NF-κB Regulates Transcription of the Mouse Telomerase Catalytic Subunit*

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Expression of the telomerase catalytic subunit (TERT) is the rate-limiting determinant of telomerase activity in most cells. Analysis of the mouse TERT promoter revealed a potential NF-κB binding site 350 base pairs upstream from the translational start site. An oligonucleotide from this region of the TERT promoter bound to proteins in a nuclear extract prepared from a mouse hepatoma cell line. These proteins were identified as NF-κB by a number of criteria: 1) the protein complex formed on the TERT oligonucleotide had an electrophoretic mobility similar to that formed on an NF-κB consensus oligonucleotide; 2) protein binding to this site was enhanced by NF-κB activators tumor necrosis factor-α, phorbol 12-myristate 13-acetate, and interleukin-1β; and 3) the complex was specific and could be supershifted with antibodies against the p50 or p65 NF-κB subunits. The NF-κB binding site from the mouse TERT promoter activated transcription when fused to a basal SV40 promoter and enhanced the activity of the native TERT promoter in mouse hepatoma cells stimulated with phorbol 12-myristate 13-acetate. Transcriptional activation by the TERT NF-κB site could also be enhanced by co-transfection with an NF-κB1 expression vector. NF-κB may therefore contribute to the activation of TERT expression observed in mouse tissue.

Telomeres are repetitive DNA sequences at the ends of eukaryotic chromosomes with a specialized structure that stabilizes chromosomes and prevents their fusion during mitosis (1, 2). Telomeres are synthesized by telomerase, a ribonucleoprotein complex that is capable of de novo synthesis of telomeric DNA. Thus far, three major components of the telomerase complex have been identified. The telomerase RNA component (TR) provides the template for telomere repeat synthesis, the telomerase-associated protein (TP1) binds to telomerase RNA and coordinates assembly of the telomerase holoenzyme (3), and the catalytic subunit of telomerase, TERT, provides the reverse transcriptase activity. Expression studies show that TR is present in most cells and tissues, whereas TERT expression is more variable (4–6).

TERT expression is often elevated when cell proliferation rates are high (4, 7–9), but TR expression remains relatively constant (9, 10). Analysis of the human and mouse TERT promoters reveals that they are regulated by a number of inducible transcription factors, including c-Myc and Sp1 (11–16). These transcription factors are likely to contribute to the observed instances of TERT gene activation.

Although both human and mouse TERT gene expression is regulated in somatic cells, the contribution of this activated expression to organism survival and health is not clear. Mice strains deficient for telomerase activity are viable without any noticeable phenotype through four generations (17, 18). It is not until the fifth and sixth generations that telomerase-deficient mice (TR−/−) begin to show signs of premature aging (19). The lag in the development of a phenotype in telomerase-deficient mice corresponds with the progressive shortening of the long mouse telomere. Because the mouse telomere can be maintained at a functionally adequate length through four generations in the absence of any telomerase activity, it is not clear why some somatic cells in the mouse activate expression of the TERT subunit. It is possible that laboratory conditions do not reveal subtle selective advantages for telomerase activation in somatic cells. Here, we provide evidence that the mouse TERT promoter is regulated by the widely expressed and highly inducible NF-κB transcription factor (20, 21). Regulation of the TERT promoter by a highly inducible transcription factor like NF-κB suggests that induction of TERT expression may play an important physiological role.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—HT-29 cells were purchased from American Type Culture Collection (Manassas, VA) and were propagated as described previously (22). Mouse Hepa 1-4C7 cells were a gift from Dr. Chen (University of Connecticut) and were propagated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, nonessential amino acids, streptomycin (50 μg/ml), and penicillin (50 units/ml). All of the medium components were purchased from Life Technologies, Inc.. TNF-α was purchased from R & D Systems and used at a final concentration of 100 μg/ml. IL-1β was purchased from Promega (Madison, WI) and used at a concentration of 2 ng/ml. Phorbol 12-myristate 13-acetate (PMA), purchased from Sigma Chemical Co., was dissolved at 250 μg/ml dimethyl sulfoxide and used at a final concentration of 200 μM.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed based on a previously reported protocol (23), with modifications as described in Inan et al. (22). The NF-κB consensus sequence agttgaggg-gaaggctgcc (Promega) was used as positive control. For supershift experiments, 1 μl (0.2 μg) of antibody was preincubated with nuclear extract on ice for 30 min. After this preincubation, the binding reaction was performed as usual. Supershifting antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). For probe competition experiments, unlabeled AP-1 or NF-κB oligonucleotides (Promega) were preincubated with nuclear extracts on ice for 30 min (at a 25– and 50-fold molar excess). After this preincubation, the binding reaction was performed as usual. The oligonucleotide from the mouse TERT promoter was cacaggggaaggctgcc. A version of this oligonucleotide

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RESULTS

NF-κB Binding Sites on the Mouse TERT Promoter—Because increases in TERT expression have been observed under conditions in which NF-κB would be activated, such as PMA treatment and UV radiation (8, 24), we searched the human and mouse TERT promoters for potential NF-κB binding sites. As shown in Fig. 1, a number of potential NF-κB binding sites were identified within the human and mouse TERT promoter regions. These sites matched the consensus NF-κB binding site for at least 9 of 10 positions. The possible NF-κB binding site identified on the mouse TERT promoter is an exact match with the NF-κB binding site on the mouse major histocompatibility complex class I promoter (21).

To determine whether any of these sites were NF-κB binding sites, DNA binding assays were performed using nuclear extracts prepared from mouse Hepa 1-4C7 cells or human HT-29 cells. As shown in Fig. 2A, the DNA sequence element from the mouse TERT promoter associated with proteins in the mouse nuclear extract and generated complexes with a mobility similar to that obtained with a consensus NF-κB binding site. Binding to both the TERT site and the consensus NF-κB binding site was enhanced by treating Hepa 1-4C7 cells with the NF-κB activator, PMA. A similar DNA binding analysis was performed using nuclear extracts from human HT-29 cells and oligonucleotides corresponding to the possible NF-κB binding sites on the human TERT promoter (Fig. 2B). As shown in Fig. 2B, no observable binding to these oligonucleotides could be detected, even when high levels of DNA binding activity were observed with the consensus NF-κB binding oligonucleotide. (Extracts were prepared from TNF-α-treated HT-29 cells.) These data indicate that the mouse TERT promoter has a binding site for NF-κB. Although NF-κB regulation of the human TERT gene cannot be ruled out, the potential binding sites tested here do not have NF-κB binding activity (at least not with the p50 and p65 complexes activated in HT-29 cells) (22).

A number of experiments were performed to confirm that the proteins binding to the mouse TERT promoter element were NF-κB. As shown in Fig. 3A, inclusion of antibodies to the NF-κB p50 or p65 subunits in the DNA binding reaction generates a supershifted complex. Moreover, binding to this site can be competed with an excess of unlabeled NF-κB oligonucleotide but not by a nonspecific oligonucleotide (an AP-1-binding oligonucleotide; Fig. 3A). In addition, the putative NF-κB site from the mouse TERT promoter can prevent complex formation on the consensus NF-κB oligonucleotide, whereas a mutated version of this oligonucleotide (predicted to preclude NF-κB

with base pair changes predicted to preclude NF-κB binding was also used for probe competition experiments. The sequence of this mutated oligonucleotide (mTERT-mutant) was cacgtgctgctgccgc. The human TERT promoter oligonucleotides were as follows: hTERT1, catggtggg-gacccctcgccgc; and hTERT2, cactccgggaggtcccgcgtgc. The human TERT oligonucleotides were as follows: hTERT1, catggtggg-cgctccctgg; and hTERT2, cactccgggaggtcccgcgtgc. The human NF-κB consensus sequence was used for probe competition experiments. The sequence of this mutated NF-κB oligonucleotide was cacccctcgccgc; and hTERT2, cactccgggaggtcccgcgtgc.

Plasmid Construction—To construct TERT-NF-κB luciferase reporter plasmid, the double-strand mouse TERT NF-κB binding oligonucleotide (used for EMSA) was ligated into the pGL3-promoter vector (Promega). The clone had five copies of the mouse TERT NF-κB binding oligonucleotide. To construct pGL3–354 and pGL3–347 luciferase reporter plasmids, DNA fragments one base pair upstream of the initiating ATG codon to the −354 or −347 position of the mouse TERT gene were Pfu amplified and inserted into the pGL3-basic vector (Promega). The NF-κB1 expression vector was constructed by subcloning the NF-κB1 coding sequence into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA).

Transient Transfection and Luciferase Activity Assays—Transient transfection of luciferase reporter plasmids was performed using LipofectAMINE (Life Technologies, Inc.) according to the protocol recommended by the manufacturer. Luciferase assays were performed using the standard luciferase assay system (Promega). All experiments were performed three times. The paired two-sample t test was used for statistical analysis.

Fig. 1. Potential NF-κB binding sites in the human and mouse TERT promoters. Two possible sites from the human TERT (hTERT1 and -2) promoter closely match the NF-κB consensus sequence. The site from the mouse TERT (mTERT) promoter is identical to the NF-κB binding site in the major histocompatibility complex class I (MHC-I) promoter.

Fig. 2. EMSA shows protein binding to the putative NF-κB site from the mouse TERT promoter. A, mouse Hepa 1-4C7 nuclear extract was used for an EMSA with a DNA oligonucleotide (−354 to −344) from the mouse TERT (mTERT) promoter. The consensus NF-κB binding site (con-κB) was used as a positive control. NF-κB activation was induced with 200 nM PMA for 30 min. B, an EMSA was performed using the possible NF-κB binding sites from the human TERT (hTERT) promoter. Nuclear extract was prepared from TNF-α-treated or untreated HT-29 cells with oligonucleotides that contained the possible NF-κB binding sites. Duplicate TNF-α samples were analyzed.
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To determine whether the NF-κB activation of the luciferase reporter in mouse Hepa 1-4C7 cells.

NF-κB vector have elevated levels of NF-κB shown in Fig. 6

SV40 basal promoter (shown schematically in Fig. 4

Enhancing NF-κB activity of the mouse TERT promoter.

TERT luciferase reporter construct was generated in which five copies of the mouse TERT promoter could activate transcription, a luciferase reporter vector containing five copies of TERT promoter (shown schematically in Fig. 4B). As shown in Fig. 4A, the TERT κB sites strongly activate expression of the luciferase reporter in mouse Hepa 1-4C7 cells. Moreover, treatment of the cells with the NF-κB activator PMA further stimulates expression of the TERT κB luciferase reporter (p < 0.05). These results indicate that the TERT κB site can activate transcription under both constitutive and PMA-activated conditions.

To determine whether the TERT κB site contributes to the regulation of the native TERT promoter, two luciferase reporter constructs were generated. One construct contained all of the previously described transcription factor binding sites (the -347 construct), whereas the other construct included these sites and the NF-κB binding site (the -354 construct; shown schematically in Fig. 5B). As shown in Fig. 5A, the NF-κB site provides a significantly higher level of luciferase reporter expression in cells treated with PMA (p < 0.01). The c-Myc binding site close to the initiator was found to have a similar contribution to the level of TERT promoter activity. These results indicate that the TERT κB site can stimulate activity of the mouse TERT promoter.

To obtain evidence that the TERT κB binding site was stimulating transcription through NF-κB, we determined how enhancing NF-κB expression in Hepa 1-4C7 cells affected activity of the luciferase reporter constructs. NF-κB activity was increased in Hepa 1-4C7 cells by transfection with a CMV-regulated NF-κB1 gene, which encodes the p50 subunit (20, 21). As shown in Fig. 6A, cells transfected with the NF-κB1 expression vector have elevated levels of NF-κB binding activity. To determine whether this increased NF-κB activity stimulated transcriptional activation by the TERT κB site, cells were transfected with the TERT κB-luciferase reporter construct (shown in Fig. 4B) in the presence or absence of the NF-κB1 expression vector. As shown in Fig. 6B, the NF-κB1 expression vector significantly enhanced transcription of the TERT κB-luciferase reporter (p < 0.03). It was also determined how the activity of the native TERT promoter was influenced by enhanced NF-κB activity. As shown in Fig. 6C, under standard conditions expression of the -354 construct (which has a κ-B site) is 1.8-fold greater than the TERT-347 construct (which has no κ-B site). However, co-transfection of these luciferase reporter constructs with the NF-κB1 expression vector results in a significant increase in luciferase activity.

2 L. Yin and C. Giardina, unpublished observation.

Fig. 3. Specificity of protein binding to the mouse TERT NF-κB binding site. A, supershift and competition analysis of the proteins binding to the mouse TERT (mTERT) κB sequence. Antibodies against the five subunits of NF-κB were included in a binding reaction as indicated. An unlabeled consensus NF-κB (κB) oligonucleotide was used as specific competitor, and an AP-1 oligonucleotide was used as a nonspecific competitor. Arrows denote supershifted DNA-protein complex. B, the putative NF-κB site from the mouse TERT (mTERT) promoter competes with the NF-κB consensus oligonucleotide for protein binding, whereas a mutant TERT oligonucleotide does not. Unlabeled competitor oligonucleotide was pre-incubated with nuclear extract at concentrations 10, 25, or 50 times higher than the labeled NF-κB consensus oligonucleotide as indicated. C, protein binding to the mouse TERT κB site is activated by the NF-κB activators TNF-α, PMA, and IL-1β.

Fig. 4. Transcriptional activation by the mouse TERT κB site. A, mouse Hepa 1-4C7 cells were transfected with the pGL3-promoter luciferase reporter vector containing five copies of TERT κB (shown schematically in B). The pGL3-promoter vector without κB sites was used as a control vector. Twenty-four hours after transfection, cells were induced with 200 nM PMA for an additional 6 h. The results shown were normalized to the control vector. B, schematic drawing of the luciferase reporters.
in the preferential activation of the -354 construct, which includes the NF-κB binding site (3.5-fold; p < 0.03). These results provide additional evidence that the NF-κB binding site on the mouse TERT promoter activates transcription through the binding of NF-κB.

**DISCUSSION**

A number of instances have been described in which expression of the TERT subunit of telomerase is activated (4, 8, 9, 25, 26). TERT expression is often found to be up-regulated when cells are proliferating and down-regulated during cell differentiation. Two inducible transcription factors have been identified that bind the human and mouse TERT promoters, Sp-1 and c-Myc/Mad (11, 14). These transcription factors are likely to contribute to the activation of TERT expression. However, many of the stimuli found to activate TERT expression in both human and mouse cells are documented to activate the transcription factor NF-κB (including PMA and UV radiation) (8, 24, 26). Analysis of the TERT promoter region revealed a functional NF-κB site on the mouse TERT promoter. Although we were unable to identify an NF-κB site on the proximal human TERT promoter, it remains possible that one exists as part of an enhancer either further upstream or downstream of the transcription start site. Alternatively, human cells may employ slightly different mechanisms to coordinate NF-κB activation and TERT expression; for example, NF-κB activation of Myc expression may lead to subsequent increases in TERT expression (13, 14, 27, 28).

The ability to generate specific gene knockouts in the mouse makes this animal an exceptional model for studying the bio-

**FIG. 5. Activation of the mouse TERT promoter by the NF-κB binding site.** A, mouse Hepa 1-4C7 cells were transfected with luciferase reporters containing the TERT promoter with (-354) or without (-347) the NF-κB site. Twenty-four hours after transfection, NF-κB activity was induced with 200 nM PMA for 6 h. The results shown were normalized to the -347 construct (*, p < 0.01). B, schematic representation of the luciferase reporters. The pGL3–354 construct included a complete NF-κB binding site, whereas pGL3–347 included only the last three bases of this site.

**FIG. 6. The mouse TERT promoter is activated by overexpression of NF-κB1.** A, EMSA was performed using nuclear extract prepared from mouse Hepa 1-4C7 cells transfected with a CMV-regulated NF-κB1 expression vector or an empty expression vector. Cells were treated with PMA (200 nM) where indicated. B, mouse Hepa 1-4C7 cells were transfected with the TERT-κB-luciferase construct with an empty CMV expression vector or with the CMV-NF-κB1 expression vector. Twenty-four hours after transfection, NF-κB activation was induced with 200 nM PMA for an additional 12 h. The results shown were normalized to the TERT-κB construct in the absence of the NF-κB1 expression vector. Inclusion of the NF-κB1 expression vector significantly increased the TERT-κB reporter (*, p < 0.03). C, mouse Hepa 1-4C7 cells were transfected with pGL3–354 or pGL3–347, with or without the CMV-regulated NF-κB1 expression vector. Twenty-four hours after transfection, the luciferase assay was performed. The results shown were normalized to the -347 construct. The NF-κB1 expression vector significantly increased expression of the -354 construct over the -347 construct (*, p < 0.05).
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logical role of telomerase. Moreover, the mouse has organ systems similar to those found in humans. Telomerase expression in the mouse is also activated under circumstances that activate the human telomerase. It is not clear, however, what benefit arises from activating TERT expression in mouse somatic cells, because telomerase activity can be eliminated from the mouse genetically without any observed effect on the animal for four to five generations (17, 18, 29). Our finding that the TERT promoter is regulated by NF-κB argues that TERT activation in somatic cells provides an advantage to the mouse because NF-κB is found in most cell types and can be activated by an impressive array of stimuli, ranging from cytokines and growth factors to stress-inducing agents such as oxidants and UV radiation (20, 21). Perhaps the conditions that reveal the benefit of telomerase expression in somatic cells have not been identified. TERT activation may be beneficial under conditions of chronic infection, injury, and/or immune and inflammatory responses. Whether or not the NF-κB activation of TERT expression plays an important role in the physiology of the mouse remains an open and interesting question. This question is of particular significance given the importance of the mouse as an experimental model.

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