

The CD40L Promoter Contains Nuclear Factor of Activated T Cells-binding Motifs Which Require AP-1 Binding for Activation of Transcription*

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Four nuclear factor of activated T cells (NF-AT) binding motifs were found in the murine CD40 ligand promoter. Electrophoretic mobility shift assays using 18-base pair (bp) long oligonucleotides corresponding to the proximal site and nuclear extracts from activated T cells revealed two complexes which were inhibited by cyclosporin A and contained NF-ATc and NF-ATp. Neither complex contained AP-1 proteins. Multimers of the 18-bp oligonucleotides were not active in transient transfection assays using luciferase reporter gene constructs. In contrast, a 30-bp long oligonucleotide bound AP-1 proteins in addition to NF-AT proteins and its multimers strongly induced luciferase gene expression. These results suggested that NF-AT proteins play an important role in the expression of the CD40L gene and that their transcriptional activity requires AP-1 binding.

The ligand for the B cell antigen CD40 (CD40L) is expressed on activated T cells and plays a critical role in T cell dependent B cell stimulation, particularly in isotype switching (1). This is highlighted by the fact that isotype switching is deficient in patients with X-linked HyperIgM syndrome (HIGM1) in whom the CD40L gene is mutated (2). Furthermore, immunoglobulin isotype switching in response to T cell-dependent antigens is deficient in mice with disruption of the CD40L gene or the CD40 gene (3–5).

CD40L is a type II membrane glycoprotein homologous to tumor necrosis factor while CD40, its counter-receptor on B cells, is a member of the tumor necrosis factor receptor/nerve growth factor receptor family (6). The expression of CD40L on T cells is tightly regulated. Both activation of protein kinase C and a rise in $[Ca^{2+}]_i$ are required for optimal CD40L gene expression (7). Following stimulation with PMA¹ and ionomycin, CD40L mRNA is detectable as early as 1 h after stimulation and disappears by 16 h. Similarly, surface expression of CD40L protein peaks at 6 h after stimulation with PMA plus

ionomycin and is barely detectable by 16 h. Surface expression of CD40L peaks 16 h following stimulation with anti-CD3 and is no more detectable by 48 h (8).

CD40L expression is developmentally regulated. Immature CD4⁺CD8⁺ human thymocytes obtained from infants (3 months to 2 years of age) fail to express CD40L upon activation. In contrast, CD4⁺ thymocytes, but not CD8⁺ thymocytes, express CD40L and can induce B cells to undergo isotype switching to IgE in the presence of IL-4 (9). Newborn T cells were found to be deficient in CD40L expression and in their ability to induce isotype switching in B cells (10, 11).

Induction of CD40L is inhibited by cyclosporin A (CsA) and its analogues (7). The ability of analogues of CsA to inhibit CD40L expression correlated with their ability to block the phosphatase activity of calcineurin. Furthermore, CsA inhibited CD40L-driven T cell dependent isotype switching. These findings suggested that calcineurin is involved in activation of CD40L gene expression.

Nuclear factor of activated T cells (NF-AT) is a transcription factor that plays a critical role in IL-2 and IL-4 gene expression and which is dependent on the phosphatase activity of calcineurin for its activation (12–14). Recent studies have shown that NF-AT expression is not restricted to T cells and is probably a feature of all activated lymphoid cells (15–17). NF-AT consists of two components, a pre-existing cytoplasmic subunit, NF-AT, and a nuclear component consisting of an AP-1 complex (18, 19). PMA causes activation of the nuclear component, whereas calcium signals induce the translocation of NF-AT to the nucleus thus explaining the requirement for two signals for the induction of NF-AT. A murine isoform of pre-existing NF-AT (NF-ATp) has been recently cloned and shown to be a 100-kDa protein containing in its carboxyl-terminal region a domain that shows homology to the Rel family of transcription factors (20). Recently, the human isoforms of NF-ATp and NF-ATc were cloned (21). NF-ATc differs from NF-ATp in that its Rel homologous domain is located in the middle of the molecule.

The promoter/enhancer regulatory elements and the transcriptional factors involved in expression of the IL-2 gene are well known, whereas little is known about the function of these factors in the control of other genes that are expressed during T cell activation. The transient expression of CD40L, its developmentally regulated expression, and its inhibition by CsA suggest that common regulatory elements are shared between IL-2 and CD40L. In this paper, we show that the upstream regulatory region of the murine CD40L gene contains four potential NF-AT binding motifs. Each of these four motifs bound nuclear factors from T cells activated with PMA and ionomycin to give rise in electrophoretic mobility shift assays (EMSA) to two complexes that comigrated with the two complexes formed with the distal NF-AT site (NF-AT1) of the

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¹ The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; IL, interleukin; CsA, cyclosporin A; NF-AT, nuclear factor of activated T cells; EMSA, electrophoretic mobility shift assay; bp, base pair(s); NM, NF-AT-like motifs; mAb, monoclonal antibody.

murine IL-2 gene and that were inhibited by cyclosporin A. Detailed analysis of the most proximal of the NF-AT homologous motifs (NM-1) using an 18-bp oligonucleotide revealed the presence of NF-ATp in the lower complex and of NF-ATc in the upper complex. Neither complex contained proteins of the Fos or Jun families. Transient transfection assays using luciferase reporter gene constructs showed that multimers of the 18-bp long NM-1 oligonucleotide were not transcriptionally active in the EL-4 thymoma cell line nor in the mature T cell hybridoma line 68-41 which can be induced to express CD40L. In contrast, multimers of a 30-bp long NM-1 oligonucleotide, which bound AP-1 proteins in addition to NF-ATp and NF-ATc from the nuclear extracts of activated T cells, strongly induced expression of the luciferase gene in the 68-41 T cell line.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—The murine T cell hybridoma 68-41 (a kind gift of Dr. M. Kubo, Tokyo University) (31, 32) and murine EL-4 thymoma (ATCC, Rockville, MD) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 μ M 2-mercaptoethanol. Rabbit antiserum to NF-ATp was a kind gift of Dr. A. Rao (Dana Farber Cancer Institute). Mouse monoclonal antibody 7A6 (IgG1) to NF-ATc was a generous gift from Dr. G. Crabtree (Stanford University, CA). Rabbit anti-Fos and anti-Jun antisera were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). These antibodies were raised to peptides which are highly conserved among individual members of the particular family and cross-reacted with all the known members of that families. Normal rabbit serum and mouse IgG1(MOPC21) were purchased from Sigma. Cyclosporin A was a gift from Sandoz Pharmaceuticals (East Hanover, NJ).

Thymocytes and Spleen T Cells—Thymocytes were obtained by preparation of the single cell suspension from the thymus of 2–3-week-old mice C57BL/6. Splenocytes were prepared from the spleen of 2-month-old C57BL/6 mice, and T cells were enriched by incubation of splenocytes with goat anti-mouse IgG magnetic beads from Perspective Technology, Cambridge, MA.

Electrophoretic Mobility Shift Assay—Cells at 10^6 /ml were either left unstimulated or were stimulated with 20 ng/ml PMA and 1 μ M ionomycin for 1 h. Nuclear extracts were prepared essentially as described in Ref. 33. Briefly, 10^7 cells were washed twice with ice-cold phosphate-buffered saline, resuspended in cold 10 mM Hepes buffer, pH 7.9, containing 0.01% Nonidet P-40, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and a protease inhibitor mixture (0.2 mM phenylmethylsulfonyl fluoride, 10 μ M leupeptin, 100 ng/ml pepstatin, 100 ng/ml chymostatin, 1 μ M antipain) and incubated for 10 min on ice. Nuclei were pelleted in a microcentrifuge for 10 s and resuspended in ice-cold 20 mM Hepes buffer, pH 7.9, containing 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, and the protease inhibitor mixture. After incubation on ice for 20 min, debris were removed by centrifugation at 14,000 rpm for 10 min at 4 °C. The supernatants were aliquoted and frozen at –80 °C. The protein concentration was estimated by the bicinchoninic acid (BCA) protein assay kit (Pierce). Where indicated, cyclosporin A was added at a final concentration of 0.1 μ g/ml 20 min prior to cell stimulation.

Single-stranded oligonucleotides were 5'-end-labeled with [γ - 32 P] ATP, using T4-polynucleotide kinase, annealed, and purified on 12% PAGE in 1 \times TAE. For each reaction, 1×10^3 cpm (~0.1 ng) of radiolabeled oligonucleotide probe was incubated with 1–5 μ g of nuclear extract in 20 μ l of binding buffer for 30 min on ice or at room temperature. The binding buffer contained 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% glycerol, 50 ng/ml poly(dI-dC), 1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, and the protease inhibitor mixture. Samples were then run on 5% polyacrylamide gel electrophoresis in 1 \times TBE. Unlabeled competitors were added in 100-fold molar excess. When indicated, the nuclear extracts were preincubated with the respective immunoreagent for 30 min before addition of radiolabeled oligonucleotides.

The sequences of the oligonucleotides used are listed in Table I. Additional oligonucleotides used in these experiments are as follows: NF- κ B site of murine IL-2 promoter, GAGGGATTTCCACC-TAAAT; consensus AP-1, CGCTTGATGAGTCAGCCG.

Transfections and Luciferase Assay—EL-4 thymoma or 68-41 T cell hybridoma cells were transfected using the CellPfect transfection kit from Pharmacia Biotech and cultured in complete medium. Four hours later, each culture of transfected cells was divided into the appropriate

TABLE I
Position and nucleotide sequence of NF-AT-binding motifs (NM) in the CD40L promoter

Sequences of the NF-AT binding oligonucleotides from the upstream regions of the different murine genes including CD40L, IL-2, and IL-4. The NF-AT-binding core sequence (TTTTC) is labeled with bold letters.

Gene	Motif	Position	Sequence
CD40L	NM-1	–74 to –57	AAGCACAT TTTTC AGGAA
	NM-2	–283 to –266	GCAGTAT TTTTC TATTG
	NM-3	–935 to –918	AAAGTCT TTTTC CTTAGC
	NM-4	–1016 to –999	AAATGAG TTTTC CAATG
	NLM-1	–327 to –310	TTTGTCA cTTTTC TTGAA
	NLM-2	–1169 to –1152	AAGCAAT TTTTC CCAGTTT
muIL-2	NF-AT1	–290 to –273	AAACAAAT TTTTC CTCTT
muIL-4	P1	–81 to –64	AATAAAAT TTTTC CAATGT

number of aliquots and left unstimulated or was stimulated by PMA and ionomycin. Cells were collected 20 h after treatment. Firefly luciferase activity was measured by the Luciferase assay kit (Promega, Madison, WI). Luciferase activity was normalized to the protein concentration estimated by the BCA protein assay kit (Pierce) and expressed as relative light intensity/ μ g of protein. Efficiency of transfection was verified by cotransfection with pCAT-Control vector (Promega). When indicated, cyclosporin A was added at 0.1 μ g/ml 20 min before cell stimulation.

Reporter Plasmid Construction—Pentamers of both 18 and 30 bp NM-1 oligonucleotides were cloned into the *Xho*I site of the pGL2-Promoter vector (Promega). Similar constructs were made with NF-AT1 pentamer and mutant NM-1 pentamer in which the TTTTC consensus sequence was replaced by TTCGAT.

Fluorescence-activated Cell Sorter Analysis—The 68-41 cells at 10^6 /ml were unstimulated or stimulated by 20 ng/ml PMA and 1 μ M ionomycin for 6 h and stained for the CD40L expression using hamster mAb MR-1 (a kind gift of Dr. R. Noelle) (8). Purified hamster IgG isotype standard antibody and anti-hamster F(ab')₂ conjugated with phycoerythrin, were purchased from PharMingen, San Diego, CA.

RESULTS

The Upstream DNA Sequence of the CD40L Gene Contains Four NF-AT-like Motifs—We have recently reported the organization of the murine CD40L gene (22). DNA sequence analysis of 1.7 kilobases upstream of the transcription start site revealed the presence of four motifs that contain the invariant TTTTC sequence found in the two NF-AT binding motifs of the mouse IL-2 promoter and in the five NF-AT binding sites (P0-P4) of the mouse IL-4 promoter (23). The sequences of these four NF-AT motifs (NM) and their positions relative to the transcription start site are shown in Table I together with the sequences of the NF-AT1 site in the mouse IL-2 promoter and the P1 site in the mouse IL-4 promoter. The NF-AT motifs in the CD40L gene were numbered 1 through 4 (NM-1 through 4) with NM-1 being the most proximal to the transcription start site. In addition, two NF-AT-like motifs (NLM), each with a single base pair substitution in the TTTTC consensus sequence were identified in the CD40L gene. The sequence of these NF-AT like motifs (NLM-1 and -2) is also shown in Table I.

EMSA Using Oligonucleotides Corresponding to the NF-AT Motifs of the CD40L Gene—Nuclear extracts were prepared from EL-4 murine thymoma cells which were either left unstimulated or were activated for 1 h with PMA and ionomycin. EMSA was performed using kinase-labeled synthetic 18-bp oligonucleotides which corresponded to DNA sequences in the CD40L gene that spanned the NM-1 through 4 motifs, as shown in Table I. Fig. 1A shows that each of these four oligonucleotides (NM-1 to NM-4) gave rise to two retarded bands with nuclear extracts from both stimulated and unstimulated EL-4 cells. The intensity of the gel shift bands was increased with nuclear extracts from PMA- and ionomycin-treated cells.

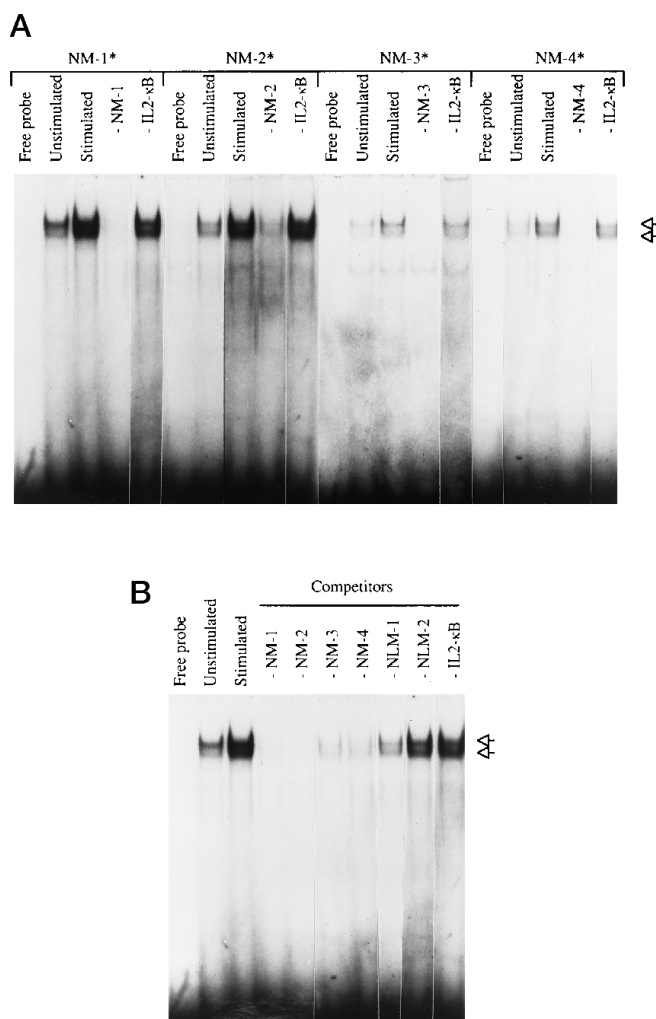


FIG. 1. EMSA using nuclear extracts of EL-4 thymoma cells and radiolabeled oligonucleotides NM-1 to NM-4. Nuclear extracts were prepared from stimulated or unstimulated EL-4 cells as described under "Experimental Procedures." The sequence of the NM oligonucleotides is shown in Table I. *A*, the arrows denote the two complexes formed between nuclear proteins and radiolabeled (*) NM oligonucleotides. These two complexes were specifically inhibited by 100-fold molar excess of unlabeled self oligonucleotide but not by oligonucleotide corresponding to the NF- κ B of the murine IL-2 promoter (IL2- κ B). *B*, cross-competition of NM-1 with other NM oligonucleotides. Unlabeled competitors were added in 100-fold molar excess. NM-1, -2, -3, and -4 oligonucleotides inhibited the mobility shift bands whereas NLM-1, NLM-2, and IL2- κ B did not.

The two gel shift bands obtained with each of the four oligonucleotides (NM-1 to 4) showed identical mobility and were competed out by a 100-fold excess of self cold competitor but not by a 100-fold excess of an oligonucleotide corresponding to the NF- κ B sequence of the mouse IL-2 promoter (Fig. 1*A*). No gel shifts were obtained with oligonucleotides corresponding to NLM-1 and NLM-2 in which the consensus TTTTCC sequence had a single base pair substitution, cTTTCC and TTTaCC, respectively (data not shown).

Given the identical gel shift pattern obtained with oligonucleotides corresponding to NM-1 through 4, we tested the capacity of these oligonucleotides to cross-compete in EMSA. Fig. 1*B* shows that both bands obtained with NM-1 oligonucleotide were completely inhibited by a 100-fold excess of cold NM-2 oligonucleotide and inhibited to a very large extent by a 100-fold excess of NM-3 and NM-4 oligonucleotides. In contrast, substantially less inhibition was obtained with NLM-1 oligo-

nucleotide and no inhibition was obtained with NLM-2 oligonucleotide or with the irrelevant NF- κ B sequence of the mouse IL-2 promoter. These results suggest that similar or identical nuclear factors may bind to the four NF-AT elements of the CD40L gene. Since NM-1 is the element most proximal to the transcription start site of the CD40L gene, we chose it for further detailed analysis.

Characterization of the NM-1 Nuclear Complex—The presence of the NF-AT core sequence TTTTCC in NM-1 prompted us to compare the NM-1 nuclear complex to the nuclear complex formed with NF-AT1. Nuclear extracts from EL-4 cells were incubated with the NM-1, NF-AT1, and P1 18-bp oligonucleotides shown in Table I and analyzed in parallel on polyacrylamide gels. Fig. 2*A* shows that the two gel shift bands obtained in EMSA with NM-1 comigrated with the two bands obtained with NF-AT1 and with P1. In each case, the two bands were competed for by a 100-fold molar excess of all three unlabeled oligonucleotides but not by a 100-fold excess of the irrelevant NF- κ B sequence of the mouse IL-2 promoter (data not shown). These results suggested that the transcription factors that bind to the NM-1 motif may be similar to those that bind to the NF-AT1 and P1 motifs.

The sequence TTTTCC has been shown to be critical for the formation of the NF-AT nuclear complex. To examine the role of the TTTTCC sequence in the formation of the NM-1 nuclear complex, we mutated this sequence in NM-1 and examined the capacity of the mutated oligonucleotide to compete with the wild type oligonucleotide in EMSA. Fig. 2*B* shows that replacement of TTCC with CGAT abolished the capacity of cold NM-1 oligonucleotide to inhibit the gel shift bands. In contrast mutating the CAC sequence located 1 bp 5' to the TTTTCC sequence in NM-1, did not affect the ability to inhibit nuclear complex formation with wild type oligonucleotide.

NF-AT complex formation has been shown to require activation of the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin (24, 25). Both calcineurin activation and NF-AT complex formation are inhibited by CsA (26). We therefore examined the effect of ionomycin and CsA on the formation of the NM-1 nuclear protein complex. Fig. 2*C* shows that ionomycin enhanced the intensity of the two gel shift bands obtained in EMSA with NM-1 oligonucleotide as well as with NF-AT1 oligonucleotide. CsA (100 ng/ml) inhibited both bands obtained with NM-1 and with NF-AT1 (Fig. 2*C*) as well as with NM-2 through 4 (data not shown). In contrast, CsA had no effect on the formation of the AP-1 complex in EL-4 cells activated with PMA and ionomycin.

The NM-1 Nuclear Protein Complex Contains NF-ATp and NF-ATc—We examined the presence of NF-AT proteins in the nuclear complexes obtained with the 18-bp oligonucleotides from NM-1 and nuclear extracts from EL-4 cells. Fig. 3*A* shows that rabbit anti-NFATp supershifted the lower band of the NM-1 complex but did not affect the migration of the upper band. In contrast, the mouse anti-NFATc mAb 7A6 supershifted the upper band of the NM-1 complex but did not affect the migration of the lower band. Similar results were obtained with the two bands generated by the 18-bp NF-AT1 oligonucleotide. The supershifts were specific because neither normal rabbit serum nor mouse IgG1 isotype control had an effect. Furthermore, neither anti-NF-ATp nor anti-NFATc altered the mobility of the AP-1 nuclear complex obtained using an AP-1 oligonucleotide and nuclear extracts from EL-4 cells treated with PMA and ionomycin (data not shown).

We next analyzed the presence of NFAT proteins in nuclear factors that bind to NM-1 in primary murine thymocytes and spleen T cells. Fig. 3*B* shows that in contrast to EL-4 cells no detectable complexes were obtained with nuclear extracts from

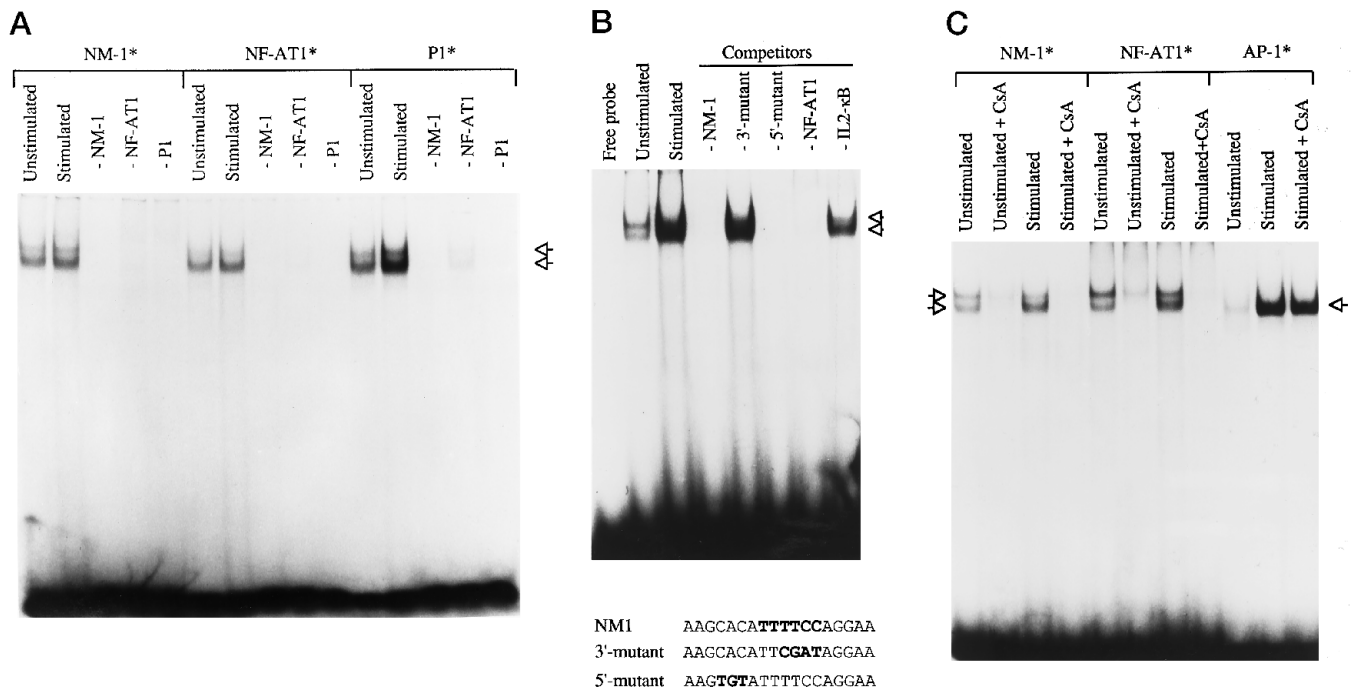


FIG. 2. Characterization of the NM-1-binding nuclear complex. A, NM-1, NF-AT1, and P1 show identical gel mobility shift pattern and cross-competition. EMSA was performed using nuclear extracts from EL-4 thymoma cells and radiolabeled (*) NM-1, NF-AT1 (murine IL-2 promoter), and P1 (murine IL-4 promoter) oligonucleotides. A 100-fold molar excess of unlabeled oligonucleotides was included in cross-competition experiment as indicated. B, mutant oligonucleotides were used to identify the region critical for protein binding in NM-1. The unlabeled 3' and 5' mutants were used as competitors of radiolabeled (*) NM-1 oligonucleotide in EMSA. C, cyclosporin A inhibits NM-1-nuclear protein complex formation. EMSA was performed using nuclear extracts from EL-4 thymoma cells and radiolabeled (*) NM-1, NF-AT1 (murine IL-2 promoter), and consensus AP-1 oligonucleotides. EL-4 cells were stimulated with ionomycin in experiments with NM-1 and NF-AT1 and PMA + ionomycin in experiments with AP-1. Where indicated cyclosporin A was added at concentration 100 ng/ml.

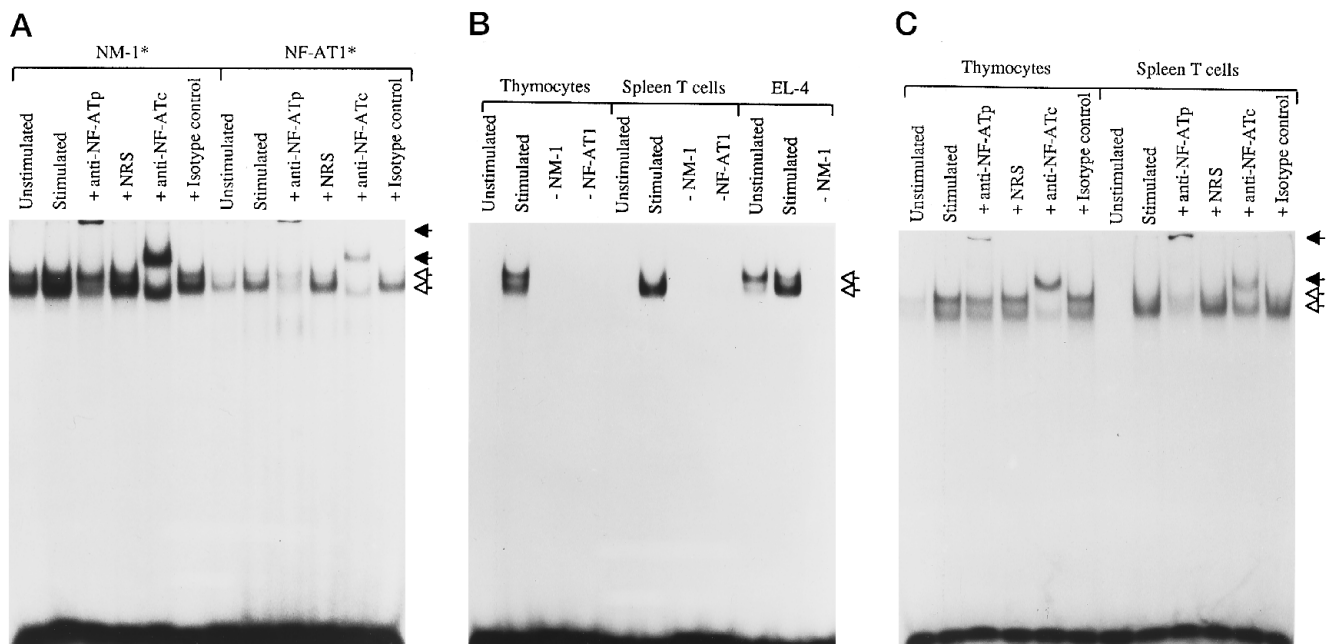


FIG. 3. Analysis of NM-1 complex using antibodies to NFATp and NFATc. EMSA was performed using radiolabeled NM-1 oligonucleotide. A, nuclear extracts from stimulated EL-4 cells were preincubated with antiserum to NF-ATp, normal rabbit serum (NRS), anti-NF-ATc mAb 7A6 and IgG1 isotype control. The same experiment was performed using radiolabeled NF-AT1 oligonucleotide. B, nuclear extracts from unstimulated and stimulated murine thymocytes and spleen T cells were used for EMSA. A 100-fold molar excess of unlabeled oligonucleotides was included in cross-competition experiment as indicated. C, supershift analysis of the complexes from murine thymocytes and spleen T cells. The open arrows denote the two complexes formed between nuclear proteins and radiolabeled NM-1 oligonucleotide. The closed arrows denote the supershifted complexes.

unstimulated thymocytes and spleen T cells. Nuclear extracts from thymocytes activated with PMA and ionomycin displayed two complexes that comigrated with the two complexes ob-

tained with nuclear extracts from EL-4 cells. Both bands were completely inhibited by cold self oligonucleotide as well as by NF-AT1 oligonucleotide. Nuclear extracts from spleen T cells

activated with PMA and ionomycin gave rise to a major complex that comigrated with the faster migrating complex obtained with nuclear extracts of EL-4 cells. A minor complex that comigrated with the slower migrating complex from EL-4 cells was evident in some but not all experiments. Both complexes were completely inhibited by cold self oligonucleotide as well as by the NF-AT1 oligonucleotide. Fig. 3C shows that the lower band obtained with nuclear extracts from normal thymocytes and splenocytes was supershifted by antiserum to murine NF-ATp but not by mAb to NFATc nor by normal rabbit serum. It should be noted that a faint upper band became clearly evident in the nuclear extracts of spleen cells following the supershift with anti-NFATp. MAb to NF-ATc, but not isotype control, supershifted the upper complex from thymocytes and spleen cells. These results indicate that both NF-ATp and NF-ATc participate in the nuclear complex from normal T cells that binds to NM-1.

AP-1 Binding Is Required for Transcriptional Activation by NM-1 Multimers—To determine the transcriptional activity of the 18-bp NM-1 oligonucleotide, pentamers of this oligonucleotide were placed upstream of the SV40 minimal promoter which drives the luciferase gene in the pGL2 vector. The plasmid construct was transiently transfected into T cell lines and 4 h later, the cells were stimulated with PMA and ionomycin. Cell lysates were prepared 24 h after transfection and assayed for luciferase activity. Since EL-4 cells do not express detectable levels of CD40L on the cell surface (<1% positive cells) and express very low amounts of CD40L mRNA, we examined NM-1 driven reporter gene expression in the EL-4 cell line as well as in the 68-41 murine T cell hybridoma. This cell line expresses CD40L on its surface following stimulation with PMA and ionomycin (Fig. 4A) and nuclear extracts from 68-41 cells exhibited two complexes in EMSA which comigrated with those obtained with EL-4 cells (Fig. 4B) and were specifically inhibited by an excess of NM-1 and NF-AT1 oligonucleotides (data not shown). Furthermore, the lower complex was supershifted by anti-NFATp antiserum, whereas the upper complex was supershifted by NF-ATc antibody (Fig. 3B). No induction of luciferase activity could be detected either in EL-4 cells or in 68-41 cells transfected with plasmids containing pentamers of the 18-bp NM-1 oligonucleotides whereas the pGL2-Control plasmid containing the SV40 minimal promoter and SV40 enhancer induced vigorous luciferase gene expression in both cell lines (data not shown). As importantly, there was no induction of luciferase activity by pentamers of the 18-bp NF-AT1 oligonucleotide derived from the murine IL-2 promoter.

The NF-AT1 oligonucleotides derived from the IL-2 promoter which have been reported to be transcriptionally active are ~30 bp long and bind a complex containing both NF-AT and AP-1 proteins (18, 20, 21, 27). We therefore examined the possibility that lack of transcriptional activity of the 18-bp NM-1 oligonucleotide is associated with lack of AP-1 binding. Fig. 5 shows that a 100-fold excess of cold AP-1 oligonucleotide did not affect the mobility of either complex formed by the 18-bp NM-1 or the 18-bp NF-AT1 oligonucleotide (data not shown) but completely inhibited the specific complex formed by an AP-1 oligonucleotide and nuclear extracts from EL-4 cells treated with PMA and ionomycin. Furthermore, neither anti-Fos nor anti-Jun antibody were able to cause supershift of the NM-1 complexes (Fig. 5), whereas both antibodies supershifted the AP-1 complex. Similar data were obtained using nuclear extracts from activated spleen cells and thymocytes (data not shown).

We next examined whether a longer NM-1 oligonucleotide may bind AP-1 and exhibit transcriptional activity. To this purpose, we synthesized a 30-bp long oligonucleotide which corresponds to bp -88 to -59 and in which the TTTTCC core

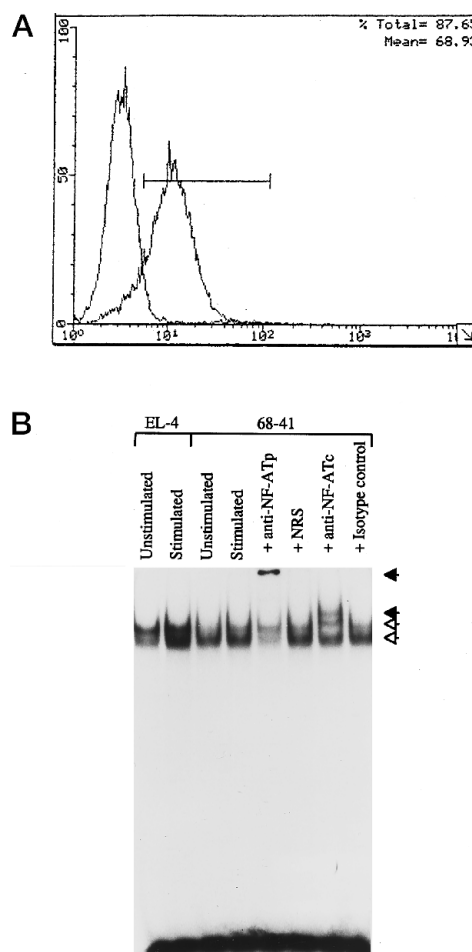


FIG. 4. Characterization of the NM-1-binding complexes from the murine T cell hybridoma, 68-41. A, fluorescence-activated cell sorter analysis of CD40L expression on the surface of 68-41 cells. Left and right peaks represent expression of CD40L on unstimulated and stimulated 68-41 cells, respectively. Cells were stimulated using PMA and ionomycin for 6 h before staining with anti-CD40L mAb MR-1. Isotype control curves virtually overlaid the curve obtained for the CD40L staining of unstimulated 68-41 cells. B, EMSA and supershift analysis using nuclear extracts from 68-41 cells and radiolabeled NM-1 oligonucleotide. NRS, normal rat serum.

sequence is flanked by 21 bp on the 5' end and by 3 bp on the 3' end as shown in Fig. 6. EMSA analysis with nuclear extracts of EL-4 cells showed the presence of a major complex (complex b) and of a closely slower migrating minor complex (complex a) as well as of a faster migrating minor complex (complex c), all of which were specifically inhibited by the unlabeled 30-bp NM-1 oligonucleotide and to a lesser extent by the 18-bp NM-1 oligonucleotide, but not by the irrelevant NF- κ B sequence of the mouse IL-2 promoter. The closely migrating complexes a and b were inhibited by AP-1, were supershifted by anti-NF-ATp and anti-c-Jun antibodies, and were disrupted by anti-c-Fos antibody and to a lesser extent by anti-NF-ATc suggesting that they contain NF-AT as well as AP-1 proteins. The lower most minor complex (complex c) comigrated with the lower complex obtained with the 18-bp NM-1 oligonucleotide, was supershifted by anti-NFATp but not by anti-c-Fos and anti-c-Jun and was not displaced by excess AP-1 oligonucleotide suggesting that it contains NF-ATp but no AP-1 proteins. These results suggest that the 30-bp NM-1 oligonucleotide binds both NF-AT and AP-1 proteins.

Fig. 7 shows that a pentamer of the 30-bp NM-1 oligonucleotide 5x(NM-1) was able to strongly drive luciferase gene expression in 68-41 cells stimulated with PMA and ionomycin. As

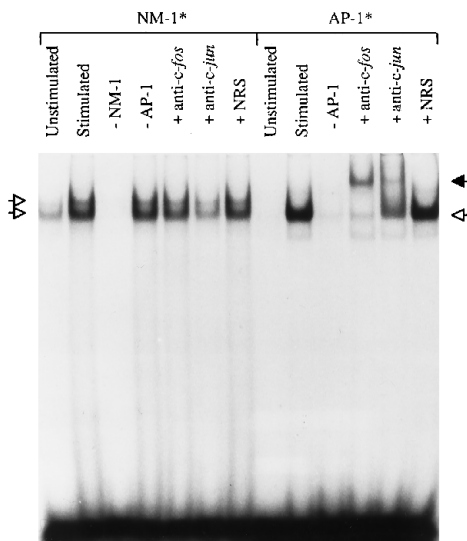


FIG. 5. **Lack of AP-1-binding to the 18-bp NM-1 oligonucleotide.** EMSA was performed using nuclear extracts from EL-4 thymoma cells and radiolabeled (*) NM-1 and consensus AP-1 oligonucleotides. A 100-fold molar excess of unlabeled oligonucleotides was included in cross-competition experiment as indicated. Nuclear extracts from stimulated EL-4 cells were preincubated with anti-c-Fos and c-Jun antisera and normal rat serum.

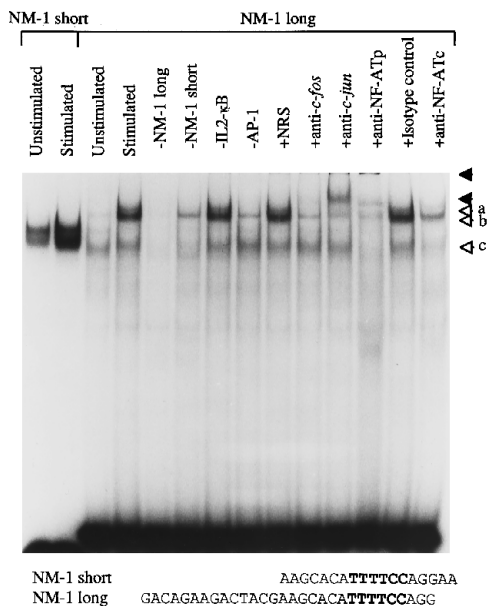


FIG. 6. **EMSA and supershift analysis of the nuclear complexes bound to 30-bp-long NM-1 oligonucleotide.** EMSA was performed using nuclear extracts from EL-4 thymoma cells and radiolabeled 18-(short) and 30-bp (long) NM-1 oligonucleotides. Unlabeled 18-bp NM-1, 30-bp NM-1, IL2- κ B, and consensus AP-1 were added in a 100-fold molar excess, respectively. For supershift analysis, nuclear extracts from stimulated EL-4 cells were preincubated with the indicated immunoreagent. The open arrows denote the three complexes (complexes a, b, c) formed between nuclear proteins and radiolabeled 30-bp NM-1 oligonucleotide. The closed arrows denote the supershifted complexes.

expected, luciferase gene expression was driven by a pentamer of the 30-bp NF-AT1 sequence (5x(NF-AT1)) identical to that used in Refs. 27 and 28, but not by the SV40 minimal promoter in pGL2. A pentamer of the 30-bp mutant NM-1 oligonucleotide described above (TTCC-CGAT) failed to induce reporter gene expression. Reporter gene expression in 68-41 cells transfected with the 5x(NM-1) reporter gene construct could be induced by ionomycin alone but not by PMA alone and was inhibited by

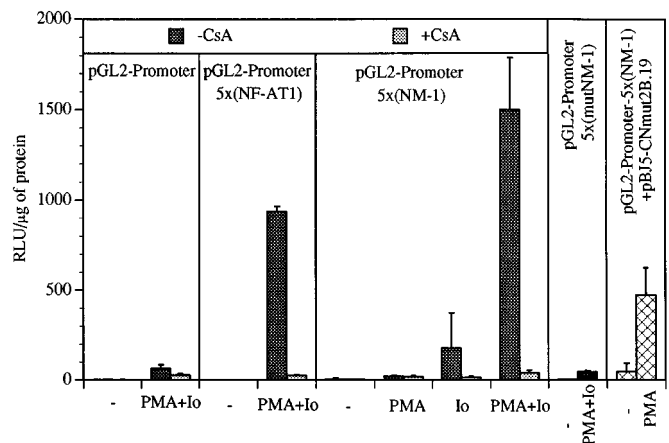


FIG. 7. **Induction of luciferase expression in stimulated 68-41 cells transfected with vectors containing pentamers of 30-bp NM-1.** The pentamers of 30-bp NF-AT1, NM-1, or mutNM-1 were placed upstream the SV40 minimal promoter in luciferase reporter vector, pGL2-Promoter. 68-41 T cells were transiently transfected with these vectors and stimulated 4 h later. After 20 h cells were collected, lysed, and luciferase activity was measured. The last two bars present a cotransfection experiment consisting of pGL2-Promoter-5x(NM-1) and expression vector with a constitutively active mutant of calcineurin A. As indicated, cyclosporin A was added at 1 μ g/ml 20 min before cell stimulation. For all panels the data shown represent the mean \pm S.D. of three independent experiments.

CsA (Fig. 7). This suggested that activation of calcineurin played an important role in the activation of the NM-1 transcriptional complex. This was confirmed by the observation that cotransfection of a plasmid pB15-CNmut2B.19 encoding for a constitutively active mutant of calcineurin A could replace ionomycin in synergizing with PMA to induce reporter gene expression.

Taken together, the results of the reporter gene assays and EMSA assays suggest that AP-1 binding to NF-AT complex formed by NM-1 oligonucleotide is critical for transcriptional activity.

DISCUSSION

In this study, we present evidence which suggests that two members of the NF-AT family of transcription factors bind to DNA elements in the regulatory region of the CD40L gene and that together with AP-1 proteins they form a transcriptionally active complex.

A consensus 6-bp sequence, TTTTCC, present in the murine and the human NF-AT binding sites of the IL-2 and IL-4 promoters was repeated four times in the upstream region of the CD40L gene transcription start site. Eighteen-bp long oligonucleotides that spanned these four sites (NM-1 to NM-4) bound identically and in a specific manner to two complexes in nuclear extracts from T cells (Fig. 1A). The intensity of these complexes markedly increased upon stimulation with PMA and ionomycin. Furthermore, complexes generated with each of the four individual NM oligonucleotides were inhibited by an excess of each of the four unlabeled oligonucleotides (Fig. 1B and data not shown). This suggested that all four NM sites bound similar if not identical nuclear factors. For this reason we used the most proximal of these sites, NM-1, for further analysis.

Several lines of evidence suggested that NF-AT participates in binding to NM-1. The nuclear complexes formed with NM-1 comigrated with those formed with the distal NF-AT sequence of the murine IL-2 promoter, NF-AT1, and with the P1 site of the IL-4 promoter (Fig. 2A). Furthermore, unlabeled NF-AT1 and P1 oligonucleotides inhibited both complexes formed by NM-1 (Fig. 2A) as well as those formed by NM-2, NM-3, and NM-4 (data not shown). Moreover, the consensus sequence

TTTTCC shown to be critical for binding to NF-AT1 was also critical for binding to NM-1. A mutated 18-bp NM-1 oligonucleotide in which the TTTTCC sequence was replaced by TTC-GAT failed to bind to nuclear factors from EL-4 cells (data not shown) and failed to inhibit complex formation by "wild type" NM-1 oligonucleotide (Fig. 2B). Furthermore, two 18-bp long oligonucleotides that correspond to two sequences in the CD40L upstream region in which the NF-AT consensus sequence is altered at only one position, cTTTCC and TTTaCC, respectively (Table I), failed to bind EL-4 nuclear proteins and failed to inhibit complex formation by NM-1. Like with NF-AT, ionomycin, but not PMA alone (data not shown), resulted in the formation of NM-1 complexes which was inhibited by CsA (Fig. 2C). These results suggest that calcium mobilization and subsequent activation of the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin was sufficient for the formation of nuclear proteins that bind to NM-1. The inability of PMA to elicit the formation of an NM-1 nuclear protein complex does not preclude a role for protein kinase C activation in CD40L expression. Indeed both protein kinase C activation and calcium mobilization are required for optimal complex formation with NM-1 (Fig. 2, *C versus B*) and for optimal CD40L expression in T cells (7).

Using antibodies specific to two members of the NF-AT family, we established both in T cell lines and in primary T cells that the upper NM-1 complex contains NF-ATc while the lower complex contains NF-ATp (Fig. 3, *A and B*). There was no evidence for the association of AP-1 proteins in complexes formed by the 18-bp long NM-1 oligonucleotide (Fig. 5). These results demonstrated that both NF-ATp and NF-ATc can bind to the NM-1 site independently of AP-1.

To our surprise we found that the control 18-bp-long NF-AT1 oligonucleotide derived from the murine IL-2 promoter also failed to bind AP-1 although it bound to both NF-ATc and NF-ATp (data not shown). Previous study using a 29-bp oligonucleotide that spans the NF-AT1 site showed binding to both NF-AT and AP-1 Fos and Jun proteins (18, 25). The failure of the 18-bp long NM-1 and NF-AT1 to bind AP-1 and the failure of the pentamers of these oligonucleotides to drive reporter gene transcription in T cell lines stimulated with PMA and ionomycin prompted us to examine whether a longer oligonucleotide that spans the NM-1 site will bind AP-1 and be transcriptionally active. A 30-bp-long NM-1 oligonucleotide was synthesized in which the regions that flanked the TTTTCC core sequence corresponded in length to those that flanked this core sequence in the 30-bp NF-AT1 oligonucleotide previously shown to bind AP-1 proteins (27, 28). This 30-bp long NM-1 oligonucleotide bound the AP-1 proteins of the Fos and Jun family in addition to NF-ATp and NF-ATc (Fig. 6). More importantly, multimers of the 30-bp oligonucleotide were able to drive expression of the luciferase reporter gene in stimulated T cell lines (Fig. 7). Induction of reporter gene activity was optimal following stimulation with PMA and ionomycin but also occurred with ionomycin alone. Furthermore, induction was inhibited by CsA suggesting an important role for calcineurin activation. This observation was confirmed by the fact that the mutated constitutively active calcineurin synergized with PMA in the induction of NM-1 dependent transcription.

It has been proposed that the formation of the AP-1 containing NF-ATp complex in the NF-AT1 site depends on the presence of a sequence bearing homology to an AP-1 consensus site located from -278 to -272 bp (18). No such sequence is found within our 30-bp-long NM-1 oligonucleotide. The ability of the 30-bp-long NM-1 oligonucleotide to drive reporter transcription could not be explained by direct binding of AP-1 because direct binding of AP-1 would have resulted in activation of transcrip-

tion by PMA alone which was clearly not the case (Fig. 7). More importantly, as in the case of the 29-bp NF-AT1, oligonucleotide binding of recombinant Fos and Jun proteins to the 30-bp NM-1 oligonucleotide could not be detected. Finally, mutating the TTTTCC core sequence in the 30-bp NM-1 oligonucleotide abolished the formation of nuclear complexes suggesting that binding of AP-1 proteins is contingent binding of NF-AT proteins.

Taken together, our findings suggest that binding of NF-AT proteins alone to DNA sites is not sufficient to form a transcriptionally active complex. The formation of a transcriptionally active complex appears to require NF-AT dependent binding of AP-1 factor to the complex. A 20-bp oligonucleotide which spans the P1 site of the IL-4 promoter and which binds NF-ATp has been reported to be transcriptionally active in the apparent absence of binding of AP-1 proteins (29). A very recent analysis of the P1 site by the same group using a longer oligonucleotide has demonstrated binding of AP-1 proteins which was contingent on NF-AT binding and which was associated with a marked enhancement of transcriptional activity (30). These recent results support the general notion that AP-1 binding to the NF-AT complex is necessary for transcriptional activation.

The requirements for the induction of reporter gene expression by multimers of the NF-AT-like NM1 site in the 5'-upstream region of the CD40L gene paralleled with the requirements for the induction of CD40L expression. This suggests that the NF-AT-like motifs in the CD40L promoter may play an important role in CD40L gene expression in activated T cells. Mutational analysis of these motifs in the context of the CD40L promoter are ongoing to define their individual roles in the regulation of CD40L expression.

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