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*J Immunol* 1999; 163:3295-3303

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An Activation-Responsive Element in Single C Motif-1/Lymphotactin Promoter Is a Site of Constitutive and Inducible DNA-Protein Interactions Involving Nuclear Factor of Activated T Cell

Tetsuya Yoshida,* Izumi Ishikawa,* Yuichi Ono,* Toshio Imai,*† Ryuji Suzuki,* and Osamu Yoshie2*†

Single C motif-1 (SCM-1)/lymphotactin is a C-type chemokine whose expression is activation dependent, cyclosporin A sensitive and restricted to CD8+ T cells, double-negative thymocytes, γδ-type T cells, and NK cells. In humans, there are two highly homologous genes encoding SCM-1α and SCM-1β. Here we examined the regulatory mechanism of the SCM-1 genes. The luciferase reporter gene under the control of the 5′ flanking region of 0.7 kb was strongly induced upon activation with anti-CD3 or PHA plus PMA only in SCM-1-producer T cell lines through a cyclosporin A-sensitive mechanism. An element termed E1 located at −108 to −95 nt relative to the major transcription start site was found to be critical for the promoter activity. In electrophoretic mobility shift assays using the E1 oligonucleotide as probe, nuclear extracts from unstimulated T and B cell lines formed a constitutive complex termed complex I, while nuclear extracts from stimulated SCM-1-producer T cell lines formed a higher mobility complex termed complex II with a concomitant decrease in complex I. The shift from complex I to complex II seen only in SCM-1-producer T cell lines upon activation was completely suppressed by cyclosporin A. Both complexes were critically dependent on the NF-AT core sequence TTTCC in the E1 element and were partially supershifted by anti-NF-ATp. One-hybrid assays in yeast isolated NF-ATp as an E1 binding protein, and transfection of NF-ATp into T and B cell lines strongly enhanced the activation-dependent SCM-1 promoter activity. Collectively, a unique mechanism involving NF-ATp appears to regulate the cell type-specific and activation-dependent expression of the SCM-1 genes. The Journal of Immunology, 1999, 163: 3295–3303.

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1 Abbreviations used in this paper: SCM-1, single C motif-1; ATAC, activation-induced, T cell-derived, and chemokine-related molecule (ATAC) is a novel cytokine produced by activated T cells (1–4). It has a significant structural homology to chemokines, especially to the CC subfamily, but lacks the first and third of the four canonical cysteine residues conserved in other chemokines. It is thus considered to represent the C subfamily of chemokine. The pathophysiological functions of SCM-1 are still mostly unknown. It was reported to be chemotactic for lymphocytes and NK cells (1, 4–6). It was found to be selectively expressed by activated CD8+ T cells and double-negative γδ-type thymocytes (1, 3, 4). Furthermore, epidermal dendritic γδ-type T cells and intestinal intraepithelial γδ-type T cells were found to produce copious amounts of this cytokine upon activation (7). IL-2-activated murine NK cells and human NK clones were also shown to express its mRNA (6).

Thus, SCM-1/lymphotactin/ATAC is a unique cytokine whose expression is highly selective for certain subsets of T cells and NK cells.

In our previous study we isolated genomic clones hybridizing with the SCM-1 cDNA. There are two highly homologous SCM-1 genes, termed SCYC1 and SCYC2, in the human genome (8). These two genes are closely mapped to human chromosome 1q23, similarly inducible in peripheral blood T cells and Jurkat T cell line upon activation, and encode SCM-1α and SCM-1β with only two amino acid differences (8). We have also identified a specific functional receptor for SCM-1α and SCM-1β, now termed XCR1, which is a seven-transmembrane G protein-coupled receptor selectively expressed in tissues such as placenta, spleen, and thymus (9).

In the present study we have investigated the molecular mechanism of SCM-1 gene expression in particular subsets of T cells. Because cyclosporin A (CsA) was shown to suppress the induction of this protein in activated CD8+ T cells (3), the NF-AT family transcription factors are likely to be involved in its expression (10).

We have found that an NF-AT-like cis-acting element in the 5′ flanking regions of the SCM-1 genes termed E1 is essential and sufficient for activation-dependent, cell type-specific, and CsA-sensitive gene expression. We have further demonstrated that E1 is a site of constitutive and activation-inducible DNA-protein interactions involving NF-AT family proteins.

Materials and Methods

Cells

Human T cell lines, Jurkat, PEER, and HPB-ALL, and a human B cell line BALL-1 were described previously (11). These cell lines and HeLa-S3 were maintained in RPMI 1640, 10% heat-inactivated FCS, and kanamycin (30 μg/ml). PBMC were isolated from heparinized venous blood from...
FIGURE 1. Nucleotide sequences of the immediate upstream regions of the two SCM-1 genes. The numbering is relative to the major transcriptional start site (see Fig. 1). PCR products were amplified from the SCM-1 genomic template by PCR using a 3'-tailed 32P-labeled probe (Dynal, Great Neck, NY) after incubation with anti-CD8 or anti-CD4 (Dako, Carpenteria, CA) for 30 min on ice, respectively. After that, total and selected populations were expanded in RPMI 1640, 10% FCS, and 100 U/mL of IL-2. After overnight culture, CD4+ and CD8+ T cells were negatively selected using magnetic beads (Dynal, Great Neck, NY) after incubation with anti-CD8 or anti-CD4 (Dako, Carpenteria, CA) for 30 min on ice, respectively. After that, whole and selected T cells were used as a test plasmid and 100 μg of a test plasmid and 5 μg of pCMV-β-gal were mixed with 32P-labeled probes. Bound complexes were separated from free probes by electrophoresis on a 5% polyacrylamide gel. Gels were dried, and complexes were visualized by autoradiography.

Plasmid construction

The promoterless luciferase plasmid pgV-B was purchased from Toyo Ink (Tokyo, Japan). To generate a series of 5' deletions, DNA fragments were amplified from the SCM-1α genomic template by PCR using a 3'-oligonucleotide tail with an Xho I site and 5'-oligonucleotides of 20 bp tailed with a 9-bp linker (5'-CTCGAGCTGTGCAAGGAGA-3'). 5' oligonucleotides of 20 bp tail with a Mlu I site and beginning at −669, −497, −279, −127, −117, −108, −98, −83, −62, −42, and −32 relative to the major transcriptional start site (see Fig. 1). PCR products were digested with MluI and XhoI and cloned into the MluI and XhoI sites of the reporter plasmid pgV-B. Base substitutions were also introduced using PCR. An N-terminally truncated 464-aa form of NF-ATp (12), which contains the domains required for DNA binding and for formation of a transcriptionally active complex with Fox and Jun, was subcloned into the NcoI site of pEBV-His (Invitrogen, San Diego, CA). To increase fidelity, all PCR amplifications were performed using Pfu polymerase (Stratagene, La Jolla, CA) for only 15 cycles with a large amount of template (100 ng/reaction). All PCR-derived sequences were checked by DNA sequencing.

Transfection and luciferase assay

pCMV-β-gal, a CMV promoter-driven β-galactosidase (β-gal) construct, was purchased from Promega (Madison, WI). It was used for normalization of transfection efficiency. For transfection of each cell line, −1 × 10^6 cells were mixed with 30 μg of a test plasmid and 5 μg of pCMV-β-gal in 200 μL of PBS and transfected by electroporation at 250 V/500 μF. For transfection of primary T lymphocytes, we followed the procedure described previously (13). In brief, about 2 × 10^6 cells were mixed with 100 μg of a test plasmid and 10 μg of pCMV-β-gal in 0.8 ml of RPMI 1640, 10% FCS, and 100 μU/ml of IL-2 and transfected by electroporation at 350 V/960 μF. Transfected cell lines were cultured in RPMI 1640 and 20% FCS for 18 h. Transfected lymphocytes were cultured in RPMI 1640, 20% FCS, and 100 μU/ml of IL-2 for 18 h. After that, cell cultures were divided and further treated without or with PHA (1/100) and PMA (50 ng/ml; Sigma, St. Louis, MO) or with anti-CD3 (OKT3) that was immobilized on plastic dishes by incubation at concentration of 1 μg/ml overnight. After additional 24 h culture, cells were washed twice in PBS and resuspended in a lysis buffer for luciferase assay (Promega). After 10 min, cell extracts were obtained by centrifugation. After normalization of transfection efficiency by β-gal assay, luciferase assay was performed using the PicaGene kit (Toyo Inki) as described previously (14). Light units were measured on a luminometer (Lumat LB9501, Berthold, Wildbad, Germany).

Electrophoretic mobility shift assay

The following double-stranded oligonucleotides were synthesized using a DNA synthesizer (Cyclon Plus DNA Synthesizer, MilliGene/Biosearch, Bedford, MA): E1, 5'-ATTGCAAGACCTTACATGCTA; μE1, 5'-ATTGCAAGACCTACATGCTA-3'; AP-1 IL-2, 5'-CTCCAAAAGGGGGAG-3'; NF-κB/ c-REL, 5'-TGTCGAATGCAAATCACTAGAA-3'; AP-1', 5'-TTCCAAAGAGGGCGGAT-3'; and AP-1, 5'-TTCCAAAGGATGATCAG-3'; and a series of mutated E1 oligonucleotides with 2-bp substitutions shown in Fig. 5E. The following oligonucleotides were purchased from Promega: Oct-1, 5'-TGTGCGAGCTTACATGCTA; SP-1, 5'-ATTCGATCGGCGGGCGGCGA-3'; and AP-1, 5'-TTCCAAAGGATGATCAG-3'. EMSA was conducted as described previously (15). In brief, cells were stimulated without or with PHA (1/100) and PMA (30 ng/ml) for 4 h in the absence or the presence of CsA (100 nm; Sandoz Research Institute, Hanover, NJ). Nuclear extracts were prepared as described previously (16). Double-stranded oligonucleotides were end labeled using [γ-32P]ATP and T4 polynucleotide kinase (Pharmacia LKB Biotechnology, Piscataway, NJ). Polyclonal anti-NF-ATp was purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti-NF-ATb and anti-NF-ATc were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Nuclear extracts (3 μg/reaction) were incubated without or with competitors or anti-NF-ATp (0.5 μg) for 15 min at room temperature and mixed with 32P-labeled probes (~3 × 10^6 cpm/mg, 1 ng/reaction) for another 20 min at room temperature in 20 μl of a solution consisting of 10 mM Tris-HCl (pH 7.3), 50 mM NaCl, 0.5 mM EDTA, 0.1% glycerol, 1% Ficoll, and 2 μg/ml poly(dI-dC). Bound complexes were separated from free probes by electrophoresis on a 5% polyacrylamide gel. Gels were dried, and complexes were visualized by autoradiography.
Yeast one-hybrid assay

The yeast one-hybrid assay (17) was conducted by using Matchmaker One-Hybrid System (Clontech, Palo Alto, CA). Briefly, the concatemer consisting of six tandem repeats of the E1 element was linked to a low activity promoter directing expression of HIS3. The reporter construct was integrated into the recipient yeast genome. This yeast strain was transformed with a Jurkat cDNA library expressing fusion proteins with the target-independent Gal4 activation domain and plated on medium lacking histidine. HIS3 colonies were isolated, plasmids were prepared, and inserts were sequenced. The same reporter system directing expression of lacZ was used to test positive clones (17).

Results

Functional analysis of the 5′ flanking regions of the SCM-1 genes

Previously, we have described the presence of two highly homologous SCM-1 genes encoding SCM-1α and SCM-1β in the human genome (8). These two genes are similarly inducible upon cell activation (8). As shown in Fig. 1, the ~0.7-kb 5′ flanking sequences of the SCM-1α and SCM-1β genes are 97% identical. Computer analysis reveals a number of potential regulatory elements. We first tested whether the 5′ flanking sequences of the SCM-1α and SCM-1β genes were similarly inducible by cell activation. We generated reporter genes by introducing the ~0.7-kb 5′ flanking regions of SCM-1α and SCM-1β in the upstream of the promoterless luciferase gene pGV-B. We transfected these reporter plasmids into Jurkat, a human CD4+ T cell line capable of expressing SCM-1 (8). At 18 h posttransfection, cells were stimulated with immobilized anti-CD3 or PHA and PMA, and cell extracts were prepared for luciferase assay after additional 24-h culture. As shown in Fig. 2A, the ~0.7-kb 5′ flanking regions of the SCM-1α and SCM-1β genes showed very low levels of basal expression, but were strongly induced upon stimulation with anti-CD3 or PHA and PMA. Thus, the ~0.7-kb 5′ flanking regions of SCM-1α genes contained essential regulatory elements for the activation-dependent gene expression. Because these ~0.7-kb 5′ flanking regions are 97% identical at the DNA level (Fig. 1), our subsequent experiments were conducted using the SCM-1α gene promoter.

In Fig. 2B we transfected the reporter plasmid with the ~0.7-kb 5′ flanking region of the SCM-1α gene (~669/luc) into total T cells, CD4+ T cells and CD8+ T cells. Upon treatment with PHA and PMA, the reporter gene was strongly induced in primary human T cells. Furthermore, a higher level of induction was consistently seen in CD8+ T cells (~90% purity) than in CD4+ (~80% purity). Thus, the ~0.7-kb 5′ flanking region of the SCM-1α gene was capable of regulating activation-dependent gene expression in
primary human T lymphocytes with preference for CD8\(^+\) T cells. Expression of the reporter gene in the CD4\(^+\) T cell populations might be due in part to the relatively low purity of CD4\(^+\) T cells (\sim 80\%) and/or to human equivalents of the murine NK1.1\(^+\) CD4\(^+\) T cells that were mentioned to express lymphotactin/SCM-1 (4).

In Fig. 2C, we further tested the cell type-specific promoter activity by using five human cell lines. The CMV-\(\beta\)-gal plasmid was used to normalize transfection efficiency by \(\beta\)-gal activity. Consistent with the induction of the endogenous SCM-1 genes (8), the treatment with PHA and PMA strongly activated the promoter in Jurkat (a CD4 single-positive T cell line) and PEER (a double-negative y8 T cell line), but not in HPB-ALL (a double-positive T cell line), BALL-1 (a B cell line), or HeLa (an epithelial cell line) (11). Thus, the \sim 0.7-kb 5' flanking sequence of the SCM-1\(\alpha\) gene is functional only in certain T cell lines that are capable of expressing the endogenous SCM-1 genes upon activation (8). We also tested the effect of CsA on the stimulation-dependent activation of the promoter in Jurkat. As shown in Fig. 2D, CsA completely abrogated the activation of the promoter by PHA and PMA with an IC\(_{50}\) of about 1 ng/ml. Collectively, these results clearly demonstrated that the \sim 0.7-kb 5' flanking region of the SCM-1\(\alpha\) genes contain major cis-acting elements that regulate the activation-dependent, cell type-specific, and CsA-sensitive expression of the SCM-1 genes.

**Deletion analysis of the 5' flanking region**

To determine the cis-acting regulatory elements in the 5' flanking region of the SCM-1\(\alpha\) gene, we transfected a series of luciferase reporter plasmids with progressively deleted 5' flanking regions into Jurkat cells. As shown in Fig. 3, all the plasmids showed a very low level of basal promoter activity. Upon PHA and PMA stimulation, \(-669\)/\(\alpha\) and \(-497\)/\(\alpha\) induced luciferase activity by about 30-fold; \(-279\)/\(\alpha\), \(-117\)/\(\alpha\) and \(-108\)/\(\alpha\) induced luciferase activity by about 15-fold; \(-98\)/\(\alpha\), \(-83\)/\(\alpha\), and \(-62\)/\(\alpha\) induced luciferase activity by about 3-fold; \(-42\)/\(\alpha\) and \(-32\)/\(\alpha\) induced luciferase activity by <2-fold. From these results, at least three regions (\(\sim 497\) to \(-279\), \(-108\) to \(-98\), and \(-62\) to \(-42\)) appeared to contribute to the full inducibility of the SCM-1\(\alpha\) promoter upon treatment with PHA and PMA, and the region between \(-108\) and \(-98\) contained a major cis-acting element. A close inspection of the sequence in and around this region revealed a sequence 5'-GAC(TT)TCCAT-3' (\(\sim 101\) to \(-92\)) that contained an inverted core sequence of the NF-AT site (TTTCC) (10). Because NF-AT is known to be involved in the expression of various cytokine genes in T cells by activation-dependent and CsA-sensitive mechanisms (10), our subsequent study was focused on this element. For the sake of convenience, we designated the sequence between \(-108\) and \(-83\) as element 1 (E1; Fig. 1).

**Role of the NF-AT core sequence in E1**

We first examined the effect of mutations in the NF-AT core sequence of E1 on the promoter activity of the \(-669\)-bp 5' flanking region. The luciferase reporter plasmids with the wild-type E1 or a mutated E1 with three base substitutions in the NF-AT core sequence (Fig. 4A) were transfected into Jurkat and PEER. As shown in Fig. 4B, the mutations at this site effectively abrogated the stimulation-dependent induction of the reporter driven by the \(-669\)-bp 5' flanking region in both cell lines. To further test whether E1 was an activation-inducible and CsA-sensitive regulatory element, we constructed an artificial reporter plasmid by inserting six tandem repeats of E1 in the upstream of the \(-42\)/\(\alpha\) (Fig. 3). As shown in Fig. 4C, the reporter driven by the tandem repeats of E1 was indeed induced by about 10-fold in transfected Jurkat by PHA and PMA. Furthermore, this induction was fully abrogated by CsA. Taken together, E1 containing the NF-AT core sequence is a critical regulatory element for the activation-dependent and CsA-sensitive expression of the SCM-1 promoter.

**Binding of nuclear proteins to E1**

To demonstrate specific interactions of nuclear proteins with E1, EMSA was conducted using the E1 probe (Fig. 5A). Unexpectedly, even the nuclear extract from unstimulated Jurkat was capable of forming a DNA-binding complex, termed complex I. On the other hand, the nuclear extract from stimulated Jurkat formed a higher mobility complex, termed complex II, with concomitant decrease in complex I. Both complexes were blocked by the wild-type E1 site probe (wt), but not by a mutated E1 site probe with three base substitutions in the NF-AT core sequence (mu, see Fig. 4A). Moreover, the mutated E1 site probe (mu-E1) failed to form any complexes with the nuclear extracts from either unstimulated or stimulated Jurkat. These results indicated that the NF-AT core sequence of the E1 site, which is critical for the enhancer activity of the E1 site (Fig. 4), is also critical for the formation of both complex I and complex II.

EMSA was also conducted to test the effect of CsA. As shown in Fig. 5B, the nuclear extract of Jurkat cells stimulated with PHA and PMA in the presence of CsA only formed complex I and failed to form complex II. Thus, the shift from complex I to complex II observed upon stimulation with PHA and PMA was effectively blocked by CsA treatment. This further supported the idea that the
shift from complex I to complex II was required for the transcriptional activation.

By cold competition experiments, we next analyzed the nature of the nuclear proteins binding to E1 in complex I and II. As shown in Fig. 5C, both complexes were clearly inhibited by the E1 probe at 10-fold excess and by the IL-2 NF-AT site probe at 10- to 30-fold excess (15). On the other hand, the IL-2 AP-1 site probe (15) hardly affected complex I even at a 100-fold excess. This probe, however, partially inhibited complex II at a 30- to 100-fold excess. For an internal control, EMSA was also conducted using the IL-2 NF-AT site probe (15). As expected, a complex was formed only with the extract from stimulated Jurkat. Furthermore, this complex was inhibited by the IL-2 NF-AT site probe (and the E1 site probe) as well as by the IL-2 AP-1 site probe (10, 15) (Fig. 5D). Collectively, these results suggested that both complexes contained proteins related to NF-AT. Furthermore, the complex II might contain a cofactor related to the AP-1 family. However, there is no consensus AP-1 site as defined by T(T/G)ANT(A/C)A (18) in or around the E1 site (Fig. 1).

To further define the critical nucleotides in the E1 site that were involved in the formation of constitutive complex I and inducible complex II, we next conducted EMSA in the presence of mutated E1 site probes with scanning-type 2-bp substitutions as cold competitors. As shown in Fig. 5E, both constitutive complex I and activation-inducible complex II showed essentially identical inhibition patterns, indicating that the same nucleotide sequence in the E1 site was involved in the formation of both complexes. Furthermore, the lack of competition by M6, M7, and M8 revealed that TTTCCA corresponding exactly to the NF-AT core sequence was essential for the formation of both complexes (10).

Cell type-specific formation of complex II

By using the E1 probe, EMSA was further conducted with nuclear extracts from Jurkat, PEER, BALL-1, and HPB-ALL (Fig. 6, upper panel). Both Jurkat and another SCM-1 producer T cell line, PEER (8), formed the constitutive complex I before activation and the inducible complex II with a concomitant decrease or disappearance of complex I upon activation with PHA and PMA. SCM-1-nonproducer cell lines BALL-1 and HPB-ALL (8) also formed complex I, but failed to form complex II even after activation with PHA and PMA. Thus, the formation of complex II was seen only with the nuclear extracts from SCM-1 producer cell lines upon stimulation with PHA and PMA (Fig. 2). As internal controls, the same nuclear extracts were probed with the IL-2 NF-AT site probe (15). In this case, the DNA-binding complex was seen only with extracts from stimulated Jurkat and HPB-ALL (Fig. 6, lower panel).

NF-ATp as a component of complexes I and II

To test the presence of the NF-AT family proteins in complexes I and II, the effect of polyclonal anti-NF-ATp was examined in EMSA. As shown in Fig. 7A, complex I formed with the nuclear extract from unstimulated Jurkat was partially supershifted by anti-NF-ATp, while both complex I and complex II formed with the nuclear extract from activated Jurkat were reduced by anti-NF-ATp (panel E1). A faint supershift band was also seen. The discrepancy of the effect of anti-NF-ATp on complex I formed by unstimulated Jurkat and that formed by stimulated Jurkat was unexpected and might indicate that they were not identical. As internal controls, the same Ab was shown to partially supershift the IL-2 NF-AT nuclear complex (panel NFAT), but to have no effect on the SP-1 nuclear complex (panel SP-1). We further examined the effects of various anti-NF-AT Abs in EMSA using the nuclear extracts from PEER. As shown in Fig. 7B, anti-NF-ATp again partially supershifted complex I formed by the nuclear extract of unstimulated PEER. It also slightly reduced and faintly supershifted the complex II formed by the nuclear extract from stimulated PEER even though the effect was less dramatic than that seen with Jurkat. No such effects were seen with anti-NF-ATc and anti-NF-ATx. Collectively, these results support that both complexes I and II contain NF-ATp or some proteins immunologically cross-reactive with anti-NF-ATp.

Binding of NF-ATp to E1

To identify proteins binding to E1, we conducted one-hybrid screening in yeast (17). The reporter construct consisted of six
FIGURE 5. Binding of nuclear proteins to the E1 site. A, The E1 site is the specific target site of constitutive and inducible DNA binding proteins. Nuclear extracts were prepared from Jurkat cells that had been mock treated or treated with PHA plus PMA. EMSA was conducted using 32P-labeled wild-type E1 or mutated E1 with three base substitutions (see Fig. 4). The specificity of the binding was tested by cold competitors at 100-fold molar excess as indicated. Constitutive complex I and inducible complex II are indicated by arrows. B, The effect of CsA on inducible complex II. Nuclear extracts were prepared from Jurkat cells that had been mock treated or treated with PHA plus PMA in the absence or the presence of CsA at 100 ng/ml. EMSA was carried our using 32P-labeled E1. Constitutive complex I and inducible complex II are indicated by arrows. C, Competition of constitutive complex I and inducible complex II by the NF-AT site and AP-1 site of the IL-2 gene (see Fig. 4). Nuclear extracts were prepared from Jurkat cells that had been mock treated or treated with PHA plus PMA. EMSA was conducted using 32P-labeled E1 in the absence or the presence of the indicated cold competitors. D, The IL-2 NF-AT site is the specific target for NF-AT and AP-1. Nuclear extracts were prepared from Jurkat cells that had been mock treated or treated with PHA plus PMA. EMSA was conducted using the 32P-labeled IL-2 NF-AT site (see Fig. 4) in the absence or the presence of indicated cold competitors at a 100-fold excess. The NF-AT complex is indicated by an arrow. E, Mapping of the binding site of nuclear proteins in E1 by scanning mutations. Nuclear extracts were prepared from Jurkat cells that had been mock treated (-) or treated with PHA plus PMA (+). EMSA was conducted with 32P-labeled E1 as a probe and each one of the cold competitors in 100-fold excess as indicated.
tandem repeats of E1 site linked to a low activity promoter directing His3 gene expression. A Jurkat cDNA library of $2 \times 10^6$ clones expressing a translational fusion with the Gal4 trans-activating domain was transformed into a his− yeast strain carrying the HIS3 reporter plasmid. About 2 million transformants were plated on the medium lacking histidine, and 20 HIS1 colonies were isolated. By partial sequencing, we found that 10 of 20 positive clones were NF-ATp. We sequenced one NF-ATp clone and found that it encoded an N-terminally truncated NF-ATp starting from the amino acid position at 401 and consisting of 521 aa fused in-frame to the transcriptional activation domain of Gal4. To measure its trans-activation activity, we conducted β-gal reporter assay in yeast using a reporter construct consisting of six tandem repeats of E1 site linked to a low activity promoter directing lacZ gene expression (17). As shown in Fig. 8, the NF-ATp-Gal4 fusion protein was capable of inducing the reporter gene by about 20-fold. Thus, NF-ATp is capable of directly binding to the E1 site in yeast.

**Discussion**

Studies to examine factors that regulate the expression of cytokines such as IL-2 and IL-4 have demonstrated that the NF-AT family of transcription factors plays an essential role in the activation-inducible and CsA-sensitive gene expression (10). There are four distinct NF-AT family members, NF-AT1/NF-ATp (12).

**Transfected NF-ATp enhances SCM-1 promoter activity**

To further examine the effect of NF-ATp on the SCM-1 promoter, we cotransfected −669/luc with wild-type E1 site (wt) or mutated E1 site (μ; see Fig. 4) and either a control vector or a vector expressing an N-terminally truncated 464-aa functional NF-ATp (12) into Jurkat and BALL-1. As shown in Fig. 9, transfection of functional NF-ATp into Jurkat significantly augmented the activity of the SCM-1 promoter with the wild-type E1 site, but not that with the mutated E1 site upon stimulation with PHA and PMA. Furthermore, transfection of NF-ATp into the SCM-1-nonproducer BALL-1 strongly induced the activity of the wild-type promoter, but not that of the mutated one upon stimulation with PHA and PMA. The latter observation may support the lack of expression of functional NF-ATp in BALL-1 (19). Thus, an N-terminally truncated functional NF-ATp (12) is capable of trans-activating the SCM-1 promoter through the E1 site upon cell activation.
NF-AT into the nucleus through dephosphorylation of NF-AT by (24, 25). Activation of T cells leads to translocation of cytoplasmic homologous DNA-binding domain that shows a weak sequence between Th1 and Th2 (31, 32), SCM-1 may provide a unique model for activation-dependent gene expression in particular subsets of T cells and NK cells. In the present study we have shown that the SCM-1 promoter has an element termed E1 between −108 and −90 relative to the transcription initiation site (Fig. 1). E1 is essential and sufficient for the activation-dependent and CsA-sensitive expression of the SCM-1 genes (Figs. 2–4). Importantly, the E1 site contains the NF-AT core sequence that is essential for its enhancer activity (Fig. 4). Unlike most other NF-AT sites (10), however, the E1 site probe formed a constitutive complex, termed complex I, with nuclear extracts from unstimulated SCM-1 producer T cell lines and even from SCM-1 nonproducer cell lines (Fig. 5). With nuclear extracts from stimulated SCM-1 producer T cell lines, however, the E1 probe formed a higher mobility complex, termed complex II, with a concomitant decrease in complex I (Figs. 5 and 6). Furthermore, the shift from complex I to complex II upon cell activation was effectively suppressed by treatment with CsA (Fig. 5). Both complexes were critically dependent on the NF-AT core sequence (Fig. 5) and were partially supershifted by anti-NF-ATp, but not by anti-NF-ATc or anti-NF-ATx (Fig. 7). Furthermore, NF-ATp was found to be a protein capable of binding to the E1 site bait in the yeast one-hybrid assay (Fig. 8) (17). Exogenously transfected N-terminally truncated functional NF-ATp (12) was further shown to be capable of trans-activating the SCM-1 promoter in the E1-dependent manner upon stimulation with PHA and PMA (Fig. 9). Collectively, these results support the following conclusions. Transcription from the SCM-1 promoter takes place through a shift from the pre-existing complex I to the activation-inducible complex II. The NF-AT core sequence (TTTCC) in E1 is critical for the formation of both complexes. NF-ATp is capable of binding to the SCM-1 promoter and to induce transcription of the SCM-1 promoter only upon stimulation with PHA and PMA.

The SCM-1 E1 site thus presents notable differences from the classical IL-2 NF-AT sites (10). In the case of IL-2 NF-AT sites, NF-AT proteins bind only after stimulation-induced nuclear translocation (10). In the case of E1, however, not only the inducible complex II but also the pre-existing complex I seem to contain NF-ATp and c-Maf in the expression of IL-4 (30). Thus, it is likely that cell type-specific cofactors assembling at a given NF-AT site determine the cell type-specific expression of various cytokines.

SCM-1/lymphotactin/ATAC is a unique cytokine whose expression is highly selective for activated CD8+ T cells, double-negative thymocytes, intradermal γδ+ dendritic T cells, intestinal intraepithelial γδ+ T cells, and NK cells (1–8). Like IL-2 and IL-4, which serve as models to examine differential transcriptional regulation between Th1 and Th2 (31, 32), SCM-1 may provide a unique model for activation-dependent gene expression in particular subsets of T cells and NK cells.

Collectively, these results support the following conclusions. Transcription from the SCM-1 promoter takes place through a shift from the pre-existing complex I to the activation-inducible complex II. The NF-AT core sequence (TTTCC) in E1 is critical for the formation of both complexes. NF-ATp is capable of binding to the SCM-1 promoter and to induce transcription of the SCM-1 promoter only upon stimulation with PHA and PMA.

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| Jurkat (A) and BALL-1 (B) were cotransfected with CMV-β-gal, wild-type −669/luc (WT), or −669/luc with the mutated E1 site (Mut; see Fig. 4) and the control expression vector or the expression vector of an N-terminally truncated functional NF-ATp (13). Transfected cells were mock treated (−) or treated with PHA plus PMA (+), and cell extracts were prepared. After normalization of transfection efficiency by β-gal assay, the luciferase assay was performed in triplicate. The mean ± SEM from three independent experiments are shown as the fold induction from unstimulated cells transfected with −669/luc. kDa), NF-AT2/NF-ATc (~80 kDa), NF-AT3 (~100 kDa), and NF-AT4/NF-ATx (~120 kDa) (12, 20–23). They share a highly homologous DNA-binding domain that shows a weak sequence similarity to the DNA-binding domain of the Rel family proteins (24, 25). Activation of T cells leads to translational cytoplasmic NF-AT into the nucleus through dephosphorylation of NF-AT by Ca2+/calmodulin-dependent phosphatase calcineurin, which is the target of the immunosuppressive drugs CsA and FK506 (26). Within nuclei, translocated NF-AT and a de novo synthesized cofactor cooperatively bind to a composite site (10). Thus, most NF-AT binding sites in various cytokine promoters are accompanied by the binding sites for auxiliary transcription factors such as AP-1 and Oct (10). For example, NF-AT and AP-1 cooperatively bind to multiple composite sites in the promoter of IL-2 for full induction in activated T cells (27). The distal NF-AT site in the IL-4 promoter also consists of a composite binding site for NF-AT and AP-1 (28). Recently, the proto-oncogene c-maf was found to be expressed highly selectively in Th2-type T cells and to act in synergy with NF-AT to trans-activate the IL-4 promoter by binding adjacent to the proximal NF-AT site (29). Furthermore, a nuclear factor designated NIP45 has been shown to interact with the Rel homology domain of NF-ATp and to act in synergy with NF-ATp and c-Maf in the expression of IL-4 (30). Thus, it is likely that cell type-specific cofactors assembling at a given NF-AT site determine the cell type-specific expression of various cytokines.
complexes of the IL-4 ARE contained proteins immunoreactive with anti-NF-ATp and anti-NF-ATc (33). In contrast to the SCM-1 E1 site, however, the AP-1 family members were clearly associated with the activation-induced complex of the IL-4 ARE (34). The same ARE was also critical for activation-dependent expression of the IL-4 gene in murine mast cells and was also shown to be bound by nuclear proteins from both unstimulated and stimulated mast cells (35). Both constitutive and inducible complexes contained proteins immunoreactive with anti-NF-ATp (35). In contrast to T cells, however, the AP-1 family members were not associated with the activation-inducible complex of mast cells (35). The observation of mast cells is thus rather similar to our present findings. In contrast to the SCM-1 E1 site, however, the constitutive complex of ARE formed by nuclear extracts from unstimulated mast cells had an electrophoretic mobility much faster than the activation-induced complex formed by nuclear extracts from stimulated mast cells (35).

Collectively, some NF-AT proteins appear to be constitutively present in the nuclei of certain types of cells and capable of binding to elements such as SCM-1 E1 or IL-4 ARE even without cell activation. In fact, a protein with a molecular mass of about 41 kDa and reactive with anti-NF-ATp was shown to be present in the nuclei of unstimulated murine mast cells (35). It remains to be seen whether this protein is encoded by an unknown gene belonging to the NF-AT family or produced by an alternative splicing or a post-translational modification of NF-ATp. Recently, the carboxy-terminal end of NF-AT/4/x has been shown to be required for its maximum trans-activation activity, and notably, the same sequence is well conserved in other NF-AT family proteins (36). Furthermore, multiple isoforms of NF-AT proteins with distinct carboxy-terminal regions and with different trans-activation activities have been shown to be generated by alternative splicing (21, 36, 37). Thus, some isoforms of NF-ATp may be capable of trans-locating into the nucleus without cell activation and may even behave as a negative factor. Upon cell activation, they may be replaced by transcriptionally active NF-ATp translocated from the cytoplasm through dephosphorylation by calcineurin. Transcriptional activation may also require some de novo synthesized cofactors specific for each cell type and to be associated with NF-ATp. The E1 element of the SCM-1 genes may be useful to explore this new aspect of transcriptional regulation by the NF-AT system.

Acknowledgments

We thank Drs. Yorio Hinuma and Masakazu Hatanaka for constant support and encouragement.

References