ETS variant transcription factor 5 and c-Myc cooperate in derepressing the human telomerase gene promoter via composite ETS/E-box motifs

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The human telomerase gene (hTERT) is repressed in most somatic cells. How transcription factors activate the hTERT promoter in its repressive chromatin environment is unknown. Here, we report that the ETS family protein ETS variant transcription factor 5 (ETV5) mediates epidermal growth factor (EGF)-induced hTERT expression in MCF10A cells. This activation required MYC proto-oncogene bHLH transcription factor (c-Myc) and depended on the chromatin state of the hTERT promoter. Using chromatinized bacterial artificial chromosome (BAC) reporters in human fibroblasts, we found that ETV5 and c-Myc/MYC-associated factor X (MAX) synergistically activate the hTERT promoter via two identical, but inverted, composite Ets/E-box motifs enclosing the core promoter. Mutations of Ets or E-box sites in either DNA motif abolished the activation and reduced or eliminated the synergism. ETV5 and c-Myc facilitated each other’s binding to the hTERT promoter. ETV5 bound to the hTERT promoter in both telomerase-negative and -positive cells, but it activated the repressed hTERT promoter and altered histone modifications only in telomerase-negative cells. The synergistic ETV5/c-Myc activation disappeared when hTERT promoter repression became relieved because of the loss of distal regulatory elements in chimeric human/mouse BAC reporters. Our results suggest that the binding of c-Myc and ETS family proteins to the Ets/E-box motifs derepresses the hTERT promoter by inducing an active promoter configuration, providing a mechanistic insight into hTERT activation during tumorigenesis.

Telomeres are protective caps of chromosomes that limit human cell proliferation during replicative aging. In pluripotent stem cells and early embryonic tissues, telomere repeats are replenished by telomerase, a nucleoprotein complex including an RNA template TERC and a catalytic telomerase reverse transcriptase TERT (1). The hTERT gene is, however, repressed in most adult tissues, resulting in low or undetectable telomerase activities in those tissues. Consequently, most somatic cells undergo progressive telomere shortening upon successful cell divisions, leading to cellular senescence. hTERT activation, as it occurs in the majority of cancers, results in telomerase activation and cellular immortalization.

Regulation of the hTERT gene involves promoter-binding transcription factors (TFs) and its chromatin context that controls promoter accessibility. Our previous studies revealed that the hTERT gene was embedded in a nuclease-resistant chromatin domain and repressed by multiple HDAC1-containing corepressor complexes in human somatic cells (2–4). The hTERT locus contained abundant repressive histone marks in human somatic cells (5). This chromatin conformation also repressed several other strong promoters, as the mTert and HSV-TK promoters became silenced in the genomic context of hTERT gene in chromatinized chimeric BAC reporters (6, 7). These data indicated that the chromatin environment plays a central role in tight repression of the hTERT gene in human somatic cells.

Whereas hTERT transcription requires a permissive chromatin context, it was also regulated by multiple TFs, such as Sp1, c-Myc, and E26 transformation-specific (ETS) family proteins, via their specific binding sites at the hTERT promoter (6, 8, 9). The five consensus GC-boxes (5'-GGGCGGGGG-3') were binding sites for Sp1 family TFs, like Sp1 and Sp3. Mutation of these sites completely eliminated hTERT promoter activity, indicating that the GC-boxes provided basic promoter function (6, 10). Other TFs, such as c-Myc, functioned to regulate the accessibility and/or chromatin environment of the hTERT promoter (9, 11, 12). Upon binding, these TFs recruited histone-modifying proteins to reprogram the promoter. These processes further increased the accessibility of the promoter, allowing the binding of additional TFs and the assembly of transcription machinery at the promoter. For example, it was shown that two canonical E-box sites (5'-CACGTG-3'), located upstream and downstream of the hTERT core promoter, respectively, played crucial roles in regulating hTERT transcription (9, 11, 12).

In addition to the E-boxes, it was also reported that ETS family TFs were involved in hTERT regulation in cancer cells. Upon induction by EGF via the MAPK pathway, ETS2 activated hTERT transcription through Ets consensus sites (5'-TTTCTTTCCG-3') at the −22 nt position upstream of the hTERT transcription start site (TSS) (13). In Ewing’s sarcomas, EWS fused with one of five ETS family members (FLI1, ERG, ETV1, E1A1, or PEA3) and up-regulated hTERT expression through the same Ets-binding sites (14). However, Xu et al. (15) showed that ETS2 maintained hTERT expression by interacting with c-Myc in breast cancer cells via a composite Ets/E-box motif at the −169 nt position. These
data indicated that ETS family proteins were critical for the regulation of hTERT promoter. However, it is worth mentioning that transiently transfected hTERT promoter reporters used in the aforementioned studies were missing a crucial downstream Ets/E-box motif at the +44 nt position.

Recently, specific mutations at the hTERT core promoter, located at positions −69 nt (C250T), −48 nt (C228T), and +21 nt relative to the TSS, were found in multiple types of cancer and correlated with higher hTERT expression in cancer cells (16–18). These cancer-specific mutations all generated new Ets-binding sites and recruited ETS factors (19, 20). Introducing any of these mutations at the endogenous hTERT promoter via genetic editing in human embryonic stem cells prevented hTERT silencing upon their differentiation (21, 22). These studies indicated that ETS family proteins played crucial roles in hTERT activation during tumorigenesis.

ETV5 (ETS variant gene 5, also known as ERM) belongs to the PEA3 subfamily of ETS proteins, which also includes ER81 (Ets-related protein 81, or ETV1) and PEA3 (polyoma enhancer activator 3, or ETV4). ETV5 is broadly expressed in tissues including brain, lung, and testis and is involved in diverse cellular processes, like neurodevelopment, cell differentiation, tumor invasion, and metastasis (23–25). In tests, ETV5 maintained the self-renewal of spermatogonial stem cells (26, 27). ETV5-null male mice were sterile due to the loss of sperm in testes (28). ETV5 was also able to bind to the C228T mutation site and activate the mutant hTERT promoter in GABP-negative thyroid cancer cells (29). Here, we report that ETV5 is a critical factor in regulating hTERT transcription in human mammary epithelial cells and fibroblasts. To study how TFs activated the hTERT promoter in a relevant genomic context, we previously developed a chromatinized reporter system, in which single-copy BACs were integrated into proviral acceptor sites in human cells by recombination-mediated BAC targeting (RMBT) (30). Using this system, we found that, whereas ETV5 bound to the hTERT promoter in both telomerase-negative (Tel−) and -positive (Tel+) human fibroblast cells, it activated the hTERT promoter only in Tel− cells. ETV5 cooperated with c-Myc to activate hTERT transcription via two identical composite Ets/E-box motifs, which surrounded the hTERT promoter symmetrically. The synergistic activation by ETV5 and c-Myc was attenuated or abolished when the Ets site or E-box in one of these motifs was mutated. ETV5 and c-Myc augmented each other’s binding to these two motifs. The activation of hTERT promoter by ETV5 was associated with histone acetylation and H3K4 methylation at the promoter. Altogether, our study indicated that ETV5 and c-Myc cooperated to relieve the hTERT promoter from repressive chromatin via two Ets/E-box motifs, supporting a model that the binding of ETS and c-Myc family proteins to the hTERT promoter led to the establishment of an active promoter configuration.

**Results**

**ETV5 mediated hTERT activation by EGF signaling**

To identify genes involved in human cell immortalization, we performed a proviral tagging experiment in pre-crisis 3C cells. 3C cells were a clonal strain of SV40 large T antigen-transformed IMR90 cells and underwent crisis after a finite number of population doublings due to telomere exhaustion (31). Following the infection of 3C cells with a replication-deficient retrovirus, MFG, individual immortal clones were isolated, and genomic DNAs were extracted. The proviral integration sites in these clones were determined by inverse PCR and sequencing. In one clone, a provirus integrated in the first intron of the ETV5 gene and resulted in its overexpression (data not shown), suggesting that ETV5 facilitated telomere maintenance and cellular immortalization.

The possibility that ETV5 regulated hTERT expression was tested in human epithelial MCF10A cells, which proliferated in an EGF-dependent manner. As shown in Fig. 1 (A–C), EGF induced the expression of ETV5 and hTERT mRNAs, as well as telomerase activity, in these cells. To understand the role of ETV5 in hTERT regulation, MCF10A cells were transduced with lentiviral small hairpin RNAs (shRNAs) against ETV5, followed by EGF treatment. Reverse transcription and quantitative PCR (RT-qPCR) and Western analyses showed that the shRNAs against ETV5 reduced its expression (Fig. 1, D and E). EGF-induced hTERT mRNA expression and telomerase activity also decreased upon ETV5 knockdown (KD), indicating the involvement of ETV5 in hTERT up-regulation by EGF (Fig. 1, F and G). In addition, the hTERT mRNA level was also elevated upon ETV5 overexpression in the absence of EGF (Fig. 1H). These data suggested that ETV5 mediated hTERT activation by EGF in MCF10A cells.

**ETV5 activated hTERT promoter in chromatinized BAC reporters**

ETV5 overexpression also activated hTERT expression in telomerase-negative fibroblast cells, GM847 (Fig. 2A). To study the role of ETV5 in hTERT activation in a relevant chromatin context, we utilized chromatinized single-copy BAC reporters that were integrated into GM847.7 cells (referred as Tel− cells), a clone of GM847 cells containing a single proviral BAC acceptor, via RMBT (30). The integrated BAC reporter H(wt), containing the entire hTERT locus and its neighboring loci CRR9 and XTRP2 (Fig. 2B), recapitulated the native chromatin context in the host cells and provided a system for studying the complex transcriptional regulation of hTERT gene (7, 9). ETV5 overexpression in Tel− cells induced hTERT promoter activity (Fig. 2C). The hTERT promoter in a similarly chromatinized H(wt) in normal human fibroblasts (NHF) was also activated by ETV5 (Fig. 2D).

The ETS family TFs are characterized by their Ets DNA-binding domains (DBDs). Their DNA-binding activities are often regulated by amino acid sequences around the DBDs (32, 33). It was reported that an N-terminal domain negatively regulated ETV5 transactivation (34), whereas a C-terminal domain mediated ETV5 protein degradation by the E26 proteasome (35). Hence, three ETV5 plasmids were constructed by deleting one or both inhibitory domains: ∆N, ∆C, and ∆NC (missing amino acids 72-298, 484-510, and both regions, respectively) (Fig. 2E). Whereas ∆C augmented ETV5’s expression level, likely due to increased protein stability, ∆N and ∆NC enhanced ETV5’s abilities to activate hTERT promoter (Fig. 2, F and G).
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**Ets and E-box sites were involved in hTERT activation by ETV5**

Multiple Ets consensus binding sites were found at the hTERT promoter, including two Ets consensus sites (GGAA), at −169 and +50 nt relative to TSS (Fig. 3A). To determine the roles of these sites in hTERT transcription, each of these sites in the BAC reporter H(wt) were mutated to generate H(etsU) and H(etsD). Each of these mutations reduced hTERT promoter activities in both Tel1 and Tel2 cells (Figs. 3B). The ability of ETV5 to activate these mutant promoters was also impaired (Fig. 3C), indicating that both Ets sites were required for ETV5 activation.

Each of these two Ets sites is juxtaposed to an E-box, the binding site for basic helix-loop-helix (bHLH) family TFs, such as c-Myc/Max (9). In fact, these sequences form two identical motifs (GCTTCCCACGTG), at positions −171 and +44 bp, surrounding the hTERT promoter symmetrically (Fig. 3A). hTERT promoter activity was reduced 40% by a mutation of the upstream E-box in H(eboxU) and 6-fold by a downstream E-box mutation in H(eboxD) in Tel1 cells (Fig. 3B). Interestingly, the ability of full-length ETV5 and ETV5ΔNC to activate the mutant promoters of H(eboxU) and H(eboxD) was diminished in Tel1 cells (Fig. 3D), indicating that the E-boxes were also involved in hTERT regulation by ETV5.

**ETV5 and c-Myc cooperated to activate hTERT transcription via two ets/E-box motifs**

To explore possible interaction between ETV5 and c-Myc, lentiviral shRNAs against c-Myc and ETV5 were transduced into MCF10A cells, and hTERT mRNA induction by EGF was measured (Fig. 1). ETV5 and c-Myc cooperation was indicated by a significant increase in hTERT mRNA levels upon co-transduction of c-Myc and ETV5 compared to either shRNA alone (Fig. 1E).

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**Figure 1. Regulation of hTERT expression and telomerase activity by ETV5 in MCF10A cells.** A–C, regulation of ETV5 and telomerase expression by EGF. MCF10A cells were treated with or without 160 ng/ml EGF for 24 h. ETV5 (A) and hTERT (B) mRNAs were measured by RT-qPCR analysis (n = 3) and normalized to those from cells without EGF treatment. Telomerase activities in 800, 160, and 32 cells (C) were determined by a TRAP assay, and numbers below represent quantitation of telomere extension bands normalized to the internal control (IC) bands. D–G, effect of ETV5 knockdown on hTERT expression. MCF10A cells were transduced with lentiviral shRNAs targeting ETV5 and cultured in the absence of EGF for 72 h, followed by stimulation with EGF for 24 h. ETV5 (D) and hTERT (F) mRNAs were assessed by RT-qPCR (n = 3) and normalized to those from cells treated with shSCR. ETV5 protein was detected by Western blot analysis (E), and telomerase activities in 800 and 160 cells were measured by a TRAP assay (G). SCR, scrambled RNA.
determined. As shown in Fig. 4A, hTERT mRNA expression decreased following inhibition of either c-Myc or ETV5, but its level did not decline further upon KD of both c-Myc and ETV5, suggesting that two TFs cooperate to activate hTERT expression. In Tel− cells with chromatinized H(wt), c-Myc and ETV5/ETV5ΔNC each activated the hTERT promoter. Co-expression of c-Myc/ETV5 or c-Myc/ETV5ΔNC synergistically stimulated the hTERT promoter (Fig. 4B). Co-transfection of a Max-expressing plasmid further increased hTERT promoter activity. Thus, ETV5 and c-Myc/Max cooperated to regulate hTERT transcription.

To determine whether the Ets/E-box motifs were involved in the cooperation between ETV5 and c-Myc, ETV5− and c-Myc−expressing plasmids were transfected into Tel− cells with chromatinized H(etsU) and H(etsD). As shown in Fig. 4C, mutation of the downstream Ets site in H(etsD) completely abolished hTERT promoter activation by c-Myc and ETV5, either alone or together. Mutation of the upstream Ets site in H(wt) did not affect hTERT promoter activity. Thus, ETV5 and c-Myc cooperated to regulate hTERT transcription.
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Also eliminated the activation by ETV5 and c-Myc, but ETV5 and c-Myc together activated the mutant promoter, albeit at a lower level compared with the WT promoter. Parallel experiments using H(etsU) and H(etsD) also showed that both the upstream and downstream E-boxes were required for synergistic activation by ETV5 and c-Myc (Fig. 4D). Next, electrophoretic mobility shift assays (EMSAs) were performed to assess the binding of ETV5 and c-Myc proteins to the upstream and downstream Ets/E-box motifs. As shown in Fig. 5 (A–C), biotinylated probes containing either Ets/E-box motif formed three bands upon incubation with 293FT nuclear extracts. The intensities of the middle band increased upon overexpression of either c-Myc or ETV5, indicating that this band was the specific c-Myc/ETV5/DNA complex. The middle band also diminished in the presence of excess unlabeled WT, but not scrambled, probes as competitors, suggesting that c-Myc and ETV5 bound directly to these motifs. Thus, our data indicated that the Ets and E-box sites in both motifs mediated co-activation of the hTERT promoter by ETV5 and c-Myc.

To determine whether cooperation between ETV5 and c-Myc depended on the adjoining Ets and E-box sites, unlabeled mutant competitors were used in EMSAs. Consistent with those in c-Myc– or ETV5–overexpressing cells, the same shifted complexes were detected upon incubation of 293FT cell nuclear lysates with biotinylated hTERT-U (Fig. 5D, lanes 2, 11, and 12). Inclusion of 10- or 5-fold unlabeled hTERT-U reduced the intensity of the DNA/protein complex (lanes 3 and 4). Similarly, the presence of unlabeled hTERT-U M1, containing an
Ets site mutation, also competed for the binding with labeled probe (lanes 5 and 6). E-box mutation (M2) reduced its ability to compete for the complex formation (lanes 7 and 8), and mutations of both the Ets site and E-box (M3) further decreased this activity (lanes 9 and 10). A parallel EMSA experiment was also performed to test the downstream motif (hTERT-D). Mutation of either the Ets site or E-box reduced the abilities of unlabeled competitors to disrupt protein binding by biotinylated hTERT-D (Fig. 5E, lanes 5–8), and mutation of both sites further diminished this activity (lanes 9 and 10). Hence, cooperative binding of ETV5 and c-Myc to the composite motifs involved both Ets sites and E-boxes.

To assess the binding of ETV5 and c-Myc to the hTERT promoter in vivo, ChIP experiments were performed using Tel2/H (wt) cells overexpressing ETV5 (with a FLAG tag) and c-Myc individually or together. In this experiment, PCR amplicons were designed to distinguish upstream and downstream sites. As shown in Fig. 5F, the binding of exogenous ETV5 to both upstream and downstream Ets/E-box motifs increased upon co-expression of ETV5 and c-Myc. The bindings of c-Myc to the upstream site and Max to the downstream site were also increased in cells expressing both ETV5 and c-Myc. Taken together, our results indicated that ETV5 and c-Myc/Max enhanced each other’s binding to upstream and downstream composite ETS/E-box motifs at the hTERT promoter.

ETV5 activated the hTERT promoter in a repressive chromatin context

Chromatin environment plays a crucial role in the regulation of the hTERT gene. As we reported previously, chromatinized BAC reporter H(wt) recapitulated the endogenous hTERT loci in Tel+ and Tel− cells (5). Whereas the hTERT promoter was stringently repressed in Tel− cells by multiple HDAC1-containing corepressor complexes, this repression was partially relieved in Tel+ cells (3). To study the impact of chromatin environment on hTERT activation, plasmids expressing ETV5 or ETV5ΔNC were transfected into Tel+ and Tel−/H (wt) cells. In Tel− cells, ectopic ETV5 or ETV5ΔNC expression alone induced hTERT promoter activity, and co-transfection of c-Myc− and ETV5− expressing plasmids led to synergistic activation of the hTERT promoter (Fig. 6A). Interestingly, in Tel+ cells, c-Myc activated the hTERT promoter 2-fold, but ETV5 or ETV5ΔNC had no effect with or without c-Myc, even though Tel+ and Tel− cells expressed similar levels of ETV...
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proteins (Fig. S1, A and B). Consistently, treatment of Tel− cells with the HDAC inhibitor trichostatin A (TSA) opened chromatin and blunted ETV5 activation (Fig. 6B). In Tel− cells, although TSA treatment also induced hTERT transcription, ETV5 had no effect with or without TSA.

Next, ChIP experiments were performed to assess ETV5 binding to the hTERT promoter in Tel− and Tel+ cells in expressing FLAG-tagged ETV5. As shown in Fig. 6C, binding of ETV5 protein to the upstream Ets/E-box motif was readily detected in both Tel− and Tel+ cells. Surprisingly, little ETV5 bound to the downstream motif in this assay. Moreover, ChIP experiments also showed that ETV5 overexpression increased histone H4 acetylation and H3K4 dimethylation at the hTERT promoter in Tel− cells (Fig. 6D), suggesting that ETV5 helped to open chromatin around the hTERT promoter, making it available for binding and activation by other TFs. Taken together, our data indicated that, whereas ETV5 binding to the hTERT promoter was not affected by chromatin environment, ETV5 activated the hTERT promoter only in the repressive chromatin in Tel− cells.

**Genomic contexts played a critical role in hTERT regulation by ETV5 and c-Myc**

Our recent studies showed that the repressive chromatin state of the hTERT promoter was established owing to the presence of human-specific distal genomic sequences and mediated by multiple HDAC1-containing repressor complexes (3, 36). As we reported (3), the hTERT promoter in chromatinized H(wt) had little activity in Tel− cells but was activated >600-fold by TSA, indicating that the hTERT promoter was stringently repressed in its native genomic context (Fig. 7A). On the other hand, the mTert promoter in a chromatinized BAC reporter containing a 135-kb syntenic genomic region around the mTert locus was active in Tel− cells and was induced 3-fold upon TSA treatment. The hTERT promoter in M(hPro), in which a 474-bp hTERT promoter fragment upstream of the ATG codon replaced the mTert promoter in M(wt), was highly active and unaffected by TSA treatment, indicating that the mouse genomic sequence gave rise to an open chromatin environment for the mTert and hTERT promoters in Tel− cells (7, 9). As shown in Fig. 7B, whereas ETV5 and c-Myc synergistically activated the hTERT promoter in H(wt), these two proteins, either alone or together, had little effect on the mTert and hTERT promoters in the mouse genomic context, M(wt) and M(hPro).

We also constructed a set of chimeric BAC reporters containing human and mouse genomic DNA sequences. These BAC reporters were integrated into the same acceptor site in Tel− cells by RMBT. Multiple regions of the hTERT locus, including the 5′ intergenic region (5IR) and intron 2 (In2), repressed the hTERT promoter when they replaced their mouse counterparts in M(wt) (Fig. 7A). To determine how distal sequences affected ETV5 activation of the hTERT promoter, plasmids expressing ETV5 and/or c-Myc were transfected into Tel− cells containing chimeric BACs. ETV5 and c-Myc did not activate the hTERT promoter in M(h5IR), in which the 22-kb 5IR replaced its counterpart in M(wt). However, ETV5ΔNC alone activated the hTERT promoters in M(h5IR+In2) and M(h5IR+TERT), in which the 11-kb human In2 and the entire body of the hTERT gene from the ATG codon to exon 16 were included in the BAC reporters, respectively (Fig. 7B). Co-expression of c-Myc further enhanced the activation to about 8-fold, suggesting that hTERT intron 2 contained regulatory sequences that were involved in hTERT activation by ETV5 and c-Myc. On the other hand, deletions of 5IR or In2 in H(Δ5IR) or H(ΔIn2) decreased hTERT repression and also reduced its co-activation by ETV5 and c-Myc. Taken together, our data indicated that there was a correlation between synergistic activation by ETV5 and c-Myc and the repressive state of the hTERT promoter, consistent with the notion that these two proteins cooperated to relieve the repression of the hTERT promoter.

**Chromatin context affected hTERT regulation by ETS family TFs**

ETS family TFs share several features, including highly conserved core DNA-binding domains and intramolecular regulatory domains and cooperation with other TFs to regulate gene expression (37). Multiple ETS TFs can regulate the hTERT gene by binding to the same or different sites at the promoter (19, 38). To address whether regulatory abilities of other ETS TFs were also affected by chromatin contexts, ETS TFs, ETS2, ETV1, and GABPα were expressed in Tel−/H(wt) and Tel+/H(wt) cells (Fig. 8). In Tel− cells, all TFs increased hTERT promoter activity, with the strongest activation by ETV5ΔNC, ETS1, and GABPα. The abilities of these TFs to activate hTERT transcription in Tel− cells were modest in general, with no more than 2-fold activation. These data indicated that the activation of the hTERT promoter by other ETS TFs was also affected by chromatin environment.

**Discussion**

Repression of the hTERT promoter results in undetectable hTERT expression in most human somatic cells. Mechanisms
of relieving hTERT promoter from repression in telomerase-positive cancer cells remain unknown. Previous studies have shown that promoter binding by ETS family TFs, like ETS1, ETS2, ETV1, and GABPα, via either native Ets sites or those resulting from cancer-specific mutations, led to the activation of hTERT transcription (15, 19, 20, 39). In this study, we showed that ETV5 and bHLH family protein c-Myc cooperated to activate the hTERT promoter in a repressive chromatin

Figure 6. Effects of chromatin states on hTERT promoter regulation by ETV5 and c-Myc. A, regulation of chromatinized hTERT promoters by ETV5 and c-Myc in Tel− and Tel+ cells. Tel−/H(wt) (top) and Tel+/H(wt) cells (bottom) were transfected with plasmids containing ETV5 variants and c-Myc and harvested for luciferase assays 72 h later (n = 3). B, effects of HDAC inhibition on hTERT promoter regulation by ETV5. Tel+/H(wt) (top) and Tel+/H(wt) cells (bottom) were transfected with plasmids expressing ETV5 or ETV5ΔNC for 72 h, followed by treatment with or without 250 nM TSA for 24 h (n = 3). Cells were then harvested for luciferase assays. C, ETV5 binding to the hTERT promoter in Tel− and Tel+ cells. Tel−/H(wt) (top) and Tel+/H(wt) cells (bottom) were transfected with pcDNA3.1 (VEC) or pcDNA3.1-FLAG-ETV5 (ETV5). Cells were harvested in 72 h, and chromatin fragments were precipitated using a FLAG antibody (n = 3). D, ETV5 induced histone modifications at the hTERT promoter. Tel−/H(wt) cells were transfected with pcDNA3.1 (VEC) or pcDNA3.1-ETV5 (ETV5), and ChIP experiments were performed using antibodies against acetylated histone H4 (top) or H3K4me2 (bottom) in 72 h (n = 3). PCR amplicons USK and U2.5K were at 3 and 2.5 kb upstream of the hTERT promoter; amplicons Pro and Rluc were at the hTERT promoter and within the Renilla luciferase cassette (about 1 kb downstream of the promoter) in the chromatinized H(wt), respectively. *, p < 0.05; **, p < 0.01, by two-tailed Student’s t test. n.s., not significant. Error bars, S.D.
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Figure 7. Effects of genomic/chromatin contexts on hTERT promoter regulation by ETV5 and c-Myc. A, chimeric BAC reporters in Tel\(^{−}\) cells. Left, diagrams of chromatinized BAC reporters. Fluc and Rluc, Firefly and Renilla luciferase cassettes inserted at the ATG codons of CRR9 and TERT genes, respectively. White and black bars represent human and mouse genomic sequences, respectively. All BAC reporters were integrated into the same acceptor site in Tel\(^{−}\) cells by RMBT. Right, luciferase expression from chromatinized BAC reporters in Tel\(^{−}\) cells. Cells were treated with or without 250 nM TSA for 24 h, and luciferase activities were measured. B, TERT promoter activities in chimeric BAC reporters in Tel\(^{−}\) cells. Luciferase expression from each chimeric BAC reporter was normalized to those transfected with empty vectors, pcDNA3.1 and pLNCX. Statistical significance of co-overexpression of ETV5 and c-Myc with each chimeric BAC reporter in Tel\(^{−}\) cells was calculated by comparing with empty vectors pcDNA3.1/pLNCX in each line (n = 3). \(-\)Fold TSA induction was from data shown in A. *, \(p < 0.05\); **, \(p < 0.01\), by two-tailed Student’s t test. Error bars, S.D.

Figure 8. Activation of the hTERT promoter in Tel\(^{−}\) and Tel\(^{+}\) cells by ETS family TFs. Tel\(^{−}/\)H(wt) (A) and Tel\(^{+}/\)H(wt) (B) cells were transfected with plasmids expressing ETS TFs and harvested for a luciferase assay 72 h later (n = 3). Statistical significance of the activation by ETS family members in Tel\(^{−}\) and Tel\(^{+}\) cells was calculated by comparing with empty vector (VEC) in Tel\(^{−}\) and Tel\(^{+}\) cells, respectively. *, \(p < 0.05\); **, \(p < 0.01\), by two-tailed Student’s t test. Error bars, S.D.
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The hTERT promoter was highly active when placed in an open chromatin environment in Tel- cells M(hPro) in Fig. 7A (3, 7). It is a TATA-less promoter and contains five GC-boxes, which are binding sites for Sp1 family TFs. The binding of Sp1 protein to the promoter likely recruits TATA-binding protein–associated factors, forming the TFII D complex and leading to RNA polymerase II–dependent transcription. The binding of Sp1 proteins to their cognate sites also induced a bend in DNA double-stranded helix, a conformational change crucial for promoter activation (43). Accordingly, we previously reported that mutations of these five GC-boxes abolished hTERT promoter function in BAC reporters (6).

How did two identical Ets/E-box motifs, both CACGTTGG-GAAGC, surrounding the promoter cooperatively mediate hTERT activation? Previously, we showed that hTERT transcription was associated with the formation of a major DNase I–hypersensitive site (DHS) at the hTERT promoter in telomerase-expressing cells (2, 31). Further micrococcal nuclease and restriction enzyme accessibility mapping of the hTERT promoter indicated the presence of a nucleosome-free region between these two motifs in HL60 cells (44). The formation of this DHS/nucleosome-free region at the hTERT promoter was likely essential for the assembly of transcription machinery. Therefore, we proposed that two events occurred at an active hTERT promoter. First, Sp1 binding to the hTERT promoter caused DNA bending and nucleosome sliding, resulting in a nucleosome-free region at the promoter. Second, the ETS/c-Myc complexes bound to the upstream and downstream Ets/E-box motifs might interact with each other directly or indirectly, stabilizing the bend in the DNA conformation, and also forming barriers to prevent nearby nucleosomes from encroaching into the nucleosome-free region (Fig. 9A). These two events together led to a stably active hTERT promoter. Mutations of the Ets site or E-box at one of the Ets/E-box motifs would destabilize the DNA bending and abolish hTERT activation.

The hTERT promoter is embedded in a nucleosomal array and repressed in most somatic cells (top). Upon hTERT activation, ETV5/Myc complexes bind to two symmetrical Ets/E-box motifs at the hTERT promoter and may interact with each other directly or indirectly, facilitating or stabilizing a DNA bend at the promoter (bottom), forming a DNase I–hypersensitive site. This bend creates optimized GC-boxes for Sp1 proteins, which recruit general transcription factors, leading to the assembly of transcriptional machinery. Genomic DNAs are represented by black lines, and nucleosomes are depicted as gray cylinders. TSSs of hTERT and hTAPAS are indicated by divergent arrows. B, DHSs at the hTERT promoter in cancer cells. Exon 1 of the hTERT gene is shown as a rectangle, and the blue portion is a part of the hTERT coding region. The maps were drawn using the Integrative Genomics Viewer with data from DNase-Seq data sets from the ENCODE database (55) (Table S4). Two vertical red dashed lines indicate the positions of two Ets/E-box motifs.

Figure 9. Chromatin structures of the hTERT promoter. A, a model of hTERT promoter activation by ETV5 and c-Myc. The hTERT promoter is embedded in a nucleosomal array and repressed in most somatic cells (top). Upon hTERT activation, ETV5/Myc complexes bind to two symmetrical Ets/E-box motifs at the hTERT promoter and may interact with each other directly or indirectly, facilitating or stabilizing a DNA bend at the promoter (bottom), forming a DNase I–hypersensitive site. This bend creates optimized GC-boxes for Sp1 proteins, which recruit general transcription factors, leading to the assembly of transcriptional machinery. Genomic DNAs are represented by black lines, and nucleosomes are depicted as gray cylinders. TSSs of hTERT and hTAPAS are indicated by divergent arrows. B, DHSs at the hTERT promoter in cancer cells. Exon 1 of the hTERT gene is shown as a rectangle, and the blue portion is a part of the hTERT coding region. The maps were drawn using the Integrative Genomics Viewer with data from DNase-Seq data sets from the ENCODE database (55) (Table S4). Two vertical red dashed lines indicate the positions of two Ets/E-box motifs.
glioblastomas, thyroid cancer, breast cancer, and hepatocellular carcinoma (49–52). The lack of an absolute requirement for this Ets site suggested that other Ets sites or binding sites for other TFs within the hTERT promoter might substitute its function.

Our previous studies showed that the hTERT gene was embedded in a nuclease-resistant chromatin domain in somatic cells (2). Within this domain, the hTERT promoter was covered by an orderly spaced nucleosomal array and repressed in an HDAC-dependent manner (44). These repressive chromatin features could be replicated by integrating a single-copy BAC reporter with a 160-kb human genomic fragment, containing the hTERT gene, into ectopic chromosomal sites (30). By using chimeric BAC reporters containing human and mouse genomic sequences, we found that multiple distal genomic sequences of the hTERT locus contributed to the repression of the hTERT promoter in Tel− cells. Our data showed that the activation of the hTERT promoter by ETV5, and its synergistic activation by ETV5 and c-Myc, correlated with TSA-activatable repression of the promoter, suggesting that a key role of ETV5 was to open up the hTERT promoter for the assembly of transcription machinery by counteracting HDAC-mediated repression. ETV5 and c-Myc synergistically activated the hTERT promoter only in Tel− cells. In Tel+ cells, the active promoter structure was already established, likely due to the binding of endogenous ETV5/c-Myc or other Ets family TF/bHLH protein combinations, as evidenced by the presence of a major DHS at the hTERT promoter in these cells (31). Other ETS TFs, like ETS2, ETV1, and GABPa, also induced a stronger activation of the hTERT promoter in Tel− cells than in Tel+ cells. The importance of ETS TFs in the activation of the hTERT promoter in a repressive chromatin environment was consistent with the finding that most cancer-specific hTERT promoter mutations, such as C228T and C250T, resulted in derepressed promoters. These mutation sites recruited ETS TFs, which derepressed the hTERT promoter in its negative chromatin environment during tumor development.

Taken together, our current study showed that ETV5 and c-Myc synergistically activate hTERT transcription in telomerase-negative cells. This synergism resulted from cooperative binding of ETV5 and c-Myc proteins to the juxtaposing Ets sites and E-boxes in two composite motifs that surrounded the hTERT promoter symmetrically. These findings provided molecular insights into telomerase activation in normal and cancer cells.

**Experimental procedures**

**BACs and plasmids**

H(wt) and M(wt) are BAC reporters that contained a 160-kb human and a 135-kb mouse genomic region, covering CRR9 (also called C1PTM11), TERT, and XTR2P (SLC6A18) genes, respectively (30). In these BAC reporters, a Firefly and a Renilla luciferase cassette were inserted at the ATG codons of CRR9 and TERT genes. All BAC modifications were done using an improved BAC recombinemerger method (53, 54). H(eboxU) and H(eboxD) were derived from H(wt) and contained point mutations at the upstream and downstream E-boxes, as described previously (3, 9). H(etsU) and H(etsD) were generated by introducing point mutations, TTCC → TCCT and GCAA → AGAA, at the upstream and downstream Ets sites, respectively. The hTERT promoters (∼392 to +77 nt, relative to the TSS) replaced the mTERT promoter in M(wt), resulting in H(mPro) (3, 9). The hTERT 5′ intergenic region (h5IR) replaced its counterpart in M(wt), resulting in M(h5IR) (36). M(h5IR+In2) contained hTERT h5IR and intron 2 (In2) in the mouse genomic context (36). M(h5IR+TERT) contained a 64-kb human genomic fragment, including 5IR and the entire hTERT gene, replacing their mouse counterparts in M(wt) (54). The 5IR and In2 were deleted in H(wt), resulting in H(Δ5IR) and H(ΔIn2), respectively. pcDNA3.1-ETV5 contained a human ETV5 cDNA with an N-terminal FLAG tag. pcDNA3.1-ETV5ΔN, -ETV5ΔC, and -ETV5ΔNC were derived from pcDNA3.1-ETV5 using a Q5 site-directed mutagenesis kit (New England Biolabs). Primers for mutagenesis are listed in Table S1. pLNCX-c-Myc was described previously (9).

**Cell culture, BAC integration, and shRNA KD**

Human fibroblasts were cultured in minimum Eagle’s medium with 10% fetal bovine serum. Human mammary epithelial MCF10A cells were cultured in Dulbecco’s modified Eagle’s medium/F12 medium with 20 μg/ml insulin, 0.5 μg/ml hydrocortisone, 40 ng/ml EGF, 100 ng/ml cholera toxin, and 5% horse serum. Acceptor loci, 3C167b3.1 (Tel+) and GM847.7 (Tel−), were reported previously (9). Single-copy BAC reporters were integrated into the acceptor loci in Tel+, Tel−, and NHF cells by RMBT (30). Transfection were performed in triplicate wells in 96-well plates using FuGENE HD reagent (Promega). hTERT promoter activities of chromatinized BAC reporters were measured using the Dual-Luciferase Assay System (Promega). Lentiviral pLKO plasmids containing shRNA clones (Table S2) were purchased from Sigma–Aldrich. Lentivirus packaging and the KD experiment were performed as described previously (3, 9). The efficiency of c-Myc KD by shcMyc was demonstrated previously (3, 9).

**ChIP and in vitro DNA-binding assay**

ChIP experiments were performed as reported previously (9), using antibodies against histones and FLAG tag (F1804, Sigma–Aldrich). Primer sequences used for ChIP are listed in Table S1 or have been published previously (6, 7). DNA/protein complexes were detected by EMSA. dsDNA probes (Table S3) were labeled using a Pierce Biotin 3′ End DNA Labeling Kit (Thermo Scientific). Nuclear proteins were extracted with NE-PER™ nuclear extract reagent (Thermo Scientific). Nuclear extract and probes were incubated in binding buffer (10 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM MgCl2, 1 mM DTT, 2.5% glycerol, and 0.05% Nonidet P-40) containing 0.8 μg/15 μl of poly(dI-dC) at room temperature for 20 min. In competition studies, unlabeled WT or mutant oligonucleotides were added to binding reactions 20 min prior to the addition of biotin-labeled probes. Incubation mixtures were analyzed on native 5% (w/v) polyacrylamide gels, transferred to nylon membrane, and detected using a chemiluminescence nucleic acid detection module (Thermo Scientific).
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Telomerase activity and gene expression analyses

Telomerase activities were determined using a modified TRAP (telomeric repeat amplification protocol) assay (36). RT-qPCR and Western analyses were performed as reported previously (6). All RT-qPCR data were normalized to 18S rRNA. Primers are shown in Table S1. Antibodies used in Western blots are ETV5 (13011–1-AP, Proteintech), vinculin (66305–1-lg, Proteintech), and tubulin (sc-5274, Santa Cruz Biotechnology, Inc.).

Statistical analyses

Data were analyzed using GraphPad Prism in two or three independent experiments, as indicated as n for each figure. Bar graphs represent the mean ± S.D. Differences in all figures were analyzed using Student’s t test: *, p < 0.05; **, p < 0.01.

Data availability

All of the data are contained within the article.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: TF, transcription factor; TSS, transcription start site; RMBT, recombination-mediated BAC targeting; H3K4, histone H3 Lys-4; EGF, epidermal growth factor; shRNA, short hairpin RNA; RT-qPCR, reverse transcription quantitative PCR; KD, knockdown; NHF, normal fibroblast; DBD, DNA-binding domain; nt, nucleotide; bHLH, basic helix-loop-helix; EMSA, electrophoretic mobility shift assay; HDAC, histone deacetylase; TSA, trichostatin A; BAC, bacterial artificial chromosome; 5IR, 5′ intergenic region; h5IR, hTERT 5′ intergenic region; In2, intron 2; DHS, DNase I–hypersensitive site; DNase-Seq, DNase I–hypersensitive site sequencing; TRAP, telomeric repeat amplification protocol.

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