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by

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[redacted]

I dedicate this work to my Mom and Dad.

They have always supported me with boundless encouragement,

patience and generosity.

I am eternally grateful and I love you both.

I could while away the hours

Conferrin' with the flowers

Consultin' with the rain

And my head, I'd be scratchin'

While my thoughts were busy hatchin'

If I only had a brain...

- L. Frank Baum

And I think of my life as vintage wine

From fine old kegs

From the brim to the dregs

It poured sweet and clear

It was a very good year

- Frank Sinatra

Acknowledgements

I came to UCSF to learn x-ray crystallography, but, to try my hands at biochemistry, I chose Keith's lab for my third rotation. I selected Keith for my thesis advisor, not merely because he is an excellent scientist, as all UCSF PIBS faculty members are great scientists, but because I wanted my scientific endeavors to reflect his distinct style and perspective. Keith displays meticulous attention to detail while maintaining a watchful eye on the distant horizon. If only a fraction of his scientific style and rigor become my own, I will have grown tremendously. Notably, when I entered graduate school, UCSF was top ranked by US News and World Report. As I leave, they are tied for 7th; causal or coincidental?

I will always remember the seemingly endless supply of unique and colorful people working in the labs of UCSF. In different ways, I have enjoyed my interactions with everyone. I will be taking particularly fond memories with me involving the many escapades, some even science-related, that involved Michael Cronin, Brian Freeman, Hussein Hadeiba, Hans Leucke and Raquel Sitcheran.

I was fortunate enough to experience several recreational eras during my graduate career. My brother Chris and my long-time friends Seth Jones and Kurtiss Takishita played critical roles in determining how much fun one can physically tolerate. Without them, I probably would have finished my thesis project sooner, although with less sanity intact, but getting there would not have been nearly as pleasant; contrast is wonderful. There were many additional players, some very good friends but too many to list, so to the supporting cast of San Francisco, and occasionally Las Vegas, I say, thank you.

Abstract

Intracellular receptor regulation of NFκB transcriptional activation activity

Robert Michael Nissen

The intracellular receptor (IR) gene family comprises a large number of transcriptional regulators governing physiological processes ranging from development and differentiation to maintenance of cellular homeostasis. IRs either activate or repress transcription rates depending on the cellular context and nucleotide sequence of DNA response elements. I sought to examine how IRs transmit hormone signals to the general transcriptional machinery at response elements not directly bound by IR:hormone complexes, termed tethering response elements. As an example of such sites, glucocorticoids repress pro-inflammatory gene transcription through response elements directly recognized by NFκB. The NFκB family of transcriptional regulators transmits both developmental and immuno-modulatory signals. In this study, I focused on characterizing the mechanisms by which the glucocorticoid receptor (GR) inhibits NFκB activation of pro-inflammatory genes. I showed that GR represses transcription of the IL-8 and ICAM-1 pro-inflammatory genes through direct protein interaction with the DNA bound RelA subunit of NFκB. Furthermore, I demonstrated that GR represses transcription after NFκB stimulation of preinitiation complex (PIC) assembly by altering the phosphorylation pattern on the carboxyl-terminal domain (CTD) of the RNA polymerase II (pol II) Rpb1 subunit. Similarly, I showed that two additional IR family members, the thyroid hormone receptor (T3R) and the retinoic acid receptor (RAR), can regulate the transcriptional activation activity of various NFκB family members *via* a tethering mode. As organisms exist within complex environments, the sensory systems they employ must triage and integrate multiple inputs; extrapolation of my results shows how two distinct gene families can integrate a potentially wide field of signals.

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Introduction

Small molecules regulate several aspects of development and cellular homeostasis. The various steroid hormones (androgens, estrogens, glucocorticoids, mineralocorticoids and progestins) circulate throughout the body via the blood stream. The steroid hormones are derivatives of cholesterol and therefore seem to freely diffuse through the lipid bilayers of cells in target tissues. In some cells, transmembrane pumps have been detected that reduce hormone potency by actively exporting particular ligands (Kralli et al. 1995; Kralli and Yamamoto 1996). Within target cells, hormone-specific soluble receptor proteins transform the chemical information embodied by steroid hormones into modified patterns of gene expression. These intracellular receptors not only bind the steroid ligands but also encode DNA binding and transcriptional regulatory activities. Furthermore, many small molecules, metabolites and derivatives (*e.g.* thyroid hormone, retinoic acid, vitamin D and arachidonic acid) serve as signaling ligands for proteins homologous to the steroid hormone receptors; collectively, these regulators constitute the intracellular receptor (IR) gene family (Yamamoto 1985; Tsai and O'Malley 1994).

The glucocorticoid receptor (GR) is the first identified and cloned IR (Miesfeld et al. 1984; Hollenberg et al. 1985; Miesfeld et al. 1986). Many years of biochemical, molecular genetic and structural analysis reveal that GR consists of three protein domains (Fig. 1A). The GR zinc binding region (ZBR) is in the center of the protein and harbors the DNA binding and protein dimerization functions of GR (Rusconi and Yamamoto 1987; Luisi et al. 1991). The GR-ZBR is essential for regulation of all known GR target genes (Schena et al. 1989). A large domain amino-terminal from the GR-ZBR is involved in many context-specific aspects of gene regulation including both

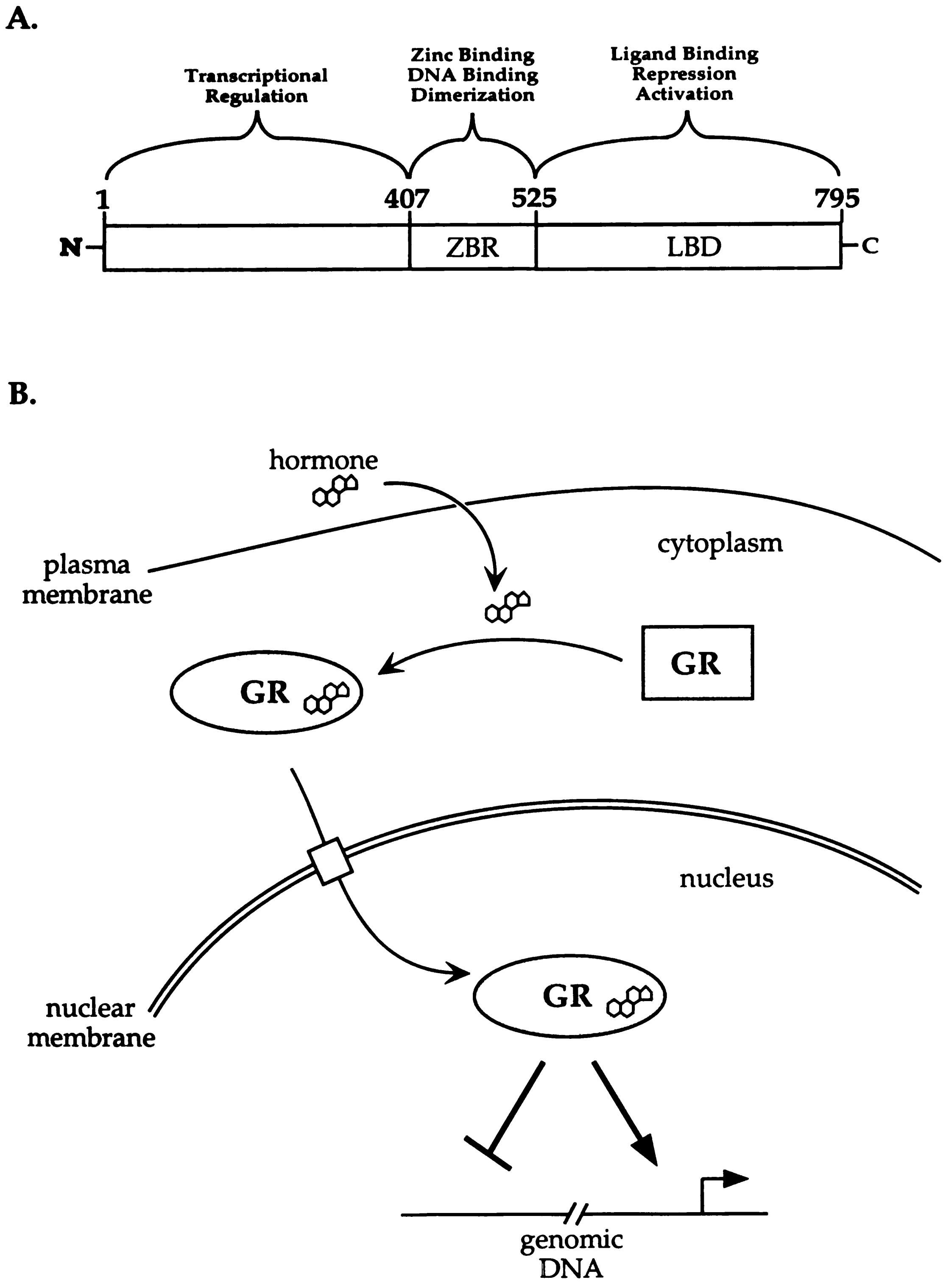

A.
Transcriptional Regulation
Zinc Binding
DNA Binding
Dimerization
Ligand Binding
Repression
Activation
1
407
525
795
N
ZBR
LBD
C
B.
hormone
plasma membrane
cytoplasm
GR
GR
nucleus
nuclear membrane
GR
genomic DNA

Figure 1.

transcriptional activation and repression (Miesfeld et al. 1987; Diamond et al. 1990; Pearce and Yamamoto 1993). Carboxyl-terminal to the GR-ZBR is the GR ligand binding domain (LBD) which directly binds hormonal ligands (Rusconi and Yamamoto 1987). Truncations that remove the LBD, but retain a functional ZBR, are constitutively nuclear and transcriptionally active (Godowski et al. 1987). In the absence of hormone, GR resides in the cytoplasm associated with several molecular chaperones. In the presence of hormone, interactions with the chaperone complex change and the receptor redistributes to the cell nucleus (Picard and Yamamoto 1987; Picard et al. 1988; Howard et al. 1990; Picard et al. 1990; Freeman et al. 2000).

GR mediates the physiological effects of glucocorticoid hormones by regulating transcription initiation rates (Fig. 1B) (Yamamoto 1985) and plays essential roles throughout life (Tronche et al. 1998). For example, GR enables newborn mammals to take their first breath by inducing synthesis of lung surfactant proteins and maintains blood-glucose levels during fasting by stimulating gluconeogenesis (Cole et al. 1995). Conditional knock-out experiments further demonstrate that GR signaling is important in the nervous system because mutant animals have reduced anxiety and show impaired responses to stress (Tronche et al. 1999). Strikingly, GR is an essential component of the negative-feedback regulatory circuit that controls the acute-phase inflammatory reaction provoked by an invading pathogen (Fig. 2) (Da Silva 1999).

The acute-phase inflammatory reaction stimulates both adherence of inflammatory cell-types (lymphocytes, neutrophils, monocytes, eosinophils, basophils, mast cells) to vascular endothelial cells and their migration into the affected region. The local response is initiated following activation of tissue macrophages, which respond by synthesizing and releasing three critical cytokines: tumor necrosis factor-α (TNFα), interleukin-1 (IL-1), and interleukin-6 (IL-6). These three cytokines activate inflammatory cell-types through binding to cell surface receptors which then activates specific signal transduction cascades. In turn, these transduction cascades stimulate the

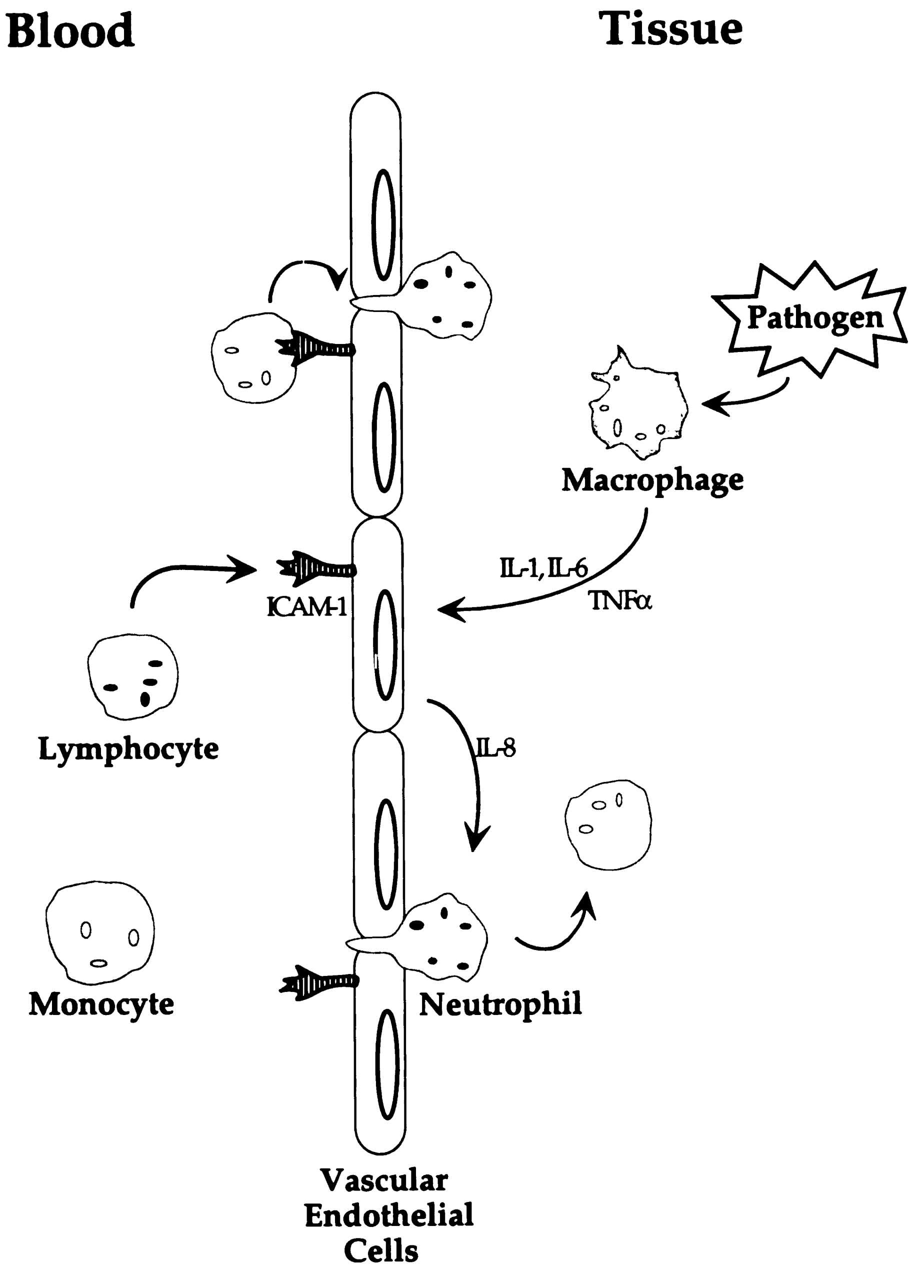

Blood
Tissue
Pathogen
Macrophage
IL-1, IL-6
TNFα
ICAM-1
Lymphocyte
IL-8
Monocyte
Neutrophil
Vascular Endothelial Cells

Figure 2.

synthesis and secretion of additional pro-inflammatory gene products including interleukin-8 (IL-8, contributing to neutrophil invasion), interferon-γ (IFNγ, a macrophage chemoattractant), intercellular adhesion molecule-1 (ICAM-1, a lymphocyte adhesion molecule), granulocyte-macrophage colony stimulating factor (GM-CSF, increases white blood cell production) which act as autocrine, paracrine and endocrine effectors, ultimately generating a full inflammatory response adhesion molecule), granulocyte-macrophage colony stimulating factor (GM-CSF, increases white blood cell production) which act by autocrine, paracrine and endocrine fashions ultimately generating a full immune response (Elenkov et al. 1999; Goldsby et al. 2000)].

The signal transduction cascades activated by TNFα, IL-1 and IL-6 all converge on the transcriptional regulator NFκB (Fig. 3). The NFκB family of transcriptional regulators transmits both developmental and immune-stimulatory signals (Baldwin 1996; Ghosh et al. 1998). The most studied form of NFκB is a heterodimer composed of two subunits, RelA and p50, that activates transcription of many pro-inflammatory genes through direct recognition of promoter-proximal DNA response elements. In the inactivated state, NFκB is sequestered in the cytoplasm by the inhibitor protein IκB. TNFα, IL-1 and IL-6 all trigger the phosphorylation, ubiquitination and proteosomal degradation of IκB (Chen et al. 1995; Scherer et al. 1995; Mercurio et al. 1997; Woronicz et al. 1997). In the absence of IκB, NFκB undergoes nuclear translocation and DNA binding (Baeuerle and Baltimore 1988a; Baeuerle and Baltimore 1988b). Once DNA bound, NFκB activates the transcription of many pro-inflammatory genes including IL-8, IFNγ, ICAM-1 and GM-CSF (van de Stolpe et al. 1993; Kunsch et al. 1994; Mukaida et al. 1994; Young 1996; Adkins et al. 1998).

The systemic component of the acute-phase inflammatory response is characterized by fever, increased white blood cell production (partly by GM-CSF), elevation of C-reactive protein (an activating component of the complement cascade)

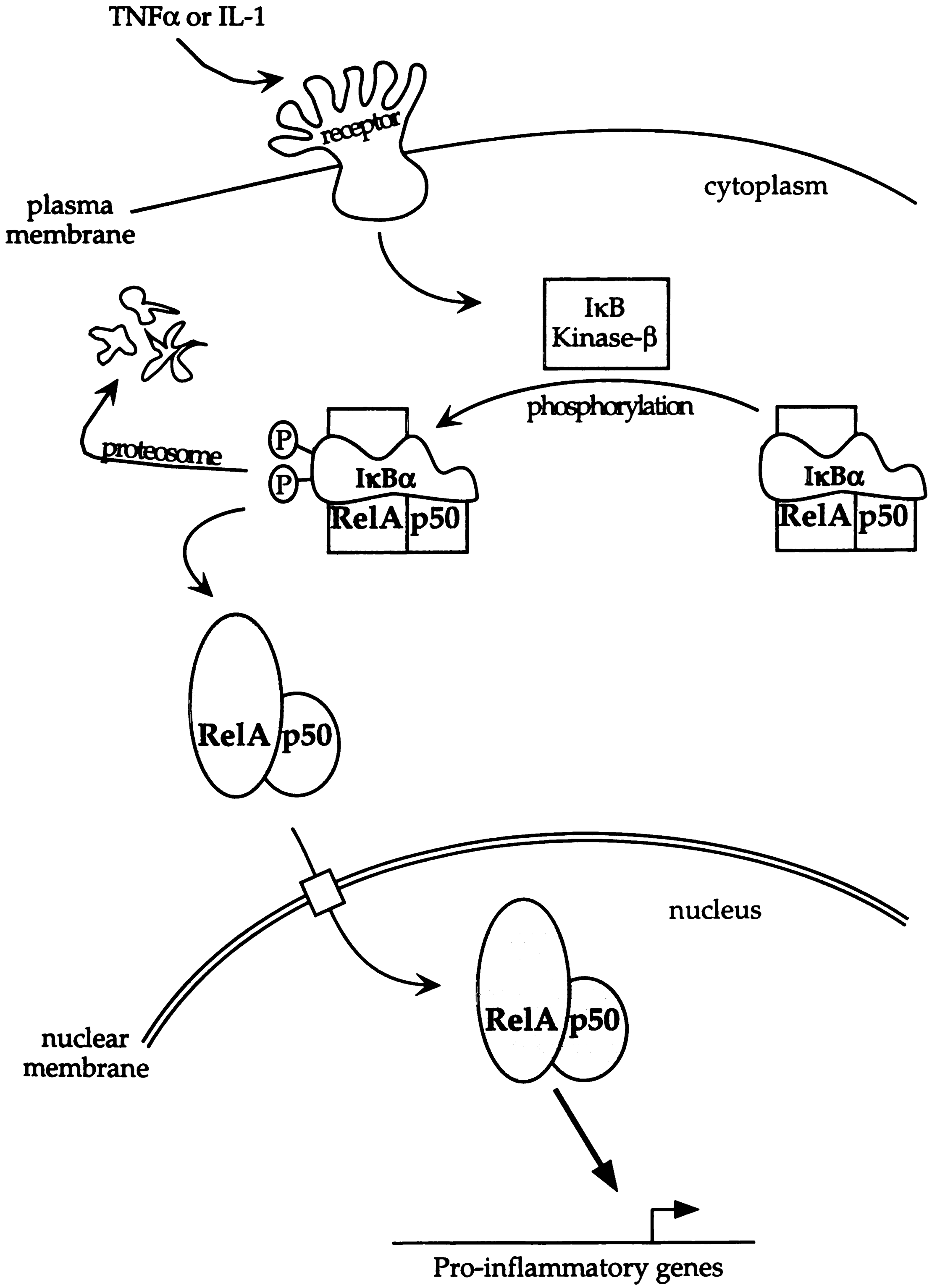

TNFα or IL-1
receptor
plasma membrane
cytoplasm
IκB Kinase-β
phosphorylation
proteosome
P
P
IκBα
RelA
p50
IκBα
RelA
p50
RelA
p50
nucleus
RelA
p50
nuclear membrane
Pro-inflammatory genes

Figure 3.

and, as part of the negative-feedback response, elevated glucocorticoid levels (Elenkov et al. 1999; Goldsby et al. 2000). Strikingly, glucocorticoids inhibit transcriptional activation of pro-inflammatory genes and the NFκB response element is the necessary and sufficient promoter sequence for inhibition (Mukaida et al. 1994; van de Stolpe et al. 1994; Caldenhoven et al. 1995).

GR either activates or represses transcription rates depending on the cellular context and nucleotide sequence of DNA response elements. Likewise, transcriptional regulators can adopt distinct regulatory modes depending on the sequence and context, cellular and physiological, of response elements (Lefstin and Yamamoto 1998; Yamamoto et al. 1998). These response elements fit into three broad categories; simple, composite and tethering. Simple response elements are directly bound by regulators with the outcome, positive or negative, independent of other factors (Jantzen et al. 1987; Heinrichs et al. 1993; Meyer et al. 1997b). Composite response elements are also directly bound but require cooperation between multiple regulators, the composition of which can influence the transcriptional effect (Diamond et al. 1990; Miner and Yamamoto 1992). In contrast, regulators indirectly bind tethering response elements via protein-protein interaction with a distinct, directly bound target regulator. For example, the AP-1 site of the collagenase gene is a tethering GR response element (GRE) (Jonat et al. 1990; Yang-Yen et al. 1990). GR inhibits transcription driven by the AP-1 site only when the AP-1 transcription factor is present, presumably tethering to the GRE *via* the observed direct interaction with AP-1 protein (Konig et al. 1992). The fact that GR represses pro-inflammatory genes via NFκB response elements suggests that those elements may also serve as tethering GREs.

Of particular interest is how IRs transmit hormone signals to the general transcriptional machinery at tethering response elements. Glucocorticoid induction of IκB gene transcription has been reported in certain cell types (Auphan et al. 1995; Scheinman et al. 1995a). However, GR inhibition of NFκB-mediated activation is independent of new protein synthesis (van de Stolpe et al. 1993; Wissink et al. 1998). Similarly, primary bovine aortic endothelial cells, the human A549 lung alveolar-like cell line, and others support GR inhibition of NFκB in the absence of glucocorticoid mediated IκBα or IκBβ gene induction (Brostjan et al. 1996; De Bosscher et al. 1997; Heck et al. 1997; Newton et al. 1998; Wissink et al. 1998; Adcock et al. 1999). Additionally, GR selectively interacts with the RelA subunit of NFκB (Ray and Prefontaine 1994; Caldenhoven et al. 1995; Scheinman et al. 1995b; Wissink et al. 1997). Therefore, I decided to re-examine GR repression of NFκB in the light of a tethering model. In principle, GR could affect NFκB DNA binding, NFκB activation domain function, RNA polymerase II (pol II) preinitiation complex (PIC) formation, pol II phosphorylation, PIC isomerization or promoter clearance.

I carried out my studies *in vitro* and in CV-1 and A549 cultured cells. In chapter I, I describe a detailed molecular analysis of GR repression of NFκB-mediated activation. Chapter II describes my discovery that two IRs critically involved in developmental processes (Brent 1994; Morriss-Kay and Sokolova 1996), the thyroid hormone receptor (T3R) and the retinoic acid receptor (RAR) can regulate NFκB family members in context-specific manners, likely *via* a tethering mode. Within that chapter, I speculate that these interactions might affect particular facets of embryonic development. In the perspectives chapter, I review our current understanding of the regulatory mechanisms that govern pol II transcription, incorporating my results, and propose a new scheme for classifying transcriptional regulators.

Chapter I

The Glucocorticoid Receptor Inhibits NFκB by Interfering with Serine-2 Phosphorylation of the RNA Polymerase II Carboxyl-Terminal Domain

Abstract

Glucocorticoids repress NFκB-mediated activation of pro-inflammatory genes such as IL-8 and ICAM-1. Our experiments suggested that the glucocorticoid receptor (GR) mediates this effect by associating through protein-protein interactions with NFκB bound at each of these genes. That is, we showed that the GR zinc binding region (ZBR) binds directly to the dimerization domain of the RelA subunit of NFκB *in vitro*, and that the ZBR is sufficient to associate with RelA bound at NFκB response elements *in vivo*. Moreover, we demonstrated *in vivo* and *in vitro* that GR does not disrupt DNA binding by NFκB. In transient transfections, we found that the GR ligand binding domain (LBD) is essential for repression of NFκB but not for association with it, and that GR can repress an NFκB derivative bearing a heterologous activation domain. We used chromatin immunoprecipitation assays in untransfected A549 cells to infer the mechanism by which the tethered GR represses NFκB-activated transcription. As expected, we found that the inflammatory signal TNFα stimulated preinitiation complex (PIC) assembly at the IL-8 and ICAM-1 promoters, and that the largest subunit of RNA polymerase II (pol II) in those complexes became phosphorylated at serines 2 and 5 in its carboxyl-terminal domain (CTD) heptapeptide repeats (YSPTSPS); these modifications are required for transcription initiation. Under repressing conditions, GR did not inhibit PIC assembly, but rather interfered with phosphorylation of serine 2 of the pol II CTD.

Introduction

Glucocorticoids are the most commonly prescribed treatment for inflammatory diseases such as rheumatoid arthritis (Da Silva 1999; Elenkov et al. 1999), and it is well established that glucocorticoids downregulate the transcription of pro-inflammatory genes (van de Stolpe et al. 1993; Mukaida et al. 1994; Ray and Prefontaine 1994). Those genes are themselves activated by the NFκB transcriptional regulator, but the mechanism by which glucocorticoids preclude activation has been a matter of debate.

NFκB is the central member of the mammalian rel gene family (Baldwin 1996; Ghosh et al. 1998); a major form of NFκB is a heterodimer of the RelA and p50 family members. In the inactivated state, RelA/p50 heterodimers are sequestered in the cytoplasm by the inhibitor protein IκB (Baeuerle and Baltimore 1988a; Baeuerle and Baltimore 1988b). Pro-inflammatory signals such as tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1), trigger IκB phosphorylation, ubiquitination and proteosomal degradation, enabling NFκB nuclear translocation and binding to specific genomic response elements (Chen et al. 1995; Scherer et al. 1995; Mercurio et al. 1997; Woronicz et al. 1997). Once DNA bound, NFκB activates the transcription of pro-inflammatory genes such as IL-8 and ICAM-1 (van de Stolpe et al. 1993; Kunsch et al. 1994; Mukaida et al. 1994). Importantly, the NFκB response elements are the necessary and sufficient promoter sequence both for induction and for glucocorticoid-mediated inhibition (Mukaida et al. 1994; van de Stolpe et al. 1994; Caldenhoven et al. 1995).

The glucocorticoid receptor (GR) is the founding member of the intracellular receptor (IR) gene family (Tsai and O'Malley 1994; Yamamoto 1995). The GR zinc binding region (ZBR) harbors the DNA binding and protein dimerization functions of GR and is essential for regulation of all known GR target genes (Schena et al. 1989; Luisi et al. 1991). In the absence of hormone, the apoGR is localized to the cytoplasm in association with a molecular chaperone complex that interacts with the GR ligand binding domain (LBD) (Picard and Yamamoto 1987; Rusconi and Yamamoto 1987; Picard et al. 1988; Howard et al. 1990). Upon hormone binding, GR releases the chaperone complex and translocates to the cell nucleus. Truncations that remove the LBD, but retain a functional ZBR, are constitutively nuclear and transcriptionally active (Godowski et al. 1987).

Transcriptional regulatory factors adopt distinct activities depending on the sequence and context, cellular and physiological, of the particular response elements with which they associate (Miner and Yamamoto 1991; Lefstin and Yamamoto 1998). Regulatory factors bind and function at response elements in three different modes: simple, composite and tethering (Yamamoto et al. 1998). A regulatory factor binds directly to a simple response element, and it is the sole DNA binding factor necessary for regulation from that element. At a composite response element, the regulatory factor similarly binds directly to DNA, but its activities are defined by the composition of heterologous regulators that also bind to the element. In contrast, the regulatory factor does not bind DNA at a tethering response element, but rather associates via protein-protein interaction with a heterologous regulator that itself is specifically bound to DNA. For example, an AP-1 site near the collagenase gene serves both as a simple AP-1 response element and as a tethering glucocorticoid response element (GRE); from that site, AP-1 activates transcription, and GR represses without disrupting AP-1 binding, apparently by direct interaction with AP-1 (Konig et al. 1992). The fact that GR represses pro-inflammatory genes via NFκB response elements suggested to us that those elements might also serve as tethering GREs.

Two reports have appeared suggesting that glucocorticoids inhibit NFκB action by inducing IκB gene transcription (Auphan et al. 1995; Scheinman et al. 1995a). However, others demonstrated that the glucocorticoid effects occur independent of new protein synthesis (van de Stolpe et al. 1993; Wissink et al. 1998), or showed directly that GR inhibition of NFκB could be observed in the absence of glucocorticoid mediated IκBα or IκBβ gene induction (Brostjan et al. 1996; De Bosscher et al. 1997; Heck et al. 1997; Newton et al. 1998; Wissink et al. 1998; Adcock et al. 1999). These findings, together with reports that GR interacts selectively with the RelA subunit of NFκB (Ray and Prefontaine 1994; Caldenhoven et al. 1995; Scheinman et al. 1995b; Wissink et al. 1997), encouraged us to re-examine the tethering model, and to explore the mechanism by which GR inhibits NFκB mediated activation. In principle, GR could affect NFκB DNA binding, NFκB activation domain function, RNA polymerase II (pol II) preinitiation complex (PIC) formation, pol II phosphorylation, PIC isomerization or promoter clearance.

We carried out our studies *in vitro* and in CV-1 and A549 cultured cells. We used an *in vitro* protein-protein interaction assay to map segments of GR and RelA that interact. Transient transfections of CV-1 cells, which lack endogenous GR and RelA, enabled characterization of GR and RelA mutations, truncations and chimeras. In contrast, A549 cells express endogenous GR and NFκB; in these cells, the pro-inflammatory stimulus, TNFα (Newton et al. 1998) potentiates NFκB-mediated activation of the endogenous IL-8 and ICAM-1 promoters, and the synthetic glucocorticoid dexamethasone triggers GR-mediated repression. Together, we used these experimental approaches to determine whether GR blocks NFκB DNA binding, whether NFκB response elements are tethering GREs, and whether GR inhibition is activation domain specific. Finally, we probed the biochemical composition of the pol II complexes during inhibition at the endogenous IL-8 and ICAM-1 gene promoters, *in vivo*.

Results

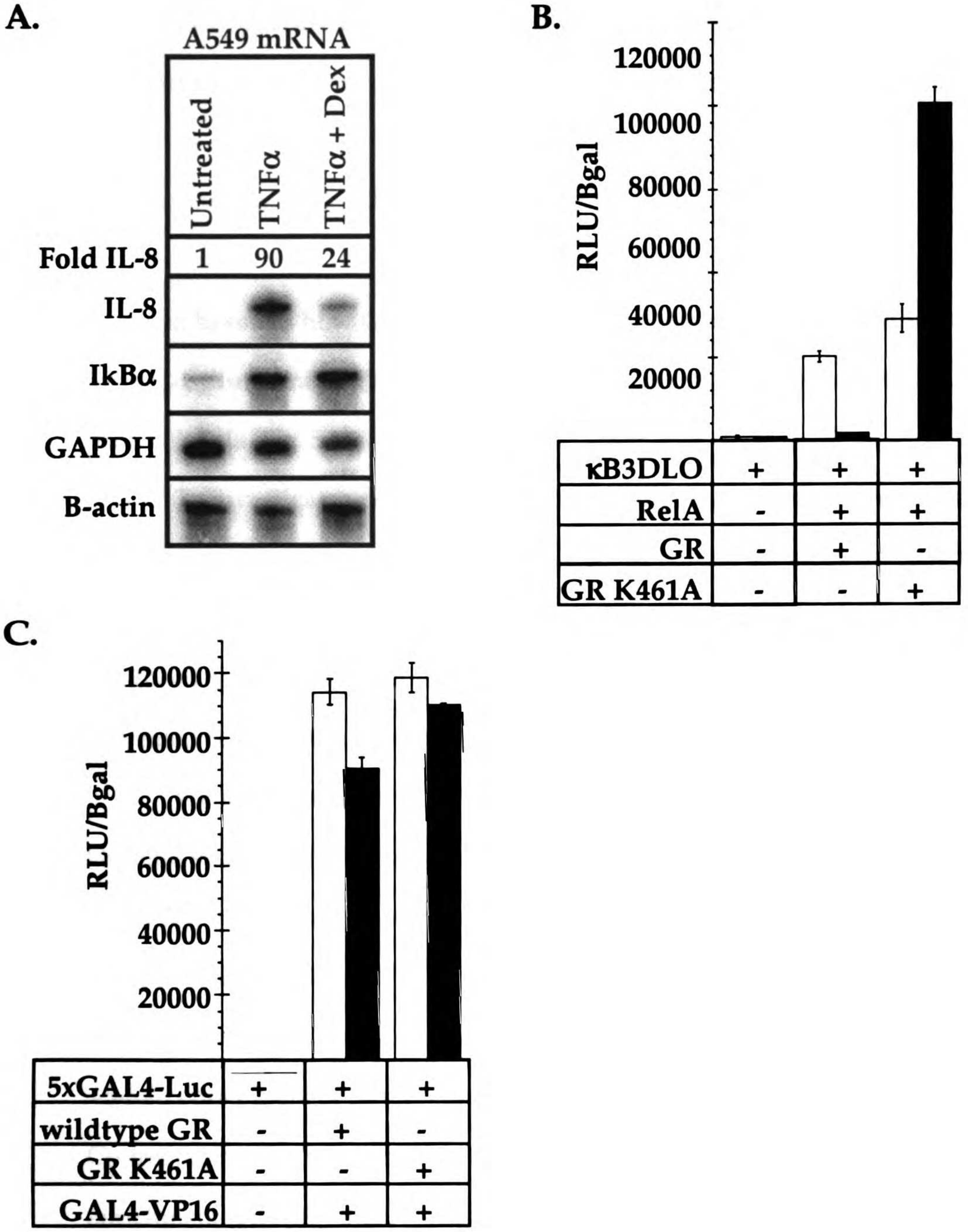
Direct repression of TNFα induced IL-8 transcription by GR

We focused on the IL-8 and ICAM-1 promoters in A549 cells because of their robust TNFα induction and glucocorticoid repression, strict dependence on NFκB response elements, and the physiological importance of IL-8 and ICAM-1 during inflammation (Mukaida et al. 1992; Harada et al. 1994; Mukaida et al. 1994; Caldenhoven et al. 1995). In these cells, which express endogenous GR, we found that IL-8 mRNA accumulation, measured by ribonuclease protection, was induced ~90-fold by TNFα, and that dexamethasone, a synthetic glucocorticoid, inhibited that induction by ~4-fold (Fig. 4A). As controls, we analyzed expression of the GAPDH and β-actin mRNAs, genes not regulated by either NFκB or GR, and did not detect significant alterations (Fig. 4A).

The GR-ZBR is required for both activation and repression of transcription (Schena et al. 1989), and response elements themselves can be allosteric effectors of GR activity (Lefstin et al. 1994; Lefstin and Yamamoto 1998). The response element signals appear to be detected or interpreted by rat GR residue K461 within the ZBR, as GR mutant K461A activates transcription from composite and tethering response elements even under conditions in which the wild type GR represses (Starr et al. 1996). Thus, if GR inhibits NFκB indirectly by inducing another factor such as IκBα, the K461A mutant should repress like wild type GR. On the other hand, if NFκB response elements are tethering GREs, then the GR mutant K461A might enhance rather than repress RelA activity.

Transient transfections of CV-1 cells revealed that the mutant indeed activated transcription from the NFκB reporter (Fig. 4B). The effect was specific to RelA as the unrelated transcriptional regulator GAL4-VP16 (Sadowski et al. 1988) was unaffected by either wild type or mutant GR (Fig. 4C). Moreover, dexamethasone treatment did not

Figure 4. The specificity of GR inhibition of NFκB activity, as demonstrated by analysis of the endogenous IL-8 gene in A549-k9 cells, and two synthetic reporter constructs in transiently transfected CV-1 cells. (A) Glucocorticoid regulation of the endogenous IL-8 gene in A549-k9 cells. A RNAase protection assay performed on total RNA harvested from A549-k9 cells left untreated (Un), treated for 2 hrs with 2.5ng/ml TNFα (TNFα), or co-treated for 2 hrs with 2.5ng/ml TNFα and 100nM dexamethasone (TNFα/Dex). (B) Inhibition of NFκB by wildtype GR and further activation by GR mutant K461A. Transiently transfected CV-1 cells comparing NFκB response element reporter alone, to cells co-transfected with RelA and GR, to cells co-transfected with RelA and GR mutant K461A. White bars are cells treated with ethanol vehicle, black bars are cells treated with 10nM dexamethasone. (C) The effect on GAL4-VP16 activity by wildtype GR or GR mutant K461A. Transiently transfected CV-1 cells comparing GAL4 response element reporter alone, to cells co-transfected with GAL4-VP16 and GR, to cells co-transfected with GAL4-VP16 and GR mutant K461A. White bars are cells treated with ethanol vehicle, black bars are cells treated with 10nM dexamethasone.


A.
A549 mRNA
Untreated
TNFα
TNFα + Dex
Fold IL-8
1
90
24
IL-8
IkBα
GAPDH
B-actin
B.
RLU/Bgal
120000
100000
80000
60000
40000
20000
κB3DLO + + +
RelA - + +
GR - + -
GR K461A - - +
C.
RLU/Bgal
120000
100000
80000
60000
40000
20000
5xGAL4-Luc + + +
wildtype GR - + -
GR K461A - - +
GAL4-VP16 - + +

significantly increase IκBα mRNA levels in the A549 cells (Fig. 4A). These results are consistent with the view that the NFκB site is a tethering GRE.

NFκB expression and DNA binding are unaffected by GR

To begin to assess how GR affects RelA function, we first examined A549 whole cell extracts by immunoblotting, and found the glucocorticoids had no effect on RelA protein levels (Fig. 5A).

To investigate whether glucocorticoids influence NFκB DNA binding *in vitro*, we analyzed A549 nuclear extracts using an electrophoretic gel mobility-shift assay with the IL-8 NFκB binding site as a probe (Fig. 5B). Extracts from TNFα-treated A549 cells produced a readily detectable RelA/p50-DNA complex relative to extracts from untreated cells, and this induced signal was undiminished by dexamethasone (Fig. 5B).

We used the chromatin immunoprecipitation assay (Braunstein et al. 1996; Orlando et al. 1997) to assess the effect of glucocorticoids on the occupancy *in vivo* of NFκB response elements associated with the IL-8 and ICAM-1 promoters (Fig. 6A). Normalized to the internal U6 snRNA control and relative to untreated cells, chromatin immunoprecipitation from TNFα treated cells with an antibody against RelA resulted in ~5-fold enrichment of sequences containing the IL-8 NFκB response element (Fig. 6B). Co-treatment of A549 cells with TNFα and dexamethasone yielded a similar ~5-fold enrichment (Fib. 3B); thus, consistent with the *in vitro* assay, glucocorticoids do not inhibit NFκB binding to these sites *in vivo*. Similarly, ICAM-1 promoter sequences were enriched ~3-fold upon gene activation and enrichment was unaffected by dexamethasone treatment (Fig. 6B). As controls, we monitored three additional sequence segments, none of which carries an NFκB site: a segment 700 base pairs upstream of the IL-8 NFκB site, and the promoter regions from the HSP70 and U6

Figure 5. The effect of glucocorticoid treatment of A549-k9 cells on the expression level and the *in vitro* DNA binding activity of the RelA protein. (A) An immunoblot performed using a RelA antibody (sc-372, Santa Cruz Biotechnology, Inc.) on equal amounts of whole cell extract from A549-k9 cells treated as described in Fig. 1A. (B) An electrophoretic gel-mobility shift assay performed using a ^{32}P-endlabeled NFκB response element from the IL-8 promoter and equal amounts of nuclear extract from A549 cells treated as in Fig. 1A. An arrow indicates the RelA/p50 heterodimer, as determined by pre-incubating the TNFα and dexamethasone co-treated extract (TD) with the RelA antibody sc-372, or the p50 antibody sc-114 (Santa Cruz Biotechnology, Inc.) blocking the specific bandshift (TD + sc-372, TD + sc-114). The normal rabbit IgG control did not affect the bandshift pattern (TD + IgG). To demonstrate the specificity of the bandshift pattern to the IL-8 probe, we added a 10-fold excess of either unlabeled IL-8 probe (10x cold) or collagenase AP-1 site (unspecific) to TD extract.

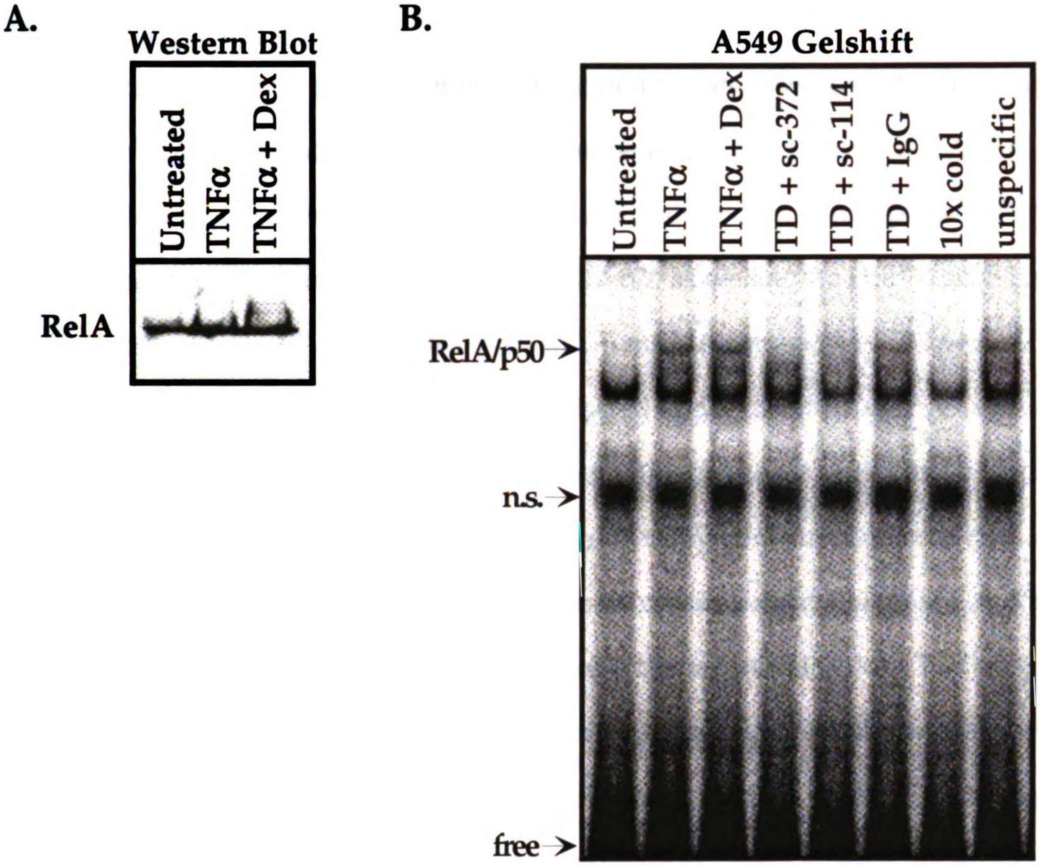
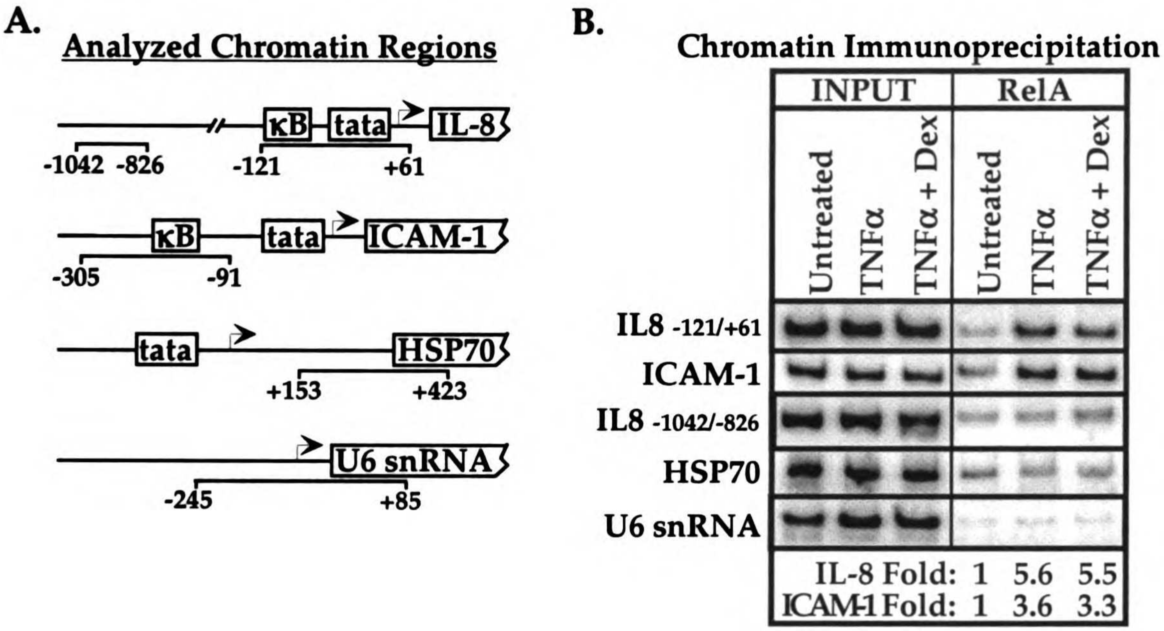

A.
Western Blot
Untreated
TNFα
TNFα + Dex
RelA
B.
A549 Gelshift
Untreated
TNFα
TNFα + Dex
TD + sc-372
TD + sc-114
TD + IgG
10x cold
unspecific
RelA/p50
n.s.
free

Figure 6. The effect of dexamethasone on NFκB response element occupancy, *in vivo*, as determined by chromatin immunoprecipitation analysis. (A) A schematic map for the analyzed chromatin regions. We chose the IL-8 promoter region -121/+61 and the ICAM-1 promoter region -305/-91 for use as the experimental probes. For controls, we chose the IL-8 promoter 5′-region -1042/-826 as well as the HSP70 gene region +153/+423 and the pol III transcribed U6 snRNA gene region -245/+85. The regions covered by the various probes are indicated under the particular gene promoter and coding region schematic. (B) The effect of GR on response element occupancy by RelA, *in vivo*. A chromatin immunoprecipitation assay performed using a RelA antibody (sc-109, Santa Cruz Biotechnology, Inc.) on chromatin extracts prepared as described in the materials and methods from A549-k9 cells treated as in Fig. 1A. The left panel (INPUT) shows that the starting chromatin extracts all had equal amounts of the probed regions, while the right panel (RelA) shows enrichment of the IL-8 and ICAM-1 experimental regions that contain the NFκB response element. The fold enrichment values for the experimental regions are shown relative to the untreated right lane and are normalized to the U6 snRNA band intensity.


A.
Analyzed Chromatin Regions
κB
tata
IL-8
-1042
-826
-121
+61
κB
tata
ICAM-1
-305
-91
tata
HSP70
+153
+423
U6 snRNA
-245
+85
B.
Chromatin Immunoprecipitation
INPUT
RelA
Untreated
TNFα
TNFα + Dex
Untreated
TNFα
TNFα + Dex
IL8 -121/+61
ICAM-1
IL8 -1042/-826
HSP70
U6 snRNA
IL-8 Fold: 1 5.6 5.5
ICAM-1 Fold: 1 3.6 3.3

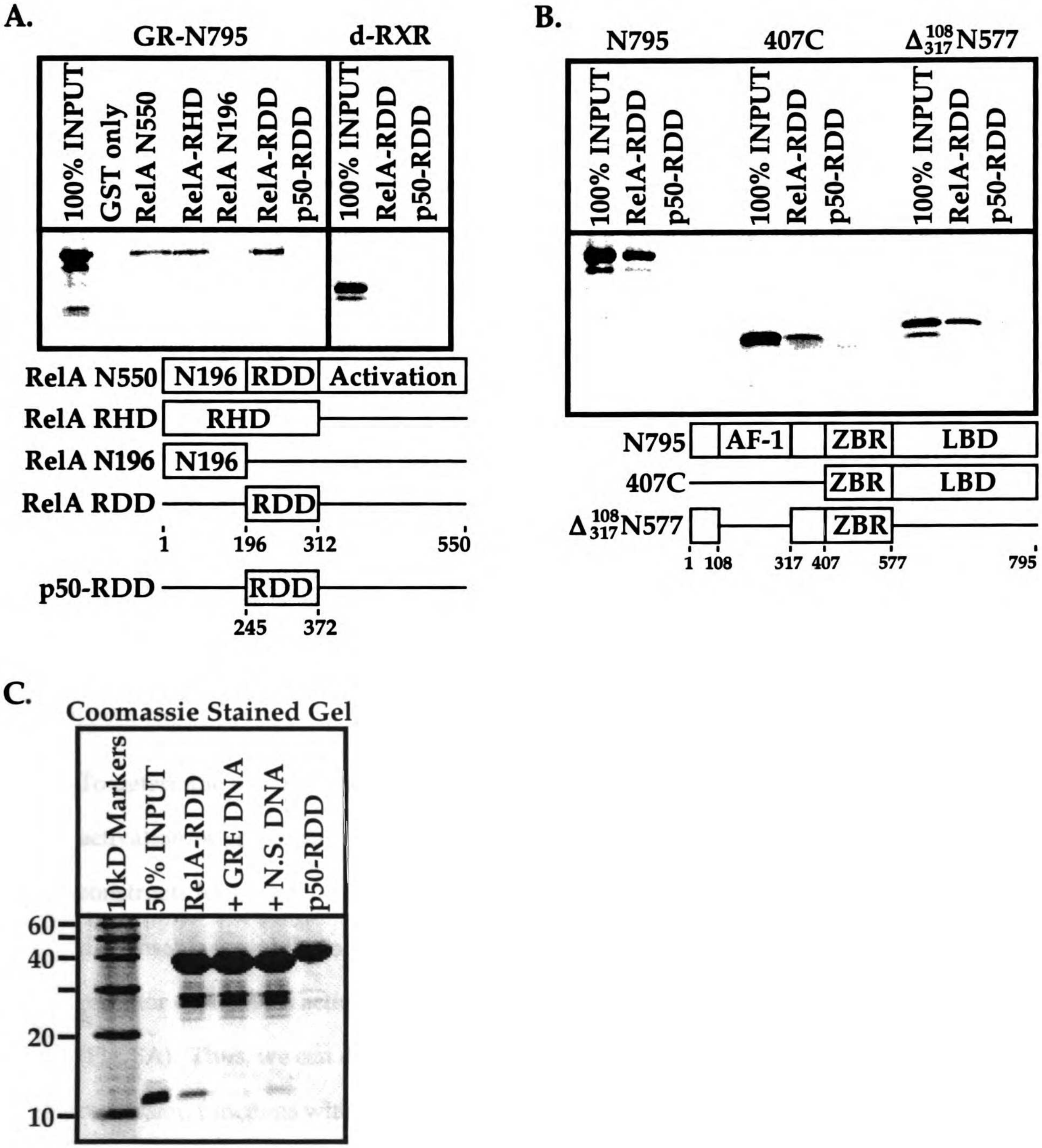
snRNA genes, which are transcribed by RNA polymerase II and III, respectively (Fig. 6B). These results lend further support to the tethering scheme, which requires site occupancy by NFκB during inhibition; it seems likely that glucocorticoids similarly affect other pro-inflammatory genes.

The GR-ZBR directly binds the RelA dimerization domain

In view of these findings, we characterized in greater detail the physical interaction between GR and RelA (Ray and Prefontaine 1994; Caldenhoven et al. 1995; Scheinman et al. 1995b; Wissink et al. 1997). We constructed a series of GST fusions bearing full length RelA, the rel homology domain (RHD) or its two subregions, the amino-terminal DNA interacting region and the rel dimerization domain (RDD) (Ghosh et al. 1995; Chen et al. 1998) (Fig. 7A); a fourth GST fusion contained the RDD from the p50 protein. In standard "pull down" assays (see Materials and Methods), we found that ^{35}S-methionine labeled GR bound to full length RelA and to the RHD, but not to the RelA amino-terminal segment; moreover, the RelA dimerization domain (RelA-RDD) was necessary and sufficient for the interaction (Fig. 7A). In contrast, the p50-RDD, which is 50% identical to the RelA-RDD, supported no significant interaction (Fig. 7A). As a negative control, the Drosophila RXR homologue, *ultraspiracle* (USP) (Oro et al. 1990), failed to interact with either RDD fusion (Fig. 7A). Interestingly, the GR mutant K461A displayed the same interaction profile as wild type GR (data not shown).

We next tested two ^{35}S-labeled GR deletion constructs for interaction with the RelA-RDD. We found that 407C, which lacks the amino-terminal 406 amino acids of GR but retaining the ZBR and LBD, and Δ(108-317)N577, which lacks most of the amino-terminus as well as the LBD (Fig. 7B), both remained competent for interaction with the RelA-RDD but not with the p50-RDD. As the two constructs have only the GR-ZBR in

Figure 7. The protein domains required for direct physical interaction between RelA and GR. (A) Protein-protein interaction domain mapping on the RelA protein. A protein-protein interaction assay performed as described in materials and methods using *in vitro* translated ^{35}S-labeled full length GR (N795) tested on full length RelA (RelA N550), the RelA-RHD, the amino-terminal 196 amino acids of RelA (RelA-N196), the RelA-RDD or the p50-RDD. The distantly related IR, d-RXR, included as a negative control, did not significantly interact with either the RelA-RDD or p50-RDD. The generated image is from a phosphorimager exposure of a SDS-PAGE gel. (B) Protein-protein interaction domain mapping on the GR protein. The same protein-protein interaction assay as in figure 7A, but using *in vitro* translated ^{35}S-labeled N795, 407C and Δ(108-317)N577 constructs of GR tested on the RelA-RDD and p50-RDD proteins. The generated image is from a phosphorimager exposure of a SDS-PAGE gel. (C) The test for direct interaction between the GR-ZBR and the RelA-RDD. The same protein-protein interaction assay as in figure 4A,B but using recombinant, purified GR-ZBR (amino acids 407-525). Where indicated, we included in the assay 200nM DNA for either an idealized simple GRE or the unrelated collagenase AP-1 site. The image is of a Coomassie stained SDS-PAGE gel.


A.
GR-N795
d-RXR
100% INPUT
GST only
RelA N550
RelA-RHD
RelA N196
RelA-RDD
p50-RDD
100% INPUT
RelA-RDD
p50-RDD
RelA N550
N196
RDD
Activation
RelA RHD
RHD
RelA N196
N196
RelA RDD
RDD
1
196
312
550
p50-RDD
RDD
245
372
B.
N795
407C
Δ^{108}_{317}N577
100% INPUT
RelA-RDD
p50-RDD
100% INPUT
RelA-RDD
p50-RDD
100% INPUT
RelA-RDD
p50-RDD
N795
AF-1
ZBR
LBD
407C
ZBR
LBD
Δ^{108}_{317}N577
ZBR
1
108
317
407
577
795
C.
Coomassie Stained Gel
10kD Markers
50% INPUT
RelA-RDD
+ GRE DNA
+ N.S. DNA
p50-RDD
60
40
20
10

common, the simplest interpretation is that the GR-ZBR associates with the RelA-RDD (Fig. 7B).

To test directly this interaction, we repeated these assays using purified recombinant components visualized by Coomassie staining (Luisi et al. 1991). The GR-ZBR, competent for DNA binding (data not shown), specifically bound to the RelA-RDD and not to the p50-RDD (Fig. 7C). Interestingly, an oligonucleotide bearing a simple GRE DNA binding site abrogated the RelA-RDD interaction, whereas a nonspecific oligonucleotide was less effective (Fig. 7C). We conclude that these regulatory proteins interact directly, and suggest that GR binding to a simple GRE or to the RelA-RDD are mutually exclusive due either to overlapping interaction surfaces or to an allosteric change elsewhere in the ZBR triggered by the response element.

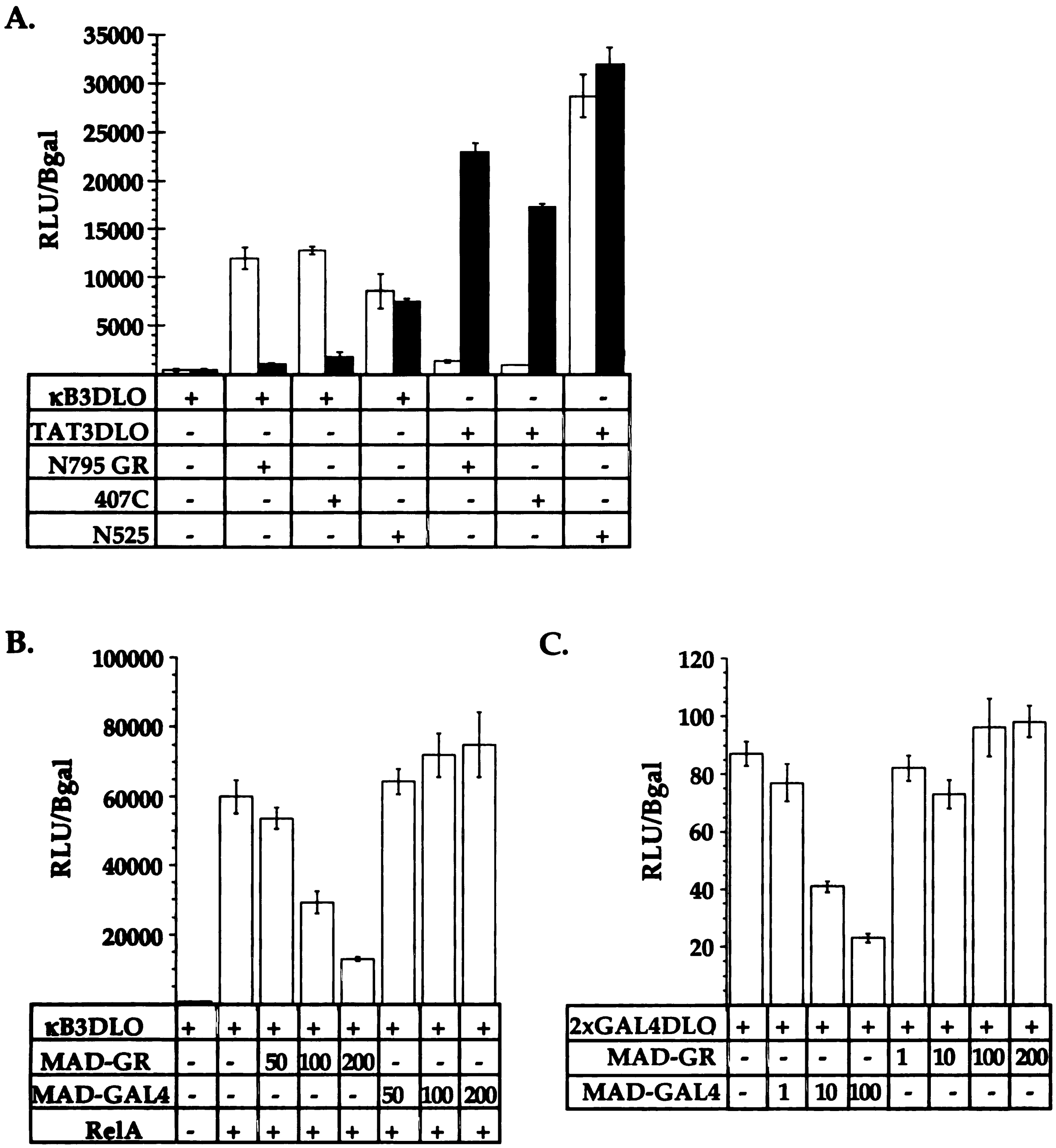
The GR-LBD harbors repression activity

To determine which domains of GR are required for inhibition of RelA mediated activation, we performed transient transfections of CV-1 cells testing GR deletion constructs for inhibition of RelA activity. The amino-terminal 406 residues of GR was dispensable for inhibition while the LBD was essential (Fig. 8A). As a control, both receptor derivatives activated transcription from the simple GRE reporter, TAT3-DLO (Fig. 8A). Thus, we can distinguish the protein-protein interaction from the RelA repression functions within GR.

The GR-ZBR associates with RelA at NFκB response elements

If the protein-protein interaction observed *in vitro* occurs *in vivo*, then fusion of the GR-ZBR to a heterologous repression domain, such as that from the Mad1 protein, might

Figure 8. Functional domain mapping on GR and functional test of the Mad-GR chimera. (A) Functional analysis of GR deletion constructs. Transiently transfected CV-1 cells comparing NFκB response element reporter alone, to cells co-transfected with RelA and GR N795, to cells co-transfected with RelA and GR deletion 407C, to cells co-transfected with RelA and GR deletion N525. Also included for comparison, are cells transiently co-transfected using simple GRE reporter (TAT3DLO) with GR N795, TAT3DLO with GR deletion 407C, and TAT3DLO with GR deletion N525. White bars are cells treated with ethanol vehicle, black bars are cells treated with 10nM dexamethasone. (B) Effect of the Mad-GR chimera on RelA activity. Transiently transfected CV-1 cells comparing NFκB response element reporter alone, to cells co-transfected with RelA and increasing amounts (in nanograms) of Mad-GR expression vector, to cells co-transfected with RelA and increasing amounts (in nanograms) of Mad-GAL4 expression vector. (C) Effect of the Mad-GAL4 chimera on basal transcription of the GAL4 response element reporter, 2xGAL4-DLO. Transiently transfected CV-1 cells comparing 2xGAL4-DLO alone, to cells co-transfected with RelA and increasing amounts (in nanograms) of Mad-GAL4 expression vector, to cells co-transfected with RelA and increasing amounts (in nanograms) of Mad-GR expression vector.



κB3DLO	+	+	+	+	-	-	-
TAT3DLO	-	-	-	-	+	+	+
N795 GR	-	+	-	-	+	-	-
407C	-	-	+	-	-	+	-
N525	-	-	-	+	-	-	+

κB3DLO	+	+	+	+	+	+	+	+
MAD-GR	-	-	50	100	200	-	-	-
MAD-GAL4	-	-	-	-	-	50	100	200
RelA	-	+	+	+	+	+	+	+

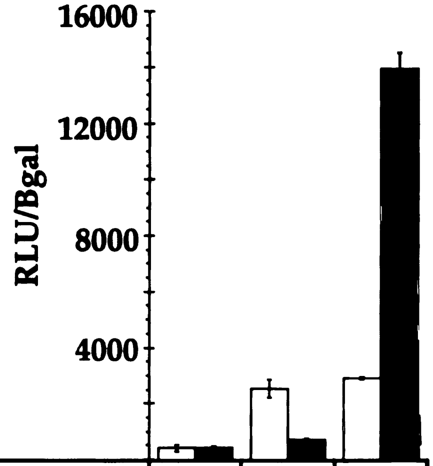
2xGAL4DLO	+	+	+	+	+	+	+	+
MAD-GR	-	-	-	-	1	10	100	200
MAD-GAL4	-	1	10	100	-	-	-	-

create a new repressor of RelA activity. Mad1 is a member of the Mad/Myc/Max family of transcriptional regulators that functions by recruiting the N-CoR/mSin-3A/SMRT histone deacetylase (HDAC) containing complex (Ayer et al. 1996; Alland et al. 1997; Hassig et al. 1997; Heinzel et al. 1997; Laherty et al. 1997). We transiently transfected CV-1 cells with constructs expressing the Mad1 repression domain fused either to the GR-ZBR (Mad-GR) or to the unrelated GAL4-DBD (Mad-GAL4) (Fig. 5B, 5C). The Mad-GR construct inhibited RelA mediated activation while the control Mad-GAL4 construct did not (Fig. 8B). Both constructs were functional and specific since Mad-GAL4 inhibited basal transcription from a GAL4 site-driven reporter while Mad-GR had no effect (Fig. 8C). Notably, Mad-GR was less potent than wildtype GR, perhaps due to intrinsic differences between the Mad1 repression domain and GR-LBD. Regardless, the GR-ZBR is necessary and sufficient to associate with RelA *in vivo.*

The GR inhibitory function operates on a heterologous activation domain

The yeast GAL80 protein negatively regulates galactose inducible genes by selectively binding and inhibiting the yeast GAL4 transcriptional activation domain (Lue et al. 1987; Melcher and Johnston 1995; Grant et al. 1997; Yano and Fukasawa 1997; Ansari et al. 1998; Sil et al. 1999). To test whether the GR-LBD repression function is similarly limited to only the cognate activation domain of RelA, we constructed a fusion between the RelA-RHD and the activation domain from the herpesvirus VP16 protein (Triezenberg et al. 1988). The VP16 activation domain was insensitive to regulation by GR when fused to the GAL4-DBD (Fig. 4B). However, in transient transfections of CV-1 cells, we found that wild type GR inhibited the RHD-VP16 fusion protein whereas GR mutant K461A activated it (Fig. 9). Thus, the inhibitory functions of GR are not dedicated to a single activation domain perhaps suggesting that it affects components of the basal transcription machinery.

Figure 9. Effect of GR on the activity of the RHD-VP16 chimera. Transiently transfected CV-1 cells comparing NFκB response element reporter alone, to cells co-transfected with RHD-VP16 chimera and GR, to cells co-transfected with RHD-VP16 chimera and GR mutant K461A. White bars are cells treated with ethanol vehicle, black bars are cells treated with 10nM dexamethasone.



κB3DLO	+	+	+
RHD-VP16	-	+	+
wildtype GR	-	+	-
GR K461A	-	-	+

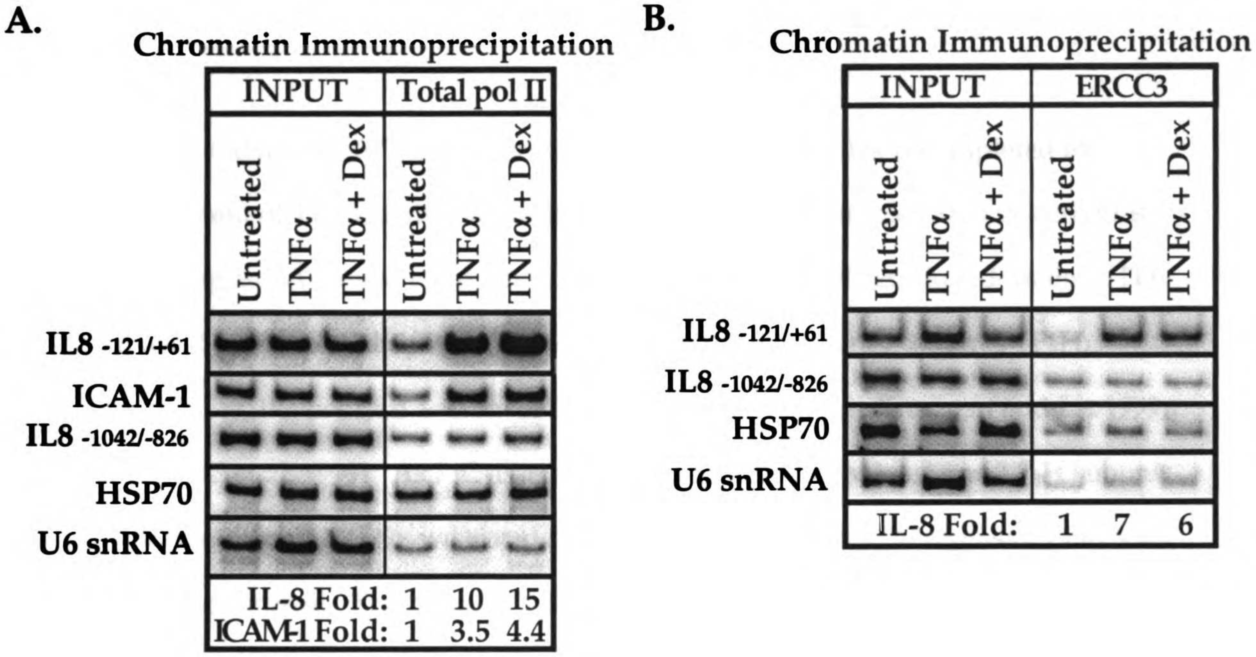
GR represses transcription after preinitiation complex (PIC) assembly

The minimal preinitiation complex (PIC) contains the general transcription factors (GTFs) $\mathrm{TF_{II}A}$, $\mathrm{TF_{II}B}$, $\mathrm{TF_{II}D}$, $\mathrm{TF_{II}F}$ and RNA polymerase II (pol II). The $\mathrm{TF_{II}D}$ complex contains the TATA-binding protein (TBP) and several TBP associated factors (TAFs) which together initiate PIC assembly by recognizing the TATA box and/or initiator promoter elements. $\mathrm{TF_{II}B}$ is incorporated second, followed by the $\mathrm{TF_{II}F}$-pol II complex (Zawel and Reinberg 1993; Roeder 1996). In an alternative view, PICs might assemble by directly recruiting a pol II "holoenzyme" containing a subset of the GTFs (Koleske and Young 1994).

To determine whether GR inhibits NFκB mediated activation by interfering with PIC assembly, we performed the chromatin immunoprecipitation assay using an antibody against the amino-terminus of the pol II large subunit (Fig. 10A). This antibody recognizes both the unphosphorylated (IIa) and the phosphorylated (IIo) forms of pol II allowing determination of total pol II (IIa + IIo) recruitment. Treatment of A549 cells with TNFα stimulated pol II occupancy of the IL-8 and ICAM-1 promoter regions by ~10-fold and ~3-fold, respectively (Fig. 10A).

Notably, under conditions of repression by dexamethasone, total pol II promoter occupancy was further increased to ~15-fold and ~4-fold, respectively (Fig. 10A). Regulated pol II recruitment was promoter region-specific as control sequences 700 base pairs upstream were not significantly enriched, and the inducing or repressing signals provoked no appreciable effects on pol II occupancy of the HSP70 or U6 snRNA genes (Fig. 10A). As expected, pol II was readily detectable at HSP70 (Fig. 10A) relative to the U6 snRNA gene (Birnstiel 1988) (Fig. 10A), as a stalled, unphosphorylated pol II complex just downstream of this promoter in non-heat shocked cells (O'Brien et al. 1994). Thus, we conclude that GR represses NFκB mediated activation by interfering with a step after PIC assembly, perhaps affecting initiation or promoter clearance.

Figure 10. Effect of TNFα and glucocorticoids on promoter recruitment of pol II and the $\mathrm{TF_{II}H}$ helicase subunit ERCC3. (A) The effect of GR on promoter occupancy by the pol II large subunit, *in vivo*. A chromatin immunoprecipitation assay performed using a pol II antibody (sc-899, Santa Cruz Biotechnology, Inc.) on chromatin extracts prepared as described in the materials and methods from A549-k9 cells treated as in Fig. 1A. The left panel (INPUT) shows that the starting chromatin extracts all had equal amounts of the probed regions, while the right panel (total pol II) shows enrichment of the IL-8 and ICAM-1 experimental regions. The fold enrichment values for the experimental regions are shown relative to the untreated right lane. (B) The effect of GR on promoter occupancy by the $\mathrm{TF_{II}H}$ helicase subunit ERCC3, *in vivo*. A chromatin immunoprecipitation assay performed using an ERCC3 antibody (sc-293, Santa Cruz Biotechnology, Inc.) on chromatin extracts prepared as described in the materials and methods from A549-k9 cells treated as in Fig. 1A. The left panel (INPUT) shows that the starting chromatin extracts all had equal amounts of the probed regions, while the right panel (ERCC3) shows enrichment of the IL-8 experimental region. The fold enrichment values are shown relative to the untreated right lane and are normalized to the U6 snRNA band intensity.


A.
Chromatin Immunoprecipitation
INPUT
Total pol II
Untreated
TNFα
TNFα + Dex
Untreated
TNFα
TNFα + Dex
IL8 -121/+61
ICAM-1
IL8 -1042/-826
HSP70
U6 snRNA
IL-8 Fold: 1 10 15
ICAM-1 Fold: 1 3.5 4.4
B.
Chromatin Immunoprecipitation
INPUT
ERCC3
Untreated
TNFα
TNFα + Dex
Untreated
TNFα
TNFα + Dex
IL8 -121/+61
IL8 -1042/-826
HSP70
U6 snRNA
IL-8 Fold: 1 7 6

PIC incorporation of $TF_{II}H$ is unaffected by GR-mediated repression

In "ordered assembly" models of PIC formation, the complex is completed by recruitment of $TF_{II}H$, a nine-subunit GTF that contains multiple catalytic activities, including an ATP-dependent DNA helicase and a carboxyl-terminal domain (CTD) kinase (Svejstrup et al. 1996). Promoter melting and clearance requires $TF_{II}H$; specifically, the helicase activity of the XPB/ERCC3 subunit (Guzman and Lis 1999; Moreland et al. 1999). As PIC assembly is independent of at least one $TF_{II}H$ subunit (Kuras and Struhl 1999), we tested the possibility that GR might repress by blocking $TF_{II}H$ recruitment. Treatment of A549 cells with TNFα induced ~7-fold recruitment of the XPB/ERCC3 DNA helicase subunit of $TF_{II}H$ to the IL-8 promoter region, and this recruitment was unaffected by co-treatment with dexamethasone (Fig. 10B). As controls, XPB/ERCC3 recruitment was promoter specific, as sequences 700 base pairs upstream from the IL-8 promoter were not enriched, and there was no appreciable effect on the HSP70 and U6 snRNA control chromatin regions (Fig. 10B). Thus, incorporation of XPB/ERCC3, and likely the whole nine-subunit $TF_{II}H$ complex, into PICs is unaffected by glucocorticoid repression of NFκB mediated activation.

Phosphorylation of CTD serine-5 is unaffected by GR-mediated repression

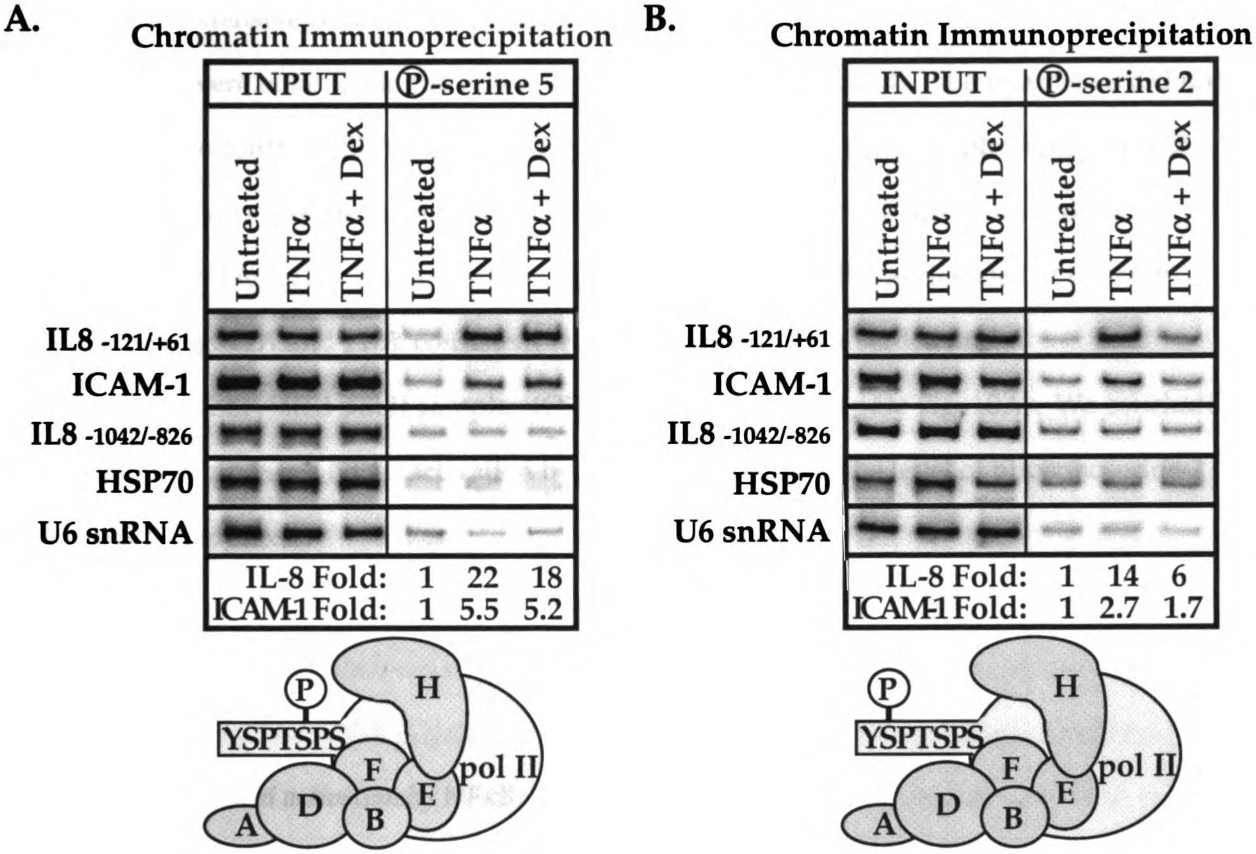
The largest subunit of mammalian pol II includes a carboxyl-terminal domain (CTD) consisting of 52 tandem repeats of a heptapeptide (YSPTSPS) that is essential for viability and is conserved among eukaryotes (Allison et al. 1985; West and Corden 1995). The CTD is unphosphorylated during PIC assembly, but initiation is accompanied by cooperative phosphorylation of the heptapeptide serine-2 and serine-5 residues (Nonet et al. 1987; Bartolomei et al. 1988; Cismowski et al. 1995; West and Corden 1995; Parada and Roeder 1996; Lee and Lis 1998; Trigon et al. 1998; Kuras and Struhl 1999). The cdk7 subunit of $TF_{II}H$ selectively phosphorylates the CTD heptapeptide at serine-5 (Trigon et al. 1998). This phosphorylation event appears to be essential for transcription of most genes (Cismowski et al. 1995; Kuras and Struhl 1999).

In chromatin immunoprecipitation assays using a monoclonal antibody specific for the phosphoserine-5 heptapeptide repeat, we found that TNFα induction provoked a ~22-fold increase in binding of pol II bearing this modification at the IL-8 promoter and that dexamethasone repression had little effect (Fig. 11A); parallel results were obtained at the ICAM-1 promoter though to a lesser extent. Control regions of the HSP70 and U6 snRNA genes were unaffected and phosphoserine-5 pol II was not observed upstream of the IL-8 promoter region (Fig. 11A). These results imply that a phosphorylation event essential for transcription initiation, possibly mediated by $TF_{II}H$-associated cdk7, occurs even under repressing conditions (Fig. 10B). Formally, however, because maintenance of phosphoserine-5 on just one of the 52 heptapeptide repeats might be sufficient for a positive signal in this assay, a potentially substantial effect of repression might go undetected.

Phosphorylation of CTD serine-2 is reduced by GR-mediated repression

Phosphorylation of the serine-2 residue of the CTD heptapeptide repeat also accompanies transcription initiation; this modification is essential for viability and has been demonstrated to be required for transcription of various genes in S. *cereviseae* (West and Corden 1995; Trigon et al. 1998; Patturajan et al. 1999). To determine whether glucocorticoids affect phosphorylation of the CTD serine-2 position, we used a monoclonal antibody specific for the phosphoserine-2 heptapeptide repeat in the chromatin immunoprecipitation assay (Fig. 11B). Treatment with TNFα induced ~14-fold recruitment of phosphoserine-2 pol II to the IL-8 gene, whereas recruitment was reduced to 6-fold under repressing conditions; as noted above, this effect corresponds to

Figure 11. Effect of TNFα and glucocorticoids on CTD-phosphorylation of the promoter bound pol II large subunit. (A) The effect of GR on CTD serine-5 phosphorylation, *in vivo*. A chromatin immunoprecipitation assay performed using a phosphoserine-5 monoclonal antibody (H14, BAbCO, Inc.) on chromatin extracts prepared as described in the materials and methods from A549-k9 cells treated as in Fig. 1A. The left panel (INPUT) shows that the starting chromatin extracts all had equal amounts of the probed regions, while the right panel (P-serine 5) shows enrichment of the IL-8 and ICAM-1 experimental regions. The fold enrichment values for the experimental regions are shown relative to the untreated right lane. (B) The effect of GR on CTD serine-2 phosphorylation, *in vivo*. A chromatin immunoprecipitation assay performed using a phosphoserine-2 monoclonal antibody (H5, BAbCo, Inc.) on chromatin extracts prepared as described in the materials and methods from A549-k9 cells treated as in Fig. 1A. The left panel (INPUT) shows that the starting chromatin extracts all had equal amounts of the probed regions, while the right panel (P-serine 2) shows enrichment of the IL-8 and ICAM-1 experimental regions. The fold enrichment values for the experimental regions are shown relative to the untreated right lane and are normalized to the U6 snRNA band intensity.


A.
Chromatin Immunoprecipitation
INPUT
Ⓟ-serine 5
Untreated
TNFα
TNFα + Dex
Untreated
TNFα
TNFα + Dex
IL8 -121/+61
ICAM-1
IL8 -1042/-826
HSP70
U6 snRNA
IL-8 Fold: 1 22 18
ICAM-1 Fold: 1 5.5 5.2
P
YSPTSPS
H
F
pol II
D
E
A
B
B.
Chromatin Immunoprecipitation
INPUT
Ⓟ-serine 2
Untreated
TNFα
TNFα + Dex
Untreated
TNFα
TNFα + Dex
IL8 -121/+61
ICAM-1
IL8 -1042/-826
HSP70
U6 snRNA
IL-8 Fold: 1 14 6
ICAM-1 Fold: 1 2.7 1.7
P
YSPTSPS
H
F
pol II
D
E
A
B

a very extensive reduction in CTD phosphorylation at the serine-2 position. Parallel results were obtained in the ICAM-1 promoter region (Fig. 11B). The treatments did not affect the control HSP70 and U6 snRNA promoter regions and phosphoserine-2 pol II binding was specific to the IL-8 promoter region relative to the upstream control segment (Fig. 11B). In addition, little phosphorylated pol II was detected at the HSP70 gene (Fig. 11A and 11B), consistent with its occupancy by a stalled and unphosphorylated pol II complex (O'Brien et al. 1994) (compare Fig. 10A). We conclude that GR represses NFκB activation by selectively reducing the level of phosphoserine-2 pol II complexes.

Discussion

Transcriptional activation by NFκB

RelA interacts with the general coactivators CBP and p300, which act in part as histone acetyltransferases (HAT) (Zhong et al. 1998), increasing factor accessibility to chromatin packaged templates (Grunstein 1997). RelA also interacts with the ARC/DRIP coactivator complex (Naar et al. 1999), which stimulates PIC assembly by forming a physical bridge between activation domains and pol II (Chiba et al. 2000). In addition, the RelA activation domain itself interacts functionally and physically with several GTFs including $TF_{II}B$, TBP and $TAF_{II}105$ (Schmitz et al. 1995; Yamit-Hezi and Dikstein 1998). Collectively, these findings imply that NFκB stimulates PIC assembly by multiple mechanisms. At the IL-8 and ICAM-1 promoters, we showed that RelA indeed enhances PIC assembly, producing a complete and fully phosphorylated promoter bound pol II complex (Figs. 7 and 8). Thus, at these response elements, we conclude that RelA achieves transcriptional activation using a multifunctional activation domain that stimulates several facets of PIC assembly, likely affecting, directly or indirectly, CTD phosphorylation as well.

Pro-inflammatory gene NFκB sites are tethering GREs

The fact that GR regulates NFκB response elements without direct binding to the DNA suggested to us that these sequences are tethering GREs. Consistent with this view, we showed in chromatin immunoprecipitation assays that the RelA protein was not displaced from promoter DNA under repressing conditions (Fig. 6B). Efforts to detect GR at tethering response elements have been only sporadically successful in our lab (R. Nissen, et al., unpublished observations). However, several lines of evidence support the conclusion that GR is indeed tethered. First, GR mutant K461A further enhanced RelA driven transcription (Fig. 4B). Second, the GR-ZBR interacted directly with the RelA-RDD in a reaction that is inhibited by a GRE oligonucleotide (Fig. 7C). Finally, the MAD-ZBR fusion protein was selectively and functionally recruited to RelA *in vivo* (Fig. 5B,C). We conclude that pro-inflammatory gene NFκB response elements serve as tethering GREs.

These findings indicate that regulators that carry DNA binding domains can be recruited into certain regulatory complexes through protein-protein rather than protein-DNA interactions. How general this mode of regulation might be is unknown, but it is clearly not limited to intracellular receptors such as GR. For example, the yeast Ste12 protein regulates a-specific genes by directly binding simple Ste12 response elements; in contrast, at α-specific genes, Ste12 tethers to the α1 protein, which, together with MCM1, binds to response element sequences (Yuan et al. 1993). Therefore, the α1/MCM1 composite response elements are tethering response elements for Ste12. Tethering demonstrates strikingly that "DNA binding domains" in fact can carry multiple functional surfaces whose utilization is contextually determined.

Repression affects a step after activation domain function

Unlike GAL80, GR can repress transcription mediated by more than one activation domain (i.e. NFκB and AP-1). Thus, it was unsurprising that the GR-LBD inhibitory function was activation domain independent (Fig. 9). Moreover, GR bypassed the multiple distinct mechanisms by which NFκB appears to stimulate PIC assembly, instead repressing initiation itself by interfering with pol II CTD phosphorylation (Fig. 7 and 8). These findings demonstrate a global repression mechanism, and underscore the notion that regulation of another regulator need not operate by enhancing or suppressing the effects of the targeted regulator.

In vivo detection of a hemi-phosphorylated pol II species?

The lack of intermediate species migrating on SDS-PAGE gels between the unphosphorylated IIa and the maximally phosphorylated IIo forms suggests that CTD-kinases and CTD-phosphatases are highly processive (Lehman and Dahmus 2000). However, it has not been proven that a single heptapeptide repeat can be phosphorylated at multiple positions. Nor has it been determined whether phosphorylation by one enzyme influences phosphorylation by another (*e.g.*, can cdk7-mediated conversion of YSPTSPS to YSPTS*PS be further processed by another kinase to YS*PTS*PS, or *vice-versa?).* It is nevertheless apparent that the different phosphorylation sites can be functionally distinct. For example, mammalian mRNA capping activity is stimulated by CTD peptides phosphorylated at serine-5 but not at serine-2 (Ho and Shuman 1999). Our findings suggest that glucocorticoids repress NFκB activity by generating a transcriptionally inactive DNA bound pol II species phosphorylated at serine-5, but not at serine-2. Notably, Bonnet et al. (1999) detected a similar pol II species, denoting it pol IIm. We speculate that pol IIm and the pol II species we detect are identical and represent one of several hemi-phosphorylated pol II isoforms. The specificity of the glucocorticoid effects reveal that differential post-translational modifications of pol II are an important facet of gene regulation.

Recruitment of a TSA resistant corepressor?

Acetylation of nucleosomes appears to facilitate gene activation by increasing factor access to genomic binding sites (Grunstein 1997; Blackwood and Kadonaga 1998). Some (Mizzen et al. 1996; Chen et al. 1997; Grant et al. 1997; Blanco et al. 1998; Kraus and Kadonaga 1998) but not all (Naar et al. 1999; Orphanides et al. 1999; Rachez et al. 1999; Ryu et al. 1999; Rachez et al. 2000) coactivator complexes harbor HAT activity. Conversely, the IR corepressors identified to date carry HDAC activity; both coactivators and corepressors interact with the LBD (Heery et al. 1997; Nagy et al. 1997; Darimont et al. 1998; Voegel et al. 1998; Perissi et al. 1999).

Repression of AP-1 mediated activation by the thyroid hormone receptor is blocked by trichostatin A (TSA) (M. Cronin and K.R.Y., unpublished observations), a general inhibitor of most HDACs (Taunton et al. 1996). In contrast, GR regulation of AP-1 activity, at the same response element used in the TR experiments, is TSA resistant, even at micromolar concentrations (M. Cronin and K.R.Y., unpublished observations). Similarly, we found that GR repression of NFκB activity was TSA resistant in A549 cells (data not shown). Together with our findings that neither PIC assembly (Fig. 10A) nor PIC incorporation of $TF_{II}H$ (Fig. 10B) are affected by glucocorticoid-mediated repression, we suggest that promoter occlusion or HDAC recruitment are unlikely. In principle, the GR-LBD might have an intrinsic activity for blocking CTD serine-2 phosphorylation, but the modularity of regulatory complexes leads us to postulate the existence of a novel corepressor, recruited by the GR-LBD, that selectively interferes with pol II CTD phosphorylation at serine-2.

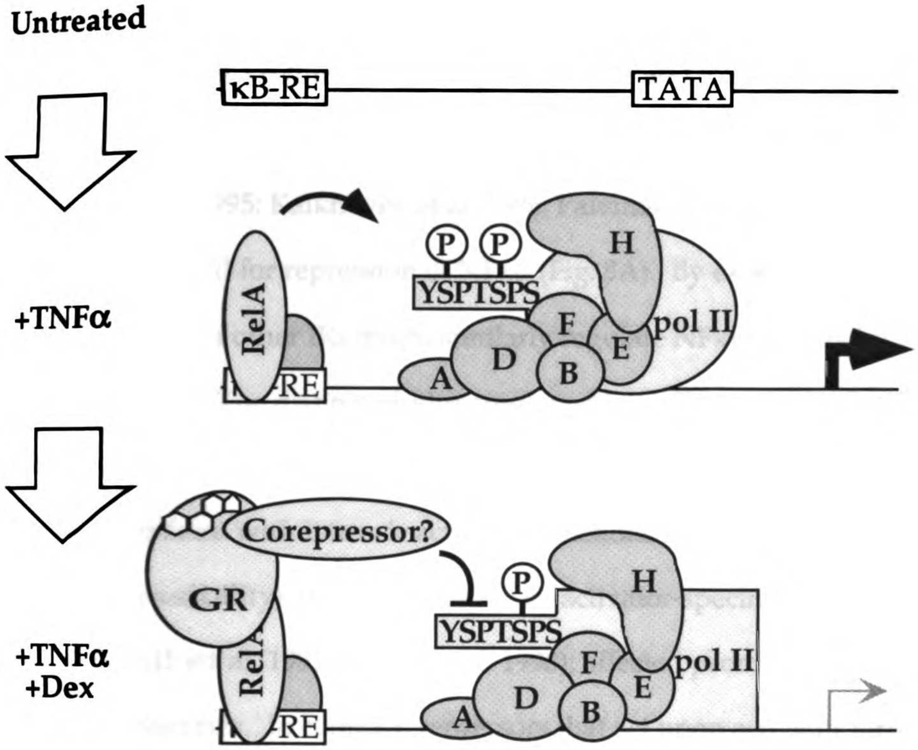
Specifically, we propose that the GR-LBD recruits the novel corepressor to tethering GREs in a manner similar to the the characterized cofactors (Fig. 12), and that the resultant regulatory complex yields complete PICs deficient in serine-2 phosphorylation, with a consequent decline in initiation or promoter clearance. The putative corepressor might be a serine-2 phosphatase or a serine-2 kinase inhibitor. The CTD phosphatase FCP1 completely dephosphorylates the CTD before assembly into PICs and thus seems unlikely to be the putative corepressor (Cho et al. 1999). However, there are four identified human FCP1 homologs (Archambault et al. 1997), one of which might encode a specific CTD serine-2 phosphatase.

An intriguing potential kinase target for GR is the mammalian CTD-kinase cdk9, a subunit of the positive transcript elongation factor (P-TEFb) (Price 2000). Cdk9 is a homolog of the yeast CTK1 gene; ctk1 deletion strains (ctk1Δ) display slow growth and cold sensitive phenotypes, with reduced transcription of various genes (Lee and Greenleaf 1991; Patturajan et al. 1999). Because ctk1Δ reduces phosphorylation of the pol II CTD at serine-2 (Patturajan et al. 1999), it is tempting to speculate that cdk9 might act similarly. Notably, CTK1 is not the only yeast serine-2 kinase (Patturajan et al. 1999); hence, higher eukaryotes are also likely to carry multiple kinases that affect this residue of the CTD repeat. Thus, GR might target, either directly or through recruitment of a kinase inhibitor, a cdk9-like factor.

Mechanisms for transcriptional repression

Several members of the IR gene family including mineralocorticoid receptor (Liden et al. 1997), estrogen receptor (ER) (Ray et al. 1994; Stein and Yang 1995), progesterone receptor (PR) (Caldenhoven et al. 1995; Kalkhoven et al. 1996), androgen receptor (AR)

Figure 12. A model for repression of NFκB mediated activation by GR. TNFα treatment induces NFκB response element binding, PIC assembly and transcription. Dexamethasone treatment induces nuclear localization of GR with concomitant tethering at RelA occupied response elements. The GR-LBD, perhaps via recruitment of a novel corepressor, bypasses RelA activation domain functions interfering with phosphorylation of the pol II CTD at serine-2.


Untreated
κB-RE
TATA
+TNFα
RelA
RE
P
P
YSPTSPS
H
F
pol II
D
A
B
E
+TNFα
+Dex
GR
Corepressor?
RelA
RE
P
YSPTSPS
H
F
pol II
D
A
B
E

(Palvimo et al. 1996), thyroid hormone receptor (Nissen and Yamamoto, unpublished data), and retinoic acid receptor (Nissen and Yamamoto, unpublished data) can regulate NFκB mediated activation. Consistent with the results with PR, ER, and AR (Stein and Yang 1995; Kalkhoven et al. 1996; Palvimo et al. 1996), we found that the GR-LBD was essential for repression of NFκB (Fig. 8A). By extension, it seems likely that these and perhaps other IRs might similarly regulate NFκB activity by affecting the pol II CTD.

The distinct modes employed by different transcriptional regulators can provide insights about the contexts in which those regulators might function. Repressors that interfere with intrinsic activator functions (e.g., DNA binding or activation domain accessibility) are more likely to be activator-specific (Baeuerle and Baltimore 1988b; Small et al. 1991; Ansari et al. 1998); affected promoters could still be activated by others activators. In contrast, repressors that act upon activator targets or initiation events downstream of those targets would function more globally on the affected promoters; repressors that affect late steps of initiation would down regulate targeted promoters regardless of the range or mechanisms of associated activators. For example, our findings indicate that GR bypasses RelA stabilization of PIC assembly by affecting CTD phosphorylation at serine-2. Likewise, the *Caenorhabditis elegans* repressor protein PIE-1 appears to inhibit all pol II transcription in germline blastomeres by preventing phosphorylation of the CTD at serine-2 (Batchelder et al. 1999). Similarly, the cdc2/cyclinB kinase complex mediates global mitotic repression of transcription by phosphorylating and inactivating the CTD-kinase of $TF_{II}H$ (Long et al. 1998).

Multiple signaling networks regulate natural promoters, requiring that pol II integrate multiple inputs from positive and negative regulators. This combinatorial approach to gene expression enables "fine tuning" of transcriptional activity at discrete chromosomal loci. Transcriptional regulators such as GR are likely to exploit a diversity of mechanisms across different cellular and promoter contexts.

Materials and Methods

Plasmid DNAs

The plasmids pSG5 (Promega), pSG5-rGR (Darimont et al. 1998), p6R-N525 (Iniguez-Lluhi et al. 1997), TAT3-DLO (Iniguez-Lluhi et al. 1997), pSG5-MAD-GR (gift of M. Cronin), pGEX4T-1 (Pharmacia), 5xGAL4-e1b-Luc (gift of R. Uht), pRS423-N577-delta108-317 (gift of B. Darimont), CMV-Bgal (Spaete and Mocarski 1985), pSG424-MAD-GAL4, and PGK-Neo are previously described. Subcloning the BspEI - BbsI fragment of GR containing the K461A mutation from p6R-K461A (Starr et al. 1996) into pSG5-rGR generated plasmid pSG5-GR-K461A. PCR amplification of nucleotide sequences encoding amino acids 407-795 of rat GR from p6R-rGR (Starr et al. 1996) using forward primer #7 5′-AAAAGGATCCATAATGTCAGTGTTTTCTAATGGG-3′ and reverse primer #8 5′-AAAAGGATCCTCATTTTTGATGAAACAGAAGC-3′ generated plasmid pSG5-GR-407C by digesting the PCR fragment with BamHI followed by ligation into pSG5 digested with BamHI. The BamHI-ScaI fragment of pRS423-N577-Δ(108-317) ligated into pSG5-rGR digested with EagI (blunt) and BamHI generated plasmid pSG5-N577-Δ(108-317). PCR amplification of the mouse RelA open reading frame from plasmid J134 (Blank et al. 1991) using forward primer #1 5′-GGCGCGAATTCATGGACGATGTGTTTCCCC-3′ and reverse primer #1 5′-GGCGCGAATTCTTAGGAGCTGATCTGACTCAAA-3′ followed by digestion with EcoRI and ligation into the EcoRI site of pSG5 generated plasmid pSG5-RelA. PCR amplification of two tandem copies of the VP16 amino acids 413-454 from plasmid pGAL4-VP16 (gift of M. Carey) using forward primer #2 5′-CCCCCGAATTCCAGCCCGGGCGATCCGCC-3′ and reverse primer #2 5′-CCCCCGGATCCTTATCTAGAGGATCTCGG-3′ with digestion using EcoRI and BamHI followed by ligation into pSG5 generated intermediate plasmid pSG5-VP16.

PCR amplification of RelA nucleotide sequences encoding amino acids 1-312 using forward primer #1 and reverse primer #3 5'-GGCGCGAATTCGATACTCTTGAAGGTCTCATAGGT-3' followed by digestion using EcoRI and ligation into pSG5-VP16 digested with EcoRI yielded plasmid pSG5-RHD-VP16.

Inserting three copies of the annealed and kinased IL-2Rα NFκB response element oligonucleotides 5'-TCGACGGAGAGGGAGATTCCCCTGCCGTC-3' and 5'-TCGAGACGGCAGGGGAATCTCCCTCTCCG-3' into the SalI site of plasmid pΔODLO (Iniguez-Lluhi et al. 1997) generated the reporter plasmid κB3-DLO. Subcloning the double GAL4 binding site oligonucleotide 5'-AGCTCGGAGGACTGTCCTCCGTTCTCGAGAACGGAGGACAGTCCTCCG-3' into the HindIII site of pΔODLO generated the reporter plasmid 2xGAL4-DLO which has a higher basal activity than 5xGAL4-e1b-Luc.

The E. coli expression vector pGEX-RelA for the GST-RelA fusion protein is previously described (Stein and Yang 1995). PCR amplification of mouse RelA nucleotide sequences encoding amino acids 1-312 using forward primer #1 and reverse primer #9 5'-GGCGCGAATTCGATACTCTTGAAGGTCTCATAGGT-3' followed by digestion using EcoRI and ligation into the EcoRI site of pGEX4T-1 generated plasmid pGEX-RelA-RHD. PCR amplification of mouse RelA sequences encoding amino acids 1-196 using forward primer #1 and reverse primer #10 5'-GACTGATCGCGGCCGCTCAGATCTTGAGCTCGGCAGT-3' followed by EcoRI-NotI digestion with ligation into the corresponding sites of pGEX4T-1 yielded plasmid pGEX-RelA-N196. PCR amplification of mouse RelA sequences encoding amino acids 192-312 using forward primer #8 5'-GGCGCGAATTCACTGCCGAGCTCAAGATC-3' and reverse primer #9 followed by EcoRI digestion with ligation into the EcoRI site of pGEX4T-1 generated plasmid pGEX-RelA-RDD. PCR amplification of mouse p50 sequences encoding amino acids 245-372 from plasmid J130 (Blank et al. 1991) using forward primer #9 5'-GAAGAGGATCCATGGCATCCAACCTGAAAATCGT-3' and reverse primer #11 5'-GAAGAGAATTCTTAGAAGCTGTCCGAGAAGTTC-3' followed by BamHI-EcoRI digestion and ligation into the corresponding sites of pGEX4T-1 yielded plasmid pGEX-p50-RDD. PCR amplification of mouse RelA sequences encoding amino acids 304-550 using forward primer #6 and reverse primer #1 followed by EcoRI digestion and ligation into the corresponding site of pGEX4T-1 generated plasmid pGEX-RelA-304-550.

Subcloning the PCR amplified human IL-8 coding sequence into pBLUESCRIPT KS+ (Stratagene) at the XhoI to SmaI sites generated the anti-sense IL-8 plasmid. Subcloning the PCR amplified human ICAM-1 exon 3 sequence into pBLUESCRIPT KS+ at the HindIII to EcoRI sites yielded the anti-sense ICAM-1 plasmid. Subcloning the XhoI-XmnI fragment of the human IκBα coding sequence into pBLUESCRIPT KS+ at the XhoI and EcoRV sites generated the anti-sense IκBα plasmid. Digestion of the anti-sense plasmids with XhoI and *in vitro* transcription from the T7 promoter yielded the anti-sense probes for RNAase protection.

Cell culture and transfections

CV-1 cells and A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum and split every third day.

The reporter stable A549-k9 was made by linearizing both the kB3DLO luciferase reporter and the PGK-Neo plasmid with XmnI followed by electroporation and selection in 1.5 mg/ml G418. Screening colonies for TNFα inducible luciferase activity identified the reporter stable A549-k9. Subsequent culturing of A549-k9 did not require G418.

For transient transfections, CV-1 cells were plated in 24 well plates at a concentration of 5x10^4 cells per well in 500μl of DMEM containing 5% charcoal-stripped fetal calf serum approximately 16 hrs before addition of lipid-DNA complexes. Serum was charcoal-stripped as described (Freeman et al. 2000). Cationic lipid stock was prepared as described (Hong et al. 1997b). A typical transfection contained 100ng luciferase reporter, 100ng beta-galactosidase control plasmid, varying amounts of the transcription factors, and empty pSG5 plasmid to 450ng total DNA in 40µl of OPTI-MEM I (Gibco-BRL). The 5mM cationic lipid stock was diluted to 0.5µl lipid per 40µl in OPTI-MEM I. Then, 40µl of the diluted lipid was added to the 40µl of DNA, mixed, and incubated at room temperature for 15 min. After incubation, the lipid-DNA complexes were further diluted with 300µl of OPTI-MEM I and the entire 380µl was added to a single aspirated well of the 24 well plate. Lipid-DNA complexes were typically prepared as a stock of 6.5 identical reactions and aliquoted onto cells for replicates. Approximately 6 hrs later, 400µl of DMEM containing 5% stripped FCS and 2X-concentrations of hormone or ethanol vehicle was added. Approximately 16 hrs post hormone addition cells were harvested and assayed for beta-galactosidase and luciferase activity as described (Iniguez-Lluhi et al. 1997).

Protein Purification

Proteins fused to GST were expressed in E. coli strain BL21 at 18°C with 1mM IPTG induction at approximately 0.3 OD600 followed by an additional 12-14 hrs of growth prior to harvest. All steps were performed at 4°C unless noted otherwise and all buffers for GST fusions contained 0.5mM PMSF and 1 µg/ml leupeptin, pepstatin and aprotinin. Cell pellets were resuspended in one volume of phosphate buffered saline containing 1mM EDTA and 14mM b-mercaptoethanol. Extracts were prepared by lysozyme treatment followed by sonication to reduce turbidity. The extracts were then ultracentrifuged in a beckman 70.1Ti rotor at 45,000 rpm for 2 hrs. The supernatant was batch bound onto 1ml of 50% slurry glutathione-agarose beads (Sigma Co.) in the same buffer adjusted to 0.1% NP-40 for 30 minutes. The beads were then loaded into a disposable column and washed with 10ml of Wash Buffer (10mM Tris pH 8, 1mM EDTA, 2M NaCl, 0.1% NP-40, 14mM b-mercaptoethanol) followed by 10ml DnaK Buffer (50mM Tris 8, 10mM MgSO4, 2mM ATP). A final 5ml wash in Binding Buffer (10mM Tris pH 8, 1mM EDTA, 150mM NaCl, 14mM b-mercaptoethanol) was then performed and the purified proteins were stored with the beads at –80°C.

GST-fusion protein interaction assays

Equal amounts, ~1µg, of each fusion protein were used as judged by coomassie gel loading titrations. GR and derivatives in the pSG5 vector were *in vitro* translated using the Promega TNT coupled transcription/translation kit in the presence of ^{35}S-methionine. Binding reactions were performed in a total volume of 100µl Binding Buffer containing 12.5µl packed glutathione beads and 5nM receptor (determined by immunoblotting analysis and comparison to known concentrations of purified GR standards). Reactions were conducted at room temperature with mild agitation to keep the beads in suspension for 45 min. Samples were then pelleted and beads were washed four times with 500µl of Binding Buffer containing 500mM NaCl. The pellets were aspirated, resuspended in 10µl of SDS-PAGE loading buffer and the entire reaction was resolved over an appropriate percentage (12% or 15%) SDS-PAGE gel. The gel was then dried down and exposed to a phosphorimager screen overnight. Molecular dynamics software was used to generate and quantify gel images.

Western blot and ribonuclease protection assays

Immunoblotting was performed on A549-k9 whole cell extracts with a RelA antibody (sc-372, Santa Cruz Biotechnology, Inc.) as per the manufacturer recommendations. The ribonuclease protection assays were performed on 20μg total RNA according to the manufacturer recommendations (Ambion).

Electrophoretic gel-mobility shift assays

Nuclear extracts were prepared from A549-k9 by the Dignam method (Dignam et al. 1983). Gel shifts were performed with 2.5μg nuclear extract in a 10μl volume of final concentrations 10mM HEPES, pH 8; 80mM NaCl; 1mM EDTA; 0.5mM EGTA; 5% glycerol; .05% NP-40; .1mg/ml poly-dG/dC (Pharmacia Biotech.). After 5 minutes at room temperature, various antibodies were added to some samples (anti-p50, sc-114, Santa Cruz Biotechnologies, Inc.). After an additional 5 min, ^{32}P-endlabeled NFκB IL-8 site probe 5'-CAAATCGTGGAAATTTCCTCTGAC was added to a final concentration of 2nM followed immediately by cold oligonucleotide or not. The non-specific probe is the collagenase AP-1 site 5'-AGTCATGAGTCAGACACCTCTGGC.

Chromatin immunoprecipitation assays

A modified protocol was developed based on several previous reports (Braunstein et al. 1996; Orlando et al. 1997; Parekh and Maniatis 1999). All antibody amounts were titrated for maximal immunoprecipitation of signal. Identical conditions were used for the RelA antibody (Santa Cruz, sc-109) and the total RNA polymerase II antibody (Santa Cruz, sc-899). Following the 2 hr treatments with various hormone combinations, approximately 5x10^{8} adherent A549-k9 cells were crosslinked at 4°C by the addition of 11X Formaldehyde Stock (50mM Hepes-KOH, pH 8; 1mM EDTA; 0.5mM EGTA; 100mM NaCl; 11% formaldehyde) to a final of 1X for 40 min. Crosslinking was stopped by the addition of glycine to a final concentration of 125mM for 5 minutes. Cell monolayers were washed in the plates with ice cold phosphate buffered saline, scraped into 50ml conical tubes and spun 600xg for 5 minutes at 4 degrees Celsius. Pellets were aspirated and resuspended in 10ml Chro-IP Lysis Buffer (50mM Hepes-KOH, pH 8; 1mM EDTA; 0.5mM EGTA; 140mM NaCl; 10% glycerol; 0.5% NP-40; 0.25% Triton X-100; 1mM PMSF; 5 μg/ml leupeptin, pepstatin A, and aprotinin) and nutated for 10 min at 4°C. Nuclei were again pelleted and resuspended in 10ml Wash Buffer (10mM Tris-HCl, pH 8; 1mM EDTA; 0.5mM EGTA; 200mM NaCl; 1mM PMSF; 5μg/ml leupeptin, pepstatin A, and aprotinin) and nutated again. Then, nuclei were re-pelleted and resuspended in 2ml of 1X RIPA Buffer (10mM Tris-HCl, pH 8; 1mM EDTA; 0.5mM EGTA; 140mM NaCl; 1% Triton X-100; 0.1% Na-deoxycholate; 0.1% SDS; 1mM PMSF; 5μg/ml leupeptin, pepstatin A, and aprotinin). Samples were sonicated with a Branson Sonifier 250 with a microtip in 20 sec constant bursts followed by 1 min of cooling on ice between bursts for a total sonication time of 3 min per sample. This procedure resulted in fragment sizes ranging from 1.5 to 0.3 kb. Samples were then centrifuged at 16,000xg for 10 min at 4°C. Appropriate antibody, 2 μg, were added to 600μl aliquots of cleared chromatin extract and incubated with rotation at 4°C for 6 hrs. Samples were then centrifuged again. Supernatant was then transferred to a fresh tube containing 20μl of precleared 50% slurry Protein A/G beads (ICN Pharmaceuticals) which had been resuspended in 1x RIPA Buffer containing 100 μg/ml sonicated salmon sperm DNA. After 3 hrs with the beads, samples were pelleted at 600xg and washed twice with 1x RIPA buffer, once with 1x RIPA buffer containing 100 μg/ml salmon sperm DNA for 5 min with rotation, 5 times with 1x RIPA buffer containing 500mM NaCl final plus 100μg/ml salmon sperm DNA for 5 min with rotation, and once with 1x RIPA buffer. Then, 100μl of digestion buffer was added (50mM Tris, pH 8; 1 mm EDTA; 100mM NaCl; 0.5% SDS; 100μg/ml proteinase K) and placed at 55°C for 3 hrs, followed by 6 hrs at 65°C to reverse crosslinks. DNA was phenol-CHCl3 extracted once, CHCl3 extracted once, and ethanol precipitated in the presence of 20μg glycogen. Pellets were resuspended in 20μl TE. PCR reactions, 50μl, were programmed for 30 cycles with 4μl of DNA sample and 50nM each of appropriate ^{32}P-endlabeled (500,000-cpm/reaction) primer oligonucleotides (Gibco BRL buffers, nucleotides and Taq enzyme). Titrations were performed to ensure a linear range of amplification. One fifth of each PCR reaction was ran on a 6% 0.5x TBE gel (19:1 acrylamide:bisacrylamide, Bio-rad), dried and exposed on a phosphorimager cassette for quantification and image generation. The IL-8 or ICAM-1 promoter intensities were first normalized by dividing them by the internal control intensity of the U6 snRNA gene. Fold inductions are defined as the ratio of the normalized intensities for the treated lanes to the untreated lane.

The $TF_{II}H$ XPB/ERCC3 subunit antibody (Santa Cruz, sc-283) was treated similarly except for the following modifications. Eight µg antibody were used per aliquot of chromatin extract and were incubated for 16-24 hrs with rotation at 4°C. Immunoprecipitates were washed twice with 1x RIPA buffer, once with 1x RIPA containing 100µg/ml salmon sperm DNA for 5 min with rotation, once with 1x RIPA containing 500mM NaCl final plus 100µg/ml salmon sperm DNA for 5 min with rotation, and once with LiCl buffer (10mM Tris-HCl, pH 8; 1mM EDTA; 0.5 mM EGTA; 250mM LiCl; 1% Triton X-100; 1% Na-deoxycholate; 1mM PMSF; 5µg/ml leupeptin, pepstatin A, and aprotinin).

The CTD phosphoserine-2 specific monoclonal antibody H5 (BAbCo, Inc.) and the CTD phosphoserine-5 specific monoclonal antibody H14 (BabCo, Inc.) were treated similarly except for the following modifications. Extracts were prepared with all buffers including the general phosphatase inhibitor 10mM Na-pyrophosphate, pH 8. Five µl of H5 ascites fluid or 10µl of H14 ascites fluid were used per aliquot of chromatin extract and incubated with rotation 16-24 hrs at 4°C. Protein A/G beads were pre-incubated with goat IgG anti-mouse IgM (2 µg/µl packed beads) overnight in 1xRIPA and washed 3 times with 1xRIPA prior to use. Immunoprecipitates were washed twice with 1x RIPA buffer, once with 1x RIPA containing 100µg/ml salmon sperm DNA for 5 min with rotation, once with 1x RIPA containing 300mM NaCl final plus 100μg/ml salmon sperm DNA for 5 min with rotation, and once with LiCl buffer.

PCR Primer sets for the chromatin immunoprecipitation assay

The human IL-8 promoter region -121/+61 was amplified with the primer pairs 5'-GGGCCATC AGTTGCAAATC and 5'-TTCCTTCCGGTGGTTTCTTC. The human IL-8 upstream region -1042/-826 was amplified with the PCR primer pairs 5'-AACAGTGGCTGAACC AGAG and 5'-AGGAGGGCTTCAATAGAGG. The human U6 snRNA promoter region -245/+85 was amplified with the PCR primer pairs 5'-GGCCTATTTCCCATGATTCC and 5'-ATTTGCGTGTCATCCTTGC. The human ICAM-1 promoter region -305/-91 was amplified with the PCR primer pairs 5'-ACCTTAGCGCGGTGTAGACC and 5'-CTCCG GAACAAATGCTGC. The human HSP70 promoter region +153/+423 was amplified with the PCR primer pairs 5'-GGATCCAGTGTTCCGTTTCC and 5'-GTCAAACACGGT GTTCTGCG.

Supplementary Information to Chapter I

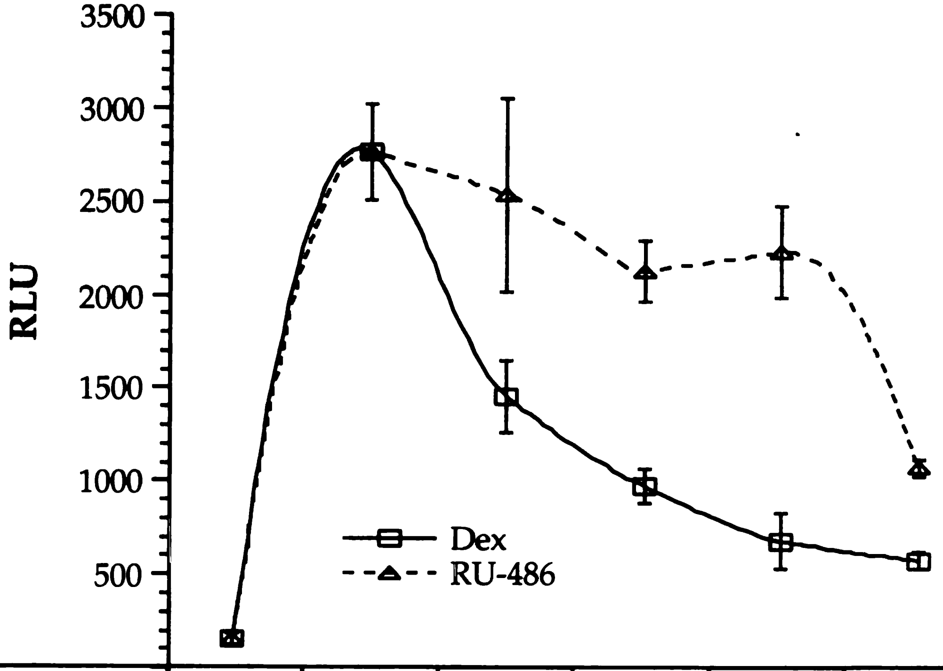
Hormone titration curves for GR inhibition of NFκB in A549-k9 cells

We used two hormones to examine the responsiveness of the NFκB reporter transgene integrated into the A549-k9 cells. The synthetic glucocorticoid dexamethasone maximally repressed TNFα stimulation of the NFκB reporter at 100nM. Notably, the ~4-fold repression of the reporter was comparable to the effects measured on the endogenous IL-8 gene (Chap. I, Fig. 4). The synthetic anti-glucocorticoid mifepristone (RU-486) had no effect up to 100nM; at 1000nM, RU-486 repressed, albeit to a lesser extent than dexamethasone (Fig. 13). The Kd for dexamethasone is ~4 nM and for RU-486 the Kd is ~5 nM. Thus, the inhibition by RU-486 at extremely high doses may reflect a nonspecific toxic effect.

Acetylation in transcriptional regulation: trichostatin A (TSA) titration on A549-k9 cells

Various regulators, including RelA, interact with one or more histone acetyltransferases such as GCN5, SRC-1, ACTR, CBP/p300, P/CAF, and the basal factor $\mathrm{TAF_{II}}$250 (Bannister and Kouzarides 1996; Mizzen et al. 1996; Ogryzko et al. 1996; Chen et al. 1997; Grant et al. 1997; Blanco et al. 1998). While acetylation of nucleosomes is generally associated with transcriptional activation (Grunstein 1997), there are notable exceptions. The nucleosomes of the MMTV promoter are constitutively acetylated and transcriptional activation by GR correlates with nucleosome deacetylation (T. Archer, personal communication). Furthermore, nucleosomes are not the only substrates for these acetyltransferases. For example, acetylation enhances transcriptional activation by the p53 (Gu and Roeder 1997), EKLF (Zhang and Bieker 1998), NF-Y (Li et al. 1998), GATA-1 (Boyes et al. 1998; Hung et al. 1999), and HIV-TAT (Kiernan et al. 1999) regulators, while acetylation of HMG I(Y) inhibits activation of the interferonβ gene (Munshi et al. 1998).

Figure 13. Analysis of glucocorticoid and anti-glucocorticoid dose response curves on inhibition of NFκB mediated activation in A549-k9 cells. We seeded 24 well plates at a density of 105 cells per well in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. The following morning, we stimulated the integrated NFκB reporter activity with either human TNFα (5 ng/ml) alone or by co-treatment with TNFα and various concentrations of steroid hormones. We harvested cells 8 hrs after hormone addition by aspirating the wells and adding 100μl of reporter lysis buffer. We assayed 20μl of cell lysate for luciferase activity; each data point was the result of a triplicate measurement.



TNFa	-	+	+	+	+	+
Hormone (nM)	-	-	1	10	100	1000
% Activity (Dex)		100	53	35	25	21
% Activity (RU-486)		100	92	77	81	39

Figure 13.

Transcriptional regulatory complexes are subject to a variety of post-translational modifications including acetylation. The general transcription factors (GTFs) TF$_{II}$E and TF$_{II}$F are acetylated by CBP/p300, P/CAF, and TAF$_{II}$250 (Imhof et al. 1997). Furthermore, acetyl coenzyme A (CoA) stimulates *in vitro* transcription in the absence of histones, increasing the DNA binding potency of TF$_{II}$D but not of recombinant TBP (Galasinski et al. 2000). The requirement for TAFs to increase TBP binding implicates the acetyltransferase activity of TAF$_{II}$250, but the putative acetyl acceptor remains unidentified. Perhaps, acetyl CoA hydrolysis ($\Delta G^{\circ\prime} = -8$ kcal/mol) serves as an energy source for an isomerization event early in the transcription cycle, analogous to ATP hydrolysis ($\Delta G^{\circ\prime} = -7.3$ kcal/mol) by the prokaryotic regulator NtrC (Wedel and Kustu 1995). The discovery that regulators and general transcription factors are acetyltransferase substrates and that acetylation does not always increase regulator efficacy highlights acetylation as a context dependent post-translational modification analogous to phosphorylation (Kouzarides 2000).

Histone deacetyltransferases (HDACs) catalyze the removal of acetyl groups from various substrates (Kouzarides 2000). Trichostatin A (TSA) is a small molecule inhibitor for several HDACs (Taunton et al. 1996). We treated A549-k9 cells with increasing amounts of TSA to determine whether TSA sensitive HDACs are required for GR inhibition of NFκB mediated activation. The basal, TNFα induced, and dexamethasone inhibited activities of the integrated reporter were largely unaffected by the TSA treatments (Fig. 14). Transient transfections of F9 cells yielded similar results (data not shown). CV-1 cells were not tested as TSA was toxic under the transfection conditions (data not shown). In any case, it appears that HDACs are not required by GR for inhibition of NFκB activity.

Figure 14. GR inhibition of NFkB mediated activation is trichostatin A resistant in A549-k9 cells. We seeded 24 well plates at a density of 105 cells per well in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. The following morning, we stimulated the integrated NFκB reporter activity with either human TNFα (5 ng/ml) alone or by co-treatment with TNFα and 100nM dexamethasone. We performed each of the three treatments with increasing concentrations of TSA. We harvested cells as described in the legend of figure 13.

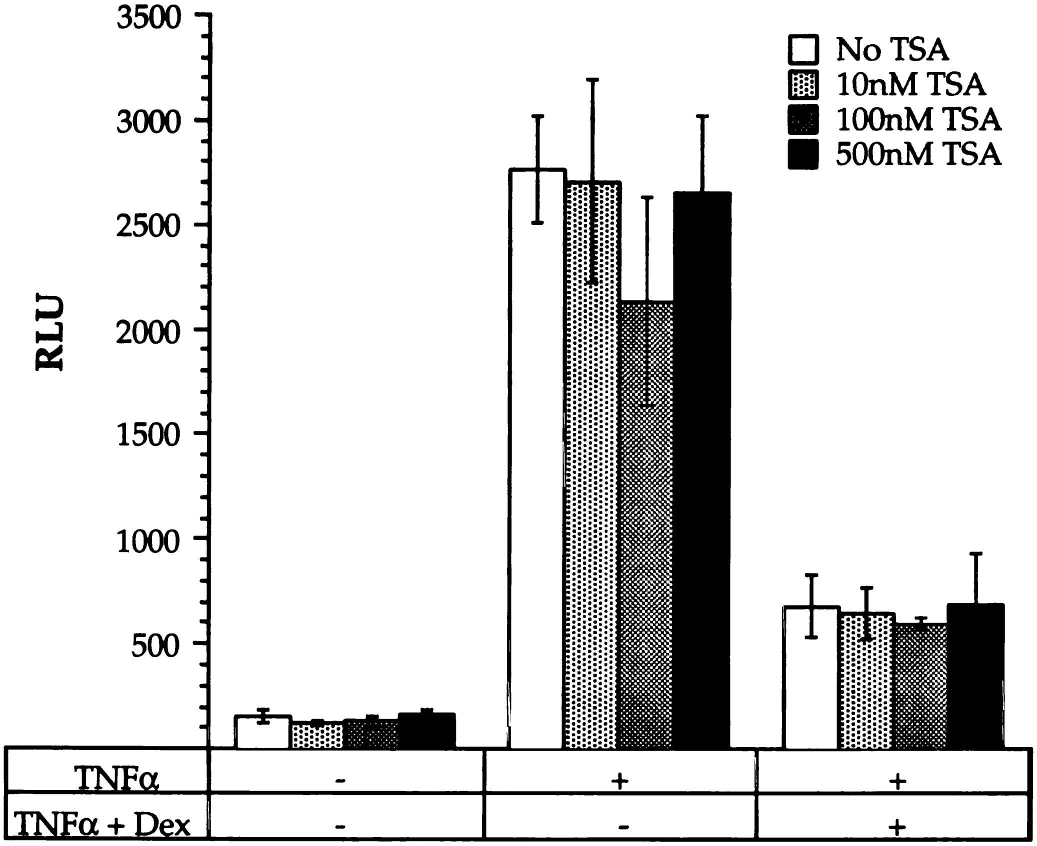

3500
3000
2500
2000
1500
1000
500
RLU
☐ No TSA
▨ 10nM TSA
▨ 100nM TSA
■ 500nM TSA
TNFα - + +
TNFα + Dex - - +

Figure 14.

Quantitative linear-range PCR for the chromatin immunoprecipitation assay

We performed PCR on a series of two-fold input DNA dilutions to show that amplification was linear under the PCR conditions used in the chromatin immunoprecipitation assay. The range used extends both above and below the quantities shown for a typical PCR reaction in the chromatin immunoprecipitation assay. Plotting the band intensities on a log scale showed that the PCR reactions are linear and, therefore, the results are quantifiable (Fig. 15). Furthermore, primers were never limiting in the reactions as <5% of the input primer was incorporated into product (data not shown).

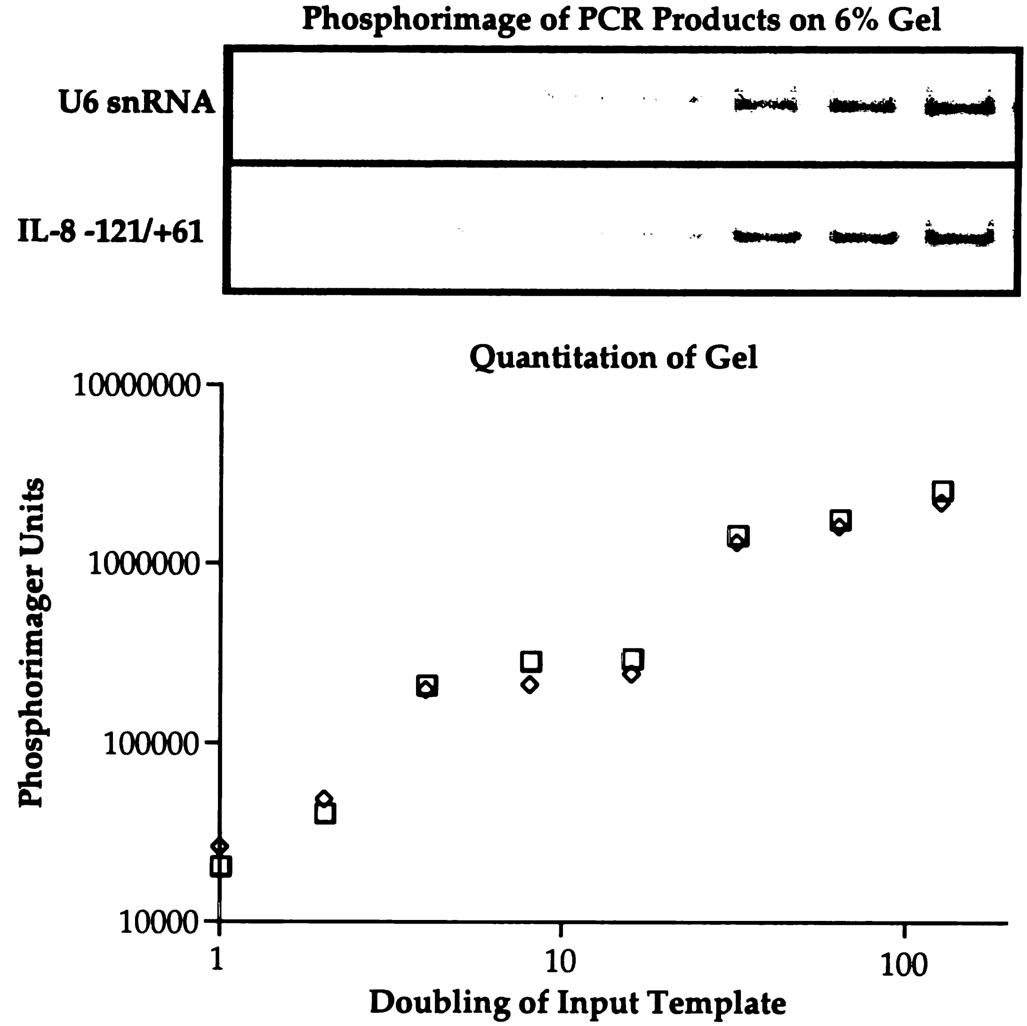
Coactivator competition test by CBP co-transfection

One report states that CBP over-expression can alleviate GR inhibition of NFκB activity (Sheppard et al. 1998), suggesting that GR functions by competing with RelA for a common coactivator. We transiently transfected CV-1 cells to ascertain whether over-expression of the CBP coactivator could interfere with GR inhibition of NFκB. Co-expression of CBP with GR and RelA led to an increase in RelA mediated activation, but had no effect on inhibition of RelA upon treatment with dexamethasone (Fig. 16). Thus, repression does not appear to involve a competition for the CBP coactivator.

GR K461A in vitro protein–interaction with the RelA-RDD

We used the GST fusion protein-protein interaction assay to explore the possibility that GR mutant K461A might display an altered interaction profile with the various fragments of RelA protein. We found that *in vitro* translated GR mutant K461A was not significantly different from wildtype GR in the interaction assay (Fig. 17A).

Figure 15. Demonstration of the quantitative and linear nature of the PCR conditions used for the chromatin immunoprecipitation assay. We purchased Taq enzyme, buffer and nucleotides from GIBCO BRL. The PCR primers were included at the typical final concentrations of 50nM forward and 50nM reverse. Reactions included 500,000 cpm of 32P end-labeled forward/reverse mix for each primer set. One-fifth of the PCR reaction was resolved on a 6% (19:1) acrylamide:bisacrylamide gel containing 0.5x TBE. After drying, the gel was exposed overnight on a molecular dynamics phosphorimager screen for quantitation and visualization.

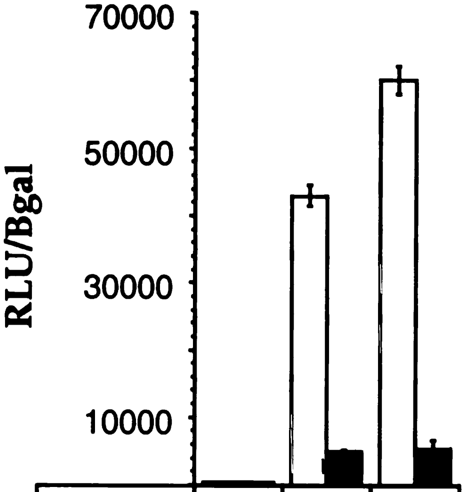


Regression Statistics

Regression Statistics	y = 21173.403x + 168023.317	$r^2 = 0.909$	□ **U6 snRNA**
	y = 17975.615x + 157622.498	$r^2 = 0.886$	◇ **IL8 -121/+61**

Figure 15.

Figure 16. Over-expression of CBP does not affect GR inhibition of RelA mediated activation in CV-1 cells. Cells were transfected, treated with hormone and harvested as described in the Materials and Methods section in Chapter I.



κB3DLO	+	+	+
RelA	-	50	50
GR	50	50	50
CBP	-	-	150

Figure 16.

Figure 17. The GR mutant K461A was indistinguishable from wildtype GR for interaction with RelA. GR and K461A mutant in the pSG5 vector were *in vitro* translated using the Promega TNT coupled transcription/translation kit in the presence of 35S-methionine. We performed binding reactions in a total volume of 100μl Binding Buffer containing 12.5μl packed glutathione beads (~1μg GST fusion protein) and 5nM receptor. We incubated reactions at room temperature with mild agitation to keep the beads in suspension for 45 min. We washed the beads four times with 500μl of Binding Buffer containing 500mM NaCl. We then aspirated and resuspended the pellets in 10 μl of SDS-PAGE loading buffer and the entire reaction was resolved over a 12% SDS-PAGE gel. The gel was then dried down and exposed to a phosphorimager screen overnight. Molecular dynamics software was used to generate and quantitate gel images. (A) GR and GR mutant K461A preferentially interact with the RelA-RDD. (B) GR and GR mutant K461A exhibit similar RelA-RDD binding curves.

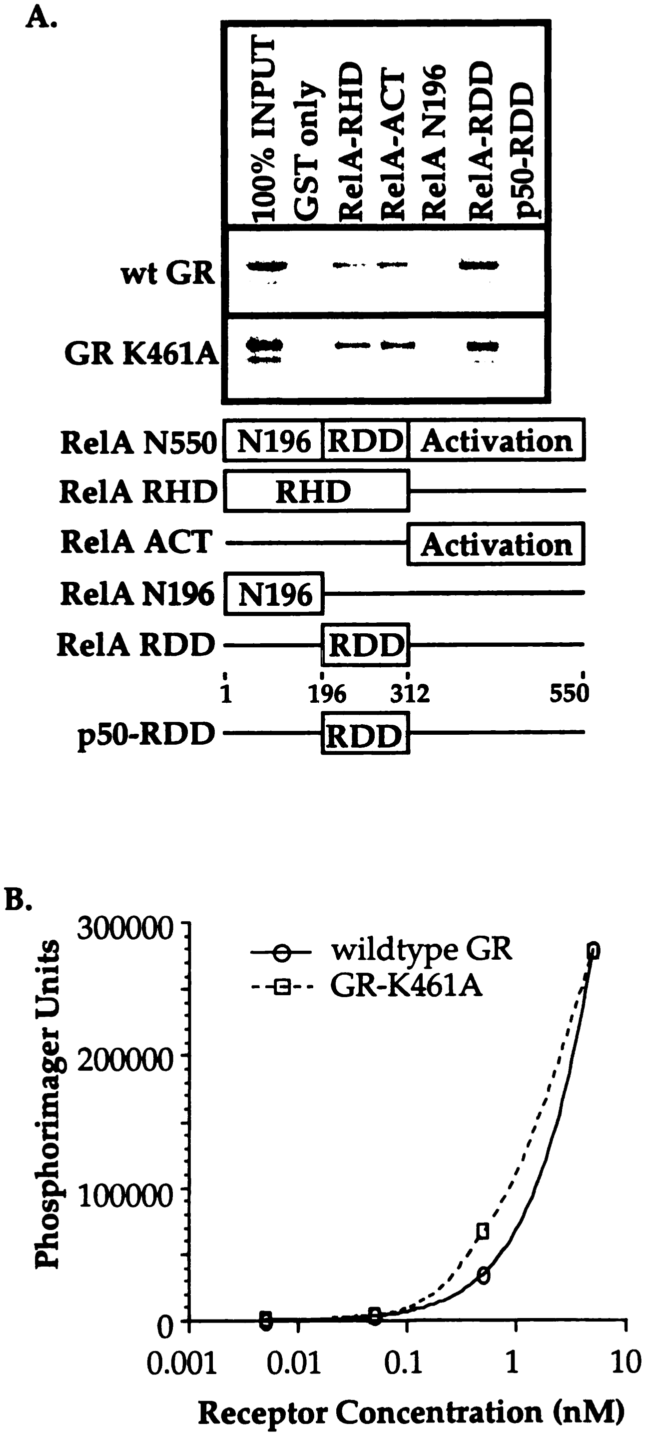

A.
100% INPUT
GST only
RelA-RHD
RelA-ACT
RelA N196
RelA-RDD
p50-RDD
wt GR
GR K461A
RelA N550
N196
RDD
Activation
RelA RHD
RHD
RelA ACT
Activation
RelA N196
N196
RelA RDD
RDD
1
196
312
550
p50-RDD
RDD
B.
Phosphorimager Units
300000
200000
100000
0
wildtype GR
GR-K461A
0.001
0.01
0.1
1
10
Receptor Concentration (nM)

Figure 17.

Furthermore, the binding curves for wildtype and mutant were not significantly different as determined by titrating the input GR (Fig. 17B). Thus, these results are consistent with the interpretation that an allosteric difference exists between wildtype and GR mutant K461A such that the mutant activates rather than inhibits transcription at tethering GREs.

Protein interaction detection using gel filtration chromatography

In an effort to quantify the interaction between the GR-ZBR and the RelA-RDD, a GR 407-525 (X525-HMK) construct with a c-terminal heart muscle kinase sequence tag followed by a HISx6 sequence tag was cloned into a pET vector for expression in E. coli. Also, an E. coli expression vector for RelA residues 192-312 followed by a HISx6 sequence tag was constructed. Both proteins were expressed and purified to >95% homogeneity by nickel chelation chromatography. The X525-HMK protein was functional, as measured by standard gel shift of a GRE oligonucleotide (comparable to DART, kindly provided by B. Maler), and was ^{32}P-labeled using heart muscle kinase (Sigma, P-2645). We used gel filtration chromatography (Pharmacia, S-75) in an attempt to detect a complex between X525-HMK-^{32}P and RelA-RDD. However, only a single fraction shift (1ml size) was detected at concentrations as high as 100µM X525-HMK and 100µM RelA-RDD (Fig. 18). The likely complication in the assay was that co-incubation of the proteins at these high concentrations generated a substantial amount of insoluble material (data not shown). Insolubility was specific to co-incubation of high concentrations of X525-HMK and RelA-RDD as both proteins are soluble indefinitely when in separate tubes or when co-incubated with the neutral proteins p50-RDD or carbonic anhydrase (data not shown).

Figure 18. Gel filtration chromatography of the RelA-RDD with X525-HMK-32P. The protein molecular weight standards are aprotinin (6.5 kD), cytochrome c (12.7 kD), carbonic anhydrase (29 kD), bovine serum albumin (66kD) and alcohol dehydrogenase (150 kD). The column volume was 20ml and the flow rate used was 0.4 ml/min with an injection volume of 100µl and a buffer system of 10mM Tris, pH 8; 1mM EDTA; 5% glycerol; 100mM NaCl. All FPLC runs were performed at 4°C. Binding reactions were centrifuged at 20,000g for 10 min prior to column loading. At 100µM X525 and RelA-RDD, approximately 60% of the input cpm were lost after sample centrifugation.

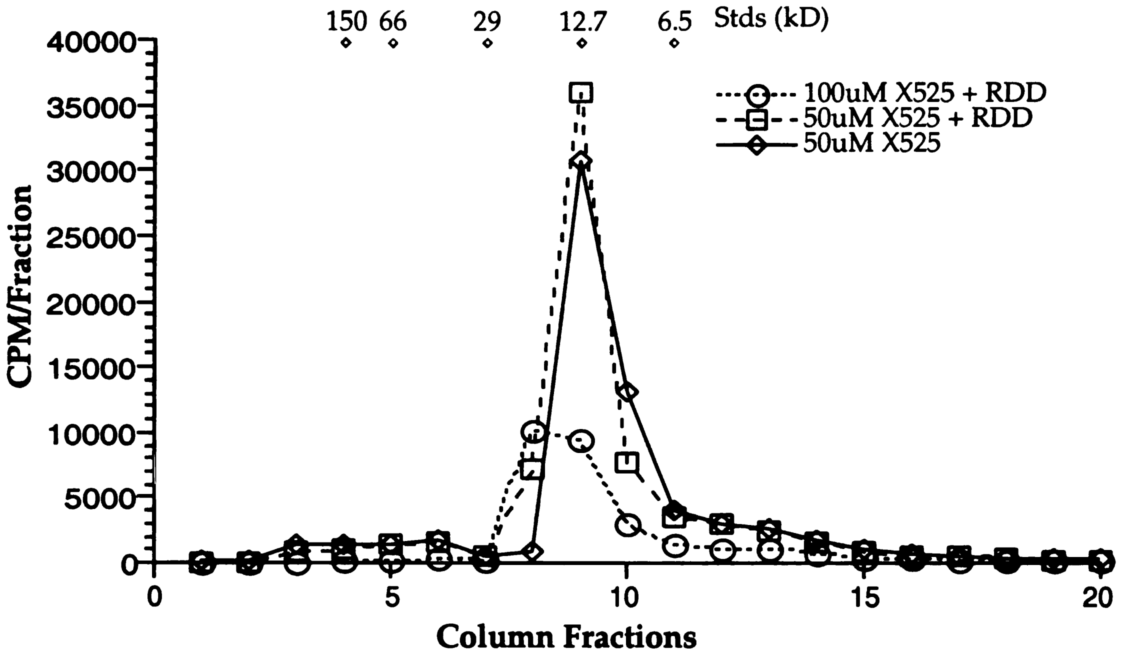

150
66
29
12.7
6.5
Stds (kD)
40000
35000
30000
25000
20000
15000
10000
5000
0
CPM/Fraction
100uM X525 + RDD
50uM X525 + RDD
50uM X525
0
5
10
15
20
Column Fractions

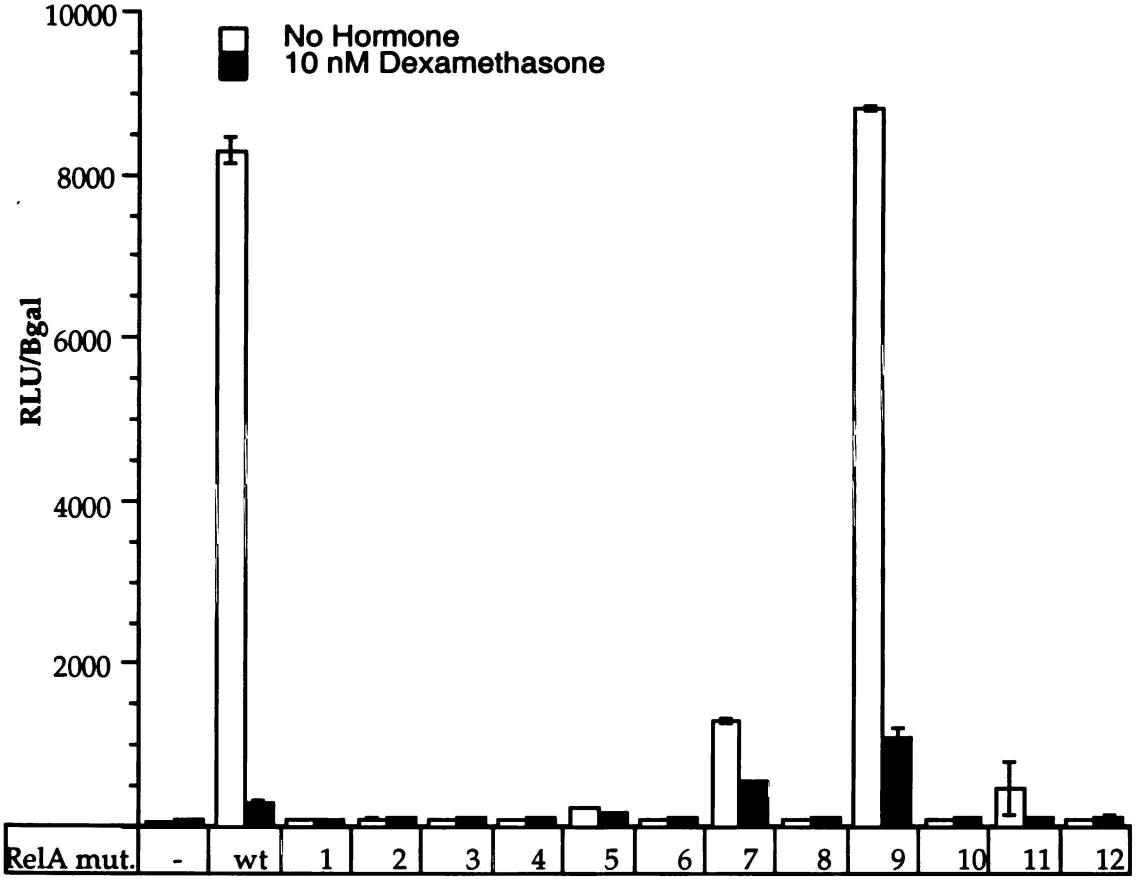
Figure 18.

The RelA-RDD alanine shave mutants

Atomic resolution structures for the p50 homodimer and RelA/p50 heterodimer bound to DNA shows the surface residues on the RelA-RDD (Ghosh et al. 1995; Chen et al. 1998). We exploited this information to generate a collection of RelA mutants with various RDD surface patches of 2 or 3 residues truncated to alanine. Standard Kunkel mutagenesis reactions were performed using mutagenic oligonucleotide primers. Surprisingly, approximately half of the alanine shave mutants failed to activate transcription in a transient transfection (Fig. 19). Therefore, we remade several of the inactive shave mutants as single mutations to alanine and tested for inhibition by wildtype GR (Fig. 20) and enhancement by GR mutant K461A (Fig. 21). The transcriptionally active mutants were regulated by both wildtype and mutant GR and *in vitro* translation of the mutant proteins, both active and inactive, yielded full length protein. Thus, this attempt to uncouple RelA interaction with GR from its activation activity was unsuccessful.

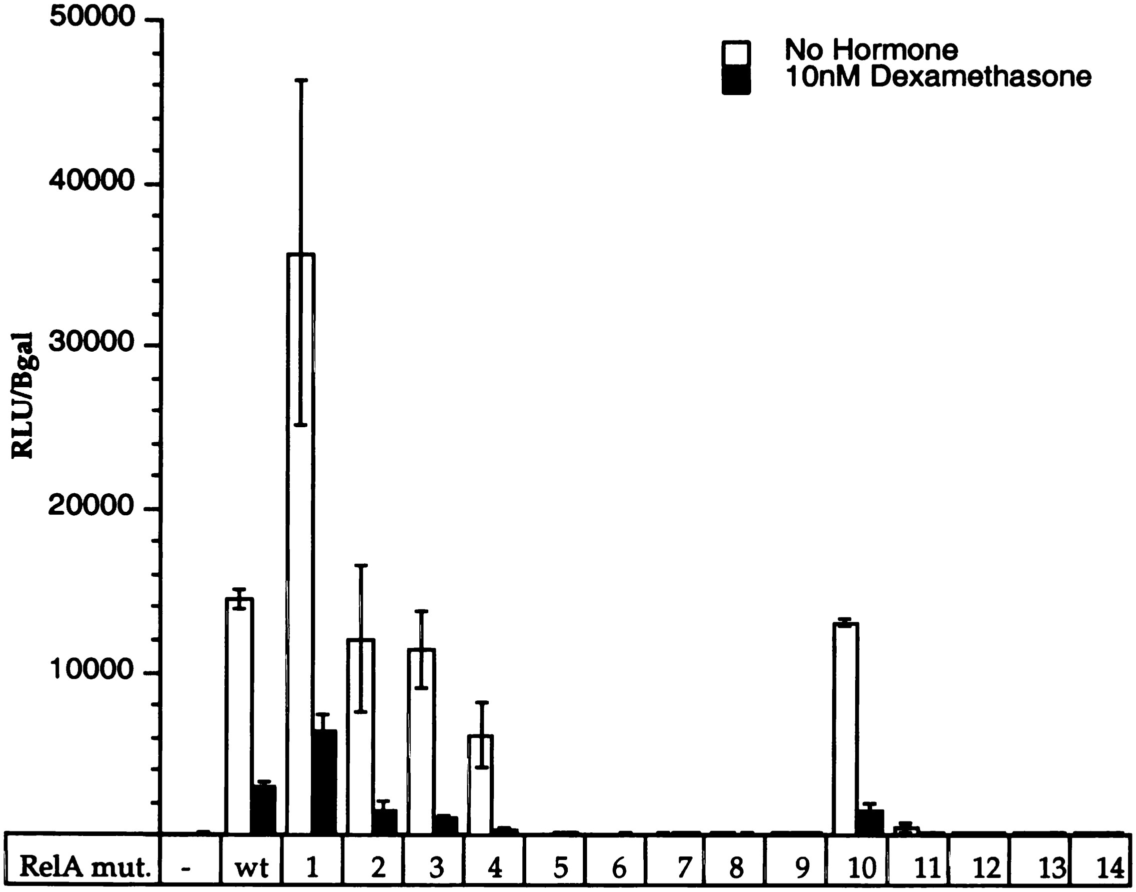
Hormone titration curves on an osteocalcin promoter stable cell line

A simple GRE that overlaps the TATA box sequence regulates the rat osteocalcin promoter. Presumably, GR inhibits transcription from this promoter by directly competing with TBP for promoter occupancy (Meyer et al. 1997a; Meyer et al. 1997b; Meyer et al. 1997c). As a potential control for the chromatin immunoprecipitation assay, we generated an A549 stable (A549-OC2) by electroporation with XmnI linearized pOC-Luc and PGK-Neo followed by G418 selection and screening for luciferase activity. The construct contains the minimal rat osteocalcin TATA box/GRE and is constitutively active due to a 5′-located SV40 enhancer. We tested the A549-OC2 derivative for sensitivity to dexamethasone and RU-486. Inhibition by dexamethasone was maximal at



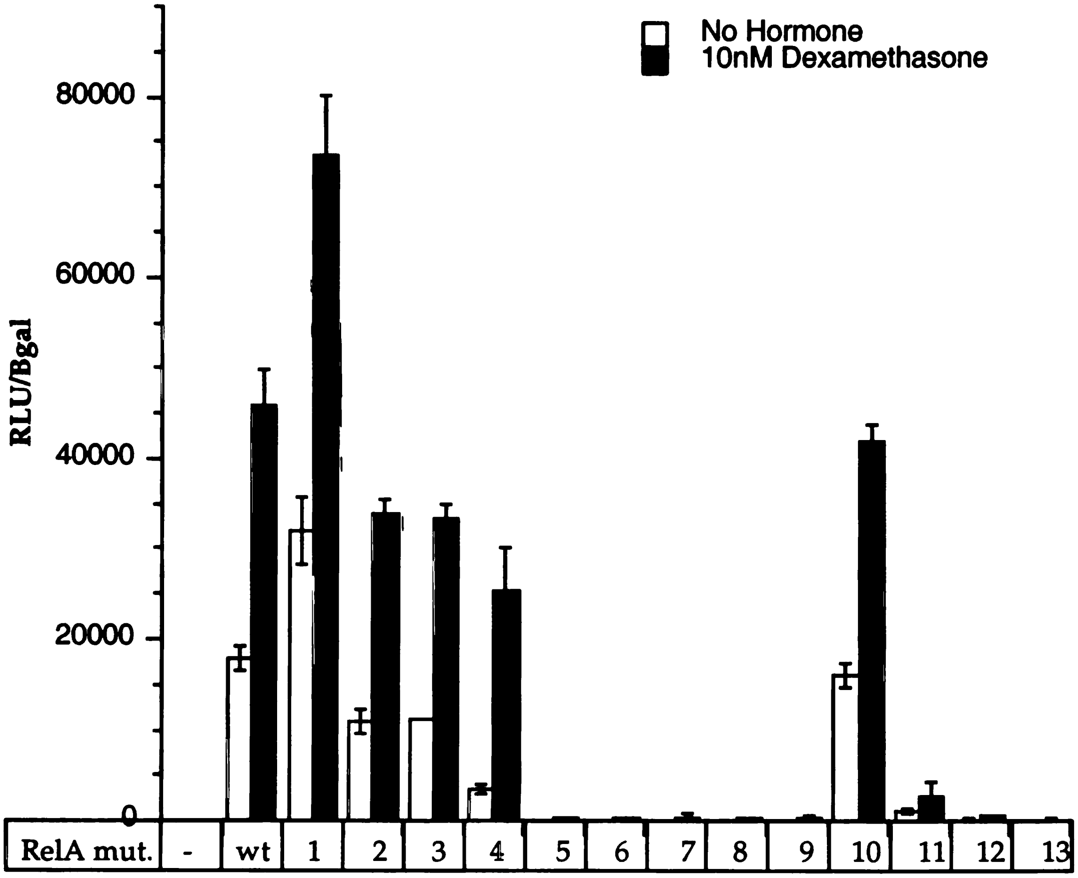
1 RelA E193A,K195A,C197A
2 RelA R201A,N202A,S203A
3 RelA L207A,D210A
4 RelA E222A,D223A,E225A,Y227A
5 RelA R236A,S238A,S240A
6 RelA R253A,P255A,P256A
7 RelA D259A,P260A
8 RelA S261A,L262A,Q263A
9 RelA R267A,S269A
10 RelA R273A,R274A
11 RelA P275A,S276A,D277A
12 RelA M284A,E285A,Q287A,L289A

Figure 19.



1 RelA E193A
2 RelA C197A
3 RelA N202A
4 RelA S203A
5 RelA D210A
6 RelA E225A
7 RelA Y227A
8 RelA T229A
9 RelA R253A
10 RelA R253A
11 RelA P256K
12 RelA R273A
13 RelA R273A
14 RelA R274A

Figure 20.



1 RelA E193A
2 RelA C197A
3 RelA N202A
4 RelA S203A
5 RelA D210A
6 RelA E225A
7 RelA Y227A
8 RelA T229A
9 RelA R253A
10 RelA R253A
11 RelA P256K
12 RelA R273A
13 RelA R273A

Figure 21.

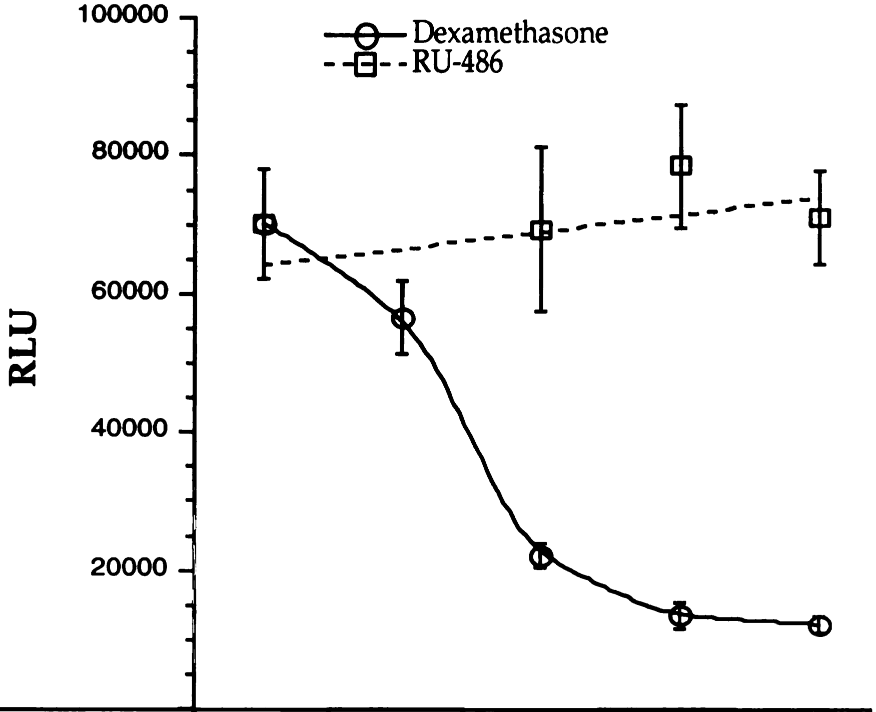
100nM while RU-486 had no effect even at 1000nM concentrations (Fig. 22). Thus, the simple GRE from the rat osteocalcin promoter can function as a stable construct in the A549 cell line. Chromatin immunoprecipitations using an antibody against total RNA polymerase II (sc-899, Santa Cruz Biotechnology, Inc.) did not show an intensity change at the integrated OC locus during inhibition (data not shown). Therefore, GR might repress the osteocalcin promoter transgene by a mechanism that does not cause pol II displacement from the promoter. However, a more likely conclusion is that transgene occupancy by pol II was not sufficient for detection using the current protocol.

TNFα recruitment of the positive transcript elongation factor-b (P-TEFb)

After initiation and promoter clearance, complete synthesis of an RNA transcript by the elongating pol II complex requires the rapid formation of an extraordinary number of phosphodiester bonds. For example, the dystrophin gene, spanning over 2,300 kilobases of chromosomal DNA, is transcribed in approximately 16 hours at an average rate of 2,400 nucleotides per minute (Tennyson et al. 1995). In contrast, *in vitro* transcription is dramatically slower, ranging from 100 to 300 bases per minute, and is a discontinuous process characterized by frequent pausing with occasional formation of permanently arrested complexes (Reines et al. 1996).

The HIV protein TAT potently activates transcription from the HIV-1 LTR by stimulating transcript elongation (Jeang et al. 1999). TAT recruits P-TEFb (Mancebo et al. 1997; Zhu et al. 1997), a 5,6-dichloro-1β-D-ribofuranosylbenzimidazole (DRB) sensitive CTD-kinase composed of a regulatory cyclin T subunit and the CTD-kinase cdk9, through physical association with the cyclin T subunit (Peng et al. 1998). Similarly, the non-heat shocked HSP70 gene promoter is occupied by a stalled and unphosphorylated pol II complex containing a short ~25 nucleotide transcript (Gilmour and Lis 1986; Rougvie and Lis 1988; O'Brien et al. 1994). Exposing cells to elevated

Figure 22. Repression of constitutive activity from A549-OC2. We seeded 24 well plates at a density of 105 cells per well in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. The following evening, we repressed the integrated osteocalcin reporter activity by treating with various concentrations of steroid hormones. We harvested cells 16 hrs after hormone addition by aspirating the wells and adding 100µl of reporter lysis buffer. We assayed 20µl of cell lysate for luciferase activity; each data point was the result of a triplicate measurement.



Hormone (nM)	-	1	10	100	1000
% Activity (Dex)	100	80	31	19	17
% Activity(RU-486)	100	-	99	112	101

Figure 22.

temperatures induces HSP70 transcript elongation that correlates with both P-TEFb localization at the HSP70 promoter and pol II hyperphosphorylation (O'Brien et al. 1994; Lis et al. 2000). Interestingly, as HSF and P-TEFb do not directly interact and co-localization can be uncoupled *in vivo*, how P-TEFb is recruited to the pol II complex is unknown. Furthermore, although P-TEFb can phosphorylate the pol II CTD, it is possible that it could facilitate re-incorporation or re-activation of a different CTD kinase. Lastly, although DRB causes global repression of transcription, direct functional evidence for P-TEFb exists for only a handful of genes.

We investigated the possibility that RelA might recruit the elongation factor P-TEFb at the IL-8 gene using the chromatin immunoprecipitation assay and an antibody against the cyclin T1 subunit of human P-TEFb (kindly provided by D. H. Price, University of Iowa, Iowa City). Chromatin extracts were prepared from A549-k9 cells as described (Chap. I) and P-TEFb was specifically detected at the IL-8 promoter upon treatment with TNFα; co-treatment with 100nM dexamethasone did not significantly affect P-TEFb recruitment (Fig. 23). Therefore, P-TEFb might function during RelA mediated activation. However, neither glucocorticoid repression of IL-8 gene transcription nor GR inhibition of pol II CTD serine-2 phosphorylation results from P-TEFb displacement.

Interestingly, *in vitro* transcription reactions using purified GTFs and pol II are insensitive to DRB while crude extracts recapitulate the sensitivity observed *in vivo*. This observation led to the identification of two cooperative elongation inhibitors, DRB sensitivity-inducing factor (DSIF), composed of a spt4/spt5 heterodimer, and negative elongation factor (NELF), composed of RD and four other proteins, which P-TEFb counteracts (Wada et al. 1998a; Wada et al. 1998b; Yamaguchi et al. 1999a; Yamaguchi et al. 1999b). How DSIF and NELF function is not clear, but the specific association with pol IIa suggests that P-TEFb phosphorylation of the CTD reverses the negative effects of DSIF and NELF by disrupting association with pol II. However, cdk9 can also

Figure 23. The transcript elongation factor P-TEFb was recruited at the IL-8 promoter upon gene induction by TNFα; glucocorticoids did not significantly affect recruitment. Chromatin immunoprecipitation was performed using 20μl of polyclonal α-human cyclin T1 antibody (kindly provided by D. H. Price) essentially as described in Materials and Methods, Chapter I.

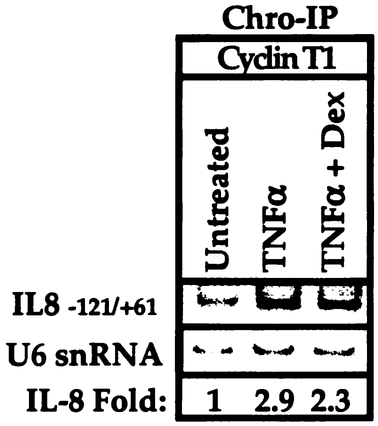

Chro-IP
Cyclin T1
Untreated
TNFα
TNFα + Dex
IL8 -121/+61
U6 snRNA
IL-8 Fold:
1
2.9
2.3

Figure 23.

phosphorylate a component of DSIF, and the recent discovery that cdk8 phosphorylates a substrate distinct from the pol II CTD and from known cdk7 substrates suggests that the various CTD-kinases will have distinct targets. Currently, it is unknown whether nuclear concentrations of DSIF and NELF are sufficient for global repression, or if regulators recruit them to promoters to induce transcript pausing.

Chapter II

Cross-regulation Between NFκB And The Intracellular Receptors For Thyroid Hormone and Retinoic Acid

Abstract

Cell-type dependent regulatory interplay between NFκB and thyroid hormone receptor (T3R) or retinoic acid receptor (RAR) had both positive and negative consequences. Both T3R and RAR inhibited transcriptional activation by transiently transfected RelA in F9 cells while only RAR inhibited RelA in CV-1 cells. Unexpectedly, T3R enhanced cRel activity in CV-1 cells while T3R inhibited cRel in F9 cells. Similarly, T3R mediated transcriptional activation in HeLa cells was inhibited by either induction of endogenous NFκB or the transient expression of RelA. The detection of a protein-protein interaction between RelA and both T3R and RAR suggested that the effect may be direct.

Introduction

Originally characterized as a developmental marker for B cell maturation (Lenardo et al. 1987; Ruben et al. 1991), the founding member of the rel transcription factor gene family is NFκB (Baldwin 1996; Ghosh et al. 1998). The RelA, cRel and p50 family members activate transcription as homodimers or heterodimers. The heterodimer of RelA and p50 is the most studied form of NFκB. In the absence of stimuli, RelA/p50 heterodimers are sequestered in the cytoplasm by the inhibitor protein IκB (Baeuerle and Baltimore 1988a; Baeuerle and Baltimore 1988b). Signaling molecules such as tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1) activate the IκB kinase complex resulting in the phosphorylation, ubiquitination and proteosomal degradation of the IκB protein (Chen et al. 1995; Scherer et al. 1995; Mercurio et al. 1997; Woronicz et al. 1997). The proteolytic digestion of IκB allows RelA/p50 to undergo nuclear translocation, DNA binding, and gene regulation.

The thyroid hormone receptor (T3R) and retinoic acid receptors (RAR) can positively or negatively regulate transcription in response to specific spatial and

temporal signals both during development and in the adult organism (Paulsen 1994; Morriss-Kay and Sokolova 1996). T3R and RAR are ligand regulated transcription factors of the intracellular receptor (IR) gene family (Tsai and O'Malley 1994; Yamamoto 1995) and are constitutively localized to the cell nucleus. Similarly, both receptors have zinc binding regions (ZBR) that mediate DNA binding while the ligand binding domains (LBD) bind either thyroid hormone (T3) for T3R or retinoic acid (RA) for RAR. Heterodimerization with one of the ubiquitously expressed retinoid X receptors (RXR) enhances DNA binding and transcriptional activity for both receptors (Yu et al. 1991).

Evidence is accumulating in support of cross-regulation between the rel and IR gene families. Several IR gene family members including mineralocorticoid receptor (Liden et al. 1997), estrogen receptor (Ray et al. 1994; Stein and Yang 1995), progesterone receptor (Caldenhoven et al. 1995; Kalkhoven et al. 1996), androgen receptor (Palvimo et al. 1996), and glucocorticoid receptor (Mukaida et al. 1994; Ray and Prefontaine 1994; van de Stolpe et al. 1994; Caldenhoven et al. 1995) regulate NFκB mediated activation. In each case, the NFκB binding site is the required promoter sequence for inhibition. Both protein synthesis dependent (Auphan et al. 1995; Scheinman et al. 1995a) and independent mechanisms of inhibition are known (van de Stolpe et al. 1993; Brostjan et al. 1996; De Bosscher et al. 1997; Heck et al. 1997; Newton et al. 1998; Wissink et al. 1998; Adcock et al. 1999). Additionally, several IRs physically bind NFκB with selectivity for RelA over p50 (Ray and Prefontaine 1994; Caldenhoven et al. 1995; Scheinman et al. 1995b; Wissink et al. 1997). Here, we report that two additional IR gene family members, T3R and RAR, display functional and physical associations with NFκB.

Results

T3R can regulate NFκB mediated activation

Transient transfection of the mouse F9 embryonic teratocarcinoma cell line with mouse RelA potently activated transcription from a NFκB responsive reporter construct (Fig. 24A). Co-transfection of F9 cells with RelA and increasing amounts of human T3Rβ expression vector revealed that the apoT3R confers a significant 3-fold further increase in NFκB reporter activity in the absence of thyroid hormone (Fig. 24A). However, exposure of the cells to T3 caused greater than 98% inhibition of RelA mediated activation (Fig. 24B). Experiments with chicken TRα yielded similar results (data not shown). Thus, and contrary to a prior report (Liden et al. 1997), RelA occupied NFκB sites can be regulated by T3R.

When directly bound to reporters driven by a DR4 response element (direct repeats of 5′-AGGTCA-3′ with a four nucleotide spacer), T3R represses in the absence of hormone and activates in the presence of hormone (Naar et al. 1991). Therefore, T3R mediated repression of NFκB is response element specific; Fig. 24C shows that a DR4 driven reporter was activated 75-fold by the holoreceptor.

Cell-type specific modulation of cRel and RelA activity

To explore the possibility that T3R regulates NFκB in a cell-type specific manner, we compared transfections in F9 cells with transfections in the African green monkey kidney cell line CV-1. In F9 cells, T3R inhibited cRel mediated activation comparable to that observed for GR (Fig. 25A), while in CV-1 cells T3R displayed ligand dependent 3-fold enhancement of cRel activation (Fig. 25B). As a control, GR inhibited cRel

Figure 24. Effects of T3R on RelA mediated activation in F9 cells. A) Unliganded TR potentiates RelA mediated activation from NFkB sites in F9 cells. B) Liganded TR represses RelA mediated activation from NFkB sites in F9 cells. C) Ligand dependent activation by TR from the DR4 response element in F9 cells. T3 is tri-iodo-thyronine.

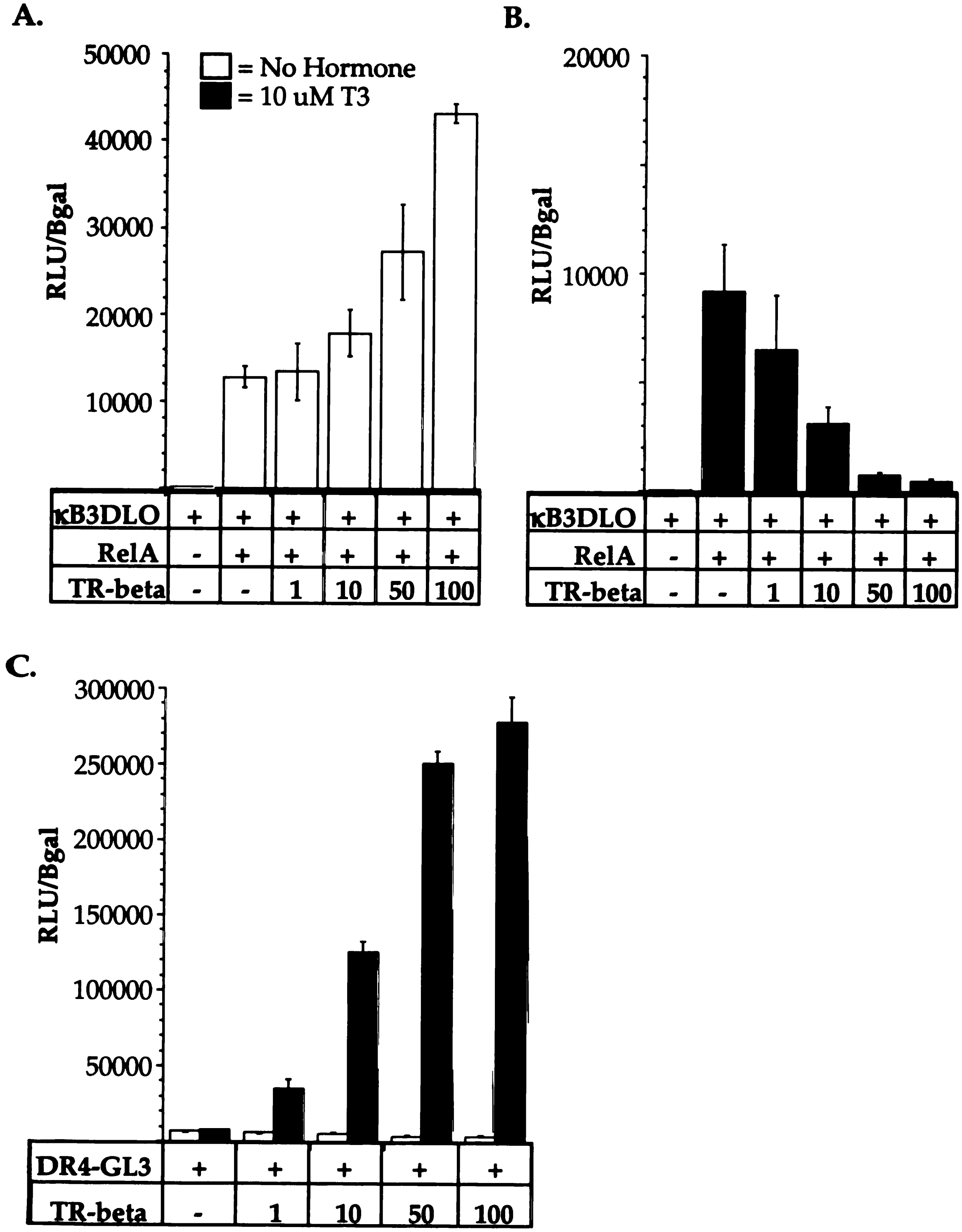
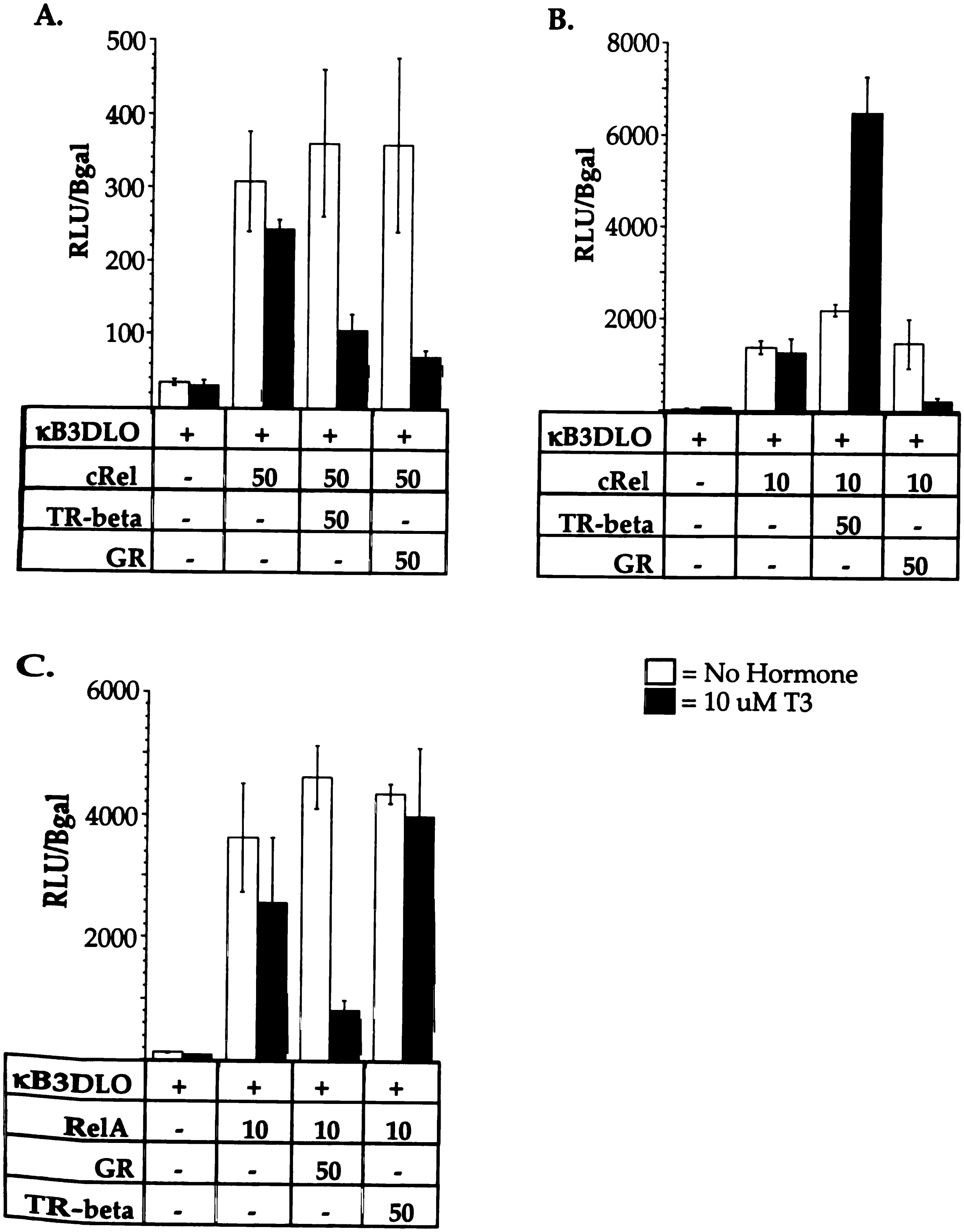

A.
B.
C.
□ = No Hormone
■ = 10 uM T3
RLU/Bgal
50000
40000
30000
20000
10000
κB3DLO + + + + + +
RelA - + + + + +
TR-beta - - 1 10 50 100
RLU/Bgal
20000
10000
κB3DLO + + + + + +
RelA - + + + + +
TR-beta - - 1 10 50 100
RLU/Bgal
300000
250000
200000
150000
100000
50000
DR4-GL3 + + + + +
TR-beta - 1 10 50 100

Figure 24.

Figure 25. Differential effects of T3R and GR on RelA and cRel in F9 and CV-1 cells. A) TR and GR both inhibit cRel in F9 cells. B) Liganded TR activates while liganded GR inhibits cRel in CV-1 cells. C) T3R has no effect on RelA in CV-1 cells.



A.

κB3DLO	+	+	+	+
cRel	-	50	50	50
TR-beta	-	-	50	-
GR	-	-	-	50

B.

κB3DLO	+	+	+	+
cRel	-	10	10	10
TR-beta	-	-	50	-
GR	-	-	-	50

C.

κB3DLO	+	+	+	+
RelA	-	10	10	10
GR	-	-	50	-
TR-beta	-	-	-	50

Figure 25.

activation in CV-1 cells (Fig. 25B). In contrast, T3R did not inhibit RelA activation in CV-1 cells (Fig. 25C). The cell-type specificity of the effects highlights the importance of cell context in gene regulation.

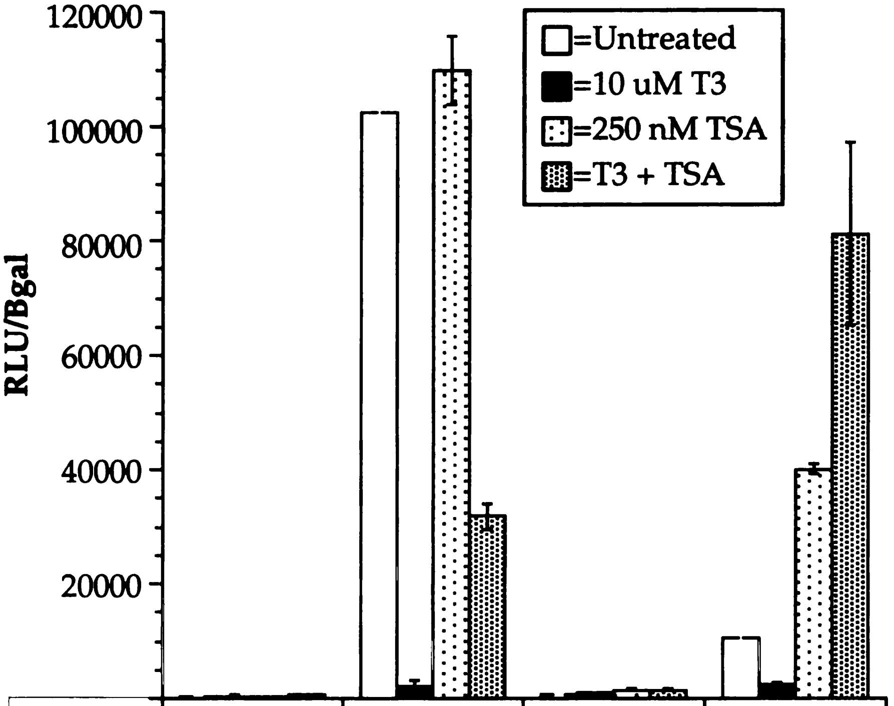
T3R inhibition of RelA is trichostatin A (TSA) resistant

The ligand independent repression of basal transcription from DR4 elements by T3R requires the activity of the histone deacetylase (HDAC) containing mSin3a/SMRT/N-CoR co-repressor complex (Ayer et al. 1996; Alland et al. 1997; Hassig et al. 1997; Heinzel et al. 1997; Laherty et al. 1997; Nagy et al. 1997; Perissi et al. 1999). Trichostatin A (TSA) is a small molecule inhibitor of several HDAC enzymes (Taunton et al. 1996). Interestingly, TSA inhibits T3R repression of AP-1 mediated activation (Cronin, in prep). To test whether T3R inhibition of RelA requires HDAC activity, we treated transiently transfected F9 cells with TSA (Fig. 26). Treatment with 250nM TSA completely abolished T3R inhibition of cJun activity, but had only a partial effect on inhibition of RelA (Fig. 26). Thus, a significant portion of T3R inhibition of RelA may be independent of HDAC activity.

NFκB can inhibit T3R mediated activity

Overexpression of RelA inhibits activation by GR and PR (Kalkhoven et al. 1996; Wissink et al. 1997). Therefore, we tested T3R mediated activation for sensitivity to NFκB activation. HeLa cells contain endogenous T3R and tumor necrosis factor-α (TNFα) inducible NFκB (Charles et al. 1975; Beg et al. 1993). Treatment of HeLa cells with T3 induced 12-fold activation of a transfected DR4 driven reporter construct and addition of TNFα inhibited 70% of the basal activity and 80% of the T3 dependent activity (Fig. 27A).

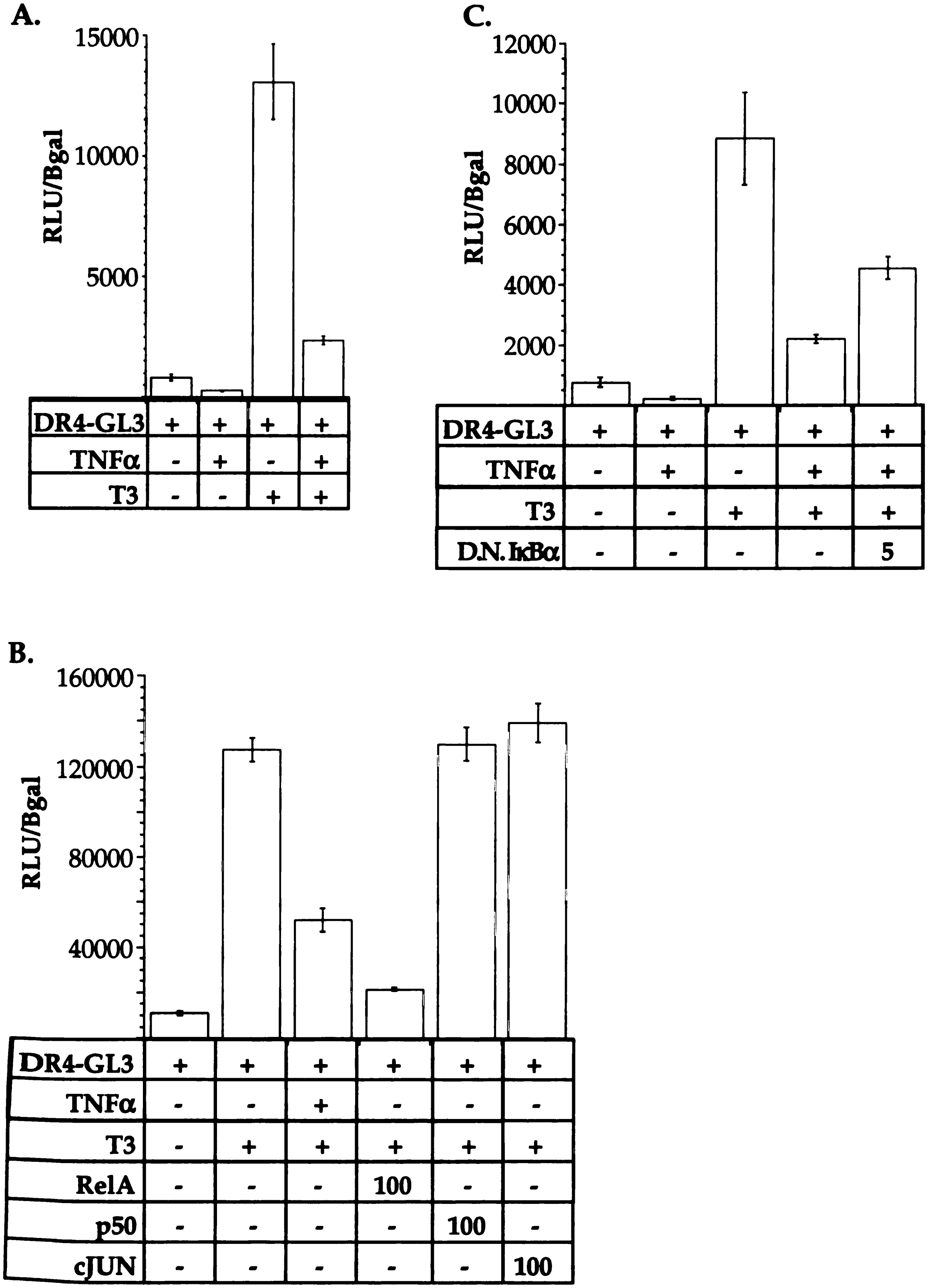
Figure 26. T3R inhibition of RelA is TSA resistant in F9 cells. Transient transfection of F9 cells in the presence of the HDAC inhibitor TSA performed as described in Materials and Methods.



κB3DLO	+	+	-	-
-73Coll	-	-	+	+
RelA	-	+	-	-
cJun	-	-	-	+
TR-beta	-	+	-	+

Figure 26.

Figure 27. Effect of TNFα and RelA on T3R mediated activation. A) Activation from the DR4 response element by endogenous TR is inhibited by co-treatment with TNFα in HeLa cells. B) Co-transfected RelA can inhibit TR mediated activation from DR response elements. C) Dominant negative IkBα can partially rescue the TNFα inhibition.



A.

DR4-GL3	+	+	+	+
TNFα	-	+	-	+
T3	-	-	+	+

C.

DR4-GL3	+	+	+	+	+
TNFα	-	+	-	+	+
T3	-	-	+	+	+
D.N. IκBα	-	-	-	-	5

B.

DR4-GL3	+	+	+	+	+	+
TNFα	-	-	+	-	-	-
T3	-	+	+	+	+	+
RelA	-	-	-	100	-	-
p50	-	-	-	-	100	-
cJUN	-	-	-	-	-	100

Figure 27.

The TNFα signal transduction pathway causes the degradation of IκBα, a negative regulator for several members of the rel gene family (Beg et al. 1993). To identify which family members are involved, we transfected HeLa cells with RelA, p50 or the unrelated transcription factor c-Jun. Interestingly, co-transfection of a RelA expression vector inhibited ~85% of T3R activation while p50 and cJun expression vectors had no effect (Fig. 27B). A cRel expression vector similarly inhibited T3R (data not shown), suggesting that either RelA or cRel mediated the TNFα effect.

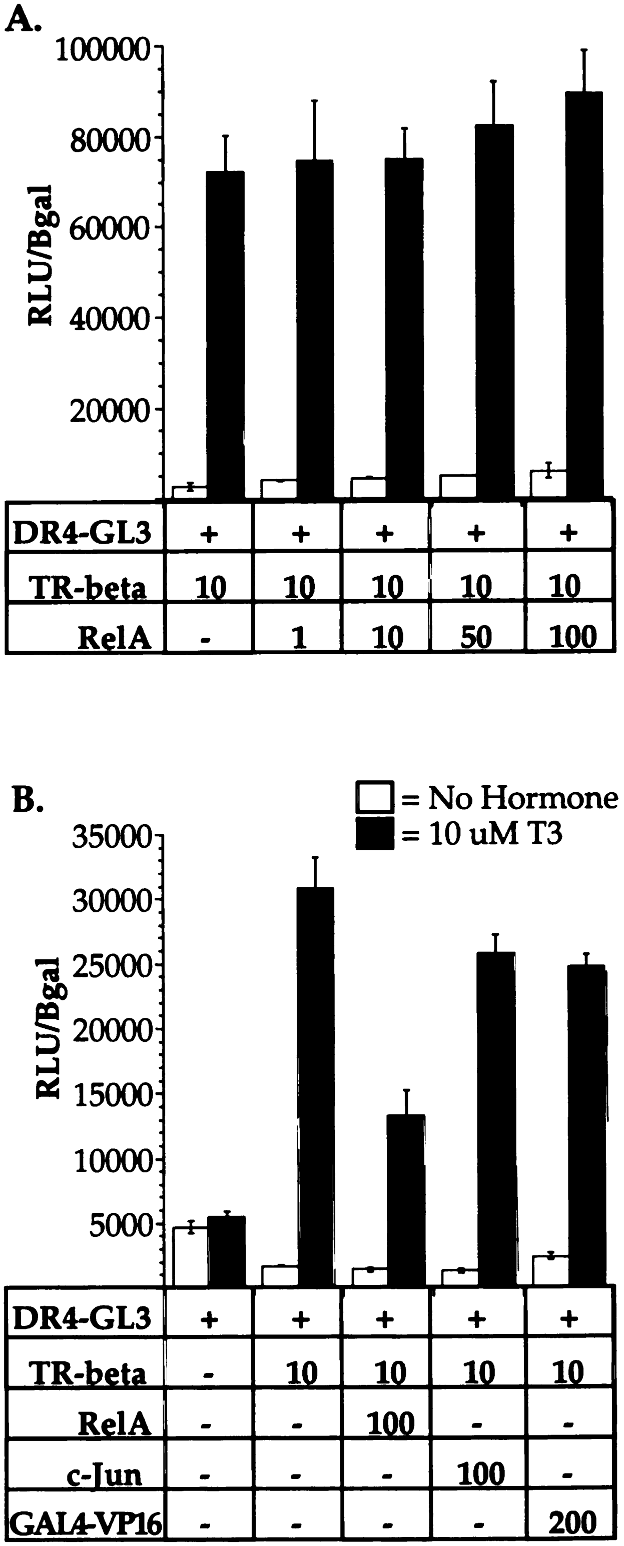
To establish whether the TNFα effect is mediated by known rel family members, we transiently expressed a dominant negative IκBα mutant (S36A/40A) in the HeLa cells (Traenckner et al. 1995). Expression of the mutant yielded ~50% rescue of the TNFα effect (Fig. 27C), suggesting a substantial contribution from known rel family members. However, RelA had no effect on T3R mediated activation in F9 cells (Fig. 28A) and only a two-fold effect in CV-1 cells (Fig. 28B). Thus, NFκB inhibition of T3R mediated activation is cell-type specific.

RAR can regulate RelA mediated activation

To determine whether another non-steroid receptor could similarly influence RelA activity, we tested RAR for functional interactions with RelA. We transfected F9 cells with a NFκB reporter and either cRel or RelA (Fig. 29A). Interestingly, exposure to RA inhibited 70% of RelA mediated activation but had no effect on cRel (Fig. 29A). Thus, RAR specifically targets RelA for inhibition.

When bound to repeats of the 5′-AGGTCA-3′ DNA half site (RAREs), RAR represses transcription in the absence of hormone and activates transcription in the presence of hormone (Yang et al. 1991). As expected, transfection of a RARE driven reporter showed RA dependent 40-fold activation, demonstrating that RA inhibition of the RelA occupied NFκB reporter is response element specific (Fig. 29A).

Figure 28. Cell-type specificity of RelA inhibition of T3R mediated activation. A) RelA does not affect TR activation from DR4 in F9 cells. B) RelA inhibits TR activation from DR4 in CV-1 cells.



A.

DR4-GL3	+	+	+	+	+
TR-beta	10	10	10	10	10
RelA	-	1	10	50	100

B.

DR4-GL3	+	+	+	+	+
TR-beta	-	10	10	10	10
RelA	-	-	100	-	-
c-Jun	-	-	-	100	-
GAL4-VP16	-	-	-	-	200

Figure 28.

Figure 29. Differential effects of RAR on RelA and cRel mediated activation. A) Inhibition of RelA activity by endogenous RAR in F9 cells. B) Inhibition of RelA by RAR is comparable to inhibition by GR in transfected CV-1 cells.

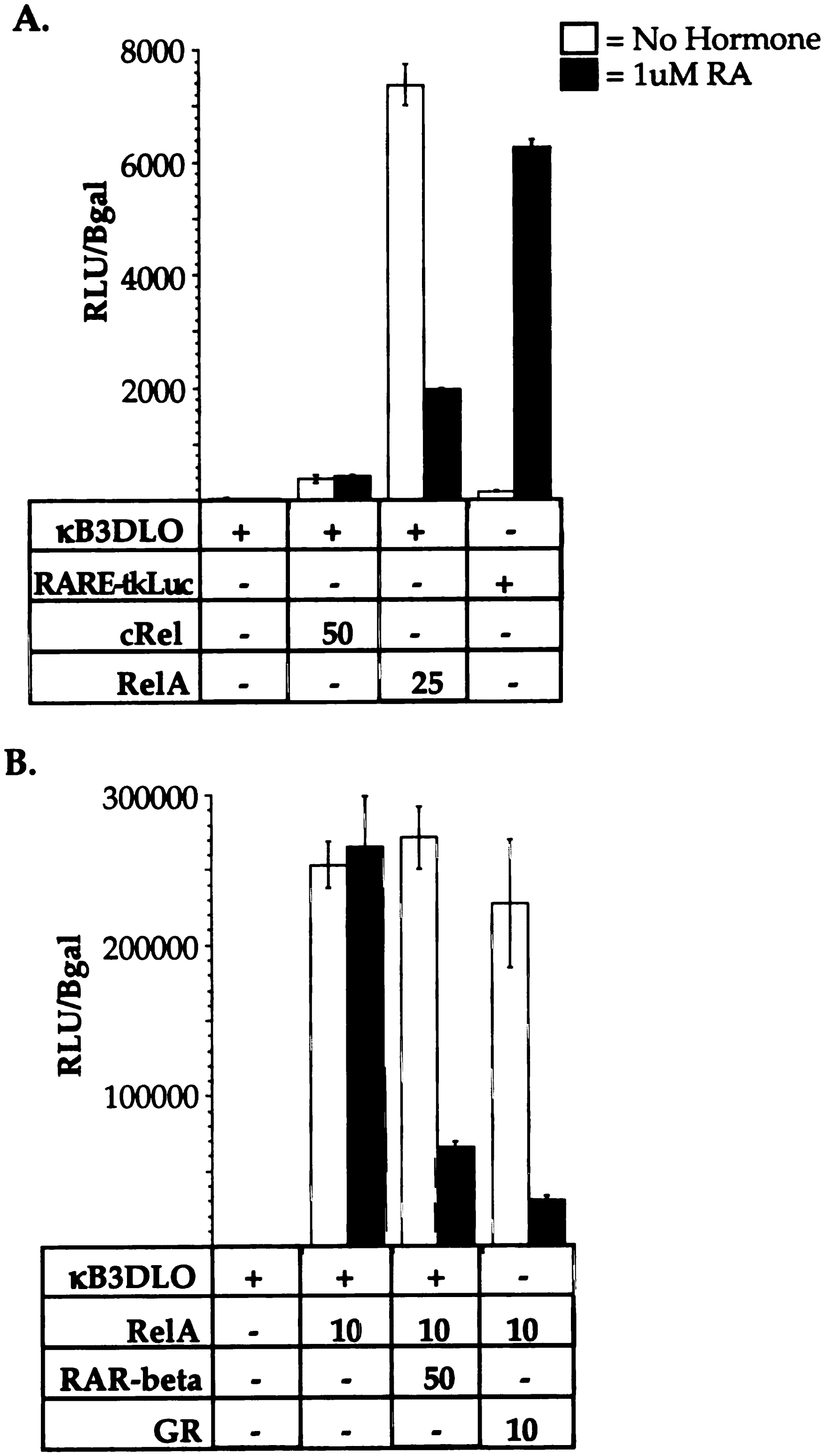

A.
8000
6000
4000
2000
RLU/Bgal
□ = No Hormone
■ = 1uM RA
κB3DLO + + + -
RARE-tkLuc - - - +
cRel - 50 - -
RelA - - 25 -
B.
300000
200000
100000
RLU/Bgal
κB3DLO + + + -
RelA - 10 10 10
RAR-beta - - 50 -
GR - - - 10

Figure 29.

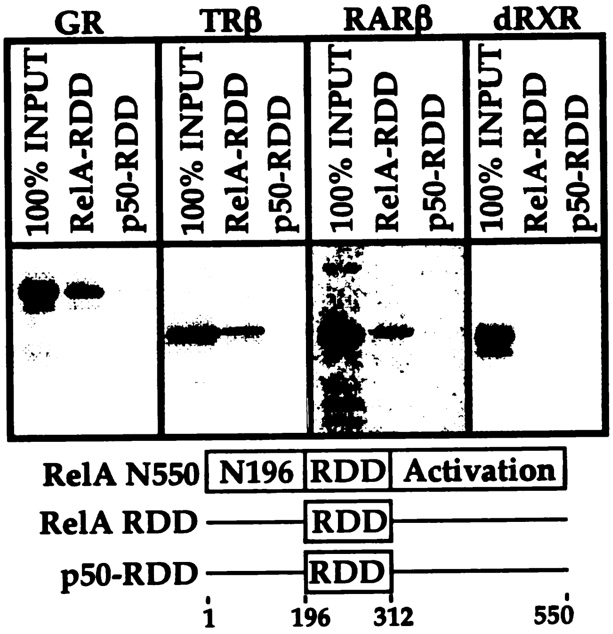
Since F9 cells express several RAR homologs (Umesono et al. 1988), dependence on a particular RAR could not be determined. To test whether the RA effect could function through a particular RAR, we transfected CV-1 cells lacking endogenous RA activity with RelA and mouse RARβ (Fig. 29B). RA treatment of CV-1 cells resulted in RARβ dependent inhibition of 75% of the RelA activity, comparable to GR mediated inhibition of RelA (Fig. 29B). Therefore, and contrary to a prior report (Caldenhoven et al. 1995), NFκB mediated activation can be repressed by a known RAR.

While treatment of HeLa cells with TNFα inhibited endogenous T3R activity from a DR4 reporter (Fig. 27A), TNFα had no effect on endogenous RAR activity from a RARE reporter (data not shown). Therefore, TNFα mediated inhibition displays IR selectivity.

Physical interaction between RelA and both T3R and RAR

Many of the IR gene family members that display functional interactions with NFκB also physically interact with RelA (Ray and Prefontaine 1994; Stein and Yang 1995; Kalkhoven et al. 1996; Palvimo et al. 1996). To determine whether the observed functional interactions between NFκB and both T3R and RAR might similarly result from physical associations, we performed the glutathione S-transferase (GST) fusion protein–protein interaction assay. We tested purified recombinant GST-RelA and GST-p50 dimerization domains (RDDs) for interaction with various IRs labeled by *in vitro* translation in the presence of ^{35}S-methionine. As a positive control, GR bound specifically to the RelA fusion and not p50 (Fig. 30). T3R and RAR displayed comparable retention with a greater than 10-fold selectivity for the RelA-RDD over the p50-RDD (Fig. 30). As a negative control, the drosophila RXR homologue, *ultraspiracle*

Figure 30. T3R and RAR can physically interact with the RelA-RDD. A) GST fusion protein interaction assay with in vitro translated intracellular receptors and GST-fused RelA-RDD or control GST-fused p50-RDD.



GR
TRβ
RARβ
dRXR
100% INPUT
RelA-RDD
p50-RDD
100% INPUT
RelA-RDD
p50-RDD
100% INPUT
RelA-RDD
p50-RDD
100% INPUT
RelA-RDD
p50-RDD
RelA N550
N196
RDD
Activation
RelA RDD
RDD
p50-RDD
RDD
1
196
312
550

Figure 30.

(USP) (Oro et al. 1990), showed no significant interaction with either fusion protein (Fig. 30). Therefore, the observed functional cross-regulatory interactions between NFκB and both T3R and RAR might result from physical association with the RelA subunit.

Discussion

Tethering: a common strategy in transcriptional regulation

Many proteins physically interact with DNA bound activators to repress transcription. The striking feature of tethering is that the DNA binding domains of regulators are multifunctional, mediating direct recognition of DNA at simple response elements and also mediating protein-protein interactions with target factors at tethering response elements. For example, the yeast pheromone response regulator STE12 regulates a-specific genes by directly binding promoter proximal simple response elements. Remarkably, STE12 also regulates α-specific genes, which lack STE12 binding sites. STE12 protein directly interacts with the α1 protein, which, together with MCM1, directly recognizes α-specific gene promoters. Therefore, the α1/MCM1 binding sites are STE12 tethering response elements (Yuan et al. 1993). Likewise, several IR family members can bind and regulate the activity of other transcription factors. Activation by AP-1 is subject to regulation by several members of the IR gene family, including GR (Jonat et al. 1990; Yang-Yen et al. 1990; Konig et al. 1992), AR (Kallio et al. 1995), ER (Gaub et al. 1990), RAR (Yang-Yen et al. 1991), and T3R (Perez et al. 1993; Schmidt et al. 1993). The functional interactions we observed add T3R and RAR to the growing number of IRs capable of regulating NFκB activity. Furthermore, the detection of physical associations is consistent with formation of DNA bound T3R-RelA and RAR-RelA tethering complexes.

Context dependent cross-regulation

IRs function in a cell-type dependent manner. For example, a FK506-sensitive transmembrane channel reduces GR mediated activation in yeast and a FK506 sensitive reduction is also found in mammalian L929 cells, but not in HeLa cells (Kralli et al. 1995; Kralli and Yamamoto 1996). Similarly, GR inhibits transcription from the proliferin gene composite response element (plfG) in CV-1 cells and activates transcription from plfG in HeLa cells (Diamond et al. 1990). Thus, it is not surprising that T3R and RAR do not affect NFκB activity in certain cell-types (Caldenhoven et al. 1995; Liden et al. 1997). While RAR inhibited RelA mediated activation in both F9 cells and CV-1 cells, RAR did not affect NFκB in HeLa cells (data not shown). Furthermore, the pluripotent F9 cell line supported T3R ligand independent enhancement and ligand dependent repression of RelA activity while the CV-1 cell line did not. Additionally, T3R inhibits cRel activity in F9 cells and enhances cRel activity in CV-1 cells. Tissue specific expression of IR-specific coactivators and corepressors could provide an explanation for the complex regulatory landscapes these results describe.

Cross-regulation during development?

The D. *melanogaster* gene *dorsal* is essential for establishing the dorsal-ventral axis and provided the first evidence of a developmental role for the rel gene family (Belvin and Anderson 1996). Surprisingly, the knockout mice for the known mammalian family members do not display early patterning defects, either separately (Beg et al. 1995a; Beg et al. 1995b; Burkly et al. 1995; Kontgen et al. 1995; Sha et al. 1995; Weih et al. 1995; Franzoso et al. 1998) or in various combinations (Iotsova et al. 1997; Mercurio and Manning 1999). However, knockout mice for the alpha subunit of the IκB kinase complex show defects in skeletal formation and keratinocyte development (Hu et al.

1999; Takeda et al. 1999). Moreover, expression of a dominant negative IκB molecule in the developing chick limb bud causes limb truncations, suggesting an unidentified NFκB-like activity is required for proper vertebrate development (Bushdid et al. 1998; Kanegae et al. 1998).

Retinoic acid can affect skeletal patterning, neurulation and limb-bud outgrowth during embryonic development (Paulsen 1994). The fact that dominant negative IκB expression causes limb truncations is particularly intriguing since prenatal RA excess has similar effects (Morriss-Kay and Sokolova 1996). RAR inhibition of NFκB activity could explain some of the negative effects that RA treatment has on limb-bud outgrowth.

A role in neuroendocrine-immune system interactions?

Thyroid hormone induces metamorphosis in X. *laevis* tadpoles (Chatterjee and Tata 1992) and plays important roles in neural development, cardiac function and pituitary hormone secretion (Brent 1994). Similarly, NFκB activity is detectable in neurons (Rattner et al. 1993; Kaltschmidt et al. 1994), cardiac muscle (Kan et al. 1999) and pituitary cells (Grandison et al. 1994; Smith et al. 1999). Curiously, a correlation exists between high TNFα levels and impaired pituitary function (Reincke et al. 1998). Although it is not yet clear whether these coincidences carry functional consequences, interactions between T3R and NFκB might modify certain physiological responses.

Materials and Methods

Plasmids

The plasmids pCMX-IκBα S36A/40A (Traenckner et al. 1995), pSG5 (Promega), DR4-GL3 (Sharif and Privalsky 1991), pSG5-hTR (Sharif and Privalsky 1991), pSG5-mRAR (Vivanco Ruiz et al. 1991), pRSV-cJun (Baichwal and Tjian 1990), pSG5-rGR (Darimont et al. 1998), CMV-Bgal (Spaete and Mocarski 1985), and RARE-tk-Luc are previously described. Plasmids kB3-DLO, pGEX-RelA-RDD, pGEX-p50-RDD, and pSG5-RelA are described in chapter I. Plasmid pSG5-cRel was constructed by subcloning the BamHI–XbaI fragment from plasmid J132 (Bull et al. 1990) into the pSG5-rGR backbone cut with BamHI–XbaI. Plasmid pSG5-p50 was constructed by subcloning the BamHI–XbaI fragment from plasmid J130 (Blank et al. 1991) into the pSG5-rGR backbone cut with BamHI–XbaI.

Cell culture and transfections

CV-1, HeLa, and F9 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum and split every second or third day. For transient transfections, CV-1, HeLa, or F9 cells were plated in 24 well plates at a concentration of $5x10^4$ cells per well in 500µl of DMEM containing 5% charcoal-stripped fetal calf serum approximately 16 hrs prior to addition of lipid-DNA complexes. Serum was charcoal-stripped as described (Freeman et al. 2000). Cationic lipid stock was prepared as described (Hong et al. 1997b). A typical transfection contained 100ng luciferase test reporter, 100ng beta-galactosidase control plasmid, varying amounts of the transcription factors, and empty pSG5 plasmid to 450ng total DNA in 40µl of OPTI-MEM I (Gibco-BRL). The 5mM cationic lipid stock was diluted to 0.5µl lipid per 40µl in

OPTI-MEM I. Then, 40μl of the diluted lipid was added to the 40μl of DNA, mixed, and incubated at room temperature for 15 min. After incubation, the lipid-DNA complexes were further diluted with 300μl of OPTI-MEM I and the entire 380μl was added to a single aspirated well of the 24 well plate. Lipid-DNA complexes were typically prepared as a stock of 6.5 identical reactions and then aliquoted onto the cells for replicates. Approximately 8-12 hrs later, hormone combinations or ethanol vehicle was added as a 2X stock in 400μl of 5% stripped FCS DMEM and approximately 8-12 hrs post hormone addition cells were harvested for beta-galactosidase and luciferase assays as described (Iniguez-Lluhi et al. 1997).

Protein expression and purification

Proteins fused to GST were expressed in E. coli strain BL21 at 18°C with 1mM IPTG induction at approximately 0.3 OD_{600} followed by an additional 12-14 hrs of growth prior to harvest. All steps were performed at 4°C unless noted otherwise and all buffers for GST fusions contained 0.5mM PMSF and 1 μg/ml leupeptin, pepstatin and aprotinin. Cell pellets were resuspended in one volume of phosphate buffered saline containing 1mM EDTA and 14mM b-mercaptoethanol. Extracts were prepared by lysozyme treatment followed by sonication to reduce turbidity. The extracts were then ultracentrifuged in a beckman 70.1Ti rotor at 45,000 rpm for 2 hrs. The supernatant was batch bound onto 1ml of 50% slurry glutathione-agarose beads (Sigma Co.) in the same buffer adjusted to 0.1% NP-40 for 30 min. The beads were then loaded into a disposable column and washed with 10ml of Wash Buffer (10mM Tris pH 8, 1mM EDTA, 2M NaCl, 0.1% NP-40, 14mM β-mercaptoethanol) followed by 10ml DnaK Buffer (50mM Tris 8, 10mM MgSO4, 2mM ATP). A final 5mL wash in Binding Buffer (10mM Tris pH 8, 1mM EDTA, 150mM NaCl, 14mM β-mercaptoethanol) was then performed and the purified proteins were stored with the beads at –80°C.

GST-fusion protein-protein interaction assay

Equal amounts, ~1 µg, of each fusion protein were used as judged by coomassie gel loading titrations. GR, T3R and RAR were *in vitro* translated using the Promega TNT coupled transcription/translation kit in the presence of ^{35}S-methionine (NEN). Binding reactions were performed in a total volume of 100µl Binding Buffer containing 12.5µl packed glutathione beads and approximately 5nM receptor. Reactions were conducted at room temperature with mild agitation to keep the beads in suspension for 45 min. Samples were then pelleted and beads were washed four times with 500µl of Binding Buffer containing 500mM NaCl. The pellets were aspirated, resuspended in 10µl of SDS-PAGE loading buffer and the entire reaction was resolved on a 12% SDS-PAGE gel. The gel was dried down and exposed to a phosphorimager screen overnight. Molecular dynamics software was used to generate and quantitate gel images.

Perspectives:

The Regulatory Modes for RNA Polymerase II Transcription

Abstract

Historically, transcriptional regulators have been categorized based on the smallest mechanistically divisible units of the transcription cycle. I propose a new scheme for classifying regulators organizing them into one of three regulatory modes drawn from the broadest steps of the transcription cycle. This classification scheme complements the more mechanistically driven models by focusing attention on how repressors interfere, antagonize and bypass the functions of activators during the early, intermediate and late stages of the transcription cycle, respectively, and how these different modes impact regulation. Coordinate input of different types of activators and repressors across different regulatory modes allows for a broad range of signal intensities. Combining distinct modes allows an organism to triage and integrate various signals into coherent and finely tunable responses.

Introduction

The eukaryotic transcription cycle involves ~60 polypeptides organized into multiple discrete macromolecular complexes including core RNA polymerase II (pol II), the general transcription factors (GTFs; $TF_{II}A$, $TF_{II}B$, $TF_{II}D$, $TF_{II}E$, $TF_{II}F$, $TF_{II}H$), the Srb/Med containing mediator complex, and several other factors whose precise roles are unclear (Kaiser and Meisterernst 1996; Roeder 1996; Verrijzer and Tjian 1996). For a transcriptional activator to function, it must successfully localize to a promoter/enhancer region and affect one of several biochemically distinct steps in the pol II transcription cycle; preinitiation complex (PIC) assembly, promoter melting, initiation or promoter clearance. The minimal PIC is a DNA bound complex of pol II and $TF_{II}A$, $TF_{II}B$, $TF_{II}D$ and $TF_{II}F$. $TF_{II}D$ is a complex of the TATA-box binding protein (TBP) and several TBP associated factors (TAFs) that nucleates PIC assembly on pol II regulated promoters (Cormack and Struhl 1992; Schultz et al. 1992; Hernandez 1993). $TF_{II}A$ stabilizes and increases TBP DNA binding by directly dissociating inactive TBP dimers (Coleman et al. 1999; Jackson-Fisher et al. 1999). $TF_{II}B$ stabilizes TBP association with DNA and, at least partly, establishes directionality to promoters through interaction with the seven nucleotide G/C-rich $TF_{II}B$ recognition element (BRE) (Tsai and Sigler 2000). Binding of $TF_{II}B$ also creates the binding site for the $TF_{II}F$-pol II complex, whose incorporation completes the PIC (Choy and Green 1993).

Contrary to the described stepwise assembly process, several reports suggest a pol II "holoenzyme" containing the Srb/Med mediator complex and a subset of the GTFs that can be directly recruited by transcriptional activators (Koleske and Young 1994; Hengartner et al. 1995; Koh et al. 1998). In agreement, TBP association with promoters *in vivo* requires the largest subunit of pol II and srb4, a component of the mediator-holoenzyme complex (Kuras and Struhl 1999). However, attempts to quantify the abundance of holoenzyme under physiological conditions yields surprisingly low

numbers (Kimura et al. 1999). The conclusion that PIC assembly is a highly cooperative process requiring activator stabilization of just one component for sufficient PIC formation reconciles these differences. Therefore, for the purposes of this discussion, we will treat PIC assembly as a highly cooperative process such that assistance or hindrance of different phases in the "step-wise" assembly model are equivalent and favor complete assembly or disassembly, respectively.

The Rpb1 subunit of pol II is particularly notable because phosphate groups are transferred to its carboxyl-terminal domain (CTD), which consists of 26-52 repeats of the YSPTSPS heptapeptide (Bartolomei et al. 1988; West and Corden 1995). Unphosphorylated pol II (IIa) is recruited into assembling PICs (Usheva et al. 1992) while hyper-phosphorylated pol II (IIo) complexes are associated with actively elongating complexes (O'Brien et al. 1994). The pol II CTD can be phosphorylated by several kinases, including a subunit of $TF_{II}H$, cdk7. The majority of pol II phosphorylation is on heptapeptide repeat positions serine-2 and serine-5, while cdk7 specifically phosphorylates serine-5 (Trigon et al. 1998). Therefore, other CTD-kinases are involved. However, the CTD-kinase responsible for phosphorylating serine-2 *in vivo* is not clear.

Promoter melting and transcript initiation requires two additional factors, $TF_{II}E$ and $TF_{II}H$. $TF_{II}E$ recruits $TF_{II}H$ into the PIC by directly associating with pol IIa (Maxon et al. 1994) and stimulates CTD-phosphorylation by the $TF_{II}H$-associated CTD-kinase, cdk7 (Drapkin et al. 1994). The $TF_{II}H$ DNA helicase subunit XPB/ERCC3 is required *in vivo* for promoter melting (Guzman and Lis 1999) and stimulates abortive initiation *in vitro* (Moreland et al. 1999). Thus, XPB/ERCC3 grants pol II access to the template strand allowing transcript initiation. Interestingly, $TF_{II}E$ inhibits the helicase activity of $TF_{II}H$ (Drapkin et al. 1994). Therefore, CTD-phosphorylation by $TF_{II}H$ or another CTD-kinase modifies $TF_{II}E$ association relieving inhibition of XPB/ERCC3 helicase activity. The requirement for XPB/ERCC3 de-repression places the conversion of IIa to IIo across

the transition from PIC assembly to promoter melting. Notably, while $TF_{II}E$ appears to deliver $TF_{II}H$ only into pol IIa containing complexes [Maxon, 1994 #277, *in vitro* crosslinking experiments demonstrate that conversion of IIa into IIo does not actually displace either $TF_{II}E$ or XPB/ERCC3 (Kim et al. 2000).

Promoter melting, initiation, and clearance are tightly coupled as all three steps require XPB/ERCC3 activity (Dvir et al. 1997; Guzman and Lis 1999; Moreland et al. 1999). Since TBP (Usheva et al. 1992) and mediator (Svejstrup et al. 1997) favor interaction with the pol IIa form, complex release from the promoter presumably coincides with conversion from IIa to IIo.

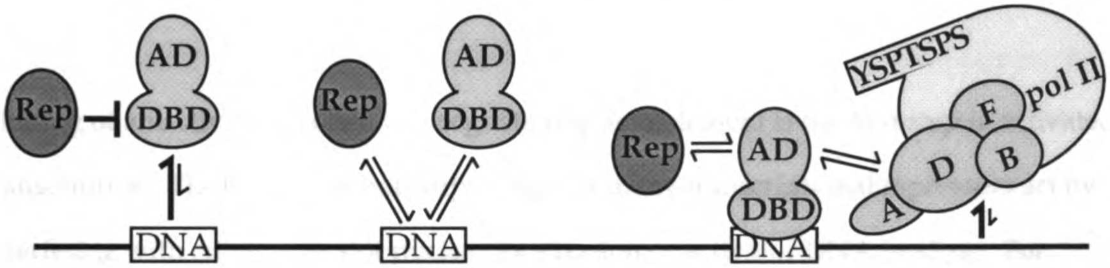
An intriguing feature of CTD phosphorylation and de-phosphorylation is the lack of intermediate species migrating between the IIa and IIo forms on SDS-PAGE gels, supporting the view that phosphate addition and removal is highly processive (Lehman and Dahmus 2000). While it is not yet clear whether a single heptapeptide repeat can be phosphorylated at multiple positions by the same or different kinases or whether phosphorylation by one enzyme precludes phosphorylation by another (*e.g.*, can cdk7-mediated conversion of YSPTSPS to YSPTS*PS be further processed by another kinase to YS*PTS*PS, or *vice-versa?)*, distinct roles for the different phosphorylation sites are suggested as mammalian mRNA capping activity is stimulated by CTD peptides phosphorylated at serine-5 but not at serine-2 (Ho and Shuman 1999). Thus, the apparent order of events during initiation and promoter clearance is conversion of IIa into IIo causing modification of $TF_{II}E$ association and de-repression of the XPB/ERCC3 helicase. The active helicase catalyzes promoter melting permitting synthesis of the first phosphodiester bonds. Simultaneously, conversion of IIa into IIo causes disengagement from TBP and mediator allowing promoter clearance catalyzed by ERCC3. The disengaged pol II complex could then incorporate general elongation factors allowing rapid synthesis of full length transcripts.

As described, the multi-megadalton complexes of the pol II transcription cycle will undoubtedly harbor many ordered enzymatic activities which will each represent a discrete step in transcription. While descriptions of these events are essential for a complete understanding of the mechanisms regulating gene expression, a broad classification allows the more general themes to emerge. Thus, I propose a classification scheme for transcriptional regulators that divides the transcription cycle into three modes: early, intermediate and late. Establishing activator presence and accessibility at a promoter region defines the earliest mode. This regulatory mode encompasses all mechanisms involving activator *interference*, that is, direct targeting of an activator by a dedicated repressor affecting either DNA binding or activation domain accessibility (Fig. 31A). After establishing DNA occupancy, activators and repressors can compete, directly or indirectly, for facilitation or suppression of PIC assembly. This intermediate mode of the transcription cycle allows for substantial "fine-tuning" of a regulatory response; countless regulators can *antagonize* one another without having to target the same aspect of PIC assembly. The regulatory output is a sum of the multi-factorial equilibria and is therefore exquisitely sensitive to the cellular concentrations of regulators and relative affinities between regulators and target factors (Fig. 31B). The late mode encompasses those mechanisms resulting in varying degrees of global repression. By definition, repressors affecting late modes of the transcription cycle *bypass* the majority of activator functions (Fig. 31C). Targeting different modes of the transcription cycle generates a regulatory gradient from the surgical precision of interference to the global nature of activator bypass. The reduced emphasis on mechanistic detail within this classification scheme highlights the different goals repressors can achieve functioning within different modes.

Figure 31. A model depicting the three functional modes of gene regulation. A) The left panel shows a repressor interfering with activator DNA binding activity by increasing the off rate. The middle panel shows direct competition between regulators for DNA occupancy. The right panel is an example of activation domain masking. The repressor directly competes with basal transcription machinery or coactivators for overlapping surfaces on an activation domain. B) An activator and a repressor stabilizing and destabilizing PIC assembly, respectively. The regulatory outcome for these competitive models hinges on the relative concentrations of regulators and affinities between factor and targets. C) Activator bypass involves repressing a late step in the transcription cycle after an activator has functioned. The example shows an activator stimulating PIC assembly and a repressor that blocks CTD phosphorylation.

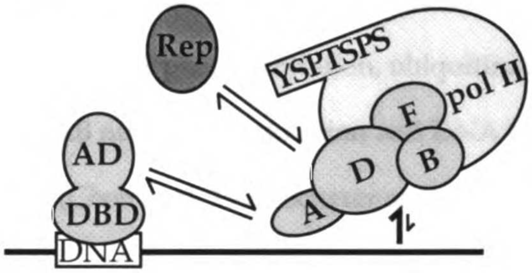
A.

The Earliest Mode: Interference



B.

The Intermediate Mode: Antagonism



C.

The Late Mode: Bypass

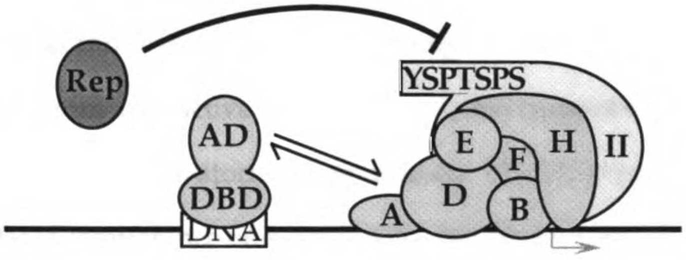


Figure 31.

The earliest mode: interference

DNA binding

Binding of an activator protein to a cognate response element is the first step in activated transcription. Therefore, it is not surprising that many transcriptional repressors act by interfering with either activator nuclear localization or activator DNA binding. For example, NFκB, a positive transcriptional regulator of the immune system, is retained in the cytoplasm by association with the dedicated inhibitory protein, IκB (Baeuerle and Baltimore 1988a; Baeuerle and Baltimore 1988b). Numerous immune-related signal transduction cascades trigger IκB phosphorylation, ubiquitination and proteosomal degradation, enabling NFκB nuclear translocation and DNA binding with subsequent transcriptional activation (Chen et al. 1995; Scherer et al. 1995; Mercurio et al. 1997; Woronicz et al. 1997).

Likewise, the unliganded glucocorticoid receptor (GR) is sequestered in the cytoplasm by the activity of an intramolecular domain, the ligand binding domain (LBD). Upon hormone addition, the LBD undergoes a conformational change allowing nuclear localization and response element binding. Strikingly, ligand binding also converts the LBD from an inactive state to a regulation competent state capable of interacting with either coactivators or corepressors. The LBD can similarly impose hormone regulated interference on heterologous fusion partners (Picard et al. 1988).

In addition to factors that alter nuclear localization, several repressors function by directly interfering with activator DNA binding. Many of these regulators have been identified and characterized in *Drosophila melanogaster* (Herschbach and Johnson 1993). Transcriptional regulators of the basic-region-helix-loop-helix (bHLH) gene family bind DNA as dimers; the HLH domain mediates dimerization while the basic region is involved in DNA binding. The *extramacrochaetae* (*emc*) protein is a bHLH family member that lacks the basic region. Thus, *emc* inhibits the bHLH activator proteins

daughterless and *achaete-scute* by forming heterodimers that cannot bind DNA (Ellis et al. 1990; Garrell and Modolell 1990; Van Doren et al. 1991; Cabrera et al. 1994).

Likewise, several repressors can directly compete with activators for binding to either identical or overlapping response elements (Herschbach and Johnson 1993). During early events of *D. melanogaster* embryo patterning, the homeodomain proteins *hunchback* (*hb*) and *bicoid* (*bcd*) activate transcription while the *giant* (*gt*) and *Krüppel* (*Kr*) homeodomain proteins repress transcription from the *even-skipped* (*eve*) stripe-2 enhancer. The DNA binding sites for *gt* and *Kr* overlap with the sites for *hb* and *bcd*. Thus, *gt* and *Kr* interfere by directly competing with *hb* and *bcd* for occupancy at the *eve* stripe-2 enhancer (Frasch and Levine 1987; Stanojevic et al. 1989; Small et al. 1991; Zuo et al. 1991; Small et al. 1992).

Activation domain masking

Although promoter occupancy by an activator protein is the first step toward activated transcription, DNA binding and transcriptional activation are separable functions for many proteins. In fact, several repressors directly bind and mask activation domains without interfering with activator DNA binding. In the presence of galactose, the *Saccharomyces cereviseae* GAL4p activates transcription of galactose metabolism genes using a carboxyl-terminal activation domain that interacts with both TBP and the histone acetyltransferase (HAT) containing Ada coactivator complex (Melcher and Johnston 1995; Grant et al. 1997). Through the GAL80p component, the yeast GAL80p-GAL3p complex is weakly bound to a central region of DNA bound GAL4p (Lue et al. 1987; Yano and Fukasawa 1997; Sil et al. 1999). In the presence of glucose, GAL3p dissociates from GAL80p allowing reorientation, TBP displacement (Ansari et al. 1998) and binding to the GAL4p activation domain (Lue et al. 1987). Thus, GAL80p directly

interferes with the GAL4p activation domain preventing contacts with basal transcription machinery and possibly with coactivator complexes.

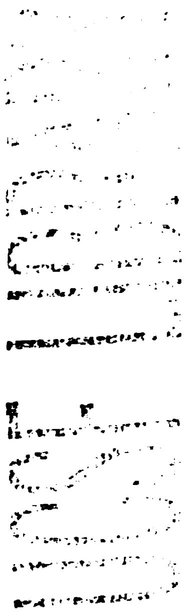
In a similar fashion, the p53 tumor suppressor protein activates transcription of G1/S cell cycle genes by interacting with both TBP and the p300 HAT coactivator (Chen et al. 1993b; Lill et al. 1997). Murine double minute 2 (mdm2) is a repressor protein that directly binds p53 (Momand et al. 1992; Chen et al. 1993a). The p53 protein can associate with either mdm2 or p300, but interaction is mutually exclusive such that mdm2 binding displaces p300 (Wadgaonkar and Collins 1999). Competition between coactivators and corepressors for overlapping interaction surfaces provides a direct mechanism for changing the regulatory mode of a single factor in a cell-type specific fashion.

Intramolecular regulatory domains are common features to diverse factors. The RelA subunit of NFκB provides a remarkable example of internal activation domain masking. The unphosphorylated amino-terminal domain of RelA silences activity by participating in an intramolecular interaction with the carboxyl-terminal activation domain. Protein kinase A phosphorylation of the RelA amino-terminal domain on serine-276 enhances activity by disrupting the intramolecular interaction. Interestingly, the coactivator CBP/p300 can bind two distinct regions within RelA but accessibility is blocked in unphosphorylated RelA. Thus, serine-276 phosphorylation relieves internal interference allowing coactivator recruitment (Zhong et al. 1998).

The intermediate mode: antagonism

PIC assembly

Given the importance of PIC assembly in gene expression, it is not surprising that many regulators facilitate and antagonize this step. For example, the vitamin D receptor (VDR), a member of the intracellular receptor gene family, activates transcription of the osteocalcin gene (Heinrichs et al. 1993). Several activators, including



the liganded VDR-LBD, associate with the ARC/DRIP coactivator, but pol II is not detected in the isolated ARC/DRIP complex (Rachez et al. 1998; Naar et al. 1999). Strikingly, the liganded VDR-LBD recruits the ARC/DRIP coactivator complex stimulating formation of the higher order ternary complex containing VDR, ARC/DRIP and a pol II enzyme that contains mediator (Rachez et al. 1998; Chiba et al. 2000). Thus, VDR appears to activate osteocalcin gene transcription by facilitating recruitment of a pol II holoenzyme. However, a GR binding site overlaps the TATA-box of the osteocalcin promoter. Thus, glucocorticoids negatively regulate the osteocalcin gene by a direct competition mechanism between GR and TBP for promoter occupancy, antagonizing VDR recruitment of pol II holoenzyme (Meyer et al. 1997a; Meyer et al. 1997b).

Regulators can also affect PIC assembly by covalently modifying components of chromatin as histone acetylation appears to facilitate TBP binding to chromatin templates (Grunstein 1997). Liganded thyroid hormone receptor (T3R) can directly interact with various members of the p160 coactivator gene family, many of which contain intrinsic histone acetyltransferase (HAT) activity (Hong et al. 1997a; Darimont et al. 1998; Feng et al. 1998). Intriguingly, the T3R-LBD can also directly interact with TRAP220 (Treuter et al. 1999), which is identical to DRIP205 (Rachez et al. 1999), suggesting recruitment of a complex similar, or identical, to the ARC/DRIP complex. Incorporating these results yields a model in which the liganded T3R-LBD might interact first with a HAT containing complex which acetylates nucleosomes, priming the template for PIC assembly followed by interaction with an ARC/DRIP-like complex which recruits pol II. Conversely, the unliganded T3R represses basal transcription by recruiting the distinct N-CoR/SMRT/mSin3a histone deacetylase (HDAC) containing complex (Chen and Evans 1995; Nagy et al. 1997). Thus, liganded T3R activates transcription by a two-step mechanism while the unliganded T3R represses transcription through recruitment of HDACs antagonizing promoter occupancy by TBP.

The multifaceted activation domain of the NFκB family member, RelA, stimulates PIC assembly by recruiting the HAT containing coactivators CBP/p300 (Zhong et al. 1998), the ARC/DRIP complex (Naar et al. 1999) and by directly interacting with several GTFs including $TF_{II}B$, TBP and $TAF_{II}105$ (Schmitz et al. 1995; Yamit-Hezi and Dikstein 1998). The dorsal switch protein 1 (DSP1) converts NFκB family members from activators to repressors in a context dependent manner by physical interaction and cooperative DNA binding with NFκB (Lehming et al. 1994; Brickman et al. 1999). DSP1 antagonizes PIC assembly by preventing incorporation of $TF_{II}A$ (Kirov et al. 1996) and also interacts with the heterochromatin protein HP1 (Lehming et al. 1998). Thus, DSP1 counters the multiple activation mechanisms NFκB employs by destabilizing PIC assembly and promoting formation of heterochromatin structures.

The late mode: bypass

Promoter clearance

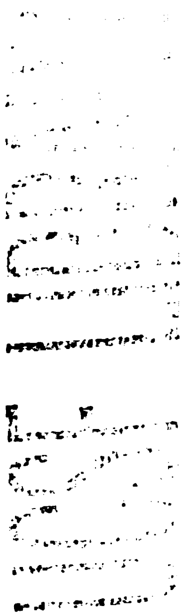
Several global regulators of transcription appear to target the functions of $TF_{II}H$. During mitosis, in addition to effects on $TF_{II}D$ (Segil et al. 1996), the cdc2/cyclinB complex mediates global transcriptional repression by phosphorylating the $TF_{II}H$ p62 and p35 subunits inhibiting the $TF_{II}H$-associated CTD-kinase, cdk7 (Long et al. 1998). As described, cdk7 is an essential CTD-kinase presumably involved in the conversion from pol IIa to IIo. Inactivation of cdk7 presumably blocks promoter melting by failing to mediated de-repression of the XPB/ERCC3 helicase.

Likewise, the NAT complex is a global negative regulator for pol II that contains the cyclin dependent kinase cdk8/Srb10 (Sun et al. 1998). Cdk8/Srb10 can inhibit transcription by prematurely phosphorylating the pol II CTD, thereby preventing PIC incorporation of the $TF_{II}F$-pol II complex (Hengartner et al. 1998; Sun et al. 1998). However, within the NAT complex, cdk8 appears to function more like cdc2/cyclinB,

phosphorylating $TF_{II}H$ and downregulating the CTD-kinase activity of cdk7 (D. Reinberg, personal communication). Although cdk8 can phosphorylate both serine-2 and serine-5 of the pol II CTD, how cdk8 phosphorylation of the CTD functionally differs from that catalyzed by cdk7 is not clear.

The *Caenorhabditis elegans* repressor PIE-1 may provide an unusual example of molecular mimicry. In early *C. elegans* embryos, mRNA synthesis is inhibited in the germline (Mello et al. 1996; Seydoux et al. 1996). The PIE-1 protein localizes to the nucleus of germline blastomeres repressing transcription by preventing phosphorylation of the pol II CTD at serine-2 by an unknown mechanism (Seydoux and Dunn 1997; Tenenhaus et al. 1998). Strikingly, the PIE-1 repression domain has a region with homology to the pol II CTD heptapeptide repeat that is required for PIE-1 function, *in vivo* (Batchelder et al. 1999; Seydoux and Strome 1999), fueling speculation for a mechanism involving competition between PIE-1 and the CTD for a common factor.

The glucocorticoid receptor (GR) can repress transcription driven by multiple distinct regulators including AP-1 and NFκB (Miner and Yamamoto 1991; Konig et al. 1992; Lefstin and Yamamoto 1998). The RelA subunit of NFκB activates, while GR represses, the transcription of many pro-inflammatory genes including IL-8 and ICAM-1 (van de Stolpe et al. 1993; Kunsch et al. 1994; Mukaida et al. 1994; Ray and Prefontaine 1994). The experiments that I have described in this thesis show that, even under glucocorticoid repressive conditions, the RelA protein binds to the cognate response elements of the IL-8 and ICAM-1 promoters, *in vivo*. Coupled with the direct interaction between GR and RelA, response element occupancy suggests that the two proteins form a ternary complex. As discussed above, the RelA activation domain primarily stimulates PIC assembly through interactions with GTFs and coactivators. Interestingly, GR inhibition of NFκB mediated activation bypasses RelA-mediated PIC assembly and $TF_{II}H$ incorporation. GR does not appear to block CTD phosphorylation at serine-5. Rather, GR blocks CTD phosphorylation at serine-2. Conceivably, phosphorylation at



serine-5 is sufficient to mediate ERCC3 de-repression and disengagement from TBP and mediator. However, it is unknown whether GR represses RelA mediated promoter melting. These results demonstrate the importance of phosphoserine-2 and highlight the need to understand the mechanistic differences between the various CTD phosphorylation events.

Conclusions

As described, transcription is an intricate process involving numerous factors. Although dissection of regulators into discrete functional domains is critical for understanding structure-activity relationships, we must keep in mind that full-length proteins within organisms will often defy the narrow mechanism-based classification schemes. The classifications offered here highlighted the contrasting modes by which a repressor can affect activated transcription. Notably, however, I could as easily have described each of the three functional categories in terms of activators overcoming repression.

While each regulatory mode encompasses a range of distinct mechanisms, the outcomes on transcription within each mode are nearly identical. For example, the regulatory scopes of the various forms of activator interference are quite similar: interference by a dedicated repressor eliminates regulatory contributions from either a single regulator or a family of highly homologous regulators (Fig. 31A). Within the intermediate mode, multiple regulators can independently target various aspects of PIC assembly. The apparent cooperative nature of PIC formation *in vivo* suggests that decorating promoter/enhancers with various regulators should allow calculation of transcriptional output based on multiple equilibria (Fig. 31B). Together, the early and intermediate modes of gene regulation allow for fine-tuning of a transcriptional response. However, regulators that affect late steps in the transcription cycle can bypass

the action of multiple activators offering the advantage of global regulation over nearly limitless regulators (Fig. 31C). Thus, organisms can prioritize physiological signals according to effects on different transcriptional modes; notably, cascades that affect late modes can override other signals.

Bibliography

Adcock, I.M., Y. Nasuhara, D.A. Stevens, and P.J. Barnes. 1999. Ligand-induced differentiation of glucocorticoid receptor (GR) trans- repression and transactivation: preferential targetting of NFκB and lack of IκB involvement. *Br J Pharmacol* **127**: 1003-1011.

Adkins, K.K., T.D. Levan, R.L. Miesfeld, and J.W. Bloom. 1998. Glucocorticoid regulation of GM-CSF: evidence for transcriptional mechanisms in airway epithelial cells. *Am J Physiol* **275**: L372-378.

Alland, L., R. Muhle, H. Hou, Jr., J. Potes, L. Chin, N. Schreiber-Agus, and R.A. DePinho. 1997. Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression [see comments]. *Nature* **387**: 49-55.

Allison, L.A., M. Moyle, M. Shales, and C.J. Ingles. 1985. Extensive homology among the largest subunits of eukaryotic and prokaryotic RNA polymerases. *Cell* **42**: 599-610.

Ansari, A.Z., R.J. Reece, and M. Ptashne. 1998. A transcriptional activating region with two contrasting modes of protein interaction. *Proc Natl Acad Sci U S A* **95**: 13543-13548.

Archambault, J., R.S. Chambers, M.S. Kobor, Y. Ho, M. Cartier, D. Bolotin, B. Andrews, C.M. Kane, and J. Greenblatt. 1997. An essential component of a C-terminal domain phosphatase that interacts with transcription factor IIF in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **94**: 14300-14305.

Auphan, N., J.A. DiDonato, C. Rosette, A. Helmberg, and M. Karin. 1995. Immunosuppression by glucocorticoids: inhibition of NFκ B activity through induction of I kappa B synthesis [see comments]. *Science* **270**: 286-290.

Ayer, D.E., C.D. Laherty, Q.A. Lawrence, A.P. Armstrong, and R.N. Eisenman. 1996. Mad proteins contain a dominant transcription repression domain. *Mol Cell Biol* **16**: 5772-5781.

Baeuerle, P.A. and D. Baltimore. 1988a. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NFκ B transcription factor. *Cell* **53**: 211-217.

Baeuerle, P.A. and D. Baltimore. 1988b. I kappa B: a specific inhibitor of the NFκ B transcription factor. *Science* **242**: 540-546.

Baichwal, V.R. and R. Tjian. 1990. Control of c-Jun activity by interaction of a cell-specific inhibitor with regulatory domain delta: differences between v- and c-Jun. *Cell* **63**: 815-825.

Baldwin, A.S., Jr. 1996. The NFκ B and I kappa B proteins: new discoveries and insights. *Annual Review of Immunology* **14**: 649-683.

Bannister, A.J. and T. Kouzarides. 1996. The CBP co-activator is a histone acetyltransferase. *Nature* **384**: 641-643.

Bartolomei, M.S., N.F. Halden, C.R. Cullen, and J.L. Corden. 1988. Genetic analysis of the repetitive carboxyl-terminal domain of the largest subunit of mouse RNA polymerase II. *Mol Cell Biol* **8**: 330-339.

Batchelder, C., M.A. Dunn, B. Choy, Y. Suh, C. Cassie, E.Y. Shim, T.H. Shin, C. Mello, G. Seydoux, and T.K. Blackwell. 1999. Transcriptional repression by the Caenorhabditis elegans germ-line protein PIE-1. *Genes Dev* **13**: 202-212.

Beg, A.A., T.S. Finco, P.V. Nantermet, and A.S. Baldwin, Jr. 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NFκ B activation. *Mol Cell Biol* **13**: 3301-3310.

Beg, A.A., W.C. Sha, R.T. Bronson, and D. Baltimore. 1995a. Constitutive NFκ B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice. *Genes Dev* **9**: 2736-2746.

Beg, A.A., W.C. Sha, R.T. Bronson, S. Ghosh, and D. Baltimore. 1995b. Embryonic lethality and liver degeneration in mice lacking the RelA component of NFκ B. *Nature* **376**: 167-170.

Belvin, M.P. and K.V. Anderson. 1996. A conserved signaling pathway: the Drosophila toll-dorsal pathway. *Annu Rev Cell Dev Biol* **12**: 393-416.

Birnstiel, M.L. 1988. Structure and function of major and minor small nuclear ribonucleoprotein particles. Springer Verlag, Berlin ; New York.

Blackwood, E.M. and J.T. Kadonaga. 1998. Going the distance: a current view of enhancer action. *Science* **281**: 61-63.

Blanco, J.C., S. Minucci, J. Lu, X.J. Yang, K.K. Walker, H. Chen, R.M. Evans, Y. Nakatani, and K. Ozato. 1998. The histone acetylase PCAF is a nuclear receptor coactivator. *Genes Dev* **12**: 1638-1651.

Blank, V., P. Kourilsky, and A. Israel. 1991. Cytoplasmic retention, DNA binding and processing of the NFκ B p50 precursor are controlled by a small region in its C-terminus. *Embo J* **10**: 4159-4167.

Boyes, J., P. Byfield, Y. Nakatani, and V. Ogryzko. 1998. Regulation of activity of the transcription factor GATA-1 by acetylation. *Nature* **396**: 594-598.

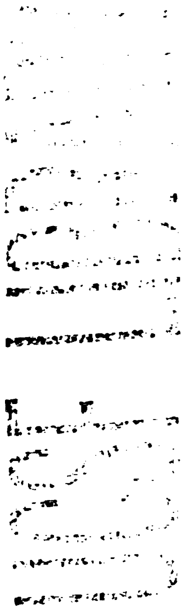
Braunstein, M., R.E. Sobel, C.D. Allis, B.M. Turner, and J.R. Broach. 1996. Efficient transcriptional silencing in Saccharomyces cerevisiae requires a heterochromatin histone acetylation pattern. *Mol Cell Biol* **16**: 4349-4356.

Brent, G.A. 1994. The molecular basis of thyroid hormone action. *N Engl J Med* **331**: 847-853.

Brickman, J.M., M. Adam, and M. Ptashne. 1999. Interactions between an HMG-1 protein and members of the Rel family. *Proc Natl Acad Sci U S A* **96**: 10679-10683.

Brostjan, C., J. Anrather, V. Csizmadia, D. Stroka, M. Soares, F.H. Bach, and H. Winkler. 1996. Glucocorticoid-mediated repression of NFkappaB activity in endothelial cells does not involve induction of IkappaBalpha synthesis. *J Biol Chem* **271**: 19612-19616.

Bull, P., K.L. Morley, M.F. Hoekstra, T. Hunter, and I.M. Verma. 1990. The mouse c-rel protein has an N-terminal regulatory domain and a C-terminal transcriptional transactivation domain. *Mol Cell Biol* **10**: 5473-5485.



Burkly, L., C. Hession, L. Ogata, C. Reilly, L.A. Marconi, D. Olson, R. Tizard, R. Cate, and D. Lo. 1995. Expression of relB is required for the development of thymic medulla and dendritic cells. *Nature* **373**: 531-536.

Bushdid, P.B., D.M. Brantley, F.E. Yull, G.L. Blaeuer, L.H. Hoffman, L. Niswander, and L.D. Kerr. 1998. Inhibition of NFκB activity results in disruption of the apical ectodermal ridge and aberrant limb morphogenesis [see comments]. *Nature* **392**: 615-618.

Cabrera, C.V., M.C. Alonso, and H. Huikeshoven. 1994. Regulation of scute function by extramacrochaete in vitro and in vivo. *Development* **120**: 3595-3603.

Caldenhoven, E., J. Liden, S. Wissink, A. Van de Stolpe, J. Raaijmakers, L. Koenderman, S. Okret, J.A. Gustafsson, and P.T. Van der Saag. 1995. Negative cross-talk between RelA and the glucocorticoid receptor: a possible mechanism for the antiinflammatory action of glucocorticoids. *Mol Endocrinol* **9**: 401-412.

Charles, M.A., G.U. Ryffel, M. Obinata, B.J. McCarthy, and J.D. Baxter. 1975. Nuclear receptors for thyroid hormone: evidence for nonrandom distribution within chromatin. *Proc Natl Acad Sci U S A* **72**: 1787-1791.

Chatterjee, V.K. and J.R. Tata. 1992. Thyroid hormone receptors and their role in development. *Cancer Surv* **14**: 147-167.

Chen, F.E., D.B. Huang, Y.Q. Chen, and G. Ghosh. 1998. Crystal structure of p50/p65 heterodimer of transcription factor NF- kappaB bound to DNA. *Nature* **391**: 410-413.

Chen, H., R.J. Lin, R.L. Schiltz, D. Chakravarti, A. Nash, L. Nagy, M.L. Privalsky, Y. Nakatani, and R.M. Evans. 1997. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* **90**: 569-580.

Chen, J., V. Marechal, and A.J. Levine. 1993a. Mapping of the p53 and mdm-2 interaction domains. *Mol Cell Biol* **13**: 4107-4114.

Chen, J.D. and R.M. Evans. 1995. A transcriptional co-repressor that interacts with nuclear hormone receptors [see comments]. *Nature* **377**: 454-457.

Chen, X., G. Farmer, H. Zhu, R. Prywes, and C. Prives. 1993b. Cooperative DNA binding of p53 with TFIID (TBP): a possible mechanism for transcriptional activation [published erratum appears in Genes Dev 1993 Dec;7(12B):2652]. *Genes Dev* **7**: 1837-1849.

Chen, Z., J. Hagler, V.J. Palombella, F. Melandri, D. Scherer, D. Ballard, and T. Maniatis. 1995. Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. *Genes Dev* **9**: 1586-1597.

Chiba, N., Z. Suldan, L.P. Freedman, and J.D. Parvin. 2000. Binding of liganded vitamin D receptor to the vitamin D receptor interacting protein coactivator complex induces interaction with RNA polymerase II holoenzyme [In Process Citation]. *J Biol Chem* **275**: 10719-10722.

Cho, H., T.K. Kim, H. Mancebo, W.S. Lane, O. Flores, and D. Reinberg. 1999. A protein phosphatase functions to recycle RNA polymerase II. *Genes Dev* **13**: 1540-1552.

Choy, B. and M.R. Green. 1993. Eukaryotic activators function during multiple steps of preinitiation complex assembly. *Nature* **366**: 531-536.

Cismowski, M.J., G.M. Laff, M.J. Solomon, and S.I. Reed. 1995. KIN28 encodes a C-terminal domain kinase that controls mRNA transcription in Saccharomyces cerevisiae but lacks cyclin-dependent kinase-activating kinase (CAK) activity. *Mol Cell Biol* **15**: 2983-2992.

Cole, T.J., J.A. Blendy, A.P. Monaghan, K. Krieglstein, W. Schmid, A. Aguzzi, G. Fantuzzi, E. Hummler, K. Unsicker, and G. Schutz. 1995. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Genes Dev* **9**: 1608-1621.

Coleman, R.A., A.K. Taggart, S. Burma, J.J. Chicca, 2nd, and B.F. Pugh. 1999. TFIIA regulates TBP and TFIID dimers. *Mol Cell* **4**: 451-457.

Cormack, B.P. and K. Struhl. 1992. The TATA-binding protein is required for transcription by all three nuclear RNA polymerases in yeast cells. *Cell* **69**: 685-696.

Da Silva, J.A. 1999. Sex hormones and glucocorticoids: interactions with the immune system. *Ann N Y Acad Sci* **876**: 102-117; discussion 117-108.

Darimont, B.D., R.L. Wagner, J.W. Apriletti, M.R. Stallcup, P.J. Kushner, J.D. Baxter, R.J. Fletterick, and K.R. Yamamoto. 1998. Structure and specificity of nuclear receptor-coactivator interactions. *Genes Dev* **12**: 3343-3356.

De Bosscher, K., M.L. Schmitz, W. Vanden Berghe, S. Plaisance, W. Fiers, and G. Haegeman. 1997. Glucocorticoid-mediated repression of nuclear factorκB-dependent transcription involves direct interference with transactivation. *Proc Natl Acad Sci U S A* **94**: 13504-13509.

Diamond, M.I., J.N. Miner, S.K. Yoshinaga, and K.R. Yamamoto. 1990. Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. *Science* **249**: 1266-1272.

Dignam, J.D., R.M. Lebovitz, and R.G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* **11**: 1475-1489.

Drapkin, R., J.T. Reardon, A. Ansari, J.C. Huang, L. Zawel, K. Ahn, A. Sancar, and D. Reinberg. 1994. Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II. *Nature* **368**: 769-772.

Dvir, A., R.C. Conaway, and J.W. Conaway. 1997. A role for TFIIH in controlling the activity of early RNA polymerase II elongation complexes. *Proc Natl Acad Sci U S A* **94**: 9006-9010.

Elenkov, I.J., E.L. Webster, D.J. Torpy, and G.P. Chrousos. 1999. Stress, corticotropin-releasing hormone, glucocorticoids, and the immune/inflammatory response: acute and chronic effects. *Ann N Y Acad Sci* **876**: 1-11; discussion 11-13.

Ellis, H.M., D.R. Spann, and J.W. Posakony. 1990. extramacrochaetae, a negative regulator of sensory organ development in Drosophila, defines a new class of helix-loop-helix proteins. *Cell* **61**: 27-38.

Feng, W., R.C. Ribeiro, R.L. Wagner, H. Nguyen, J.W. Apriletti, R.J. Fletterick, J.D. Baxter, P.J. Kushner, and B.L. West. 1998. Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. *Science* **280**: 1747-1749.

Franzoso, G., L. Carlson, L. Poljak, E.W. Shores, S. Epstein, A. Leonardi, A. Grinberg, T. Tran, T. Scharton-Kersten, M. Anver, P. Love, K. Brown, and U. Siebenlist. 1998. Mice deficient in nuclear factor (NF)κ B/p52 present with defects in humoral responses, germinal center reactions, and splenic microarchitecture. *J Exp Med* **187**: 147-159.

Frasch, M. and M. Levine. 1987. Complementary patterns of even-skipped and fushi tarazu expression involve their differential regulation by a common set of segmentation genes in Drosophila. *Genes Dev* **1**: 981-995.

Freeman, B.C., S.J. Felts, D.O. Toft, and K.R. Yamamoto. 2000. The p23 molecular chaperones act at a late step in intracellular receptor action to differentially affect ligand efficacies. *Genes Dev* **14**: 422-434.

Galasinski, S.K., T.N. Lively, A. Grebe De Barron, and J.A. Goodrich. 2000. Acetyl coenzyme A stimulates RNA polymerase II transcription and promoter binding by transcription factor IID in the absence of histones. *Mol Cell Biol* **20**: 1923-1930.

Garrell, J. and J. Modolell. 1990. The Drosophila extramacrochaetae locus, an antagonist of proneural genes that, like these genes, encodes a helix-loop-helix protein. *Cell* **61**: 39-48.

Gaub, M.P., M. Bellard, I. Scheuer, P. Chambon, and P. Sassone-Corsi. 1990. Activation of the ovalbumin gene by the estrogen receptor involves the fos-jun complex. *Cell* **63**: 1267-1276.

Ghosh, G., G. van Duyne, S. Ghosh, and P.B. Sigler. 1995. Structure of NFκ B p50 homodimer bound to a kappa B site [see comments]. *Nature* **373**: 303-310.

Ghosh, S., M.J. May, and E.B. Kopp. 1998. NFκ B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* **16**: 225-260.

Gilmour, D.S. and J.T. Lis. 1986. RNA polymerase II interacts with the promoter region of the noninduced hsp70 gene in Drosophila melanogaster cells. *Mol Cell Biol* **6**: 3984-3989.

Godowski, P.J., S. Rusconi, R. Miesfeld, and K.R. Yamamoto. 1987. Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement [published erratum appears in Nature 1987 Mar 5-11;326(6108):105]. *Nature* **325**: 365-368.

Goldsby, R.A., T.J. Kindt, J. Kuby, and B.A. Osborne. 2000. Kuby immunology. W.H. Freeman, New York.

Grandison, L., G.P. Nolan, and D.W. Pfaff. 1994. Activation of the transcription factor NF-KB in GH3 pituitary cells. *Mol Cell Endocrinol* **106**: 9-15.

Grant, P.A., L. Duggan, J. Cote, S.M. Roberts, J.E. Brownell, R. Candau, R. Ohba, T. Owen-Hughes, C.D. Allis, F. Winston, S.L. Berger, and J.L. Workman. 1997. Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. *Genes Dev* **11**: 1640-1650.

Grunstein, M. 1997. Histone acetylation in chromatin structure and transcription. *Nature* **389**: 349-352.

Gu, W. and R.G. Roeder. 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* **90**: 595-606.

Guzman, E. and J.T. Lis. 1999. Transcription factor TFIIH is required for promoter melting in vivo. *Mol Cell Biol* **19**: 5652-5658.

Harada, A., N. Sekido, T. Akahoshi, T. Wada, N. Mukaida, and K. Matsushima. 1994. Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J Leukoc Biol* **56**: 559-564.

Hassig, C.A., T.C. Fleischer, A.N. Billin, S.L. Schreiber, and D.E. Ayer. 1997. Histone deacetylase activity is required for full transcriptional repression by mSin3A. *Cell* **89**: 341-347.

Heck, S., K. Bender, M. Kullmann, M. Gottlicher, P. Herrlich, and A.C. Cato. 1997. I kappaB alpha-independent downregulation of NFκB activity by glucocorticoid receptor. *Embo J* **16**: 4698-4707.

Heery, D.M., E. Kalkhoven, S. Hoare, and M.G. Parker. 1997. A signature motif in transcriptional co-activators mediates binding to nuclear receptors [see comments]. *Nature* **387**: 733-736.

Heinrichs, A.A., R. Bortell, S. Rahman, J.L. Stein, E.S. Alnemri, G. Litwack, J.B. Lian, and G.S. Stein. 1993. Identification of multiple glucocorticoid receptor binding sites in the rat osteocalcin gene promoter. *Biochemistry* **32**: 11436-11444.

Heinzel, T., R.M. Lavinsky, T.M. Mullen, M. Soderstrom, C.D. Laherty, J. Torchia, W.M. Yang, G. Brard, S.D. Ngo, J.R. Davie, E. Seto, R.N. Eisenman, D.W. Rose, C.K. Glass, and M.G. Rosenfeld. 1997. A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression [see comments]. *Nature* **387**: 43-48.

Hengartner, C.J., V.E. Myer, S.M. Liao, C.J. Wilson, S.S. Koh, and R.A. Young. 1998. Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. *Mol Cell* **2**: 43-53.

Hengartner, C.J., C.M. Thompson, J. Zhang, D.M. Chao, S.M. Liao, A.J. Koleske, S. Okamura, and R.A. Young. 1995. Association of an activator with an RNA polymerase II holoenzyme. *Genes Dev* **9**: 897-910.

Hernandez, N. 1993. TBP, a universal eukaryotic transcription factor? *Genes Dev* **7**: 1291-1308.

Herschbach, B.M. and A.D. Johnson. 1993. Transcriptional repression in eukaryotes. *Annu Rev Cell Biol* **9**: 479-509.

Ho, C.K. and S. Shuman. 1999. Distinct roles for CTD Ser-2 and Ser-5 phosphorylation in the recruitment and allosteric activation of mammalian mRNA capping enzyme. *Mol Cell* **3**: 405-411.

Hong, H., K. Kohli, M.J. Garabedian, and M.R. Stallcup. 1997a. GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. *Mol Cell Biol* **17**: 2735-2744.

Hong, K., W. Zheng, A. Baker, and D. Papahadjopoulos. 1997b. Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly(ethylene glycol)-phospholipid conjugates for efficient in vivo gene delivery. *FEBS Lett* **400**: 233-237.

Howard, K.J., S.J. Holley, K.R. Yamamoto, and C.W. Distelhorst. 1990. Mapping the HSP90 binding region of the glucocorticoid receptor. *J Biol Chem* **265**: 11928-11935.

Hu, Y., V. Baud, M. Delhase, P. Zhang, T. Deerinck, M. Ellisman, R. Johnson, and M. Karin. 1999. Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase [see comments]. *Science* **284**: 316-320.

Hung, H.L., J. Lau, A.Y. Kim, M.J. Weiss, and G.A. Blobel. 1999. CREB-Binding protein acetylates hematopoietic transcription factor GATA-1 at functionally important sites. *Mol Cell Biol* **19**: 3496-3505.

Imhof, A., X.J. Yang, V.V. Ogryzko, Y. Nakatani, A.P. Wolffe, and H. Ge. 1997. Acetylation of general transcription factors by histone acetyltransferases. *Curr Biol* **7**: 689-692.

Iniguez-Lluhi, J.A., D.Y. Lou, and K.R. Yamamoto. 1997. Three amino acid substitutions selectively disrupt the activation but not the repression function of the glucocorticoid receptor N terminus. *J Biol Chem* **272**: 4149-4156.

Iotsova, V., J. Caamano, J. Loy, Y. Yang, A. Lewin, and R. Bravo. 1997. Osteopetrosis in mice lacking NFκB1 and NFκB2 [see comments]. *Nat Med* **3**: 1285-1289.

Jackson-Fisher, A.J., C. Chitikila, M. Mitra, and B.F. Pugh. 1999. A role for TBP dimerization in preventing unregulated gene expression. *Mol Cell* **3**: 717-727.

Jantzen, H.M., U. Strahle, B. Gloss, F. Stewart, W. Schmid, M. Boshart, R. Miksicek, and G. Schutz. 1987. Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. *Cell* **49**: 29-38.

Jeang, K.T., H. Xiao, and E.A. Rich. 1999. Multifaceted activities of the HIV-1 transactivator of transcription, Tat. *J Biol Chem* **274**: 28837-28840.

Jonat, C., H.J. Rahmsdorf, K.K. Park, A.C. Cato, S. Gebel, H. Ponta, and P. Herrlich. 1990. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell* **62**: 1189-1204.

Kaiser, K. and M. Meisterernst. 1996. The human general co-factors [see comments]. *Trends Biochem Sci* **21**: 342-345.

Kalkhoven, E., S. Wissink, P.T. van der Saag, and B. van der Burg. 1996. Negative interaction between the RelA(p65) subunit of NFκB and the progesterone receptor. *J Biol Chem* **271**: 6217-6224.

Kallio, P.J., H. Poukka, A. Moilanen, O.A. Janne, and J.J. Palvimo. 1995. Androgen receptor-mediated transcriptional regulation in the absence of direct interaction with a specific DNA element. *Mol Endocrinol* **9**: 1017-1028.

Kaltschmidt, C., B. Kaltschmidt, H. Neumann, H. Wekerle, and P.A. Baeuerle. 1994. Constitutive NFκ B activity in neurons. *Mol Cell Biol* **14**: 3981-3992.

Kan, H., Z. Xie, and M.S. Finkel. 1999. TNFα enhances cardiac myocyte NO production through MAP kinase-mediated NFκB activation. *Am J Physiol* **277**: H1641-1646.

Kanegae, Y., A.T. Tavares, J.C. Izpisua Belmonte, and I.M. Verma. 1998. Role of Rel/NFκB transcription factors during the outgrowth of the vertebrate limb [see comments]. *Nature* **392**: 611-614.

Kiernan, R.E., C. Vanhulle, L. Schiltz, E. Adam, H. Xiao, F. Maudoux, C. Calomme, A. Burny, Y. Nakatani, K.T. Jeang, M. Benkirane, and C. Van Lint. 1999. HIV-1 tat transcriptional activity is regulated by acetylation. *Embo J* **18**: 6106-6118.

Kim, T.K., R.H. Ebright, and D. Reinberg. 2000. Mechanism of ATP-dependent promoter melting by transcription factor IIH [In Process Citation]. *Science* **288**: 1418-1422.

Kimura, H., Y. Tao, R.G. Roeder, and P.R. Cook. 1999. Quantitation of RNA polymerase II and its transcription factors in an HeLa cell: little soluble holoenzyme but significant amounts of polymerases attached to the nuclear substructure. *Mol Cell Biol* **19**: 5383-5392.

Kirov, N.C., P.M. Lieberman, and C. Rushlow. 1996. The transcriptional corepressor DSP1 inhibits activated transcription by disrupting TFIIA-TBP complex formation. *Embo J* **15**: 7079-7087.

Koh, S.S., A.Z. Ansari, M. Ptashne, and R.A. Young. 1998. An activator target in the RNA polymerase II holoenzyme. *Mol Cell* **1**: 895-904.

Koleske, A.J. and R.A. Young. 1994. An RNA polymerase II holoenzyme responsive to activators [see comments]. *Nature* **368**: 466-469.

Konig, H., H. Ponta, H.J. Rahmsdorf, and P. Herrlich. 1992. Interference between pathway-specific transcription factors: glucocorticoids antagonize phorbol ester-induced AP-1 activity without altering AP-1 site occupation in vivo. *Embo J* **11**: 2241-2246.

Kontgen, F., R.J. Grumont, A. Strasser, D. Metcalf, R. Li, D. Tarlinton, and S. Gerondakis. 1995. Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. *Genes Dev* **9**: 1965-1977.

Kouzarides, T. 2000. Acetylation: a regulatory modification to rival phosphorylation? *Embo J* **19**: 1176-1179.

Kralli, A., S.P. Bohen, and K.R. Yamamoto. 1995. LEM1, an ATP-binding-cassette transporter, selectively modulates the biological potency of steroid hormones. *Proc Natl Acad Sci U S A* **92**: 4701-4705.

Kralli, A. and K.R. Yamamoto. 1996. An FK506-sensitive transporter selectively decreases intracellular levels and potency of steroid hormones. *J Biol Chem* **271**: 17152-17156.

Kraus, W.L. and J.T. Kadonaga. 1998. p300 and estrogen receptor cooperatively activate transcription via differential enhancement of initiation and reinitiation. *Genes Dev* **12**: 331-342.

Kunsch, C., R.K. Lang, C.A. Rosen, and M.F. Shannon. 1994. Synergistic transcriptional activation of the IL-8 gene by NFκ B p65 (RelA) and NF-IL-6. *J Immunol* **153**: 153-164.

Kuras, L. and K. Struhl. 1999. Binding of TBP to promoters in vivo is stimulated by activators and requires Pol II holoenzyme. *Nature* **399**: 609-613.

Laherty, C.D., W.M. Yang, J.M. Sun, J.R. Davie, E. Seto, and R.N. Eisenman. 1997. Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. *Cell* **89**: 349-356.

Lee, D. and J.T. Lis. 1998. Transcriptional activation independent of TFIIH kinase and the RNA polymerase II mediator in vivo. *Nature* **393**: 389-392.

Lee, J.M. and A.L. Greenleaf. 1991. CTD kinase large subunit is encoded by CTK1, a gene required for normal growth of Saccharomyces cerevisiae. *Gene Expr* **1**: 149-167.

Lefstin, J.A., J.R. Thomas, and K.R. Yamamoto. 1994. Influence of a steroid receptor DNA-binding domain on transcriptional regulatory functions. *Genes Dev* **8**: 2842-2856.

Lefstin, J.A. and K.R. Yamamoto. 1998. Allosteric effects of DNA on transcriptional regulators. *Nature* **392**: 885-888.

Lehman, A.L. and M.E. Dahmus. 2000. The sensitivity of RNA polymerase II in elongation complexes to C-terminal domain phosphatase [In Process Citation]. *J Biol Chem* **275**: 14923-14932.

Lehming, N., A. Le Saux, J. Schuller, and M. Ptashne. 1998. Chromatin components as part of a putative transcriptional repressing complex. *Proc Natl Acad Sci U S A* **95**: 7322-7326.

Lehming, N., D. Thanos, J.M. Brickman, J. Ma, T. Maniatis, and M. Ptashne. 1994. An HMG-like protein that can switch a transcriptional activator to a repressor. *Nature* **371**: 175-179.

Lenardo, M., J.W. Pierce, and D. Baltimore. 1987. Protein-binding sites in Ig gene enhancers determine transcriptional activity and inducibility. *Science* **236**: 1573-1577.

Li, Q., M. Herrler, N. Landsberger, N. Kaludov, V.V. Ogryzko, Y. Nakatani, and A.P. Wolffe. 1998. Xenopus NF-Y pre-sets chromatin to potentiate p300 and acetylation-responsive transcription from the Xenopus hsp70 promoter in vivo. *Embo J* **17**: 6300-6315.

Liden, J., F. Delaunay, I. Rafter, J. Gustafsson, and S. Okret. 1997. A new function for the C-terminal zinc finger of the glucocorticoid receptor. Repression of RelA transactivation. *J Biol Chem* **272**: 21467-21472.

Lill, N.L., S.R. Grossman, D. Ginsberg, J. DeCaprio, and D.M. Livingston. 1997. Binding and modulation of p53 by p300/CBP coactivators. *Nature* **387**: 823-827.

Lis, J.T., P. Mason, J. Peng, D.H. Price, and J. Werner. 2000. P-TEFb kinase recruitment and function at heat shock loci [In Process Citation]. *Genes Dev* **14**: 792-803.

Long, J.J., A. Leresche, R.W. Kriwacki, and J.M. Gottesfeld. 1998. Repression of TFIIH transcriptional activity and TFIIH-associated cdk7 kinase activity at mitosis. *Mol Cell Biol* **18**: 1467-1476.

Lue, N.F., D.I. Chasman, A.R. Buchman, and R.D. Kornberg. 1987. Interaction of GAL4 and GAL80 gene regulatory proteins in vitro. *Mol Cell Biol* **7**: 3446-3451.

Luisi, B.F., W.X. Xu, Z. Otwinowski, L.P. Freedman, K.R. Yamamoto, and P.B. Sigler. 1991. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA [see comments]. *Nature* **352**: 497-505.

Mancebo, H.S., G. Lee, J. Flygare, J. Tomassini, P. Luu, Y. Zhu, J. Peng, C. Blau, D. Hazuda, D. Price, and O. Flores. 1997. P-TEFb kinase is required for HIV Tat transcriptional activation in vivo and in vitro. *Genes Dev* **11**: 2633-2644.

Maxon, M.E., J.A. Goodrich, and R. Tjian. 1994. Transcription factor IIE binds preferentially to RNA polymerase IIa and recruits TFIIH: a model for promoter clearance. *Genes Dev* **8**: 515-524.

Melcher, K. and S.A. Johnston. 1995. GAL4 interacts with TATA-binding protein and coactivators. *Mol Cell Biol* **15**: 2839-2848.

Mello, C.C., C. Schubert, B. Draper, W. Zhang, R. Lobel, and J.R. Priess. 1996. The PIE-1 protein and germline specification in C. elegans embryos [letter]. *Nature* **382**: 710-712.

Mercurio, F. and A.M. Manning. 1999. Multiple signals converging on NFκB. *Curr Opin Cell Biol* **11**: 226-232.

Mercurio, F., H. Zhu, B.W. Murray, A. Shevchenko, B.L. Bennett, J. Li, D.B. Young, M. Barbosa, M. Mann, A. Manning, and A. Rao. 1997. IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF- kappaB activation [see comments]. *Science* **278**: 860-866.

Meyer, T., J. Carlstedt-Duke, and D.B. Starr. 1997a. A weak TATA box is a prerequisite for glucocorticoid-dependent repression of the osteocalcin gene. *J Biol Chem* **272**: 30709-30714.

Meyer, T., J.A. Gustafsson, and J. Carlstedt-Duke. 1997b. Glucocorticoid-dependent transcriptional repression of the osteocalcin gene by competitive binding at the TATA box. *DNA Cell Biol* **16**: 919-927.

Meyer, T., D.B. Starr, and J. Carlstedt-Duke. 1997c. The rat glucocorticoid receptor mutant K461A differentiates between two different mechanisms of transrepression. *J Biol Chem* **272**: 21090-21095.

Miesfeld, R., P.J. Godowski, B.A. Maler, and K.R. Yamamoto. 1987. Glucocorticoid receptor mutants that define a small region sufficient for enhancer activation. *Science* **236**: 423-427.

Miesfeld, R., S. Okret, A.C. Wikstrom, O. Wrange, J.A. Gustafsson, and K.R. Yamamoto. 1984. Characterization of a steroid hormone receptor gene and mRNA in wild-type and mutant cells. *Nature* **312**: 779-781.

Miesfeld, R., S. Rusconi, P.J. Godowski, B.A. Maler, S. Okret, A.C. Wikstrom, J.A. Gustafsson, and K.R. Yamamoto. 1986. Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor cDNA. *Cell* **46**: 389-399.

Miner, J.N. and K.R. Yamamoto. 1991. Regulatory crosstalk at composite response elements. *Trends Biochem Sci* **16**: 423-426.

Miner, J.N. and K.R. Yamamoto. 1992. The basic region of AP-1 specifies glucocorticoid receptor activity at a composite response element. *Genes Dev* **6**: 2491-2501.

Mizzen, C.A., X.J. Yang, T. Kokubo, J.E. Brownell, A.J. Bannister, T. Owen-Hughes, J. Workman, L. Wang, S.L. Berger, T. Kouzarides, Y. Nakatani, and C.D. Allis. 1996. The TAF(II)250 subunit of TFIID has histone acetyltransferase activity. *Cell* **87**: 1261-1270.

Momand, J., G.P. Zambetti, D.C. Olson, D. George, and A.J. Levine. 1992. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* **69**: 1237-1245.

Moreland, R.J., F. Tirode, Q. Yan, J.W. Conaway, J.M. Egly, and R.C. Conaway. 1999. A role for the TFIIH XPB DNA helicase in promoter escape by RNA polymerase II. *J Biol Chem* **274**: 22127-22130.

Morriss-Kay, G.M. and N. Sokolova. 1996. Embryonic development and pattern formation. *Faseb J* **10**: 961-968.

Mukaida, N., G.L. Gussella, T. Kasahara, Y. Ko, C.O. Zachariae, T. Kawai, and K. Matsushima. 1992. Molecular analysis of the inhibition of interleukin-8 production by dexamethasone in a human fibrosarcoma cell line. *Immunology* **75**: 674-679.

Mukaida, N., M. Morita, Y. Ishikawa, N. Rice, S. Okamoto, T. Kasahara, and K. Matsushima. 1994. Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factorκ B is target for glucocorticoid-mediated interleukin 8 gene repression. *J Biol Chem* **269**: 13289-13295.

Munshi, N., M. Merika, J. Yie, K. Senger, G. Chen, and D. Thanos. 1998. Acetylation of HMG I(Y) by CBP turns off IFN beta expression by disrupting the enhanceosome. *Mol Cell* **2**: 457-467.

Naar, A.M., P.A. Beaurang, S. Zhou, S. Abraham, W. Solomon, and R. Tjian. 1999. Composite co-activator ARC mediates chromatin-directed transcriptional activation. *Nature* **398**: 828-832.

Naar, A.M., J.M. Boutin, S.M. Lipkin, V.C. Yu, J.M. Holloway, C.K. Glass, and M.G. Rosenfeld. 1991. The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. *Cell* **65**: 1267-1279.

Nagy, L., H.Y. Kao, D. Chakravarti, R.J. Lin, C.A. Hassig, D.E. Ayer, S.L. Schreiber, and R.M. Evans. 1997. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* **89**: 373-380.

Newton, R., L.A. Hart, D.A. Stevens, M. Bergmann, L.E. Donnelly, I.M. Adcock, and P.J. Barnes. 1998. Effect of dexamethasone on interleukin-1beta-(IL-1beta)-induced nuclear factorκB (NFκB) and kappaB-dependent transcription in epithelial cells. *Eur J Biochem* **254**: 81-89.

Nonet, M., D. Sweetser, and R.A. Young. 1987. Functional redundancy and structural polymorphism in the large subunit of RNA polymerase II. *Cell* **50**: 909-915.

O'Brien, T., S. Hardin, A. Greenleaf, and J.T. Lis. 1994. Phosphorylation of RNA polymerase II C-terminal domain and transcriptional elongation. *Nature* **370**: 75-77.

Ogryzko, V.V., R.L. Schiltz, V. Russanova, B.H. Howard, and Y. Nakatani. 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**: 953-959.

Orlando, V., H. Strutt, and R. Paro. 1997. Analysis of chromatin structure by in vivo formaldehyde cross-linking. *Methods* **11**: 205-214.

Oro, A.E., M. McKeown, and R.M. Evans. 1990. Relationship between the product of the Drosophila ultraspiracle locus and the vertebrate retinoid X receptor. *Nature* **347**: 298-301.

Orphanides, G., W.H. Wu, W.S. Lane, M. Hampsey, and D. Reinberg. 1999. The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins. *Nature* **400**: 284-288.

Palvimo, J.J., P. Reinikainen, T. Ikonen, P.J. Kallio, A. Moilanen, and O.A. Janne. 1996. Mutual transcriptional interference between RelA and androgen receptor. *J Biol Chem* **271**: 24151-24156.

Parada, C.A. and R.G. Roeder. 1996. Enhanced processivity of RNA polymerase II triggered by Tat-induced phosphorylation of its carboxy-terminal domain. *Nature* **384**: 375-378.

Parekh, B.S. and T. Maniatis. 1999. Virus infection leads to localized hyperacetylation of histones H3 and H4 at the IFNβ promoter. *Mol Cell* **3**: 125-129.

Patturajan, M., N.K. Conrad, D.B. Bregman, and J.L. Corden. 1999. Yeast carboxyl-terminal domain kinase I positively and negatively regulates RNA polymerase II carboxyl-terminal domain phosphorylation. *J Biol Chem* **274**: 27823-27828.

Paulsen, D.F. 1994. Retinoic acid in limb-bud outgrowth: review and hypothesis. *Anat Embryol (Berl)* **190**: 399-415.

Pearce, D. and K.R. Yamamoto. 1993. Mineralocorticoid and glucocorticoid receptor activities distinguished by nonreceptor factors at a composite response element [see comments]. *Science* **259**: 1161-1165.

Peng, J., Y. Zhu, J.T. Milton, and D.H. Price. 1998. Identification of multiple cyclin subunits of human P-TEFb. *Genes Dev* **12**: 755-762.

Perez, P., A. Schonthal, and A. Aranda. 1993. Repression of c-fos gene expression by thyroid hormone and retinoic acid receptors. *J Biol Chem* **268**: 23538-23543.

Perissi, V., L.M. Staszewski, E.M. McInerney, R. Kurokawa, A. Krones, D.W. Rose, M.H. Lambert, M.V. Milburn, C.K. Glass, and M.G. Rosenfeld. 1999. Molecular determinants of nuclear receptor-corepressor interaction. *Genes Dev* **13**: 3198-3208.

Picard, D., B. Khursheed, M.J. Garabedian, M.G. Fortin, S. Lindquist, and K.R. Yamamoto. 1990. Reduced levels of hsp90 compromise steroid receptor action in vivo. *Nature* **348**: 166-168.

Picard, D., S.J. Salser, and K.R. Yamamoto. 1988. A movable and regulable inactivation function within the steroid binding domain of the glucocorticoid receptor. *Cell* **54**: 1073-1080.

Picard, D. and K.R. Yamamoto. 1987. Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *Embo J* **6**: 3333-3340.

Price, D.H. 2000. P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. *Mol Cell Biol* **20**: 2629-2634.

Rachez, C., M. Gamble, C.P. Chang, G.B. Atkins, M.A. Lazar, and L.P. Freedman. 2000. The DRIP complex and SRC-1/p160 coactivators share similar nuclear receptor binding determinants but constitute functionally distinct complexes. *Mol Cell Biol* **20**: 2718-2726.

Rachez, C., B.D. Lemon, Z. Suldan, V. Bromleigh, M. Gamble, A.M. Naar, H. Erdjument-Bromage, P. Tempst, and L.P. Freedman. 1999. Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. *Nature* **398**: 824-828.

Rachez, C., Z. Suldan, J. Ward, C.P. Chang, D. Burakov, H. Erdjument-Bromage, P. Tempst, and L.P. Freedman. 1998. A novel protein complex that interacts with the vitamin D3 receptor in a ligand-dependent manner and enhances VDR transactivation in a cell- free system. *Genes Dev* **12**: 1787-1800.

Rattner, A., M. Korner, M.D. Walker, and Y. Citri. 1993. NFκ B activates the HIV promoter in neurons. *Embo J* **12**: 4261-4267.

Ray, A. and K.E. Prefontaine. 1994. Physical association and functional antagonism between the p65 subunit of transcription factor NFκ B and the glucocorticoid receptor. *Proc Natl Acad Sci U S A* **91**: 752-756.

Ray, A., K.E. Prefontaine, and P. Ray. 1994. Down-modulation of interleukin-6 gene expression by 17 beta-estradiol in the absence of high affinity DNA binding by the estrogen receptor. *J Biol Chem* **269**: 12940-12946.

Reincke, M., W. Arlt, C. Heppner, F. Petzke, G.P. Chrousos, and B. Allolio. 1998. Neuroendocrine dysfunction in African trypanosomiasis. The role of cytokines. *Ann N Y Acad Sci* **840**: 809-821.

Reines, D., J.W. Conaway, and R.C. Conaway. 1996. The RNA polymerase II general elongation factors. *Trends Biochem Sci* **21**: 351-355.

Roeder, R.G. 1996. The role of general initiation factors in transcription by RNA polymerase II. *Trends Biochem Sci* **21**: 327-335.

Rougvie, A.E. and J.T. Lis. 1988. The RNA polymerase II molecule at the 5' end of the uninduced hsp70 gene of D. melanogaster is transcriptionally engaged. *Cell* **54**: 795-804.

Ruben, S.M., P.J. Dillon, R. Schreck, T. Henkel, C.H. Chen, M. Maher, P.A. Baeuerle, and C.A. Rosen. 1991. Isolation of a rel-related human cDNA that potentially encodes the 65-kD subunit of NFκ B [published erratum appears in Science 1991 Oct 4;254(5028):11]. *Science* **251**: 1490-1493.

Rusconi, S. and K.R. Yamamoto. 1987. Functional dissection of the hormone and DNA binding activities of the glucocorticoid receptor. *Embo J* **6**: 1309-1315.

Ryu, S., S. Zhou, A.G. Ladurner, and R. Tjian. 1999. The transcriptional cofactor complex CRSP is required for activity of the enhancer-binding protein Sp1. *Nature* **397**: 446-450.

Sadowski, I., J. Ma, S. Triezenberg, and M. Ptashne. 1988. GAL4-VP16 is an unusually potent transcriptional activator. *Nature* **335**: 563-564.

Scheinman, R.I., P.C. Cogswell, A.K. Lofquist, and A.S. Baldwin, Jr. 1995a. Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids [see comments]. *Science* **270**: 283-286.

Scheinman, R.I., A. Gualberto, C.M. Jewell, J.A. Cidlowski, and A.S. Baldwin, Jr. 1995b. Characterization of mechanisms involved in transrepression of NFκ B by activated glucocorticoid receptors. *Mol Cell Biol* **15**: 943-953.

Schena, M., L.P. Freedman, and K.R. Yamamoto. 1989. Mutations in the glucocorticoid receptor zinc finger region that distinguish interdigitated DNA binding and transcriptional enhancement activities. *Genes Dev* **3**: 1590-1601.

Scherer, D.C., J.A. Brockman, Z. Chen, T. Maniatis, and D.W. Ballard. 1995. Signal-induced degradation of I kappa B alpha requires site-specific ubiquitination. *Proc Natl Acad Sci U S A* **92**: 11259-11263.

Schmidt, E.D., S.J. Cramer, and R. Offringa. 1993. The thyroid hormone receptor interferes with transcriptional activation via the AP-1 complex. *Biochem Biophys Res Commun* **192**: 151-160.

Schmitz, M.L., G. Stelzer, H. Altmann, M. Meisterernst, and P.A. Baeuerle. 1995. Interaction of the COOH-terminal transactivation domain of p65 NFκ B with TATA-binding protein, transcription factor IIB, and coactivators. *J Biol Chem* **270**: 7219-7226.

Schultz, M.C., R.H. Reeder, and S. Hahn. 1992. Variants of the TATA-binding protein can distinguish subsets of RNA polymerase I, II, and III promoters. *Cell* **69**: 697-702.

Segil, N., M. Guermah, A. Hoffmann, R.G. Roeder, and N. Heintz. 1996. Mitotic regulation of TFIID: inhibition of activator-dependent transcription and changes in subcellular localization. *Genes Dev* **10**: 2389-2400.

Seydoux, G. and M.A. Dunn. 1997. Transcriptionally repressed germ cells lack a subpopulation of phosphorylated RNA polymerase II in early embryos of Caenorhabditis elegans and Drosophila melanogaster. *Development* **124**: 2191-2201.

Seydoux, G., C.C. Mello, J. Pettitt, W.B. Wood, J.R. Priess, and A. Fire. 1996. Repression of gene expression in the embryonic germ lineage of C. elegans [letter]. *Nature* **382**: 713-716.

Seydoux, G. and S. Strome. 1999. Launching the germline in Caenorhabditis elegans: regulation of gene expression in early germ cells. *Development* **126**: 3275-3283.

Sha, W.C., H.C. Liou, E.I. Tuomanen, and D. Baltimore. 1995. Targeted disruption of the p50 subunit of NFκ B leads to multifocal defects in immune responses. *Cell* **80**: 321-330.

Sharif, M. and M.L. Privalsky. 1991. v-erbA oncogene function in neoplasia correlates with its ability to repress retinoic acid receptor action. *Cell* **66**: 885-893.

Sheppard, K.A., K.M. Phelps, A.J. Williams, D. Thanos, C.K. Glass, M.G. Rosenfeld, M.E. Gerritsen, and T. Collins. 1998. Nuclear integration of glucocorticoid receptor and nuclear factor- kappaB signaling by CREB-binding protein and steroid receptor coactivator-1. *J Biol Chem* **273**: 29291-29294.

Sil, A.K., S. Alam, P. Xin, L. Ma, M. Morgan, C.M. Lebo, M.P. Woods, and J.E. Hopper. 1999. The Gal3p-Gal80p-Gal4p transcription switch of yeast: Gal3p destabilizes the Gal80p-Gal4p complex in response to galactose and ATP. *Mol Cell Biol* **19**: 7828-7840.

Small, S., A. Blair, and M. Levine. 1992. Regulation of even-skipped stripe 2 in the Drosophila embryo. *Embo J* **11**: 4047-4057.

Small, S., R. Kraut, T. Hoey, R. Warrior, and M. Levine. 1991. Transcriptional regulation of a pair-rule stripe in Drosophila. *Genes Dev* **5**: 827-839.

Smith, E.M., P. Cadet, G.B. Stefano, M.R. Opp, and T.K. Hughes, Jr. 1999. IL-10 as a mediator in the HPA axis and brain. *J Neuroimmunol* **100**: 140-148.

Spaete, R.R. and E.S. Mocarski. 1985. Regulation of cytomegalovirus gene expression: alpha and beta promoters are trans activated by viral functions in permissive human fibroblasts. *J Virol* **56**: 135-143.

Stanojevic, D., T. Hoey, and M. Levine. 1989. Sequence-specific DNA-binding activities of the gap proteins encoded by hunchback and Kruppel in Drosophila. *Nature* **341**: 331-335.

Starr, D.B., W. Matsui, J.R. Thomas, and K.R. Yamamoto. 1996. Intracellular receptors use a common mechanism to interpret signaling information at response elements. *Genes Dev* **10**: 1271-1283.

Stein, B. and M.X. Yang. 1995. Repression of the interleukin-6 promoter by estrogen receptor is mediated by NFκ B and C/EBP beta. *Mol Cell Biol* **15**: 4971-4979.

Sun, X., Y. Zhang, H. Cho, P. Rickert, E. Lees, W. Lane, and D. Reinberg. 1998. NAT, a human complex containing Srb polypeptides that functions as a negative regulator of activated transcription. *Mol Cell* **2**: 213-222.

Svejstrup, J.Q., Y. Li, J. Fellows, A. Gnatt, S. Bjorklund, and R.D. Kornberg. 1997. Evidence for a mediator cycle at the initiation of transcription. *Proc Natl Acad Sci U S A* **94**: 6075-6078.

Svejstrup, J.Q., P. Vichi, and J.M. Egly. 1996. The multiple roles of transcription/repair factor TFIIH. *Trends Biochem Sci* **21**: 346-350.

Takeda, K., O. Takeuchi, T. Tsujimura, S. Itami, O. Adachi, T. Kawai, H. Sanjo, K. Yoshikawa, N. Terada, and S. Akira. 1999. Limb and skin abnormalities in mice lacking IKKalpha [see comments]. *Science* **284**: 313-316.

Taunton, J., C.A. Hassig, and S.L. Schreiber. 1996. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p [see comments]. *Science* **272**: 408-411.

Tenenhaus, C., C. Schubert, and G. Seydoux. 1998. Genetic requirements for PIE-1 localization and inhibition of gene expression in the embryonic germ lineage of Caenorhabditis elegans. *Dev Biol* **200**: 212-224.

Tennyson, C.N., H.J. Klamut, and R.G. Worton. 1995. The human dystrophin gene requires 16 hours to be transcribed and is cotranscriptionally spliced. *Nat Genet* **9**: 184-190.

Traenckner, E.B., H.L. Pahl, T. Henkel, K.N. Schmidt, S. Wilk, and P.A. Baeuerle. 1995. Phosphorylation of human I kappa Bα on serines 32 and 36 controls I kappa Bα proteolysis and NFκ B activation in response to diverse stimuli. *Embo J* **14**: 2876-2883.

Treuter, E., L. Johansson, J.S. Thomsen, A. Warnmark, J. Leers, M. Pelto-Huikko, M. Sjoberg, A.P. Wright, G. Spyrou, and J.A. Gustafsson. 1999. Competition between thyroid hormone receptor-associated protein (TRAP) 220 and transcriptional intermediary factor (TIF) 2 for binding to nuclear receptors. Implications for the recruitment of TRAP and p160 coactivator complexes. *J Biol Chem* **274**: 6667-6677.

Triezenberg, S.J., R.C. Kingsbury, and S.L. McKnight. 1988. Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. *Genes Dev* **2**: 718-729.

Trigon, S., H. Serizawa, J.W. Conaway, R.C. Conaway, S.P. Jackson, and M. Morange. 1998. Characterization of the residues phosphorylated in vitro by different C- terminal domain kinases. *J Biol Chem* **273**: 6769-6775.

Tronche, F., C. Kellendonk, O. Kretz, P. Gass, K. Anlag, P.C. Orban, R. Bock, R. Klein, and G. Schutz. 1999. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet* **23**: 99-103.

Tronche, F., C. Kellendonk, H.M. Reichardt, and G. Schutz. 1998. Genetic dissection of glucocorticoid receptor function in mice. *Curr Opin Genet Dev* **8**: 532-538.

Tsai, F.T. and P.B. Sigler. 2000. Structural basis of preinitiation complex assembly on human pol II promoters. *Embo J* **19**: 25-36.

Tsai, M.J. and B.W. O'Malley. 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* **63**: 451-486.

Umesono, K., V. Giguere, C.K. Glass, M.G. Rosenfeld, and R.M. Evans. 1988. Retinoic acid and thyroid hormone induce gene expression through a common responsive element. *Nature* **336**: 262-265.

Usheva, A., E. Maldonado, A. Goldring, H. Lu, C. Houbavi, D. Reinberg, and Y. Aloni. 1992. Specific interaction between the nonphosphorylated form of RNA polymerase II and the TATA-binding protein. *Cell* **69**: 871-881.

van de Stolpe, A., E. Caldenhoven, J.A. Raaijmakers, P.T. van der Saag, and L. Koenderman. 1993. Glucocorticoid-mediated repression of intercellular adhesion molecule-1 expression in human monocytic and bronchial epithelial cell lines. *Am J Respir Cell Mol Biol* **8**: 340-347.

van de Stolpe, A., E. Caldenhoven, B.G. Stade, L. Koenderman, J.A. Raaijmakers, J.P. Johnson, and P.T. van der Saag. 1994. 12-O-tetradecanoylphorbol-13-acetate- and tumor

necrosis factor alpha- mediated induction of intercellular adhesion molecule-1 is inhibited by dexamethasone. Functional analysis of the human intercellular adhesion molecular-1 promoter. *J Biol Chem* **269**: 6185-6192.

Van Doren, M., H.M. Ellis, and J.W. Posakony. 1991. The Drosophila extramacrochaetae protein antagonizes sequence-specific DNA binding by daughterless/achaete-scute protein complexes. *Development* **113**: 245-255.

Verrijzer, C.P. and R. Tjian. 1996. TAFs mediate transcriptional activation and promoter selectivity [see comments]. *Trends Biochem Sci* **21**: 338-342.

Vivanco Ruiz, M.M., T.H. Bugge, P. Hirschmann, and H.G. Stunnenberg. 1991. Functional characterization of a natural retinoic acid responsive element. *Embo J* **10**: 3829-3838.

Voegel, J.J., M.J. Heine, M. Tini, V. Vivat, P. Chambon, and H. Gronemeyer. 1998. The coactivator TIF2 contains three nuclear receptor-binding motifs and mediates transactivation through CBP binding-dependent and -independent pathways. *Embo J* **17**: 507-519.

Wada, T., T. Takagi, Y. Yamaguchi, A. Ferdous, T. Imai, S. Hirose, S. Sugimoto, K. Yano, G.A. Hartzog, F. Winston, S. Buratowski, and H. Handa. 1998a. DSIF, a novel transcription elongation factor that regulates RNA polymerase II processivity, is composed of human Spt4 and Spt5 homologs. *Genes Dev* **12**: 343-356.

Wada, T., T. Takagi, Y. Yamaguchi, D. Watanabe, and H. Handa. 1998b. Evidence that P-TEFb alleviates the negative effect of DSIF on RNA polymerase II-dependent transcription in vitro. *Embo J* **17**: 7395-7403.

Wadgaonkar, R. and T. Collins. 1999. Murine double minute (MDM2) blocks p53-coactivator interaction, a new mechanism for inhibition of p53-dependent gene expression. *J Biol Chem* **274**: 13760-13767.

Wedel, A. and S. Kustu. 1995. The bacterial enhancer-binding protein NTRC is a molecular machine: ATP hydrolysis is coupled to transcriptional activation. *Genes Dev* **9**: 2042-2052.

Weih, F., D. Carrasco, S.K. Durham, D.S. Barton, C.A. Rizzo, R.P. Ryseck, S.A. Lira, and R. Bravo. 1995. Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NFκ B/Rel family. *Cell* **80**: 331-340.

West, M.L. and J.L. Corden. 1995. Construction and analysis of yeast RNA polymerase II CTD deletion and substitution mutations. *Genetics* **140**: 1223-1233.

Wissink, S., E.C. van Heerde, M.L. Schmitz, E. Kalkhoven, B. van der Burg, P.A. Baeuerle, and P.T. van der Saag. 1997. Distinct domains of the RelA NFκB subunit are required for negative cross-talk and direct interaction with the glucocorticoid receptor. *J Biol Chem* **272**: 22278-22284.

Wissink, S., E.C. van Heerde, B. vand der Burg, and P.T. van der Saag. 1998. A dual mechanism mediates repression of NFκB activity by glucocorticoids. *Mol Endocrinol* **12**: 355-363.

Woronicz, J.D., X. Gao, Z. Cao, M. Rothe, and D.V. Goeddel. 1997. IkappaB kinaseβ: NFκB activation and complex formation with IkappaB kinaseα and NIK [see comments]. *Science* **278**: 866-869.

Yamaguchi, Y., T. Takagi, T. Wada, K. Yano, A. Furuya, S. Sugimoto, J. Hasegawa, and H. Handa. 1999a. NELF, a multisubunit complex containing RD, cooperates with DSIF to repress RNA polymerase II elongation. *Cell* **97**: 41-51.

Yamaguchi, Y., T. Wada, D. Watanabe, T. Takagi, J. Hasegawa, and H. Handa. 1999b. Structure and function of the human transcription elongation factor DSIF. *J Biol Chem* **274**: 8085-8092.

Yamamoto, K.R. 1985. Steroid receptor regulated transcription of specific genes and gene networks. *Annu Rev Genet* **19**: 209-252.

Yamamoto, K.R. 1995. Multilayered control of intracellular receptor function. *Harvey Lect* **91**: 1-19.

Yamamoto, K.R., B.D. Darimont, R.L. Wagner, and J.A. Iniguez-Lluhi. 1998. Building transcriptional regulatory complexes: signals and surfaces. *Cold Spring Harb Symp Quant Biol* **63**: 587-598.

Yamit-Hezi, A. and R. Dikstein. 1998. TAFII105 mediates activation of anti-apoptotic genes by NFκB. *Embo J* **17**: 5161-5169.

Yang, N., R. Schule, D.J. Mangelsdorf, and R.M. Evans. 1991. Characterization of DNA binding and retinoic acid binding properties of retinoic acid receptor. *Proc Natl Acad Sci U S A* **88**: 3559-3563.

Yang-Yen, H.F., J.C. Chambard, Y.L. Sun, T. Smeal, T.J. Schmidt, J. Drouin, and M. Karin. 1990. Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein- protein interaction. *Cell* **62**: 1205-1215.

Yang-Yen, H.F., X.K. Zhang, G. Graupner, M. Tzukerman, B. Sakamoto, M. Karin, and M. Pfahl. 1991. Antagonism between retinoic acid receptors and AP-1: implications for tumor promotion and inflammation. *New Biol* **3**: 1206-1219.

Yano, K. and T. Fukasawa. 1997. Galactose-dependent reversible interaction of Gal3p with Gal80p in the induction pathway of Gal4p-activated genes of Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **94**: 1721-1726.

Young, H.A. 1996. Regulation of interferon-gamma gene expression. *J Interferon Cytokine Res* **16**: 563-568.

Yu, V.C., C. Delsert, B. Andersen, J.M. Holloway, O.V. Devary, A.M. Naar, S.Y. Kim, J.M. Boutin, C.K. Glass, and M.G. Rosenfeld. 1991. RXR beta: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* **67**: 1251-1266.

Yuan, Y.O., I.L. Stroke, and S. Fields. 1993. Coupling of cell identity to signal response in yeast: interaction between the alpha 1 and STE12 proteins. *Genes Dev* **7**: 1584-1597.

Zawel, L. and D. Reinberg. 1993. Initiation of transcription by RNA polymerase II: a multi-step process. *Prog Nucleic Acid Res Mol Biol* **44**: 67-108.

Zhang, W. and J.J. Bieker. 1998. Acetylation and modulation of erythroid Kruppel-like factor (EKLF) activity by interaction with histone acetyltransferases. *Proc Natl Acad Sci U S A* **95**: 9855-9860.

Zhong, H., R.E. Voll, and S. Ghosh. 1998. Phosphorylation of NFκ B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol Cell* **1**: 661-671.

Zhu, Y., T. Pe'ery, J. Peng, Y. Ramanathan, N. Marshall, T. Marshall, B. Amendt, M.B. Mathews, and D.H. Price. 1997. Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation in vitro. *Genes Dev* **11**: 2622-2632.

Zuo, P., D. Stanojevic, J. Colgan, K. Han, M. Levine, and J.L. Manley. 1991. Activation and repression of transcription by the gap proteins hunchback and Kruppel in cultured Drosophila cells. *Genes Dev* **5**: 254-264.

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