We observed that the human CD40 ligand (CD40L) gene 5'-flanking region conferred weak promoter activity in activated CD4 T cells, suggesting that additional regions are required for optimal CD40L gene transcription. We therefore examined a 3'-flanking segment of the CD40L gene, which contained a putative NF-xB/Rel cis-element, for its ability to enhance CD40L promoter function. This segment augmented CD40L promoter activity in an orientation-independent manner in CD4 T-lineage cells but not in human B cell or monocyte cell lines. Mapping of CD4 T-lineage cell nuclei identified a DNase I-hypersensitive site in the flanking region near the NF-xB/Rel sequence, suggesting a transcriptional regulatory role. This was further supported by truncation analysis and site-directed mutagenesis, which indicated that the CD40L 3'-flanking NF-xB/Rel cis-element was critical for enhancer function. Electrophoretic mobility shift assays showed that the cis-element preferentially bound the p50 form of the NF-xB1 gene contained in human T cell nuclear protein extracts. This binding also appeared to occur in vivo in CD4 T cells based on chromatin immunoprecipitation assays using NF-xB p50-specific antisemur. Together, these results suggest that the CD40L gene 3'-flanking region acts as a T cell-specific classical transcriptional enhancer by a NF-xB p50-dependent mechanism.

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NF-xB/Rel cis-elements have been identified in the 5′-flanking region and appear to be important for TNF expression by mononuclear phagocytes (20). However, in contrast to the NF-xB site of the IL-2 gene, the NF-xB/Rel elements in the TNF promoter do not appear to regulate gene expression in T cells (21). Rather, the concerted binding of NFAT and leucine zipper proteins to the 5′-flanking region of the TNF gene appears to be essential for expression in activated CD4 T cells (21).

Like the TNF and IL-2 genes, CD40L gene transcription in activated T cells requires the cooperative binding of NFAT and leucine zipper proteins to the 5′-flanking region (22–24). A putative NF-xB/Rel cis-element, GGGATTTCGA, has been identified in the 5′-flanking region of the CD40L gene based on sequence analysis (25) and has recently been reported to enhance promoter activity, possibly by binding NF-xB p65 (26). Interestingly, a CD28 response element has also been identified in the CD40L promoter, identifying a role for CD28 engagement in the regulation of the CD40L promoter (27). However, no enhancer elements contributing to CD40L gene transcription by T cells or other cells have been described.

Here, we show that the human CD40L gene contains a T cell-specific, NF-xB/Rel protein-dependent, classical enhancer in its 3′-flanking region that may be important in the regulation of CD40L transcription.

**EXPERIMENTAL PROCEDURES**

**Cells**—Human CD4 T cells isolated from the peripheral blood of normal adult donors were either used directly (freshly isolated cells) or primed by *in vitro* treatment with mitogen (concanavalin A (Amersham Biosciences, Inc.) and recombinant human IL-2 (Proleukin, Promega, Madison, WI) as previously described (28). Jurkat thymoma cells (J.D.NAX subline (29)), Jurkat D1.1 (gift of Dr. Seth Lederman, Columbia University, New York), 8.1.6 Epstein-Barr virus-transformed human B cells (30), and U937 human monoblastoid cells (31) were maintained in RPMI 1640 complete medium containing 10% fetal calf serum (Hyline Laboratories, Logan UT), 100 units/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml gentamicin, 25 µM Hepes, and 2 mM L-glutamine.

**Time PCR Analysis of mRNA**—Total cellular RNA was isolated (32) from CD T cells after 3 h of culture with or without activating stimuli (1.5 µM ionomycin (Calbiochem) plus 50 ng/ml PMA (Sigma)). Random hexamer-primed reverse transcription (2 µg of total RNA/sample) was performed using the reagents contained in a TqiMan Gold RT-PCR kit (PerkinElmer Life Sciences) following the manufacturer's instructions. Each reaction was subsequently amplified to real time quantitative PCR in an ABI Prism 5700 Sequence Detection System (PerkinElmer Life Sciences). Amplifications were performed in 96-well plates using oligonucleotide primers for transcript amplification of human CD40L (sense, 5′-CCAGGTTGTCTGGTTGTGT-GT-3′; antisense, 5′-ATGTCATTGCTGGTGTTT-A-3′) (33), human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (proprietary primers purchased from PerkinElmer Life Sciences). For all primer sets, the reverse-transcribed and potentially amplifiable by PCR was similar in the initial RNA sample (34). This value was determined using software included as part of the ABI Prism 5700 Sequence Detector System, following the manufacturer's instructions. The Ct values for GAPDH transcripts in the stimulated and unstimulated RNA samples were used as a control, and results were normalized to GAPDH. Comparable GAPDH cycle threshold values indicated that the amount of mRNA reverse transcribed and potentially amplifiable by PCR was similar in unstimulated and stimulated samples.

**Reporter Gene Plasmids**—The human CD40L gene sequence has been previously published (35). The 1284-bp 3′-flanking region of the CD40L gene analyzed in this study is shown in Fig. 1A. Maps of the luciferase reporter and fragments of the CD40L promoter were previously published (36). The cloned luciferase promoter contains the human CD40L promoter and 3′-flanking segments, omitting reference to the intervening luciferase reporter gene. pCD40L was created by subcloning a 1.3-kb HindIII/HindIII fragment containing the human CD40L promoter 5′-flanking region and immediately upstream of the luciferase cDNA segment of pGL2-Basic. The pGL2-PL-4, and pIL-13 constructs directed by the 5′-flanking segments of the human IL-2 (0.6-kb), IL-4 (0.6-kb), and IL-13 (1.1-kb) genes, respectively, were similarly derived from pGL2-Basic, as previously described (22, 28, 36). pSV40, in which the luciferase cDNA is directed by a 0.2-kb segment of the SV40 large T antigen promoter, was purchased (pGL2 Promoter Vector; Promega). The 1284-bp segment of the human CD40L gene’s 3′-flanking region (3′ FL) was subcloned into pCD40L at adjacent BamHI and SalI cloning sites, located 1 kb 3′ of the luciferase gene, creating pCD40L.3′FL (Fig. 2B). Subcloning used a naturally occurring 5′ BamHI site and a 3′ SalI site generated by PCR using Vent polymerase (New England Biolabs) and a human CD40L genomic λ plaque clone as template. This 1284-bp 3′-flanking segment begins 14 bp 3′ of the CD40L termination codon and ends 401 bp after the final exon of exon 5 (Fig. 1, A and B (35)). Constructs containing the entire 1284-bp 3′-flanking segment or truncations of this region are numbered using the first residue of the BamHI site as position 1. A similar approach was used to produce a construct containing the 3′ segment in the reverse orientation (pCD40L.R3′FL). An endogenous PstI site was utilized to generate truncations of the full-length segment. Both the 5′ (BamHI/PstI site (bp 1–808)) and the 3′ (PstI/SalI site (bp 809–1284)) fragments were subcloned into the pBS/KS cloning vector (Stratagene). This was followed by secondary subcloning into pCD40L using BamHI and a polylinker-derivd SalI site (5′ fragment) or a polylinker-derived BamHI and SalI site. The construct containing the subcloning fragment (pCD40L.3′FL.1–808) or the 3′ fragment (pCD40L.3′FL.809-1284) retained their original orientation with respect to the CD40L promoter. The 1284-bp CD40L 3′-flanking segment was also subcloned at the BamHI/SalI site 3′ of the luciferase cDNA segment in pGL2-PL-2, pGL-13, and pSV40 constructs to create pIL-2.3′FL, pIL-13.3′FL, and pSV40.3′FL, respectively. All PCR generated segments were sequenced to ensure that no polymerase-induced mutations occurred. In some experiments, transfection efficiency was assayed by co-transfection of a Renilla luciferase reporter gene construct, in which expression was driven by either the human β-actin promoter (β-actin-RL, a gift of M. Sweetser, University of Washington) or by a minimal T7 bacteriophage promoter (Promega).

**Site-directed Mutagenesis of the pCD40L.3′FL Plasmid**—Site-directed mutagenesis was performed using a commercial kit (QuikChange; Stratagene) to introduce dinucleotide substitution mutations into the NF-xB/Rel sites of the pCD40L.3′FL luciferase reporter gene. The mutagenesis was performed by amplification of a 1284-bp SalI-fragment containing the 3′-flanking segment of the CD40L gene of a Renilla luciferase reporter gene construct, in which expression was driven by either the human β-actin promoter (β-actin-RL, a gift of M. Sweetser, University of Washington) or by a minimal T7 bacteriophage promoter (Promega). The site-directed substitution segment was subcloned at the BamHI/SalI site 3′ of the luciferase cDNA segment in pGL2-PL-2, pGL-13, and pSV40 constructs to create pIL-2.3′FL, pIL-13.3′FL, and pSV40.3′FL, respectively. All PCR generated segments were sequenced to ensure that no polymerase-induced mutations occurred. In some experiments, transfection efficiency was assayed by co-transfection of a Renilla luciferase reporter gene construct, in which expression was driven by either the human β-actin promoter (β-actin-RL, a gift of M. Sweetser, University of Washington) or by a minimal T7 bacteriophage promoter (Promega).

**Transient Transfection and Reporter Gene Analysis**—Ten µg of firefly luciferase reporter gene plasmid DNA in complete RPMI medium was used for transient transfection by electroporation. The conditions used for the electroporation of primary CD4 T cells have been described (22, 28, 37). Jurkat cells (1.0 x 10⁶ cells/condition) were transiently transfected by electroporation at 260 V and 960 microfarads using a Gene-pulsor electroporator (Bio-Rad). Electroporation conditions for 8.1.6 Epstein-Barr virus-transformed human B cells (2.5 x 10⁶ cells/condition) were 210 V and 960 microfarads, and for U937 human monocyte-like cells (2.5 x 10⁶ cells/condition), conditions were 250 V and 960 microfarads. Following electroporation, cells were incubated for 20–24 h at 37 °C, harvested, and plated at a density of 1.0 x 10⁶ viable cells/well, in 96-well U bottom microtitre plates (Corning Glass). Cells were left untreated or stimulated with ionomycin (1.5 µM; Calbiochem) plus PMA (25 ng/ml; Sigma) or with the combination of the antibodies (mAbs) directed against CD4 (mAb 24, 64.1 (38)), 3,500 dilution of sterile ascites and CD28 (mAb 9.3 (39)), 1,200 dilution of sterile ascites, for 6 h prior to harvesting. CD3 and CD28 mAbs were purchased from Bristol-Myers Squibb Co. Cells were harvested and analyzed for firefly luciferase activity as previously described (28). For Jurkat cell lysates, a Monolight 1500 luminometer (Analytical Luminescence Laboratories, Ann Arbor, MI) was used. Primary human CD4
T cells have a relatively low transfection efficiency following electroporation (40), so a more sensitive luminometer, Berthold model LB9507, was used. For the data presented, at least one experiment was carried out in which either 0.1 μg of pβ-actin-RL was included with transfections of Jurkat thymoma cells or 0.5 μg of pRL-null was included with transfections of human CD4 T cell lysates. DNA from these cells was assayed sequentially for firefly and Renilla luciferase activity using the reagents of the Dual Luciferase Assay Kit (Promega). Renilla activity between samples varied less than 10% in these experiments. Therefore, the data for firefly luciferase activity are presented without correction for transfection efficiency.

Nuclear Protein Extract Preparation and Electromobility Shift Assays (EMSA)—Nuclear protein extracts were prepared as previously described (29) from CD4 T cells activated by incubation for 2 h with CD3 and CD28 mAb, and their protein concentration was determined by the Bradford method using a commercial kit (Ferry). For EMSAs, SDS-PAGE-purified double-stranded oligonucleotides (purchased from Invitrogen) were used as either 32P-labeled probes or as unlabeled competitors. For each oligonucleotide, only the coding strand sequence is shown, with the NF-κB binding sites underlined and mutated residues indicated in boldface type (see Table I). The CD40L 3′-flanking NF-κB oligonucleotide, 5′-TGAGGAGAATTTCCACCAC-3′ (+941 to +960 bp of the 1284-bp 3′-flanking segment (Fig. 1A)), was radio labeled using T4 polynucleotide kinase and (γ-32P)ATP and then annealed. Competitors containing the wild type 3′-flanking NF-κB oligonucleotide and derivatives containing either a single dinucleotide substitution mutation, 5′-TGAGGTAAATTTCAACCAC-3′ (single mutant), or two dinucleotide mutations, 5′-TGAGGTTAATTTCAACCAC-3′ (double mutant), or a 5′-flanking CD40L gene oligonucleotide containing a putative NF-κB element, 5′-TGAGGGATTTCCACCAC-G-3′ (bp −1203 to −1187 bp with respect to the transcription start site (22, 23)), were generated. Oligonucleotides encompassing NF-κB reporter constructs amplified by PCR from pCD40L-BLuc DNA as template. Samples were run in duplicate as described above for the real-time PCR analysis of mRNA levels using the reagents contained in a SYBR Green PCR Core Reagents kit (PerkinElmer Life Sciences). Fluorescence signals were detected during each of 40 cycles (denaturing for 15 s at 95 °C, annealing/extension for 1 min at 60 °C) as determined by binding of SYBR green to double-stranded DNA products.

RESULTS

CD40L 5′-Flanking Promoter Activity Is Lower than That of the IL-2 Promoter in Polyclonally Activated CD4 T Cells—The promoter activity of the 1.3-kb 5′-flanking segment of the CD40L gene (pCD40L) or a 0.6-kb 5′-flanking segment of the IL-2 gene (pIL-2) was assessed using luciferase reporter constructs. After transfection of these constructs into peripheral blood CD4 T cells, cells were either treated with medium alone (unstimulated) or polyclonally activated using ionomycin and PMA at concentrations that yield maximal levels of CD40L and IL-2 gene transcription and mRNA accumulation (5, 14, 15). Transfection with the promotorless parent plasmid, pGL2, served as a negative control and produced barely detectable luciferase expression (Fig. 2A). Transfection of pCD40L or pIL-2 resulted in reporter gene activity that was markedly enhanced by CD4 T cell stimulation with ionomycin and PMA. We consistently observed that pCD40L-mediated luciferase expression was considerably lower than reporter gene expression driven by pIL-2 in both freshly isolated T cells (Fig. 2A) and in CD4 T cells that had previously been primed in vitro (data not shown). The relatively low promoter activity of the 1.3-kb 5′-flanking CD40L gene segment does not appear to be due to strong negative regulatory elements, since we have previously shown that truncation of the 5′ region of this segment does not increase promoter activity (22).

Polyclonally Activated CD4 T Cells Accumulate Similar or Higher Levels of CD40L Transcripts than IL-2 Transcripts—The observation that pCD40L-mediated reporter gene expression was lower than pIL-2-driven reporter expression suggested that the level of CD40L transcripts would also be reduced in comparison with IL-2 mRNA levels. This assumed that the half-lives of CD40L and IL-2 mRNA are similar in ionomycin and PMA-stimulated CD4 T cells, which has been verified. To address this, total RNA was isolated from peripheral blood CD4 T cells 3 h after culture, with or without ionomycin and PMA stimulation, and reverse-transcribed RNA was analyzed for CD40L, IL-2, and GAPDH mRNA levels using real-time PCR.
time PCR. A lower Ct value for CD40L and IL-2 transcripts in unstimulated cells was not due to degraded template, since similar levels of GAPDH transcripts were detected in unstimulated and stimulated T cells (data not shown). In contrast to the low level of activity of the CD40L promoter in transfectant transfection experiments, the Ct values in activated CD T cells indicated that CD40L transcripts were present in equal or greater amounts than IL-2 transcripts (Fig. 2B). This is unlikely to be an artifact resulting from more efficient amplification of the CD40L product, since both CD40L and IL-2 PCR products were 50 bp in size and were amplified using identical reaction conditions. Together, these findings suggest that regions of the CD40L gene other than the 1.3-kb 3′-flanking region are necessary for optimal transcription in polyclonally activated CD T cells.

A 1284-bp Segment of the CD40L 3′-Flanking Region Acts as an Enhancer of CD40L Promoter Activity in CD4 T Cells—Because the 3′-flanking region of many genes expressed by T lymphocytes, such as the genes encoding CD2 and IL-4 (49), contain transcriptional enhancers, we investigated whether the 3′-flanking region of the CD40L gene might contribute to its transcriptional activation in CD4 T-lineage cells. Examination of the 3′-flanking region DNA sequence revealed a potential NF-κB/Rel cis-element, GGAATTTCCT, at bp 946–956 with respect to the BamHI site of exon 5, which matched 9 of 10 residues of the ideal NF-κB/Rel consensus sequence, GGGRNYYCC (50) (see Table I). Since NF-κB/Rel family proteins have been implicated in the activation-dependent expression of several T cell-derived cytokines, including IL-2 and IL-3 (50), we tested the ability of a 1284-bp segment containing this putative NF-κB/Rel binding site to modulate the activity of the CD40L promoter. This segment was subcloned downstream of the 3′-flanking region. The 3′-flanking segment of the CD40L promoter independent of the orientation of the 3′-flanking segment and pCD40L to yield pCD40L.3′FL (Fig. 1B). In order to evaluate orientation dependence, a reporter gene construct with the 3′-flanking segment subcloned in the reverse direction (pCD40L.R3′FL) was generated.

The 3′-flanking segment enhanced basal CD40L promoter activity. However, in Jurkat CD4 cells stimulated with ionomycin and PMA, or with CD3 mAb alone or in combination with CD28 mAb (51), the 3′-flanking segment greatly increased the CD40L promoter independent of the orientation of the 3′-flanking segment (Fig. 3A). These data suggest that this 3′-flanking segment acted as a classical (orientation-independent) enhancer (52). Transformed human CD4 T-lineage cell lines, such as Jurkat, have been reported to differ from primary human CD4 T cells in their transcriptional regulation of activation-dependent genes, such as IL-2 (37). Therefore, we tested whether the 3′-flanking segment could modulate CD40L promoter activity in

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**TABLE I**

Comparison of NF-κB/Rel sites described in this study

| NF-κB/Rel site | Sequence | Source |
|---------------|----------|--------|
| Human CD40L 3′-flanking region, bp 946–956 | GGAATTTCCT | Ref. 36, and see Fig. 2B |
| Human CD40L 3′-flanking region, single dinucleotide mutant | TAATT ~CTTT | This study |
| Human CD40L 3′-flanking region, double dinucleotide mutant | TATAATTT | This study |
| Human CD40L 3′-flanking region | TTTTTTT | Ref. 24 |
| Human NF-κB/Rel site | TTTTATT | Ref. 19 |
| Human IL-2 5′-flanking region | GGGGATTYCCC | Ref. 71 |
| Murine Ig kappa chain enhancer | GGGCACTTTCC | Ref. 42 |
| F50 homodimer high affinity | GGGGATYCCC | Ref. 55 |
| NF-κB/Rel high affinity consensus site | GGGRNYYCC | Ref. 48 |

*Residues that are mutated from the wild type sequence are indicated in boldface type.*

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**Fig. 1.** Reporter gene constructs used to define enhancer activity of the human CD40L gene 3′-flanking region. A, sequence of the entire 1284-bp segment of the human CD40L 3′-flanking region (36) used in constructs, with the natural BamHI site (bp 1–6) in italic type, the NF-κB/Rel cis-element (bp 946–956) underlined, and the 3′-untranslated region of the fifth exon indicated in boldface type. B, design of the reporter gene constructs. The entire 1284-bp segment was included in CD40L.3′FL and in reverse orientation in the CD40L.R3′FL construct. pCD40L.3′FL/1–808 and pCD40L.3′FL/809–1284 are CD40L promoter-directed luciferase constructs that contain either bp 1–808 or 809–1284, respectively, of the 3′-flanking CD40L segment.
T Cell-specific Enhancer of the CD40 Ligand Gene

Fig. 2. CD40L promoter activity but not transcript abundance is substantially lower than that for the IL-2 gene in activated CD4 T cells. A, luciferase activity in CD4 T cells transiently transfected with pGL2-based reporter gene constructs directed by either no promoter segment (pGL2), the 1.3 kb of the 5′-flanking region of the human CD40L gene (pcDNA4L), or 0.6 kb of the human IL-2 gene 5′-flanking region (pIL-2). Following transfection, CD4 T cells were either stimulated with ionomycin and PMA (Ionomycin + PMA) or with medium alone (Unstimulated) for 6 h prior to assaying for luciferase activity. B, CD40L and IL-2 mRNA levels, normalized against GAPDH, in CD4 T cells after 3 h of stimulation with ionomycin and PMA as determined by real time PCR. For each transcript type, the accumulation of product with each cycle of PCR and the Ct value are shown.

Freshly isolated CD4 T cells. In the absence of stimulation, luciferase expression was not detected for any of the CD40L promoter-directed constructs or for the promoterless pGL2 parent vector in CD4 T cells (Fig. 2A, and data not shown). However, similar to Jurkat T cells, the 3′-flanking segment increased CD40L promoter activity in stimulated CD4 T cells in an orientation-independent manner (Fig. 2B).

The CD40L 3′-Flanking Region Enhances the Activity of Heterologous Cytokine Promoters in a T Cell-specific Manner—We next assessed the specificity of the transcriptional enhancer activity for CD40L versus other activation-dependent genes in CD4 T-lineage cells by inclusion of the CD40L 3′ segment in firefly luciferase reporter constructs directed by the promoters for cytokine genes IL-2 and IL-13 and for the large T antigen of the SV40 virus. The 3′-flanking segment of the CD40L gene augmented ionomycin- and PMA-stimulated activity of the IL-2 and IL-13 promoters in both Jurkat cells and in freshly isolated peripheral blood mononuclear cells, used as a source of T cells in this experiment (Fig. 4). In contrast, this segment failed to enhance SV40 promoter activity in either source of T cells, suggesting that the 3′-flanking segment is not a generic enhancer of promoters that are active in CD4 T-lineage cells.

To evaluate the tissue specificity of the transcriptional enhancer activity of the 3′-flanking region of the CD40L gene, a human Epstein-Barr virus-transformed B cell line, 8.1.6, or the monocyte-like cell line, U937, was transfected with pcDNA4L or pcDNA4L3′FL and evaluated for reporter gene activity. Low amounts of CD40L promoter activity were detectable in both of these cell lines in the absence of stimulation, but this increased 1.5–2-fold in response to ionomycin and PMA treatment (data not shown). However, there was no significant increase in ionomycin- and PMA-stimulated transcriptional activity mediated by the 3′-flanking segment (Fig. 4) in the context of either the CD40L promoter or the heterologous cytokine promoters.

Similar to Jurkat and nontransformed T cells, the CD40L 3′-segment did not augment SV40-promoter activity in 8.1.6 or U937 cells. These results suggest that activation-induced enhancer activity of the 3′-flanking region of the CD40L gene is relatively T cell-specific.

A DNase I-hypersensitive Site Is Present in the 3′-Flanking Region of CD40L—For many genes, key transcriptional regulatory regions are identified based on their hypersensitivity to digestion with DNase I (53). To determine whether this applied to the CD40L 3′-flanking region, we isolated nuclei from a CD40L-expressing CD4 T-lineage cell line, subjected these to DNase I treatment, and evaluated the 3′-flanking region for hypersensitive sites using Southern blotting and an appropriate probe. In the absence of DNase I treatment, a full-length 2.2-kb BamHI fragment was detected (Fig. 5). Treatment of nuclei with increasing concentrations of DNase I revealed a 1.4-kb band (Fig. 5, lane 4), which mapped to the 3′-flanking region that contained the putative NF-κB/Rel site. These findings indicated that the NF-κB/Rel site was contained within a region having an open chromatin configuration, consistent with this region playing a role in transcriptional regulation of the CD40L gene.

A Functional NF-κB/Rel Site in the 3′-Flanking Region Is Required for Enhancement of CD40L Promoter Activity in CD4 T Cells—To determine whether the putative NF-κB/Rel site was important for 3′-flanking region enhancement of CD40L transcription, we compared the enhancement of the CD40L promoter by truncations of the 1284-bp segment that either lacked (bp 1–809 segment) or contained (bp 809–1284) this site (Fig. 1B). While the presence of the 1–809 bp segment did not consistently increase CD40L promoter activity by CD4 T cells (Fig. 6A), additional truncations within this region suggested the presence of both positive and negative regulatory elements capable of affecting CD40L promoter activity (data not shown). In contrast, the 809–1284-bp segment was sufficient to substantially augment CD40L promoter activity in CD4 T cells, indicating that it contained an important transcriptional activation element.

We next tested whether the putative NF-κB/Rel-binding segment of the 1284-bp 3′-flanking region was essential for enhancement of CD40L promoter activity. This segment AGGGAAATTTCccc includes not only a 9 of 10 match (underlined) for the consensus NF-κB decamer-binding site (GGGRNNYCC (50)) but also contains a 7 of 7 match (italic type) on the noncoding strand for an NFAT binding site (HGGAAAA (54)). This finding could be of functional importance, since there are examples where NF-κB and NFAT transcription factors can independently contribute to transcriptional activation by binding to such NF-κB/NFAT composite cis-elements (e.g. the human immunodeficiency virus-1 long terminal repeat promoter (55, 56).

To distinguish the role of these two transcription factor families we used site-directed mutagenesis. Two full-length mutant enhancers were created. We compared the enhancement of CD40L promoter activity by the wild type 1284-bp segment with that conferred by either single (GTTAATTTCccc) or double dinucleotide mutations (GTTAATTTCACC). The single dinucleotide mutation was expected to effectively abrogate binding by most NF-κB/Rel proteins, based on the results of in vitro studies using recombinant NF-κB/Rel proteins to define ideal binding sites (57), while leaving the NFAT binding site intact. In contrast, the double mutant was expected to abrogate both NF-κB/Rel and NFAT binding (54, 57). We found that the single dinucleotide mutation resulted in more than 50% loss of the enhancement of CD40L activity by the 1284-bp 3′-flanking region in CD4 T cells (Fig. 6B). On the other hand, the double
A dinucleotide mutation led to only a slight additional decrease in enhancement, suggesting that NFAT binding at this site made a minor contribution to enhancer activity. These results suggested that NF-κB/Rel proteins, rather than NFAT, were crucial for the enhancer activity mediated by the NF-κB/Rel site.

The NF-κB/Rel Site Binds CD4 T Cell p50 NF-κB1 in Vitro and in Vivo—Using EMSAs, we found that an oligonucleotide probe containing the NF-κB/Rel site of the CD40L 3′-flanking region formed a complex with nuclear protein from CD3 and CD28 mAb-activated CD4 T cells (Fig. 7A, lane 1). Formation of this complex was inhibited by either a 20- or 200-fold molar excess of unlabeled oligonucleotide (Fig. 7A, lanes 2 and 3, Self). In contrast, a 20- or 200-fold molar excess of oligonucleotides containing mutations of the NF-κB/Rel site were ineffective in competing complex (Fig. 7A, lanes 4–7). This indicated that complex formation at the NF-κB/Rel site with nuclear
protein correlated with the ability of this site to contribute to CD40L enhancer activity. The specific complex was also effectively competed by an oligonucleotide containing the canonical NF-κB/Rel site of the murine κ immunoglobulin chain, indicating that it might bind NF-κB/Rel proteins (Fig. 7A, lanes 8 and 9). In contrast, the putative NF-κB/Rel element located in the 5′-flanking region of the human CD40L gene (24) was ineffective as a competitor (Fig. 7B, lanes 1–4), suggesting that it did not specifically bind the same proteins as the 3′-flanking site.

The inclusion of specific antisera reactive with the p50 form of the NF-κB1 gene product resulted in a virtually complete supershift of the specific complex formed with the CD40L 3′-flanking NF-κB oligonucleotide, suggesting that p50 was a major component (Fig. 7B, lanes 5 and 6). In contrast, antisera to p52 (NF-κB2), p65 (RelA), c-Rel, or NFAT1, the major NFAT protein contained in these extracts (28), had little or no effect on the formation or the mobility of the complex (Fig. 7B, lanes 7–10). We also failed to obtain supershifts using a variety of commercial antisera or mAbs specific for p65 and c-Rel. Together, these results indicated that p50 homodimers were likely to be the predominant NF-κB/Rel species in the DNA-binding complex and that the CD40L 3′-flanking NF-κB/Rel site was an important cis-element for the enhancement of CD40L promoter activity by binding NF-κB p50.

To address whether binding of NF-κB1 p50 to the 3′-flanking NF-κB/Rel site occurred in intact CD4 T cells, ChIP assays were performed. Primary CD4 T cells were briefly stimulated with CD3 and CD28 mAbs, and, after formaldehyde cross-linkage and sonication, proteins were immunoprecipitated with preimmune antiserum or an antisemur specific for the N-terminal region of NF-κB p50. CD40L 3′-flanking region primers encompassing the NF-κB/Rel site were then used to amplify immunoprecipitated DNA in a real time PCR assay. The Ct value (Ct = 27.6) obtained with NF-κB p50 antisemur was consistently lower than that obtained with preimmune antisemur (Ct = 29). This indicated that the NF-κB p50 antisemur specifically immunoprecipitated the CD40L 3′ DNA template containing the NF-κB/Rel site (Fig. 8) and that NF-κB p50 binds to this site in vivo in human CD4 T cells.

FIG. 6. The NF-κB/Rel binding site is a key element for CD40L 3′-flanking enhancer activity in nontransformed T cells. A, luciferase activity directed by either the CD40L promoter alone (pCD40L) or by the CD40L promoter in conjunction with either bp 1–1284 (pCD40L.3′ FL), 1–808 (pCD40L.3′ FL/1–808), or 809–1284 (pCD40L.3′ FL/809–1284) of the CD40L 3′-flanking segment. B, luciferase activity directed by pCD40L, pCD40L.3′ FL, or a pCD40L.3′ FL construct containing either one (pCD40L.3′ FL/mut1) or two (pCD40L.3′ FL/mut2) dinucleotide substitution mutations of the canonical NF-κB/Rel site (see *Experimental Procedures*).

CD40L gene expression is crucial in both the initiation and progression of various immune responses, particularly for those involving T cells and B cells, as demonstrated by the severe immunological consequences of CD40L genetic deficiency. Ligation of CD40 is an important step for regulation of expression of cytokines, adhesion molecules, apoptotic mediators, and microbicidal activities by a number of cell types. In addition, CD40L plays a key role in pathogenesis of chronic inflammatory diseases such as autoimmune diseases, host disease, atherosclerosis, and neurodegenerative disorders (1, 58–61). Definition of the critical regulatory regions of the CD40L gene is therefore not only of great importance in understanding the events invoked in the generation of the adaptive immune response, but may allow the development of novel therapies that target this important protein in a variety of diseases.

We found that the 3′-flanking region of the CD40L gene substantially enhanced the activity of the CD40L promoter in transformed CD4 T-lineage cell lines as well as in primary peripheral blood CD4 T cells. This DNA segment enhanced transcription in either orientation, indicating that it acted as a classic enhancer (52). The enhancement of CD40L promoter activity by the 3′-flanking region was observed with activation using CD3 mAb alone or a combination of CD3 and CD28 mAbs, indicating that it was not unique to pharmacological stimulation. These results suggest that such enhancement is likely to apply to physiological activation of CD4 T cells following engagement of the αβ-T cell receptor-CD3 complex by antigenic peptide bound to class II MHC molecules or by microbe-derived superantigens.

The core 475-bp CD40L enhancer segment contained a decameric sequence, GGAATTTTCC, that is similar to well characterized and functional NF-κB/Rel cis-elements of other genes (see Table I and references therein). In addition, a DNase I hypersensitivity site that corresponded to the NF-κB/Rel element within the enhancer was identified. Consistent with the functional importance of the NF-κB/Rel site, an oligonucleotide containing the NF-κB/Rel element formed a specific complex with nuclear protein contained in CD4 T cells, of which NF-κB p50 was a prominent component. ChIP assays confirmed the association of NF-κB p50 with the 3′-enhancer site. Importantly, the ability of this element to form an NF-κB-containing protein complex with CD4 T cells strictly correlated with the ability of the 3′-flanking region to enhance CD40L promoter activity in this cell type. In contrast, the presence of an additional mutation in the 3′ region of the element, which would be expected to also prevent binding of NFAT proteins (12), only led to a modest additional decrease in enhancer activity. This suggests that the binding of NF-κB/Rel proteins, rather than NFAT proteins, is necessary for most of the enhancer activity of this element in CD4 T cells and is consistent with our inability to detect NFAT proteins in CD4 T cell nuclear protein complexes formed with the CD40L 3′-flanking NF-κB/Rel element (data not shown). In support of our findings, NF-κB p50 was noted to be required for the induction of CD40L expression, based on studies using mice with genetic disruption of the NF-κB1 gene (63). However, these results do not formally exclude the possibility that NFAT proteins may not act independently but only cooperatively with NF-κB/Rel proteins in mediating enhancer activity. While there are clear examples of composite NF-κB/Rel and NFAT cis-elements in which both transcription factor families independently contribute to transcriptional activation in T-lineage cells (e.g., the human immunodeficiency virus long terminal repeat promoter (55, 56) and the first intron interferon-gamma gene enhancer (64)), we conclude that this does not appear to apply to the CD40L 3′-flanking enhancer.

DISCUSSION
The active DNA-binding forms of NF-κB/Rel proteins are dimers, which may consist of any two members of the NF-κB/Rel family, including p50 (a derivative of the NF-κB1 gene), p52 (a derivative of the NF-κB2 gene), p65, c-Rel, and RelB (50, 57, 65). With the exception of RelB, all of these NF-κB/Rel proteins are expressed by T cells (65). We found that the p50 product of the NF-κB1 gene was the predominant type of NF-κB/Rel protein contained in the nuclear extracts of human CD4 T cells that bound to the 3′-flanking enhancer site in vitro and that p50 antisera immunoprecipitated the enhancer region of the CD40L 3′-flanking segment. No detectable binding of p52, p65, or c-Rel was found, although the nuclear protein extracts we employed contain substantial amounts of these proteins (data not shown). p50 homodimers have a tendency to bind with high affinity to palindromic NF-κB/Rel sites (57), as defined by using in vitro binding site selection assays (66). It is also interesting to note that, with the exception of one nucleotide indicated in italics, the NF-κB/Rel element consists of an 11-bp palindromic sequence, GGGAATTTC CCC, and that a 11-bp palindromic sequence, found in the enhancer of the class I MHC gene (see Table I), has previously been shown to preferentially bind p50 homodimers (67). These results, taken together, suggest that p50, probably in the form of homodimers, enhances CD40L promoter activity by binding to a 3′-flanking cis-activation element.

Analysis of enhancer activity in peripheral blood CD4 T cells revealed that most of the activity of the 3′-flanking segment was localized to a 475-bp subregion. This truncation, in which only 75 bp of the 3′-untranslated region remained, actually further increased enhancer activity compared with the initial 1248-bp segment, suggesting the presence of inhibitory regions in the 3′-untranslated region (see Figs. 1B and 5A). Determination of whether the 3′-untranslated region also contains transcriptional activation regions that contribute to enhancer activity will require additional study, but our preliminary results with additional truncation constructs are consistent with this possibility.³

³ L. A. Schubert, R. Q. Cron, and D. B. Lewis, unpublished results.
T Cell-specific Enhancer of the CD40 Ligand Gene

Our results contrast with several well described transcriptional contexts in which p50 homodimer binding correlates with the inhibition rather than the activation of transcription (e.g. in the 5'-flanking promoters of the class I MHC (67) and IL-2 genes (68)). However, p50 homodimers have been shown in other contexts to act as transcriptional activators, such as in the induction of transcription of the long terminal repeat promoter of the human immunodeficiency virus, type 1 (69). Although p50 lacks the Rel homology domain found in p65, c-Rel, and RelB (65), which associates with co-activator proteins (70), p50 has been shown to contain other domains that mediate transcriptional activation (71, 72). p50 can also associate with other non-NF-κB/Rel DNA-binding proteins in mediating transcription (65), such as members of the CCAAT enhancer-binding protein, CREB/ATF, HMG, and SP-1 families, and with the proto-oncogene product, bel-3, which can act as a transcriptional co-activator (73). Therefore, it is plausible that p50 homodimers could contribute to increased CD40L gene transcription in T-lineage cells. This is particularly the case, since such homodimers are a major component of the nuclear extracts of freshly isolated T cells (74), which have a high capacity to rapidly express the CD40L gene (6, 7).

Both basal and activation-induced transcription were increased by the 3'-flanking enhancer in Jurkat cells, while in primary cells, such as purified T cells or unfractonated peripheral blood mononuclear cells, enhancer function was strictly activation-dependent. Since CD40L mRNA and transcription by primary T cells are usually undetectable in the absence of activation (6, 7, 14, 15), these results suggest that the transcriptional regulatory environment of Jurkat cells may not fully replicate the normal inhibition of CD40L gene transcription that occurs in the absence of T cell activation. Further, although selected Jurkat cell lines have been identified that constitutively express CD40L at high levels (58), we are unaware of any Jurkat or other transformed T-lineage cell lines in which most CD40L gene expression is regulated in a physiological, activation-dependent manner. For these reasons, as well as previous studies documenting substantial differences between Jurkat cells and primary T cells in the transcriptional regulation of the IL-2 gene (37), we relied on primary circulating leukocyte populations containing T cells to further define regions of the CD40L 3' flanking segment that were necessary for enhancer activity.

Studies of the IL-2 gene as well as the genes encoding other proteins, such as granulocyte-macrophage colony-stimulating factor and TNF, have shown that CD28 engagement increases both the rate of cytokine gene transcription and cytokine mRNA stability, resulting in marked increases in cytokine production (75, 76). Our data using reporter gene constructs is consistent with CD28 engagement having a modest effect on CD40L gene transcription but does not exclude the possibility that CD40L mRNA stability may also be enhanced in this context. The modest increase in CD40L promoter activity that occurred with the combination of CD3 and CD28 mAbs compared with CD3 mAb alone is also consistent with previous reports finding only a significant but modest increase in CD40L surface expression by CD4 T cells achieved by the combination of CD3 and CD28 engagement (77). These results also suggest that the 5'-flanking region of the CD40L gene contains a functional CD28 response element, as recently reported (27), but does not rule out the possibility that the 3'-flanking enhancer may also contain such a cis-element with such responsiveness.

There is precedence both for 3'-flanking enhancers of genes expressed by T lymphocytes, such as the CD2 protein (45) and IL-4 (49), and for 3'-flanking region enhancers utilizing NF-κB/Rel cis-elements (e.g. immunoglobulin chain genes (78)) in B-lineage cells. However, to the best of our knowledge, the enhancer of the CD40L gene described here is the first in which a NF-κB/Rel element located in the 3'-flanking region has been implicated in activation-dependent gene expression by T cells. This is in contrast to previously reported NF-κB/Rel sites utilized by T cell activation-dependent genes, which have been identified either in the 5'-flanking promoter (e.g. the IL-2 (79) and granulocyte-macrophage colony-stimulating factor genes (80)) or the first intron, such as for the interferon-γ gene (64). As discussed above, the 3'-flanking NF-κB/Rel site we identified in the CD40L gene also differs from these other sites in that it appears to preferentially bind p50 homodimers rather than other NF-κB/Rel complexes commonly found in human T cells, such as heterodimers of p50 or p52 with Rel A (p65) or with c-Rel (74).

Our results suggest that the 3'-flanking region of other activation-dependent genes expressed by T cells, such as other members of the TNF ligand superfamily, could play a role in transcriptional activation. Interestingly, TNF has been reported (18) to contain a 3'-flanking transcriptional enhancer that is active in monocyte lineage cells stimulated with lipopolysaccharide or the exotoxin, toxic shock syndrome toxin-1. Like the CD40L enhancer, this TNF enhancer segment includes a NF-κB/Rel cis-element in the 3' genomic region immediately flanking the last exon, and this element may augment lipopolysaccharide-induced TNF transcription by mononuclear phagocyte lineage cells (19). This suggests the possibility that such NF-κB/Rel-dependent 3'-flanking enhancers may be a previously unappreciated feature of other members of the TNF ligand superfamily. Whether this 3'-flanking TNF enhancer and its NF-κB/Rel cis-element also have activity in T-lineage cells is not known but, given our results, is of potential interest.

Finally, the observation that a relatively small region of the CD40L gene 3'-flanking region acts in a relatively T cell-specific and activation-specific manner may have usefulness as a means to augment activation-dependent transcription by T cells in a number of contexts. Such an approach might be considered for gene therapy treatment of inherited CD40L deficiency (3, 81) or other immune deficient states in which expression of TNF ligands or hematopoietin cytokines by T cells is reduced, such as during early postnatal life (7). However, the importance of maintaining the normal physiologic pattern of CD40L gene expression in such therapy is suggested by the development of T cell lymphomas in mice following the administration of retroviruses in which CD40L expression is directed by a constitutively active promoter (62). Therefore, it will be of interest to determine whether including authentic transcriptional regulatory elements, such as the CD40L 3'-flanking enhancer region, in gene expression vectors results in higher levels of T cell activation-dependent expression of CD40L in vivo.

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