

Regulation of expression of the IL-2 and IL-5 genes and the role of proteins related to nuclear factor of activated T cells

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Cyclic adenosine monophosphate (cAMP) inhibits phorbol 12-myristate 13-acetate (PMA)-induced IL-2 production while it inhibits IL-5 production at the transcriptional level in EL-4, a mouse lymphoma line. The -321 to +46 region of the mouse IL-2 promoter is required for activation by PMA and inhibition by cAMP. This promoter region contains several elements that interact with transcription factors, such as nuclear factor of activated T cells (NF-AT), NF- κ B, AP-1, and octamer. With use of reporter plasmid carrying multiple copies of each element, we found that the construct that contained the NF-AT site was most effective for responding to PMA activation and cAMP inhibition. In electrophoretic mobility shift assay (EMSA), PMA-induced NF-AT binding complex was altered by cAMP. Furthermore, overexpression of the cytoplasmic component of NF-AT abrogated the inhibitory action of cAMP. These results indicate that the NF-AT site is a target of the inhibitory action of cAMP. We have previously reported that the -1200 to +33 region of the mouse IL-5 promoter can mediate transcriptional stimulation by PMA and cAMP in EL-4 cells. Here we identified the element IL-5P, which is required for maximal activation of the IL-5 promoter. We found that this element is homologous to the binding site for NF-AT and interacted with NF-AT-related factors induced by PMA and cAMP. Thus it appears that an NF-AT factor is involved in the regulation of IL-5 gene transcription. (*J ALLERGY CLIN IMMUNOL* 1995;96:1126-35.)

Key words: IL-2 gene, IL-5 gene, nuclear factor of activate T cells, helper T cells, cyclic adenosine monophosphate

Mouse helper T (Th) cells can be classified into two subsets, Th1 and Th2, on the basis of their pattern of cytokine production.¹ Th1 cells produce IL-2 and interferon- γ and mediate cellular immunity, whereas Th2 cells produce IL-4, IL-5, and IL-10 and stimulate humoral immunity.² Th1 and Th2 cells may differ either in the signal transduction pathways for cytokine production or in the transcription factors that regulate cytokine production. Differential sensitivity of Th1 and Th2 cells to reagents that elevate intracellular cyclic adenosine

Abbreviations used

AP-1p:	proximal AP-1
Bt ² :	N ⁶ , O ² -dibutyl
cAMP:	Cyclic adenosine monophosphate
CLE:	conserved lymphokine element
EMSA:	electrophoretic mobility shift assay
NF-AT:	nuclear factor of activated T cells
Octp:	proximal octamer
PMA:	phorbol 12-myristate 13-acetate
Th:	helper T

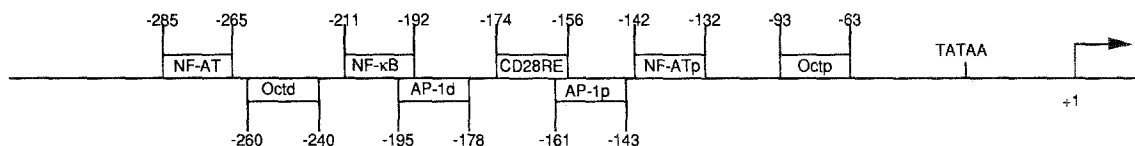
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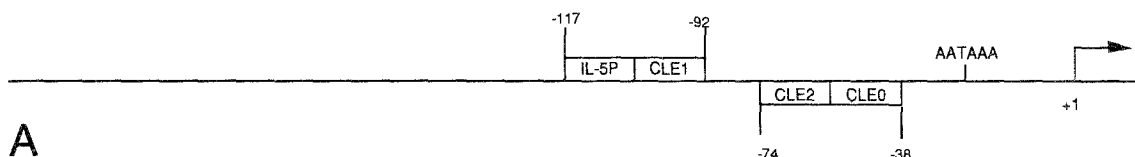
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monophosphate (cAMP) such as prostaglandin E₂, cholera toxin, and forskolin is in keeping with these views.^{3,4} After elevation of intracellular cAMP, cytokine production from Th1 clones but not Th2 clones is inhibited, whereas IL-5 production is increased³ (Y. Naito, et al., submitted). Therefore, signaling molecules or transcription factors that are differentially required for Th1- and

IL-2 promoter



IL-5 promoter



A

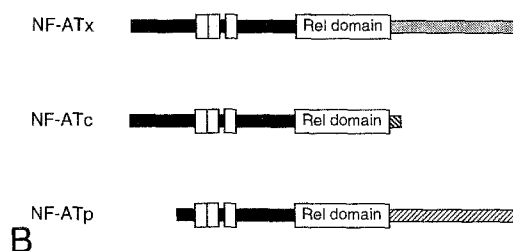


FIG. 1. A, Schematic representation of *cis*-regulatory elements in the mouse IL-2 and IL-5 promoter.⁹ Binding sites for the trans-acting factors are boxed. Numbers indicate the base pair and IL-5 positions relative to the transcription initiation site. CLE, Conserved lymphokine element. **B,** Schematic representation of the structure of NF-ATx, NF-ATc, and NF-ATp. Rel homology domains are labeled. SP boxes are represented by small rectangles. Solid black bars indicate greater than 30% identical residues. The available sequence for murine NFATp is derived from a partial clone.¹⁵ (From Masuda ES, et al. *Mol Cell Biol* 1994;15:2697-706, with permission of The American Society for Microbiology.)

Th2-type cytokine production may be the target of cAMP actions. Elucidation of the mechanism of reciprocal action of cAMP on cytokine production by Th1 and Th2 cells will shed light on the molecular basis for differences between Th1 and Th2 cells.

T cells are activated to proliferate and to produce cytokines, including IL-2 and IL-5, by interaction of T-cell receptors and antigen with the major histocompatibility complex molecules on antigen-presenting cells. Triggering of T-cell receptors has been shown to result in hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol and inositol 1,4,5-tris-phosphate. Diacylglycerol activates protein kinase C and inositol 1,4,5-tris-phosphate induces calcium mobilization. These events can be mimicked by phorbol ester phorbol 12-myristate 13-acetate (PMA) and calcium ionophore (A23187).⁵

The mouse lymphoma line EL-4 produces both Th1- and Th2-type cytokines such as IL-2, IL-3, IL-4, IL-5, IL-10, and granulocyte-macrophage colony stimulating factor, in response to PMA

without adding A23187. We reported that IL-2 production at messenger RNA (mRNA) and protein levels in EL-4 cells are induced by PMA and are inhibited almost completely by the addition of N⁶, O²-dibutyryl (Bt²) cAMP. In contrast, cAMP augmented PMA-induced IL-5 production more than tenfold. Transient transfection assays showed that cAMP repressed IL-2 promoter activity while it augmented IL-5 promoter activity. These findings strongly suggest that cAMP exerts its action mainly at the transcriptional level. We have also shown that cAMP exerts its effect via the catalytic subunit of cAMP-dependent protein kinase.⁶

Induction of IL-2 promoter activity depends on the 300 base pair region adjacent to the transcription initiation site.⁷⁻⁹ This region contains the binding sites for nuclear factor of activated T cells (NF-AT) NF-κB, AP-1, and octamer, and cooperation of these transcription factors is required for maximal activation of the IL-2 promoter¹⁰ (Fig. 1, A). NF-AT is thought to control tissue-specific expression of the IL-2 gene.¹¹ NF-AT is assembled

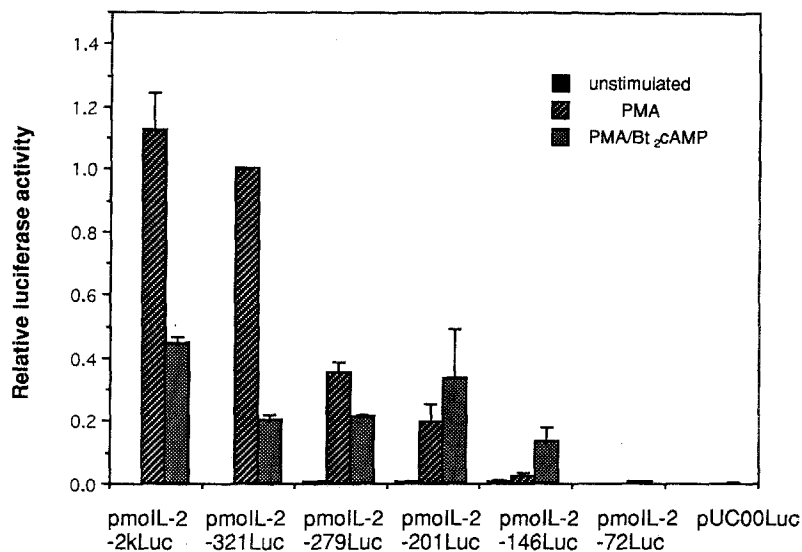


FIG. 2. Effects of cAMP on the 5'-deleted IL-2 promoter activity in transient transfection assay. pmolL-2-2kLuc, pmolL-2-321Luc, pmolL-2-279Luc, pmolL-2-201Luc, and pmolL-2-146Luc contain from -2k, -321, -279, -201, and -146 to +46 of the IL-2 promoter, respectively. pUC00Luc contains no promoter. EL-4 cells were transfected with these deletion mutant constructs and stimulated by PMA (20 ng/ml) or by the combination of PMA and Bt² cAMP (1 mmol/L). Each construct was transfected at least three times, and the average of data normalized to the value of PMA-stimulated pmolL-2-321Luc is shown as relative luciferase activity ($\pm$ SD). (From Tsuruta L, et al. *J Immunol* 1995;154:5255-64. Copyright 1995, The American Association of Immunologists.)

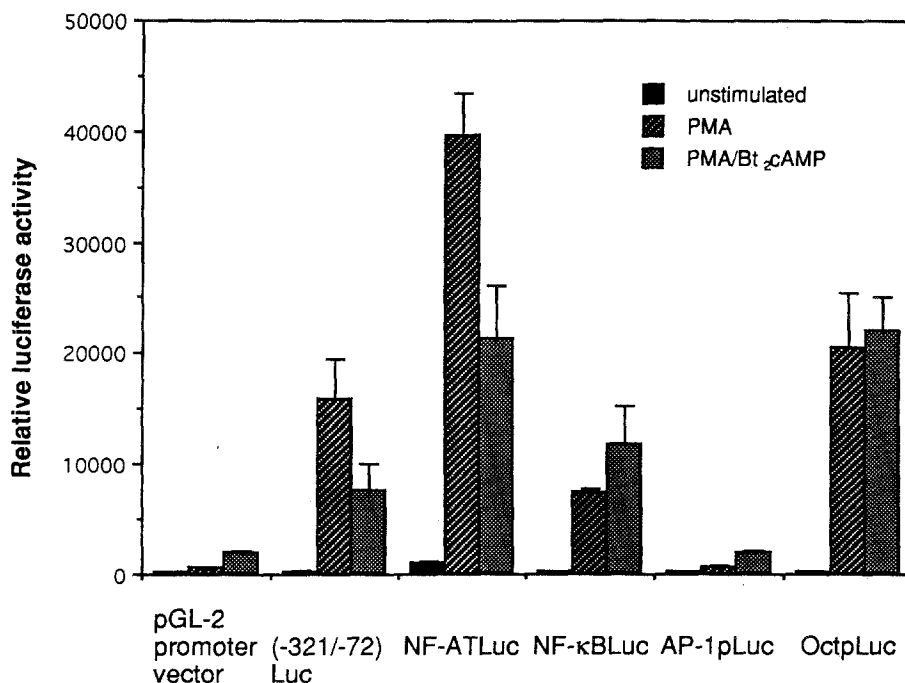


FIG. 3. Effect of cAMP on the transcriptional activity driven by the -321/-72 region or multicopies of each factor binding site in the IL-2 promoter fused to the enhancer-less basic SV40 promoter. NF-ATLuc, NF- κ BLuc, AP-1pLuc, and OctpLuc contain seven copies of the NF-AT site, eight copies of the NF- κ B site, eight copies of the AP-1p site, and seven copies of the Octp site, respectively. EL-4 cells were transfected with indicated constructs and stimulated by PMA (20 ng/ml) or the combination of PMA and Bt² cAMP (1 mmol/L). pGL2-promoter vector, which contains the enhancer-less basic SV40 promoter and the luciferase gene, was used as a control. Each construct was transfected at least three times and the average is shown as relative luciferase activity ($\pm$ SD). (From Tsuruta L, et al. *J Immunol* 1995;154:5255-64. Copyright 1995, The American Association of Immunologists.)

in the nuclei of activated T cells from a preexisting cytoplasmic component and a ubiquitous nuclear component AP-1.^{12,13} The nuclear component AP-1 can be induced by activation of protein kinase C activation. On the other hand, the cytoplasmic component of NF-AT exists before stimulation in the cytoplasm and translocates to the nucleus upon increase in intracellular calcium.¹² This translocation depends on the activity of calcium-dependent and the calmodulin-dependent phosphatase, calcineurin. Immunosuppressive drugs such as cyclosporin A (CSA) and FK506 block translocation of the cytoplasmic component of NF-AT into the nucleus by inhibiting the activity of calcineurin.^{12,14} Several distinct members of the cytoplasmic component of NF-AT, such as NF-ATp, NF-ATc, and NF-ATx have recently been cloned, which indicates that NF-AT constitutes a family of transcription factors (Fig. 1, *B*). All of these molecules contain a well-conserved homology domain that is distantly related to a domain present in the Rel family. NF-ATc and NF-ATx are capable of activating IL-2 transcription in non-T cells when stimulated with phorbol ester and calcium ionophore.¹⁵⁻¹⁷

In contrast to the IL-2 gene, details on the transcriptional control of the IL-5 gene have not been explored at the molecular level. Within the 5'-flanking region, the IL-5 gene is associated with the conserved lymphokine elements (CLE)0, CLE1, and CLE2, which are also found in the promoters of IL-3, granulocyte-macrophage colony stimulating factor and IL-4 genes and may have a role in the regulation of coordinated lymphokine expression^{18,19} (Fig. 1, *A*).

In this work we evaluated the effect of cAMP on regulatory elements of the IL-2 promoter and found that the NF-AT site is a target of cAMP actions and that transfection of NF-AT complementary DNA (cDNA) abrogates the inhibitory action of cAMP in EL-4 cells. We also identified the element necessary for full activity of the IL-5 promoter in response to PMA and Bt² cAMP stimulation in EL-4. We showed that this element, the IL-5P element, shares homology with the binding site of NF-AT of the IL-2 gene and interacts with the inducible nuclear factor NFIL-5P, which is closely related to the transcription factor NF-AT.

RESULTS

Inhibitory effects of cAMP on IL-2 transcription are mediated through the promoter region -321 to +46

To delineate the region of the IL-2 promoter responding to cAMP in EL-4 cells, we used a

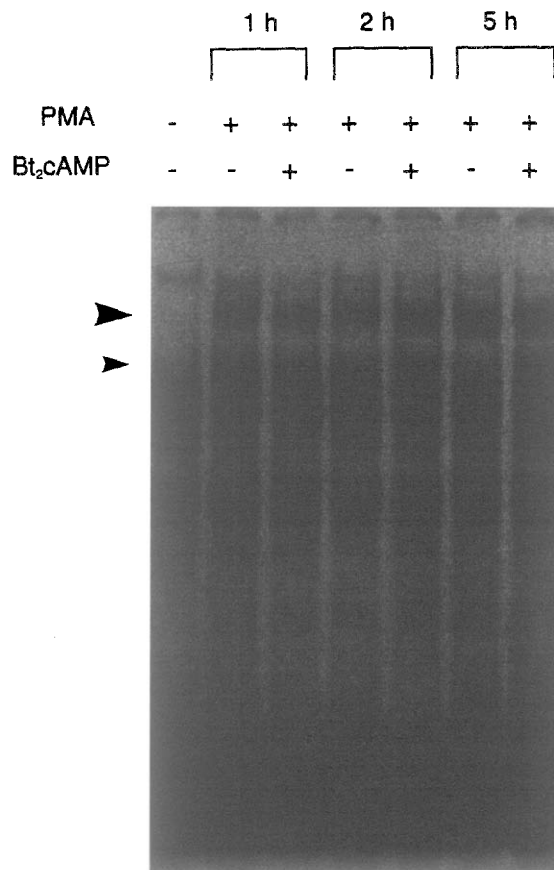


FIG. 4. Effect of cAMP on the binding activities of the proteins to the NF-AT site in the IL-2 promoter. EL-4 cells were unstimulated, stimulated with PMA (20 ng/ml), or stimulated with the combination of PMA and Bt² cAMP (1 mmol/L) for 1, 2, or 5 hours, and EMSAs were performed with nuclear extracts. Bands indicated by arrowheads were shown to be specific by competition analysis, with use of homologous and heterologous oligonucleotides (data not shown). Nuclear complexes binding to the NF-AT site. *Large arrowhead* indicates the slow migrating binding complex induced by PMA. *Small arrowhead* indicates the fast migrating binding complex induced only by the combination of PMA and Bt² cAMP. (From Tsuruta L, et al. J Immunol 1995;154:5255-64. Copyright 1995, The American Association of Immunologists.)

transient transfection assay with luciferase reporter plasmids carrying a series of 5'-deletions in the IL-2 promoter. Twenty-four hours after transfection, cells were treated with either PMA or the combination of PMA and Bt² cAMP and incubated for an additional 16 hours; luciferase activity was then determined. Transcription of pmoIL-2-2kLuc and pmoIL-2-321Luc increased approximately 2000-fold after PMA stimulation, and the addition of Bt² cAMP suppressed activities as much as 60% and 80%, respectively (Fig. 2). Similar results were obtained with CAT gene constructs used as a reporter (data not shown). In pmoIL-2-279Luc, which lacked the NF-AT site, response to PMA

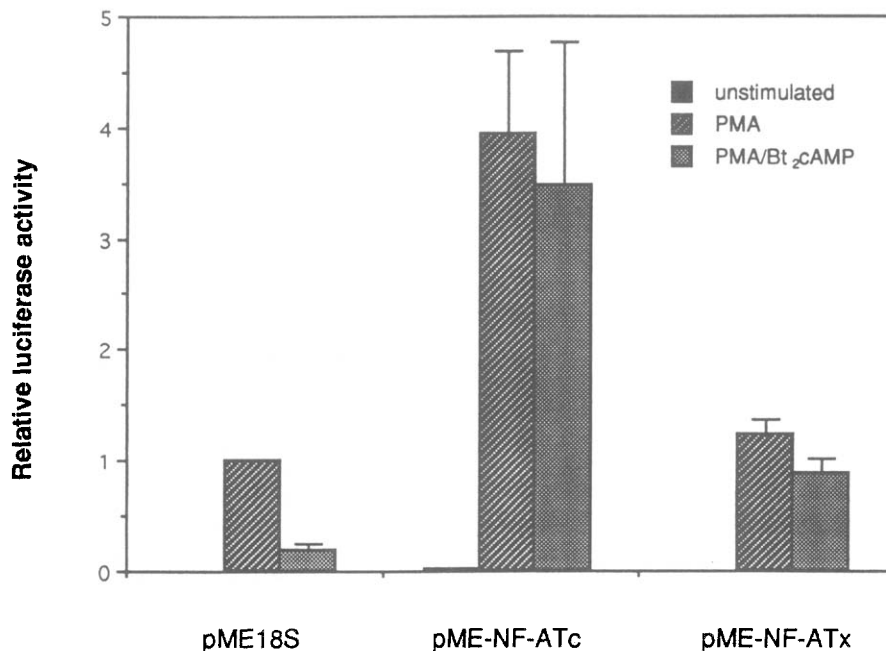


FIG. 5. Overexpression of NF-ATc or NF-ATx suppresses the inhibitory effect of cAMP on the IL-2 promoter. pmolIL-2-321Luc was co-transfected with an expression plasmid pME18S containing no insert, pME-NF-ATc, or pME-NF-ATx and stimulated by PMA (20 ng/ml) or the combination of PMA and Bt² cAMP (1 mmol/L). All data are presented as a percentage of the luciferase activity of PMA-stimulated pmolIL-2-321Luc. Each construct was transfected at least three times, and the average of the data normalized to the value obtained when pmolIL-2-321Luc and pME18S were co-transfected and stimulated by PMA is shown as relative luciferase activity ($\pm$ SD). (From Tsuruta L, et al. *J Immunol* 1995;154:5255-64. Copyright 1995, The American Association of Immunologists.)

and sensitivity to cAMP dramatically decreased. In pmolIL-2-201Luc, which lacked the NF- κ B site, response to PMA further decreased, and suppression by cAMP was no longer evident.²⁰ These results are in accord with reported data²¹ and suggest that the region responding positively to PMA and negatively to cAMP is located between positions -321 and +46 region of the IL-2 promoter. Furthermore, the data indicate that at least one of the target sites of cAMP is located at the -321 to -201 region.

The NF-AT site is a target for the inhibitory effect of cAMP

The -321/+46 region of the IL-2 promoter contains several binding sites for nuclear factors such as NF-AT, NF- κ B, AP-1, and octamer. To further delineate the element responding to cAMP, we examined the effect of cAMP on the activity of a single element in the context of heterologous promoter. We fused multiple copies of either the NF-AT, NF- κ B, proximal AP-1 (AP-1p), or proximal octamer (Octp) site to the basic enhancer-less SV40 promoter vector and examined the response of each construct to PMA and cAMP (Fig. 3). Activities of NF-ATLuc, NF- κ BLuc, and

OctpLuc, but not AP-1pLuc, were significantly enhanced by PMA. Among them, repression by cAMP was observed only with NF-ATLuc, whereas cAMP failed to repress activities directed by NF- κ BLuc and OctpLuc. The extent of repression observed with NF-ATLuc was comparable to that observed with (-321/-72)Luc, in which the -321/-72 region of the IL-2 promoter was fused to the enhancer-less basic SV40 promoter. These results suggest that the NF-AT site is a target of the inhibitory cAMP signal. It should be noted, however, that empty vector (pGL2-promoter vector) was upregulated as much as fourfold by cAMP. Apparently, insertion of the NF- κ B or Octp site to the vector reduced the upregulation induced by cAMP. Therefore, the possibility that the NF- κ B and Octp sites also mediate the inhibitory action of cAMP would need to be considered.

Effect of cAMP on formation of the protein complexed with the NF-AT site

We next attempted to determine whether exposure of EL-4 cells to cAMP would affect the formation of binding factors complexed with the NF-AT site. Nuclear extracts from EL-4 cells

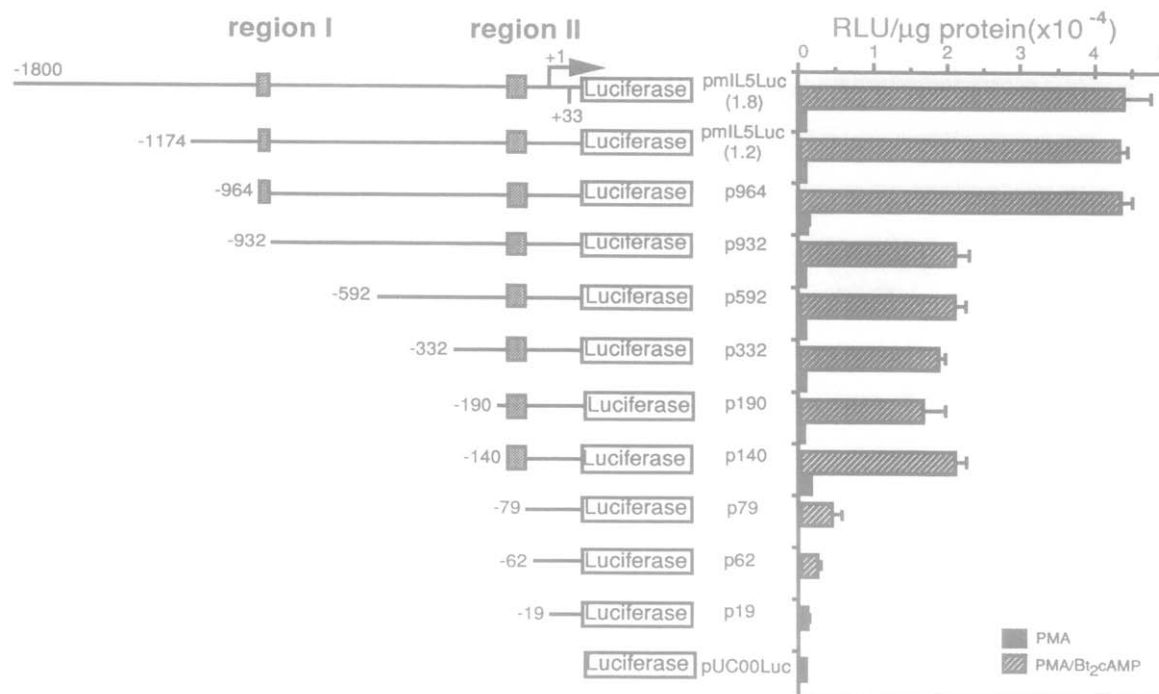


FIG. 6. Deletion analysis of the 5'-upstream region of the mouse IL-5 promoter. EL-4 cells were transfected with the serial deletion mutants shown schematically in the *left panel* and stimulated by PMA (20 ng/ml) or the combination of PMA and Bt² cAMP (1 mmol/L). Results represent the mean value of duplicate transfections. Two other independent experiments gave similar results. The regions responding to PMA and Bt² cAMP are indicated by shadowed boxes. (From Lee HJ, et al. J Biol Chem 1995;270:17541-50.)

stimulated with either PMA or the combination of PMA and Bt² cAMP for 1, 2, and 5 hours were tested for their potential to bind to the NF-AT site by electrophoretic mobility shift assay (EMSA).

The bands indicated by arrowheads (Fig. 4) represent the NF-AT sequence-specific DNA binding complexes that can be inhibited with excess unlabeled NF-AT oligonucleotides (data not shown). A slow migrating complex (indicated by the large arrowhead) was induced by PMA in the presence or absence of Bt² cAMP. After 1 or 2 hours of stimulation by PMA, the slow migrating complex consisted of two complexes of a similar mobility, and by addition of Bt² cAMP, the amount of the faster migrating complex of the two was increased. After 5 hours of stimulation by PMA, without Bt² cAMP, the slow migrating complex showed a slightly faster mobility than that detected at 1 or 2 hours, whereas in the presence of Bt² cAMP, a binding complex with a slower mobility than that induced by PMA alone was detected. A fast migrating complex (indicated by the small arrowhead) also specifically bound to the NF-AT site and was detected after stimulation by the combination of PMA and Bt² cAMP. However, the appearance of this

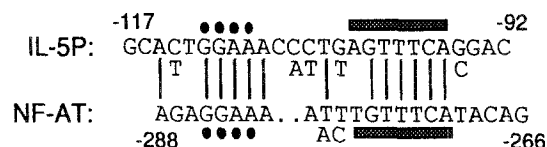


FIG. 7. Comparison of the nucleotide sequence of IL-5P with that of the distal NF-AT site of the IL-2 promoter. Gaps are introduced into the NF-AT site to maximize matches. Differences in the human sequence are shown below the murine sequence. The purine-rich sequence and an AP1-like site are indicated by closed circles and shadowed bars, respectively. (From Lee HJ, et al. J Biol Chem 1995;270:17541-50.)

complex varied between nuclear extract preparations and was not reproducible.

Transfected NF-AT cDNA abrogates the inhibitory effect of cAMP on IL-2 promoter activity

The aforementioned data indicated that the NF-AT site is a target of the inhibitory effects of cAMP. It is therefore possible that overexpression of NF-AT renders EL-4 cells more resistant to

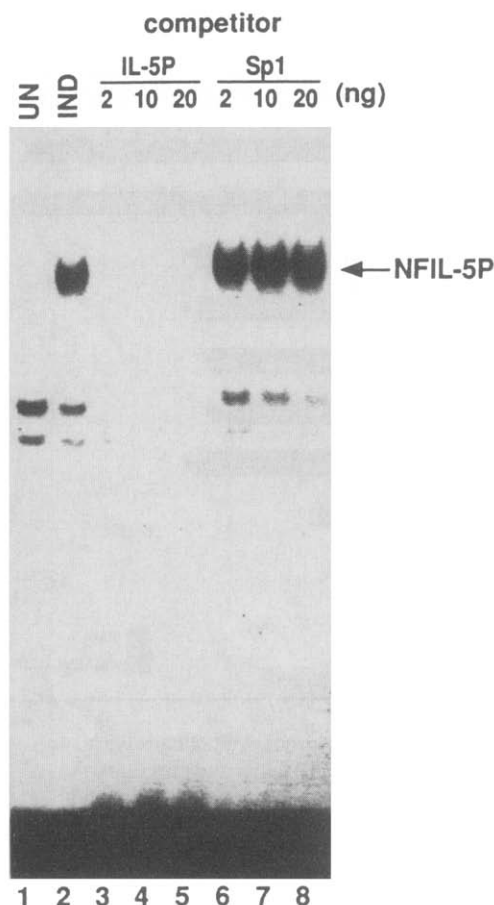


FIG. 8. Binding of nuclear factors to the IL-5P sequence. NFIL-5P is the inducible, sequence-specific factor that binds to the IL-5P sequence. EMSAs were done with unstimulated (lane 1) or PMA-Bt² cAMP stimulated (lanes 2 to 8) EL-4 nuclear extracts in the presence of increasing amounts of unlabeled IL-5P (lanes 3, 4, and 5) or Sp1 (lanes 6, 7, and 8) oligonucleotides, as indicated. (From Lee HJ, et al. *J Biol Chem* 1995;270:17541-50.)

cAMP. Consequently, we transfected EL-4 cells with the IL-2 promoter reporter plasmid and NF-ATc or NF-ATx expression plasmids. In the absence of PMA, neither NF-ATc nor NF-ATx alone activated the IL-2 promoter. In the presence of PMA, NF-ATc activated the IL-2 promoter about fourfold and suppressed the inhibitory effect of cAMP on the IL-2 promoter (Fig. 5). Although NF-ATx showed only a little stimulatory effect even in the presence of PMA, it also suppressed the inhibitory effect of cAMP on the IL-2 promoter. These results provide further evidence that the NF-AT site mediates the inhibitory effect of cAMP. They also suggest that cAMP exerts effects via a signaling pathway that regulates NF-AT family proteins.

Functional analysis of the 5'-flanking region of the IL-5 gene

We previously reported that the 5'-flanking region of the IL-5 promoter extending from -1200 to +33 relative to the transcription initiation site mediated induction by PMA and cAMP in EL-4 cells.⁶ To identify the region responsible for inducible expression of the IL-5 promoter in response to PMA and cAMP, the luciferase reporter plasmids carrying sequential 5' deletions of the mouse IL-5 promoter were transiently transfected into EL-4 cells. The plasmid pUC00Luc, which has no upstream sequence derived from the IL-5 gene,⁶ showed an almost negligible response to PMA or a combination of PMA and cAMP (Fig. 6). Removal of the 5' sequence up to position 964 did not affect the promoter activity responding to PMA and cAMP, but deletions beyond this position through -932 reduced the response by 50%. Further deletions up to position -140 led to no further reduction. Remarkably, the subsequent removal of nucleotides -140 through -80 diminished the promoter activity by 90%. Thus the response to a combination of PMA and cAMP appears to decline in two stages, at -964 to -933 (region I) and at -140 to -80 (region II). On the other hand, the IL-5 promoter responded weakly to PMA alone, and removal of the 5' sequences up to position -140 did not affect promoter activity responding to PMA, whereas deletions beyond this position almost completely abolished the response. Thus the sequences between -140 to -80 (region II) of the IL-5 promoter are required for a response, albeit a weak one, to the PMA signal. Taken together, these results indicate that the region between -140 to -80 (region II) is indispensable for promoter activity that responds to PMA as well as to PMA and Bt² cAMP, whereas the region between -964 to -933 (region I) is required only for the activity responding to PMA and cAMP. Further analysis by introduction of mutations in region II identified region -117 to -92 (designated as IL-5P) as responding to PMA and cAMP (data not shown).²²

IL-5P interacts with NF-AT-related factor

Deletion and mutation analyses revealed that downstream sequences of -124 are critical for IL-5 promoter activity. Interestingly, closer inspection revealed that IL-5P is homologous to the binding site for NF-AT (Fig. 7). NF-AT has also been implicated in transcriptional regulation of other lymphokine genes.^{22,23} The aforementioned data indicate that the NF-AT site is a major target of the inhibitory action of cAMP on the IL-2 promoter.

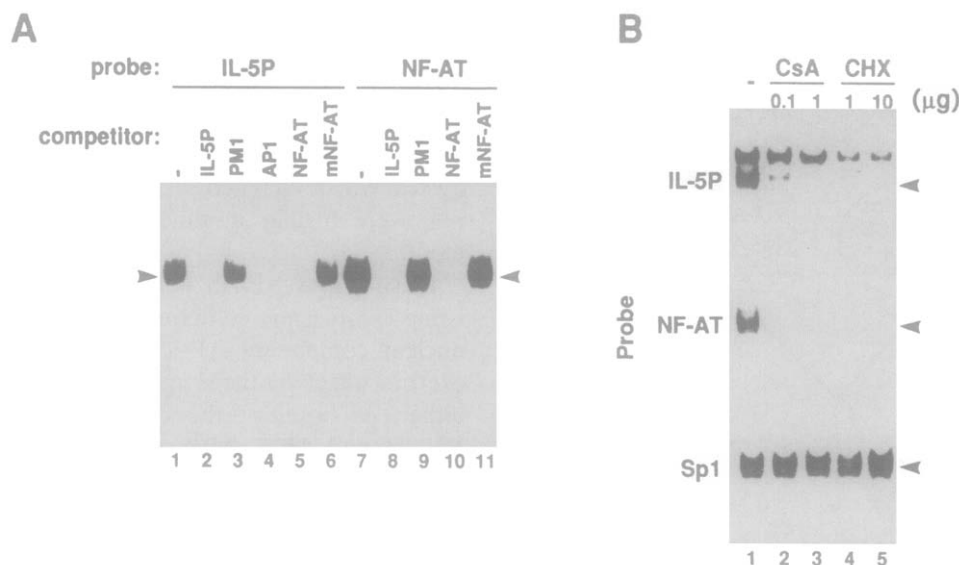


FIG. 9. Relationship between NFIL-5P and NF-AT. **A**, Nuclear extracts from EL-4 cells stimulated with PMA (20 ng/ml) and Bt² cAMP (1 mmol/L) were incubated with the IL-5P probes (lanes 1 to 6) or the NF-AT probes (lanes 7 to 11) in the presence of 20 ng of the unlabeled competitor oligonucleotides. *Left and right arrowheads* indicate positions of NFIL-5P and NF-AT complexes, respectively. **B**, Nuclear extracts were prepared from EL-4 cells stimulated with PMA and Bt² cAMP in the presence of different concentrations of cyclosporine or cycloheximide, as indicated. EMSAs were performed with IL-5P, NF-AT, and Sp1 probes. The positions of NFIL-5P, NF-AT, and Sp1 complexes are indicated by *arrowheads*. *CsA*, Cyclosporin A; *CHX*, cycloheximide. (From Lee HJ, et al. J Biol Chem 1995;270:17541-50.)

Thus we speculated that IL-5P may be involved in coordinated and differential regulation of the IL-5 gene and focused on further characterization of IL-5P.

To identify nuclear factors that can mediate inducibility through IL-5P, EMSAs were done with use of a probe corresponding to the region extending from -119 to -89 of the IL-5 promoter. Nuclear extracts prepared from EL-4 cells stimulated with a combination of PMA and Bt² cAMP formed several complexes with the IL-5P probe (Fig. 8). The major complex (designated NFIL-5P) appeared only when we used extracts prepared from stimulated cells (compare lanes 1 and 2), and this complex formation was specifically inhibited in a dose-dependent manner by excess unlabeled IL-5P oligonucleotide (lanes 2 to 5) but not by the Sp1 oligonucleotide (lanes 6 to 8). Other minor complexes with different mobilities could be detected but appearance was variable, depending on different nuclear extract preparations, and they did not seem to be specific for IL-5P because binding was also inhibited by the Sp1 oligonucleotide (lanes 6 to 8).

We next asked whether NFIL-5P and NF-AT were related. In EMSAs, with use of nuclear extracts from EL-4 cells stimulated with PMA and Bt² cAMP, NFIL-5P and NF-AT complexes

migrated with a similar mobility (Fig. 9, A, lanes 1 and 7). NFIL-5P complex formation was completely inhibited by excess unlabeled NF-AT and by IL-5P oligonucleotides (lanes 2 and 5) but not by those mutated in GGA sequences (lanes 3 and 6). As expected, AP1 oligonucleotide also inhibited NFIL-5P complex formation (lane 4). Conversely, NF-AT binding was inhibited specifically by both excess unlabeled NF-AT and IL-5P oligonucleotides, which indicates that NFIL-5P is an NF-AT-related complex (lanes 7 to 11).

NF-AT binding is known to be inhibited by either cyclosporin A or the protein synthesis inhibitor cycloheximide.^{24, 25} We then tested sensitivity of the NFIL-5P complex to cyclosporin A and cycloheximide; treatment of cyclosporin A inhibited the induction of NFIL-5P complex in cells stimulated with PMA and Bt² cAMP, in a dose-dependent manner similar to that seen for NF-AT binding (Fig. 9, B, top and central panels, lanes 2 and 3). Induction of both the NFIL-5P and NF-AT complexes was almost completely inhibited by treatment with 10 mg cycloheximide (lanes 4 and 5). This inhibition was not caused by nonspecific effects of these reagents on nuclear proteins because there was no change in Sp1 binding under the same conditions (bottom panel).

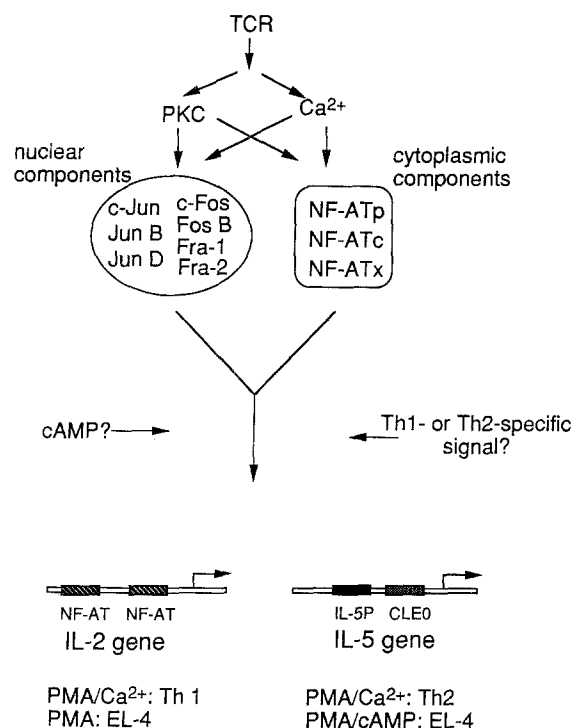


Fig. 10. Heterogenous NF-AT complexes. NF-AT is composed of AP-1 and cytoplasmic components, which are regulated by PMA and Ca^{2+} signals, respectively. NF-AT-like sequences are found in the regulatory regions of the IL-2 and IL-5 genes. Distinct NF-AT complexes with different transactivation functions may be formed by different combinations of AP-1 and cytoplasmic components, depending on signals and cell types. TCR, T-cell receptor; PKC, protein kinase C.

DISCUSSION

We reported earlier that cAMP inhibits the production of IL-2 at mRNA and protein levels in EL-4 cells.⁶ In this study we examined the mechanism of inhibition of IL-2 gene expression by cAMP. 5'-deletion analysis revealed that the region encompassing positions -321/+46 is sufficient to respond to PMA and cAMP (Fig. 2), findings consistent with the previous report.²¹ This region contains the NF-AT, NF- κ B, AP-1p, and Octp sites. On a closer look, the -321/-279 region, which contains the NF-AT site, decreased the positive response to PMA and sensitivity to cAMP. Among the *cis*-regulatory elements examined, the NF-AT site in tandem copies was most effective in responding to PMA activation and to cAMP inhibitory signaling (Fig. 3). Thus, in EL-4 cells, the NF-AT site appears to be a target for cAMP-mediated inhibition. This notion was further supported by the finding that transfection of EL-4 cells with cDNA encoding cytoplasmic subunits of NF-AT rendered the IL-2 promoter resis-

tant to cAMP (Fig. 5). EMSA indicated that properties of the NF-AT complex were altered after Bt^2 cAMP treatment. The slow migrating complexes were induced by PMA treatment (Fig. 4). The intensity and the mobility of these complexes, which changed in a time-dependent manner, were further modified by Bt^2 cAMP treatment.

Inasmuch as NF-AT is composed of the preexisting cytoplasmic component and the ubiquitous nuclear component AP-1,^{12,13} cAMP is likely to exert its effect via the signaling pathway regulating either, or both, of the cytoplasmic subunits of NF-AT and AP-1. AP-1 consists of Fos and Jun proteins, which include c-Jun, JunB, JunD, c-Fos, Fra1, Fra2, and FosB. The cytoplasmic subunit of NF-AT also consists of a family of related proteins.¹⁵⁻¹⁷ Therefore, cAMP may regulate the NF-AT complex via alteration of the cytoplasmic component of NF-AT or AP-1 in the NF-AT complex. Furthermore, both AP-1 and the cytoplasmic component of NF-AT can be modified by phosphorylation and dephosphorylation. Thus it is possible that cAMP exerts its inhibitory effect through the modification of either or both of these molecules.

In EL-4 cells, PMA and cAMP are required for IL-5 gene expression.⁶ In this study we identified a *cis*-regulatory element, designated IL-5P, that is important for the full activity of the IL-5 promoter in response to PMA and cAMP. We found that this element bears homology to the NF-AT site in the IL-2 promoter and that an inducible nuclear factor (NFIL-5P) bound to the IL-5P element (Fig. 8). NFIL-5P is similar to the transcription factor NF-AT, because an excess NF-AT oligonucleotide inhibits NFIL-5P binding to IL-5P and the formation of NFIL-5P is inhibited by cyclosporin A and cycloheximide (Fig. 9).

EL-4 cells used in this study produce both Th1- and Th2-type cytokines, and production of the Th1-specific cytokine IL-2 is inhibited, whereas production of the Th2-specific cytokine IL-5 is augmented by cAMP.⁶ In this article we demonstrated that the NF-AT site plays an important role in the inhibition of IL-2 promoter activity by cAMP and activation of the IL-5 promoter by PMA and cAMP. Thus there is a possibility that NF-AT may be involved in the differential effect of cAMP on IL-2 and IL-5 promoter activity. IL-5P and the IL-2 promoter NF-AT site, although they are closely related, may be regulated differently by cAMP. PMA and cAMP may induce distinct NF-AT-related complexes that have different tran-

scriptional activity on the IL-5P and NF-AT sites. Furthermore, PMA and cAMP may differentially affect the components of the NF-AT-related complexes by protein modifications to either promote or inhibit their transcriptional activation functions. Th1 and Th2 cells express selectively the IL-2 and IL-5 gene, respectively. As described earlier, it is possible that the mechanism for subset-specific expression of IL-2 and IL-5 genes may be related to that of differential regulation of IL-2 and IL-5 genes by cAMP in EL-4 cells. It is therefore tempting to speculate that NF-AT-related factors regulate cytokine gene expression in a Th1- or Th2-specific manner (Fig. 10).

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