilden, Germany) and sequenced (Seqlab, Göttingen, Germany).
FIG. 1. Promoter analysis of RASSF1A and RASSF1C. (A) Map of the RASSF1 locus. The locations of exons are shown. The CpG islands are indicated relative to the translational start sites. CpG islands were determined by CpGplot (http://www.ebi.ac.uk). Obs/Exp sets the minimum average observed-to-expected ratio of C plus G to CpG in a set of 10 windows that are required before a CpG island is reported. (B) Luciferase reporter assay of the RASSF1A (left panel) and RASSF1C (right panel) promoters. A 511-bp upstream fragment (A-511) including the translation start site of RASSF1A and four Sp1 binding sites was cloned in pRL-null vector and in vitro methylated (A-511 Methy.). The indicated promoter deletion (A-213 and A-Δ129) and mutation (A-mSp1) were generated. A 527-bp fragment of the RASSF1C promoter (C-527) including five Sp1 sites was cloned into pRL-null vector, and its activity was compared to that of the fragment with mutated Sp1 sites (C-mSp1). The transcriptional activities of the RASSF1A and RASSF1C fragments were determined relative to the promoterless pRL-null vector (set at 1) in three independent assays. (C) EMSA of four Sp1 sites (A, B, C, and D) located in the RASSF1A promoter. Twenty-two-bp labeled unmethylated (U) and in vitro methylated (M) oligonucleotides were incubated with nuclear extract of HeLa cells and analyzed by EMSA. Additionally, the probes were incubated with anti-Sp1 or XPA antibody (supershift is indicated by arrowheads) and competitor oligonucleotides. Mutated (m) competitors were
FIG. 2. Methylation analysis of the RASSF1 locus. (A) Map of the RASSF1 locus. The arrows indicate the transcriptional start sites of the RASSF1 isoforms and the white boxes the exons of RASSF1. Black boxes represent the exons of the BLU gene. Additional DNA elements (Alu, MER1, and LINE2) were located by RepeatMasker (http://ftp.genome.washington.edu/RM/RepeatMasker.html). The indicated 12 PCR fragments of the 7-kb locus were analyzed by combined bisulfite restriction analysis (COBRA). The coding DNA strand was deaminated in silico, and the restriction cutting sites of the CpG-containing sequence are shown (HpyCH4IV, TaqI, and BstUI). Primer sequences, PCR conditions, and restriction enzymes are listed in Table S1 in the supplemental material. (B) Representative COBRA analysis of normal blood DNA, human fibroblasts (HF), and cancer cell lines. PCR products of bisulfite-treated DNA were digested (+) or mock digested (−) with the appropriate enzymes. (C) The relative methylation level of the COBRA was plotted for the indicated cancer cell lines and HF.
passage 3; poststasis cells showed a 90% reduction in RASSF1A expression compared to growing prestasis cells (Fig. 4B). The RASSF1C expression did not significantly change in prestasis and poststasis HMEC (data not shown). In human fibroblasts, HeLa cells, and monocytes, which are unmethylated at the RASSF1A CpG island, the highest levels of RASSF1A transcripts (Fig. 4A) were detected. In the breast cancer cell lines ZR75-1, T47D, and MCF7, RASSF1A expression was dramatically reduced, and treatment with 10 μM Aza reactivated the expression of RASSF1A significantly (Fig. 4A).

In the quiescent mammary gland, the expression of RASSF1A was comparable to that in the cells at stasis. Treatment of HMEC at passage 13 with Aza and TSA increased the expression of RASSF1A 3.5 times (Fig. 4B).

Epigenetic inactivation and decrease of Sp1 binding occur during silencing of the RASSF1A promoter. We next analyzed the DNA methylation pattern of the RASSF1A locus in different passages of HMEC by COBRA (Fig. 5). Prestasis HMEC exhibited frequent methylation of the fragments U1, D1, and D2, which flank the transcription initiation site (Fig. 5B and C), and a further increase in DNA methylation was observed in later passages of both HMEC cultures. However, the CpGs located in the RA region, which contains three Sp1 binding sites and the transcription start site, were completely unmethylated in prestasis and stasis HMEC (Fig. 5B and C). Aberrant methylation occurred only in the poststasis cells. This observation may be attributed to a spreading of de novo DNA methylation from the methylated upstream and downstream regions into the RASSF1A CpG island promoter. To verify these data at several CpG sites, single PCR fragments of bisulfite-modified DNA were subcloned and analyzed by sequencing (Fig. 6). For the RA and U1 fragments, we examined 16 and 7 CpGs, respectively, obtained from several independent clones (Fig. 6B). In the human fibroblasts and monocytes, the RA region was completely unmethylated, and in the U1 products, two methylated CpGs located in the Sp1 binding site were detected. In the breast cancer cell line MCF7, almost all analyzed CpGs were methylated (Fig. 6B). Then we investigated the methylation pattern of the U1 and RA fragments in HMEC. In concordance with the COBRA results, a spreading of DNA methylation occurred with increased passage of HMEC (Fig. 6B). The upstream fragment was heavily methylated in early passages. In contrast, the RA fragment located in the CpG island was unmethylated in prestasis cells (Fig. 6B, p3), and methylated sites were found in poststasis cells (Fig. 6B, p8). Interestingly, the methylation density was significantly lower than in the breast cancer cells.

Subsequently, we investigated the acetylated histone H3 and trimethylated K9 histone H3 at three regions of the RASSF1A promoter in different passages of HMEC (Fig. 6C). At all three fragments, we detected high levels of acetylated histone H3 in passage 4. We observed that the histone H3 acetylation de-
creased at these fragments in the poststasis passage 8 and was not detected at the downstream A-1 fragment. In contrast, trimethylated K9-H3 was frequently found at the A-1 fragment in stasis and poststasis HMEC but not observed in passage 4 (Fig. 6C). The levels of H3-K9 trimethylation increased in stasis and poststasis at all three analyzed regions compared to the preceding passage 4. This indicates that the silencing of RASSF1A occurs together with deacetylation and methylation of histone H3. Interestingly, these histone modifications were already found at the unmethylated CpGs of the promoter, suggesting that altered chromatin structure at the RASSF1A promoter may precede de novo DNA methylation. Interestingly, we observed that levels of H3-K9 methylation were more than 10 times higher during the progressive inactivation of RASSF1A in HMEC (stasis and poststasis) than levels found in the breast cancer cell line ZR75-1. Thus, H3-K9 methylation may play a crucial role in the establishment of inactive chromatin at the RASSF1A promoter.

To investigate whether these epigenetic modifications are responsible for the occlusion of Sp1 binding at the RASSF1A promoter, we analyzed the abundance of Sp1 by real-time PCR. The expression levels were quantified in three independent experiments and plotted.

FIG. 4. Expression analysis of RASSF1A. (A) The expression of RASSF1A was analyzed in 0.5 μg of RNA isolated from monocytes, HeLa cells, human fibroblasts (HF), normal mammary gland, HMEC (prestasis, stasis, and poststasis), and three breast cancer cell lines (T47D, MCF7, and ZR75-1) by real-time PCR. The expression data of three prestasis and stasis HMEC isolates (184, 48R, and 141) and four poststasis HMEC isolates (184, 48R, 219, and 1001) were combined. The breast cancer cells were treated for 4 days with 10 μM Aza. The expression levels were quantified in three independent experiments and plotted relative to an internal standard of 0.5 μg of fibroblast RNA (set at 100%) as described in the text. (B) RASSF1A expression in HMEC. HMEC-184 and HMEC-48R were grown for increasing passages (p), and RASSF1A expression was determined by real-time PCR. Passage 13 of HMEC-184 was treated with 10 μM Aza and 0.3 μM TSA. The expression levels were quantified in three independent experiments and plotted.
FIG. 6. Methylation and chromatin pattern of the RASSF1A CpG island in HMEC. (A) Map of the RASSF1A promoter region and the analyzed fragments. The 5' end and the 3' end of the mRNA of RASSF1A and BLU are indicated. CpGs and Sp1 binding sites are marked by bars. (B) Seven and 16 CpG sites of the U1 and RA fragments, respectively, were analyzed in human fibroblasts (HF), monocytes, mammary cells, HMEC-184 (p3, stasis, and p8) cells, and MCF7 cells. Boxed CpGs indicate the Sp1 sites. Amplified PCR products were subcloned, and several independent clones were sequenced. Black and white dots represent methylated and unmethylated CpGs, respectively. Dots marked with a cross were not analyzable by sequencing. (C) For three fragments of the RASSF1A promoter (A-1, A-1.2, and A-2), the abundance of acetylated histone H3 (Ac-H3) and histone H3 K9 trimethylation (H3-K9 Me) were analyzed in consecutive passages (p) of HMEC by ChIP and real-time PCR in three
to passages 5 and 9. The occlusion of Sp1 binding at the upstream-located site A-2 occurred earlier in passage 9. Treatment of poststasis HMEC with Aza and TSA increased the binding of Sp1 at both analyzed regions (A-2 and A-1), and the expression of RASSF1A increased 3.5-fold (Fig. 4 and 6D). Interestingly, we identified similar binding of Sp1 in the A-2 fragments in HeLa cells and pretestis HMEC (Fig. 1 and 6D). However, the A-2 sequence (U1) is frequently methylated in HMEC but not in HeLa. These data indicate that DNA methylation per se is not responsible for the occlusion of Sp1 and suggest that a repressed chromatin state at the RASSF1A promoter is primarily responsible for the decrease in Sp1 binding.

**DISCUSSION**

In our previous work, we found frequent epigenetic inactivation of the RASSF1A CpG island in human cancers, and this silencing was associated with a hypermethylation of the CpG islands (7). However, the epigenetic mechanisms, which are responsible for the silencing of the RASSF1A promoter, were unknown. Our data indicate that histone H3 deacetylation and histone H3-K9 trimethylation precede de novo DNA methylation during the progressive inactivation of RASSF1A in HMEC. This observation is consistent with results from studies of Neurospora crassa and Arabidopsis and with results obtained in the pericentric heterochromatin of mice, which indicated that H3-K9 methylation can direct DNA methylation (21, 28, 45). Interestingly, we observed that levels of H3-K9 methylation were higher during the progressive inactivation of RASSF1A in HMEC than in the breast cancer cell line ZR75-1. Thus, H3-K9 methylation may play a crucial role in the establishment of inactive chromatin at the RASSF1A promoter. A recent study has suggested that during the silencing of a transgene, H3-K9 methylation and DNA methylation occur late and follow histone deacetylation and loss of H3-K4 methylation (30). Here, we analyzed a bona fide CpG island promoter of a tumor suppressor gene, and therefore our results may represent the mechanism of epigenetic silencing which occurs in carcinogenesis. In order to identify crucial transcriptional activators of RASSF1A, we determined four Sp1 binding sites in the RASSF1A promoter. Three of these Sp1 sites were located in the unmethylated RASSF1A CpG island, and an additional Sp1 site was detected upstream in a region with low CpG content. This upper Sp1 binding sequence can be methylated in normal mammary cells and fibroblasts, and this methylation does not affect the binding of Sp1 directly. This observation confirms that the binding of Sp1 is DNA methylation insensitive (18, 34). We proposed that the altered binding of Sp1 at the RASSF1A promoter is mediated by the repressed chromatin state and not by DNA methylation per se.

It has been shown that methylated CpG sites attract methyl-CpG binding domain proteins (MBDs) that interact with the corepressor complex Sin3, including histone deacetylases (25, 32, 33). Other studies indicate that DNA methyltransferase and MBDs interact with histone methyltransferase (14, 15). The methylated CpGs located upstream and downstream of the RASSF1A CpG island may attract histone methyltransferase and deacetylase and lead to the repressed chromatin. For the glutathione S-transferase (GSTP1) promoter, neither removal of the Sp1 binding sites nor seeds of DNA methylation alone are sufficient to achieve promoter hypermethylation (39). Recently, it has been proposed that gene silencing of GSTP1 promotes DNA hypermethylation through a sequential change in chromatin modification (43). It has been suggested that a dramatic stop in transcription is the critical precursor in cancer, which is followed by de novo DNA methylation and ends with a complete cessation of gene expression (6, 47). Our results for RASSF1A are consistent with this hypothesis. In HMEC, a drastic decrease in RASSF1A expression was detected, and this was associated with a repressed chromatin state. In these cells, the methylation pattern of the RASSF1A CpG island is completely different from that in breast cancer cell lines. We propose that the epigenetic inactivation of RASSF1A occurs in distinct steps (Fig. 7). In senescent cells, silencing of RASSF1A transcription may occur through a DNA methylation-independent mechanism involving mainly histone deacetylation and methylation (Fig. 7). In cancer cells, inactivation of RASSF1A is manifested by de novo DNA methylation of its promoter (Fig. 7). This may lead to silencing of RASSF1A, which is irreversible, since the presence of an active DNA demethylase in mammalian cells is not evident. Thus, in the proliferating poststasis HMEC, a new transcriptional pattern is established by a repressed chromatin state, and then in tumor cells, this aberrant expression profile is locked by DNA methylation and the heterochromatic state is maintained by DNA methyltransferase, methyl-CpG binding proteins, and heterochromatin protein 1 (H1p1) (Fig. 7). It has been shown that the H1p1 isoforms bind to methylated H3-K9 residues (1, 26, 31). Recently, a link between the Suv39h-H1p1 histone methylation system and the DNA methyltransferase 3b in mammals was demonstrated (28). H1p1 and MBD may be responsible for locking and maintaining the repressed chromatin together with the DNA methyltransferases in silenced cells (Fig. 7). It will be interesting to analyze other histone modification and chromatin components, such as methylation of histone H3 lysine 4, MBD, and H1p1, during the inactivation of RASSF1A.

Epigenetic inactivation of tumor suppressor gene promoters plays a fundamental role in the etiology of cancer (24). We and others have analyzed RASSF1A inactivation in primary breast carcinoma and found 49 to 65% RASSF1A CpG island methylation (9, 10). Recently, DNA hypermethylation of RASSF1A was associated with poor prognosis for breast cancer patients (29). RASSF1A inactivation has also been demonstrated in independent experiments. (D) Binding of Sp1 to the RASSF1A promoter in mammary epithelial cells. HMEC-184 cells were grown, cross-linked with formaldehyde, and analyzed by ChIP. Protein-DNA complexes were precipitated with Sp1 antibody, and the abundance was determined by real-time PCR in three independent experiments. Three regions (A-2, A-1.2, and A-1) of the RASSF1A promoter are plotted. The bound-to-input ratio (B/I) was plotted against input chromatin (100%) and no-antibody probe (0%). HMEC-184 cells were treated with 0.3 μM TSA and 10 μM Aza for 3 days, and the relative changes (B/I) in Sp1 binding were compared to the untreated controls (set at 1) by real-time PCR in three independent experiments and plotted.
epithelial hyperplasia and intraductal papillomas but has not been detected in lymphocytes, stromal tissue, normal breast epithelium, lactating breast tissue, or apocrine metaplasia (27). It has been reported that RASSF1A blocks cell cycle progression, including histone deacetylation and H3-K9 methylation (H-Me), which is accomplished by histone deacteylase (HDAC) and histone methyltransferase (HMT), respectively. The repressed chromatin structure triggers the de novo methylation of CpGps (black dots) by DNA methyltransferase (DNMT). In cancer cells, the inactive state of RASSF1A is locked and maintained by methyl-CpG binding domain proteins (MBP) and heterochromatin protein 1 (Hp1).

In summary, our data show that the aberrant transcriptional silencing of RASSF1A preceded its CpG island promoter hypermethylation, and this may be triggered by inactivating chromatin modification, including histone deacetylation and H3-K9 methylation. Since RASSF1A blocks cell cycle progression, the silencing of RASSF1A may be a critical step in tumorogenesis. It will be interesting to analyze the influence of inhibitors of DNA methylation and histone deacetylase on the reactivation of the RASSF1A gene in silenced cells.

FIG. 7. Model of the progressive epigenetic silencing of RASSF1A. In normal epithelial cells, the RASSF1A promoter is transcriptionally active, histones are acetylated (H-Ac), and the CpG island is unmethylated (white dots). The open chromatin structure allows binding of the transcription factor Sp1. In senescent cells, silencing of RASSF1A is associated with histone deactetylation and H3-K9 methylation (H-Me), which is accomplished by histone deacteylase (HDAC) and histone methyltransferase (HMT), respectively. The repressed chromatin structure triggers the de novo methylation of CpGps (black dots) by DNA methyltransferase (DNMT). In cancer cells, the inactive state of RASSF1A is locked and maintained by methyl-CpG binding domain proteins (MBP) and heterochromatin protein 1 (Hp1).

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