Regulation of Telomerase Activity by Interferon Regulatory Factors 4 and 8 in Immune Cells[▽]

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Telomerase activity is downregulated in somatic cells but is upregulated during the activation of cells of the immune system. The mechanism of this reactivation is not well understood. In this study, we demonstrated that interferon regulatory factor 4 (IRF-4) and, to a lesser extent, IRF-8 induce telomerase activity. The suppression of IRF-4 results in decreased levels of TERT (telomerase reverse transcriptase) mRNA and telomerase activity and reduces cell proliferation. The overexpression of TERT compensates for this proliferation defect, suggesting that telomerase contributes to the regulation of cell proliferation by IRF-4. The induction of telomerase by IRF-4 and IRF-8 correlates with the activation of the TERT promoter. IRF-4 binds the interferon response-stimulated element and the gamma interferon-activated sequence composite binding site in the TERT core promoter region in vivo. Additionally, the binding of Sp1, Sp3, USF-1, USF-2, and c-Myc to the TERT promoter is elevated in cells expressing IRF-4. IRF-4, but not IRF-8, synergistically cooperates with Sp1 and Sp3 in the activation of the TERT promoter. Collectively, these results indicate that IRF-4 and IRF-8, two lymphoid cell-specific transcription factors, increase telomerase activity by activating TERT transcription in immune cells.

Telomerase is a ribonucleoprotein complex with reverse transcriptase enzymatic activity which is responsible for adding TTAGGG telomeric repeats to the ends of chromosomes (15). Telomerase activity is high during embryogenesis and in stem cells but is downregulated in adult organisms, resulting in the gradual shortening of telomeres, which eventually leads to cell senescence or apoptosis (13). The downregulation of telomerase in adult tissues is considered to play a role in protection against cancer, because high telomerase activity is a prerequisite for indefinite growth of tumor cells and their protection against apoptosis (5, 56). More than 90% of human tumors express high telomerase activity (27). However, telomerase is also reactivated during wound healing and is necessary for the development and function of the immune system (11, 18, 69). Telomerase is upregulated during B- and T-cell activation, and high levels of telomerase activity are expressed in thymocytes and germinal-center B cells (37, 70). A dramatic increase in telomerase activity has also been observed during dendritic-cell differentiation (51). The most important component of the telomerase complex, which is responsible for enzymatic activity, is TERT (telomerase reverse transcriptase) (15). Several transcription factors, including c-Myc, Sp1, Sp3, and estrogens, play a role in the regulation of the telomerase promoter during cell proliferation and reproduction (43, 48, 66). However, the mechanism by which telomerase activity is transcriptionally regulated during the immune response is not known.

Interferon regulatory factors 4 and 8 (IRF-4 and IRF-8) belong to a family of transcription factors with a helix-turn-

helix DNA-binding motif, which bind to promoter elements that contain AANNGAAA consensus binding sites and which either stimulate or repress transcription (12). These two factors are closely related, and their expression is principally restricted to cells of the immune system, where they regulate different stages of B-cell, T-cell, dendritic-cell, and macrophage development (62).

In this study, we report that TERT is transcriptionally regulated by IRF-4 and IRF-8. The expression of IRF-4 and IRF-8 resulted in increased levels of TERT mRNA and telomerase activity in chicken embryonic fibroblasts (CEFs) and the HD11 macrophage cell line. IRF-4 expression also upregulates telomerase activity in splenic lymphocytes and the DT40 B-cell line. Suppression of endogenous IRF-4 leads to a decrease in telomerase activity and cell proliferation. The overexpression of TERT, but not a catalytically inactive mutant, abolishes the proliferation defect in cells where IRF-4 is suppressed. The expression of IRF-4 and IRF-8 activates the TERT promoter. IRF-4 binds the interferon response-stimulated element (ISRE) and gamma interferon-activated sequence (GAS) composite binding site in the TERT core promoter region in vivo. Additionally, electrophoretic mobility shift assays (EMSAs) and chromatin immunoprecipitation (ChIP) experiments demonstrated that Sp1, Sp3, USF-1, USF-2, and c-Myc binding to the TERT promoter is elevated in IRF-4-expressing cells. Moreover, IRF-4, but not IRF-8, synergistically cooperated with Sp1 and Sp3 in activation of the TERT promoter. Together, these results indicate that IRF-4 and IRF-8, two lymphoid cell-specific transcription factors, increase telomerase activity by activating TERT transcription in immune cells.

MATERIALS AND METHODS

Expression vectors. The open reading frame portions of chicken genes encoding IRF-1, IRF-8, USF-1, and USF-2 were amplified from splenic lymphocyte RNA by reverse transcription-PCR (RT-PCR) and cloned into pGEM-T Easy (Promega, Madison, WI). The pcDNA expression plasmids were generated by

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cloning these genes as well as the previously described chicken IRF-4 and c-Myc genes (20, 21) between the XhoI and BamHI (IRF-1, -4, and -8 and c-Myc), XhoI and HindIII (USF-2), and EcoRI (USF-1) sites of pcDNA3.1 with the appropriate orientation (Invitrogen, Carlsbad, CA). The pcDNA plasmids expressing chicken Sp1 and Sp3 were constructed by cloning the HindIII-NotI (Sp1) and HindIII-EcoRI (Sp3) fragments from pH-ΔGC Sp1 and pH-ΔGC Sp3 (8) into pcDNA3.1(+) (Invitrogen). The REV-T-based retroviral constructs pREV-IRF-4 and pREV-TERT, the pREV-0 empty vector, and pCSV11S3, containing an infectious genomic clone of chicken syncytial virus (CSV), have been described (20, 21, 47). pREV-IRF-8 was constructed by inserting the XhoI-NotI fragment from pGEM-IRF-8 into pREV-0. pREV-Sp1 and pREV-Sp3 were constructed by inserting NheI-XhoI fragments from pcDNA-Sp1 and pcDNA-Sp3 between SpeI and XhoI sites of pREV-SNs (pREV-0 with a modified polylinker). pREV-DN-TERT was constructed by site-directed mutagenesis of the chicken TERT variant that is missing 17 chicken-specific N-terminal amino acids. Two codons encoding aspartic acids in the RT domain corresponding to amino acids 1083 to 1084 of TERT (GenBank no. NP_001026178.1) were converted to two codons encoding alanines. The REV stocks were generated by cotransfection of CEFs with pREV-IRF-4, pREV-IRF-8, pREV-Sp1, pREV-Sp3, pREV-TERT, or pREV-DN-TERT with pCSV11S3, and the CSV was generated by transfection of the pCSV11S3 plasmid alone.

TERT promoter luciferase constructs. To analyze the activity of the chicken TERT promoter, a 3.9-kb region upstream of the translation start site was obtained by PCR amplification from chicken genomic DNA isolated from CEFs. A series of 5'-deletion mutants was constructed from the cloned PCR fragment by digestion-ligation using conveniently located sites for ApaI, PvuII, HindIII, and BstXI and/or by PCR amplification with various primers. These deletion mutants as well as the complete 3.9-kb region of TERT were cloned upstream of the luciferase reporter gene into the pGL3-basic vector (Promega, Madison, WI) between the XhoI and NcoI sites, generating the constructs CP-32, CP-99, CP-378, CP-567, CP-1353, CP-1719, CP-2603, CP-3318, and CP-3899. For the construction of the luciferase reporter plasmids with mutated USF/Myc, GAS/ISRE, ISRE-like, and Sp1/Sp3 binding sites, site-directed mutagenesis was performed on the CP-378 plasmid using the QuikChange Multi site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Antibodies. Several polyclonal and monoclonal antibodies were used in Western blot analysis, EMSA, and ChIP analysis. The A14-6 polyclonal antiserum specific to chicken IRF-4 was described previously (20). Rabbit polyclonal immunoglobulin G (IgG) antibodies sc-8983X against USF-1 (H-86), sc-862X against USF-2 (C-20), sc-59 against Sp1 (PEP2), sc-644 against Sp3 (D-20), sc-764X against chicken and human c-Myc (N-262), and sc-1698 against Stat6 and mouse monoclonal antibody sc-42 against c-Myc (C-33) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Normal rabbit IgG (sc-2027; Santa Cruz) and normal rabbit serum were used as controls. The A18-5 polyclonal antiserum against chicken IRF-8 was raised in a rabbit immunized with a TrpE/IRF-8 fusion protein purified from C600 bacteria transformed by the pATH-IR8#5 expression plasmid. The TrpE/chIRF-8 fusion protein contains amino acids 101 to 357 of chicken IRF-8. For Western blot and ChIP analyses of Sp1, the rabbit antiserum was used (8).

Tissue culture procedures. Embryonated eggs of pathogen-free White Leghorn chickens were obtained from Charles River SPAFAS, North Franklin, CT. CEFs were prepared from 10-day-old embryos. Spleens from 20-day-old chickens were mechanically dissociated with a nylon mesh, and splenic lymphocytes were purified using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). HD11 is a macrophage cell line derived from bone marrow cells infected by myelocytomatosis virus (4). DT40 is B-cell line established from a bursal tumor (2). C4-1 is a v-Rel-transformed non-virus-producing B-cell line (20).

Cells were cultured with Dulbecco's modified Eagle's medium supplemented with 5% fetal calf 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 CEFs were used for transfection of plasmid DNA by a calcium phosphate method as described previously (20). Viruses were harvested between 5 and 7 days after transfection.

Transfections and luciferase assays. Transient transfections were performed using the Eugene HD transfection reagent (Roche Diagnostics, Indianapolis, IN) in Opti-MEM I (Invitrogen) according to the manufacturer's protocol. After 36 h, cell lysates were prepared, and luciferase activity was determined using the Dual-Glo luciferase assay system (Promega). *Renilla* luciferase plasmids were cotransfected as controls to standardize the transfection efficiency.

Cell cycle analysis. Cells were washed and resuspended in 300 µl 5 mM EDTA in phosphate-buffered saline (PBS). Then, 700 µl of 100% ice-cold ethanol was added, and cells were stored at 4°C before the 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 mg/ml) was added, and dye incorporation was determined with a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA). Data were analyzed by CellQuest software provided by the manufacturer. The fluorescence emitted by cell aggregates was excluded based on the theory of doublet discrimination (FACSCalibur systems user's guide; BD Immunocytometry Systems).

EMSAs. Preparation of nuclear extracts and oligonucleotide probes and binding assays were done as described previously (45) with several modifications as specified below. Briefly, 10^7 PBS-washed cells were pelleted and resuspended in 200 µl of an ice-cold solution A [10 mM N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid (HEPES) (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.1 mM dithiothreitol (DTT)] and a set of inhibitors which included 2×-concentrated Complete protease inhibitor cocktail (Roche), 2 mM phenylmethylsulfonyl fluoride, 48 µM calpain inhibitor I, and aprotinin (Sigma, St. Louis, MO). The cells were incubated for 2 min on ice, and then 12 µl of 10% Nonidet P-40 was added, the mixture was vortexed, and nuclei were isolated by centrifugation. The nuclei were washed twice with solution A and resuspended in 200 µl solution C (20 mM HEPES [pH 7.9], 0.4 M KCl, 1 mM EDTA, 1 mM EGTA, 15% glycerol, 1 mM DTT, and the set of inhibitors used to prepare solution A). The nuclei were extracted for 15 min at 4°C and centrifuged at 4°C for 10 min at $10,000 \times g$. The supernatant fluids were aliquoted and stored at -70°C. The concentration of total protein was determined using the Bio-Rad protein assay kit II (Bio-Rad, Hercules, CA).

Oligonucleotide probes were prepared by annealing the oligonucleotide templates to primers complementary to nine nucleotides at the 3' end of the templates, followed by primer extension with the Klenow fragment of DNA polymerase I (Invitrogen) in the presence of [α - 32 P]dCTP. The following templates were used: M (5'-GTGCCACATCCACGTGGCTCTTGAAC-3'), *mutM* (5'-GTGCCACATCCACCGGCTCTTGAAC-3'), G (5'-CACCCTCTTTCCGAAGAAATCTGTTCCTAA-3'), *mutG* (5'-CACCCTCTTCCGCGCAGCGAAGCGTCTGTTCCTAA-3'), I (5'-CCGTGGCAGCCAAACCGCAGCGCAGC-3'), *mutI* (5'-CCGTGGCAGCGCTCCGCGCAGCGCAGC-3'), S (5'-CCTGCCCTCCGCCCGCCCGCCCGCCCTCACCT-3'), and *mutS* (5'-CCTGCCCTCCATTACCACATACCACCTACCTCACCT-3').

For binding assays, 10-µg aliquots of the nuclear extracts were incubated with 2 µg of poly(dC-dI) · poly(dC-dI) in binding buffer for 10 min on ice; then, 10^5 cpm of the probe was added, and the mixture was incubated for an additional 30 min at room temperature. Two binding buffers were used. The HS binding buffer contained 20 mM Tris-HCl [pH 7.8], 1 mM EDTA, 0.1 M KCl, 5% glycerol, 0.1% Nonidet P-40, 1 mg/ml acetylated bovine serum albumin, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1×-concentrated Complete protease inhibitor cocktail (Roche). The MZ binding buffer had the same composition except that the concentration of KCl was decreased to 0.045 M and the buffer was supplemented with 5 mM MgCl₂ and 5 mM ZnSO₄. For supershift analysis, 2 to 6 µl of rabbit antiserum or purified immunoglobulins was added, and incubation was continued for 25 min at room temperature. The complexes were separated from the free DNA probe by electrophoresis, and the dried gels were analyzed by autoradiography or were scanned by an FX molecular imager (Bio-Rad) and quantified using ImageQuant TL (Amersham Biosciences, Piscataway, NJ).

ChIPs. ChIPs were carried out using ChIP-IT Express (Active Motif, Carlsbad, CA). Immunoprecipitated chromatin was analyzed by PCR with the following primers: F1 (5'-CCACCCCCACCTGCCACGAACTCAGTG-3') and R1 (5'-TACATTGCGGAGCCGCGCAGCGCGACTCC-3') for the endogenous TERT promoter, PROMF (5'-TTACGGCTGCTAGCCCGGGCTCGAGATC TG-3') and PROMR (5'-CCAGCGGTTCATCTTCCAGCGGATAGAAATG GC-3') for the exogenous promoter, and GAPDH1 and GAPDH2 (21) as negative-control primers.

RNA interference. Small interfering RNAs (siRNAs), designated IF3 and IF4, targeted the IRF-4 sequences AAGACCAGAUUGAGGUGUCUUU and AACAAGAGCAACGACUUUGAAGA, located 265 and 289 nucleotides downstream of the start codon. These siRNAs and a negative control, 1NC (negative control 1; Ambion, Austin, TX) were synthesized using an siRNA construction kit (Ambion). siRNAs (4 µg) were electroporated into exponentially growing cells (10^5) using siPORT siRNA electroporation buffer (Ambion) at 1 µF and 0.4 kV. After electroporation, cells were placed in regular medium, and the levels of TERT mRNA and telomerase activity were analyzed 24 h later. RNA was isolated by the RNAqueous-Micro kit (Ambion), and the expression level of TERT mRNA was determined by RT-PCR as described below.

Identification of TERT by semiquantitative RT-PCR. cDNA synthesis was performed as described previously (21). For detection of avian TERT, IRF-4, and GAPDH by RT-PCR, 2 µl of the first-strand synthesis reaction mixture was

used together with 1 μ l of Advantage cDNA polymerase mix (BD Clontech Biosciences, Mountain View, CA) or 2.5 U Herculase Hotstart DNA polymerase (Stratagene) and C14F and C16B primers for avian TERT (5'-CTGATACTGC TTGATGCTGCTATTTTATCC-3' and 5'-GATGGTTCGTCACCGTCTTC AGCAGTTC-3') and GAPDH1 and GAPDH2 primers for the GAPDH gene (21). The following primers were used for detection of IRF-4 mRNA: 5'-GAG GAATTTCCAGATCCACAGAGACAGCGA-3' and 5'-TGAACCTTTGATC CCCC AACACATCCAGC-3'. PCRs were performed as follows: 5 cycles of 30 s at 94°C and 3 min at 72°C; 5 cycles of 30 s at 94°C, 30 s at 70°C, and 3 min at 72°C; and 25 to 50 cycles of 30 s at 94°C, 30 s at 65°C, and 3 min at 72°C.

Northern analysis and probes. Total RNA was isolated by RNeasy or TRI reagent (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 [α -³²P]dCTP by nick translation. Membranes were hybridized with these probes using UltraHyb solution (Ambion) at 55°C. The probes used are listed below, with GenBank accession numbers and the positions of the first and last nucleotides of the fragment based on the GenBank sequence in parentheses: Sp1 (AJ317960; 60 to 855), Sp3 (AJ317961; 204 to 711), USF1 (NM_001007485; 31 to 401), and USF2 (AY620401; 45 to 387).

Western analysis. Western analysis was performed as described previously, with several modifications (21). Harvested cells were lysed in sodium dodecyl sulfate (SDS) sample buffer supplemented with the inhibitor set used for preparation of nuclear extracts. For electrophoresis, EZ-Run prestained molecular weight markers from Thermo Fisher Scientific (Waltham, MA) were employed. Blotted proteins were visualized either by colorimetric detection as described previously or with Western Lightning Plus chemiluminescence reagent (PerkinElmer, Waltham, MA) (21).

Coimmunoprecipitation. Nuclear cell extracts were prepared with a nuclear complex Co-IP kit (Active Motif). Protein extracts (500 μ g) were immunoprecipitated with an anti-IRF-4 (5 μ l) antibody, anti-Stat6 antibody (5 μ g), or normal rabbit IgG control (5 μ g) using IP buffer with low stringency. The immunoprecipitates were resolved by 7.5% SDS-polyacrylamide gel electrophoresis and blotted, and the proteins were detected by Western analysis.

TRAP. The level of telomerase activity was evaluated using the telomerase repeat amplification protocol (TRAP) assay as described previously (21). Whole-cell extracts were prepared with CHAPS buffer (27). Protein concentrations were determined by the Bradford method with the Bio-Rad protein assay reagent (Bio-Rad). Protein extracts (0.8 to 20 μ g of total protein) were first incubated with 0.5 μ g of the TS primer and all four deoxynucleoside triphosphates (1 mM each) in 1 $\times$ TRAP reaction buffer, 0.8 mM spermidine, and 5 mM β -mercaptoethanol in a total reaction volume of 50 μ l for 30 min at 37°C. The reaction was stopped by incubation at 94°C for 2 min. Aliquots of synthesis (2.5 μ l) were then PCR amplified as described by Kim and Wu (28), except that 0.1 μ g unlabeled TS primer and 1 μ l of Advantage cDNA polymerase mixture (BD Biosciences Clontech) were used. PCR amplification started with 94°C for 2 min, followed with 33 to 35 cycles (30 s at 94°C, 30 s at 50°C, and 1 min at 72°C). The TRAP PCR products were separated on 7.5% acrylamide gels (ratio of acrylamide to bis-acrylamide, 19:1). Gels were stained with Vista green (Amersham Biosciences), and images were captured using an FX molecular imager (Bio-Rad). For molecular weight determination, a 10-bp ladder (Invitrogen) was used.

Nucleotide sequence accession number. The sequence of the chicken TERT promoter was submitted to GenBank under accession number EU650197.

RESULTS

Telomerase activation by IRF-4 and IRF-8 correlates with an increase in TERT transcription. Telomerase is activated during the immune response, but the factors involved in the regulation of telomerase activity in immune cells have not been identified. Our previous work demonstrated that overexpression of IRF-4 significantly prolongs the life span of primary avian cells (20). IRF-4 and its closely related family member, IRF-8, are transcription factors specifically expressed in cells of the immune system and therefore may participate in the regulation of telomerase activity. To test whether IRF-4 and IRF-8 contribute to the telomerase regulation, the levels of telomerase activity were determined in cells expressing IRF-4 or IRF-8.

Initially, the analyses were performed with CEFs and the macrophage cell line HD11. CEFs are primary cells and have low telomerase activity relative to most transformed cell lines. The HD11 cells were included because this macrophage cell line is the important model for the study of activation of macrophages, the process in which IRF-4 and IRF-8 play an important role (42). HD11 cells represent nonactivated macrophages with low endogenous levels of IRF-4, which are increased together with telomerase activity during activation (data not shown).

CEFs and the macrophage cell line HD11 were infected with retroviral vectors expressing IRF-4 or IRF-8 or with helper virus, and the cells were harvested 3 days after infection. Western blot analysis confirmed that IRF-4 or IRF-8 was overexpressed in these cells (Fig. 1A). Telomerase activity was determined by the TRAP assay. The levels of telomerase activity were significantly increased in both cell types expressing IRF-4 or IRF-8 relative to control cells. IRF-4 activated telomerase activity more efficiently than IRF-8.

Reverse transcriptase (TERT) is an essential component of the telomerase enzymatic complex. To assess the mechanism by which IRF-4 and IRF-8 increase telomerase activity, the levels of TERT mRNA were analyzed by RT-PCR in cells expressing IRF-4 or IRF-8 (Fig. 1B). Total RNA was harvested from CEFs and HD11 cells 3 days after infection with retroviruses expressing IRF-4 or IRF-8 and from control cells infected with the helper virus. The level of TERT mRNA was significantly increased in IRF-4- and IRF-8-expressing cells, suggesting that the activation of TERT transcription contributes to the elevated activity of telomerase observed in IRF-4- or IRF-8-expressing cells.

The induction of telomerase activity by IRF-4 and IRF-8 was also evaluated in chicken splenic lymphocytes and in the chicken lymphoid cell line DT40 (Fig. 1C). Splenic lymphocytes were chosen because they are primary cells, and IRF-4 plays a key role during their activation (50). The DT40 B-cell line represents an important IRF-4 cellular target. Splenic lymphocytes from a 20-day-old chicken and DT40 cells were infected with retroviruses expressing IRF-4 or a helper virus control. Cells were assayed for telomerase activity 48 h after infection. The increased expression of IRF-4 correlated with induction of telomerase activity, demonstrating that in these hematopoietic cells, IRF-4 induces telomerase.

Telomerase contributes to IRF-4-mediated proliferation. To determine whether the endogenous levels of IRF-4 maintain telomerase activity, IRF-4 expression was decreased by siRNA technology in the v-Rel-transformed B-cell line C4-1. siRNAs targeting two different regions of IRF-4 were transfected into C4-1 cells, and TERT mRNA levels and telomerase activity were determined after 24 h (Fig. 2A). The results indicate that the levels of TERT mRNA and telomerase activity were reduced simultaneously with the reduction in IRF-4 expression, suggesting that the expression of endogenous IRF-4 contributes to the level of telomerase activity in immune cells. Moreover, the cells with decreased IRF-4 levels proliferated more slowly, which is in agreement with our previous observations that the proliferation of C4-1 B cells is significantly decreased by inhibition of IRF-4 expression by antisense IRF-4 RNA (20). Fluorescence-activated cell sorting analysis of the percentage of cells with a DNA content lower than that of the G₁

FIG. 1. Increased telomerase activity in cells expressing IRF-4 or IRF-8 correlates with enhanced expression of TERT mRNA. (A) IRF-4 and IRF-8 expression increased telomerase activity. CEFs and HD11 cells were infected with retroviruses expressing either IRF-4 or IRF-8 or with helper virus (H). Extracts for the TRAP assays were prepared 3 days after infection. Products of the TRAP assays were visualized in the acrylamide gel by Vistra green staining (top). Molecular sizes (in thousands) are on the left, and the positions of the TRAP PCR products (6 bp ladder starting with 50-bp band) and a 36-bp PCR internal control (IC) are shown on the right. The levels of IRF-4 and IRF-8 protein expression were determined by Western blot analysis in whole-cell lysates (bottom). (B) Induction of TERT mRNA expression by IRF-4 and IRF-8. The levels of TERT mRNA were determined by RT-PCR. The expression of GAPDH was analyzed as a control. Aliquots from the PCR were taken every fifth cycle, beginning with cycle 30 for the amplification of TERT and cycle 20 for GAPDH, and the PCR products were resolved on agarose gels and visualized with ethidium bromide. Gray triangles indicate an increasing number of PCR cycles. (C) Chicken splenic lymphocytes (LYMPH.) and DT40 cells were infected with retroviruses expressing IRF-4 or with helper virus (H). Extracts for the TRAP and Western analyses were prepared 2 days after infection. The TRAP analysis was performed as described above. The levels of IRF-4 protein expression were determined by Western blot analysis in whole-cell lysates.

phase indicated that apoptosis was not increased in cells with decreased IRF-4 level (data not shown). Cell cycle analysis further demonstrated that more cells accumulated in S/G₂ phase, indicating that the decrease in IRF-4 levels resulted in a partial block in the transition through S/G₂ (Fig. 2A). To support this conclusion, CEFs overexpressing IRF-4, which have increased proliferation rates, were also analyzed. In these cells, the S/G₂ phase was significantly shorter than that in control cells, suggesting that IRF-4 accelerates the transition of cells through the cell cycle.

Telomerase activity has also been implicated in the enhancement of cell proliferation (1, 14, 54, 57). Therefore, we determined whether the overexpression of TERT could overcome the C4-1 cell proliferation defect induced by decreasing IRF-4 levels. C4-1 cells overexpressing TERT or a catalytically inactive dominant negative mutant of TERT (DN-TERT) were established (Fig. 2B). Telomerase activity in TERT-overex-

pressing cells was significantly higher than in the parental C4-1 cells, while it was slightly decreased in cells expressing DN-TERT. Subsequently, the expression of IRF-4 in these cell lines was decreased by siRNA, as described for the previous experiment, and cell numbers were determined after 48 h. Cell numbers were reduced in the parental C4-1 cell line and in C4-1 cells expressing DN-TERT by 36 to 41%, depending on the siRNA employed. By contrast, treatment of C4-1 cells overexpressing TERT with siRNA targeting IRF-4 did not result in a decrease in cell proliferation. These results indicate that telomerase activity is able to compensate for the proliferation defect caused by depletion of IRF-4 and suggest that the IRF-4-mediated induction of telomerase contributes to its regulation of proliferation.

IRF-4 and IRF-8 activate the TERT promoter. To examine the roles of IRF-4 and IRF-8 in the regulation of TERT expression, the chicken TERT promoter was cloned based on a

FIG. 2. IRF-4-mediated induction of telomerase contributes to proliferation of v-Rel transformed cells. (A) Suppression of IRF-4 expression by siRNA leads to a decrease in TERT mRNA and telomerase activity in a v-Rel cell line. C4-1 B cells were electroporated with siRNAs targeting IRF-4 (IF3 and IF4) or a 1NC siRNA (4 μ g). RNA and TRAP extracts were harvested after 24 h. The levels of IRF-4, TERT, and GAPDH mRNA were analyzed by RT-PCR as described in the legend for Fig. 1. The first aliquot from the PCR amplification of IRF-4 was taken at cycle 25. Gray triangles indicate an increasing number of PCR cycles. Telomerase activity was determined by the TRAP assay. Cell cycle analysis was performed as described in Materials and Methods. C4-1 cells were harvested 44 h after transfection by siRNAs (IF3 and 1NC) and CEFs were harvested 7 days after infection with retroviruses expressing IRF-4 or with helper virus (H). For the analysis, gating was set to exclude cells with DNA content lower than that of the G₁ phase. Percentages of cells in G₁, S, and G₂ phases are indicated. (B) Suppression of IRF-4 expression by siRNA leads to a decrease in proliferation of C4-1 cells, and this defect can be compensated for by the overexpression of TERT. C4-1 cells were infected with retroviruses expressing TERT, a dominant negative TERT mutant (DN-TERT), or helper virus (H), and telomerase activity was determined 5 days after infection by a TRAP assay (left). C4-1 cells with and without exogenous expression of TERT or DN-TERT were then electroporated with IRF-3, IRF-4, or a control 1NC siRNA (4 μ g), and cell numbers were determined after 48 h using a hemocytometer (right). Means and standard errors were calculated from three independent experiments.

FIG. 3. IRF-4 and IRF-8 activate the TERT promoter. Various lengths of the TERT promoter were cloned into the pGL3-basic luciferase reporter vector. 5' boundaries of the deletion constructs are indicated in the diagram of the TERT promoter (top). The positions of the transcription start site (TSS) and the translation start site (ATG) are based on previously published data (9). The reporter plasmids were cotransfected with an empty pcDNA expression vector (CTRL) or pcDNA expressing IRF-4 or IRF-8 into HD11 cells. Luciferase activity was measured 36 h after transfection in relative luciferase units (RLU). Firefly luciferase activity was standardized to *Renilla* luciferase activity, expressed by the cotransfected pRL-TK reporter. The means and standard errors from four to six independent transfections are shown.

previous description of this promoter and the chicken genome project database (9, 24). A 3.9-kb fragment of the sequence upstream of the TERT start codon was cloned into a luciferase reporter vector, and a series of 5' promoter deletions was constructed. These constructs were transfected into HD11 cells together with vectors expressing IRF-4 or IRF-8 or an empty vector control (Fig. 3). IRF-expressing cells exhibited an increased activity of the TERT promoter. The most active promoter construct (CP-378) showed approximately a sixfold activation by IRF-4 and a threefold activation by IRF-8. The profiles of luciferase activity of the 5'-end-deleted TERT promoter constructs were similar in cells overexpressing IRF-4 and IRF-8. These results demonstrate that IRF-4 and IRF-8 activate the TERT promoter and that the region 378 bp upstream of the translation start codon contains the promoter sequences sufficient for both baseline and IRF-4- and IRF-8-induced activity. This region is referred to as the core promoter.

Identification of the elements in the TERT promoter responsible for its inducibility in IRF-4- and IRF-8-expressing cells. To identify which transcription factors bind and regulate the TERT promoter in IRF-4-expressing cells, gel shift assays were performed with oligonucleotide probes that covered the entire core promoter (data not shown). Four regions with differential binding were detected using these probes with nuclear extracts from HD11 cells and CEFs expressing either IRF-4 or the helper virus. Additional experiments employing shorter probes narrowed the binding sites required for these activities to the 26- to 36-bp regions designated M, G, I, and S

(Fig. 4A). Sequence analysis indicated that region M contained a canonical binding site for Myc/upstream stimulatory factor (USF). Region G contained a GAS site followed by a single-copy ISRE. In region I, a symmetrical ISRE-like GCCAACCG site was found. Finally, region S was formed by four Sp1/Sp3 binding sites arranged in tandem. Further EMSAs were done to quantify differences in the DNA-binding profiles to these sites between cells infected with viruses expressing IRF-4 and cells infected with the helper virus and to identify the binding factors (Fig. 4B). These assays demonstrated that exogenous expression of IRF-4 increased the binding to the M and S probes by 50 to 80% in HD11 cells and by 30% in CEFs. Because the patterns of binding complexes were identical in both cell systems and the binding increased in IRF4-expressing cells of both types, only the data from HD11 cells are shown for M and S probes (Fig. 4, lanes 1, 2, 25, and 26). Differences in binding to the G and I probes were, however, cell type specific. Changes in the binding profiles for the G probe were different in HD11 cells and CEFs (Fig. 4, lanes 13, 14, 17, and 18). Binding to the I probe increased in HD11 cells but was not altered in CEFs expressing IRF-4 (Fig. 4, lanes 19, 20, 23, and 24).

The composition of the complexes bound to the Myc/USF and Sp1/Sp3 sites in the nuclei of HD11 cells was determined using supershift analyses (Fig. 4, lanes 5 to 12 and 29 to 36). Two binding complexes were detected with the M probe (Fig. 4, lanes 5 to 12). Both complexes were supershifted by antibody specific for USF-1, while antibodies against USF-2 and against Myc each supershifted about half of the upper C2 complexes.

FIG. 4. Binding sites in the TERT core promoter are functionally important for induction by IRF-4 or IRF-8. (A) Structure of the core promoter of chicken TERT. The locations of four binding sites (Myc/USF, GAS/ISRE, ISRE-like, and Sp1/3), the transcription start site (TSS), and the translation start site (ATG) of TERT are shown. Below are indicated the positions of four EMSA probes (M, G, I, and S) as well as upstream boundaries of the CP-32, CP-99, and CP-378 deletion mutants used in the promoter reporter assays. Nucleotide sequences of the binding sites and their mutated counterparts are shown in the box below. (B) Gel shift assays with nuclear extracts from HD11 cells and CEFs infected with REV-IRF-4 (IRF4) or CSV helper virus (H). EMSA probes are indicated below the gel shift images. Two binding buffers described in Materials and Methods were used: HS (lanes 1 to 24) and MZ (lanes 25 to 36). Antibodies used for supershifts are indicated above the images (aUSF1, aUSF2, aMyc, aSp1, and aSp3). The positions of the shifted (C and C1 to C3) and supershifted (S) complexes are indicated to the right of the gels. (C) Luciferase reporter assays were performed with wild-type (*wt*) and mutated TERT CP-378 promoter constructs (*mutM*, *mutG*, *mutI*, *mutS*, and *mutMGS*) in HD11 cells transfected with empty pcDNA vector (CTRL) or pcDNA expressing IRF-4 or IRF-8. Luciferase activity was determined as described in the legend for Fig. 3. The means and standard errors from four to six independent transfections are shown.

These results suggest that the lower C1 complex is composed of USF-1 homodimers while the upper C2 complexes contain USF-1/USF-2 heterodimers. Both USF-1- and USF-2-binding activities increased in the IRF-4-expressing cells. The C2 complexes also contained the Myc protein, which substantially increased in abundance in cells expressing IRF-4. Myc was likely associated with USF-1, as all the binding activity in C2 complexes was supershifted with the USF-1 antibody. With the S probe (Fig. 4, lanes 29 to 36), two very closely migrating com-

plexes were detected: the lower C1 complex contained Sp1, and the upper C2 complex contained Sp3. In control cells, Sp1 was less abundant in the complex than Sp3, but its abundance increased more dramatically (2-fold) than that of Sp3 (1.25-fold) in IRF-4-expressing cells. Nuclear extracts from CEFs contained Myc/USF and Sp1/Sp3 site-binding complexes identical to those detected in HD11 cells (data not shown). To determine if Myc/USF, GAS/ISRE, ISRE-like, and Sp1/Sp3 sites functionally contribute to the increased transcrip-

tional activity of the TERT promoter in IRF-4-expressing cells, mutations were introduced by site-directed mutagenesis in the CP-378 reporter vector, creating a series of mutant reporter constructs (Fig. 4A). To verify that the mutated DNA-binding sites failed to bind the appropriate factors, EMSAs were performed with the mutated probes (*mutM*, *mutG*, *mutI*, and *mutS*) and nuclear extracts from HD11 cells expressing either IRF-4 or the helper virus (Fig. 4B). These mutations eliminated the binding of proteins present in HD11 nuclear extracts on *mutM*, *mutI*, and *mutS* sites (Fig. 4, compare lanes 1 and 2 with lanes 3 and 4, lanes 19 and 20 with lanes 21 and 22, and lanes 25 and 26 with lanes 27 and 28). The *mutG* mutation substantially decreased the binding of the C2 and C3 complexes (Fig. 4, compare lanes 13 and 14 with lanes 15 and 16). The activity of mutant and wild-type reporter constructs was then evaluated in HD11 cells expressing IRF-4 and IRF-8 and in control cells (Fig. 4C). While the mutations in Myc/USF (*mutM*), ISRE-like (*mutI*), and Sp1/Sp3 sites (*mutS*) decreased the activity of the promoter in both control cells and IRF-expressing cells, the extent of the decrease was much more profound and statistically significant in cells expressing either IRF-4 or IRF-8. Mutations in the Sp1/Sp3 sites decreased the induction by IRFs most strongly. Mutations in the GAS/ISRE element (*mutG*) did not decrease the activity in control cells but significantly decreased the induction in IRF-expressing cells. Finally, combined mutations in M, G, and S sites abolished the inducible effect of IRF-4 and IRF-8.

These results indicate that several transcription factors, including Sp1, Sp3, Myc, USF-1, USF-2, and factors binding to the GAS/ISRE and ISRE-like elements, increase their binding to the TERT promoter in IRF-4-expressing cells. Furthermore, this binding is important for the inducibility of the TERT promoter by IRF-4 and IRF-8, and the binding of Sp1/Sp3 factors to the tandem site has the most profound effect on promoter activity.

The role of Sp1 and Sp3 factors in the regulation of the TERT promoter by IRF-4. In IRF-4-expressing cells, the binding to the TERT promoter of Sp1, Sp3, USF-1, USF-2, and c-Myc significantly increased (Fig. 4). To elucidate the mechanism by which IRF-4 increases the binding of these factors, their mRNA and protein levels were determined by Northern and Western blot analyses. The levels of Sp1, Sp3, USF-1, and USF-2 mRNA were induced by IRF-4 expression in CEFs and more significantly in HD11 cells (Fig. 5A). The increase in mRNA levels for these factors corresponded to an increase in their protein levels in the nucleus, indicating that the increase of Sp and USF factor DNA binding to the TERT promoter in IRF-4-expressing cells may be a direct result of an increase in their mRNA and protein levels. The mRNA and protein levels of c-Myc were not changed in either cell type, suggesting that increased c-Myc DNA binding to the E-box is due to its activation rather than increased levels (data not shown). These results identified new important targets of IRF-4 transcriptional regulation—Sp1, Sp3, USF-1, and USF-2—that may mediate many IRF-4 functions.

To determine whether these factors cooperate with IRF-4 in the transactivation of the TERT promoter, we compared their activities in transient-reporter assays alone and in combination with IRF-4 or IRF-8. IRF-4 cooperated only with Sp1 and Sp3 and failed to cooperate with USF-1, USF-2, and c-Myc (Fig. 5B

and data not shown). In the absence of IRF-4, Sp1 and Sp3 slightly increased the activity of the telomerase promoter. However, in the presence of IRF-4, TERT promoter activity was synergistically increased, indicating that these factors cooperate with IRF-4. This effect was specific for IRF-4, since cooperation was not observed between IRF-8 and Sp1 or Sp3. Interestingly, the cooperation between IRF-4 and Sp1/Sp3 factors occurred even when promoter constructs without the putative IRF-binding sites located in the G and I elements (CP-99) were employed, although the promoter was activated to a lesser extent than with the core promoter.

To determine whether the cooperation between IRF-4 and Sp factors also occurs *in vivo*, HD11 cells were infected with retroviruses expressing IRF-4, Sp1, and Sp3 alone or in combination at a 1:1 ratio. The expression promoted proliferation of all Sp1- and Sp3-overexpressing cells, including those coinfecting with IRF-4, suggesting that there was an increase in the level of functional Sp factors. TRAP assays demonstrated that Sp1 and Sp3 alone induced telomerase and that the cells coexpressing IRF-4 with Sp3 had the highest level of telomerase activity (Fig. 5C, lanes 1 to 6). To extend these results, CEFs were infected with retroviruses expressing IRF-4, Sp1, and Sp3 alone or in combination at a 1:5 (IRF-4:Sp1 or IRF-4:Sp3) ratio. Lower multiplicity of infection by IRF-4-expressing virus resulted in low telomerase activity in IRF-4-expressing cells 2 days after infection (lane 9). However, the coexpression of either of the two Sp factors significantly increased telomerase activity well above the levels detected in cells expressing Sp1, Sp3, or IRF-4 alone (lanes 7 to 11).

Collectively, these results suggest that while the activation of basic helix-loop-helix (bHLH) factors (USF-1, USF-2, and c-Myc) by IRF-4 may contribute to the regulation of telomerase activity, the cooperation of IRF-4 with Sp1/Sp3 transcription factors plays a more important role.

The mechanism of TERT regulation in IRF-4-expressing cells. Transactivation experiments identified a 378-bp core sequence within the TERT promoter necessary for IRF-4 inducibility. Sequence analysis combined with EMSA identified putative GAS/ISRE and ISRE-like sites for binding of the IRF factors, but we did not detect IRF-4 bound on these probes by supershift analyses. However, the detection of IRF-4 DNA binding by supershift analysis is often difficult due to the complexity of its binding to specific sites (49). Therefore, the binding of IRF-4 *in vivo* under physiologically relevant conditions was examined by ChIP analysis (Fig. 6A). HD11 cells were infected with retroviruses expressing IRF-4 or helper virus, and complexes bound to chromatin were harvested from cells 3 days after infection. The ChIP assay demonstrated that IRF-4 bound to a 357-bp region in the core promoter, while IRF-4 binding was not detected in control cells.

The bHLH factors (USF-1, USF-2, and c-Myc) as well as Sp1 and Sp3 factors increased their binding to their appropriate sites in IRF-4-expressing cells by EMSAs (Fig. 4). To address whether these assays reflect their binding *in vivo*, ChIP analysis was performed with antibodies targeting these proteins (Fig. 6A). A strong increase in the binding of Sp3 to the TERT core promoter was observed in IRF-4-expressing cells relative to control cells. An increase in the binding of Sp1 was also detected. The increased binding of USF-1, USF-2, and c-Myc was detected in IRF-4-expressing cells, suggesting that

FIG. 5. Sp1 and Sp3 transcription factors cooperate with IRF-4 in the regulation of telomerase activity. (A) Northern blots of RNA from IRF-4-expressing-virus-infected (IRF4) or helper virus-infected (H) CEF or HD11 cells were hybridized with Sp1, Sp3, USF-1, and USF-2 probes, and gels were stained with ethidium bromide as a loading control. Western blots of the nuclear extracts prepared from the same cells were stained with antibodies against Sp1, Sp3, USF-1, or USF-2, and the signal was detected chemiluminescently. Molecular masses (in thousands) are on the left. (B) Three reporter plasmids (pGL3basic, CP-99, and CP-378) were cotransfected with an empty pcDNA expression vector (CTRL) or pcDNAs expressing IRF-4, IRF-8, Sp1, and Sp3 in various combinations into HD11 cells. Luciferase activity was determined as described in the legend for Fig. 3. (C) HD11 cells and CEFs were infected with retroviruses expressing IRF-4, Sp1, and Sp3, either alone or in various combinations, or with helper virus (H). Extracts from HD11 cells for the TRAP assays were prepared 3 days after infection and extracts from CEFs 2 days after infection. Extracts were analyzed as described in the legend to Fig. 1.

binding to the E-box site in the TERT promoter core region is activated by IRF-4 in vivo.

In order to determine which potential site in the 378-bp TERT promoter core region is bound by IRF-4 in vivo, ChIP analysis was performed with TERT promoter mutants (Fig. 6B). HD11 cells overexpressing IRF-4 were transfected with the CP-378 reporter vector or this vector with mutations in the GAS/ISRE composite site (*mutG*) or in the ISRE-like site (*mutI*). The cells were subjected to ChIP analysis using IRF-4 antiserum. The wild-type construct and the construct with mutation in the ISRE-like site were immunoprecipitated with IRF-4 antiserum. In contrast, the promoter region with a mutation in the GAS/ISRE composite site failed to be immuno-

precipitated, indicating that this site is the principal IRF-4 binding site in core TERT promoter region in vivo.

Transactivation assays demonstrate a strong cooperation between IRF-4 and Sp factors in the activation of the TERT promoter (Fig. 5). Stat factors have been demonstrated to cooperate with Sp transcription factors to regulate gene expression (38). Moreover, Stat6 is a transcription factor which binds GAS sites specifically with IRF-4 in human cells (17). Therefore, we examined whether Stat6 also interacts with IRF-4 in chicken cells (Fig. 6C). Coimmunoprecipitation experiments using nuclear extracts from HD11 cells expressing IRF-4 demonstrated the association of IRF-4 and Stat6.

The analysis of the chicken TERT promoter indicates that

FIG. 6. Mechanism of regulation of TERT promoter in vivo. (A) Structure of the core promoter of chicken TERT and ChIP analysis of transcription factor binding. Schematic representation of the chicken proximal TERT promoter with the PCR primer locations (FP and RP) is shown at the top. The locations of binding sites (Myc/USF, GAS/ISRE, ISRE-LIKE, and Sp1/3), the transcription start site (TSS), and the translation start (ATG) of TERT are also shown. HD11 cells were infected with IRF-4-expressing retrovirus (4) or helper virus (H), and the chromatin bound by transcriptional factors IRF-4, Sp1, Sp3, USF-1, USF-2, and c-Myc were analyzed by a ChIP assay as described in Materials and Methods. Antibodies used for ChIP assay are indicated above the images (aIRF4, aSp1, aSp3, aUSF1, aUSF2, aMyc). The PCR products from genomic DNA (INPUT) and from DNA precipitated with preimmune serum (PREIM) or normal rabbit IgG were included as positive and negative controls, respectively. The precipitated chromatin was also analyzed with primers against the GAPDH gene. (B) ChIP analysis with TERT promoter mutants. HD11 cells were infected with retrovirus expressing IRF-4 5 days before transfection. The reporter plasmids were transfected into the cells and analyzed by a ChIP assay 2 days later. (C) Interaction of IRF-4 with Stat6. HD11 cells were infected with retroviruses expressing IRF-4, and nuclear extracts were harvested 5 days after infection. The immunoprecipitation was carried out under reducing (R) or nonreducing (N) conditions with antibody against IRF-4 or Stat6 or normal rabbit IgG overnight at 4°C. The immunoprecipitates (IP) were resolved by SDS-polyacrylamide gel electrophoresis under reducing or nonreducing conditions and analyzed by Western blot analysis using antibody against IRF-4. The lysate from IRF-4-expressing cells (IRF-4 lysate) was analyzed as a control. The migration of IRF-4 and immunoglobulin heavy chain (Ig) is indicated on the right. (D) Structure of the core region of human TERT promoter. The locations of binding sites (Myc/USF and Sp1/3), the transcription start site (TSS), and the translation start site (ATG) of TERT were described previously (61). The locations of two single-copy ISRE sites, one canonical site (ISRE1) and one noncanonical site (ISRE2), are also indicated.

IRF-4 transcriptionally regulates telomerase activity involving GAS/ISRE, Myc/USF, and Sp sites in the core region. The regulation of TERT transcription and binding sites for transcription factors are well conserved among vertebrates (48). A schematic representation of a proximal part of the human TERT promoter demonstrating the presence of these sites is provided (Fig. 6D).

Collectively, these results implicate IRF-4, Sp1/Sp3, Stat6, and bHLH transcription factors in the cooperative regulation of TERT transcription.

DISCUSSION

The regulation of telomerase activity during the immune response. The identification of IRF-4 and IRF-8 as positive regulators of TERT transcription provides a clue to the mechanism by which telomerase is regulated in immune cells. Both

factors are expressed predominantly in hematopoietic cells, where they display a largely overlapping expression pattern, but they differ in their interactions with other transcription factors (46, 62). IRF-8 cooperates with IRF-1 in the induction of a large set of interferon-responsive genes (10). On the other hand, IRF-4 suppresses most of the IRF-1-driven gene expression and cooperates with IRF-8 in the control of a different transcription program (52, 71). IRF-4 and IRF-8 also differ in their responsiveness to external signals. IRF-4 responds to antigen-receptor ligation, lipopolysaccharide (LPS), and specific cytokines such as interleukin 4 but not to interferons. In contrast, IRF-8 also responds to interferons together with IRF-1 and other IRF family members (62). In this way, the interplay between the interferons and B- or T-cell receptor signaling coordinates innate and adaptive immunity through the differential activation of IRF-1, IRF-4, and IRF-8. Since

IRF-1 and interferons are negative regulators of telomerase activity (33, 34), and IRF-1 efficiently suppresses IRF-4- or IRF-8-mediated activation of the TERT promoter (data not shown), the relative ratios of IRF-4 and IRF-8 to IRF-1 are important for the differential regulation of TERT expression. Therefore, telomerase activity would be positively regulated by IRF-4/8 in cells where their influence prevails over IRF-1, as in the humoral branch of adaptive immunity. The expression of IRF-4/8 and telomerase has been reported in the same differentiation stages in various immune cell lineages. IRF-4 and IRF-8 cooperate in the development, activation, and maturation of cells in the B-cell lineage. IRF-4 and IRF-8 alternate their relative abundance during the sequence of events leading to the maturation of naïve B cells in germinal centers (GC) into effector plasma cells (7). Interestingly, telomerase activity follows the pattern of IRF-4/8 expression during these stages of B-cell terminal differentiation (68). IRF-4 expression is low in naïve cells, where telomerase activity is also very low (35). IRF-8 expression is at its highest level in GC centroblasts, while IRF-4 is highly expressed in centrocytes (25, 29, 32, 39, 53, 64). In both these cell types, telomerase is strongly activated (68). Finally, telomerase activity, which is expressed at very low levels in memory cells and at medium levels in plasma cells, correlates with the IRF-4 expression pattern in these cells (30, 68).

The expression of IRF-4/8 also coincides with telomerase activation during the differentiation of dendritic cells (DC) and macrophages. IRF-4 and IRF-8 are essential for DC differentiation, each controlling the development of different DC subsets (60, 63). Interestingly, telomerase activity is significantly increased during maturation of bone marrow-derived DC (51). Furthermore, IRF-4 and IRF-8 are activated by LPS in macrophages (42). Likewise, telomerase activity of peritoneal macrophages is increased after stimulation of these cells by LPS *in vivo* (51).

Our results demonstrate that telomerase activation by IRF-4 and IRF-8 is the result of the transcriptional regulation of TERT. IRF-4 and IRF-8 are transcription factors specifically expressed during the immune response, suggesting that they are key regulators of telomerase activity during this process.

Role of telomerase activity in IRF-4-associated proliferation. A role for IRF-4 in the regulation of the cell cycle seems to be cell type specific; however, in most cell types, IRF-4 promotes cell proliferation (39, 64). IRF-4 overexpression enhances the proliferation of fibroblast-derived cell lines, primary chicken fibroblasts, and the murine pro-B-cell line Ba/F3 (20, 22, 65). IRF-4-deficient splenic B cells have a proliferation defect and low responsiveness to stimulation with LPS or anti-IgM (44). Accordingly, a decrease in IRF-4 levels by antisense or siRNA technology in a lymphoid B-cell line is associated with a reduction in cell proliferation (reference 20 and this study). The identification of telomerase as a target of IRF-4 is important to elucidate the mechanism of its promotional effect on proliferation. First, telomerase itself directly enhances cell proliferation, exhibiting the ability to mediate some of the IRF-4 cell proliferation effect (1, 14, 54, 57). Our results demonstrate that elevated telomerase activity can compensate for proliferation defects resulting from suppression of IRF-4 expression, suggesting that telomerase activity induced by IRF-4 may, at least in part, participate in IRF-4-enhanced prolifera-

tion. IRF-4 may also increase the levels of other important genes (such as those for the transcription factors Sp1, Sp3, USF-1, and USF-2, identified in this study) which may directly affect cell proliferation. Second, IRF-4-induced telomerase activity would have an important function during the proliferation of activated lymphocytes in reducing the rate of telomere shortening.

Role of telomerase regulation by IRF-4 and IRF-8 in cancer. Telomerase activation is a common step in tumorigenesis (5). IRF-4 plays an important role in several human malignancies of hematopoietic cell origin and melanomas (16, 22, 23, 72). In most types of malignancies, elevated expression of IRF-4 indicates that the tumors were derived during lymphocyte activation, and this finding is associated with an unfavorable prognosis (3, 6, 41, 58, 59). The identification of TERT as an IRF-4 transcriptional target may explain why IRF-4 is activated in so many histologically distinct cancers and how it may contribute to carcinogenesis.

In contrast, IRF-8 appears to function as a tumor suppressor, because IRF-8-deficient mice develop a myeloproliferative syndrome, and the expression of IRF-8 is impaired in human myeloid leukemia (19, 55). However, IRF-8 function in cancer may be cell type dependent. Several proviral insertions of murine leukemia virus have been found within the IRF-8 locus in mature B-cell lymphomas or plasmacytomas, suggesting a role for IRF-8 in their establishment (40). IRF-8 is also highly expressed in centroblasts and centroblast-derived tumors, where it induces the expression of the proto-oncogene Bcl6 (32). Its ability to activate telomerase may, therefore, contribute to the development of lymphomas, in which the cells are more differentiated.

Mechanism of telomerase activation by IRF-4. Our results demonstrate that IRF-4 directly binds to the TERT promoter *in vivo*. IRF-4 was also able to orchestrate changes in two transcriptional systems known to regulate TERT: bHLH factors and Sp1/Sp3 factors. IRF-4 expression increased the levels of Sp1/Sp3, USF-1, and USF-2 mRNA and proteins. Moreover, ChIP analysis detected the *in vivo* binding of these proteins to the TERT promoter. Collectively, these results suggest that in IRF-4-expressing cells, IRF-4 directly activates the TERT promoter in cooperation with Sp factors, predominantly Sp3, and bHLH factors.

IRF-4 and IRF-8 poorly bind DNA, and their full transactivation activity is often dependent on their interaction with other transcription factors (62). Although a direct interaction between IRF and Sp factors was not detected, several instances of cooperation between GAS (bound by IRF together with Stat factors) and GC sites (bound by Sp factors) have been described (36, 38, 73), and coimmunoprecipitation experiments have also demonstrated a direct interaction between Sp1 and Stat1 (73). Finally, the complex of ISGF-3 (Stat1, Stat2, and IRF-9) with Sp1, which binds the *PKR* promoter, is replaced after interferon activation by the ISGF-3/Sp3 complex (67). However, the role of interferon-inducible Stat1 and Stat2, in response to interferons, is taken on by Stat6 in response to IRF-4-specific activation signals, antigen-receptor engagement in B cells, and interleukin 4 treatment (26, 31). The cooperation of IRF-4 with Stat6 on the GAS site on the CD23 promoter is through their physical interaction (17). Our experiments confirmed that Stat6 associates with IRF-4. Therefore,

Stat6 would likely mediate IRF-4 and Sp factor cooperative binding on the TERT promoter.

In conclusion, our results demonstrate that IRF-4 and IRF-8 activate telomerase transcriptionally regulating TERT. This finding partially defines telomerase regulation during immune responses, in which IRF-4 and IRF-8 play the principal roles. Additionally, the identification of TERT as a direct target of IRF-4 contributes to understanding how this factor promotes cell proliferation and cancer development.

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