Mechanism of Telomerase Activation by v-Rel and Its Contribution to Transformation

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Telomerase is activated during the transformation of lymphoid cells and fibroblasts by v-Rel, the oncogenic member of the Rel/NF-κB family of transcription factors. v-Rel-transformed cell lines have longer telomeres than untransformed chicken lymphoid cells and have high levels of telomerase activity. v-Rel-mediated activation of telomerase is achieved by multiple mechanisms. The expression of the gene encoding the catalytic subunit of telomerase (TERT) was directly upregulated by v-Rel. Moreover, the expression of v-Rel altered the ratio of alternatively spliced and full-length TERT transcripts in favor of the full-length forms. The activation of telomerase by v-Rel in lymphocytes was also accompanied by inactivation of nuclear inhibitors. The inhibition of telomerase activity in v-Rel-transformed cell lines led to apoptosis within 24 h. The expression of v-Rel in a macrophage cell line resulted in elevated levels of reactive oxygen species (ROS), increased telomerase activity, and increased sensitivity to telomerase inhibitors. In contrast, the ectopic expression of TERT decreased the extent of apoptosis induced by ROS. The activation of telomerase by v-Rel may, therefore, partially protect the transformed cells from apoptosis induced by ROS.

Special DNA-protein complexes, referred to as telomeres, are present at the ends of linear chromosomes. Telomeres protect chromosomal ends from degradation as well as prevent their fusion or illegitimate recombination with other chromosomes (for a review, see reference 12). In vertebrates, telomeric DNA is composed of multiple repeats of a short sequence (5'-TTAGGG-3'). Telomeric DNA, ranging from 10 to >50 kb in length, is complexed with multiple proteins which control the stability of telomeres. Telomeric DNA is synthesized by a multisubunit enzymatic complex, telomerase, consisting of the telomerase reverse transcriptase (TERT), an RNA component (TR) which acts as a template, and other associated proteins. Telomerase adds repeated sequences to the ends of linear chromosomes during cell division to overcome the “end replication problem” of linear DNA molecules. Recently, additional functions in cellular proliferation, survival, and carcinogenesis have been ascribed to telomerase (31, 54, 55). Telomerase activity protects cells from apoptosis induced by serum deprivation or agents which induce double-stranded DNA breaks, such as hydroxyl radicals (4, 68, 95). The expression of TERT also enhances genomic stability and DNA repair (70). Finally, the overexpression of TERT leads to transcriptional alterations in the expression of a subset of genes, some of which enhance cell proliferation (38, 70, 74).

The activity of telomerase is high in germinal cells, contributing to their immortality, but is low in most somatic cells of homoeothermic vertebrates (28). As a result, these somatic cells can divide for only a limited number of times before undergoing senescence or apoptosis. This reduction in telomerase activity reduces the risk of unrestricted somatic cell proliferation and malignant tumor growth. Telomerase, however, may be reactivated during tissue repair or the immune response (28, 86). Telomerase also becomes reactivated in 90% of human tumors, and telomerase activity increases with tumor progression (34, 47, 79). Telomerase is often reactivated in tumor cells by the abrogation of its original repression in somatic cells. The shortening of telomeres during proliferation of tumor cells leads to genomic instability, resulting in aberrations, such as partial chromosomal deletions and amplifications (71). Chromosomal deletions often result in telomerase reactivation by removing loci encoding telomerase repressors (24). The transcriptional repressor Mad1/c-Myc, the transforming growth factor beta pathway, and the tumor suppressor Menin are involved in telomerase repression (51). In contrast, only a few oncogenes, including v-Myc, Bmi-1, E6 and E7 of human papilloma viruses (HPV 16 and HPV 18), and the Bcl-2 proto-oncogene, directly upregulate telomerase activity without a requirement of genomic instability induced by telomere shortening (23, 44, 53, 84).

The activity of telomerase in normal and neoplastic cells is regulated at several different levels (20). Most of the known regulatory mechanisms operate by controlling the transcription or posttranslational modifications of the TERT subunit. Several transcription factors have been shown to regulate the TERT promoter, including c-Myc, Sp1, Sp3, and the estrogen receptor (56, 62, 84). The activity of the human telomerase complexes also appears to be regulated by alternative splicing (33, 40). Several variants of TERT are generated by splicing within the reverse transcriptase region and the C-terminal part of the TERT gene. The most frequently described spliced forms (α and β) lack telomerase activity, and the α-splice variant may function as an endogenous dominant-negative regulator of telomerase activity (19, 90). The phosphorylation of TERT by Akt has been shown to play a regulatory role at the posttranslational level (37). There are suggestions that Rel/
NF-κB transcription factors may also play a role in the regulation of TERT. Rel/NF-κB binding sites are present in the mouse TERT promoter, and a synthetic inhibitor of the IκB kinase abrogates the constitutive and cytokine-induced up-regulation of telomerase activity (2, 91). The Rel/NF-κB family member p65 (RelA) induces nuclear translocation of human TERT after both p65 and TERT become phosphorylated following the exposure of cells to tumor necrosis factor alpha (TNF-α) (3). However, a role for telomerase activation by Rel/NF-κB family members in cell transformation has not been established.

v-Rel, a member of the Rel/NF-κB family of transcription factors, is the oncogene expressed by avian reticuloendotheliosis virus (REV-T) (75, 88). REV-T rapidly induces a fatal lymphoma in chickens and efficiently immortalizes splenic lymphocytes in vitro (49). Moreover, REV-T transforms primary chicken fibroblasts and induces sarcomas in experimentally infected chickens (30, 57). Because of the rapidity and efficiency with which v-Rel transforms cells and induces a fatal neoplastic disease, the v-Rel system provides a unique model for studying the role of Rel/NF-κB family members in carcinogenesis.

The altered expression of hundreds of genes contributes to tumor development; however, only a few genes are known to directly activate telomerase (23, 44, 53, 84). In this report we demonstrate that v-Rel, in contrast to most other oncogenes, directly activate telomerase (23, 44, 53, 84). In this report we demonstrate that v-Rel, in contrast to most other oncogenes, directly activate telomerase.

**MATERIALS AND METHODS**

**Cloning of avian TERT and TR.** The chicken TERT gene was cloned from RNA obtained from a 5-day-old chicken embryos and inserted into the vector pTZ-18R, creating pTZ-TERT. A portion of the TR gene was reverse transcribed (RT-PCR) amplified from chicken fibroblast genomic DNA using the GC-melt heDNA Advantage kit (BD Clontech Biosciences, Mountain View, CA) and primers TRP1 and TRP3 (Table 1), which were designed based on the available chicken TR sequence (18). This fragment was cloned into the vector pGEM-T Easy, creating pGEM-TR.

**TABLE 1. Oligonucleotide primers used for RT-PCR analyses**

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene product</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB1</td>
<td>TERT</td>
<td>5′-GATGTGTTCGTCACCGCTCTACAGTTTC-3′</td>
</tr>
<tr>
<td>T4</td>
<td>TERT</td>
<td>5′-CCAGATTTGCGGGATATGCGTTGTG-3′</td>
</tr>
<tr>
<td>KbSpA</td>
<td>TERT</td>
<td>5′-CAGCTTCTGGCAAATGGAATTCTAGG-3′</td>
</tr>
<tr>
<td>KbSpWT</td>
<td>TERT</td>
<td>5′-AGTTAAGTACTGGCTATGGAGCTCTGC-3′</td>
</tr>
<tr>
<td>T6</td>
<td>TERT</td>
<td>5′-CCACACATAGATCAACGCACAACTC-3′</td>
</tr>
<tr>
<td>TRP1</td>
<td>TR</td>
<td>5′-AGGGTCCGCTGTCCTCCTACCTC-3′</td>
</tr>
<tr>
<td>TRP3</td>
<td>TR</td>
<td>5′-TGCTTGGGACACACTCTTGCATCTC-3′</td>
</tr>
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**Retroviral expression vectors.** Two retroviral expression systems were used: one based on REV-T and the other on Rous sarcoma virus (RSV). The REV-T-based plasmid pREV-TW, which encodes v-Rel, has been described previously (36, 60). pREV-Ts-1 was created by a G-37→E mutation of v-Rel using the v-G37E construct described by White et al. (87). The chicken TERT gene was first modified by adding a Kozak sequence and XhoI cloning site before the start codon and an MluI cloning site 15 nucleotides (nt) downstream of the STOP codon. pREV-TERT was then created by cloning the XhoI-MluI TERT fragment into a REV-based retroviral construct, pREV-0 (36). pCSV11S3 contains an infectious genomic clone of chicken synctial virus (CSV) (27). The virus stocks of REV-T, REV-Ts-1, and REV-TERT were generated by cotransfection of pCSV11S3 with the respective plasmids pREV-TW, pREV-Ts-1, and pREV-TERT. CSV viruses were generated by transfection of the pCSV11S3 plasmid alone.

The plasmids pDS3 and pREP-A together encode an RSV-based replication-competent retroviral vector derived from a genomic clone of the Schmidt-Ruppin strain of Rous sarcoma virus (subgroup A) (22, 61). The pDS3 plasmid was further modified to obtain pTZZ-Ts-XB by cloning a 2.1 kb Sal-Sac fragment of pDS3 into a polylinker in pTZZ-18R (Amersham Biosciences Corp., Piscataway, NJ) and inserting an XhoI-NotI-SpeI-BglII-BssHII adaptor into an unique BglII site. Coding sequences for v-Rel and v-Myc were cloned into pTZZS-XB to generate pTZZS-vRel and pTZZS-vMyc. The v-Rel coding sequence was derived from a previously described plasmid, pV-relc (36, 60). pTZZS-vMyc was derived from a MC29 v-myc-encoding plasmid provided by Thomas Gilmore, Boston University, MA (6, 85). DS(A), DS-vRel(A), and DS-vMyc(A) viruses were generated by transfection of concennters of the respective pTZZS constructs ligated with pREP-A as described previously (35).

**Chickens and tissue culture procedures.** Embryonated eggs from pathogen-free White Leghorn chickens were obtained from Charles River SPAFAS, North Franklin, CT. Chicken splenic lymphocytes were purified from 5-week-old chicken embryos. Cells were cultured with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 5% chicken serum (Invitrogen, Carlsbad, CA), 100 U of penicillin, and 50 μg of streptomycin per ml. Secondary cultures of fibroblasts were established as described previously (59). Fibroblasts were used for transfection of plasmid DNA by a calcium-phosphate method as described previously (35). Viruses were harvested between 5 and 7 days after transfection and infectious titers of Rel-expressing viruses determined by an immunohemocytometric titration assay (36, 76).

**Cell lines.** 123/12, 160/8, 160/2, 123/6, 123/6T, and 123/8 are v-rel tumor-derived cell lines. 123/12 is a B-cell line, 160/8 and 160/2 are T-cell lines, and 123/6, 123/6T, and 123/8 are non-B/non-T lymphoid cell lines which express several myeloid markers (36). DT40 and DT95 are B-cell lines established from chickens bearing bursa-derived lymphoid tumors (5, 59). DS(A), DS-vRel(A), and DS-vMyc(A) viruses were generated by transfection of concennters of the respective pTZZS constructs ligated with pREP-A as described previously (35).

**siRNA electroporation.** Four chicken TERT-specific small interfering RNAs (siRNAs) were designed using guidelines proposed for the optimization of siRNAs and siRNA duplexes (94). Preliminary experiments determined that two of these siRNAs decreased TERT mRNA levels. These double-stranded oligonucleotides, designated TE2 and TE4, have the following sequences: 5′-CCAGATTTGCGGGATATGCGTTGTG-3′ (NC), 5′-CAACCATAGATCAACGCACAACTC-3′ (TE2), 5′-AGGGTCCGCTGTCCTCCTACCTC-3′ (TE4), and 5′-TGCTTGGGACACACTCTTGCATCTC-3′ (TE5).
ACAGUU-3', and target the regions of TERT mRNA located 2.865 and 3.881 nucleotides downstream of the start codon. These siRNAs and a negative control, INC (negative control #1; Ambion, Austin, TX) were synthesized using the siRNA construction kit (Ambion). Additionally, we employed an siRNA chemically synthesized by Ambion that targets chicken TR (5'-GCGUCGACCAACCCUAUACUCU-3' and 5'-GAUAGGAGCGGAAGCACGCAGG-3'). This siRNA binds to the region of TR that is used as the template for the synthesis of telomeres (26). siRNAs (6 μg) were electroporated into exponentially growing cells (10^6) using siPORT siRNA electroporation buffer (Ambion) at 1 μF and 0.4 kV. After electroporation, cells were placed into regular medium, and the levels of TERT mRNA and the extent of apoptosis were analyzed 24 h later. RNA was isolated using an RNaseq-activated-Micro kit (Ambion), and TERT mRNA was measured by RT-PCR as described below.

**Determination of cell resistance to oxidative challenge.** Exponentially growing HD11 cells expressing TERT or helper virus (0.5 × 10^6) were washed, resuspended in 1 ml of culture media, and seeded in 12-well plates. Freshly diluted r-butyl hydroperoxide (r-BHP) (Sigma) was added for 24 h, and the extent of apoptosis in these cells was determined by measuring DNA content in ethanol-fixed cells by propidium iodide staining and flow cytometry.

**Determination of apoptosis by flow cytometry.** Cells were washed and resuspended in 300 μl of 5 mM EDTA in phosphate-buffered saline (PBS) (10 μl EDTA). Then, 700 μl of 100% ice-cold ethanol was added and cells were stored at 4°C before analysis. Cells were pelleted and resuspended in 500 μl of PBS-EDTA, and RNA was digested by incubation with 50 μl of RNase A (10 mg/ml) for 30 min at room temperature. Then, 500 μl of propidium iodide solution (100 μg/ml in PBS) was added, and dye incorporation was determined with a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA). The percentage of cells in the early and late apoptotic stage was determined based on the theory of doublet discrimination as described by the manufacturer (FACS Calibur system user’s guide; BD Immunocytometry Systems; 1996). The percentage of cells with a DNA content lower than that of the G1 phase was calculated using the CellQuest software provided with the FACSCalibur instrument.

**TRAP.** The level of telomerase activity was evaluated using the telomerase repeat amplification protocol (TRAP) assay. Whole-cell extracts were prepared with CHAPS buffer (42). Cells (1 × 10^7 to 2 × 10^7) were extracted with 200 μl of CHAPS buffer on ice for 30 min and centrifuged at 10,000 g at 18°C for 30 min. Supernatant fluids were stored at -70°C. Extracts from cytoplasmic and nuclear fractions were prepared using a nuclear extract kit (Active Motif, Carlsbad, CA) with 2 mM phenylmethylsulfonyl fluoride added to the hypotonic buffer. Protein concentrations were determined by the Bradford method with protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

Two different TRAP assays were performed. For lymphoid cells, with high telomerase activity, single-tube extension PCR TRAP assays were used, while for fibroblasts and HD11 cells, which generally have low telomerase activity, the two-tube extension PCR protocol was employed. In the single-tube extension PCR TRAP assay, the protein extracts (100 μg of total protein or less) were incubated with primers, control template, deoxyinosine triphosphates (dNTPs), polymerase, and 1 × TRAP reaction buffer as described by Kim and Wu (43) except that 0.1 μg unlabeled TS primer and 2 μl Platinum Taq polymerase (Invitrogen) were used. Incubation for 30 min at 50°C was followed by a 2-min incubation at 94°C and by PCR amplification with 33 cycles (30 s at 94°C, 30 s at 50°C, 1 min at 72°C). In the two-tube extension PCR TRAP assay, protein extracts (20 μg of total protein or less) were first incubated with 0.5 μg of the TS primer and all four dNTPs (1 mM each) in 1 × TRAP reaction buffer, 0.8 mM spermidine, 5 mM β-mercaptoethanol in a total reaction volume of 50 μl for 30 min at 37°C. The reaction was stopped by incubation at 94°C for 2 min. Aliquots of synthesis (2.5 μl) were then PCR amplified as described by Kim and Wu (43) except that 0.1 μg unlabeled TS primer and 1 μl Advantage cDNA polymerase mixture (BD Biosciences Clontech) were employed. PCR amplification started with 94°C for 2 min followed by 33 cycles to 35 cycles (30 s at 94°C, 30 s at 50°C, 1 min at 72°C). The TRAP PCR products were then separated on 5% polyacrylamide gels (ratio of acrylamide to bisacrylamide, 19:1) in 0.5× TBE (TBE is 0.09 M Tris-borate, 2 mM EDTA, pH 8.0). Gels were stained with Vistra Green (Amerham Biosciences) diluted 1:4000 in 0.5× TBE for 1 h, and images were captured using a Fluorimag (Amerham Biosciences). For molecular weight determination, a 10-bp ladder was used (Invitrogen).

Terminal restriction fragment (TRF) length analysis. High-molecular-weight genomic DNA was digested with a cocktail of restriction enzymes (HinfI, HaeIII, MspI, and RsaI). DNA samples (0.2 μg) were then separated in 1% TBE in a 0.7% agarose gel. Undigested λ phage DNA mixed with λ digested with EcoRI and HindIII was used as a marker. DNA was transferred by using a standard Southern blot technique to a Hybond-N+ membrane (Amersham Biosciences) and hybridized to the telomeric probe (CCTTTAAGGGCGG, end-labeled with [γ-32P]ATP at 4°C using the Ultrasnap solution (Ambion). Blots were washed as suggested by the Ultrasnap manufacturer. The blots were autoradiographed at -70°C using intensifying screens. Subsequently, the blots were rehybridized with a λ probe to visualize the position of the marker.

**Western analysis.** Western analysis was performed as described previously (36). Briefly, harvested cells were washed, resuspended, boiled in sodium dodecyl sulfate sample buffer, and the total protein concentration in the lysate was determined by the Bradford method with a Bio-Rad protein assay reagent (Bio-Rad Laboratories). Volumes of lysate lower than 1 μl were used in the total reaction volume of 1 ml to avoid interference of the Bradford assay reagent with chemicals in the lysates. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a PolyScreen polynvulidine difluoride membrane (PerkinElmer Life Sciences, Boston, MA). Monoclonal antibody antibody HY87 was used to detect the expression of v-Rel and avian c-Rel (36).

**Northern analysis and probes.** Total RNA was isolated by RNAzol (Ambion). RNA was separated by electrophoresis in a 1% agarose gel in 20 mM MOPS (3-[N-morpholino]propanesulfonic acid buffer) and transferred to a Hybond N+ membrane (Amersham Biosciences). DNA fragments of the chicken genes were labeled with [γ-32P]ATP by nick translation. Membranes were hybridized with probe blots (Ambion) that were hybridized on nylon membrane (Hybond-N+) and then washed. Genes listed below, beginning with the name of the gene, followed by the GenBank accession number and position of the first and last nucleotides of the fragment based on the GenBank sequence: Non2 (BG717333; 918 nt downstream from nt 614 to 1483 nt downstream from nt 614), p40(phox) (AJ725555; 123 to 741), p47(phox) (AJ179555; 522 to 888), Rac1 (NM_205017; 10 to 612), Rac2 (B606602; 1 to 572 plus approximately 130 nt downstream), inducible nitric oxide synthase (NOS) (U01437; 480 to 2490), manganese superoxide dismutase (MnSOD) (B085995; 1 to 587 plus 86 nt downstream), catalase (BG711409; 382 to 638 plus 542 nt downstream), c-Rel (X52193; 16 to 1865), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM_204035; 381 to 664), and env (AF246698; 6031 to 6676).

**Determination of TRAP reaction products by radiolabeled-quantitative reverse transcription-PCR.** Total RNA was isolated by RNAzol (Ambion). The samples of RNA used for detection of wild-type and alternatively spliced variants of TERT and avian GAPDH by RT-PCR were first pretreated with DNase I (“DNA-free” reagent; Ambion) to remove traces of DNA in the RNA preparations. Total RNA (1 μg) together with 1 μl of 10 μM LDP primer were denatured for 10 min at 70°C in 1 μl of water (35). First-strand cDNA synthesis was carried out with 15 μl of Thermoscript reverse transcriptase (Invitrogen), 2 μl of 10 μM dNTP, and 2 μl of 100 μM dithiothreitol at 50°C for 1 h. The reaction was stopped by a 5-min incubation at 85°C. The RNA was destroyed by RNase H treatment, and the reaction was diluted with 100 μl of water. For detection of avian TERT, its alternative spliced variants, and GAPDH by RT-PCR, 2 μl of the first-strand synthesis reaction was used together with 1 μl Advantage cDNA polymerase mixture (BD Clontech Biosciences) and with primers, which include T4 and S1 primers for avian TERT, KbspWT or KbspA and T6 primers for the alternatively spliced variants of TERT, and GAPDH1 and GAPDH2 primers for the GAPDH gene (77) (Table 1). KbspWT and T6 primers detect only TERT without alternative splicing A, because the KbspA primer anneals at the junction created by alternative splicing A and is not suitable to determine the synthesis of the TERT. PCR products were performed as follows: 4 cycles (30 s at 94°C and 3 min at 72°C), 5 cycles (30 s at 94°C, 30 s at 70°C, and 3 min at 72°C), and 25 to 50 cycles (30 s at 94°C, 30 s at 65°C, and 3 min at 72°C). The PCR for detection of chicken TR was performed in the same way with primers TRP1 and TRP3 for the TR gene (Table 1), but 1 μl of Advantage-GC.
FIG. 1. Telomere length and telomerase activity in cells transformed by v-Rel. (A) Telomere length was determined by TRF length analysis. This analysis included high-molecular-weight DNA isolated from first-passage chicken embryonic fibroblasts (lane 1), purified splenic lymphocytes obtained from a 2-week-old chicken (lane 2), four v-Rel-transformed cell lines (123/12, 160/2, 160/8, and 123/6T) (lanes 3 to 6), and the macrophage HD11 and lymphoid MSB1 chicken cell lines (lanes 7 and 8). Longer exposure of the lane with HD11 DNA is also shown (lane 9). Human genomic DNA (S0950; BD Biosciences Clontech) is shown for comparison (lane 10). The positions of molecular weight standards are indicated. (B) Telomerase activity in avian cell lines of hematopoietic origin. v-Rel cell lines of non-B/non-T phenotype (nonB/T), T-cell origin (T), or B-cell origin (B) were isolated from REV-T-induced splenic tumors (lanes 1 to 6). Cell lines transformed by other oncogenes include the erythroblastoid (E) line AEV1, cell lines expressing a macrophage phenotype (Mf), HD11 and BM2, T-cell lines RP1 and MSB1, and B-cell lines DT40 and DT95 (lanes 7 to 13). Purified splenic lymphocytes were obtained from a 3-week-old chicken (lane 14). Telomerase activity was determined by single-tube TRAP assay using aliquots of the extracts containing 40 ng of total protein. Molecular weights are indicated in the left margin, and positions of the TRAP PCR products (6-bp ladder starting with 50-bp band) and a 36-bp PCR internal control (IC) are shown in the right margin. (C) Telomerase activity during in vitro transformation of splenic cells by v-Rel. Purified splenic lymphocytes obtained from a 3-week-old chicken were infected with REV-T at a multiplicity of 1. Extracts for the TRAP assays were harvested from cells before infection (day 0) and at various times after infection (lanes 1 to 12). In negative control assays, the samples were pretreated by heating at 85°C for 15 min before subjecting them to the TRAP assay (lanes 13 to 14). In an independent experiment, the telomerase activity detected by TRAP and v-Rel expression detected by...
cDNA polymerase mix with 10 µl GC-melt was added instead of the regular Advantage cDNA polymerase mix to facilitate the amplification of GC-rich sequences (BD Biosciences Clontech). The annealing temperature was increased to 68°C.

**Electrophoretic mobility shift assays.** The assays were performed as described previously (45). The TERT eB1 probe was prepared by annealing oligonucleotides (5'-GGTTCATACGTCAGAGTTCTCTCATACAA-3' and 5'-TTTGAAGAGAAGACTTCTCATGAAAC-3'), followed by end labeling with [γ-32P]ATP. The TERT eB2 probe was prepared by annealing oligonucleotides (5'-TCCAAGCAGTGATCAGCCTGGCTT-3' and 5'-CAAGGGAGGG-3'), followed by a Klenow fill-in reaction using [γ-32P]dCTP as described previously (45).

**GenBank accession numbers.** Newly determined nucleotide sequences were submitted to GenBank [chicken TERT], X42623, [chicken TR], DQ144859 to DQ144873, and DQ177327 [alternatively spliced isoforms of chicken TERT].

## RESULTS

Telomeres of v-Rel-transformed cell lines are longer than telomeres of normal chicken cells. Cells transformed by the majority of oncogenes are able to escape mortality 1 (M1) crisis, a growth arrest triggered by the shortening of telomeres (72). These cells, however, usually enter the second (M2) crisis phase when extremely short or absent telomeres are unable to protect chromosome ends. At this stage a few M2 cells may emerge in which telomerase (or the alternative lengthening of telomere mechanism) has been activated and telomere length stabilized. Consequently, telomeres in immortal transformed cells are generally shorter than telomeres in normal cells (48). Lymphoid cells transformed by the v-rel oncogene do not enter a crisis phase prior to immortalization (36). To determine how the absence of crisis affects telomeres in v-Rel-immortalized cells, telomere lengths in four v-Rel cell lines established from tumors were determined by TRF length analysis (Fig. 1A). The average length of telomeres in v-Rel-immortalized cells was 50 kb or greater, while splenic lymphocytes from 2-week-old chickens or chicken embryonic fibroblasts had an average telomere length of 20 to 50 kb. The avian cell line MSB1, which is transformed by Marek’s disease virus, also had longer telomeres than nontransformed chicken cells. Marek’s disease virus, however, has acquired a functional copy of the avian TR gene, which is likely responsible for the long telomeres in cells transformed by this herpesvirus (29). By contrast, the HD11 tumor cell line, which has low telomerase activity, has very short telomeres (some as short as 2 kb). Human DNA had significantly shorter telomeres (about 10 kb) than chicken cells. In summary, v-Rel transformation of hematopoietic cells results in the maintenance and extension of telomeres, which is consistent with the observation that v-Rel transforms these cells without undergoing crisis induced by extremely short telomeres.

**v-Rel increases telomerase activity during transformation of splenics and fibroblasts.** To determine whether the long telomeres in v-Rel-transformed cells correlate with telomerase activity in these cells, we examined the levels of telomerase in immortalized v-Rel-transformed cell lines. All lymphoid v-Rel cell lines examined showed significant levels of telomerase activity (Fig. 1B, lanes 1 to 6). Differences in telomerase activity were not detected among the different phenotypic classes of v-Rel-transformed cells: B, T, or non-B/non-T. Telomerase activity in most of these cell lines was two to three times higher than the activity detected in splenic lymphocytes obtained from young chickens (lane 14). In contrast, telomerase activity in other transformed avian cell lines of hematopoietic origin varied greatly, from undetectable or low (HD11, MSB1; lanes 8 and 11) to very high activity (AEV1, RP1; lanes 7 and 10). These results indicate that v-Rel-immortalized cells maintain high telomerase activity.

Transformed immortal lymphoid cell lines can be readily established by the infection of splenic cells in vitro with the v-Rel-expressing retrovirus REV-T. To assess changes in telomerase activity during the in vitro transformation process, splenic lymphocytes were infected with REV-T. Telomerase activity in these cells was determined 2, 6, and 12 days after infection and compared to that of uninfected splenic cells harvested at the beginning of the experiment (Fig. 1C, lanes 1 to 12). Since telomerase is a heat-sensitive enzyme, extracts were also heated to 85°C before the TRAP assay was performed to demonstrate that the PCR-amplified products were the result of TERT enzymatic activity (lanes 13 and 14). Initially the cultures contained a substantial proportion of v-Rel-negative cells, because REV-T can replicate in only a small proportion of the splenic cells (36). The majority of the cells undergo apoptosis in vitro, while v-Rel-expressing cells proliferate. By day 6, the majority of cells expressed v-Rel, displayed a transformed morphology, and exhibited a dramatic increase in telomerase activity. The telomerase activity in transformed cells exceeded the activity in the initial cell population 4-fold at day 6 (lanes 7 to 9) and more than 10-fold at day 12 (lanes 10 to 12). This increase in telomerase activity temporally correlated with the increased expression of v-Rel in the lymphocyte cell population (lanes 15 to 18). These results demonstrate that
a dramatic increase in telomerase activity accompanies the establishment of lymphoid cell transformation by v-Rel.

v-Rel also transforms chicken fibroblasts in culture and induces tumors of connective tissue origin (30, 57). Therefore, the level of telomerase activity was evaluated in v-Rel-transformed CEF cultures (Fig. 1D). CEF cultures (second passage) were infected with RSV subgroup A-based retroviruses expressing v-Rel or an empty vector control (VC) or left uninfected. CEF cultures were also infected with the same retroviral vector expressing v-Myc, a well established activator of telomerase (25). Telomerase activity was determined 7 days after infection. Consistent with the previously published re-

FIG. 2. The induction of TERT and TR expression during transformation by v-Rel. (A) Steady-state levels of TERT mRNA and TR RNA in splenic lymphocytes transformed by v-Rel. The levels of TERT mRNA in chicken splenic lymphocytes before infection (UN) and 12 days after infection with REV-T (v-Rel) were determined by RT-PCR. The expression of GAPDH, a housekeeping gene, was analyzed as a control. The primers for RT-PCR of chicken TERT were designed based on a region which is not subject to known alternative splicing. Aliquots from the PCR were taken every seventh cycle beginning with cycle 30 for the amplification of TERT and GAPDH and 39 for the amplification of TR, and PCR products were resolved on agarose gels and visualized with ethidium bromide. The calibration of TERT and TR RT-PCR is shown in the right panel. Indicated amounts of plasmid DNA templates pTZ-TERT and pGEM-TR were PCR amplified under the same conditions as the cDNAs in the experiment. (B) The direct induction of TERT mRNA by ts-1 v-Rel at permissive temperature. HD11 cells expressing v-Rel or the ts-1 v-Rel mutant were incubated for 4 days at permissive temperature (36.5°C) and transferred to nonpermissive temperature (41.5°C) for 24 h. RNA was harvested from some cultures. The remaining cultures were shifted to permissive temperature and grown in the presence of cycloheximide (CHX) (10 μg/ml) for 3 h, and RNA was harvested again. The levels of TERT and the GAPDH housekeeping gene were determined by RT-PCR. The experiment presented is a representative example of one of two similar experiments. Expression of v-Rel and its ts mutant (ts-1 v-Rel) was determined by Western analysis and is shown in the right panel. Western analysis also included HD11 cells infected with CSV (VC). (C) Multiple κB sites in the TERT promoter are bound by v-Rel. Nuclear extracts were isolated from CEF cultures expressing the helper virus CSV (VC) or those transformed by REV-T (v-Rel). Electrophoretic mobility shift assays were performed with 2.5 μg of nuclear extracts and 32P-labeled probes encompassing the κB1 (left panel) and κB2 (right panel) sites found in the TERT promoter. Supershift analysis was performed by preincubating the nuclear extract from v-Rel-expressing cells with normal rabbit serum (NRS) or antisera specific for v-Rel (v-Rel) prior to the addition of probe. Free probe (FP) and the binding reactions were resolved on a 5% nondenaturing polyacrylamide gel. The four bands that were supershifted by the addition of v-Rel-specific antisera are indicated. Asterisks indicate the locations of the supershifted v-Rel-containing complexes.
The expression of v-Rel directly increases the level of TERT.

The reverse transcriptase (TERT) and the RNA component of telomerase (TR) are two essential components of the telomerase enzymatic complex. Since v-Rel is a transcription factor, we evaluated whether the steady-state levels of TERT mRNA and TR RNA were altered in response to v-Rel expression (Fig. 2A). Total RNA was harvested from transformed splenocytes 12 days after infection with REV-T and from control cells before infection. RT-PCR analysis demonstrated that in v-Rel-transformed lymphocytes, the level of TERT mRNA was increased approximately 10-fold and TR expression more than 10-fold (Fig. 2A). These results suggest that the increased levels of TERT and TR RNA may contribute to the elevated activity of telomerase observed in v-Rel-transformed cells.

To assess whether the increased levels of TERT were a direct effect of v-Rel expression, a temperature-sensitive (ts) transformation mutant of v-Rel (ts-1 v-Rel) was employed (Fig. 2B) (87). HD11 cells were infected with retroviruses expressing v-Rel or ts-1 v-Rel and incubated for 4 days at the permissive temperature (36.5°C), and the expression of v-Rel was confirmed by Western blot analysis. RNA from some of these cultures was harvested for analysis of TERT mRNA levels. Other cultures were transferred to the nonpermissive temperature (41.5°C) for 24 h, and RNA was harvested. At the nonpermissive temperature, the ts-1 v-Rel protein became inactivated and TERT mRNA was degraded (Fig. 2B), whereas in cells expressing wild-type v-Rel, TERT mRNA continued to be synthesized. To determine if the change in TERT mRNA levels was a direct consequence of v-Rel activity, the remaining cultures were transferred to permissive temperature in the presence of the protein synthesis inhibitor cycloheximide (10 μg/ml), and RNA was isolated after 3 h. After a shift to the permissive temperature, the ts-1 v-Rel protein becomes active but other proteins are not synthesized due to the presence of the protein synthesis inhibitor. Therefore, any increase in the levels of TERT mRNA would be the result of the activation of ts-1 v-Rel. The ts-1 v-Rel mutant induced the expression of TERT in cells, while the levels of TERT mRNA remained the same in control cultures. The increase of TERT RNA levels is, therefore, a direct result of v-Rel activation.

To further investigate the role of v-Rel in the activation of TERT expression, we analyzed the promoter of chicken TERT (21). Two potential κB sites, at 189 and 535 bp upstream of the transcription start site, were identified. Electrophoretic mobility shift assays were performed to define whether v-Rel binds to either of these κB sites. As shown in Fig. 2C, multiple complexes in nuclear extracts from v-Rel-transformed CEF cultures bound to both of these κB sites. Four complexes were supershifted by antisera specific for v-Rel. Binding of v-Rel to these κB sites further supports the notion that v-Rel directly upregulates the expression of the chicken TERT gene.

Changes in the splicing pattern of TERT result in higher abundance of full-length and in-frame-spliced forms in v-Rel-transformed cells. Telomerase activity in human cells correlates with the presence of full-length TERT transcripts (80). Seven alternatively spliced transcripts of the human TERT gene that encode proteins with internal deletions in the reverse transcriptase domain or truncations of the C terminus have been identified (33, 40). The expression of these alternatively
spliced forms in both normal and malignant cells does not appear to be sufficient for telomerase activity (19, 90). Since these studies indicate that alternative splicing of TERT is an important mechanism in the regulation of human telomerase activity, we investigated whether TERT transcripts are also alternatively spliced in the chicken. Analysis of chicken splenic lymphocytes and v-Rel-transformed lymphoid cells revealed the presence of 16 alternatively spliced variants, which included forms with exon skipping, inclusion of a new exon cassette, intron retention, and the modification of exon boundaries as a result of employing an alternative splice donor or acceptor site (Table 2). Six of these splicing events introduced novel stop codons which would lead to prematurely terminated proteins during translation. In most of the remaining forms, these splicing alterations resulted in the removal or disruption of functional RNA interaction domain 2 or reverse transcriptase motifs.

Subsequently, we analyzed the frequency and combinatorial pattern of these splicing events in naive splenic cells, concanavalin A (ConA)-stimulated cells, splenic cells 12 days after transformation by v-Rel, and v-Rel-transformed cell lines of splenic origin in order to determine if differences in the telomerase activity in these cells correlate with the frequency of alternatively spliced forms (Table 3). The percentages of the isoforms with alternative splicing A and without A were established in separate measurements. The alternatively spliced forms were the most abundant. Thirty-six distinct combinations of one to six splicing events per molecule were detected. Full-length wild-type (WT) forms were not detected in naive splenic cells, suggesting that they represented less than 10% of the TERT mRNA. Activation of splenic cells by ConA was accompanied by an activation of Rel/NF-κB and resulted in a fivefold increase in telomerase activity (data not shown). Simultaneously, in these cells the amount of the WT TERT mRNA increased to 10% of that of the A forms. The transformation of splenic cells with v-Rel led to more than a 10-fold increase in telomerase activity (Fig. 1C), and the percentage of WT forms was elevated to 60% of the A forms. In established v-Rel-transformed splenic cell lines (123/12, 160/2, and 123/6T), high percentages of WT TERT transcripts were also detected (10 to 50% of A forms), albeit at a lower frequency than in recently transformed cells. The somewhat lower abundance of the WT TERT transcripts correlated with the level of telomerase activity, which was also lower in established cell lines.

TABLE 3. Pattern of alternatively spliced isoforms of TERT in chicken cells

<table>
<thead>
<tr>
<th>Cell group</th>
<th>Analysis of TERT transcripts&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Without splicing A (A&lt;sup&gt;–&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>With splicing A (A&lt;sup&gt;+&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>IF form (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>PT form (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ratio&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spleen</td>
<td>WT (0)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>BD2 (10)</td>
<td>60:40</td>
</tr>
<tr>
<td></td>
<td>B (50)</td>
<td>BD2F (10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D (10)</td>
<td>CD (10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CDF (10)</td>
<td>CD (10)</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>WT (10)</td>
<td>BCE (20)</td>
<td>70:30</td>
</tr>
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<td>B (20)</td>
<td>AB (60)</td>
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<tr>
<td></td>
<td>H (10)</td>
<td>ADF (20)</td>
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<td></td>
<td>ABD (60)</td>
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<td>BLE (8)</td>
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<td>I (31)</td>
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<td>160/2</td>
<td>WT (10)</td>
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<td></td>
<td>ABD (25)</td>
<td>AIF (9)</td>
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<tr>
<td></td>
<td>AI2F (9)</td>
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<sup>a</sup>The regions of TERT gene spanning all the known alternative splicing sites were RT-PCR amplified and cloned and 10 to 15 clones from each sample sequenced.  
<sup>b</sup>Lymphocytes from spleens of 3-week-old chickens, freshly isolated, stimulated for 2 days with ConA (50 μg/ml), or infected for 12 days with REV-T, as well as three v-Rel cell lines (123/12, 160/2, and 123/6T), were analyzed.  
<sup>c</sup>The alternatively spliced variants with and without alternative splicing A were detected in two separate PCR amplifications utilizing the same 3' primer and two different 5' primers as described in Materials and Methods.  
<sup>d</sup>TERT isoforms spliced in-frame.  
<sup>e</sup>TERT isoforms which encode prematurely terminated proteins.  
<sup>f</sup>Ratio of IF forms to PT forms.  
<sup>g</sup>WT, full-length wild-type forms.
FIG. 3. Decrease of nuclear inhibitors of telomerase during v-Rel transformation. Telomerase activity was determined using the cytoplasmic (CYTO) and nuclear (NUCL) fractions obtained from purified splenic lymphocytes before infection (lanes 1 to 8), and at 3 days (lanes 9 to 16) and 7 days after infection (lanes 17 to 24) with REV-T. Single-tube extension-PCR TRAP assays were performed using aliquots of cytoplasmic or nuclear extracts containing 100 ng (dilution 1), 20 ng (dilution 1/5), 4 ng (dilution 1/25), and 800 pg (dilution 1/125) of total protein. Molecular weights, positions of the TRAP PCR products, and a PCR internal control (IC) are indicated in the margins.

of lymphocytes is increased by the suppression of telomerase inhibitors present in uninfected cells.

Telomerase activity protects v-Rel-transformed cells against apoptosis. To determine whether telomerase expression is important for the maintenance of v-Rel-transformed lymphoid cells, telomerase activity in v-Rel-transformed cells was inhibited with 2,6-bis[3-(N-piperidino)propionamido]anthracene-9,10-dione (telomerase inhibitor V; Calbiochem, La Jolla, CA) and their survival evaluated (Fig. 4; Table 4). Telomerase inhibitor V belongs to the new generation of telomerase inhibitors from the G-quadruplex DNA ligand family (64). Telomeres contain a G-rich 3' overhang that can adopt an intramolecular G-quadruplex structure, which blocks the catalytic reaction of telomerase when stabilized by a G-quadruplex ligand. The exponentially growing v-Rel lymphoid cell lines 123/12, 160/2, 160/8, 123/6, and 123/6T were treated with a 5, 10, or 20 μM concentration of the inhibitor dissolved in culture medium or left untreated. The avian macrophage-like cell line HD11 and the T-cell line MSB1, which both express undetectable or very low levels of telomerase activity, served as controls. Telomerase activity was determined at 24 h after treatment using the TRAP assay, and the extent of apoptosis was determined by measuring the DNA content of these cells by flow cytometry 48 h after treatment with the inhibitor (Fig. 4A and B; Table 4). Telomerase activity was inhibited in cells exposed to the telomerase inhibitor, and an increased number of apoptotic cells was detected in v-Rel-transformed cell lines at a dose as low as 5 μM. The proportion of apoptotic cells increased significantly with increasing concentrations of the telomerase inhibitor (Table 4). At a concentration of 20 μM, the inhibitor induced apoptosis in 37 to 85% of the v-Rel-transformed cells. By contrast, the MSB1 and HD11 cell lines were not affected by exposure to even the highest concentrations of the inhibitor evaluated. DNA fragmentation analysis was performed to confirm that death of the v-Rel-transformed cells was due to apoptosis (data not shown).

Telomerase was also inhibited by decreasing TERT and TR RNA by siRNA technology (Fig. 4C and D). Two siRNAs specific for chicken TERT sequences and one TR-specific siRNA were delivered alone or in combination into the 123/12 v-Rel-transformed cell line. The level of TERT mRNA was decreased up to 40% (Fig. 4C). The level of TR decreased under the detection threshold. This inhibition correlated with the induction of apoptosis in these cells, while negative control siRNA had no effect on cell viability. The highest increase of apoptosis (120% above negative control) was reached by transfection of TE2 siRNA. These results suggest that telomerase activity in v-Rel lymphoid cell lines is necessary for maintenance of transformation by protecting these cells against apoptosis.

Expression of v-Rel increases sensitivity of cells to telomerase inhibition. In contrast to the HD11 and MSB1 cell lines, the v-Rel cell lines are extremely sensitive to inhibition of telomerase. There are two possibilities: either the target cells for v-Rel transformation are very sensitive to telomerase inhibition or v-Rel expression increases this sensitivity. To determine whether v-Rel expression is directly responsible for the high sensitivity to telomerase inhibition, HD11 cultures were infected by v-Rel-expressing retroviruses or control helper virus, and sensitivity to telomerase inhibitor V was determined.
FIG. 4. Induction of apoptosis in v-Rel-transformed cells as a result of inhibition of telomerase activity. (A) Telomerase activity in whole-cell extracts from v-Rel cell line 123/6T after a 24-h treatment with various concentrations of the telomerase inhibitor V. Telomerase activity was determined by the single-tube extension-PCR TRAP assay with extract aliquots containing 30 ng of total protein. TRAP products were quantitated with a Fluorimager, and the percentage of surviving telomerase activity is shown in the top part of the panel. Molecular sizes, positions of the TRAP PCR products, and a PCR internal control (IC) are indicated in the margins. Similar results were obtained with 123/12 and 160/2 v-Rel cell lines. (B) Apoptosis in v-Rel cell lines treated with telomerase inhibitor V. The extent of apoptosis in the 123/12, 160/2, and 123/6T v-Rel cell lines and the HD11 cell line after a 48-h treatment with telomerase inhibitor V was determined by measuring the DNA content in ethanol-fixed cells by propidium iodide staining and flow cytometry. The data shown in these graphs correspond to the numerical results presented in Table 4. (C) Decrease in expression of TERT and TR in the 123/12 v-Rel cell line after siRNA treatment. The 123/12 cell line was electroporated with TE2, TE4, and TR1 siRNAs (2 μg each) or a control 1NC siRNA (6 μg). RNA was harvested 24 h after electroporation, and the levels of TERT, TR, and GAPDH were analyzed by RT-PCR as described in the legend to Fig. 2. (D) Apoptosis of the 123/12 v-Rel-transformed cell line treated with TERT and TR siRNA. The 123/12 cell line was electroporated individually with TE2, TE4, TR1, and 1NC (negative control) (6 μg/reaction) or with a combination of TE2, TE4, and TR1 siRNAs (2 μg of each/reaction). Cells were harvested 24 h after electroporation for analysis of apoptosis by flow cytometry. Results are shown as percentages of an increase in apoptosis above negative control level (0%). The mean and standard error were calculated from three independent experiments. (E) v-Rel increases the sensitivity of cells to telomerase inhibition. HD11 cells were infected by REV-T (v-Rel) or infected with CSV (VC). Five days after infection, cells were treated with a 20 μM concentration of telomerase inhibitor V or left untreated. The extent of apoptosis in these cell lines was determined after a 48-h treatment with the inhibitor by measuring DNA content in ethanol-fixed cells by propidium iodide staining and flow cytometry.
example of one of three similar experiments.

itor V for 48 h or left untreated. The experiment presented is a representative example of one of three similar experiments.

Table 4. Apoptotic death in cell lines treated with telomerase inhibitor

<table>
<thead>
<tr>
<th>Cell line</th>
<th>v-Rel</th>
<th>% of apoptotic cells at the following concn of inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 μM</td>
</tr>
<tr>
<td>MSB1</td>
<td>–</td>
<td>0.7</td>
</tr>
<tr>
<td>HD11</td>
<td>–</td>
<td>4.3</td>
</tr>
<tr>
<td>123/12</td>
<td>+</td>
<td>3.7</td>
</tr>
<tr>
<td>160/2</td>
<td>+</td>
<td>3.0</td>
</tr>
<tr>
<td>160/8</td>
<td>+</td>
<td>10.4</td>
</tr>
<tr>
<td>123/6</td>
<td>+</td>
<td>4.2</td>
</tr>
<tr>
<td>123/6T</td>
<td>+</td>
<td>6.0</td>
</tr>
</tbody>
</table>

A Cell lines were treated with various concentrations of the telomerase inhibitor V for 48 h or left untreated. The experiment presented is a representative example of one of three similar experiments.

B Five v-Rel-transformed cell lines and two control cell lines without v-Rel were employed.

Phenotypes: B, B lymphocytes; T, T lymphocytes; nBnT, non-B/non-T lymphocytes; Mf, macrophages.

D Cells were fixed in ethanol, treated with RNase, and stained by propidium iodide, and DNA content was determined by flow cytometry. Data show the proportion of cells (%) that had a DNA content lower than cells in the G1 phase of the cell cycle and were, therefore, considered apoptotic.

(Fig. 4E). Five days after infection, HD11 cells expressed high levels of v-Rel and telomerase activity increased more than 20 times relative to that of cells expressing the helper virus alone (Fig. 1E). The level of apoptosis in these v-Rel-expressing cells was higher (19.2%) than that in cells infected with the helper virus (1.7%) (Fig. 4E). When v-Rel-expressing cells were exposed to a 20 μM concentration of the telomerase inhibitor, the extent of apoptosis increased to 81.1% as determined by flow cytometry after 2 days of treatment. By contrast, the number of apoptotic cells in the control cultures showed only a slight increase (from 1.7% to 2.7%). These results demonstrate that v-Rel expression increases the sensitivity of cells to telomerase inhibition.

v-Rel induces reactive oxygen species. The above results suggest that transformation of lymphoid cells by v-Rel may lead to the induction of proapoptotic factors which may be partially negated by the simultaneous induction of telomerase activity. Reactive oxygen species (ROS) are one candidate with these characteristics. Increased production of ROS is used during defense reactions, in which members of the Rel/NF-κB family play a critical role (17). ROS also induce telomere damage followed by apoptosis, while telomerase has the ability to protect against hydroxyl radical-induced apoptosis (68, 82). To establish if v-Rel-transformed lymphoid cells have high levels of ROS, the levels of reactive oxygen molecules in five v-Rel-transformed cell lines and in three other avian lymphoid cell lines (MSB1, DT95, and DT40) were determined by flow cytometry of dichlorodihydrofluorescein diacetate (H2DCFDA)-exposed cells (Fig. 5A). The level of ROS was high in all v-Rel-transformed cell lines examined relative to that in MSB1 T cells, and four out of five cell lines had higher ROS levels than DT95 and DT40 B cells. The levels of ROS in v-Rel cell lines correlated with their sensitivity to apoptosis induced by the telomerase inhibitor. The 160/8 cell line, which is the most sensitive to exposure to the telomerase inhibitor, had the highest levels of ROS (Table 4; Fig. 5A). To evaluate whether v-Rel expression leads to higher production of ROS in cells, ROS levels were determined in CEFs and in HD11 cells after their transformation by v-Rel (Fig. 5B). The levels of ROS were more than twofold higher in v-Rel-transformed CEFs than in untransformed cells and four times higher in HD11 cells expressing v-Rel than in control cells, indicating that v-Rel expression increases ROS levels in different cell types.

To assess the mechanism by which v-Rel induces ROS, the levels of mRNA of several genes involved in the production of ROS were determined in HD11 macrophage cells expressing v-Rel (Fig. 5C). NADPH oxidase represents the major system responsible for increased production of ROS in phagocytes during a defense response against invading microorganisms (16). The steady-state mRNA levels of several subunits of the NADPH complex specific to hematopoietic cells (p40phox, p47phox, and Rac1) increased as a result of v-Rel expression. Another type of reactive molecules produced in immune cells and detected by H2DCFDA staining is peroxynitrite anions, which are formed by the reaction of ROS with nitric oxide (R. P. Haugland, Handbook of fluorescent probes and research products, 9th edition; Molecular Probes, Inc., Eugene, OR). The nitric oxide present in immune cells is a product of iNOS (15). Northern analysis showed that in v-Rel-expressing cells, the level of iNOS mRNA was strongly upregulated. Finally, we analyzed the expression of genes whose products are responsible for ROS degradation. The majority of ROS are degraded by MnSOD and catalase. MnSOD converts the superoxide anion to H2O2, a reactive intermediate product, which is further processed by catalase (8). c-Rel is known to increase MnSOD expression without increasing of the levels of catalase, leading to the accumulation of H2O2 and subsequent proliferation arrest or apoptosis of c-Rel-overexpressing cells (8, 9). Similarly, in our experiment v-Rel induced the expression of MnSOD, eliminating the negative effect of helper virus and increasing the MnSOD level above that detected in uninfected cells. v-Rel did not, however, alter the level of expression of catalase, possibly allowing for H2O2 accumulation because the ROS increased by the NADPH system would not be properly degraded. Collectively, these results demonstrate that v-Rel upregulates the mRNA of several members of the oxygen and nitrogen species regulating systems, which could lead to an increase in their levels in cells.

The expression of TERT increases resistance of chicken cells to challenge by ROS. TERT expression has been shown to protect several cell types against apoptosis induced by oxidative challenge (70). Therefore, it is plausible that v-Rel-induced telomerase activity contributes to protection of transformed cells against apoptosis resulting from increased levels of ROS in v-Rel-expressing cells. However, the ability of chicken TERT to protect against ROS has not been established. Therefore, we cloned the avian TERT gene, overexpressed it from a retrovirus vector in HD11 cells, and challenged these cells with t-butyl hydroperoxide (t-BHP), which produces oxidative radicals (Fig. 6). HD11 cells infected with the retrovirus expressing TERT had high telomerase activity. The extent of apoptosis in these cells was increased only two-fold after treatment by t-BHP, while t-BHP treatment of HD11 cells infected with the helper virus resulted in a fivefold increase in apoptosis. These results demonstrate that like human TERT, chicken TERT is able to protect cells against apoptosis induced by oxidative radicals.
DISCUSSION

In this report we demonstrated that chromosomes in v-Rel-transformed cells have longer telomeres than in normal chicken cells and that immortalized v-Rel-transformed cell lines have uniformly high levels of telomerase. v-Rel induces telomerase during the transformation of lymphoid cells by directly upregulating the mRNA levels of the TERT gene and by regulating the pattern of alternative splicing of TERT transcripts. Telomerase activity protects v-Rel-transformed cells from ROS-induced apoptosis.

v-Rel induces the expression of TERT mRNA and TR. The elevated activity of telomerase observed in v-Rel-transformed cells is consistent with the increased expression of TERT and TR subunits of the telomerase complex. The TERT subunit is the main target of the pathways which regulate telomerase activity (20). The increase in TERT expression is a direct result of the activity of v-Rel, since v-Rel is able to upregulate TERT expression independently of protein synthesis. v-Rel binds to the two κB sites located in the TERT promoter and may also increase the expression of TERT by using binding sites of other transcription factors. v-Rel and other Rel/NF-κB proteins interact with the Sp1 transcription factor, and v-Rel is capable of increasing transcription activity from Sp1 binding sites (63, 73). Several Sp1 sites are located close to the transcription start site in the promoter region of the chicken TERT gene.

The expression of TR RNA was substantially increased in v-Rel-transformed lymphocytes. Levels of TR are also often elevated in human tumors (65, 93). The increased steady-state levels of TR transcripts detected in v-Rel-transformed cells may be due to both increased expression and the stabilization of TR RNA by the increased levels of TERT.

v-Rel regulates the TERT gene by alternative splicing. The chicken TERT gene is extensively regulated by alternative splicing. In normal splenic cells, alternative spliced forms of TERT which encode nonfunctional molecules are by far the most abundant transcripts detected. In contrast, in v-Rel tumor cells, which contain elevated levels of telomerase activity, a large proportion of the TERT transcripts would encode func-

FIG. 5. Increased levels of ROS in v-Rel-transformed cells. (A) Quantitative comparison of ROS levels in five v-Rel-transformed lymphoid cell lines (123/6, 123/6T, 160/2, 160/8, and 123/12) with the levels of ROS in avian lymphoid cell lines transformed by other mechanisms (MSB1, DT95, and DT40). Cells were incubated in 5 μM H2DCFDA for 30 min, and the conversion of the nonfluorescent to the fluorescent form was determined by flow cytometry. The mean and standard error were calculated from three independent experiments. (B) Levels of reactive oxygen species in fibroblasts (CEF) and in HD11 cells expressing v-Rel. ROS levels were determined in uninfected (uninf.) fibroblasts and in CEF cultures infected for 10 days with DS-vRel(A) (v-Rel) or the corresponding vector control DS(A) (VC) by H2DCFDA staining and flow cytometry. The measured fluorescence intensities of v-Rel-transformed and vector control-infected cells from three independent experiments were standardized using the fluorescence intensity of a parallel culture of uninfected fibroblasts treated identically. The mean and standard error were then calculated for infected cultures and are shown as a bar graph. The horizontal line represents the relative fluorescent intensity of uninfected cultures. HD11 cells were infected by REV-T (v-Rel) or infected with the helper virus CSV (VC). The levels of ROS were determined 5 days after infection as described for fibroblasts except that standardization to the fluorescence intensity of uninfected HD11 cells was not used. (C) Changes in the expression of genes responsible for ROS production (ROS produc.) and degradation (ROS degrad.) in v-Rel-expressing HD11 cells. RNA was isolated from HD11 cells (UN) or HD11 cells infected by CSV (VC) or REV-T (v-Rel) 4 days after infection and analyzed by Northern blotting using 10 μg total RNA per lane. The probes used for detection are indicated.
tional TERT. Likewise, in most human cells that have low telomerase activity, alternatively spliced TERT transcripts are the most abundant; and in human tumors with high telomerase activity, WT TERT mRNA becomes the dominant transcript (46, 80, 92). The pathways which regulate the alternative splicing of TERT have not been identified.

Sixteen distinct alternatively spliced chicken TERT transcripts have been detected. The alternatively spliced variants of chicken TERT are all distinct from the seven described human forms. While both genes contain 16 exons, the chicken splice variants involve exons 2 to 11 while the human splice variants described involve exons 5 to 14 (33, 40). All of the alternatively spliced human forms are suggested to be inactive or to function as dominant-negative forms (19, 90). Interestingly, the alternatively spliced forms of TERT, containing new stop codons which would encode prematurely terminated proteins, are replaced by alternatively spliced forms with in-frame-spliced TERT transcripts during transformation or activation of chicken lymphocytes. The prematurely terminated mRNA may be recognized by the nonsense-mediated decay mechanism during initial translation and be rapidly degraded (32). In contrast, in-frame-spliced TERT transcripts could be translated. A recent report indicates that TERT protects cells against apoptosis in a p53-dependent manner and does not require its catalytic domain (66). Therefore, it is possible that some of the chicken alternative spliced in-frame forms may protect against apoptosis in a similar manner or may acquire full telomerase activity by assembling complementing dimers.

Role of telomerase activity in v-Rel transformation. Telomerase activity contributes to transformation in two ways (13, 14, 54). First, by adding telomeric repeats to the ends of chromosomes, telomerase prevents cells from undergoing apoptosis or senescence triggered by the shortening of the telomeres. Secondly, telomerase also inhibits apoptosis triggered by DNA-damaging agents by a still largely unknown mechanism. v-Rel-induced telomerase activity apparently is important for cell transformation in both ways. Telomeres in v-Rel cell lines are longer than those in nontransformed chicken lymphocytes, suggesting that v-Rel transformation of hematopoietic cells is not accompanied by a mortality crisis induced by short telomeres. However, despite their “well-maintained” telomeres, v-Rel cell lines require the constitutive antiapoptotic function of telomerase for their survival. The apoptotic death of v-Rel-transformed lymphoid cells within 24 h after telomerase inhibition is unlikely to be the result of the shortening of telomeric sequences below a critical level due to the end replication problem. The increased levels of ROS induced by v-Rel coincide with the increased sensitivity of these cells to inhibition of telomerase, and the ectopic expression of TERT protects cells against oxidative challenge. Taken together, these results are consistent with a model which suggests that telomerase activity is necessary to increase the threshold for an apoptotic response induced by ROS. Higher telomerase activity would increase the maintenance of telomeres otherwise damaged by free radicals, which often result in apoptosis or senescence (31, 83). Telomeric DNA is known to be more sensitive to damage by oxidative stress than nontelomeric DNA; therefore, telomeric DNA may function as a sensor of DNA damage (83). Differential levels of telomerase may establish the sensitivity of this sensor. Reducing the sensitivity of cells to ROS by increasing telomerase activity would therefore protect v-Rel-transformed cells from apoptosis induced by high levels of ROS.

Parallel between v-Rel transformation and chronic inflammation. The expression of most oncogenes does not result in the activation of telomerase (84). The rapid induction of telomerase during v-Rel-mediated transformation is consistent with the unprecedented ability of v-Rel to efficiently transform and immortalize lymphoid cells in vitro. The Rel/NF-κB family plays a major role in the activation of genes leading to an inflammatory response (81). The events which occur in cells during transformation by v-Rel resemble many of the events which occur during an inflammatory response. v-Rel induces the expression of inflammatory cytokines, such as interleukin 1 (IL-1), IL-6, IL-8 (CEF-4), and MIP1β and increases the levels of ROS (64a; also data not shown). The parallels with human carcinogenesis are significant. The activation of human telomerase by Rel/NF-κB is attributed to IL-6 and TNF-α signaling (2, 3). These pathways are accompanied by increased levels of ROS. In human tumors, telomerase activity is accompanied by the expression of inflammatory cytokines which occur during an inflammatory response. v-Rel-induced telomerase activity apparently is important for cell transformation in two ways (13, 14, 54). First, by adding telomeric repeats to the ends of chromosomes, telomerase prevents cells from undergoing apoptosis or senescence triggered by the shortening of the telomeres. Secondly, telomerase also inhibits apoptosis triggered by DNA-damaging agents by a still largely unknown mechanism. v-Rel-induced telomerase activity apparently is important for cell transformation in both ways. Telomeres in v-Rel cell lines are longer than those in nontransformed chicken lymphocytes, suggesting that v-Rel transformation of hematopoietic cells is not accompanied by a mortality crisis induced by short telomeres. However, despite their “well-maintained” telomeres, v-Rel cell lines require the constitutive antiapoptotic function of telomerase for their survival. The apoptotic death of v-Rel-transformed lymphoid cells within 24 h after telomerase inhibition is unlikely to be the result of the shortening of telomeric sequences below a critical level due to the end replication problem. The increased levels of ROS induced by v-Rel coincide with the increased sensitivity of these cells to inhibition of telomerase, and the ectopic expression of TERT protects cells against oxidative challenge. Taken together, these results are consistent with a model which suggests that telomerase activity is necessary to increase the threshold for an apoptotic response induced by ROS. Higher telomerase activity would increase the maintenance of telomeres otherwise damaged by free radicals, which often result in apoptosis or senescence (31, 83). Telomeric DNA is known to be more sensitive to damage by oxidative stress than nontelomeric DNA; therefore, telomeric DNA may function as a sensor of DNA damage (83). Differential levels of telomerase may establish the sensitivity of this sensor. Reducing the sensitivity of cells to ROS by increasing telomerase activity would therefore protect v-Rel-transformed cells from apoptosis induced by high levels of ROS.

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of ROS and consequently an increased probability that the cells will undergo apoptosis. The activation of telomerase by Rel/NF-κB may partially protect these cells from the negative effects of ROS by delaying the induction of apoptosis to allow for DNA repair as well as contributing to telomere maintenance. However, the long-term activation of genes involved in the inflammatory response may eventually result in DNA mutations which ultimately lead to cancer development (89). Chronic inflammation is frequently associated with the development of many cancer types, including pancreatic, liver, stomach, and lung (1). The fact that v-Rel, the oncogenic member of the Rel/NF-κB family, simultaneously activates ROS and telomerase provides an additional rationale for the recent focus of anticancer therapies based on the inhibition of Rel/NF-κB (11, 39, 50).

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