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Transcriptional Activity of the Distal CD40 Ligand Promoter[1]

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CD40 ligand (CD40L, CD154) is a T cell cytokine with highly regulated expression that requires the transcription factor nuclear factor of activated T cells (NF-AT) to bind at two sites in the proximal CD40L promoter. We have determined that the distal CD40L promoter (−500 to −1300 bp from start of transcription) conveys superior promoter activity in reporter gene assays. Within the distal promoter, we have identified a third NF-AT binding site, at −761 to −756. Oligonucleotides incorporating each of the three NF-AT sites cross-compete for binding of nuclear extracts from activated T cells and bind NF-ATc2 by antibody supershift. Mutation of the distal NF-AT site reduces activity of the 1300 bp CD40L promoter construct to that of the proximal 500 bp construct, which includes only two NF-AT sites. This suggests that the newly identified NF-AT site is the major mediator of transcriptional activation in the distal CD40L promoter.

Key Words: **CD40 ligand; NF-AT; T lymphocyte; cytokine; gene expression.**

CD40 ligand (CD40L), a member of the TNF family of cytokines, is a potent T lymphocyte-derived mediator of the inflammatory response. The events induced by the interaction of CD40L and CD40 include B cell growth and differentiation (reviewed in (1)), upregulation of costimulatory molecules on B cells and antigen presenting cells (2), and IL-12 production by dendritic cells (3). CD40L displays a high degree of regulation in its cell surface expression. CD40L is expressed early after *in vitro* T cell activation by fixed CD3 mAb, appearing on the surface by 4 h after stimulation, reaching peak expression from 6 to 8 h, and returning to baseline by 24 to 48 h (4). CD40L mRNA is detectable 30 min after *in vitro* T cell stimulation by antigen/MHC, peaking at 2 to 3 h, and diminishing after 15 h (5). Expression is developmentally regulated, being restricted to mature, single-positive thymocytes (6) and is almost exclusively limited to $CD4^+$ T cells, occurring in only a small percentage of $CD8^+$ cells (7).

Transcriptional activation of CD40L demonstrates an absolute requirement for the transcription factor NF-AT (8, 9), which plays a pivotal role in the transcription of a variety of inducible cytokines and transcription factors expressed early after T cell stimulation (10, 11). The actions of NF-AT have been studied extensively in the IL-2 promoter (12, 13). NF-AT exists in an inactive, phosphorylated state in the cytoplasm of resting T cells. Following T cell activation, elevated intracellular calcium levels activate calcineurin by a calmodulin-dependent mechanism, enabling calcineurin to dephosphorylate NF-AT, which then can translocate into the nucleus. There, NF-AT and AP-1 protein complexes bind cooperatively in the IL-2 promoter to activate transcription.

At least four isoforms of NF-AT have been identified, with unique tissue distribution and gene specificities (11, 14, 15). Electrophoretic mobility shift assays (EMSA) employing antibody supershifting have implicated NF-ATc_2 (NF-ATp) in CD40L promoter activation (16)) and have demonstrated the requirement for coordinate binding of an AP-1 protein composed of *c-Fos* and *c-Jun*. A similar requirement for cooperative binding of NF-AT and AP-1 protein elements has been demonstrated for promoter activation of TNF-α (17, 18) and Fas ligand (19), both members of the TNF family. In addition to IL-2 and TNF family members, the calcineurin-NF-AT signaling pathway also is impor-

Abbreviations used: CD40L, CD40 ligand; NF-AT, nuclear factor of activated T cells; XHIM, X-linked hyper IgM; EMSA, electrophoretic mobility shift assay; CsA, cyclosporin A; bp, base pair(s).

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tant for the activation of a broad array of functionally distinct cytokines. These include T lymphocyte- and mast cell-derived IL-4 (20) and IL-5 (21, 22), as well as T lymphocyte-, endothelial cell-, and NK cell-derived GM-CSF (23, 24), and T lymphocyte-derived IFN-γ (25).

Previous studies have identified two NF-AT binding motifs in the CD40L promoter at -62 to -69 and -258 to -265 relative to the start of transcription site (9). Both NF-AT sites have been determined to be necessary for promoter activation in reporter gene assays in which mutation of either site abolished inducible activity. CD40L expression can be inhibited completely by the calcineurin inhibitor cyclosporin A (CsA) (26), providing further evidence that calcineurin and NF-AT are necessary for promoter activation.

Definition of the critical regulatory regions of the CD40L promoter is of great importance to understanding the sequence of cytokine events invoked in the early stages of the inflammatory response. In addition, delineation of the transcription factors and signaling pathways that regulate CD40L expression may allow for the rational design of therapies that target this potent inflammatory cytokine. Finally, the composition of potential gene therapy constructs for the disease X-linked hyper IgM syndrome (XHIM), a primary immunodeficiency that results from mutations in CD40L (1), will depend on a thorough familiarity with the positive and negative regulatory elements of the CD40L promoter.

In this study we determine that the distal CD40L promoter significantly enhances the overall inducible activity of the CD40L promoter. This enhanced activity is mediated by a third, previously unstudied NF-AT binding motif in the distal CD40L promoter that binds NF-ATc_2.

MATERIALS AND METHODS

Cell lines. All transfections were carried out in Jurkat thymoma cells (American Type Culture Collection, Rockville, MD) grown in RPMI supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium).

Reporter plasmid construction. Reporter plasmid constructs incorporating varying lengths of the CD40L promoter were prepared as follows: Fragments of the CD40L promoter originating at -1227 and -500 relative to the start of transcription site (9) of the CD40L gene were derived by PCR using the indicated sense primers fitted with Hind III sites (underlined): -1227 bp, 5′-AAGCTTTCTAGACCAGGTTTGGCATG-3′; and -500 bp, 5′-AAGCTTCCTGCCAGGCTTTCATTGAGTTT-3. The proximal 240 bp of the CD40L promoter was derived by PCR using the indicated sense primer: -240 bp, 5′-GAAGTCTATGACATTTCAAGGCAAG-3′. The proximal 61 bp of the CD40L promoter was derived by PCR using the indicated sense primer fitted with an *Xho*I site (underlined): -61 bp, 5′-CTCGAGGAAGTGTGGGCTGCAACGATTGTG-3′. These primers were paired with an antisense primer, the 3′ terminus of which corresponds with +67 bp relative to the start of transcription: 3′(antisense) primer, 5′-AAGCTTGCTGTGTTAAAGTTGAAATG-3′. The 1227 bp and 500 bp PCR-amplified fragments were subcloned into the *Hind*III site upstream of a luciferase reporter gene in the PGL3 basic vector (Promega, Madison, WI), while the -61 bp fragment was subcloned into PGL3B at the *Xho*I and *Hind*III sites. The 240 bp fragment was subcloned into PGL3 basic by blunt-ended ligation of the 5′ terminus at the *Sma*I site and complementary ligation of the 3′ terminus at the *Hind*III site. A construct incorporating the CD40L promoter originating at -100 was derived by restriction enzyme digestion of the -500 bp construct using the Sna BI enzyme.

Transient transfection and luciferase reporter gene assays. Transient transfection was performed by electroporation as previously described (27). Briefly, 5×10^6 Jurkat cells in a volume of 250 μl of complete medium were transfected with equimolar quantities of each construct by electroporation at 250v and 950 μF in 0.4 cm cuvettes. To correct for transfection efficiency, 2 μg of a β-galactosidase reporter plasmid driven by the β-actin promoter were cotransfected in each cuvette. After electroporation, cells were cultured in complete medium for 1–2 h. Where indicated, cyclosporin A (100 ng/ml) (Calbiochem, San Diego, CA) was added to the cultures for 60 min prior to stimulation. Where indicated, transfected cells were stimulated with plate-bound anti-CD3 (10 μg/ml OKT3 mAb incubated in wells overnight at 4°C) or by 20 ng/ml phorbol myristate acetate (PMA) and 1.5 μM ionomycin for 24 h. Luciferase activity was measured in 25 μl of lysate after the addition of 50 μl luciferin substrate (Promega). To correct for transfection efficiency, β-galactosidase activity was measured spectrophotometrically from the same lysates after the addition of a chloro-phenol red-conjugated substrate (28). Relative luciferase reporter gene activity was calculated as the ratio of absolute luciferase activity to absolute β-galactosidase activity from an individual sample.

Electromobility shift assays. Nuclear extracts were prepared as previously described (29) from Jurkat T cells that were stimulated for 1 h with 20 ng/ml phorbol myristate acetate (PMA) and 1.5 μM ionomycin or left unstimulated. EMSA were performed using a commercially available chemiluminescent detection system (DIG Gel Shift Kit, Boehringer Mannheim, Indianapolis, IN) using 5 μg of nuclear extract protein per condition. Competition assays were performed with 100-fold excess of unlabeled oligonucleotide, and antibody supershifting was conducted with 0.5 μg of NF-ATc2 mAb or IgG1 isotype control (both from Pharmingen, San Diego, CA). The following oligonucleotides were synthesized at a core oligonucleotide synthesis facility in the Department of Pathology of Yale University School of Medicine using an Applied Biosystems 394 synthesizer (sense sequences indicated, with introduced mutations shown in bold): NFAT 1, 5′-AAGCACATTTTCCAGGAG-3′; NFAT 2, 5′-TGATAGGAAAATACTGCC-3′; NFAT 3, 5′-CAGCCTATTTTCCTATTC-3′; and NFAT 3 mutant, 5′-CAGCCTATTTT**AA**TATTC-3′.

Site-directed mutagenesis. Site-directed mutagenesis of the distal NF-AT site from TTTTCC to TTGGCC was performed using a commercially available kit (Quickchange, Stratagene, La Jolla, CA), employing the following sense primer and a complimentary antisense primer (introduced mutations shown in bold): 5′-CTTAACTGCAGCCTATT**GG**CCCTATTCTGAACTGTTACATCAGC-3′.

RESULTS

Design of reporter gene constructs containing the distal CD40L promoter. In order to determine the relative and cumulative contributions of the distal CD40L promoter to activation of CD40L gene transcription, we generated a series of luciferase reporter gene constructs containing progressively larger fragments of the CD40L promoter site (9) (Fig. 1). The two largest constructs, consisting of 1227 bp and 500 bp 5′ of the start of transcription site, contained the two previously characterized NF-AT motifs, at -69 to -62 and -265 to -258 relative to the start of transcription site. The

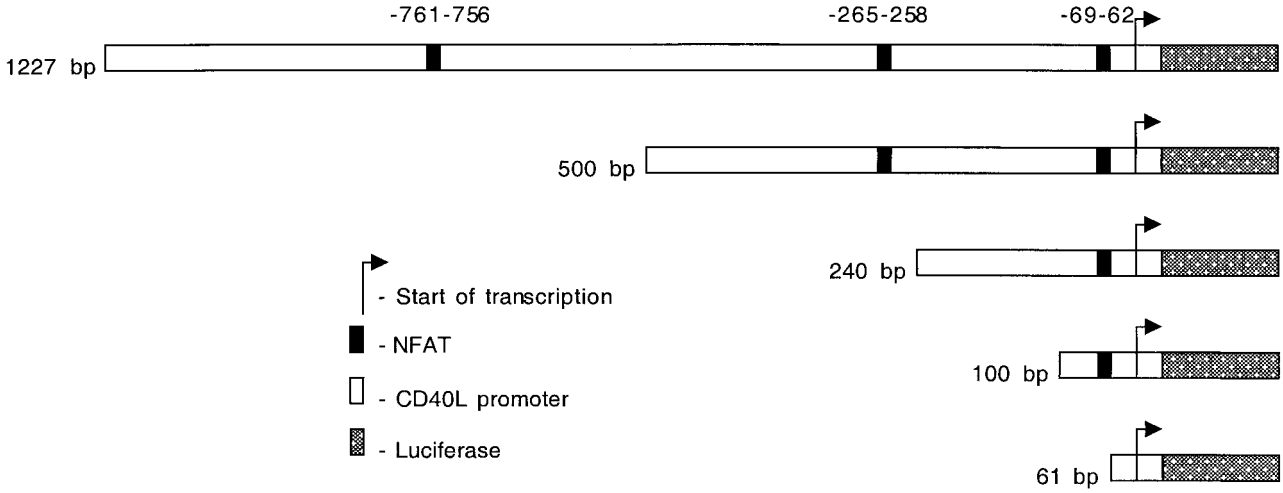

FIG. 1. Design of reporter gene constructs containing 5′ deleted elements of the CD40L promoter. 1227, 500, 240, and 61 bp promoter fragments were generated by PCR using primers described under Materials and Methods. The 100 bp promoter construct was generated using restriction enzyme digestion of the 500 bp fragment as described under Materials and Methods. Sequence numbers are counted from the CD40L start of transcription site (indicated with arrow).

constructs with the proximal 240 bp and 100 bp of promoter sequence provided only the most proximal NF-AT site. We also generated a construct with the proximal 61 bp of the CD40L promoter, which eliminates the most proximal NF-AT site, in order to demonstrate the minimal active CD40L promoter.

The −1227 bp promoter generates the highest activity, which is sensitive to CsA. In order to determine the role of the distal promoter in overall activity, we compared the inducible activities of the luciferase reporter gene constructs containing varying lengths of the CD40L promoter and tested the effect of CsA on these activities. After transient transfection with the indicated reporter gene constructs, Jurkat T cell lines were incubated either in the presence of CsA or in medium alone, followed by stimulation for 24 h with PMA and ionomycin, or with fixed CD3 mAb. Compared with the empty parent vector, the CD40L promoter-containing constructs yielded inducible promoter activity that increased with progressive inclusion of 5′ promoter sequence (Fig. 2). In all constructs, the activity induced by PMA and ionomycin was two to threefold higher than that induced by anti-CD3. The construct containing 1227 bp of promoter sequence generated the highest activity. The 500 bp-containing construct, which includes the proximal two NF-AT sites, generated approximately 45% of the reporter activity of the 1227 bp construct, while the 240 bp-containing construct, which only contains the most proximal NF-AT site, generated 35%. The 100 bp construct, which also includes only the most proximal NF-AT site, gave the lowest activity at approximately 15% of the 1227 bp construct. The superior activity of the 240 bp construct in comparison to the 100 bp construct, both of which contain only one NF-AT site, sug-

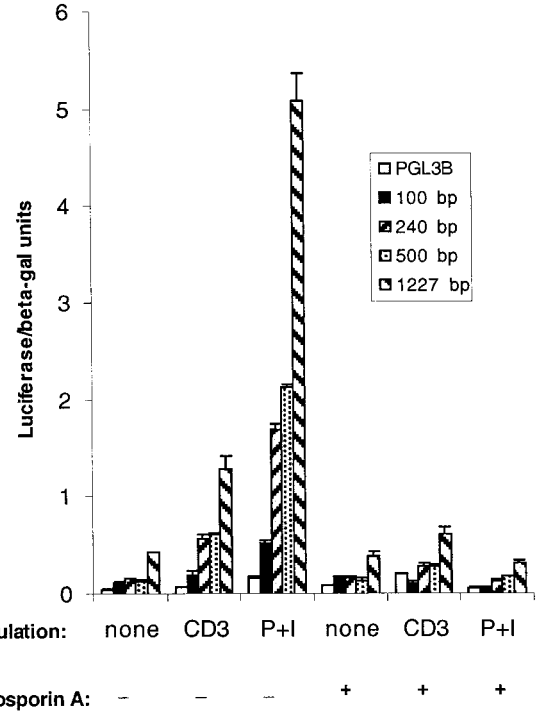

FIG. 2. Inclusion of the distal CD40L promoter conveys superior inducible promoter activity by reporter gene assay. Jurkat T cells were transfected with molar equivalents of the indicated promoter constructs (15 μg of 61 bp construct) along with a fixed amount (2 μg) of a construct containing the β-galactosidase gene driven by the CMV promoter. Transfected cells then were left unstimulated or stimulated with 20 ng/ml PMA and 1.5 μM ionomycin. As indicated, 100 ng/ml of cyclosporin A were added to the cultures 30 min prior to stimulation. After 24 h, luciferase activity was determined for triplicate samples and corrected for transfection efficiency using β-galactosidase activity as described under Materials and Methods. Each bar represents the mean ± SEM of corrected luciferase activity for triplicate samples from an experiment representative of four independent experiments.

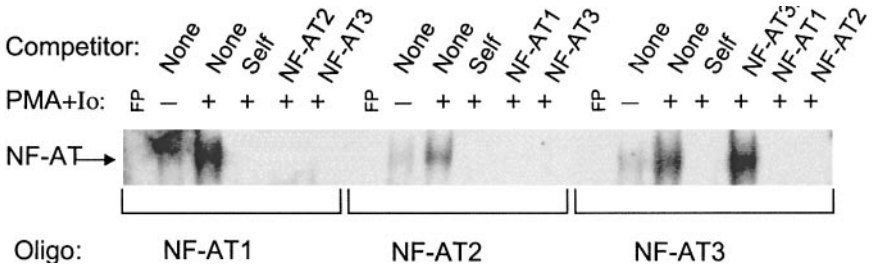

FIG. 3. All three NF-AT sites in the CD40L promoter bind similar nuclear extract proteins. Nuclear extracts from unstimulated or PMA and ionomycin-stimulated Jurkat T cells were incubated with labeled oligonucleotides containing either the most proximal (NF-AT1), intermediate (NF-AT2), or distal (NF-AT3) NF-AT sites and were analyzed by EMSA. Where indicated, competition was conducted with a 100-fold excess of unlabeled oligonucleotides including the three used as labeled probes, as well as an oligo containing a 2 bp mutation (see Materials and Methods) in the consensus NF-AT binding site within the NF-AT3 oligo (NF-AT3M). FP indicates free probe run without incubation with nuclear extracts.

gests that enhancing regulatory elements other than NFAT may be present in the sequence from −100 to −240. We also have tested the activity of a 61 bp construct, which contains the most proximal promoter sequence up to, but not including, the first NF-AT site. The activity of this construct was similar to the empty parent vector, showing no inducible activity (data not shown). CsA treatment reduced both CD3 mAb-induced and PMA and ionomycin-induced activities of all of the constructs, indicating that calcineurin-mediated signaling, and hence NF-AT, was essential for their activation. Based on previous dose-response studies, the dose of CsA in these experiments was sufficient for complete ablation of CD40L protein expression (26, 27). The minor increase in CsA-insensitive activity seen with progressive increase in the promoter length further suggests that elements other than NF-AT may be involved, although they would appear to make a relatively small contribution to overall promoter activity.

The distal CD40L promoter contains an NF-AT site that shares protein binding characteristics with proximal NF-AT sites. We analyzed the distal CD40L promoter for consensus binding motifs of factors that are known to activate cytokine transcription. This analysis revealed an NF-AT binding motif (TTTTCC) at −761 to −756 that was identical to the proximal NF-AT site, at −62 to −69. In view of the sequence identity of the most proximal and distal NF-AT sites, we predicted that they would bind to similar proteins found in the nuclear extracts of activated T cells. In order to test this hypothesis, we conducted EMSA using nuclear extracts from unstimulated Jurkat T cells or cells 1 h after stimulation with PMA and ionomycin. Labeled oligonucleotide probes for the NF-AT sites at −62 to −69 (NF-AT1), −258 to −265 (NF-AT 2), and the newly identified site at −761 to −756 (NF-AT3) all bound nuclear proteins migrating at the same position, but only derived from nuclear extracts of stimulated cells (Fig. 3). Unlabeled probes from each site competed successfully with each labeled probe, indicating that all probes were binding the same nuclear protein. The probe incorporating the TT to GG mutation at −758 to −759 of the NF-ATM3 construct was unsuccessful in competition with the NF-AT3 probe, demonstrating that mutation of the predicted NF-AT binding site abolished binding of the probe to nuclear proteins from activated lymphocytes.

The distal NF-AT site binds NF-ATc$_2$. In order to define the proteins bound by the distal NF-AT site and to compare them with the protein-binding characteristics of the previously defined NF-AT sites, we performed EMSA with antibody supershifting. A monoclonal antibody recognizing the NF-ATc$_2$ isoform, but noncross-reactive with other NF-AT isoforms, was incubated with nuclear extracts from PMA and ionomycin-stimulated Jurkat T cell nuclear extracts prior to the addition of the labeled probes. The NF-ATc$_2$ mAb specifically supershifted the protein/oligonucleotide complex formed both by the NF-AT1 probe and by the NF-AT3 probe (Fig. 4), indicating that both sites bind the NF-ATc$_2$ isoform.

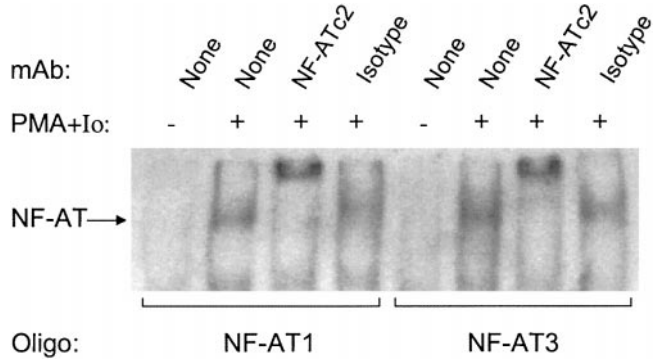

FIG. 4. The distal NF-AT site binds the NF-ATc$_2$ isoform of NF-AT. As indicated, nuclear extracts from unstimulated or PMA and ionomycin-stimulated Jurkat T cells were preincubated for 30 min with 0.5 μg of an NF-ATc$_2$ mAb that is noncross-reactive with other NF-AT isoforms, or with 0.5 μg of an isotype control. Labeled oligonucleotides (NF-AT1 and NF-AT3) then were added and EMSA analysis was performed.

CD40L promoter activity is reduced by site-directed mutation of the distal NF-AT site. In order to confirm that the NF-AT site at −761 to −756 contributes to the superior activity observed in the 1227 bp promoter construct, we performed site-directed mutation of the distal NF-AT site. Within the 1227 bp construct, the consensus sequence TTTTCC was mutated to TTGGCC to generate the NF-ATM3 construct. Jurkat T cells were transfected with the intact 1227 bp construct, the 500 bp construct, or NF-ATM3. Activity was compared 24 h after stimulation with PMA and ionomycin. Mutation of the distal NF-AT site resulted in a reduction of inducible activity to the level observed with the 500 bp construct (Fig. 5). This suggests that the distal NF-AT site is the major mediator of inducible activity in the promoter sequence from −500 to −1227. Furthermore, it supports a model in which multiple NF-AT sites in series are capable of synergistic augmentation of overall promoter activation.

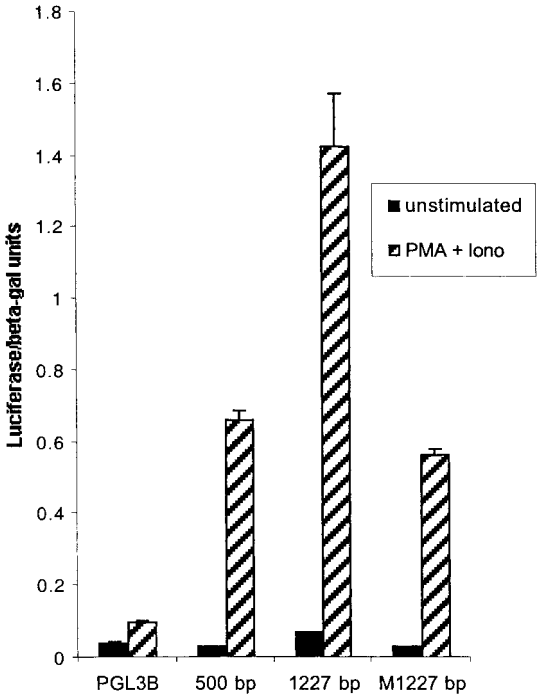

FIG. 5. Mutation of the distal NF-AT site ablates superior activity of the 1227 bp promoter. Jurkat T cells were transfected with molar equivalents of the indicated promoter constructs (15 μg of 500 bp construct) along with a fixed amount (2 μg) of a construct containing the β-galactosidase gene driven by the CMV promoter. Transfected cells then were left unstimulated or stimulated with 20 ng/ml PMA and 1.5 μM ionomycin. After 24 h, luciferase activity was determined for triplicate samples and corrected for transfection efficiency using β-galactosidase activity as described under Materials and Methods. Each bar represents the mean ± SEM of corrected luciferase activity for triplicate samples from an experiment representative of three independent experiments.

DISCUSSION

The expression of CD40L after T cell receptor-mediated lymphocyte activation is a critical step in the adaptive immune response. Failure to express functional CD40L results in the congenital disease XHIM (30–34), which is characterized by recurrent sinopulmonary bacterial infections, opportunistic infections with *Pneumocystis carinii* and *Cryptosporidium*, by autoimmune complications such as sclerosing cholangitis, and by malignancies of lymphoid, neuroendocrine, and hepatobiliary tissues (35). XHIM, like several other primary immunodeficiencies, is an attractive candidate for gene therapy due to the accessibility of the immune system for *ex vivo* manipulation and to the observation that only partial expression of the normal gene is sufficient for normal immune function (36). Gene therapy, however, will require a thorough understanding of the regulation of CD40L expression at all levels, from promoter through cell surface protein. The dangers of poorly regulated expression of CD40L are illustrated by the association of systemic lupus erythematosus (37) with unregulated CD40L expression, as well as the apparent induction of thymic lymphoproliferative disease in mice treated with a retroviral-vector-mediated gene therapy that induced constitutive CD40L expression (38). These results may be explained by the complex regulation of CD40L expression, which permits transient, high level expression after T cell activation, but silences expression at rest.

In this study we have identified a novel NF-AT site in the distal CD40L promoter that mediates most, if not all, of the distal promoter's quantifiable activity. This site bears the consensus sequence for NF-AT binding sites and is identical to core sequence of the most proximal site. Our study demonstrates the ability of serial NF-AT sites to act in synergy to activate gene transcription. This finding is consistent with the demonstrated capacity of multimerized 30 bp sequences spanning the proximal NF-AT site of the CD40L promoter to induce transcriptional activation (16). There is no evidence from our experiments that any one of the three identified NF-AT sites in the CD40L promoter contributes more to the overall promoter activity. Furthermore, it appears that the newly identified site contributes significantly to promoter activation but is not required for minimal promoter activity as shown by the reporter gene assays employing a mutation of the distal NF-AT site. In contrast, activity of the IL-2 promoter is abolished by mutation of any of the five NF-AT sites identified within it (39).

To date, the only transcription factors that have been demonstrated to activate CD40L transcription are NF-AT and AP-1 proteins. We recently defined a novel role for the calcium/calmodulin-dependent kinase IV in transducing calcium-dependent activation signals to promote CD40L gene transcription (27). It is possible, however, that the mechanism by which calcium/calmodulin-dependent kinase IV mediates CD40L

transcriptional activation is by its previously demonstrated ability to activate AP-1 proteins (40) rather than by a novel transcription factor. The finding that the 240 bp promoter conveyed activity superior to the 100 bp promoter despite the inclusion of only one NFAT site in both constructs suggests that there may be enhancing elements contained in the promoter sequence between −100 and −240. This is the subject of ongoing investigations in our laboratory. Nevertheless, our study provides further evidence for the primacy of NF-AT in the activation of CD40L gene expression. It also suggests that gene therapy constructs designed to replicate the native regulation of CD40L expression in XHIM patients should include the distal CD40L promoter.

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