MCAF1/AM Is Involved in Sp1-mediated Maintenance of Cancer-associated Telomerase Activity

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Telomerase maintains telomere length and is implicated in senescence and immortalization of mammalian cells. Two essential components for this enzyme are telomerase reverse transcriptase (TERT) and the telomerase RNA component (encoded by the TERC gene). These telomerase subunit genes are known to be mainly expressed by specificity protein 1 (Sp1). MBD1-containing chromatin-associated factor 1 (MCAF1), also known as ATFα-associated modulator (AM) and activating transcription factor 7-interacting protein (ATF7IP), mediates gene regulation, although the precise function of MCAF1 remains to be elucidated. Here, we report that MCAF1 is involved in Sp1-dependent maintenance of telomerase activity in cancer cells. Two evolutionarily conserved domains of MCAF1 directly interact with Sp1 and the general transcriptional apparatus. Selective depletion of MCAF1 or Sp1 down-regulates TERT and TERC genes in cultured cells, which results in decreased telomerase activity. The transcriptionally active form of RNA polymerase II and the general transcription factor ERCC3 decreased in the TERT promoter under the loss of MCAF1 or Sp1. Consistently, MCAF1 is found to be frequently overexpressed in naturally occurring cancers that originate in different tissues. Our data suggest that transcriptional function of MCAF1 facilitates telomerase expression by Sp1, which may be a common mechanism in proliferative cancer cells.

Telomerase is a multiple subunit complex that contains RNA-dependent DNA polymerase that adds telomeric hexamer repeats to the ends of chromosomes (1, 2). This enzymatic activity is normally absent in most somatic cells, which results in a gradual loss of telomeres through cell division. In contrast, more than 85% of cancerous cells and in vitro immortalized cells gain telomerase activity, which is required for unlimited proliferation (3–6). The reactivation of telomerase activity may contribute to cell transformation by increasing the potential risk of genetic and epigenetic defects (7). The telomerase enzyme consists of two essential components, telomerase reverse transcriptase (TERT) (encoded by the TERT gene) and the telomerase RNA component (TR; encoded by the TERC gene), which serves as a template for telomeric DNA synthesis. TR is ubiquitously expressed in most tissues, whereas TERT is expressed only in telomerase-positive cells (8, 9). Introduction of exogenous TERT can immortalize primary human cells (10, 11), and overexpression of both TERT and TR synergistically increases telomerase activity (12). Because the telomerase subunit genes must be appropriately regulated in vivo, abnormal expression of TERT and TERC genes induces cell deregulation.

The specificity protein 1 (Sp1) plays a crucial role in transcriptional control of the TERT gene (13). There are five GC boxes that are consecutive binding sites of Sp1 in the TERT core promoter (14). In addition, Sp1 is known to cooperate with c-Myc to transactivate the TERT gene in a cell type-specific manner (15). Although Sp1 is a ubiquitous protein that targets both core promoter and enhancers, it is interesting that this protein is highly expressed in various cancers of the stomach, colon and rectum, pancreas, breast, and thyroid (16). Thus, Sp1-dependent expression of the TERT gene may provide a frequent pathway toward cancer development. However, the precise mechanism of Sp1-dependent control of telomerase expression remains to be elucidated.

We initially found that methylated DNA-binding domain protein 1 (MBD1) interacts with MBD1-containing chromatin-associated factor 1 (MCAF1) (17), also known as ATFα-associated modulator (AM) and activating transcription factor 7-interacting protein (ATF7IP) (18). MCAF1 binds the transcriptional repression domain of MBD1 and further recruits SET domain bifurcated 1 (SETDB1) for gene silencing in methylated DNA regions (17, 19). AM/MCAF1 facilitates conversion of dimethyl to trimethyl histone H3 at lysine residue 9 (H3K9) by euchromatic gene-associated protein with SET domain, a murine homologue of human SETDB1 (20). Thus, MCAF1 is involved in MBD1-dependent transcriptional...
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repression via heterochromatin assembly. Previous observations have suggested that MCAF1 and Sp1 are functionally related (17, 19, 21); for instance, MCAF1 enhances Sp1-mediated transcription by Rta protein of Epstein-Barr virus (22). In addition, Sp1 is known to interact with basal transcriptional factors including TFIIB, TFIIE, TBP/TFIID, and further with the transcriptional mediators such as the cofactor required for Sp1 (23). The transcriptional mediators transduce regulatory information between Sp1 and the basal transcription machinery including the core RNA polymerase II (24). It is of great interest to investigate the transcriptional role of MCAF1 and Sp1 in the telomerase subunit genes.

During our investigations, we have found that MCAF1 is highly expressed in human cancers that originated in many different tissues, suggesting that MCAF1 has an important role in tumorigenesis. Consistently, our data demonstrate that MCAF1 is required for Sp1-dependent maintenance of telomerase activity in cancer cells. Specific knockdown of MCAF1 or Sp1 reduced both TERT and TERC expression, which resulted in a decrease of telomerase activity. The active form of RNA polymerase II and general transcription factor were released from the TERT promoter by the loss of MCAF1 or Sp1. Two evolutionarily conserved domains of MCAF1 were responsible for direct interaction with Sp1 and the general transcriptional apparatus. These data shed light on the transcriptional mediator-like function of MCAF1 that facilitates Sp1-dependent expression of the telomerase subunit genes, which may be a common mechanism in oncogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s minimum essential medium and Ham’s F-12 nutrient medium (Sigma) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and penicillin/streptomycin.

Antibodies—Anti-MCAF1 antibodies were as described previously (17). The antibodies utilized were anti-Sp1 (Santa Cruz Biotechnology), anti-β-tubulin (Amersham Biosciences Bioscience), anti-GST (DAKO), anti-RNA polymerase II CTD4H8 (Upstate Biotechnology), anti-His (Qiagen), anti-ERCC3 (Santa Cruz Biotechnology), anti-ERCC2 (Santa Cruz Biotechnology), anti-TFIIEα (Santa Cruz Biotechnology), and anti-TFIIEβ (Santa Cruz Biotechnology). Western blot and immunofluorescence analyses were carried out as described elsewhere (17, 19).

Glutathione S-Transferase (GST) Pull-down Assay—Bacterially expressed GST and GST fusion proteins (1 μg) were immobilized on glutathione-agarose beads, and incubated with HeLa nuclear extract (60 μg) or His-tagged proteins (1 μg) in buffer that contained 0.1% Triton X-100, 50 mM HEPES (pH 7.4), 100 mM NaCl, 5% glycerol, 2 mM dithiothreitol, and protease inhibitors for 1 h at 4 °C. The input indicated 10% of the proteins in the reaction mixture.

Immunoprecipitation—HeLa nuclear extract (Promega) was diluted 10 times with buffer (40 mM Tris-HCl (pH 7.8), 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 25% glycerol), and mixed with anti-MCAF1 antibodies, anti-Sp1 antibodies, or control IgG for 1 h at 4 °C, followed by incubation for 1 h after adding 30 μl of protein A/G-agarose beads (Amersham Biosciences). After washing, the bound proteins were detected by Western blot analysis.

siRNA-mediated Knockdown of Sp1, MCAF1, and TERT—HeLa cells were transfected with siRNAs with Lipofectamine RNAiMAX (Invitrogen) and harvested at 60 h after transfection. Twenty-one nucleotide siRNA duplexes were designed to target mRNAs that encoded human Sp1, MCAF1, or TERT. The selected siRNA target sequences were submitted to human genome and EST databases to ensure the target specificities. These sequences are described in supplemental Table S1. The siRNAs for GL3 were as described previously (19).

Chromatin Immunoprecipitation (ChIP) and Quantitative PCR—HeLa cells (1 × 10^6) were cross-linked with 1% formaldehyde at 37 °C for 10 min. Crude cell lysates were sonicated to generate 200–1000-bp DNA fragments. ChIP was performed with anti-Sp1 or anti-MCAF1 antibodies, or control IgG, according to the manufacturer’s protocols (Upstate Biotechnology). PCR amplification of the TERT gene promoter was carried out for 20 (the first PCR) and 26 cycles (the second PCR) under conditions of 20 s at 98 °C, 30 s at 65 °C, and 30 s at 72 °C. Amplification of the TERC gene promoter was performed for 35 cycles under conditions of 20 s at 98 °C, 30 s at 58 °C, and 30 s at 72 °C. Amplification of the ~8 kb upstream of the TERT gene was performed for 30 cycles under conditions of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Quantitative PCR was performed on the products of ChIP in triplicate by the SYBR Green method using the Power SYBR Green PCR Master Mix and ABI 7300 Real Time PCR System (Applied Biosystems). Standard curves were generated using serial dilutions of input DNA (10.0, 2.0, 0.4, and 0.08%). The C_T of each reaction was quantified against the standard curve. The primer sets are listed in supplemental Table S2.

Quantitative RT-PCR—Total RNA was isolated using Isogen (Nippon Gene). For cDNA synthesis, 3 μg of total RNA was reverse-transcribed with Superscript III (Invitrogen) using oligo(dT) primers. Quantitative PCR of the target cDNAs was performed by the SYBR Green method using Power SYBR Green PCR Master Mix (Applied Biosystems). Each experiment was carried out at least three times. The fold relative enrichment was quantified, together with normalization by the β-actin level. The primer sets are listed in supplemental Table S2.

Measurement of Telomerase Activity—Telomerase activity was measured using the quantitative telomeric repeat amplification protocol (Q-TRAP) assay (25). Briefly, cell pellets were resuspended in a lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM EGTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS, and 10% glycerol) and incubated for 30 min on ice. After centrifugation at 12,000 × g for 20 min at 4 °C, aliquots of the supernatant were rapidly frozen and stored at −80 °C. Protein concentration of lysates was determined with GeneQuant Pro (Bio-Rad). To prepare a control lysate, telomerase was inactivated by incubating at 85 °C for 10 min. The SYBR Green Q-TRAP assay was conducted with cell lysates (1.0, 0.5, 0.2, and 0.1 μg), 0.1 μg of telomerase primer TS, and 0.1 μg of anchored return primer ACX, in 25 μl with SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences are listed in supplemental Table S2. Using the ABI PRISM 7300 thermal cycler (Applied Biosystems), samples
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**RESULTS**

MCAF1 Complexes with Sp1 via the Evolutionarily Conserved Domains—The MCAF/AM family of proteins is characterized by two functional domains, Domain 1 and Domain 2, which are evolutionarily conserved from *Drosophila* to humans (Fig. 1A) (17, 19). There is no DNA binding motif found in MCAF1. On the other hand, Sp1 has two transcriptional activation domains and a region that is mainly composed of three zinc finger motifs (27).

Previous observations have suggested that MCAF1 and Sp1 are functionally related (17, 19, 21, 22). To examine the direct interaction between MCAF1 and Sp1 at the domain levels, we prepared bacterially expressed deletion mutants of MCAF1 and Sp1, which were fused to GST or His-tagged. The representatives included MCAF1 (∆10, ∆18, and ∆34), and Sp1 (∆5, ∆6, and ∆7). GST and GST-fused portions of MCAF1 were immobilized on glutathione-agarose beads and incubated with the HeLa nuclear extract (Fig. 1B, left). Sp1 bound both Domain 1 (∆18) and Domain 2 (∆34), but not the amino-terminal region (∆10) of MCAF1. To further determine the region of Sp1 responsible for interacting with MCAF1, we used GST-fused portions of Sp1 and His-tagged MCAF1 (Fig. 1B, right). GST and GST-fused portions of Sp1 on glutathione-agarose beads were mixed with His-tagged MCAF1 (∆18 or ∆34). Domain 1 (∆18) of MCAF1 bound ∆5 and ∆6, whereas Domain 2 (∆34) bound ∆6 and ∆7 of Sp1. The amino-terminal region of Sp1 that contained activation domains did not bind MCAF1, and the

were incubated for 20 min at 25 °C, and amplified in 35 cycles of 30 s at 95 °C and 90 s at 60 °C. Samples, inactivated samples, and lysis buffer were used on all PCR plates. Each sample was analyzed at least in duplicate. Telomerase activity is shown relative to that in control cells.

**Bisulfite Genomic DNA Sequencing**—Genomic DNAs were purified and treated with sodium bisulfite using the BisulFast DNA Modification Kit (Toyobo) (26), followed by PCR amplification with each of the specific primers, which contained no CpG sequences, using LA Taq polymerase (Takara). The amplified region corresponded to nucleotides 3791–4105 of the unmodified human TERT gene sequences (GenBank™ AF097365). PCR was carried out for 32 cycles under conditions of 30 s at 98°C, 30 s at 56°C, and 60 s at 72°C. PCR products were subcloned into pGEM-T easy vector (Promega) and sequenced (at least 15 individual clones). Primers used are shown in supplemental Table S2.

**Immunohistochemistry**—Immunohistochemistry was performed with human Tumor Tissue Arrays including stomach, breast, and lung tumors, and various normal tissues (BioChain Institute, Inc. and ISU Abaxis Co., Ltd.). The array slides were deparaffinized and then incubated in methanol with 0.3% hydrogen peroxide for 30 min to block the endogeneous peroxidase activity. Thereafter, tissue sections were immersed in 1.0% Block-Ace (Dainippon Sumitomo Pharma Co., Ltd.) in phosphate-buffered saline for 30 min, covered with anti-MCAF1 antibodies, and incubated 1 h at room temperature. Visualization of the immunoreaction was performed using Histofine Simple Stain MAX-PO (Nichirei Bioscience) and 3,3-diaminobenzidine tetrahydrochloride (Dako). The slides were counterstained with hematoxylin and mounted with Malinol (Muto Pure Chemicals).

Statistical Analysis—Differences between groups were analyzed using Student’s *t* test. *p* < 0.05 was considered statistically significant.

**FIGURE 1.** MCAF1 complexes with Sp1 via the evolutionarily conserved domains. A, functional domains of MCAF1 and Sp1. MCAF1 has two evolutionarily conserved domains, Domain 1 and Domain 2. Sp1 contains two transcriptional activation domains with serine and threonine-rich regions (S/T), and the three zinc fingers with DNA binding activity. Bacterially expressed portions of MCAF1 and Sp1 are indicated for a pull-down assay. B, direct interaction between MCAF1 and Sp1. Immobilized GST-fused MCAF1 (∆10, ∆18, and ∆34) was incubated with HeLa nuclear extracts (left). Both Domains 1 (∆18) and 2 (∆34) of MCAF1 bound to the carboxy-terminal region of Sp1 (right). C, complex formation of MCAF1 and Sp1. Endogenous MCAF1 and Sp1 were immunoprecipitated in HeLa cells. Two isoforms of Sp1 (95 and 105 kDa) are normally detected. The full-length Sp1, which were fused to GST or His-tagged. The representatives included MCAF1 (∆10, ∆18, and ∆34) and Sp1 (∆5, ∆6, and ∆7). GST and GST-fused portions of MCAF1 were immobilized on glutathione-agarose beads and incubated with the HeLa nuclear extract (Fig. 1B, left). Sp1 bound both Domain 1 (∆18) and Domain 2 (∆34), but not the amino-terminal region (∆10) of MCAF1. To further determine the region of Sp1 responsible for interacting with MCAF1, we used GST-fused portions of Sp1 and His-tagged MCAF1 (Fig. 1B, right). GST and GST-fused portions of Sp1 on glutathione-agarose beads were mixed with His-tagged MCAF1 (∆18 or ∆34). Domain 1 (∆18) of MCAF1 bound ∆5 and ∆6, whereas Domain 2 (∆34) bound ∆6 and ∆7 of Sp1. The amino-terminal region of Sp1 that contained activation domains did not bind MCAF1, and the
regions of MCAF1 except Domains 1 and 2 did not bind Sp1 (data not shown). To confirm whether MCAF1 formed a complex with Sp1, immunoprecipitation analysis using anti-MCAF1 and anti-Sp1 antibodies were performed in HeLa cells (Fig. 1C). MCAF1 was found in the immunoprecipitates with Sp1. Conversely, Sp1 was present in the MCAF1 immunoprecipitates. These data suggest that the conserved domains of MCAF1 directly bind the carboxyl-terminal region of Sp1, resulting in the formation of an MCAF1-Sp1 complex.

**Formation of MCAF1-Sp1 Complex in Telomerase Subunit Gene Promoters**—To investigate the involvement of the MCAF1-Sp1 complex in endogenous gene regulation, ChIP analysis was carried out on HeLa cells. Telomerase-associated TERT and TERC genes are reported to be transactivated by Sp1, through five and four binding sites within the core promoter regions, respectively (Fig. 2A) (28, 29). After formaldehyde-based cross-linking of protein and DNA, chromatin fragmented by sonication was immunoprecipitated with anti-MCAF1 or anti-Sp1 antibodies, followed by PCR amplification using specific primers for the promoter sequences (Fig. 2B). MCAF1 and Sp1 were found in the TERT and TERC promoter regions. In addition, the ~8 kb upstream site of the TERT gene was not bound by these proteins. Species-matched IgG was used for immunoprecipitation as a control. In addition, both proteins were also found in the distinct Sp1-dependent BRCA1 gene promoter (supplemental Fig. S1). These data show that MCAF1 and Sp1 coexist in the TERT and TERC gene promoters (open bars, \( p < 0.01 \)), compared with the controls. This suggests that MCAF1 is localized to the TERT gene promoter in an Sp1-dependent manner, via complex formation of these proteins.

**MCAF1 and Sp1 Are Required for Maintaining Telomerase Activity**—To clarify the role of MCAF1 and Sp1 in transcriptional control of telomerase components, we performed a quantitative RT-PCR for TERT and TERC mRNAs (Fig. 3A). The transcripts of TERT and TERC genes were significantly down-regulated by the knockdown of MCAF1 or Sp1 in HeLa cells (\( p < 0.01 \) or \( p < 0.05 \)). Similar results were obtained in HCT116 cells (supplemental Fig. S2). The data suggest that MCAF1 cooperates with Sp1 to activate transcription from the TERT and TERC genes. We had difficulty detecting the TERT protein, probably because of recognition inefficiency of anti-TERT antibodies; therefore, we assessed the effect of MCAF1 and Sp1 on endogenous telomerase activity, using a Q-TRAP assay (25). The cell lysates were prepared from HeLa cells under the knockdown of MCAF1 or Sp1, and were serially diluted for the TRAP assay, followed by quantitative PCR using specific primers for telomeric repeats (Fig. 3B). Compared with the controls, telomerase activity markedly decreased in the MCAF1 or Sp1 knockdown cells (\( p < 0.01 \) or \( p < 0.05 \)). These results suggest that the MCAF1-Sp1 complex maintains telomerase activity by inducing TERT and TERC expression.

To further evaluate the effect of the MCAF1-Sp1 complex in telomerase expression, we counted the cell numbers under
knockdown of MCAF1 or Sp1 (Fig. 3C). MCAF1- or Sp1-depleted cells showed lower proliferative activity in comparison to the controls. These data were very analogous to the TERT knockdown by using two specific siRNAs (Fig. 3D). As expected, the knockdown of TERT diminished the known mRNAs and the resulting telomerase activity (supplemental Fig. 3). Collectively, these data suggest that the MCAF1-Sp1 complex is required for maintaining telomerase activity and proliferation in the cancer cells examined.

MCAF1-Sp1 Complex Is Involved in Maintaining the Localization of Phosphorylated RNA Polymerase II and General Transcription Factor in the TERT Promoter—To examine transcriptional control by the MCAF1-Sp1 complex, we analyzed the status of modified histones in the TERT promoter, by a quantitative ChIP analysis using specific antibodies (supplemental Fig. S4). Among the transcriptionally active marks tested, H3 acetylation alone was diminished in the Sp1 knockdown cells, whereas MCAF1 or Sp1 depletion showed no significant changes in H3K4 methylation and H4 acetylation. These results may have been caused by the originally low levels of these modified histones in the TERT promoter. Because MCAF1 and Sp1 are associated with RNA polymerase II and general transcription factors (18, 23), we then tested for the presence of the transcriptionally active form of RNA polymerase II (Pol II [Ser 5-P]) and the excision-repair, complementing defective, in Chinese hamster 3/xeroderma pigmentosum B (ERCC3/XPB), an essential component of TFIIH, in the TERT promoter (Fig. 4, A and B). Knockdown of MCAF1 or Sp1 resulted in significant reduction of both Pol II (Ser 5-P) and ERCC3 in the gene promoter ($p < 0.01$ or $p < 0.05$). Thus, the MCAF1-Sp1 complex maintains the localization of phosphorylated RNA polymerase II and ERCC3 in the TERT core promoter.

To confirm the fundamental role of MCAF1, we characterized the ability of the protein to bind the general transcription apparatus. MCAF1 in the HeLa nuclear extract was immunoprecipitated with anti-MCAF1 antibodies, and cellular ERCC3 and Pol II were found in the MCAF1 immunoprecipitates (Fig. 4C, left). The MCAF1 immunoprecipitates also contained TFIIe, TFIIE, and ERCC2/XPD (data not shown). We next immunoprecipitated ERCC3, Pol II, TFII-e, and TFIIE from the nuclear extract with appropriate antibodies, and the presence of MCAF1 was detected in these immunoprecipitates (Fig. 4C, right). Because TFIIID is known to interact with Sp1, we examined whether the TBP box-binding protein (TBP), an essential component of TFIIID, interacts with MCAF1 (supplemental Fig. S5). Sp1, but not MCAF1, was found in the TBP immunoprecipitates, suggesting that TBP preferentially coexists with Sp1. To further determine the region of MCAF1 that interacted with general transcription factors, GST-MCAF1 deletions were used for a pull-down assay with the nuclear extract (Fig. 4D). Both ERCC3 and ERCC2 were associated with Domain 2 ($\Delta$8) of MCAF1. In contrast, TFII-e and TFII-e bound both Domain 1 ($\Delta$18) and Domain 2 ($\Delta$8) of the protein. To check the direct interaction between MCAF1 and general transcription factors, a pull-down analysis was performed using purified proteins (Fig. 4E). GST-fused ERCC3 and ERCC2 on glutathione-agarose beads were incubated with His-tagged MCAF1 ($\Delta$10 and $\Delta$8). Both ERCC3 and ERCC2 directly bound the $\Delta$8 of MCAF1 but not $\Delta$10, which was consistent with the data in Fig. 4D. Conversely, immobilized GST-MCAF1 ($\Delta$10, $\Delta$18, and $\Delta$8) was mixed with His-tagged TFII-e and TFII-e. TFII-e was found to bind $\Delta$18 alone, whereas TFII-e bound both $\Delta$18 and $\Delta$8 of MCAF1. Taken together, these data suggest that two conserved domains of MCAF1 directly bind Sp1 and the general transcription apparatus, like the transcriptional mediators.
MCAF1-Sp1 Complex May Affect CpG Methylation Status in the TERT Promoter Region—Recent studies have reported that the human TERT promoter region is uniquely methylated in telomerase-positive cell lines (3, 30), and that the unmethylated region just upstream of the transcriptional start site may permit low transcriptional activity of the TERT gene in tumor cells (31, 32). In addition, it has been shown that the repressor protein CCCTC-binding factor (CTCF) binds the unmethylated motifs within the first exon to inhibit TERT transcription, whereas CpG methylation at the binding site inhibits CTCF binding, leading to TERT gene transcription in cancer cells (32). To test the epigenetic role of the MCAF1-Sp1 complex, we investigated the methylation status at 38 CpG dinucleotides in the promoter and first exon of the TERT gene (Fig. 5), using bisulfite genomic sequencing. Compared with the control cells, the CpG sites 5–9 between the Sp1-binding motifs tended to be selectively methylated in MCAF1 or Sp1 knockdown HeLa cells, in which TERT expression was reduced. In addition, the first exon region, which contained the CTCF binding site, was densely methylated in the controls, whereas it tended to be partially unmethylated at some CpG sites under MCAF1 or Sp1 depletion. Thus, the loss of the MCAF1-Sp1 complex may affect the DNA methylation status in the TERT gene. The molecular mechanism underlying the methylation changes on selective CpG sites under the loss of MCAF1 or Sp1 is unknown, but it may result from transcriptional down-regulation of the TERT gene.

It is reported that MCAF1 cooperates with MBD1 and SETDB1 for gene silencing in methylated DNA regions (17, 19). To test the implication of the MCAF1-MBD1-SETDB1 complex in the TERT gene, quantitative ChIP analysis was performed with specific antibodies (supplemental Fig. S6). MBD1 was detected in the TERT promoter, which was not affected by depletion of MCAF1 or Sp1. In contrast, SETDB1 did not appear to be significantly enriched in this promoter, in agreement with the low levels of H3K9 trimethylation, suggesting that the MCAF1-MBD1-SETDB1 complex unlikely affects TERT regulation. In addition, the knockdown of MCAF1 or Sp1 did not significantly change CTCF binding to the target site in the TERT gene.

MCAF1 Is Overexpressed in Human Cancer Tissues—To clarify the involvement of MCAF1 in cancer development, we finally investigated the expression of MCAF1 in human cancer tissues,
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To investigate the correlation of MCAF1 and Sp1 in human cancer, immunohistochemical analysis was carried out using anti-Sp1 antibodies (supplemental Fig. S8). In agreement with previous studies (16), Sp1 was positively stained in more than 80% of adenocarcinoma of the stomach examined, whereas this protein was detected at low levels in non-cancerous tissues. Both MCAF1 and Sp1 tended to coexist in these gastric cancer tissues. To further test the relationship between MCAF1, Sp1, and the telomere state, we performed a telomere-FISH with the same tissue samples (supplemental Fig. S9). Multiple telomere signals were detected in adenocarcinoma tissues, rather than in non-cancerous tissues. We observed that high expression of MCAF1 and Sp1 was found in telomere-positive cancer cells, suggesting that telomeres may be well maintained in cancer cells that overexpress MCAF1 and Sp1.

To clarify the role of MCAF1 and Sp1 in telomerase activities, we assessed the mRNA levels of TERT, TERC, MCAF1, and Sp1 in telomerase positive cancer cell lines, using quantitative RT-PCR analysis (supplemental Fig. S10). The expression levels of these genes were significantly higher than those in control primary fibroblasts (p < 0.01). The association between two relative mRNA levels was analyzed using the Pearson correlation coefficient (r), which varies from a perfect negative correlation (−1) to a perfect positive correlation (+1). A significantly correlated expression was found between TERT and TERC (TERT-TERC; r = 0.93), whereas there was no correlation of TERT-GAPDH or TERC-GAPDH. Moderate correlations were found in TERT-MCAF1 (r = 0.41), TERT-Sp1 (r = 0.33), TERC-MCAF1 (r = 0.30), and TERC-Sp1 (r = 0.48) (supplemental Fig. 10F). Thus, these data may support the transcriptional role of MCAF1 and Sp1 in regulating the telomerase subunit genes.

DISCUSSION

The mechanism responsible for maintaining telomerase activity is crucial for understanding cellular immortalization and cancer development (3, 33, 34). Our present study reveals
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FIGURE 6. Overexpression of MCAF1 in human cancer tissues. Serial sections of non-cancerous and cancerous tissues; stomach (A, panels a–d), breast (B, panels e–h), and lung (C, panels i–n). Normal tissues show undetectable or very low expression of MCAF1 (panels b, f, and j). In contrast, MCAF1 was highly stained in adenocarcinoma of the stomach (panel d), invasive ductal carcinoma of the breast (panel h), and adenocarcinoma and squamous cell carcinoma of the lung (panels l and n, respectively). Hematoxylin and eosin staining are shown in panels a, c, e, g, i, k, and m; immunostaining for MCAF1 are shown in panels b, d, f, h, j, l, and n. Scale bar, 200 μm. The correlation analyses of MCAF1, Sp1, and telomeres are shown in supplemental Figs. S7–S10.

that: (i) MCAF1 and Sp1 maintain expression of the TERT and TERC genes, which results in positive telomerase activity; (ii) Sp1 substantially tethers MCAF1 to the TERT promoter; (iii) MCAF1-SP1 complex maintains the localization of transcriptionally active RNA polymerase II and ERCC3 in the TERT promoter; (iv) two evolutionarily conserved domains of MCAF1 directly bind Sp1 and the general transcriptional apparatus; and (v) MCAF1 is frequently expressed at high levels in most cancer tissues. Thus, overexpressed MCAF1 mediates Sp1-dependent maintenance of telomerase activity, by facilitating the localization of the general transcriptional apparatus and active form of RNA polymerase II in telomerase subunit genes, which may be a common mechanism in proliferative cancer cells.

MCAF1 Mediates Sp1-dependent Expression of Telomerase Subunit Genes—The increase of telomerase activity is characteristic of cancer and prevents telomere shortening and senescence of tumor cells, which are critical for oncogenesis (3, 34, 35). There are at least two possible mechanisms for telomerase up-regulation in cancer. One is the increased copy number of the TERT and TERC genes (36), which are located at human chromosomes 5p15.33 and 3q26.3, respectively. Chromosomal gains and amplifications that involve 5p (13.2%) and 3q (16.4%) have been detected in solid tumors, using comparative genomic hybridization (37). The other mechanism is increased expression of the telomerase subunit genes. Exogenous expression of both TERT and TR, compared with either alone, markedly augmented telomerase activity. For this reason, it is of interest that Sp1 commonly activates the TERT and TERC genes (15, 38).

Because expression of TERT is essential for positive telomerase activity in cancer cells (5, 39), we focused on characterizing transcriptional regulation of the TERT gene by Sp1. Although the TERT promoter lacks either a TATA box or TATA-like sequence, it has been demonstrated that TATA-less promoters still utilize TATA factors for transcriptional initiation of these genes (40), and that Sp1 tethers TATA factors that lead to transcriptional initiation of TATA-less promoters (41). In this study, we showed that MCAF1 and Sp1 are required for maintaining the localization of transcriptionally active RNA polymerase II and general transcriptional factors in the TERT promoter, suggesting the possibility that MCAF1, together with Sp1, may stimulate the conversion of unphosphorylated to phosphorylated forms of RNA polymerase II. MCAF1 did not affect the DNA-binding ability of Sp1, whereas it was recruited to the TERT promoter by Sp1. Thus, MCAF1 synergistically participates in transcriptional activation of the telomerase subunit genes by interacting with Sp1, which is necessary for maintenance of telomerase activity in cancer. These data suggest that up-regulation of MCAF1 and Sp1 may be a fundamental event in telomerase maintenance in cancer.

Epigenetic states in the TERT promoter region are important for transcriptional regulation, including trimethylated histone H3K4, and acetylated H3 and H4 in transcribed genes (42). In telomerase-positive cancer cells, however, we found that these modified histones were originally at low levels and did not significantly change under MCAF1 or Sp1 knockdown. Nevertheless, acetylated H3 alone tended to decline in Sp1-depleted cells, probably due to histone acetyltransferases involved in Sp1-mediated gene activation (43). Several studies have re-
ported that promoter methylation is also one of the important mechanisms in the TERT regulation in cancer cells (32, 44). CpG methylation of the TERT promoter region is necessary for transcription by blocking access of repressor CTCF to the binding site within the first exon of the gene. In addition, partially unmethylated CpG sites just upstream of the transcription start site of the TERT gene permit transcription (31, 32). Consistent with these studies, our results showed that depletion of MCAF1 or Sp1 reduced TERT transcription, together with alterations in CpG methylation status, which suggests that the MCAF1-Sp1 complex affects DNA methylation of the TERT gene. Our findings that depletion of MCAF1 or Sp1 reduced telomerase activity and inhibited cell proliferation suggest the active involvement of MCAF1 in both telomerase and proliferative activities via cooperating with Sp1.

MCAF1 Has a Transcriptional Mediator-like Function Coupled with Sp1—Our previous studies have shown that the MBD1-MCAF1-SETDB1 complex plays an important role in transcriptionally repressive chromatin formation (17, 19). In contrast, we showed here that MCAF1 facilitated transcriptional activation by Sp1, via interacting with RNA polymerase II and general transcriptional factors. MCAF1 may function as a positive and negative modulator, depending on the interacting partners and on DNA context. Promoter regions of RNA polymerase II-transcribed genes often possess discrete CpG islands, usually containing several GC-box sequences that are targeted by Sp1 (27, 45). The transcriptional mediators control gene expression through direct interactions with RNA polymerase II, activators with DNA-binding ability, and the general transcriptional factors loaded onto the core promoter (24, 46). Our results indicated that MCAF1 interacted with Sp1, RNA polymerase II, and general transcriptional factors, through its two evolutionarily conserved domains. The MCAF1-Sp1 complex recruits the general transcriptional apparatus to the TERT gene, which results in subsequent production of telomerase activity. Thus, MCAF1 facilitates transcription by scaffolding functionally distinct transcriptional regulatory molecules to the TERT gene. This suggests that MCAF1 has a transcriptional mediator-like function to stably couple Sp1 to the general transcription apparatus.

The MBD1-MCAF1-SETDB1 complex is recruited by CpG methylation for transcriptional repression (17, 19), whereas the CpG island-associated promoter is activated by the MCAF1-Sp1 complex together with the general transcriptional apparatus. In HeLa cells, the CpG-methylated p53BP2 tumor suppressor gene is inactivated by the involvement of the MBD1-MCAF1-SETDB1 complex (19), whereas the telomerase subunit genes are transactivated by the MCAF1-Sp1 complex coupled with general transcriptional machinery. These findings suggest that both roles of MCAF1 synergistically contribute to cancer phenotype. Furthermore, the association between MCAF1 and MBD1 is enhanced by SUMO modification of MBD1 (47). Some modifications such as phosphorylation, acetylation, and SUMOylation of Sp1 may induce the recruitment of MCAF1, and certain modifications of MCAF1 itself may determine the interacting partners. The results of the present study shed light on the mediator-like function of MCAF1, which contributes to maintaining cancer-associated telomerase activity in an Sp1-dependent manner. Overexpression of MCAF1 may enable Sp1 to enhance the telomerase subunit genes as a common feature in naturally occurring human cancer.

Overexpression of MCAF1 in Human Cancer—We verified that MCAF1 is overexpressed in cancer that originates from many different tissues, but not in non-cancerous tissues, which suggests a role for MCAF1 in oncogenesis. MCAF1 interacts with Sp1, which regulates expression of many genes involved in cell growth and cell cycle progression, including vascular endothelial growth factor, transforming growth factor-β and its receptor, E2F1 and cyclin D1, and telomerase components (16, 48). Our data further demonstrated that up-regulation of MCAF1 participates in transcription activation of the telomerase subunit genes by Sp1. In addition, cancer progression requires resistance to apoptosis and stimulation of angiogenesis (49). Because the promoters of many pro-apoptotic, anti-apoptotic, and angiogenesis genes have been found to contain Sp1-binding sites (50), overexpressed MCAF1 may also affect these genes, which therefore accelerates cancer development. Currently, the mechanism of MCAF1 overexpression in tumor cells is unknown, but it may be one of the common events in human cancer that are required for maintaining proliferative phenotypes.

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