The up-regulated expression and telomerase activity of human telomerase reverse transcriptase (hTERT) are hallmarks of tumorigenesis. The hTERT promoter has been shown to promote hTERT gene expression selectively in tumor cells but not in normal cells. However, little is known about how tumor cells differentially activate hTERT transcription and induce telomerase activity. In this study, we identified activating enhancer-binding protein-2β (AP-2β) as a novel transcription factor that specifically binds to and activates the hTERT promoter in human lung cancer cells. AP-2β was detected in hTERT promoter DNA-protein complexes formed in nuclear extracts prepared only from lung cancer cells but not from normal cells. We verified the tumor-specific binding activity of AP-2β for the hTERT promoter in vitro and in vivo and detected high expression levels of AP-2β in lung cancer cells. We found that ectopic expression of AP-2β reactivated hTERT promoter-driven reporter green fluorescent protein (GFP) gene and endogenous hTERT gene expression in normal cells, enhanced GFP gene expression in lung cancer cells, and prolonged the life span of primary lung bronchial epithelial cells. Furthermore, we found that inhibition of endogenous AP-2β expression by AP-2β gene-specific small interfering RNAs effectively attenuated hTERT promoter-driven GFP expression, suppressed telomerase activity, accelerated telomere shortening, and inhibited tumor cell growth by induction of apoptosis in lung cancer cells. Our results demonstrate the tumor-specific activation of the hTERT promoter by AP-2β and imply the potential of AP-2β as a novel tumor marker or a cancer therapeutic target.
AP-2β, which was detected in the hTERT promoter DNA-protein complexes in nuclear extracts prepared only from lung cancer cells but not from normal cells.

AP-2β is a member of the AP-2 transcription factor family, the members of which bind GC-rich consensus sequences and regulate the expression of many downstream genes. The AP-2 family consists of five different isoforms known as AP-2α, AP-2β, AP-2γ, AP-2δ, and AP-2ε. They are encoded by separate genes and have different biologic functions (22–25). The expression of AP-2 is tissue- and cell-specific. AP-2α and AP-2βγ have been shown to be capable of controlling the expression of many cancer-related genes such as HER-2 (26), p21 (27), c-kit (28), bcl-2 (29), vascular endothelial growth factor (30), MUC18 (31), and p53 (32). Recent studies have also shown that AP-2α and AP-2γ have tumor-suppressive activity in breast cancer, melanoma, and prostate cancer cells (30, 32–38).

Decreased expression of AP-2α or AP-2γ has been found in these tumor cells and is associated with disease progression and the metastatic capabilities of the tumors. Restoration of AP-2α or AP-2γ expression reduces tumorigenesis and inhibits tumor cell growth. However, so far, the role of AP-2β in regulating cancer-related gene expression is largely unknown. Here we show that AP-2β specifically bound to the hTERT promoter and activated hTERT promoter-driven gene expression in human cancer cells. In vitro and in vivo DNA-protein binding assays revealed the tumor cell–selective binding of AP-2β to the hTERT promoter. Reverse transcription (RT)-PCR and immunoblot analyses also showed high expression of AP-2β at the mRNA and protein levels in various lung cancer cells. Moreover, we found that exogenous overexpression of AP-2β considerably enhanced hTERT promoter-driven green fluorescent protein (GFP) gene expression in human lung cancer cells and reactivated GFP gene expression in normal lung cells cotransfected with AP-2β- and hTERT-GFP-expressing plasmids.

However, treatment with AP-2β-specific small interfering RNA (siRNA) largely attenuated hTERT promoter-mediated gene expression in lung cancer cells. Furthermore, we found that the AP-2β siRNA could greatly inhibit telomerase activity and tumor cell growth in human lung cancer cells. Because the expression of hTERT is closely related to tumorigenesis and tightly regulated at the transcriptional level, our identification of AP-2β as a tumor-specific hTERT promoter activator suggests that the AP-2β protein might serve as a biomarker for diagnosing cancer or as a cancer therapeutic target by inhibiting hTERT activity and tumor cell growth.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Human non-small cell lung cancer (NSCLC) cell lines (H1299, A549, and H322) and an immortalized human embryonic kidney cell line (293) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 5% glutamine. Telomerase-transformed and immortalized normal human bronchial epithelial (HBE) and primary HBE (PHBE) cells (Clonetics, Walkersville, MD) were cultured in the medium supplied by the manufacturer according to the instructions provided. Normal human lung fibroblast WI-38 and VA13 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. All cells were incubated in a humidified incubator supplied with 5% carbon dioxide.

**Plasmid Vectors**—Recombinant plasmid vectors expressing wild-type AP-2β (driven by a cytomegalovirus (CMV) promoter) and a GFP reporter (driven by a CMV or an hTERT promoter) were used in the transfection experiments. The plasmid vector LacZ, which contains a β-galactosidase gene, was used as a nonspecific negative control. These recombinant plasmid vectors were constructed at our laboratory.

**DNA-Protein Binding Assay**—DNA-protein binding assays were performed using a streptavidin-agarose bead pulldown assay as described previously (39, 40). Briefly, 80–90% confluent cells were harvested, and nuclear extracts were prepared. The biotin-labeled double-stranded oligonucleotide probes were synthesized by Sigma based on hTERT promoter sequence −378 to +60 or the AP-2 consensus sequence (sense, 5′-GATCGAAGTGGGCCCGAGCGG-3′). A non-relevant biotinylated sequence (5′-AGAAGTCGACTAACCCCTCTG-3′) was included as a control. The binding assay was performed by mixing 1 mg of the nuclear proteins, 10 μg of the biotin-labeled DNA oligonucleotides, and 100 μl of streptavidin-agarose beads (Sigma). The mixture was incubated at room temperature for 2 h with shaking. The beads were then pelleted by centrifugation at 500 × g in a microcentrifuge for 1 min and washed three times with cold phosphate-buffered saline (PBS). The bound proteins were eluted for further analysis.

**Identification of hTERT Promoter-binding Proteins**—The hTERT promoter-binding proteins were separated by 4–15% SDS-PAGE and visualized by silver staining. The protein bands of interest were cut and digested with trypsin on a gel or chip. The digested samples were subjected to peptide mapping and N-terminal amino acid sequencing using surface-enhanced laser desorption ionization interfaced with new high-end quadrupole time-of-flight tandem mass spectrometry (MS/MS) (Ciphergen Biosystems, Inc., Fremont, CA) and other microprotein sequencing methods. The identities of the proteins were searched and verified via available databases and software such as TagIdent (ca.expasy.org) for calculated protein PI and mass, ProFound (prowl.rockefeller.edu/) for peptide mass, and Biomarker Wizard (Ciphergen Biosystems, Inc.) for MS-based peptide mapping.

**Chromatin Immunoprecipitation (ChIP)**—The ChIP assay was done as described previously (39, 40). Briefly, 1% formaldehyde was added to the culture medium; after incubation for 20 min at 37 °C, the cells were washed twice with PBS, scraped, and lysed for 10 min at 4 °C in lysis buffer (10 mM Tris–HCl (pH 8.0), 1% SDS, 1 mM phenylmethylsulfonyl fluoride, pepstatin A, and aprotinin). Lysates were sonicated three times for 10 s each, and the debris was removed by centrifugation. One-third of the lysate was used as the DNA input control. The remaining two-thirds of the lysate was diluted 10-fold with dilution buffer (10 mM Tris–HCl (pH 8.0), 50 mM NaCl, 0.01% SDS, 1 Triton X-100, and 1 mM EDTA), followed by incubation overnight at 4 °C with anti-AP-2β antibody or non-immune rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoprecipitated complexes were collected using protein A/G-agarose beads (Santa Cruz Biotechnology, Inc.). The precipitates were
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extensively washed and incubated in elution buffer (1% SDS and 0.1 M NaHCO₃) at room temperature for 20 min. Cross-linking of DNA-protein complexes was reversed at 65 °C for 5 h, followed by treatment with 100 μg/ml protease K for 3 h at 50 °C. The DNA was extracted three times with phenol/chloroform and precipitated with ethanol. Pellets were resuspended in Tris/EDTA buffer and subjected to PCR amplification using specific hTERT promoter primers (5′-primer, −378-TGGCCCTCCCTCCGGTTAC−359, and 3′-primer, +60-CCAGGGCTTCCCACGTGCGC+441). The resulting product of 438 bp for the hTERT promoter was separated by 2% agarose gel electrophoresis.

Transient Transfection—The cells were transfected with N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammoniummethyl sulfate/cholesterol-encapsulated plasmid DNA nanoparticles. In brief, 2 μl of N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammoniummethyl sulfate/cholesterol and 4 μg of plasmid DNA were mixed, and the mixture was slowly added to each well in a 6-well plate and incubated for 72 h. The GFP-expressing plasmid vector was used as a transfection control to assess the transfection efficiency. The transfection efficiency was in the range of 40–60% in these cell lines.

Western Blot Analysis—Western blots were probed with a specific antibody to AP-2, general transcription factor IIB, or β-actin (Santa Cruz Biotechnology, Inc.). The protein bands were detected by enhanced chemiluminescence.

RT-PCR—Total cellular RNA was isolated using TriReagent RNA isolation reagent (Invitrogen) according to the manufacturer’s instructions. Aliquots (1 μg) of total RNA were used to produce cDNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and oligo(dT)₁₅ primer (Roche Applied Science) in a final volume of 20 μl. The cDNA products (1 μl) obtained were used for the PCR amplification of AP-2β. The sense and antisense primers used for AP-2β were as follows −378-TGGCCCTCCCTCCGGTTAC−359 (5′-primer) and +60-CCAGGGCTTCCCACGTGCGC+441 (3′-primer), respectively. The 438-bp DNA fragment amplified by PCR was obtained. The PCR conditions were 95 °C for 5 min; 30 amplification cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min; with a final extension at 72 °C for 5 min. The PCR products from each sample were subjected to electrophoresis on 2% agarose gels, stained with ethidium bromide, and photographed.

Cell Viability and Apoptosis Assay—Cell growth inhibition and apoptosis analysis was performed as described previously (41). Cell viability was analyzed by counting viable cells using a trypan blue exclusion assay. The percentage of viable cells was calculated in terms of the number of treated cells relative to the number of control cells. Apoptosis was measured by flow cytometry using a terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL)-based fluorescence-activated cell sorting (FACS) assay. The relative number of apoptotic cells was calculated in terms of the TUNEL-positive values in cells. Experiments were repeated three times with quadruplicate samples for each treatment in each experiment.

siRNAs—The siRNAs expressing vector were prepared with a transcription-based method using a Silencer siRNA construction kit (Ambion, Inc., Austin, TX) according to the manufacturer’s instructions. Cells were transfected with siRNA duplexes using Oligofectamine reagent (Invitrogen). The sequences of 21-nucleotide sense and antisense RNAs were as follows: siAP-2β-1, 5′-GGUGUUCACUAGCUAGGGTT-3′ (sense) and 5′-CCCUCAGAGCACUGGT-3′ (antisense) for the AP-2β gene (−998 to −978); siAP-2β-2, 5′-GGUGUUCACUAGCUAGGGTT-3′ (sense) and 5′-CCCUCAGAGCACUGGT-3′ (antisense); and nonspecific control siRNA (siNSC), 5′-AAUGUUCGAACUAGUGUUU-3′ (sense) and 5′-AAAAACACUAGAUUCGAACCA-3′ (antisense) (as a non-specific control scrambled siRNA). These siRNAs were prepared with a transcription-based method using the Silencer siRNA construction kit according to manufacturer’s instructions. Cells were transfected with siRNA duplexes using Oligofectamine reagent.

Electrophoretic Mobility Shift Assay (EMSA)—The AP-2 consensus oligonucleotides (sense, 5′-GATCGAACCTGACGGCCGCGGCGGT-3′; and antisense, 5′-CAGGCGCAGGCTGGTGCGCTG-3′) were obtained from Promega Corp. (Madison, WI). The hTERT promoter oligonucleotides with an AP-2-binding site (−43 to −18) were synthesized by Sigma. The probes were end-labeled with [γ-32P]ATP using T4 kinase (Promega Corp.). EMSA was performed by incubating 10 μg of nuclear extract with a labeled probe (10,000 cpm, −10 fmol) in binding buffer containing 15 mM Tris-HCl (pH 7.5) 100 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 12% glycerol, 5 μg of bovine serum albumin, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 1.5 μg of poly(dI-dC). To assess the specificity of DNA-protein binding, up to a 50-fold molar excess of unlabeled wild-type or mutant oligonucleotide was added. The mixture was applied to 4% polyacrylamide gel and subjected to electrophoresis at 300 V for 45 min, and the complex was detected by autoradiography.

Telomerase Activity Assays—Telomerase activity was analyzed by a highly sensitive qualitative photometric enzyme immunoassay utilizing the telomeric repeat amplification protocol with a TeloTAGGG telomerase PCR enzyme-linked immunosorbent assay kit (Roche Applied Science) according to the manufacturer’s instructions.

Telomere Length Assay—Telomere length was measured by a highly sensitive nonradioactive chemiluminescence assay utilizing Southern blot analysis of terminal restriction fragments obtained by digestion of genomic DNA with frequently cutting restriction enzymes (Hinfl/Rsal) with a TeloTAGGG telomere PCR length assay kit (Roche Applied Science) according to the manufacturer’s instructions. Telomeric smears were revealed by exposure on x-ray film.

Statistical Analyses—All experiments were performed at least three times with triplicate samples. Analysis of variance and Student’s t test were used to compare the values of the test and control samples in vitro and in vivo. p < 0.05 was considered statistically significant. StatView 5.0 (Abacus Concepts, Inc., Berkeley, CA) and SAS software were used for all statistical analyses. The significance was evaluated by the paired t test.

RESULTS

Detection and Identification of Tumor-specific hTERT Promoter-binding Proteins—The streptavidin–agarose bead pull-down assay is a useful and feasible approach for detecting DNA-
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binding proteins such as transcription activators and coactivators (42). In this study, we also used this technique to discover and detect the tumor-specific cellular protein factors that specifically bind to the hTERT promoter. We designed and synthesized a 438-bp biotin-labeled double-stranded oligonucleotide corresponding to the 5′-flanking sequence of the hTERT gene from −378 to +60 as a DNA probe (Fig. 1A) to assess the binding of cellular protein factors to the hTERT promoter region. Nuclear extracts prepared from human lung cancer cells (H1299, A549, and H322) and normal lung cell lines (HBE, WI-38, and VA13) were incubated with the biotin-labeled hTERT oligonucleotide probes and streptavidin-agarose beads. Nuclear proteins from the immortalized cell line 293 was used as a positive control. The DNA-protein complexes were separated by SDS-PAGE, and protein bands were visualized by silver staining. The protein of interest was dissected from the gel and digested with trypsin. The peptide digests were loaded onto an H50 ProteinChip array and analyzed by surface-enhanced laser desorption ionization MS. The hTERT promoter-binding protein was identified by peptide mapping using the ProFound program. A, the schematic illustration of the prototypic probe used in this study shows a biotin-labeled double-stranded DNA corresponding to the hTERT promoter sequence −378 to +60. Key regulatory elements are shown. B, the SDS-PAGE image shows hTERT promoter-binding proteins. The arrow indicates the tumor cell-selective hTERT promoter-binding protein.

FIGURE 1. Detection and identification of tumor-specific hTERT promoter-binding proteins. Nuclear proteins prepared from lung cancer and normal lung cells were incubated with biotin-labeled hTERT oligonucleotide probes and streptavidin-agarose beads. Nuclear protein from the immortalized cell line 293 was used as a positive control. The DNA-protein complexes were separated by SDS-PAGE, and protein bands were visualized by silver staining. The protein of interest was dissected from the gel and digested with trypsin. The peptide digests were loaded onto an H50 ProteinChip array and analyzed by surface-enhanced laser desorption ionization MS. The hTERT promoter-binding protein was identified by peptide mapping using the ProFound program. A, the schematic illustration of the prototypic probe used in this study shows a biotin-labeled double-stranded DNA corresponding to the hTERT promoter sequence −378 to +60. Key regulatory elements are shown. B, the SDS-PAGE image shows hTERT promoter-binding proteins. The arrow indicates the tumor cell-selective hTERT promoter-binding protein.

To determine whether the tumor-specific binding of AP-2β to the hTERT promoter observed in vitro could be reproduced under a physiological condition, we also analyzed the binding of AP-2β to the chromatin hTERT promoter in living cells by a ChIP assay using a specific antibody against AP-2β. Normal IgG and AP-2α were used as negative and nonspecific positive controls, respectively. As expected, we detected a high degree of binding of AP-2β to the AP-2 consensus probe was detected in lung cancer cells but not in normal cells (Fig. 2A). Thus, these results show the specificity of AP-2β binding to the hTERT promoter in human lung cancer cells. Because a computer-aided transcription factor-binding site analysis revealed a consensus AP-2-binding site in the hTERT promoter, we also detected the binding of AP-2β to a specific AP-2 consensus sequence probe. Consistent with the results observed above, a high level of binding of AP-2β to the AP-2 consensus probe was detected in lung cancer cells but not in normal cells (Fig. 2B), supporting the finding that AP-2β binding is tumor-specific.

Because EMSA is a conventional approach for detecting binding between transcription factors and promoter DNA, we
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**A.** Normal and lung cancer cells expressing AP-2β detected in lung cancer and normal cells. However, the protein-detection of AP-2β was also evaluated using anti-AP-2β antibodies, the hTERT promoter probe, and nuclear proteins from normal lung cells (WI-38 and HBE) (Fig. 2D).

The specificity of AP-2β binding to the radiolabeled hTERT promoter probe was also demonstrated by the inhibition of binding by a 50-fold molar excess of unlabeled wild-type AP-2 probe but not by a 50-fold molar excess of unlabeled mutant probe in the presence or absence of anti-AP-2β antibodies (Fig. 2D). Taken together, these results show that the transcription factor AP-2β specifically binds to the hTERT promoter in human lung cancer cells.

Endogenous Expression of AP-2 in Human Lung Cancer Cells—To determine whether the tumor-specific binding of AP-2β to the hTERT promoter is due to tumor-selectively elevated expression of AP-2β, we detected AP-2β expression at the mRNA and protein levels in human lung cancer and normal lung cells by RT-PCR and Western blot analysis, respectively. We detected the expression of AP-2β mRNA (Fig. 3A) and protein in both the cytosol (Fig. 3B) and nuclei (Fig. 3C) in all three lung cancer cell lines and the immortalized cell line 293. The expression of AP-2β at the mRNA and protein levels was also detected in the normal lung cell lines WI-38 and HBE. However, a 3–4-fold reduction in AP-2β mRNA and protein levels was observed compared with the cancer cell lines (Fig. 3A–C). These results suggest that the function of AP-2β in regulating hTERT or other cancer-related gene expression may be controlled not just by AP-2β protein abundance or expression level but mainly by its nuclear translocation or DNA binding activity.

**Activation of hTERT Promoter-driven Gene Expression by AP-2β Overexpression—**To understand the specific function of AP-2β in regulation of hTERT promoter-mediated gene expression, we cotransfected normal lung cells (WI-38 and HBE) and lung cancer cells (H1299 and H322) with plasmids expressing AP-2β driven by a CMV promoter and a GFP reporter driven by the hTERT or CMV promoter. LacZ was used as a transfection control. The expression of the GFP gene was detected by fluorescence microscopy, and the GFP-positive cell population and intensity of the sorted GFP cells were analyzed by a FACS assay. Exogenous expression of AP-2β reactivated hTERT promoter-mediated GFP expression in normal cells and greatly enhanced GFP expression in lung cancer cells (Fig. 4A), resulting in a considerable increase in both the GFP-positive cell population (Fig. 4B) and the fluorescence intensity of the GFP protein (Fig. 4C) in cells cotransfected with AP-2β and hTERT-GFP plasmids compared with those in cells cotransfected with LacZ and hTERT-GFP plasmids. In contrast, overexpression of AP-2β did not alter CMV promoter-driven GFP expression in normal lung and lung cancer cells cotransfected with AP-2β and CMV-GFP plasmids (Fig. 4A, A–C). To confirm the role of AP-2β in regulating hTERT promoter activity, we further evaluated the effect of ectopic expression of AP-2β on endogenous hTERT expression and hTERT activity in normal WI-38 and HBE cells. We found that ectopic expression of AP-2β up-regulated the expression of the endogenous hTERT gene in AP-2β-transfected WI-38 and HBE cells compared with LacZ-transfected control cells as indicated by a semiquantitative RT-PCR assay (Fig. 4D). Ectopic expression of
AP-2 also markedly activated hTERT as indicated by a 40–50% increase in telomerase activity in AP-2/H9252-transfected WI-38 and HBE cells compared with LacZ-transfected control cells (Fig. 4E), suggesting that AP-2/H9252 specifically regulates hTERT promoter activity and thus activates hTERT promoter-driven gene expression.

Inhibition of hTERT Promoter-driven Gene Expression by AP-2β siRNA—To further confirm the role of AP-2β in activation of hTERT promoter-mediated gene expression, we blocked the expression of endogenous AP-2β using siRNA technology in human lung cancer cells and analyzed the resultant effects on hTERT promoter-mediated gene expression. Transfection of lung cancer cells (H1299) with an AP-2β-specific siRNA (referred to as siAP-2β-1 or siAP-2β-2) expressing plasmid dramatically inhibited the expression of the AP-2β protein (Fig. 5A) and blocked the binding of AP-2β to a biotinylated hTERT promoter probe in vitro (Fig. 5B) and to the hTERT promoter in the chromatin structure in vivo (Fig. 5C) compared with transfection with siNSC. Inhibition of AP-2β expression by siAP-2β-1 or siAP-2β-2 attenuated hTERT promoter-driven GFP expression in H1299 and H322 cells cotransfected with hTERT promoter-driven GFP and siAP-2β-1- or siAP-2β-2-expressing plasmids (Fig. 5D). Moreover, FACS
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A.  

B.  

C.  

D.  

E.  

F.  

FIGURE 5. Effect of AP-2β siRNA on hTERT promoter-driven gene expression. Human lung cancer cells (H1299 and H322) were cotransfected with AP-2β-specific siRNA (siAP-2β-1 or siAP-2β-2) and hTERT promoter-driven GFP-expressing plasmids. PBS and siNSC were used as negative and transfection controls, respectively. At 72 h after transfection, the expression of nuclear AP-2β in H1299 cells (A) was detected by Western blotting. General transcription factor IIb (TFIIb) was used as a loading control. The binding of AP-2β to a biotinylated hTERT promoter probe (B) or to the chromatin hTERT promoter in living cells (C) was analyzed in H1299 cells by streptavidin-agarose pull-down and ChIP assays, respectively. The expression of GFP was detected by fluorescence microscopy (magnification ×40) (D). The percentage of the GFP-positive cell population (E) and the fluorescence intensity of the GFP protein derived from 5000 cells (F) were measured by FACS analysis. siNSC was used as a control. At least three separate experiments done in duplicate were performed. The error bars represent S.E. from three separate experiments. NSP, nonspecific oligonucleotide probe; Abs, antibodies; Ctrl, control.

analysis showed a dramatic reduction in the GFP-positive cell population (Fig. 5E) and in the fluorescence intensity of the GFP protein (Fig. 5F) by siAP-2β-1 or siAP-2β-2 compared with PBS or siNSC. However, transfection with AP-2β siRNA did not affect CMV promoter-driven GFP expression in lung cancer cells (data not shown). These results confirmed that AP-2β is essential for hTERT promoter-driven gene expression and suggest that AP-2β transcriptionally up-regulates hTERT promoter-driven gene expression by activating hTERT promoter activity in human lung cancer cells.

Telomerase Activity Inhibition and Tumor Suppression by AP-2β siRNA—Telomerase is a novel target for potential anticancer therapy, so we also explored the biologic effects of the AP-2β-specific siRNA on telomerase activity and antitumor activity. Transfection with the siRNA of AP-2β (siAP-2β-1 or siAP-2β-2) dramatically suppressed telomerase activity in the lung cancer cell lines H322 and H1299 but had no effect on the normal lung fibroblast WI-38 cells compared with transfection with siNSC (Fig. 6A). The normal HBE cells were immortalized by transformation with a CMV promoter-driven hTERT-expressing plasmid vector and showed a relatively high hTERT enzyme activity, which was not affected by transfection with AP-2β siRNAs, suggesting the specificity of interaction of AP-2β with the hTERT promoter. It was interesting that we also found that the AP-2β siRNA showed strong tumor-suppressive activity: both siAP-2β-1 and siAP-2β-2 inhibited cell growth (Fig. 6B) and induced apoptosis (Fig. 6C) in lung cancer H1299 and H322 cells but not in normal lung HBE and WI-38 cells.

To further verify the involvement of hTERT expression and activity in AP-2β siRNA-mediated tumor suppression, we constructed a ponasterone A-inducible hTERT-expressing plasmid and used it to establish an H1299 subline stably expressing hTERT. We determined the effect of AP-2β siRNAs on telomerase activity and tumor suppression in the absence and presence of induced ectopic expression of hTERT in hTERT-expressing stable H1299 cells. The PBS- and siNSC-treated cells were used as negative controls. Consistent with the results observed in the parental H1299 cells, a considerable inhibition of both telomerase activity (Fig. 7A) and cell viability (Fig. 7B) and elevated induction of apoptosis (Fig. 7C) were detected in the hTERT-expressing stable H1299 cells treated with AP-2β siRNAs in the absence of the ponasterone A inducer. However, the AP-2β siRNA-induced telomerase activity inhibition (Fig. 7A) and tumor suppression (Fig. 7B and C) clearly appeared to be partially rescued by the induced ectopic expression of AP-2β in these siRNA-treated stable H1299 cells in the presence of 10 μM ponasterone A. These results suggest that one of the pathways in the AP-2β siRNA-induced inhibition of tumor cell growth and apoptosis is mediated by a specific inhibition of hTERT activity.

Accelerated Telomere Shortening of NSCLC Cells by AP-2β Inhibition and Prolonged Life Span of Normal Cells by AP-2β Activation—Telomeres play an essential role in the stable maintenance of eukaryotic chromosomes within a cell by specifically binding to structural proteins. Maintaining stable telomere length is associated with activation of hTERT. To better understand the biologic role of AP-2β in regulating activation of hTERT, we determined the effects of inhibition of AP-2β expression by AP-2β-specific siRNAs on telomere length in NSCLC cells and of ectopic expression of AP-2β on...
cell expansion and transformation in normal PHBE cells. We continuously treated NSCLC H1299 cells with AP-2β siRNAs or transfected PHBE cells with AP-2β-expressing plasmid vectors over 28 days through seven cell passages and then determined the effect of AP-2β on telomere length in viable cells and on cell life span in PHBE cells, respectively. Long-term treatment of H1299 cells with AP-2β siRNA (siAP-2β-1) effectively inhibited AP-2β expression (Fig. 8A) and telomerase activity (Fig. 8B) and induced telomere shortening (Fig. 8C) compared with treatment with PBS or siNSC. In contrast, normal PHBE cells transfected with AP-2β-expressing plasmids (at the third passage) exhibited a
have demonstrated that the streptavidin-agarose pulldown assay is a useful approach for discovering and detecting tumor-specific individual protein factors in a large protein complex that binds to the hTERT promoter region. Using this DNA-protein binding assay, we clearly detected an array of proteins that interact with the biotinylated hTERT promoter probe in a tumor cell-selective manner. Evidence for detection of the tumor-specific binding of AP-2β to the hTERT promoter includes the following. 1) The binding of other AP-2 isoforms such as AP-2α was not different between lung cancer cell lines and normal lung cells; 2) the binding of the protein complex was undetectable when the AP-2 sites in the hTERT promoter were mutated or a non-relevant probe was used; and 3) neither non-immune IgG nor anti-von Willebrand factor antibody detected any protein that was pulled down by the streptavidin-agarose beads. Although EMSA is conventionally used to qualitatively assess DNA-protein complex formation, this technique is inadequate for identifying the individual proteins in a large complex because the DNA-protein complex is too large to be resolved by gel electrophoresis (40). The streptavidin-agarose pulldown assay has recently been used successfully to analyze the specific binding of transactivators and coactivators to the cancer-related genes COX-2 and NOS-2 (39, 40, 43). The method allows the rapid, sensitive, and scalable detection of relative binding activities and specificities directly from cellular extracts. This binding assay also offers other advantages over conventional EMSA. For example, it does not require the use of 32P or other radioisotopes. In this study, we also showed the feasibility of using the streptavidin-based DNA-protein binding assay for detecting individual proteins that bind to the biotinylated hTERT promoter DNA probe. Because the expression of cancer-related genes is regulated by a large complex of proteins that bind to the promoter/enhancer region of a gene, our results suggest that this streptavidin-agarose pulldown assay will be valuable for discovering and detecting tumor-specific cellular factors in the complex comprising transactivators, coactivators, mediators, and general transcription factors.

In this study, we used the hTERT promoter as a model because high expression of hTERT is an important hallmark of tumorigenesis. Recently, hTERT was proposed as a therapeutic target for cancer because of its nearly universal expression in human tumors and its critical functional role in oncogenesis (44). Consequently, it is rather reasonable to hypothesize that the tumor-specific activation of the hTERT promoter may be regulated by various cellular factors such as transcription factors and effectors, which are differentially activated in tumor cells or repressed in normal cells. These tumor-specific cellular factors can either specifically bind to the hTERT promoter or interact with its effectors to differentially regulate hTERT transcription. A large class of cancer-linked genes can be assigned to the DNA-binding proteins, the function of which is controlled not just by their abundance or expression level, but mainly at the level of their activity in terms of their interactions with DNA and protein targets. Because the AP-2β protein was selectively detected in DNA-binding protein complexes from lung cancer cells but not from normal cells, we postulated that the up-regulated hTERT promoter activity in those tumor cells was due to an increase in the binding activity of AP-2β or to the
increased import of AP-2β into the nucleus. The results from our in vitro and in vivo experiments confirmed this notion and illustrate the high specificity of AP-2β in tumor-specific telomerase activation, although the exact molecular mechanism controlling this import process is unknown. Moreover, because we did not detect an obvious difference in the binding of AP-2α (another isoform of the AP-2 family) between lung cancer and normal lung cells, it seems unlikely that the AP-2α isoform plays a key role in regulating hTERT gene expression in lung cancer development and progression. Taken together, our results indicate the specificity of AP-2β binding to the hTERT promoter in lung cancer cells and imply that AP-2β might be a potential biomarker for the diagnosis of lung cancer.

Furthermore, our findings shed light on the biologic roles of AP-2β in regulating activation of the hTERT promoter. The results from our transfection experiments indicate that exogenous expression of AP-2β not only greatly enhances the activity of the hTERT promoter in tumor cells but also reactivates the hTERT promoter in normal cells. Overexpression of AP-2β elevated the expression of the hTERT promoter-driven GFP reporter gene without altering the expression of the CMV promoter-driven GFP gene. We also found that ectopic expression of AP-2β in primary normal bronchial epithelial cells increased endogenous telomerase activity and prolonged cell proliferation and survival, although no measurable loss of telomeres was detected in untransfected and LacZ-transfected cell controls. The data from our siRNA experiments in lung cancer cells further confirmed the role of AP-2β in selectively regulating telomerase activity and altering telomere length in tumor cells. In view of the broad involvement of AP-2β in the transcriptional activation of the hTERT promoter, AP-2β is likely to control a large number of cancer-linked genes, notably oncogenic genes. The AP-2 family members AP-2α and AP-2γ have been implicated in the progression, vascularization, metastasis, and/or recurrence of tumors, and they constitute good prognostic factors for melanoma and breast and prostate cancers (34–38). Our study has revealed that the effect of AP-2β on the tumor cell-selective activation of the hTERT promoter is not simply a question of expression level but rather is defined by tumor-selective activation of the hTERT promoter. Because telomerase enzyme is a novel target for potential anticancer therapy and stem cell expansion, we explored the biologic effects of AP-2β siRNAs by evaluating their effects on telomerase activity and tumor suppression. An important finding of this study is that we observed that the siRNA of AP-2β dramatically inhibited telomerase activity in lung cancer cells, largely suppressed tumor cell growth, and induced apoptosis, suggesting that it is a potential therapeutic drug for cancer treatment. The observed immediate effects of AP-2β siRNAs on tumor growth inhibition and apoptosis induction may be partially interpreted by the fact that AP-2β is also a general transcription activator that controls or regulates multiple biologically important gene activities and that knockdown of AP-2β expression by siRNA may therefore block other cell proliferation-associated genes under the influence of AP-2β in addition to activation of the hTERT promoter. Additional studies are needed to elucidate the role of AP-2β siRNA in tumor suppression in association with other genes and other types of human cancers.

In summary, we discovered and identified the transcription factor AP-2β as a tumor-specific hTERT promoter-binding protein in human lung cancer cells. Our results demonstrate that AP-2β specifically activates hTERT promoter activity and up-regulates hTERT promoter-driven gene expression. Moreover, AP-2β siRNA suppresses telomerase activity and tumor cell growth in lung cancer cells. Because activation of telomerase is tightly regulated at the transcriptional level, these data demonstrate that AP-2β could serve as a molecular marker for tumor detection or as a therapeutic target for anticancer therapy.

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