Binding of C/EBP and RBP (CBF1) to Overlapping Sites Regulates Interleukin-6 Gene Expression*

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Lynne D. Vales‡ and Erika M. Friedl
From the Department of Biochemistry, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854-5635

The ILRE (interleukin response element) contained within the promoter of the interleukin-6 (IL-6) gene is defined as the site recognized by the p65 NF-κB transcriptional activator and is crucial for activation of the IL-6 gene. The region of the promoter containing the ILRE is complex containing a CCAAT enhancer-binding protein (C/EBP) site immediately upstream of the ILRE, which is required for optimal activation of the IL-6 gene. Additionally, the ILRE overlaps a site that is recognized by the mammalian transcriptional repressor RBP (CBF1), and RBP binding within the ILRE region represses activated IL-6 expression. In this study, the complexity of this region is further revealed by the identification of a second nested C/EBP site, which overlaps that of RBP and therefore also the ILRE. Optimal activation requires both the upstream and newly identified C/EBP sites in conjunction with the p65 NF-κB binding site. We previously reported that RBP represses IL-6 activation but does not target p65. We extend these analyses here to show that RBP binding does not occlude p65 from binding but instead directly overlaps the newly identified downstream C/EBP site, thereby impeding p65-C/EBP-mediated co-activation. This result suggests a role for RBP in the repression of other genes containing a C/EBP site that exhibits sequence overlap with the RBP site.

After the response to stimuli, the strong activation of transcription of cellular genes is followed by a reestablishment of basal or uninduced expression. The maintenance and/or reestablishment of such basal expression was previously believed to be a passive process involving the loss of activity of the transcriptional activators. However, the reestablishment of basal gene expression is now being reevaluated given the newly appreciated role of transcriptional repression in regulating eukaryotic gene expression. The cellular gene encoding the cytokine interleukin-6 (IL-6)1 is an excellent model for such analyses, as its activation has been studied extensively (for review, see Ref. 1). Deregulated IL-6 expression has been correlated with the pathogenesis of several diseases including rheumatoid arthritis, Castleman’s disease, and certain types of tumors (for review, see Ref. 2). Finally, the cellular transcriptional repressor RBP (also known as CBF1 or CSL) has been found to repress activated IL-6 expression (3, 4).

Previous studies of the IL-6 promoter identified an element designated the interleukin response element (ILRE), which is crucial for IL-6 activation (5, 6). This element was identified as the NF-κB site; optimal IL-6 activation correlated with the p65 NF-κB species alone both in vivo and in vitro (7). Subsequent studies identified a C/EBP site immediately upstream of the ILRE, and these two elements gave rise to optimal IL-6 activation in the presence of stimuli that induced NF-κB and C/EBP (8–10). IL-6 gene activation involves the rapid transport of preexisting p65 to the nucleus after release from a complex with IκB in the cytosol and the synthesis of C/EBP-β and -δ. C/EBP-β activity is regulated by post-translational modifications (11, 12). The C/EBP family of basic region-leucine zipper proteins α, β, and δ have been shown capable of functioning similarly in IL-6 activation in transient expression assays using reporter constructs containing the IL-6 promoter (Ref. 13, and this study). However, it is the β and δ species that are activated and function in conjunction with p65 NF-κB with resultant increased IL-6 expression as a consequence of cellular exposure to tumor necrosis factor-α, lipopolysaccharides, or cytokines such as interleukin-1 (for reviews, see Refs. 1, 14, and 15).

The relatively small region of the IL-6 promoter containing the ILRE and contiguous upstream C/EBP site is extremely important for rapid and strong expression of the IL-6 gene. We previously showed (3) that the ILRE overlaps almost entirely the recognition site for the cellular transcriptional repressor, RBP. RBP binding within the ILRE repressed activated IL-6 expression in the presence of C/EBP-β and p65 NF-κB. RBP-mediated repression required binding within the ILRE. RBP appeared to be involved in reestablishing or retaining basal IL-6 expression (3).

The functional role of cellular RBP in transcriptional repression in mammalian cells was originally revealed through studies of viruses that sequester its activity during infection (16, 17). The Drosophila homologue of RBP, Suppressor of Hairless, recently has been shown to function in repression as well (18). RBP has been shown to be the target of virally encoded proteins and cellular proteins that interact with RBP to modulate its activity. One notable example is the role of RBP in Notch signal transduction that involves RBP/Notch interaction and provides the molecular basis for the long appreciated genetic interaction of these factors in Drosophila neuron development (19–22) (for reviews, see Refs. 23 and 24). Similar to other transcriptional repressors, RBP exhibits more than one mechanism to impede transcription, and these mechanisms can also involve RBP...
interaction with cellular factors. RBP was shown to repress transcription through its interaction with corepressors NCoR/SMRT and histone deacetylase HDAC1 (25) and corepressors CIR/SAP30, which facilitate interaction with HDAC2 (26). In the case of the adenoviral pIX gene in which the natural position of the RBP site is immediately upstream of the TATA motif, the position of the RBP site was a determinant in repression. RBP was shown to interact directly with two adjacent transcriptional co-activators, TFIIA and TFIID, and thwarts activated pIX transcription (27).

In the case of the IL-6 gene, we reported that RBP binds within the ILRE of the IL-6 promoter and represses activated IL-6 expression (3). These studies involved transient expression assays with IL-6 reporter constructs containing the ILRE in the presence or absence of the upstream C/EBP site along with expression vectors for p65, C/EBP-β, and RBP. In our study (3), RBP-mediated repression required RBP binding within the ILRE. A reporter construct containing a mutation within the ILRE that diminished RBP binding (RBPM) resulted in loss of repression. However, repression required not only RBP binding but also RBP binding specifically within the ILRE. A newly positioned RBP site did not restore repression in the case of RBPM. These studies also demonstrated that RBP did not repress p65-activated IL-6 expression. Instead, our results suggested that RBP targeted C/EBP-β alone or co-activation between p65 and C/EBP-β (3).

Two subsequent reports on the role of RBP in IL-6 gene expression resulted in some discrepancy as to the target of RBP in IL-6 gene repression and the actual role of RBP as repressor or activator of the IL-6 gene. In the first case (4), RBP was shown to functionally repress IL-6 activation, but the target of RBP in this repression was identified as p65. In the second report (28), a mutation in the IL-6 promoter that disrupted RBP binding also resulted in decreased IL-6 activation. This result led the authors to propose that RBP functions in IL-6 gene repression and the actual role of RBP as repressor or transcriptional co-activator as described previously (28). A mutation in the IL-6 promoter that disrupted RBP in this repression was identified as p65. In the second report (28), a mutation in the IL-6 promoter that disrupted RBP binding also resulted in decreased IL-6 activation. This result led the authors to propose that RBP functions in IL-6 gene repression and the actual role of RBP as repressor or transcriptional co-activator as described previously (28).

**Experimental Procedures**

**Cells and Transfection Assays**—COS-7 cells were propagated and transfected using the calcium phosphate procedure under serum stimulation conditions as described previously (3). HEPG2 cells were also propagated in Dulbecco’s modified Eagle medium containing 10% fetal calf serum and transfected similarly except that cells were not serum-supplemented before and after transfection. The calcium phosphate precipitates routinely contained 10 μg of reporter plasmid and cells were harvested after overnight incubation. The calcium phosphate precipitates were renatured by step dialysis in buffer C (25 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 10% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.05% Triton X-100) was fractionated on a 20-ml S-Sepharose (Sigma) column equilibrated in buffer B, 4 M urea. A gradient of 0.1–1.0 M NaCl in buffer B, 4 μl mouse (7 column volumes) was used, and 2-m fractions were collected. C/EBP-β eluted at a salt concentration of 150–400 mM NaCl/urea. Pooled fractions were renatured by step dialysis in buffer C (25 mM Tris, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.05% Triton X-100) containing 2, 1, and 0.5 mM NaCl/urea, respectively. The last dialysis step was performed in buffer C containing 60 mM KCl and without urea. Renatured C/EBP-β was further purified on a DEAE-5PW column (TosoHaas) using a gradient of 60–600 mM KCl in buffer C without detergent. Purified C/EBP-β protein was obtained in the column flow through.

**Reconstituted Transcription Assays**—Transcription reactions were reconstituted as described previously (27, 30). The reactions contained two different templates with G-less cassettes of different lengths. The IL-6 template contained the IL-6 promoter (~87 to +5, relative to the start site of transcription) with a G-less cassette of 261 nucleotides. The control template, p50, contained the AdMLP (~50 to +10 nt, relative to the start site of transcription) with a G-less cassette of 392 nucleotides. The control template is devoid of consensus sites for p65 NF-κB and RBP. The detectable increase in transcription obtained from this template in the presence of C/EBP-β may be due to the presence of a cryptic C/EBP site between ~40 and ~50 nt of the AdMLP (5’-TCAGGAGAC-3’) or within the pGEM3 vector itself. The basal transcription factors used in the reconstituted system were bacterially expressed TFIIA, TFIIF, TFIIF, TFIIE, and highly purified mammalian TFIIH and RNA polymerase II (31).

**RNA Preparation and Analysis**—The isolation of poly(A)-containing RNA from transfected cells and antisense probe analysis were performed using TRIzol reagent (Life Technologies). The antisense probe analysis was performed using RNAs One from Promega and scores for the levels of expression of the IL-6 reporter and internal control within the same sample (3).

**Nuclear Extract Preparation and EMSAs**—The preparation of nuclear extracts from transfected COS-7 cells and the conditions for gel shift assays using crude extracts were described previously (3, 16).

Antibodies specific for p65, C/EBP-α, C/EBP-β, and C/EBP-δ were purchased from Santa Cruz Biotechnologies. The antibodies were obtained from Santa Cruz Biotechnologies. The IL-6 promoter, was selected as the standard for normalization. The antisense probe is derived from the promoter region of the IL-6 gene, has been described previously (3). The internal control used for normalization experiments was the SV40 promoter, which contains a minimal palindromic sequence of the SV40 promoter ligated to ~20 nt relative to the start site of transcription of the IL-6 gene (3). The antisense probe was derived from ~87/14/IL-6/d91 and was described previously (3). The derivation of ~87/4/IL-6/d91 and was described previously (3). The derivation of ~87/4/IL-6/d91 was performed in a manner similar to that used to obtain the wild type constructs using oligonucleotide ligation to ~45/IL-6/d91 as described previously (3).

**Plasmid Constructions**—The construction of reporter constructs ~87/14/IL-6/d91 and ~74/14/IL-6/d91, which contain the indicated IL-6 promoter relative to the start site of IL-6 transcription ligated to +90 nt relative to the start site of transcription of the adenovirus pIX gene, has been described previously (3). The internal control used for normalization experiments was the SV40 promoter, which contains a minimal palindromic sequence of the SV40 promoter ligated to ~20 nt relative to the start site of transcription of the IL-6 gene (3). The antisense probe was derived from ~87/14/IL-6/d91 and was described previously (3).

**Purification of Recombinant Proteins**—The transcriptional repressor protein RBP was expressed in Escherichia coli and purified by conventional chromatography as described previously (27). p65 (RelA) was expressed as a His-tagged protein in baculovirus-infected SF9 cells and purified using nickel/nitritriacetic acidagarose (28). C/EBP-β was expressed in baculovirus-infected SF9 cells. Cell lysates were obtained under denaturing conditions using buffer A (8 μl urea, 0.1 mM Na2HPO4, 10 mM Tris, pH 6.3). Cell pellets were stirred in buffer A for 60 min and then centrifuged at 10,000 x g for 30 min at room temperature. The C/EBP-β-containing lysate was dialyzed to 4 M urea in two steps of 6 and 4 M urea in buffer B (20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 10% glycerol). The C/EBP-β lyase (buffer B/4 M urea) was fractionated on a 20-ml S-Sepharose (Pharmacia) column equilibrated in buffer B, 4 M urea. A gradient of 0.1–1.0 M NaCl in buffer B, 4 μA (7 column volumes) was used, and 2-m fractions were collected. C/EBP-β eluted at a salt concentration of 150–400 mM NaCl/urea. Pooled fractions were renatured by step dialysis in buffer C (25 mM Tris, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.05% Triton X-100) containing 2, 1, and 0.5 mM NaCl, respectively. The last dialysis step was performed in buffer C containing 60 mM KCl and without urea. Renatured C/EBP-β was further purified on a DEAE-5PW column (TosoHaas) using a gradient of 60–600 mM KCl in buffer C without detergent. Purified C/EBP-β protein was obtained in the column flow through.
RESULTS

RBP Targets p65 and C/EBP-β Co-activation in Vitro—In our previous studies, we examined IL-6–activated transcription using transient expression assays. The reporter constructs and mutant derivatives contained the regions of the IL-6 promoter extending from either −74 or −87 nt to +14 nt relative to the start sites for IL-6 expression. As shown in Fig. 1A, the difference between these two constructs is the absence (−74) or presence (−87) of the previously identified C/EBP site that is immediately upstream of the ILRE. Fig. 1A also shows the RBP consensus site, which is overlapped by the ILRE and is contained in both constructs. We previously showed that overexpression of p65 resulted in activated IL-6 expression but the co-addition of RBP was ineffectual. On the other hand, overexpression of p65 and C/EBP-β resulted in greater levels of activation as expected, and the co-addition of RBP resulted in decreased levels of IL-6 expression (Ref. 3 and see below). We also found, however, that p65 binding was required for C/EBP-β co-activation. A reporter construct containing a mutation in the ILRE that inhibited p65 binding, but not RBP binding, did not exhibit activation upon overexpression of C/EBP-β. In addition, our results showed that RBP binding was required for repression of p65/C/EBP-β co-activated IL-6 expression. A construct containing a mutation in the RBP site that disrupted RBP but not p65 binding was defective in RBP-mediated repression, although p65/C/EBP-β co-activation levels appeared similar to that of the wild type (Ref. 3 and see below). On the other hand, restoring a wild type RBP site 20 nt downstream from its normal position did not restore repression in the case of the IL-6 promoter containing a mutation in the normal RBP binding site. From these and other results, we concluded that RBP represses IL-6 activated transcription dependent upon the presence of the RBP site within the ILRE and that the target of repression is C/EBP-β alone or co-activation by p65 and C/EBP-β.

A subsequent report (4) also demonstrated that RBP repressed activated IL-6 expression; however, these authors found that RBP repressed activated transcription from constructs containing a portion of the IL-6 promoter that was devoid of the upstream C/EBP site. In their case, NF-κB activity was provided endogenously after tumor necrosis factor-α or IL-1-β treatment of the transfected pool of cells. As the IL-6 constructs employed were devoid of the upstream C/EBP site and the activation obtained was repressed by RBP, the results were consistent with RBP targeting p65. Additionally, the authors (4) found that the off-rates of RBP and p65 binding in crude extracts were disparate and inconsistent with RBP and p65 co-binding to the IL-6 promoter. They therefore concluded that RBP binding to the ILRE occludes p65 binding and thereby represses activated IL-6 expression (4).

To clarify the controversy in the mechanism by which RBP represses IL-6 expression, we first sought an independent assay system to determine whether the identity of the target of RBP in mediating IL-6 repression was consistent with any of the previous reports. We employed the reconstituted transcription assay using purified p65 and C/EBP-β activators and purified RBP repressor, along with the general transcription factors and RNA polymerase II. Fig. 2 shows the levels of IL-6 transcription achieved during titration of each activator alone or together. Similar to previous studies performed in vivo, neither p65 nor C/EBP-β alone gave rise to substantial IL-6 transcription (lanes 1–5) (3, 32). However, the addition of both activators appeared to act synergistically, giving rise to substantial levels of co-activated IL-6 transcription (lanes 6 and 7). The addition of RBP alone was ineffective (lanes 8–10). Also, the co-addition of RBP with either p65 alone or C/EBP-β alone was ineffective (compare lane 3 with lanes 17–19 and lane 5 with lanes 20–22, respectively). However, the co-addition of increasing amounts of RBP led to a substantial reduction of IL-6 transcription when both activators were present (compare lane 6 with lanes 11–13 and lane 7 with lanes 14–16). This result shows that RBP does not repress p65 activation alone and, further, that RBP does not target C/EBP-β alone. The result of this independent assay is consistent with our previously reported studies using transient expression assays (3).

RBP and p65 Co-bind the IL-6 Promoter—Because the results of the transcription reaction performed in vitro shown above are consistent with our previous studies (3) showing that RBP does not target p65 in vivo, we next tested whether purified p65 and RBP proteins were capable of co-binding the IL-6 promoter. Fig. 3 shows the results of a gel shift assay using purified preparations of RBP and p65 in the unlabeled probe containing −87 to −45 nt of the IL-6 promoter relative to the start sites of IL-6 transcription. Panel A shows each candidate protein alone in the gel shift assay. RBP alone gives rise to two complexes that are inhibited by excess oligonucleotides containing either the RBP site or the ILRE; RBP binding is not inhibited by excess oligonucleotide containing the TATA motif, as expected. Both complexes are supershifted in the presence of
RBP Binding Overlaps That of C/EBP in the IL-6 Promoter

We next investigated the contribution of C/EBP to complex formation on the IL-6 promoter. Similar to a previous report, we observed that the α, β, and δ members of the C/EBP family of transcription factors were all capable of co-activating IL-6 expression in the presence of p65 (Ref. 13 and Fig. 4). The co-addition of RBP gave rise to repression in all three cases. However, repression in the presence of C/EBP-α was the least effective. Using crude extracts derived from COS-7 cells transfected with expression vectors for p65 and C/EBP-α, we examined complex formation using probe containing the wild type IL-6 promoter from −87 to −45 nt and therefore containing the ILRE and upstream C/EBP site. Fig. 5A shows the presence of complexes containing p65 alone, C/EBP-α alone, and the two proteins in complex together. Complex containing p65 alone was supershifted with antibody specific to p65 but was unaffected by the addition of antibody specific to C/EBP-α or control antibody specific for AP1. Complexes containing C/EBP-α alone were supershifted by antibody specific to C/EBP-α but unaffected by antibody specific to p65 or AP1. The slowest migrating complex was supershifted both by antibody specific to p65 and by antibody specific to C/EBP-α but not by control antibody. This result indicates that this complex contains both p65 and C/EBP-α binding activities. We also examined complex formation on probe containing a mutation in the p65 binding site that we reported previously to be defective in p65 binding, p65 activation, and p65/C/EBP-β co-activation (3). Fig. 5A shows that complex containing p65 protein alone or in conjunction with C/EBP-α is not detectable in this case. However, complex containing C/EBP-α alone is present at levels similar to that of the wild type probe.

Similar results were obtained using extracts derived from COS-7 cells transfected with p65 and C/EBP-δ (Fig. 5B). Complexes containing p65, C/EBP-δ, or p65 and C/EBP-δ were observed as indicated. The interpretation of similar analyses performed with extracts containing p65 with C/EBP-δ was hampered by a diffuse banding pattern exhibited by C/EBP-δ. Several species of C/EBP-δ that arise from internal translation start sites and from proteolytic degradation during extract preparation have been reported, and these may account for the diffuse banding pattern we observed (Refs. 33 and 34, respectively). Therefore, extracts containing p65 in conjunction with C/EBP-δ and C/EBP-β were analyzed. The levels of co-activation of IL-6 expression achieved with p65 and C/EBP-δ and p65 together are similar to those obtained with p65 and either species alone, as is targeting by RBP in vivo (data not shown). Fig. 5C shows that complexes containing C/EBP-β and δ species were clearly discernable, and the results obtained were similar to those obtained with p65 and C/EBP-α or C/EBP-δ. The presence of complex containing both p65 and C/EBP family members is consistent with that previously reported in the case of the gene encoding IL-8. Although the NF-κB sites of the IL-8 and IL-6 promoters are disparate, in each case there is a closely
positioned C/EBP site. Previous studies have shown that p65 and C/EBP-β bind cooperatively to the IL-8 promoter (35).

**A Previously Unidentified Downstream C/EBP Site**—We next examined the pattern of complex formation using probe containing the ILRE but lacking the upstream C/EBP site (−74 to −45 nt, Fig. 6). Complex containing p65 alone was evident, as expected. To our surprise, complex containing C/EBP-α alone was also present. This was unexpected, because the probe did not contain the previously reported C/EBP site upstream of the ILRE. Although complexes containing either p65 or C/EBP-α were evident, the slow mobility complex containing both proteins, which we observed using probe containing the upstream C/EBP site (Fig. 5A), was not detectable. Therefore, cooperative binding between p65 and C/EBP-α requires the presence of the upstream C/EBP site. Similar results were obtained with extracts containing p65 and C/EBP-δ or C/EBP-β in combination with C/EBP-β (data not shown, and see below). This result shows that C/EBP family members exhibit binding activity to the IL-6 promoter contained within −74 to −45 nt that is devoid of the previously identified C/EBP site upstream of the ILRE.

We first tested for the functional relevance of C/EBP binding to the IL-6 promoter devoid of the upstream C/EBP site using HepG2 cells. Unlike COS-7 cells, HepG2 cells do not require treatment to obtain C/EBP-β-mediated co-activation with p65 in transient expression assays. Therefore, to ensure that serum stimulation of COS-7 cells used to obtain C/EBP-β co-activation with p65 was not contributing to IL-6-activated expression in a manner independent of C/EBP-β, we first tested HepG2 cells. HepG2 cells were transfected with the −74/+14 IL-6/dl9 reporter construct and expression vectors for p65 alone or in combination with each C/EBP family member (Fig. 7). Indeed, the addition of any of the C/EBP family members resulted in increased IL-6 expression relative to the presence of p65 alone, demonstrating the ability of C/EBP to co-activate the IL-6 promoter in the absence of the upstream C/EBP site. The addition of RBP gave rise to repression in each case. However, p65-C/EBP-α co-activated IL-6 transcription was the least responsive, similar to the results obtained in COS-7 cells shown in Fig. 4 above using the −87/+14/IL-6/dl9 construct containing the upstream C/EBP site under conditions of serum stimulation.

**The Relative Resistance of p65-C/EBP-α to Repression Correlates with Greater Levels of C/EBP-α Binding to the Downstream C/EBP Site**—Fig. 8 shows a comparison of C/EBP-α versus C/EBP-δ binding to the upstream C/EBP site, the downstream C/EBP site, and the C/EBP consensus site. The levels of C/EBP-δ binding to the upstream and downstream C/EBP sites within the IL-6 promoter were similar and greatly reduced relative to those obtained with the C/EBP consensus site (Fig. 8A). On the other hand, although the levels of C/EBP-α binding to the upstream C/EBP site within the IL-6 promoter were weak and similar to those obtained with C/EBP-δ, C/EBP-α binding to the downstream site was considerably greater, similar to its level of binding to the C/EBP consensus site. This difference in binding activity between C/EBP family members weak to C/EBP sites has been reported for other C/EBP sites (for example, see Ref. 36). In the case of the IL-6 promoter, the greater levels of C/EBP-α binding to the downstream site, relative to that of C/EBP-δ, correlates with greater levels of co-activation with p65 and greater resistance to RBP repression (Figs. 4 and 7, and see above).

**The Downstream C/EBP Site Overlaps That for RBP and Is Functionally Relevant to IL-6 Gene Activation**—Another report of the role of RBP in IL-6 gene regulation described a specific mutation within the ILRE that disrupted RBP DNA binding.

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**Fig. 5. Complexes containing p65 and/or C/EBP family members and the IL-6 promoter.** The results of EMSAs performed with crude extracts prepared from COS-7 cells overexpressing protein factors as indicated below the panels. The addition of specific antibodies are shown above the lanes. The proteins contained within specific complexes, as evidenced by reactivity to specific antibody, are indicated. A, complexes containing p65 without C/EBP-α, C/EBP-α without p65, or both proteins together are shown. The probes used contained −87 to −45 nt relative to the start sites of transcription of the wild type IL-6 promoter (−87/wt) or the IL-6 promoter containing a mutation in the ILRE, which results in defective p65 binding activity and p65 activation (−87/p65M (3)) as indicated below the panel. Asterisks indicate the position of supershifted complexes formed in the presence of antibody specific to C/EBP-α. B, complexes containing p65 without C/EBP-δ, C/EBP-δ without p65, or both proteins together are indicated. The free probe is indicated (−87/wt). C, complexes containing p65 without C/EBP-δ or -β, C/EBP-δ and -β without p65, or all three proteins together are indicated.
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activity. IL-6 reporter constructs containing this mutation, designated IL-6-κB-mt3, were found to be defective in IL-6 gene expression when cells were treated with IL-1β (25). The authors concluded that the defect in RBP binding correlated with a defect in IL-6 gene expression and that RBP may actually function to activate IL-6 gene expression (25). In light of our findings that C/EBP can bind to the IL-6 promoter region contained within −74 to −45 nt, which also contains the p65 and RBP binding sites, we next analyzed this reported mutation in the RBP site, within the context of −74 to −45, for the level of C/EBP binding. The middle portion of Fig. 6 shows that p65 binding to probe containing this mutation is similar to that of the wild type. However, C/EBP-α binding activity is barely detectable. Similar results were obtained for C/EBP-δ and for heterodimers containing C/EBP-β and -δ (data not shown). This result indicates that although this mutant promoter may be defective in RBP binding, it is also defective in C/EBP binding. Therefore, we designated this mutation as RBPM/EBPM2 to denote a defect in RBP and C/EBP binding activities. The defect in C/EBP binding, rather than RBP binding, to this mutant promoter may explain the reported suboptimal levels of IL-6 expression achieved from this promoter relative to the wild type case (see below). Moreover, that this promoter mutation results in decreased RBP and C/EBP binding activities indicates overlap of the RBP site with this downstream C/EBP site.

We also examined the levels of C/EBP binding to the IL-6 mutant construct that we previously had reported to be defective in RBP binding and, therefore, in repression (designated previously as RBPM and for purposes of clarity redesignated RBPM1 here). The presence of this mutation in the RBP site within the context of the −74 to −45 probe appeared to give rise to similar levels of p65 binding, as shown previously, and also gives rise to similar levels of C/EBP binding activity relative to the wild type (Fig. 6). Therefore, this mutation appears to affect only RBP binding.

We further tested the functional relevance of the newly identified downstream C/EBP site by comparing the levels of p65-C/EBP-β co-activation achieved in HepG2 cells from IL-6 reporter constructs devoid of the upstream C/EBP site and containing either the wild type or mutated IL-6 promoters within the context of the −74 to +14/IL-6/dl9 (Fig. 9). Using transient expression assays performed in HepG2 cells and expression vectors for p65, C/EBP-β, and RBP, the RBPM1 construct showed similar levels of co-activation relative to the wild type. This is consistent with the wild type levels of p65 and C/EBP binding to the downstream site obtained in RBPM1. Co-addition of RBP gave rise to decreased levels of IL-6 activation in the wild type but not in RBPM1. This was expected, as we previously reported that this mutation within the RBP site, in the −87 to +14 context of the IL-6 promoter, gave rise to wild type levels of p65-C/EBP-β co-activation but was defective in RBP repression due to a defect in RBP binding (3). The result with RBPM1 shown in the −74 context here confirms that this mutation does not disrupt C/EBP binding to the downstream site. On the other hand, the levels of p65-C/EBP-β co-activated IL-6 expression achieved from the RBPM/EBPM2 construct is greatly reduced relative to the wild type and RBPM1 constructs. The reduced levels of IL-6 expression obtained correlate with the reduced levels of C/EBP binding obtained with this mutated promoter. This result shows that the downstream C/EBP site is functionally relevant to IL-6 activation.

We next compared the relative contributions of the upstream and downstream C/EBP sites with respect to the levels of activated IL-6 expression achieved in COS-7 cells, under the conditions of serum stimulation used in our previous report (3) and in Fig. 4. Fig. 10 shows that both sites contribute to optimal IL-6 co-activation by p65 and C/EBP-β. The −74/+14/IL-6/dl9 construct, which is devoid of the upstream C/EBP site, and the RBPM/EBPM2 mutant within the context of the upstream C/EBP site are similarly defective for co-activation relative to the wild type having both sites intact (−87/+14/IL-6/dl9). Therefore, the downstream C/EBP site contributes to p65-C/EBP co-activation in both COS-7 and HepG2 cells.

Given our findings that RBP binding does not occlude the ability of p65 to bind, as well as our findings that the RBP site overlaps a downstream C/EBP site, our results strongly suggest that RBP binding would have an effect on activated IL-6 expression similar to that of a mutation that disrupts downstream C/EBP binding. Overlapping RBP binding disrupts downstream C/EBP binding, thereby disrupting p65-C/EBP co-activated IL-6 expression.
DISCUSSION

In an attempt to understand the discrepancies between the published reports regarding the role of RBP in IL-6 gene regulation, we have uncovered a downstream C/EBP site that overlaps the ILRE and also the RBP site within the ILRE. This site is functionally relevant to optimal levels of IL-6 activation and to repression mediated by overlapping RBP binding activity. In the absence of the upstream C/EBP site, p65 and C/EBP family members co-activate IL-6 gene expression. In the absence or presence of the upstream C/EBP site, a mutation within the IL-6 promoter that results in deficient C/EBP binding to the downstream site is also deficient in the levels of activated IL-6 gene expression achieved in the presence of p65 and C/EBP family members. This mutation also disrupts RBP binding, demonstrating an overlap between RBP and downstream C/EBP sites. A previous study (32) also demonstrated that the NF-κB site of the IL-6 promoter was sufficient to result in co-activation; however, the authors did not report the presence of a specific C/EBP binding site that overlaps the ILRE. They suggested that co-activation may be facilitated by protein-protein interactions between p65 and C/EBP-α or -δ (32). Our results have shown here that C/EBP family members bind specifically to a site overlapping the ILRE and RBP site. To our knowledge this study is the first to examine the nature of complexes formed on the IL-6 promoter in the presence of p65 and C/EBP family members.

Previous analyses of IL-6 gene regulation have examined cells that were transfected with reporter constructs containing only the ILRE or multiple copies of the ILRE and that were then treated with IL-1 or tumor necrosis factor-α to activate endogenous p65 (4, 28). As these constructs did not contain the known upstream C/EBP site, activated IL-6 expression from the reporter constructs was presumed to be due to p65 alone. However, these treatments also activate endogenous C/EBP-β and -δ. Therefore, based on our findings of a functionally relevant C/EBP site overlapping the ILRE, we believe that the levels of activation obtained from these IL-6 reporter con-
structs were due to co-activation by p65 and C/EBP family members. For example, we found that the mutation within the RBP site that was reported previously to be deficient in IL-6 activation due to the deficiency in RBP binding is also deficient in C/EBP binding to the overlapping RBP site. Our results indicate that this deficiency in C/EBP binding to the mutation, rather than the deficiency in RBP binding, correlates with decreased IL-6 activation.

In our previous study (3) of the role of RBP in IL-6 gene regulation, we examined constructs containing the ILRE in the absence and presence of the upstream C/EBP site. Our results demonstrated that RBP does not repress IL-6 activation in the presence of p65 alone. However, we also found that p65 binding to the ILRE was nonetheless important in achieving repression. Using a construct defective in p65 binding and containing the upstream C/EBP site, we found that the low but detectable levels of C/EBP-β-activated IL-6 expression obtained were not detectably targeted by RBP in repression. We concluded that RBP targeted co-activated IL-6 expression. Our results here showed that p65 and C/EBP-β, or δ-β bind cooperatively to the IL-6 promoter containing the upstream C/EBP site. We also showed that C/EBP-α binds at greater levels to the downstream C/EBP site than to the upstream site, whereas C/EBP-δ binds weakly and similarly to both sites. In a similar analysis with C/EBP-β, binding to the upstream site was very weak and to the downstream site barely detectable relative to the C/EBP consensus site (data not shown). Yet, as shown here, these two sites are important for p65-C/EBP-β-co-activated IL-6 expression. Therefore, the interaction between p65 and C/EBP-β may be critical in facilitating C/EBP-β binding to these two weak C/EBP sites.

The consensus sites for RBP and C/EBP binding are 5'-GTGGGAA/c-3' and 5'-TT/GNNGAAAT/G-3', respectively (Refs. 37 and 1, respectively). The RBP site within the IL-6 promoter contains the sequence 5'-ATGGGAAA-3'. Excess amounts of oligonucleotide containing the ILRE (5'-CTAGATGGGATTCCCCAG-3'); NFκB site is underlined) inhibit p65 and RBP binding but do not inhibit C/EBP binding to the downstream C/EBP site (data not shown). However, oligonucleotide containing the addition of the T residue 3' to the ILRE (5'-CTAGATGGGATTTTTCCCAG-3') now effectively inhibits C/EBP binding activity (data not shown). The RBPM/EBCM2 promoter contained a substitution of the AT residues 3’ to the ILRE, resulting in defective C/EBP and RBP binding activities. The possibility exists that other RBP sites may function as C/EBP sites dependent upon the sequence surrounding the core RBP consensus.

In our previous studies of the role of RBP in repression of the adenovirus pIX gene and the IL-6 gene (Refs. 27 and 3, respectively), we attempted to retain the natural promoter as much as possible. In both cases, we found that resaturating the natural RBP site resulted in a loss of repression. In the case of the pIX gene, RBP binds immediately upstream of the TATA motif and between the TATA motif and the binding site for the SP1 activator. This facilitates RBP interaction with TFIIA and the dTARf110 subunit of TFID to the detriment of SP1-mediated interactions that facilitate activation. RBP binding does not occlude binding of these other factors, and resaturating the RBP site upstream of that for SP1 relieves RBP-mediated repression (27). In the case of the IL-6 gene, we showed that resaturating the RBP site outside of the context of the ILRE also relieves repression (3). In this case, a derivative of the −87/RBPM1 construct contained a newly positioned RBP site. We have shown here that C/EBP family members bind to RBPM1 at levels similar to the wild type. In light of our findings that RBP overlaps the downstream C/EBP site in the wild type, repression was likely thwarted when the RBP site was resaturated in the RBPM1 construct, as RBP binding no longer competed for that of C/EBP at the ILRE.

Our results demonstrate that RBP does not inhibit activated IL-6 expression by p65 alone and that RBP can co-bind with p65. On the other hand, the RBP site does overlaps a C/EBP site. Although RBP binding may compete with that of C/EBP, C/EBP may still remain in complex with p65 with resultant repression. Interaction between p65 and C/EBP-β has been found to involve the Rel and bZIP domains, respectively (38), although the dimer/monomer state of each member in the complex has not been clearly defined yet (39). Previous studies showed that complex containing p65 and C/EBP-β together can bind either a p65 site or a C/EBP site (38). Interestingly, this previous study also showed that the nature of the p65-C/EBP protein complex bound to a single site is such that p65 activation results when the complex binds the C/EBP site, but p65 repression results when the complex binds the NF-κB site. Therefore, C/EBP is inhibitory to p65 activation when the complex binds without an adjacent C/EBP site. Fig. 11 presents a model in which RBP binding may repress IL-6 expression by occluding the overlapping C/EBP site but without dislodging C/EBP from complex with p65. In this model, the occlusion of C/EBP binding to the downstream site results in an altered complex that maintains C/EBP interaction with p65 but is now defective in mediating activation.

We show here that the levels of co-activated IL-6 expression are greater in the presence of C/EBP-α, relative to β- or δ, and p65. This correlated with substantially greater C/EBP-α binding activity to the downstream C/EBP site, which in turn correlated with greater resistance to RBP-mediated repression. RBP is less likely to be able to compete with C/EBP-α for the overlapping binding sites. This specificity in RBP repression may be relevant. Previous studies of C/EBP-β knockout mice revealed no defect in IL-6 gene activation; however, interestingly, IL-6 expression was constitutive (40). These authors suggested that other C/EBP family members may compensate for the lack of C/EBP-β activity (40). The possibility exists that C/EBP-α may partially compensate in these mice and that IL-6 constitutive levels may be attributable to the specificity of RBP in IL-6 gene repression.

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Binding of C/EBP and RBP (CBF1) to Overlapping Sites Regulates Interleukin-6 Gene Expression

Lynne D. Vales and Erika M. Friedl

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