stantially decrease. HeLa cultures were metabolically labeled in the absence or presence of DEX for several hours. The labeling medium was then replaced with chase medium containing DEX and TNF-α. Cultures were harvested and extracts were immunoprecipitated with the p65-specific antibody. Under these conditions, the IκBα signal rapidly disappeared (Fig. 4B). Thus, DEX does not appear to alter the signal transduction pathway leading to the induced dissociation of NF-κB and IκBα.

Together, these data indicate that DEX treatment induces the transcription of the IκBα gene. This induction results in the increased synthesis of IκBα protein. This increase in protein synthesis leads to the rapid turnover of IκBα protein associated with preexisting NF-κB complexes. In the presence of an activator such as TNF-α, newly released NF-κB reassociates with the DEX-induced IκBα and thus reduces the amount of NF-κB translocating to the nucleus. Additionally, newly synthesized IκBα may enter the nucleus and inhibit NF-κB DNA binding (*15*). A model of this process is shown in Fig. 5. Consistent with this model, we show that the GC-mediated inhibition of NF-κB induction by means of TNF-α is blocked by CHX (Fig. 1). Previously we and others showed that activated GR could physically associate with NF-κB subunits and that DEX represses the DNA binding activity of nuclear NF-κB (*5*, *6*). Here we demonstrate a second independent mechanism through which the NF-κB and GC signal transduction systems interact. As NF-κB is a critical regulator of cytokine genes, the inhibition of the activity of this transcription factor would effectively block cytokine secretion, thus explaining an immunosuppressive function of GCs. It has also been reported recently that salicylates, at concentrations corresponding to doses prescribed for arthritis patients, also block NF-κB activity (*16*). Thus, NF-κB activation serves as a target for two distinct immunosuppressive therapies. The presence of multiple levels of interaction between the NF-κB and GC systems suggests that these interactions may have evolved to serve a physiological role in the development of the immune system and in modulation of the immune response.

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Immunosuppression by Glucocorticoids: Inhibition of NF-κB Activity Through Induction of IκB Synthesis

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Glucocorticoids are among the most potent anti-inflammatory and immunosuppressive agents. They inhibit synthesis of almost all known cytokines and of several cell surface molecules required for immune function, but the mechanism underlying this activity has been unclear. Here it is shown that glucocorticoids are potent inhibitors of nuclear factor kappa B (NF-κB) activation in mice and cultured cells. This inhibition is mediated by induction of the IκBα inhibitory protein, which traps activated NF-κB in inactive cytoplasmic complexes. Because NF-κB activates many immunoregulatory genes in response to pro-inflammatory stimuli, the inhibition of its activity can be a major component of the anti-inflammatory activity of glucocorticoids.

Glucocorticoids (GCs) are physiological inhibitors of inflammatory responses and are widely used as immunosuppressive and anti-inflammatory agents (*1*). Interference with GC action or synthesis increases animal mortality after challenge with bacterial superantigens (*2*). GCs induce lymphocyte apoptosis (*1*, *3*) and inhibit synthesis of lymphokines (*4*) and cell surface molecules required for immune functions (*5*). In spite of the widespread use of GCs, the molecular mechanisms that underlie their therapeutic effects are poorly understood (*1*). GCs induce target genes through the glucocorticoid receptor (GR), a ligand-activated transcription factor (*6*). GCs repress gene expression through transcriptional interfer-

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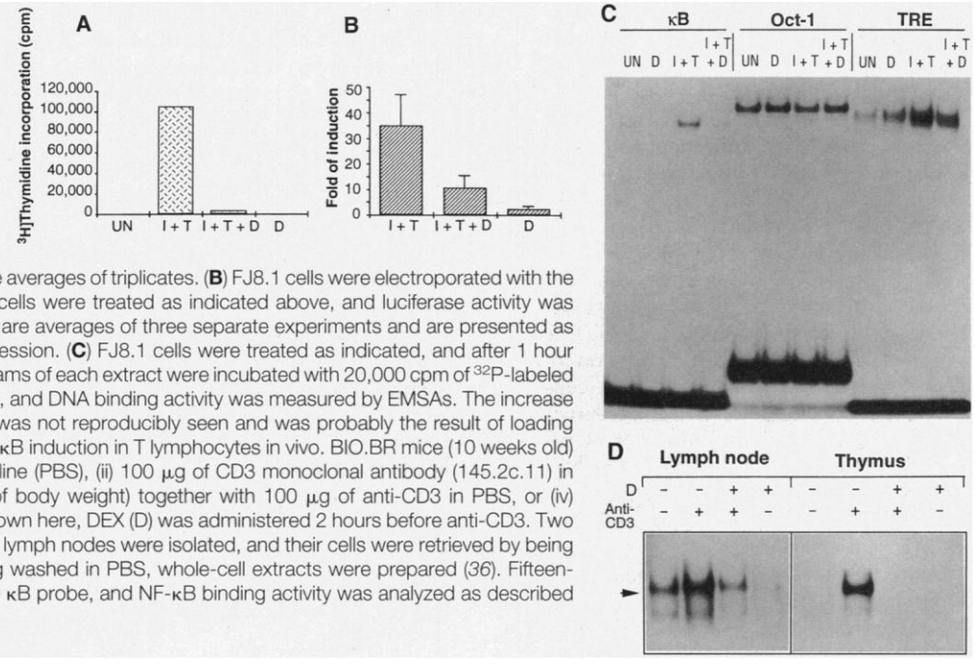

Fig. 1. Inhibition of IL-2 promoter activation by DEX correlates with decreased DNA binding of both AP-1 and NF-κB. (**A**) FJ8.1 cells (*16*) were seeded into microtiter plates (2×10^4 cells per well) and incubated for 24 hours with either medium alone (UN) or with 4 μM ionomycin (I) and TPA (T) (100 ng/ml) in the absence or presence of 1 μM DEX (D). Culture supernatants were harvested, and their IL-2 content was measured through its mitogenic effect on the T cell line HT-2 (*16*). The results shown are averages of triplicates. (**B**) FJ8.1 cells were electroporated with the IL-2–LUC plasmid (*17, 18*). After 12 hours, cells were treated as indicated above, and luciferase activity was determined 7 hours later. The results shown are averages of three separate experiments and are presented as fold of induction above basal IL-2–LUC expression. (**C**) FJ8.1 cells were treated as indicated, and after 1 hour nuclear extracts were prepared. Five micrograms of each extract were incubated with 20,000 cpm of ^{32}P-labeled κB, Oct-1, or AP-1 (TRE) binding site probes, and DNA binding activity was measured by EMSAs. The increase in AP-1 binding activity in response to DEX was not reproducibly seen and was probably the result of loading more protein in that lane. (**D**) Inhibition of NF-κB induction in T lymphocytes in vivo. BIO.BR mice (10 weeks old) were injected with (i) phosphate-buffered saline (PBS), (ii) 100 μg of CD3 monoclonal antibody (145.2c.11) in PBS, (iii) DEX-acetate (1 mg per kilogram of body weight) together with 100 μg of anti-CD3 in PBS, or (iv) DEX-acetate (1 mg/kg). In the experiment shown here, DEX (D) was administered 2 hours before anti-CD3. Two hours after anti-CD3 injection, thymuses and lymph nodes were isolated, and their cells were retrieved by being squeezed through a nylon mesh. After being washed in PBS, whole-cell extracts were prepared (*36*). Fifteen-microgram samples were incubated with the κB probe, and NF-κB binding activity was analyzed as described above.

ence between the activated GR and other transcription factors, most notably AP-1, by a mechanism likely to involve protein-protein interactions (*7, 8*). Although AP-1 is involved in lymphokine gene induction (*9*), interference with AP-1 activity cannot account for the full spectrum of immunoregulatory genes affected by GCs. Another transcription factor that activates immunoregulatory genes is NF-κB, whose predominant form is a heterodimer composed of p50 and p65 subunits (*10*). In unstimulated cells, the NF-κB heterodimer is kept as an inactive cytoplasmic complex by inhibitory proteins, such as IκBα and IκBβ (*11*). After cell stimulation, the IκBs are rapidly degraded (*12, 13*) and free NF-κB dimers translocate to the nucleus and activate target genes (*10, 14*). This process is transient and is terminated through delayed NF-κB–mediated IκBα induction (*12, 14*). Because of its role in the activation of lymphokine genes and other immunoregulatory genes, we examined whether NF-κB activity is inhibited by GCs. Here we show that GCs are potent inhibitors of NF-κB activation. This inhibition is the result of induction of IκBα expression, followed by sequestration of NF-κB dimers in the cytoplasm.

The interleukin-2 (IL-2) promoter contains cis elements required for maximal induction during T cell activation, which are recognized by AP-1, NF-κB, NF of activated T cells, and octamer-binding proteins (OBP) (*9, 15*). We investigated which of these factors mediate the inhibitory effect of the synthetic GC dexamethasone (DEX) on IL-2 production by a murine T cell hybridoma (*16*). As shown previously (*17, 18*), DEX inhibited induction of IL-2 secretion (Fig. 1A) and transcription, as measured by an IL-2–luciferase reporter plasmid (*19*) (Fig. 1B). Electrophoretic mobility-shift assays (EMSAs) (*20*) revealed that both AP-1 and NF-κB DNA binding activities were elevated in nuclear extracts of activated cells (Fig. 1C). DEX inhibited induction of NF-κB binding activity and reduced the amount of AP-1 binding activity, whereas OBP binding activity was unaffected. DEX also inhibited induction of NF-κB in mouse T lymphocytes in vivo (Fig. 1D). Administered either 2 hours before or simultaneously with anti-CD3, a potent T cell–activating monoclonal antibody (*21*), DEX inhibited induction of NF-

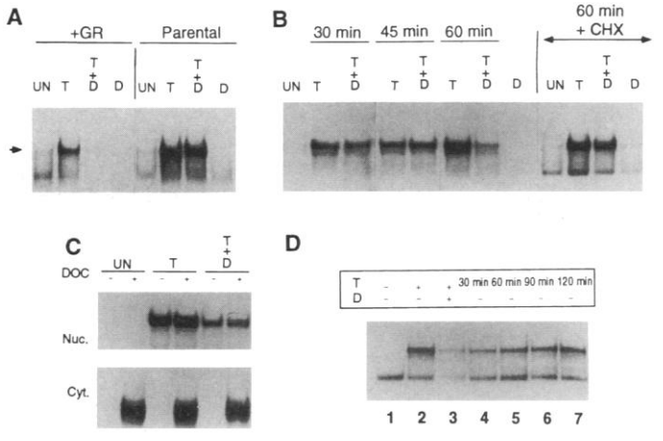

Fig. 2. Inhibition of NF-κB activation requires induction of a short-lived inhibitory protein. (**A**) A GR$^-$ subclone of Jurkat cells (parental) or Jurkat cells stably transfected with an expression vector encoding rat GR and expressing 20,000 receptors per cell (+GR) were incubated with TPA (T) (100 ng/ml), DEX (D) (1 μM), a combination of TPA plus DEX, or no further addition (UN). After 1 hour, nuclear extracts were prepared and 5-μg samples were examined for NF-κB binding activity as described above. The arrowhead indicates the NF-κB–DNA complex. (**B**) GR$^+$ Jurkat cells were treated with TPA (T), TPA plus DEX, or DEX (D) for the indicated times. Untreated (UN) and DEX-treated cells were harvested after 60 min. When indicated, cells were pretreated with CHX (10 μg/ml) for 30 min and then incubated with TPA, TPA plus DEX, or DEX in the presence of CHX. NF-κB DNA binding activity in nuclear extracts was assayed as described above. (**C**) GR$^+$ Jurkat cells were either untreated (UN) or incubated in the presence of TPA (T) or TPA plus DEX (D) as described above. After 60 min, nuclear and cytoplasmic extracts were prepared. Samples of nuclear (Nuc.) (5 μg) and cytosolic (Cyt.) (10 μg) extracts were treated with 0.8% Na-deoxycholate (DOC) and 1.2% NP-40 and analyzed for NF-κB binding activity by EMSA. (**D**) GR$^+$ Jurkat cells were incubated in medium alone (lane 1), in medium plus TPA (T) (lane 2), or in TPA plus DEX (D) (lanes 3 to 7) for 90 min. After two washes, cells treated with TPA plus DEX were divided into aliquots and incubated for the indicated times with either TPA (lanes 4 to 7) or TPA plus DEX (lane 3) for 120 min. In lanes 1 and 2, cells were maintained under the same conditions (lane 1, medium; lane 2, TPA) for another 120 min. Nuclear extracts were prepared and analyzed for NF-κB binding activity.

κB activity in both thymocytes and lymph nodes. In the latter, DEX also inhibited basal NF-κB binding activity. In addition, DEX inhibited induction of NF-κB activity in splenocytes of mice injected with lipopolysaccharide (*22*). Inhibition of NF-κB activation by DEX, with a median inhibitory dose (IC_{50}) of 5 nM (*22*), was also observed in a Jurkat human T cell leukemia line stably transfected with a GR expression vector (Fig. 2A). No inhibition was observed in the parental GR Jurkat subclone. Therefore, the inhibitory effect of DEX is mediated through the GR. Inhibition of NF-κB activity was also observed in cells stimulated by either 12-*O*-tetradecanoylphorbol 13-acetate (TPA) alone or by tumor necrosis factor (TNF-α) (*23*). The NF-κB binding activity inhibited by DEX was composed mostly of the p50 and p65 (RelA) subunits (*23*).

Inhibition of NF-κB activity required 45 to 60 min of DEX treatment (Fig. 2B). Because initial induction of NF-κB binding activity was unaffected, DEX is unlikely to interfere with the signaling cascade that leads to NF-κB activation. Inhibition of NF-κB activity required new protein synthesis; it was prevented by cycloheximide (CHX) (Fig. 2B). The inactive, cytoplasmic NF-κB–IκB complex dissociates after deoxycholate (DOC) treatment (*24*). DOC treatment of cytoplasmic extracts of DEX-treated cells revealed the same amount of NF-κB binding activity as in untreated cells (Fig. 2C). Thus, inhibition of NF-κB binding activity is not caused by degradation or covalent modification of any of its constituents. Upon DEX removal, but in the continued presence of TPA, NF-κB binding activity reappeared after 30 min and peaked at 60 min (Fig. 2D). However, if DEX was left on the cells for 5 hours, NF-κB activity remained repressed. These results suggest that inhibition of NF-κB is mediated by a short-lived DEX-induced protein.

A possible mediator of the inhibitory action of DEX is IκBα, encoded by the *MAD-3* gene (*25*), as it is a short-lived protein whose degradation is further enhanced by various NF-κB inducers (*12*, *13*). These inducers also cause IkBα resynthesis by stimulating *MAD-3* transcription, a feedback response that terminates NF-κB activation (*12*). The effect of DEX on IκBα metabolism was examined by immunoblotting with an antibody to IκBα. Treatment with TPA led to the partial disappearance of IκBα, which reached 20% of its basal level after 30 min (Fig. 3A). At 10 and 20 min, DEX had an insignificant effect on the amount of IκBα in TPA-treated cells, but after 30 min the amount of IκBα in cells treated with TPA plus DEX was similar to that in unstimulated cells, and after 45 min it was 50% higher than in unstimulated cells. The kinetics of DEX-induced IκBα accumulation paralleled the kinetics of NF-κB inhibition. Sensitivity to CHX indicated that increased IκBα abundance in cells treated with TPA plus DEX required new protein synthesis, whereas the TPA-stimulated degradation of IκBα did not. None of the treatments affected p65 abundance. Similar results were obtained in vivo: Injection of mice with anti-CD3 caused degradation of thymic IκBα, but simultaneous administration of DEX resulted in IκBα levels similar to those in untreated animals (Fig. 3B). Examination of GR^+ Jurkat cells (*26*) or HeLa cells stained with an antibody to p65 by indirect immunofluorescence indicated that p65 was cytoplasmic in unstimulated cells, but after 10 min of TNF-α stimulation, most of it was nuclear (Fig. 3C). Treatment of TNF-α–stimulated cells with DEX resulted in retention of p65 in the cytoplasm. Staining with antibody to GR indicated that DEX induced nuclear translocation of GR, regardless of whether the cells were stimulated with TNF-α or not. Recently a second form of IκB, IκBβ, was molecularly cloned (*27*). Immunoblot analysis of HeLa cell extracts with antibodies to IκBα did not reveal any effect of DEX on IκBα expression (*28*).

Because DEX did not prevent IκBα degradation at early time points, the increase in IκBα abundance in DEX-treated cells is probably the result of increased IκBα synthesis. Northern (RNA) blot analysis verified this assumption and indicated that DEX treatment of GR^+ Jurkat cells, but not the GR^- parental cell line, led to sevenfold in-

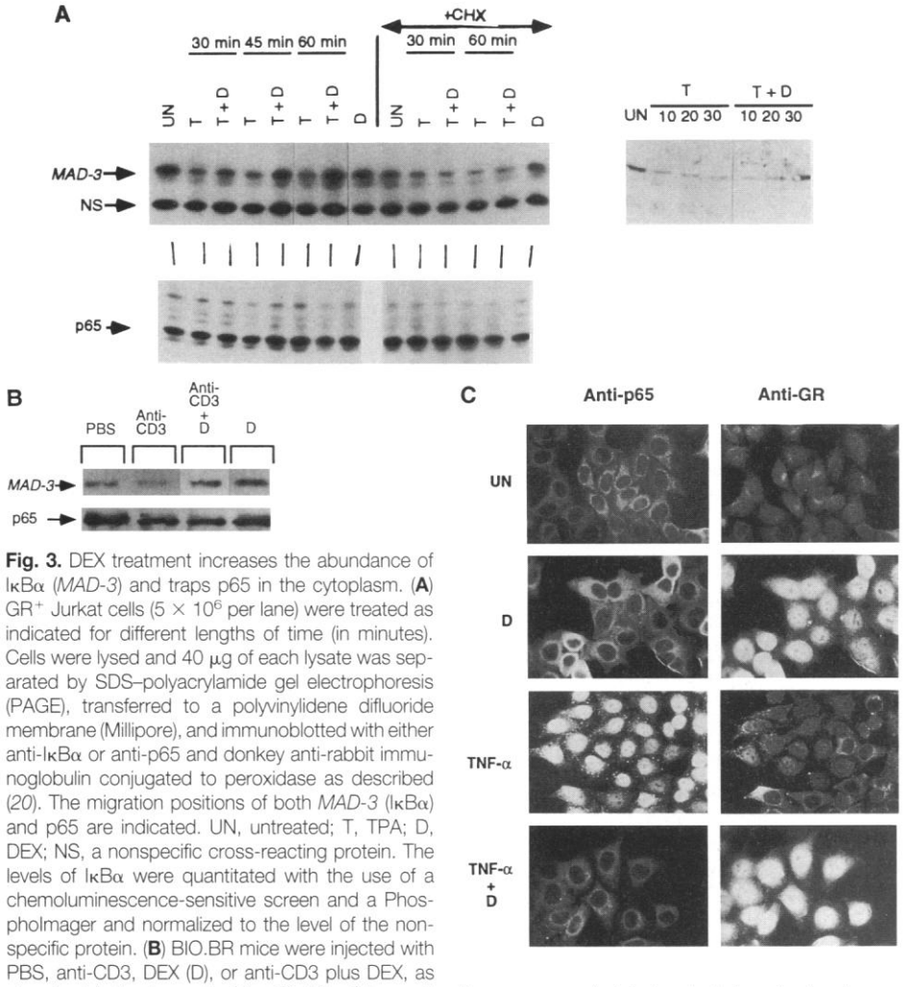

Fig. 3. DEX treatment increases the abundance of IκBα (*MAD-3*) and traps p65 in the cytoplasm. (**A**) GR^+ Jurkat cells (5×10^6 per lane) were treated as indicated for different lengths of time (in minutes). Cells were lysed and 40 μg of each lysate was separated by SDS–polyacrylamide gel electrophoresis (PAGE), transferred to a polyvinylidene difluoride membrane (Millipore), and immunoblotted with either anti-IκBα or anti-p65 and donkey anti-rabbit immunoglobulin conjugated to peroxidase as described (*20*). The migration positions of both *MAD-3* (IκBα) and p65 are indicated. UN, untreated; T, TPA; D, DEX; NS, a nonspecific cross-reacting protein. The levels of IκBα were quantitated with the use of a chemoluminescence-sensitive screen and a PhosphoImager and normalized to the level of the nonspecific protein. (**B**) BIO.BR mice were injected with PBS, anti-CD3, DEX (D), or anti-CD3 plus DEX, as described in the legend to Fig. 1D. After 2 hours, the thymuses were isolated and whole-cell extracts were prepared. Fifty micrograms of each extract were separated by SDS-PAGE, transferred to Immbilon P membrane, and probed with antibodies to IκBα and p65. (**C**) HeLa cells were plated on glass cover slips and were left untreated (UN) or were treated with DEX (D) for 30 min, TNF-α for 10 min, or DEX for 30 min and then TNF-α for 10 min. After treatment, the cells were fixed and the intracellular locations of p65 and GR were determined by indirect immunofluorescence with the use of anti-p65 (Pharmingen, La Jolla, California) and anti-GR, as described (*37*). Shown are typical fields of stained HeLa cells showing the exclusive cytoplasmic location of p65 in untreated, DEX-treated, or TNF-α–plus–DEX–treated cells and its nuclear translocation in TNF-α–treated cells.

duction of *MAD-3* (IκBα) mRNA (Fig. 4A). Stimulation of either GR^+ or GR^- cells with TPA resulted in sixfold induction of *MAD-3* mRNA after 1 hour. Treatment with both TPA and DEX resulted in a synergistic induction (23-fold), but only in the GR^+ subclone. None of the treatments had a substantial effect on expression of p65 (RelA) mRNA. Induction of IκBα expression by both TPA and DEX is most likely the result of increased transcription because it was inhibited by actinomycin D (Fig. 4B). In an accompanying report (*29*), Scheinman *et al.*, who obtained similar results, demonstrate that DEX increases the transcription rate of the *MAD-3* gene. Actinomycin D also prevented DEX-induced inhibition of NF-κB binding activity (Fig. 4C).

Collectively, these results define a simple mechanism through which GCs repress NF-κB activity. Unlike previously described transcriptional interference (*8*), inhibition of NF-κB activity does not rely on direct interaction between the activated GR and any NF-κB constituent. Rather, the inhibition is based on induction of IκBα expression. Induction of *MAD-3* mRNA results in faster reappearance of IκBα in activated cells treated with DEX than in activated cells not exposed to DEX. Reappearance of IκBα correlates with termination of NF-κB activation. Even a modest increase in the amount of IκBα, not greatly exceeding its basal level in unstimulated cells, is sufficient to cause redistribution of active p65 from the nucleus to the cytoplasm, where it is sequestered as an inactive complex.

It was previously suggested that inhibition of NF-κB activation by DEX is mediated by physical interactions between the activated GR and the p65 component of NF-κB (*30*). However, those results were obtained by transient cotransfection experiments, and although it is feasible that once overexpressed the GR and p65 can directly interact, this mechanism is not necessary to explain the results described above that were obtained under physiological conditions. Furthermore, DEX treatment causes translocation of p65 from the nuclei of stimulated cells to the cytoplasm, whereas the activated GR remains nuclear. It is therefore unlikely that direct binding of GR to p65 mediates cytoplasmic sequestration of p65. Because DEX induces *MAD-3* expression in nonactivated T cells, the induction response itself does not appear to rely on direct interaction between the activated GR and NF-κB. As is consistent with other work (*31*), the simplest explanation of the current results is that newly synthesized IκBα translocates to the nucleus, where, as shown in vitro (*32*), it can sequester free NF-κB and thereby promote net dissociation of DNA-bound NF-κB. This is followed by translocation of IκBα- and p65-containing complexes to the cytoplasm. This mechanism also differs from the one proposed to explain inhibition of NF-κB activation by the relatively weak anti-inflammatory agent aspirin (*33*).

Inhibition of NF-κB activation can account for many of the immunosuppressive and anti-inflammatory activities of GCs, which are amongst the most potent anti-inflammatory agents known. NF-κB plays a central role in induction of a large number of important immunoregulatory genes, including those encoding IL-1, IL-2, IL-3, IL-6, IL-8, TNF-α, interferon γ (IFN-γ), granulocyte-macrophage colony-stimulating factor, class I and class II major histocompatibility complexes, the κ light chain, and endothelial leukocyte adhesion molecule 1 and intercellular adhesion molecule 1 (*10*). Several of these genes are also regulated by AP-1 (*9*), which synergizes with NF-κB (*34*). Because GCs inhibit both NF-κB and AP-1 activities, albeit through different mechanisms, it is no longer a surprise that they repress expression of a very wide spectrum of immunoregulatory genes. Indeed, administration of GC prevents systemic release of IL-2, IL-6, IFN-γ, and TNF-α in response to anti-CD3 or to superantigen (*2*, *35*). It is anticipated that other effective inhibitors of NF-κB and AP-1 may also turn out to be useful immunosuppressive and anti-inflammatory agents.

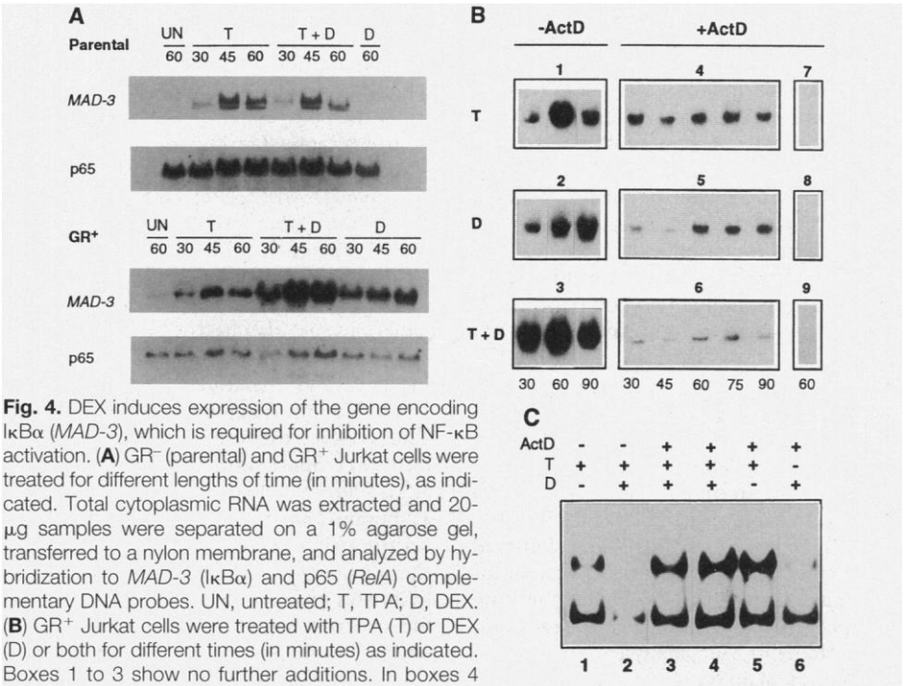

Fig. 4. DEX induces expression of the gene encoding IκBα (*MAD-3*), which is required for inhibition of NF-κB activation. (**A**) GR^- (parental) and GR^+ Jurkat cells were treated for different lengths of time (in minutes), as indicated. Total cytoplasmic RNA was extracted and 20-μg samples were separated on a 1% agarose gel, transferred to a nylon membrane, and analyzed by hybridization to *MAD-3* (IκBα) and p65 (*RelA*) complementary DNA probes. UN, untreated; T, TPA; D, DEX. (**B**) GR^+ Jurkat cells were treated with TPA (T) or DEX (D) or both for different times (in minutes) as indicated. Boxes 1 to 3 show no further additions. In boxes 4 and 5, actinomycin D (ActD; 3 μg/ml), was added 15 min after the initial exposure to TPA or DEX, then cells were harvested every 15 min as indicated. In box 6, cells were pretreated with DEX for 15 min, followed by ActD, and after 15 min TPA was added. In boxes 7 to 9, ActD was added 15 min before incubation with TPA or DEX or both. Total cellular RNA was prepared and 30-μg samples were separated on an agarose gel, transferred to nylon membrane, and analyzed for the presence of *MAD-3* (IκBα) mRNA. (**C**) GR^+ Jurkat cells were incubated with DEX (D) for 15 min, then ActD (3 μg/ml) was either added (lane 3) or not (lane 2), and after 15 min TPA (T) was added. As a control, ActD was also added 15 min before incubation with TPA plus DEX (lane 4), TPA (lane 5), or DEX (lane 6). In addition, TPA was added alone (lane 1). Nuclear extracts were prepared, and NF-κB binding activity was examined.

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Altered DNA Recognition and Bending by Insertions in the α2 Tail of the Yeast a1/α2 Homeodomain Heterodimer

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The yeast MATα2 and MAT**a**1 homeodomain proteins bind cooperatively as a heterodimer to sites upstream of haploid-specific genes, repressing their transcription. In the crystal structure of α2 and **a**1 bound to DNA, each homeodomain makes independent base-specific contacts with the DNA and the two proteins contact each other through an extended tail region of α2 that tethers the two homeodomains to one another. Because this extended region may be flexible, the ability of the heterodimer to discriminate among DNA sites with altered spacing between α2 and **a**1 binding sites was examined. Spacing between the half sites was critical for specific DNA binding and transcriptional repression by the complex. However, amino acid insertions in the tail region of α2 suppressed the effect of altering an **a**1/α2 site by increasing the spacing between the half sites. Insertions in the tail also decreased DNA bending by **a**1/α2. Thus tethering the two homeodomains contributes to DNA bending by **a**1/α2, but the precise nature of the resulting bend is not essential for repression.

Homeodomain proteins are found in a wide range of eukaryotic organisms and form a large family of transcription factors that function in many different cellular processes (*1*). Although many homeodomain proteins bind DNA with relatively low sequence specificity in vitro, they often confer very specific regulatory activities in vivo (*2*). One mechanism that homeodomain proteins use to achieve their biological specificity is through interactions with additional protein factors (*3*). Such protein-protein interactions increase DNA binding affinity and specificity to the target sites. For example, in the yeast *Saccharomyces cerevisiae*, the α2 homeodomain protein interacts with two other proteins to regulate cell-type specific gene expression (*4*). In haploid α cells, the α2 protein acts in combination with MCM1, a MADS box protein, to bind DNA as a heterotetramer to repress **a**-specific genes (*asg*) (*5*). In **a**/α diploid cells, the α2 protein also interacts with **a**1, another homeodomain protein, to bind DNA as a heterodimer and repress haploid-specific genes (*hsg*) (*6*). Therefore, MCM1 and **a**1 contribute to the target site selection of α2.

In *asg* repression, the MCM1 protein contributes to the sequence specific binding of α2 in two different ways. First, the cooperative interactions between the two proteins increases the apparent DNA binding affinity of the complex (*5*, *7*). Second, interactions with MCM1 dictate the spacing and orientation of the α2 binding sites, which increases the DNA binding specificity of the complex (*8*).

In *hsg* repression, the **a**1 protein appears to play a similar role in helping α2 bind selectively to the **a**1/α2 target sites. It has been shown that protein-protein interactions between α2 and **a**1 contribute to the DNA binding affinity (*9*). However, we wondered whether these protein-protein interactions also dictate the spacing and orientation of the homeodomain DNA binding sites and therefore contribute to the DNA binding specificity of the complex. The crystal structure of the **a**1/α2/DNA ternary complex shows that although there are no direct contacts between the α2 and **a**1 homeodomains, the two proteins interact through a region immediately following the α2 homeodomain that forms a short α helix and makes a set of mainly hydrophobic contacts with **a**1 (*10*). This helix is tethered to the end of the third helix in the α2 homeodomain by an extended linker region (residues 59 to 63 in the crystal structure, residues 189 to 193 of the intact protein) which does not appear to be making contacts with either protein or DNA. Because both NMR and x-ray studies suggest that the linker is extended, it is possible that the linker is somewhat flexible (*10*, *11*). The **a**1 protein may therefore not de-

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