Interleukin 2 Transcription Factors as Molecular Targets of cAMP Inhibition: Delayed Inhibition Kinetics and Combinatorial Transcription Roles

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Summary

Elevation of cAMP can cause gene-specific inhibition of interleukin 2 (IL-2) expression. To investigate the mechanism of this effect, we have combined electrophoretic mobility shift assays and in vivo genomic footprinting to assess both the availability of putative IL-2 transcription factors in forskolin-treated cells and the functional capacity of these factors to engage their sites in vivo. All observed effects of forskolin depended upon protein kinase A, for they were blocked by introduction of a dominant negative mutant subunit of protein kinase A. In the EL4.E1 cell line, we report specific inhibitory effects of cAMP elevation both on NF-κB/Rel family factors binding at -200 bp, and on a novel, biochemically distinct "TGGGC" factor binding at -225 bp with respect to the IL-2 transcriptional start site. Neither NF-AT nor AP-1 binding activities are detectably inhibited in gel mobility shift assays. Elevation of cAMP inhibits NF-κB activity with delayed kinetics in association with a delayed inhibition of IL-2 RNA accumulation. Activation of cells in the presence of forskolin prevents the maintenance of stable protein–DNA interactions in vivo, not only at the NF-κB and TGGGC sites of the IL-2 enhancer, but also at the NF-AT, AP-1, and other sites. This result, and similar results in cyclosporin A–treated cells, imply that individual IL-2 transcription factors cannot stably bind their target sequences in vivo without coengagement of all other distinct factors at neighboring sites. It is proposed that nonhierarchical, cooperative enhancement of binding is a structural basis of combinatorial transcription factor action at the IL-2 locus.

Activation of T helper cells results in secretion of diverse lymphokines. The precise combination of lymphokines produced is variable, depending on the circumstances of the activation process. Different effector mechanisms are triggered by different lymphokines. Locally and systemically, the relative levels of different lymphokines with respect to one another strongly affect in vivo outcomes. Thus, the type of immune response that is mobilized ultimately depends on the differential transcriptional regulation of lymphokine genes. The major stimulatory signal for lymphokine production is delivered by engagement of the TCR with antigen in the context of MHC on APCs. However, engagement of accessory molecules on the T cell with additional ligands, either on APCs or in the microenvironment, may also deliver other kinds of signals that affect lymphokine gene expression differentially. Expression of the T cell growth factor, IL-2, is particularly susceptible to this kind of modulation. Glucocorticoids inhibit IL-2 induction preferentially (1–3), and contact of a T cell with antigen in the absence of a CD28 ligand can drive the T cell into an anergic state in which IFN-γ can be expressed, but not IL-2 (4, 5). Agonists that elevate cAMP levels, such as prostaglandin E_1 and E_2, give especially

clear evidence for selective inhibition of IL-2 expression (6, 7), as they can inhibit IL-2 expression in cases where there is no inhibition of IL-4 expression at all (8–10). The effects of cAMP on T cells can be exerted at both cell- and gene-specific levels, depending on the cells and stimuli used. In certain IL-2–producing cells, cAMP elevation can uncouple the TCR/CD3 complex from its downstream signaling mediators, preventing the generation of Ca^{2+} and protein kinase C signals (11–13). On the other hand, even when Ca^{2+} and protein kinase C signals are provided pharmacologically, cAMP elevation can still interfere selectively with induction of IL-2 gene expression, yet allowing other responses to proceed. It is the mechanism of this second, gene-specific effect that we investigate here.

The IL-2 gene regulatory region consists of compact, clustered binding sites for an assortment of transcription factors that are disparately regulated (14). In most or all cases studied, IL-2 expression is induced only when all the known factors are present, and the IL-2 promoter only works at maximal efficiency when all the major binding sites for these factors are intact. However, in physiological situations, data seem to indicate that certain factors play more critical roles than

others. Two of them, AP-1 and NF-AT, appear repeatedly to be rate limiting for IL-2 expression. When selective down-regulation of IL-2 production is caused by exposing T cells to glucocorticoids, the possible mechanisms are a protein–protein interaction that sequesters AP-1 by dimerization of AP-1 components with the glucocorticoid receptor (2), or a specific inhibition of interaction between AP-1 and NF-AT (3). When IL-2–producing cells have been anergized and become unable to express IL-2, their failure appears to be due to either a general block of NF-AT, NF-κB, and AP-1 activation (15), or a selective inability to activate AP-1 (16). The potent immune-suppressive drugs cyclosporin A (CsA)[1] and FK 506 completely block IL-2 transcription by blocking the nuclear translocation of one component of NF-AT (17). Whereas this is an example of strictly pharmacological inhibition, defects in both NF-AT and AP-1 mobilization also appear to be responsible for a normal developmental block in the ability to express IL-2, namely in cortical thymocytes (18). A similar phenotype is observed in a pre-mast cell line that can express IL-4 but not IL-2 (19). Thus, an important question is whether all physiological regulation of IL-2 expression is exerted through effects on AP-1 or NF-AT, or whether cAMP-elevating agonists might inhibit IL-2 expression through effects on different factors.

To address these questions, we have chosen EL4.E1 thymoma cells as a model T cell system, and the Ca^{2+} ionophore A23187 and the phorbol ester PMA (TPA) as stimulants that can bypass any effects of cAMP on signal generation at the cell membrane. Using this system previously, we showed that cAMP could inhibit IL-2 transcription (9). We also obtained initial indications that elevation of cAMP caused a slight decrease in the binding activity of NF-κB, but did not inhibit NF-AT or AP-1 site binding activities (20). In our present studies, we have clearly defined two specific effects of cAMP on factor-binding activities and examined the resulting protein–DNA interactions at the IL-2 locus in vivo. Our results confirm that cAMP, acting via activation of protein kinase A (PKA), selectively affects the in vitro DNA binding activities of NF-κB and a newly described TGGGC binding factor, and that it does so with novel and distinctively delayed kinetics. No evidence was found for any negative effect on NF-AT or AP-1 binding. However, cells stimulated in the presence of forskolin failed to sustain stable protein-DNA contacts at a broad array of sites spanning the entire IL-2 enhancer region. The lack of occupancy may be evidence for a new role for NF-κB and TGGGC binding activities, as required partners in transcription complex assembly.

Materials and Methods

Cells. EL4.E1 cells (EL4) were grown in RPMI-1640 in the presence of 2 mM L-glutamine, 50 mM β-ME, antibiotics, and 6%

fetal bovine serum (Hyclone, Logan, UT). Cells were cultured to confluence before stimulation and were stimulated at $1–2 \times 10^6$ cells/ml using 17 nM TPA and 120 nM A23187 in the presence or absence of 10 μM of forskolin (Sigma Chemical Co., St. Louis, MO), which raises the cAMP level by directly activating adenylate cyclase. Recombinant human IL-1α (Genzyme Corp., Cambridge, MA) was used at 50 U/ml.

IL-2 Analysis. RNase probe protection analysis to quantitate IL-2 RNA was performed as previously described (19, 21). For each sample, 10 μg of RNA was used to hybridize with the IL-2 probe (22), and 1 μg RNA was used to hybridize with either an actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (23).

Cyclic AMP Assay. Levels of cAMP were measured in EL4 cells as described previously using an Amersham dual-range cAMP kit (9), except that nonacetylated standards and samples were used.

Gel Mobility Shift Assays. Nuclear extracts of EL4 cells were prepared as before (18). Double-stranded oligonucleotide corresponding to portions of the mouse IL-2 gene regulatory region were labeled by end filling with α-$[^{32}P]$deoxynucleoside triphosphates. The detailed sequences and corresponding sites relative to the transcription start site were described elsewhere (18–20). Oligonucleotides used for this experiment were synthesized in the California Institute of Technology Biology Microchemical Facility. The binding reaction and gel electrophoresis procedures were exactly as reported before (18). Note that these conditions for detection of NF-AT site binding activity differ from those we used in initial studies (20), as poly (dI-dC) is used as a nonspecific competitor in place of poly (dA-dT). In our hands, this substitution yields a spectrum of gel-shift complexes more consistent with NF-AT complexes described elsewhere, and less dominated by highly protease-sensitive complexes. However, the insensitivity of NF-AT site binding factors to forskolin reported in our earlier study (20) is confirmed here.

In vivo Footprinting Analysis. The genomic footprinting analysis was done as before with the same set of IL-2 gene primers previously reported (19). Briefly, 3×10^7 cells were concentrated into 1 ml medium, and treated with 0.1% of dimethylsulfate at 37°C for 1 min. The reaction was stopped by transferring the cells to a tube containing 49 ml of ice-cold PBS, and the cells were pelleted and washed once more with 50 ml PBS. The cell pellet was then resuspended in 3 ml of lysis buffer (300 mM NaCl, 50 mM Tris, pH 8.0, 25 mM EDTA, pH 8.0, 200 μg/ml proteinase K, and 0.2% SDS), and incubated at 37°C overnight. Nucleic acids were obtained by phenol/chloroform extraction and isopropanol precipitation, then subjected to piperidine cleavage and subsequent ligation-mediated PCR (24). The final labeled PCR products were separated on a 6% polyacrylamide sequencing gel.

Transfection and Selection. 10 μg of linearized HL-REV$_{ABp}$KOneo (designated pΔPKA in the text) (a gift from G. S. McKnight, University of Washington, Seattle, WA) was electroporated at 960 μF and 320 V into 10^7 EL4.E1 cells in 1 ml of serum-free RPMI-1640 medium with supplements and 14.4 mM β-ME. The cells were then diluted into 50 ml of medium and plated in two 24-well plates (1 ml/well). 2 d after plating, G418 (GIBCO BRL, Gaithersburg, MD) was added to the wells at 400 μg/ml. Positive wells were identified 7–10 d after selection and the cells were expanded in 200 μg/ml G418 for further analysis. The plasmid DNAs, pBR322 and pSV2neo (25), were used in transfection as negative and positive controls. Integration of the mutant PKA construct in established transfectant lines was confirmed by Southern blot analysis and its expression was further confirmed by Northern blot analysis. One mutant PKA transfectant line, IIA4, and two pSV2neo control lines were used in the subsequent experiments.

Results

Forskolin Affects DNA-binding Activities of a Specific Subset of Factors. To investigate the effects of cAMP on IL-2 transcriptional control, EL4 cells were stimulated with TPA+A23187 in the presence or absence of 10 μM of forskolin, and the IL-2 mRNA level in the cells was examined 5 h after induction (Fig. 1 *A*). Addition of forskolin to the TPA+A23187 stimuli significantly decreased the level of IL-2 mRNA (Fig. 1 *A*, lane *3*). In previous work (9), we reported that forskolin could also inhibit inducible expression of a reporter gene under the control of the minimal 321-bp IL-2 promoter/enhancer. This indicated that at least part of the effect of forskolin was mediated through the 5' regulatory sequence of the IL-2 gene, presumably at the level of transcriptional initiation, and that the ~300 bp minimal promoter/enhancer sequence was sufficient to produce this response. Therefore, to determine the mechanism of the forskolin

inhibition effect, we focused on protein–DNA interactions in this 300-bp region.

To ascertain whether forskolin inhibits the binding of particular factors to the IL-2 regulatory region, we compared the levels of most DNA binding activities known to be correlated with IL-2 gene activation in nuclear extracts from stimulated cells and from cells stimulated in the presence of forskolin. Nuclear extracts were prepared from EL4 cells incubated under different conditions for 4–5 h, and these extracts were analyzed for sequence-specific binding activities by electrophoretic mobility shift assays (EMSAs) (Fig. 1, *B* and *C*). Double stranded oligonucleotide probes were chosen to span all the major sites of protein–DNA contact in IL-2-producing EL4 cells, as previously identified by in vivo footprinting (19). As shown in Fig. 1 *C*, the DNA binding activities of factors that are constitutively present, such as the
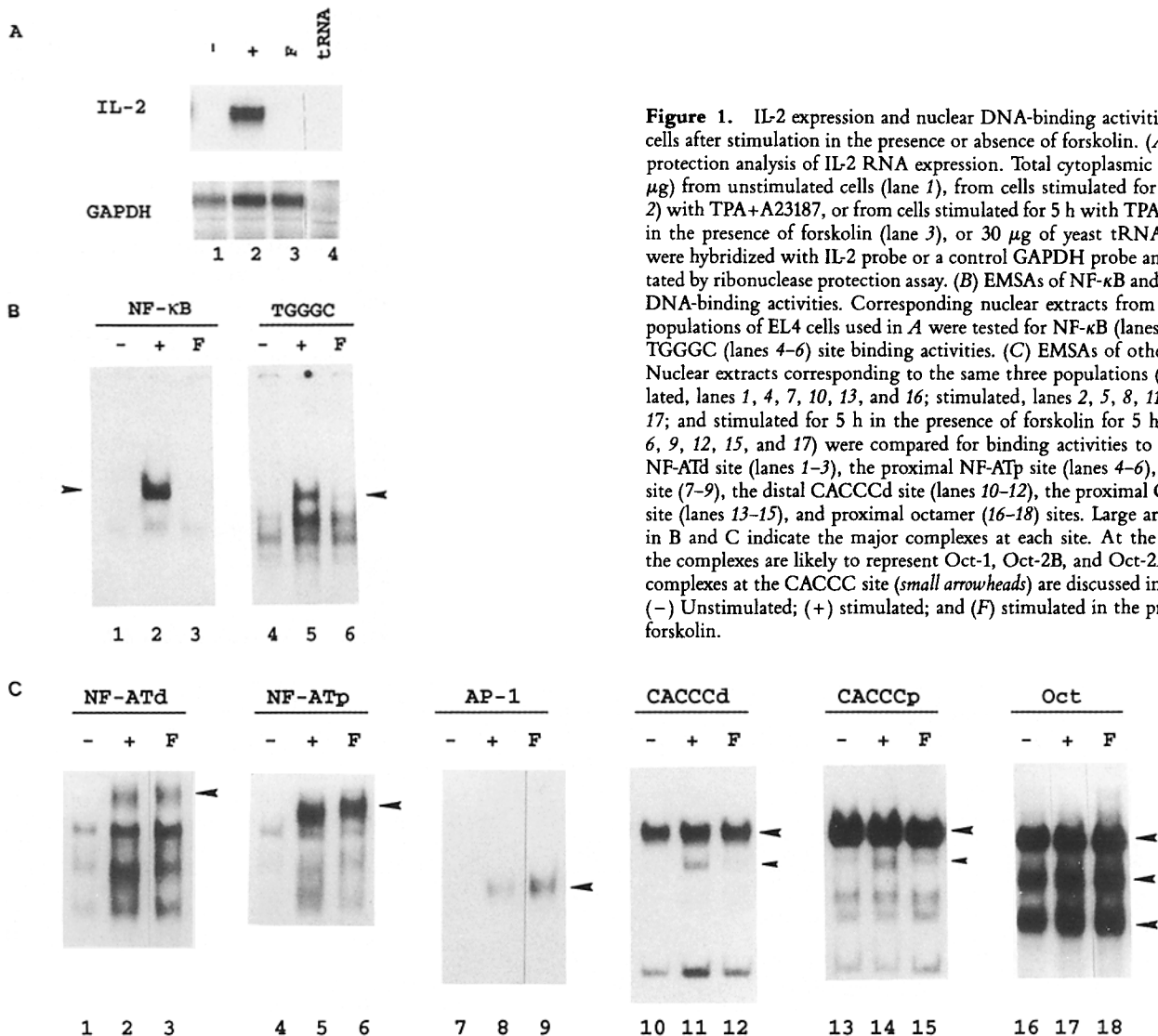

Figure 1. IL-2 expression and nuclear DNA-binding activities in EL4 cells after stimulation in the presence or absence of forskolin. (*A*) RNase protection analysis of IL-2 RNA expression. Total cytoplasmic RNA (10 μg) from unstimulated cells (lane *1*), from cells stimulated for 5 h (lane *2*) with TPA+A23187, or from cells stimulated for 5 h with TPA+A23187 in the presence of forskolin (lane *3*), or 30 μg of yeast tRNA (lane *4*) were hybridized with IL-2 probe or a control GAPDH probe and quantitated by ribonuclease protection assay. (*B*) EMSAs of NF-κB and TGGGC DNA-binding activities. Corresponding nuclear extracts from the same populations of EL4 cells used in *A* were tested for NF-κB (lanes *1–3*) and TGGGC (lanes *4–6*) site binding activities. (*C*) EMSAs of other factors. Nuclear extracts corresponding to the same three populations (unstimulated, lanes *1, 4, 7, 10, 13,* and *16*; stimulated, lanes *2, 5, 8, 11, 14,* and *17*; and stimulated for 5 h in the presence of forskolin for 5 h, lanes *3, 6, 9, 12, 15,* and *17*) were compared for binding activities to the distal NF-ATd site (lanes *1–3*), the proximal NF-ATp site (lanes *4–6*), the AP-1 site (*7–9*), the distal CACCCd site (lanes *10–12*), the proximal CACCCp site (lanes *13–15*), and proximal octamer (*16–18*) sites. Large arrowheads in B and C indicate the major complexes at each site. At the Oct site, the complexes are likely to represent Oct-1, Oct-2B, and Oct-2A. Minor complexes at the CACCC site (*small arrowheads*) are discussed in the text. (−) Unstimulated; (+) stimulated; and (*F*) stimulated in the presence of forskolin.

major binding factors for the CACCC sites (18, 19), and the proximal octamer site, were not affected by forskolin (Fig. 1 C, lanes *10–18, large arrowheads*). Similarly, some induction-dependent activities, such as the major inducible complexes binding the NF-AT and AP-1 sites, were unaffected or even slightly enhanced by stimulation in the presence of forskolin (Fig. 1 C, lanes *1–9, large arrowheads*). These results are in agreement with our previous conclusions from more limited studies (20). However, as shown in Fig. 1 B, binding activities of several other inducible factors were significantly reduced in nuclear extracts from cells that were activated in the presence of forskolin. Most prominent among these were the upper complexes binding to the NF-κB and TGGGC sites (Fig. 1 B, lanes *2, 3* and *5, 6*). In addition, we noted effects of forskolin on minor complexes binding the two CACCC sites, which in both cases were of faster mobility than the major CACCC complex (Fig. 1 C, lanes *11, 12*, and *14, 15, small arrowheads*). The identities of the lower inducible complexes bound to the CACCC sites are not known. They could not be competed by a consensus Sp-1 oligonucleotide as could the major CACCC binding complexes (18, and data not shown). The nature of the TGGGC binding factor is also not known. As it appeared to show a similar electrophoretic mobility to that of the upper NF-κB complex, it was subjected to some further characterization.

Although both NF-κB and TGGGC binding activities were coinhibited by forskolin, the protein components involved were clearly different. This was confirmed by the demonstration, (*a*), that the TGGGC and NF-κB binding sites could not cross-compete in mobility shift assays, and (*b*), by antibody supershift experiments in which anti-p65, anti-c-rel, and anti-p50 antibodies that could supershift or inhibit the NF-κB complexes were all completely unable to affect the TGGGC complexes (data not shown). Furthermore, whereas TNF-α could "rescue" NF-κB binding activity in cells stimulated in the presence of forskolin, there was no rescue of the TGGGC factor (data not shown). These results, and data presented below, show that the regulation of activation of these two binding activities was different.

The presence of forskolin during stimulation appeared to result in a reduction in the total amount of NF-κB complexes per cell, not simply a sequestration of NF-κB activity (26). This was shown both by Western blotting of nuclear and cytoplasmic extracts with antibodies against p65, p50, and c-rel, and by using deoxycholate to dissociate NF-κB/IκB complexes, thus revealing previously masked NF-κB binding activities (27). Stimulation for 4 h led to a sharp increase of p65/p50 and c-rel/p50 complexes in the nucleus, and this increase was profoundly antagonized by the presence of forskolin. However, there was no compensatory increase in NF-κB components or binding activities in the cytoplasm (data not shown). Thus, forskolin may inhibit NF-κB synthesis or promote NF-κB degradation.

Delayed Inhibition of IL-2 Expression Via Delayed Inhibition of NF-κB. The effects of forskolin on NF-κB appear to be paradoxical, for it has been reported that PKA can activate NF-κB, and our own earlier study (20) did not show consistent reduction of NF-κB binding at 2 h of forskolin treatment. To clarify this contradiction, we did kinetic studies to see whether the reduction we observed here is an early event or a possible secondary effect. First, we examined the kinetics of cAMP elevation after forskolin treatment. A significant increase in cAMP levels could be seen 15 minutes after administration of forskolin, reaching a maximum within an hour. High levels of cAMP were then retained for up to 6 h of forskolin treatment (Fig. 2 A). The addition of TPA+A23187 did not affect the initial elevation or the later accumulation of cAMP (Fig. 2 B). Pretreatment of cells for 2 h with TPA+A23187 before addition of forskolin also did not significantly affect the subsequent elevation of cAMP (Fig. 2 C). The high levels of cAMP sustained for many hours made it possible that cAMP-dependent mechanisms could exert effects at late time points, not just immediately after stimulation.

In fact, the effects of forskolin on NF-κB and TGGGC binding factor induction showed strikingly different time courses. When cells were activated in the presence of forskolin, mobilization of the factors binding to the TGGGC
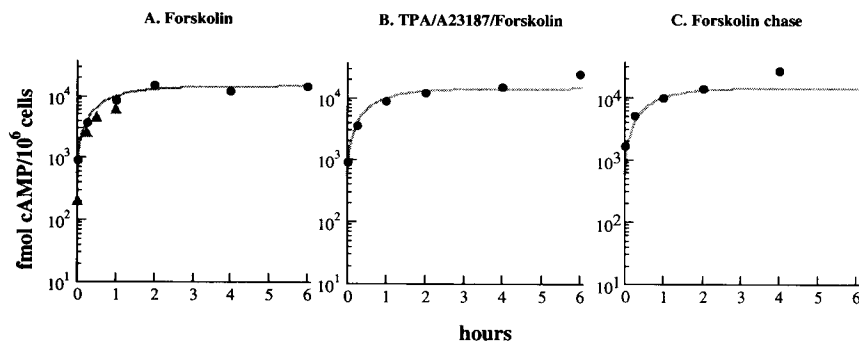

Figure 2. Sustained elevation of cAMP as a function of duration of forskolin treatment. The figure shows cAMP levels in EL4 cells that were cultured in the presence of forskolin alone (*solid and dotted curve, A; solid curve, B and C*); with TPA+A23187+ forskolin added simultaneously (*dots, B*); or with forskolin added (time 0) after 2 h of pretreatment with TPA+A23187 (*dots, C*). Levels of cAMP are also shown for IIA4 transfectants (see Fig. 4) that were cultured with forskolin alone (*triangles, A*). The incubation was stopped at different time points and cAMP was measured in aliquots of 10^5 cells. As a reference for the data in *A–C*, the data from EL4 cells treated with forskolin alone (*A*) were used

to calculate the solid curve shown in all three panels. Individual data points were plotted as triangles or dots to compare with the solid curve. This comparison emphasizes the similarity in the kinetics of cAMP accumulations with or without stimulation. The experimental data for forskolin-treated EL4 cells (*solid curve*) are well-represented by the equation $C_{(t)} = K_s/K_d - (K_s/K_d - C_0)\exp(-K_d t)$, where $C_{(t)}$ is the amount (fmol) of cAMP/10^6 cells at time t (h). C_0 (=568 fmol/10^6 cells) is the amount of cAMP at time 0, K_s (=1.6 × 10^4 fmol/10^6 cells h) is the calculated cAMP synthesis rate constant in the presence of forskolin. K_d (=1.14 h^{-1}) is the cAMP decay rate constant, from which we compute $t_{1/2}$ = 0.79 h.
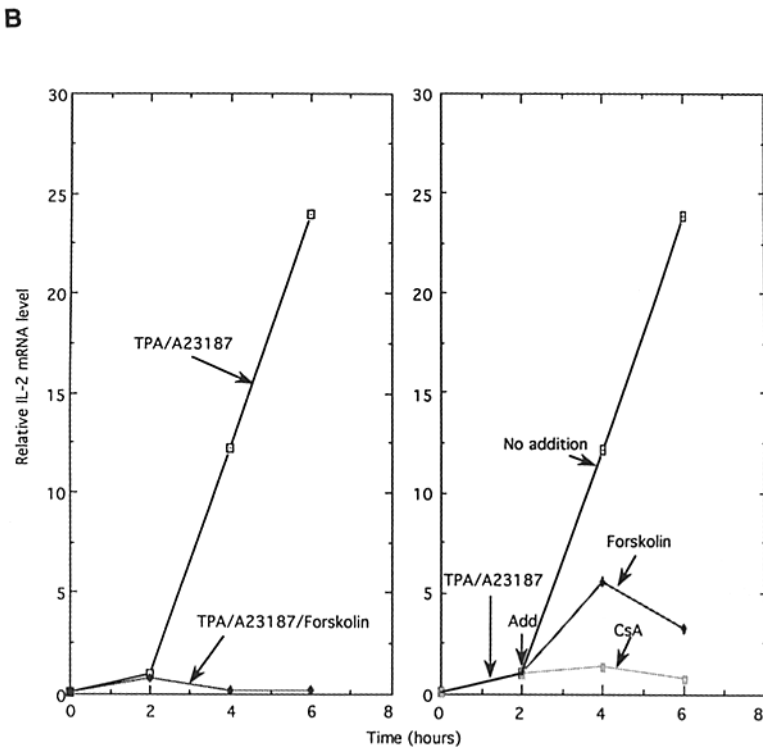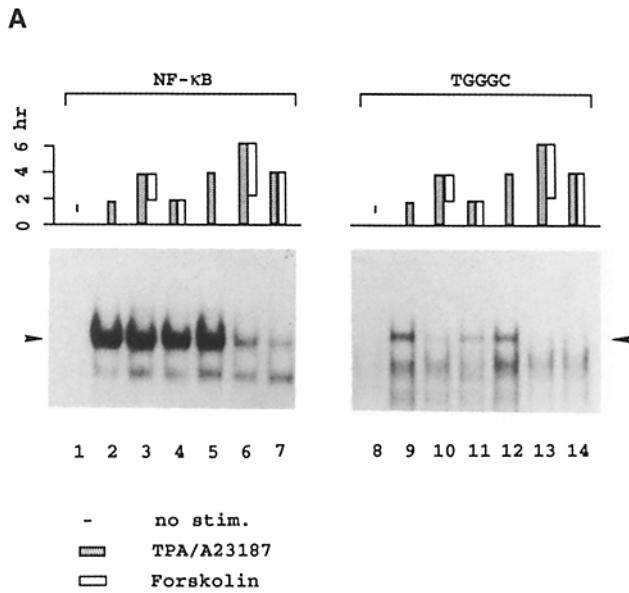
A

- no stim.
▦ TPA/A23187
▱ Forskolin

B

Figure 3. Delayed inhibition of IL-2 RNA accumulation correlated with delayed inhibition of NF-κB binding activity. (*A*) NF-κB and TGGGC site binding activities were measured at different intervals of stimulation (▦), in the presence (▱), or absence of forskolin, as indicated in the bar graph over each lane. Note that it took 4 h of forskolin treatment to reduce NF-κB binding activity, whether forskolin addition was delayed or not (lanes 6 and 7), but only 2 h to reduce TGGGC binding activity significantly (lanes 10 and 11). (*B*) IL-2 mRNA levels at various times of stimulation were measured by RNase protection analysis. Data were collected from two independent experiments. (*Left*) Cells stimulated for the indicated lengths of time in the absence (□) or presence (◆) of forskolin from time 0. (*Right*) Forskolin and CsA chase experiments. All samples were stimulated from time 0. Forskolin (◆) or CsA (▨) was added to the culture 2 h after initiating the stimulation with TPA+A23187.

site was inhibited both at 2 and at 4 h of treatment (Fig. 3 *A*, compare lanes *9* and *11*, *12*, and *14*), indicating that this inhibition is an early event. However, when analyzed after 2 h of stimulation, NF-κB binding activities appeared to be induced equally well whether forskolin was present or not (Fig. 3 *A*, compare lanes *2* and *4*). Only after at least 4 h of stimulation in the presence of forskolin did we see the characteristic decrease of the NF-κB site binding activity (Fig. 3 *A*, compare lanes *5* and *7*). These findings indicated that elevation of cAMP does not inhibit the initial dissociation of NF-κB from IκB or its subsequent nuclear localiza-

tion. They also suggested either that the effects of forskolin on NF-κB were intrinsically subject to delay, or that NF-κB proteins only became susceptible to forskolin-dependent inhibition at some point at least 2 h after initial exposure to TPA+A23187. To test these possibilities, we first stimulated the cells with TPA and A23187 for 2 h, to make all the necessary *trans*-acting factors available and initiate transcription of IL-2 RNA, then we introduced 10 μM forskolin to the culture and continued the culture for another 2 or 4 h. As shown in Fig. 3 *A* (lanes *3* and *6*), forskolin "chasing" for 2 h still had little effect on NF-κB binding, but chasing for 4 h greatly

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reduced the binding. These results suggested that PKA activation had an intrinsically delayed effect on NF-κB binding activity.

The delayed and asynchronous effects of cAMP elevation on the NF-κB and TGGGC site binding factors were associated with delayed inhibition of IL-2 mRNA expression, as shown in Fig. 3 B. IL-2 mRNA accumulation in the stimulated control cells followed biphasic kinetics with a relatively low rate of accumulation in the first 2 h, which then shifted to a higher rate that was sustained for the next 4 h. When forskolin was present at the start of stimulation, IL-2 mRNA initially accumulated almost as fast as in the control cells without forskolin, but then declined to baseline between 2 and 4 h of stimulation (Fig. 3 B, left). When the addition of forskolin was delayed until after 2 h of stimulation, the rate of IL-2 mRNA accumulation was significantly reduced as compared with the control samples without forskolin, and inhibitory effects were evident within the first 2 h of forskolin chasing (Fig. 3 B, right). However, this inhibition was slow as compared with the immediate shut-off of IL-2 mRNA accumulation when CsA, instead of forskolin, was added after 2 h of stimulation (Fig. 3 B, right). Thus, some early effects of forskolin which inhibit IL-2 activation may be exerted before its effects on NF-κB, suggesting that a lack of factors other than NF-κB, including the TGGGC binding factor, may be sufficient to hinder the transcription but not to block it fully. The reduced NF-κB binding activity seen at relatively later time points would then be associated with the complete arrest of IL-2 mRNA accumulation.

Effects of Forskolin Are Mediated by PKA. Forskolin elevates intracellular cAMP by directly activating adenylate cyclase, and cAMP in turn activates cAMP-dependent protein kinases (PKA). To ascertain whether all the effects of forskolin depend on the activation of PKA, we introduced a dominant negative mutant PKA regulatory subunit cDNA construct, driven by the Harvey sarcoma virus LTR, into EL4 cells (28). The product encoded by this construct has two point mutations at cAMP-binding sites which block its binding to cAMP but still allow it to associate with the catalytic subunits in competition with endogenous regulatory subunits. Once bound, the mutant regulatory subunit will not dissociate from the catalytic subunit in response to cAMP and therefore acts as a dominant negative regulator of PKA activity. One stable transfectant line showing high expression of the mutant subunit, IIA4, and two pSV2neo transfectant control lines, were tested to compare their forskolin responses. As shown in Fig. 2 A (triangles), the introduction of the mutant subunit had no effect on the ability of the cells to elevate their cAMP levels in response to forskolin. However, the mutant subunit completely protected the induction of IL-2 mRNA from forskolin inhibition in the IIA4 line, in contrast to the significant inhibition of IL-2 expression by forskolin in control lines as measured 4 h after stimulation (Fig. 4 A). In agreement with its effects on IL-2 mRNA expression, expression of the dominant negative mutant PKA also relieved the inhibitory effects of forskolin on NF-κB and TGGGC binding activity (Fig. 4 B). The NF-κB and TGGGC
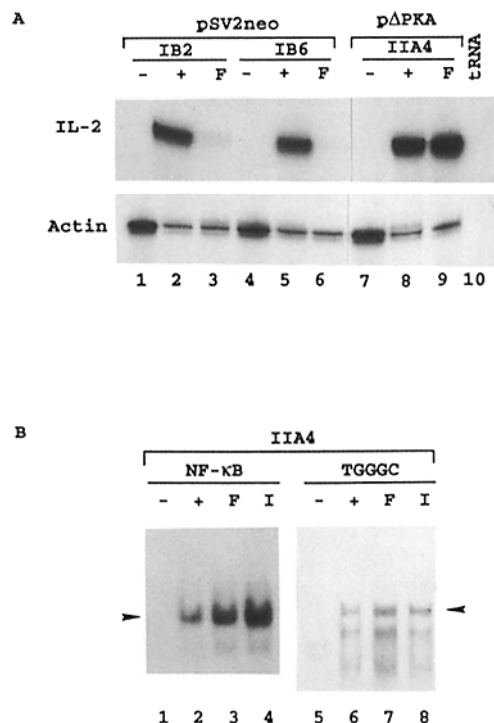

Figure 4. Dominant negative mutant PKA transfected cells are resistant to the forskolin inhibitory effect. (A) Levels of IL-2 mRNA induced in two control lines of pSV2neo transfectants, IB2 and IB6, and in one line of mutant PKA transfectants, IIA4, were compared by RNase protection analysis with 10 μg of RNA from uninduced cells (−), or cells after 4-h stimulations (+) in the absence or presence (F) of forskolin. 1 μg of RNA from each sample was hybridized with actin probe to verify comparability and integrity of the RNA. (B) Nuclear extracts from IIA4 cells were tested for NF-κB and TGGGC binding activities. All stimulations were for 4 h. (−) Uninduced; (+) induced with TPA and A23187; (F) induced with TPA and A23187 in the presence of forskolin; (I) induced with TPA and A23187 in the presence of 50 U/ml IL-1.

binding complexes, and the two lower inducible CACCC binding complexes, were fully inducible in the mutant PKA-containing cell line IIA4 whether forskolin was added or not (compare Figs. 4 B and 1 B; and data not shown). The binding activities of other factors in forskolin-treated IIA4 cells remained the same as in cells stimulated without forskolin (data not shown). The mutant cells were not simply refractory to modulation of NF-κB activity, for they remained capable of upregulating their NF-κB site binding activity upon stimulation in the presence of 50 U/ml IL-1 (Fig. 4 B, lane 4) (20). Therefore, it is clear that PKA is a mediator of the inhibitory effects of forskolin on DNA-binding activities.

Forskolin Inhibits Stable In Vivo Assembly of the IL-2 DNA–Protein Complex. The highly selective effects of forskolin on particular DNA-binding activities raised the question of whether the binding activities that remain are capable of forming stable contacts with IL-2 regulatory DNA in the nuclei of forskolin-treated, stimulated cells. To resolve this issue, living cells were treated with dimethyl sulfate (DMS), which methylates G residues (met-G) in the major groove.

Intimate protein contacts with these residues can either protect them from methylation or make them hypersensitive to methylation. The subsequent piperidine cleavage of the extracted DNA at met-Gs followed by ligation-mediated PCR amplification generates a G-specific sequence ladder. In fact, this technique has revealed specific major groove contacts at most or all of the sites known to be required for IL-2 expression (19). In agreement with the results we have shown previously (19), there is no obvious difference in the G ladder pattern between the DNA from DMS-treated, uninduced cells and naked control DNA which was DMS treated after purification (Fig. 5 A, lanes 1, 2, and 5). However, multiple footprints spanning almost the entire 300-bp upstream sequence were found in DNA from cells stimulated for 4 h with TPA+A23187 in the absence of forskolin, which corresponded to the well-characterized cis elements noted previously (Fig. 5, compare lanes 2 and 3 in A and lanes 1 and 2 in B). Whereas these DMS footprinting results do not rule out some form of protein–DNA associations at the IL-2 locus in uninduced cells, such associations clearly do not include the diagnostic, specific contacts that are formed at regulatory sites in induced cells. As noted above, these specific sites of occupancy define the oligonucleotide probes used in Fig. 1. Each site was revealed in vivo by protection (O►), hypersensitivity (➤), or a characteristic pattern of both; examples are shown in Fig. 5 C. As previously discussed (19), G residues in the footprint are never completely protected from cleavage, and this effect is exacerbated by the asynchrony of IL-2 transcription in the stimulated population, in contrast with the more complete protection seen in the regulatory sequence of a constitutively expressed gene such as metallothionein-I (19, and data not shown). However, the pattern of protections and hypersensitivities at the IL-2 locus in stimulated cells was still highly reproducible.

When examining the DNA from cells induced for 4 h in the presence of forskolin, pronounced differences from the characteristic induced pattern were noted. First, at almost every cis element, signs of occupancy were absent (Fig. 5, A, lane 4 and B, lane 3). Not only were the NF-κB and TGGGC sites empty, but the two NF-AT sites (Fig. 5 C), the AP-1 site and the proximal CACCC site also remained unoccupied, even though the factors binding these latter sites were present in the nucleus and were able to bind to their sites individually as shown by in vitro mobility shift studies (see above, Fig. 1). The global blockade of stable footprint formation is reminiscent of the CsA effect, which tightly shuts off IL-2 transcription and completely eliminates all evidence for protein–DNA interaction in footprinting analysis (19 and see below). Only one sign of protein–DNA interaction was consistently seen in the cells stimulated in the presence of forskolin: a persistent hypersensitivity at the 5'-most G in the distal CACCC element. The significance of this isolated feature, at the border of the known IL-2 enhancer, is unknown.

The elimination of protein–DNA contacts in cells that had been stimulated in the presence of forskolin was dependent on PKA activity, as shown with DNA samples from mutant PKA transfectants. Like the parental cells, IIA4 cells established a full range of protein–DNA contacts in response to TPA+A23187 (Fig. 5, A, lane 6; B, lane 5). In contrast to the wild-type parental EL4 cells, treatment of IIA4 cells with forskolin did not affect any of the TPA+A23187-induced protections and hypersensitivities on the DNA sequence ladder (Fig. 5, compare lanes 4 and 7 in A and lanes 3 and 6 in B). Thus the disruption of protein–DNA contacts at the IL-2 locus was a specific effect of PKA activation.

The effect of cAMP elevation on protein–DNA contacts differed from that of CsA, described previously (19), in that the initial establishment of footprints proceeded normally in the presence of TPA+A23187+forskolin, for example as measured after 2 h of stimulation (data not shown). The absence of footprints at the later time points thus reflected the net disassembly of preexisting complexes. To confirm the ability of forskolin treatment to block continuation of occupancy, we examined DNA samples from two independent forskolin chasing experiments (Fig. 6). In agreement with the binding studies and RNA analysis, the results showed that 2 h of forskolin chasing after 2 h of stimulation did not change the preexisting footprints formed after 2-h stimulation (Fig. 6, lane 3). By contrast, the same 2-h chase with CsA completely reversed the stimulated footprint pattern to an "uninduced" one (Fig. 6, lane 4). When forskolin chasing was extended to 4 h, however, a gradual weakening of protein–DNA interactions was seen (Fig. 6, lane 9). Notably, footprints weakened at all sites concomitantly, with the unstimulated pattern reemerging as quickly at the NF-AT site as at the NF-κB site. Thus, even preformed IL-2 DNA–protein complexes are not stable in vivo in activated cells without the continued contribution of forskolin-sensitive components.

Discussion

Agonists that elevate intracellular cAMP are likely modulators of immune responses in vivo (6–10). We and others have previously shown that they selectively interfere with IL-2 expression. Here we also report that the inhibition can follow a distinctively delayed time course, allowing an initial limited burst of IL-2 expression before transcription ceases. These qualitative and kinetic effects on cytokine gene expression resulting from the elevation of cAMP could be used in vivo to play a role in the shaping of a Th2 vs. Th1 response.

In this work, we present molecular evidence for the way elevation of cAMP downregulates expression of the IL-2 gene in a model cell line. At least two transcription factors associated with IL-2 activation, including NF-κB and the newly described TGGGC-binding factor, were specifically reduced in nuclear extracts from cells that were activated in the presence of forskolin. This pattern of transcription factor inhibition differed markedly from the patterns of inhibition by CsA treatment or anergy induction, in that neither NF-AT nor AP-1 binding activities were detectably reduced. With a closer look at protein–DNA interactions by in vivo footprinting, we discovered that the loss of the NF-κB and TGGGC binding activities was correlated with the disappearance of stable pro-
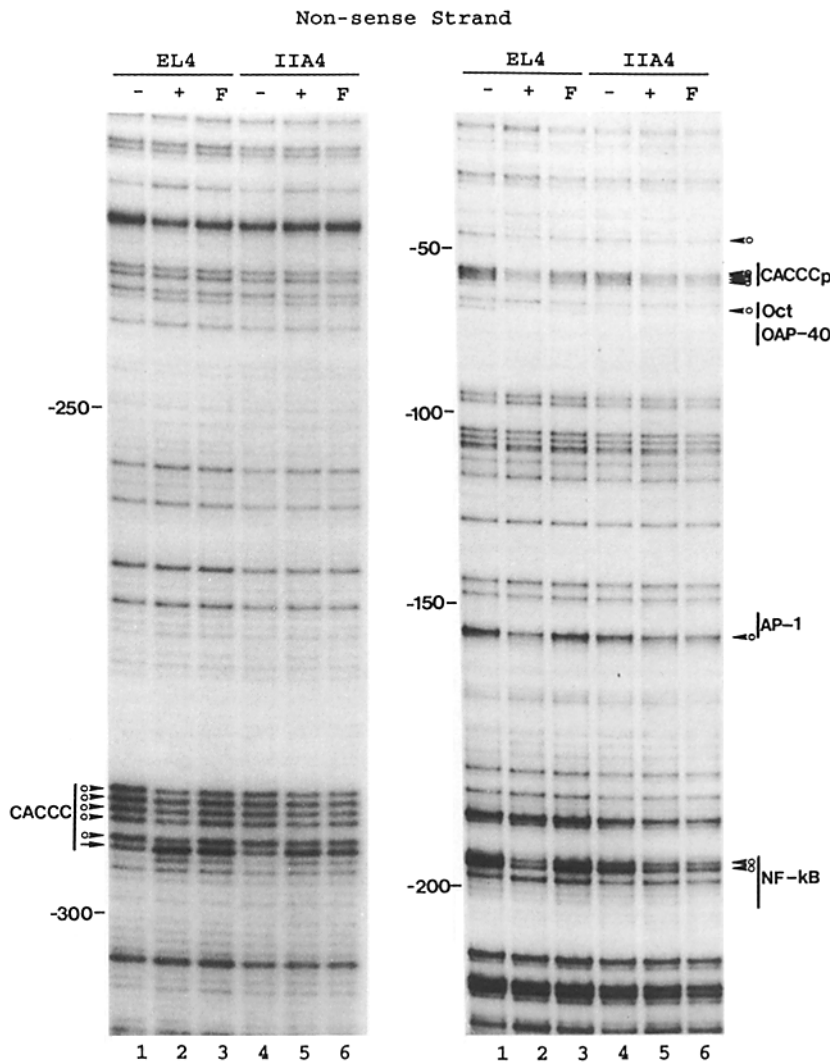
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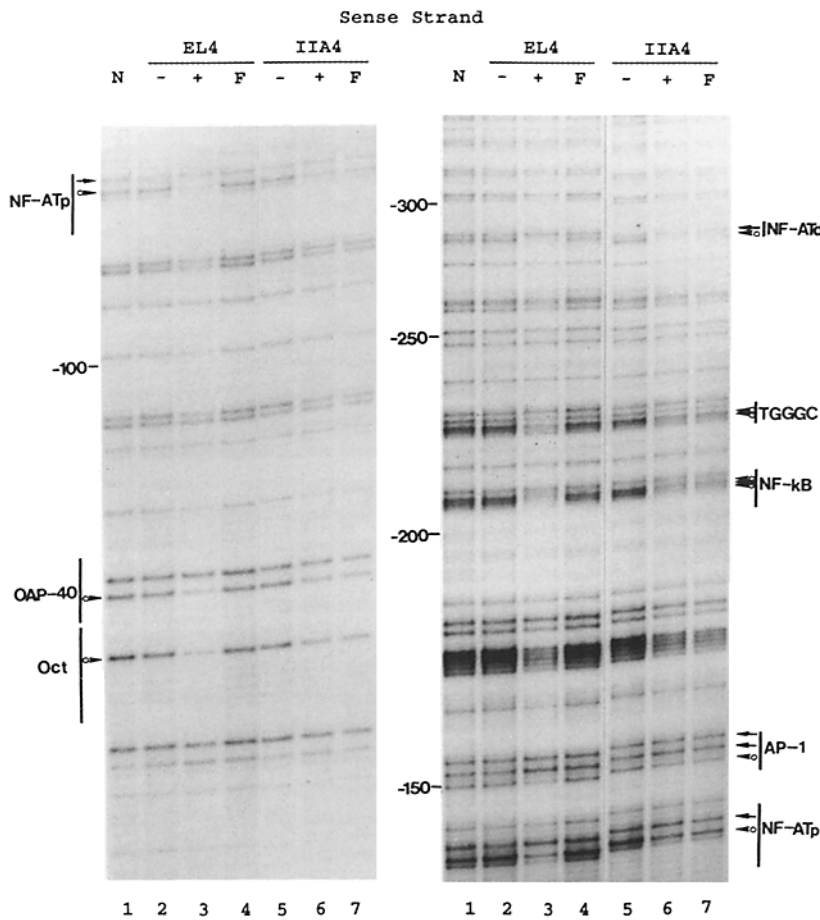
tein–DNA contacts at virtually all detectable sites in the IL-2 enhancer. Thus, cAMP-sensitive transcription factors appear to be as necessary as the CsA-sensitive factors described previously (19) for the maintenance of transcription factor complexes at the IL-2 locus.

Mechanism of Forskolin Effects on NF-κB. All the inhibitory effects of forskolin treatment studied here were executed by cAMP-dependent protein kinases, since cells expressing a dominant negative PKA mutant were completely resistant to forskolin effects. These cells preserved a high level of IL-2 mRNA accumulation, full NF-κB and TGGGC binding activities in their nuclear extracts, and persistent high affinity protein–DNA interactions at the IL-2 locus even in the presence of forskolin. In some cellular contexts, NF-κB can be activated by PKA (26, 29–31), but in stimulated EL4 cells, the net role of PKA is to inhibit NF-κB availability. Because it was not a complete failure of NF-κB induction but rather an inability to sustain NF-κB activation that was observed, it remains possible that early PKA activation might initially synergize with PKC to activate NF-κB through immediate IκB phosphorylation (27, 29–31). However, recent work has described additional pathways for PKA that provide a basis

for delayed effects. It was reported that it takes 30 min for the catalytic subunit of PKA to translocate to the nucleus, and phosphorylation of the cAMP response factor CREB and CREB-dependent gene activation occur only afterwards (32). Thus a delayed effect of cAMP elevation on NF-κB levels would be expected if the inhibition were dependent on de novo transcription of some target gene. Preliminary experiments did not reveal qualitative differences between the types of NF-κB/c-Rel complexes induced at 2 h, at 4 h, and at 2 h in the presence of forskolin (data not shown). However, any sustained NF-κB activation, as observed in stimulated EL4 cells, requires both new synthesis and ongoing nuclear translocation of NF-κB molecules (33). Forskolin could interfere with this by (*a*) causing direct cAMP-dependent repression of ongoing NF-κB subunit transcription; (*b*) causing cAMP-dependent overexpression of an appropriate form of IκB; or (*c*) directly or indirectly stimulating NF-κB proteolysis. The degree to which each of these mechanisms contributes to the lagged inhibition of NF-κB binding activity remains to be determined.

How Are Reduced NF-κB and TGGGC Binding Activities Related to the Inhibition of IL-2 Gene Expression? In our
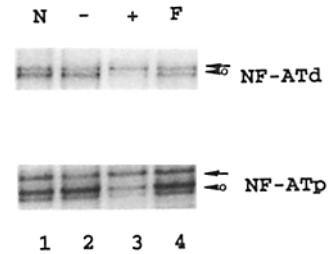
B

Non-sense Strand

EL4   IIA4   EL4   IIA4
- + F - + F   - + F - + F

-250-   -50-   ◄o
                                          ◄◄|CACCCp
                              -100-   ◄o|Oct
                                           |OAP-40

                              -150- |AP-1
                                          ◄o

CACCC   ◄8|NF-kB
                              -200-

-300-

1 2 3 4 5 6   1 2 3 4 5 6

C

N - + F

◄o NF-ATd

◄ NF-ATp
◄o

1   2   3   4

Figure 5. Forskolin affects the formation of specific DNA binding complexes at multiple sites of the IL-2 promoter/enhancer region. In vivo genomic footprinting on IL-2 300-bp promoter/enhancer regions was performed with DNA samples from EL4 (lanes 2-4, A and lanes 1-3, B) and IIA4 cells (lanes 5-7, A and lanes 4-6, B) cultured under different conditions. Resulting ladders for the coding strand are shown in A, and for the noncoding strand in B. (C) A close view of the effects of forskolin treatment on footprints at the distal and proximal NF-AT sites. (A and B) Specific sites and the distance relative to the transcription start site are as indicated. (N) Naked DNA sample from EL4 cells; (−) unstimulated cells; (+) induced with TPA+A23187 for 4 h; (F) induced with TPA+A23187+forskolin for 4 h. Comparing lanes between the uninduced and induced samples, multiple footprints throughout the whole 300-bp region are clearly observed, consisting of reproducible partial protections (O►) and hypersensitivities (→►). In the forskolin-treated EL4 lane, the sequence ladder generally resembles the uninduced one, with the exception of the hypersensitive G in the distal CACCC site (nonsense strand), as noted in the text. However, as seen in lanes 5-7 for both strands, forskolin did not affect the footprint formation in IIA4 cells. This subline exhibited relatively weak footprints in general, but there was no significant difference between the footprints in induced samples of the mutant cells whether forskolin was present or not.

previous studies (19), we described three extreme situations: when the IL-2 gene was silent, fully activated, or suppressed by CsA. Each condition was associated with the availability of a different spectrum of IL-2 DNA binding proteins as assayed in nuclear extracts. However, in vivo footprints revealed a simpler dichotomy, with either no detectable occupancy or a fully occupied footprinting pattern (Fig. 7). This indicated that in vivo, transcription factors interact with IL-2 regulatory DNA in a highly coordinated, all-or-none fashion. The effects of forskolin treatment on in vivo footprints at the IL-2 locus enhance the previous conclusion in three ways.

First, the key finding is that exposure to forskolin during stimulation blocks the formation of stable footprints at virtually all the sites of protein–DNA contact that we detect in the IL-2 enhancer. In nuclear extracts from forskolin-treated cells, almost all the major binding activities for these sites appeared to be available at normal levels; only two major com-

plexes, the upper NF-κB and TGGGC complexes, were affected. Yet in the nuclei of living, forskolin-treated cells, none of the binding activities could in fact engage their target sites in the context of the full IL-2 enhancer (Fig. 7). In vivo, the binding of forskolin-insensitive factors like NF-AT was therefore contingent upon the binding of forskolin-sensitive factors like NF-κB. This finding parallels the effect of CsA on the establishment of stable contacts (19, and Fig. 6). However, as summarized in Fig. 7, a different subset of factors is prominently affected by each inhibitor. The effect of CsA treatment, considered alone, made it possible to speculate that a single CsA-sensitive factor, like NF-AT, might play a unique role in opening the IL-2 chromatin structure for transcription. However, considered in light of the similar effects of forskolin treatment, such a model is much less likely. A probable inference from the results summarized in Fig. 7 is that the assembly of stable transcription factor complexes at the
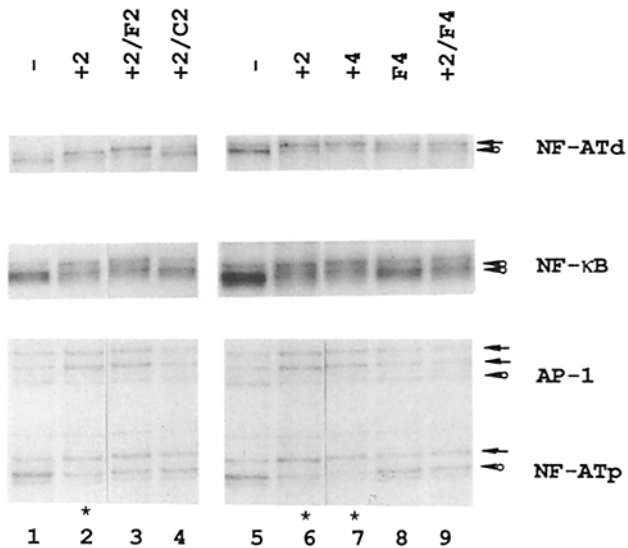
Figure 6. Forskolin has delayed effects on the stability of preformed protein–DNA complexes. In vivo footprints at three regions of the IL-2 regulatory sequence (*sense* strand) are shown. The data in lanes *1–4* and in lanes *5–9* are from two independent experiments. Lanes *1* and *5* contain DNA from uninduced cells. Lanes *2* and *6* contain DNA from cells induced with TPA+A23187 for 2 h. In lanes *3* and *4*, cells were stimulated for 2 h, and then forskolin (lane *3*) or CsA (lane *4*) were added for another 2 h. In lane *3*, the inducible footprints seen in lane *2* remain identifiable. However, in lane *4*, those inducible footprints seen in lanes *2* and *3* disappear and the overall sequence ladder approaches the uninduced state seen in lane *1*. In lane *7*, cells were stimulated for 4 h without inhibitor. In lane *8*, cells were stimulated for 4 h with forskolin added from time 0. In lane *9*, cells were prestimulated for 2 h, then incubated with forskolin for another 4 h. Stimulated samples without inhibitors (lanes *2*, *6*, and *7*) are indicated with asterisks.

IL-2 locus requires multivalent protein–protein interactions as well as the protein–DNA interactions at the individual sites. Thus, a major component of the combinatorial action of these transcription factors may be the cooperativity required for their binding.

Second, a further clue to support the need for cooperative binding in IL-2 regulation may be provided by the persistence of a sole residual site of activation-associated protein–DNA contact in nuclei of forskolin-treated cells, long after other footprints have disappeared. The transcription factor or nucleosomal structure responsible for this feature is unknown. The isolated DMS hypersensitivity at the distal CACCC site is a structure that is not only distinguished from the resting state and the fully activated state of the IL-2 locus, but also distinguished from the state of the IL-2 locus in cells activated in the presence of CsA. The equivalence of the CsA-

treated pattern with the resting (uninduced) pattern had not allowed us to distinguish whether limited chromatin accessibility or a requirement for cooperative binding actually restricted occupancy. However, it is less likely that "accessibility" is limiting after PKA activation, since residual CACCC occupancy is still permitted in forskolin-treated cells.

Finally, the effect of forskolin differs from that of CsA (19) in that the presence of forskolin does not block transcription factor binding initially. In fact, the time course of its effect on in vivo protein–DNA contacts is delayed in parallel with the effect on NF-κB availability. This indicates that a key role is played by NF-κB or by a factor(s) inhibited with a similar time course. The transient assembly and disassembly of complexes in forskolin-treated cells suggests that "stable" complex formation could be a misnomer in general. Instead, the accessibility of the IL-2 gene to sharply discontinuous regulation may result from rapid cycling of transcription factors between disassembled and reassembled states. The rate of transcriptional initiation, by this model, would depend on the rate with which the most limiting component could be engaged. Such a model would explain how forskolin could first inhibit IL-2 expression to a modest degree, through reduction in the TGGGC factor, and then more severely as NF-κB complexes also became rare. Further support for this interpretation is provided by the results of the experiments in which cells were stimulated normally, then subjected to a inhibitor chase. Forskolin and CsA work through completely different mechanisms, resulting in blocking the availability of different subsets of factors, yet both can shift the equilibrium to dissociation of active IL-2 transcription complexes.

This study adds a third set of transcription factors to those already known to be critical for physiological regulation of IL-2. As discussed in the introduction, inhibition of NF-AT and inhibition of AP-1 separately can block IL-2 expression. Neither of these factors is detectably inhibited by forskolin. Our results suggest a similar need for NF-κB, the TGGGC factors, and/or other factors that are sensitive to PKA activation. In vivo footprinting data are not yet available for any of the cases where AP-1 is selectively inhibited. However, generalizing from the effects of forskolin and the effects of CsA, we could predict that selective AP-1 inactivation would also destabilize the whole protein–DNA assembly at the IL-2 enhancer. This would confirm a nonhierarchical requirement for the various IL-2 regulatory factors. Such a mechanism vividly illustrates how, as a cell-specific and activation-dependent response, IL-2 expression is rendered vulnerable to multiple signaling events.

	CACCCd	NF-ATd	TGGGC	NF-κB	AP-1	NF-ATp	OAP40	Oct	CACCCp	Footprints
Unstim.	+	−	−	−	−	−	−	+	+	None
Stim.	+	+	+	+	+	+	+	+	+	All
+CsA	+	−	±	±	+	−	−	+	+	None
+FKL	+	+	−	−	+	+	(+)	+	+	None

Figure 7. Summary of the availability and in vivo binding to the IL-2 enhancer of different transcription factors, as measured by gel mobility shift assays and in vivo footprinting in EL4 cells under different stimulation conditions. (*Stim.*) TPA+A23187; (*+CsA*) TPA+A23187+CsA; (*+FKL*) TPA+A23187+forskolin. The pattern shown represents results obtained when stimulation, in the presence or absence of the indicated agents, is carried out for at least 4 h.

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