MOLECULAR AND CELLULAR BIOLOGY, Jan. 1997, p. 1–9
0270-7306/97/$04.00+0

Vol. 17, No. 1

The Mammalian Transcriptional Repressor RBP (CBF1) Regulates Interleukin-6 Gene Expression

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Received 14 June 1996/Returned for modification 9 August 1996/Accepted 3 October 1996

The cellular interleukin-6 (IL-6) gene contains a target site for the mammalian transcriptional repressor RBP. The target site is contained within the interleukin response element (ILRE), which mediates IL-6 activation by NF-κB. In this study, we show by using transient-expression assays that RBP represses activated transcription from the IL-6 gene. The presence and position of the RBP target site are crucial in mediating repression by RBP. While RBP binds within the ILRE, it does not target NF-κB alone; nonetheless, NF-κB binding to the ILRE is required for repression. Our results indicate that RBP represses coactivation by NF-κB and another cellular transcription factor, C/EBP-β.

Appreciation of the role of transcriptional repression in regulating gene expression in eucaryotes is evolving rapidly based on numerous findings reported relatively recently (for examples and a review, see reference 15). Repression at the transcriptional level may restrict cellular gene expression more stringently than lack of activation; in addition, a rapid cellular response to changing conditions may be achieved at the transcriptional level by decreased activation in conjunction with active repression. Studies performed with procaryotes have provided numerous precedents for understanding the molecular mechanisms underlying transcriptional repression in eucaryotes. Based on these precedents and the further complexity inherent in eucaryotic transcription, examples of repressors which act to inhibit one or more of the various participants in the transcription process have been documented. Previous studies described repressors of basal or activated transcription that mediate inhibition by direct hindrance to RNA polymerase, competitive interference with activators (either by overlapping DNA target sites or sequestration of a common factor[s]), or direct impediment to activator function by domain masking or conformational alterations (see reference 19 and references therein).

RBP (also designated RBP-Jκ or CBF1) is a previously identified cellular protein that has been recognized recently to be a transcriptional repressor in mammalian cells. Identification of the functional role of RBP as a transcriptional repressor was established by studies involving viral gene expression. Our previous analyses identified a repressive element in the promoter region of the adenovirus gene that encodes polypeptide IX (pIX) (7). The presence of this element was required to silence pIX gene expression before adenovirus DNA replication. DNA binding activity specific to the pIX-repressive element was identified in extracts from uninfected cells. The protein was purified to near homogeneity and found to be identical to RBP. RBP is a 60-kDa protein isolated previously as a possible recombinase (RBP-Jκ) (13).

Our results demonstrated that RBP actually functions as a transcriptional repressor of the adenovirus pIX gene in infection and transfection assays performed in vivo and in transcription assays performed with purified RBP protein in vitro. RBP-mediated repression was dependent upon the presence of the pIX-repressive element that was specifically targeted by RBP. The RBP target site (5′-TGGGAAA-3′ [7, 39]) lies immediately upstream of the TATA box within the pIX promoter. However, the sites were not overlapping, and RBP and the TATA binding protein (TBP) were shown to be capable of cobinding the pIX promoter region. Repression of pIX transcription did not involve RBP-mediated occlusion of adjacent TBP binding (7).

Subsequent studies revealed that Epstein-Barr virus also took advantage of the presence of the cellular RBP protein. The transcriptional activator EBNA2 encoded by Epstein-Barr virus does not bind DNA by itself. Instead, complexes containing EBNA2 and RBP target promoters containing an RBP consensus site (12, 14, 17, 22, 41, 43); furthermore, EBNA2 interaction with RBP masks the RBP repressive domain (17). Therefore, viral EBNA2 mediates specific gene activation by interaction with cellular RBP protein. Based on the analyses with adenovirus and Epstein-Barr virus, which appropriate RBP during infection, RBP has been recognized as a transcriptional repressor in mammalian cells.

Intriguingly, RBP functions as a repressor in mammalian cells and as an activator in *Drosophila melanogaster* (6). The *Drosophila* homolog of RBP [Su(H) (Suppressor of Hairless)] is one determinant to formation of the sensory organ precursor from which neuronal and accessory cells are generated in the development of the peripheral nervous system (9, 32; for a review, see reference 28). This developmental pathway includes Notch and Enhancer of Split and is opposed by Hairless. Advances in the elucidation of this pathway directly implicate *Drosophila* RBP in the Notch receptor signalling pathway and include the following findings. RBP interaction with Notch mediates cytoplasmic localization of RBP (8), and Notch receptor activation results in nuclear localization of RBP with concommitant transcriptional activation of Enhancer of Split (4, 23). On the other hand, RBP interaction with Hairless inhibits RBP binding to its DNA target site (6; for reviews, see references 1 and 16). The interaction between RBP and other cellular proteins may explain the discrepancy between the functional roles of RBP as a transcriptional repressor in mammalian cells and as a transcriptional activator in *Drosophila*. In fact, RBP interaction with active forms of mouse or human

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Notch1 results in transcriptional activation through the RBP DNA binding site in mammalian cells (18, 26). Thus, the role of RBP during development and also in the adult stage may be determined by RBP interaction with other proteins, thereby regulating its activity.

In the case of mammalian cells, adenovirus and Epstein-Barr virus evolved to sequester RBP activity during infection, yet the cellular targets of RBP repression have yet to be identified functionally. In this report, we show that RBP represses activated transcription from a cellular gene, that for interleukin-6 (IL-6). IL-6 gene expression can be induced in different types of cells by a variety of factors, including cytokines, IL-1, and tumor necrosis factor alpha (31). The interleukin response element (ILRE) within the IL-6 promoter is crucial for IL-1 and tumor necrosis factor alpha induction of IL-6 expression in vivo and consists of the recognition site for the NF-κB transcription factor (24, 33).

NF-κB has been shown to be a latent transcription factor that is retained in the cytoplasm of many different cell types by association with the cellular repressor protein I-κB (for reviews, see references 5 and 25). Stimulation of cells with cytokines, tetradecanoyl phorbol acetate, and many other agents leads to disruption of I-κB association and concommitant migration of NF-κB to the nucleus. NF-κB is composed of a family of transcription factors, including the p65 and p50 subunits (for reviews, see references 3, 10, and 11). In the case of the IL-6 gene, the p65 NF-κB member alone optimally activates expression. Previous studies showed that steroid- or hormone-mediated antagonism of IL-6 expression is elicited, at least in part, by direct inhibition of p65 activation (30, 37).

In this study, we show that RBP, which is present in many different cell types (13), modulates expression of the NF-κB-responsive IL-6 gene. Our results show that RBP binds within the ILRE and represses activated IL-6 expression. Repression is dependent upon the presence and position of the RBP target site within the IL-6 promoter. RBP did not target NF-κB activation directly but instead mediated repression by hindering NF-κB coactivation with another factor.

MATERIALS AND METHODS

Cells and transfection assays. Undifferentiated F9 cells were grown as monolayer cultures in Dulbecco's modified Eagle medium containing 10% defined calf serum, and COS-7 cells were grown as monolayer cultures in Dulbecco's modified Eagle medium containing 10% fetal calf serum (FCS). Subconfluent cultures were transfected by the calcium phosphate procedure as described previously (7, 40). DNA precipitates were added directly to the media and left for approximately 6 h. In the case of F9 cells, the medium was then replenished, and cells were harvested 24 h later as described previously (40). In the case of COS-7 cells, after precipitate removal, cells were starved for serum in medium containing 0.5% FCS for 24 h and then serum induced in medium containing 10% FCS for 12 h, at which time cells were harvested.

RNA preparation and analysis. Harvested cells were lysed, and cytoplasmic poly(A) RNA was isolated as described previously (40). RNase protection assays with RNase T_2 were performed as described previously (40). An antisense RNA probe specific to IL-6/dl9 (221 nucleotides [nt]) was derived from pGem-1 containing a fragment of IL-6/dl9 plasmid which encompassed the promoter of IL-6 (*Xba*I; 5′ to nt −87 of IL-6) and a portion of the fusion with pIX (*Pst*I; adenovirus nt 3790). The probe spans the start site of IL-6 transcription and the fusion with pIX. IL-6/dl9 mRNA protects a fragment of 134 nt. An antisense probe specific to p65 mRNA (245 nt) was derived from the coding region of CMV/p65 (nt 1270 to 1480) and protected a fragment of 210 nt. An antisense probe specific to RBP spanned the first 420 nt of the RBP-coding region (*Pvu*II); RBP mRNA is expected to protect a fragment of 420 nt.

Gel retardation assay. The preparation of nuclear extracts and conditions for the gel mobility shift assay were as described previously (7). The sequences of the RBP and ILRE oligonucleotides used in excess are, respectively, 5′-TGGGAA AGAA-3′ and 5′-TGGGATTTTCCCA-3′.

Plasmid constructions. The construct −87/+14/IL-6/dl9, containing a portion of the IL-6 promoter fused to a portion of the adenovirus pIX gene was derived as follows. The IL-6 promoter region contained within the *Hin*dIII and *Xho*I restriction sites (nt −45 to +14) was derived from −45/IL-6/CAT (33), and the *Xho*I site was blunt ended with Klenow fragment. This fragment was ligated to dl9 DNA (2) after digestion with *Bam*HI, blunt end formation, and subsequent digestion with *Hin*dIII. The resulting plasmid (−45/+14/IL-6/dl9) contains nt −45 to +14 of the IL-6 promoter fused to the pIX gene from adenovirus nt 3670 to 5640. Oligonucleotides containing nt −87 or −74 to −45 of the IL-6 promoter were ligated to −45/IL-6/dl9 after digestion with *Xba*I and *Bam*HI to derive −87/+14/IL-6/dl9 and −74/+14/IL-6/dl9, respectively. The DNA sequence of the oligonucleotide containing nt −87 to −45 is as follows: 5′-*Xba*I-TTTTTA TCAAATGTGGGATTTTCCCATGAGTCTCAATATTAG-*Bam*HI-3′ (the ILRE is underlined). The −87/+14/RBPM/dl9 and −87/+14/p65−/dl9 constructs were derived similarly by using oligonucleotides (nt −87 to −45) containing, respectively, either a mutation in the RBP target site within the ILRE (5′-TGGGATTTTCCgg-3′) or a mutation in the p65 recognition site within the ILRE (5′-TcccATTTTCCCA-3′). The −87/RBPM/RBP/dl9 construct was derived by using an oligonucleotide similar to −87/−45/RBPM but also containing a substitution of sequence downstream of the ILRE for a consensus RBP site. This construct therefore restores RBP binding, but at a position 10 nt downstream of the ILRE. The DNA sequence of this oligonucleotide is 5′-*Xba*I-TT TTTATCAAATGTGGGATTTTCCggatcctttcccaATTA-3′) (the RBP target site is underlined). The ILREX3 construct contains a spacer sequence of 10 nt (*Bgl*II linker) between each ILRE. CMV/RBP was derived by insertion of the coding region for RBP from SV40/RBP into CMV-5. The construct SV40/dl17 was described previously (40).

RESULTS

RBP represses activated IL-6 expression. The ILRE contained within the promoter of the IL-6 gene is composed of the site targeted by the cellular transcription factor p65 NF-κB (24, 33). The ILRE has been shown to be a crucial element in induction of IL-6 expression by a number of noxious agents which induce expression of NF-κB. We observed that the IL-6 promoter contains a consensus target site for the mammalian transcriptional repressor RBP within the ILRE (Fig. 1A). Crude extracts prepared from F9 cells exhibited a complex specific for RBP or p65 NF-κB binding activities in gel retardation assays with a radiolabeled probe containing the IL-6 promoter region from nt −87 to −45 (Fig. 1B). The complex containing RBP binding activity was specifically inhibited by excess amounts of oligonucleotides containing either the RBP target sequence or the ILRE; an antibody specific to RBP eliminated this complex. However, the complex containing p65 NF-κB binding activity was specifically inhibited with excess oligonucleotide containing the ILRE, not the RBP target site; this complex was supershifted with an antibody specific to p65. Therefore, while RBP and p65 NF-κB proteins specifically bind to the ILRE, the portions of the ILRE targeted by each differ. We next tested whether RBP binding to the ILRE was functionally relevant to the complex regulation of IL-6 gene expression by using transient-expression assays and expression vectors for RBP and NF-κB proteins.

Figure 2 shows a schematic representation of reporter construct containing a portion of the IL-6 promoter including the ILRE and the start sites for IL-6 transcription. The internal control for transfection efficiency is also shown and consists of the minimal simian virus 40 (SV40) early promoter that does not contain an RBP target site. The IL-6 and SV40 promoters were fused to different portions of the adenovirus pIX-coding region. The levels of mRNA derived during transient expression from both the internal control and reporter constructs were then gauged by using an antisense probe which spanned the start sites of IL-6 transcription in the RNase T_2 protection assay (see Materials and Methods) (Fig. 2).

Optimal IL-6 expression was achieved with the exogenous addition of p65 NF-κB alone in several cell lines tested (undifferentiated F9, NIH 3T3, and COS-7 cells); coexpression of p50 NF-κB gave rise to reduced IL-6 expression (references 30 and 37 and data not shown). Therefore, we tested for the effect, if any, of RBP on basal and p65-activated IL-6 expression from an IL-6 construct containing the NF-κB and RBP sites extending from nt −87 to +14 (Fig. 3). The addition of

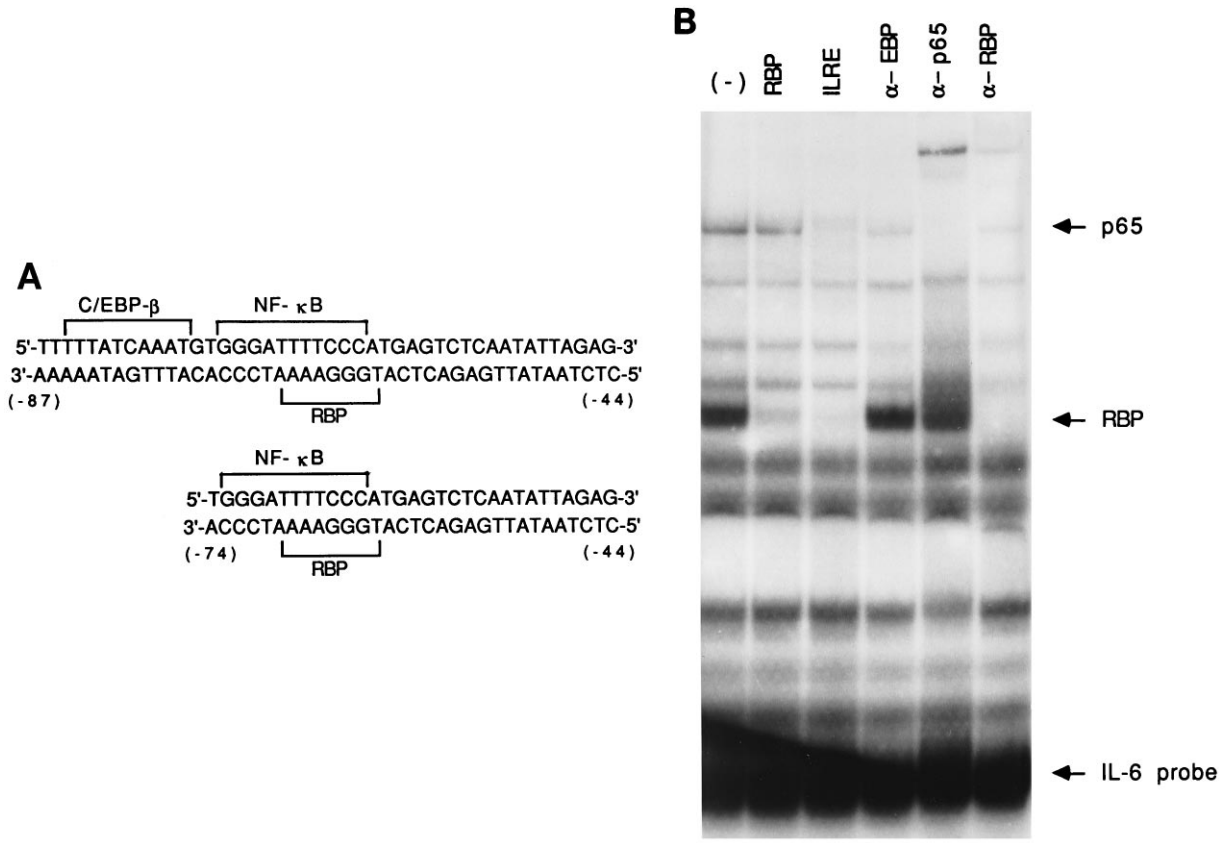

FIG. 1. (A) Portion of the IL-6 promoter region extending from nt −87 to −44 relative to the start site of transcription. The ILRE is composed of the binding site for NF-κB and is contained within nt −73 to −63 of the IL-6 promoter. The consensus target sites for the transcription facors C/EBP-β, NF-κB, and RBP are shown in brackets. Also shown is the portion of the IL-6 promoter containing the ILRE without upstream sequence. (B) Gel retardation assay with a radiolabeled probe containing the IL-6 promoter region from nt −87 to −45 and crude extract prepared from F9 cells. The addition of specific competitor oligonucleotides or an antibody (α) specific to C/EBP-β, p65, or RBP protein is indicated above the lanes. The arrows indicate specific complexes associated with RBP or p65 proteins. In the case of RBP, excess amounts of oligonucleotides containing the RBP target site or the ILRE inhibited binding, and antibody specific to RBP eliminated the complex. In the case of p65, only the oligonucleotide containing ILRE, not that containing the RBP target sequence, specifically inhibited binding, and antibody specific to p65 gave rise to a supershifted complex. None of the complexes were detectably affected upon addition of the antibody specific to C/EBP-β under these assay conditions. (−), no addition.

RBP did not result in a detectable difference in the level of basal IL-6 expression (Fig. 3, compare lanes 1, 2, and 3). As expected, addition of p65 resulted in activation from the IL-6 promoter (Fig. 3, lane 4). However, this activated transcription was markedly reduced upon the addition of increasing amounts of expression vector for RBP (Fig. 3, compare lanes 4, 5, and 6). The addition of RBP did not affect mRNA levels obtained from the internal control. This result suggested that coexpression of RBP specifically repressed p65-activated IL-6 expression.

Repression requires the RBP target site within the ILRE. In order to determine if RBP repression required the presence of its target site within the ILRE, a mutant construct which contained a 2-bp substitution within the RBP target site was prepared (−87/+14/RBPM/dl9 [see Materials and Methods]); this mutation resulted in greatly diminished levels of the RBP-specific complex, without a detectable effect on the p65-specific complex, in gel retardation assays (data not shown). The levels of p65-activated transcription from the wild type and mutant constructs were similar (Fig. 4, compare lanes 2 and 6). However, while p65-activated levels of wild-type IL-6 expression were reduced in the presence of RBP (Fig. 4, compare lanes 2, 3, and 4), no effect on activated IL-6 expression from the mutant construct was detected (compare lanes 6, 7, and 8). The inhibition of IL-6 expression from the wild-type construct was not due to decreased p65 mRNA levels which were similar throughout the experiment (Fig. 4, compare lanes 2, 3, and 4 and lanes 6, 7, and 8). As well, the lack of inhibition of IL-6 expression from the mutant construct was not due to discrepancies in RBP mRNA levels, which were similar throughout the experiment (Fig. 4, compare lanes 3 and 7 and lanes 4 and 8). This result suggested that RBP repressed activated IL-6 expression in a manner dependent upon the presence of its target site within the IL-6 promoter, similar to that observed previously for the adenovirus pIX gene (7).

The ILRE is not sufficient for repression by RBP. While RBP repressed activated IL-6 expression in the wild-type case, we observed that repression to basal levels was not achieved. In fact, detectable levels of activated expression were consistently retained in the presence of RBP. The possibility existed that the levels of RBP expression may be limiting; activated IL-6/dl9 levels were further decreased upon the addition of increasing amounts of SV40/RBP (Fig. 3 and 4, compare 1× and 3× levels). Therefore, we examined activated IL-6 expression in the presence of the CMV/RBP expression vector. Addition of CMV/RBP gave rise to elevated levels of RBP mRNA relative to those with SV40/RBP (compare 1× and 3× levels of RBP mRNA, relative to p65 mRNA levels, in lanes 3 and 4 of Fig.

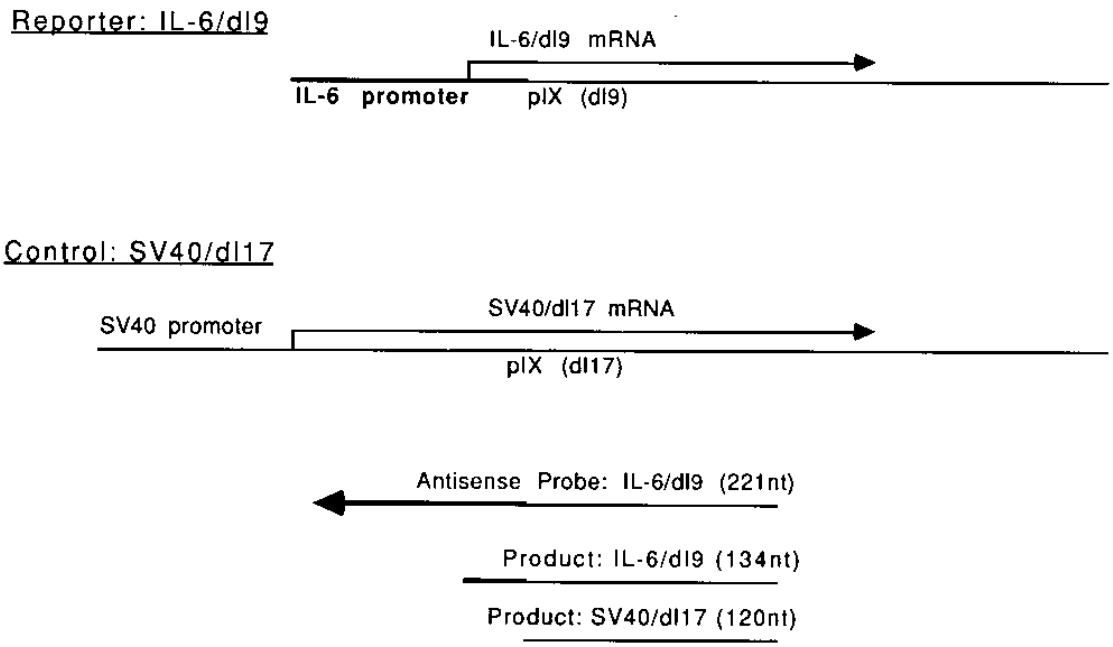

FIG. 2. Schematic representation of the plasmid constructs used in transient-expression assays. The construct IL-6/dl9 contains the IL-6 promoter region (nt −87 to +14 or nt −74 to +14 as indicated in the text) fused to a portion of the adenovirus pIX gene from adenovirus type 5 nt 3670 to 5640 (dl9) (2); mRNA transcribed from this construct is designated IL-6/dl9. The construct SV40/dl17 is used to score for transfection efficiency and contains the minimal SV40 promoter fused to a portion of the adenovirus pIX gene from adenovirus type 5 nt 3560 to 5640; mRNA transcribed from this construct is designated SV40/dl17 (40). The antisense probe specific to IL-6/dl9 spans the start site for IL-6 transcription. IL-6/dl9 and SV40/dl17 mRNAs are scored with the same aliquot of RNA and are expected to protect fragments of 134 and 120 nt, respectively, from RNase T_2 digestion after hybridization to the antisense probe (see Materials and Methods).

4 and 5). As well, CMV/RBP gave rise to saturating levels of repression; IL-6 expression from −87/+14/dl9 was not further reduced in the case of 3× versus 1× amounts of CMV/RBP (Fig. 5, compare lanes 2, 3, and 4). RBP expression did not affect mRNA levels from CMV/p65 or the internal control. Therefore, the residual level of activated IL-6 expression observed was not due to limiting amounts of RBP mRNA.

On the other hand, the IL-6 promoter region used in these initial experiments contained sequence upstream of the 5′ end of the ILRE which had been determined previously to be a consensus site for the transcriptional activator C/EBP-β (also termed NF-IL-6) (37, 42). In this case, endogenous C/EBP-β activity may contribute to the activated levels of IL-6 expression observed in the presence of p65 added exogenously. The residual IL-6 expression observed in the presence of RBP may then be due to one of the following possibilities. First, if endogenous C/EBP-β did contribute to activated IL-6 expression, RBP may target only one of the activators, p65 or C/EBP-β; in this case, residual IL-6 expression is due to the untargeted activator. Second, RBP may not repress IL-6 expression to basal levels under these assay conditions or in general.

In order to test if RBP specifically targeted p65, we examined a truncated construct containing only the ILRE at the 5′ end (−74/+14/IL-6/pIX [Fig. 1]). Figure 5 shows a comparison of the original and truncated IL-6 constructs with respect to p65-activated levels of transcription in the absence and presence of RBP. The levels of p65-activated transcription from the truncated construct were markedly reduced relative to those from the original IL-6 promoter tested (Fig. 5, compare lanes 2 and 6); the levels of p65 mRNA produced in the two cases were similar. This result suggested that endogenous C/EBP-β contributed to p65-activated transcription in the case of the extended IL-6 construct. More significantly, while the presence of RBP led to reduced IL-6 expression from the original construct in the presence of p65 (Fig. 5, lanes 2 to 4), there was no detectable effect of RBP on activated transcription from the truncated construct (lanes 6 to 8); once again, the levels of RBP mRNA achieved were similar in both cases. Remarkably, the level of activated IL-6 expression from the truncated construct, unaffected by the presence of RBP, was similar to the residual level of IL-6 expression from the original construct, in the presence of RBP.

This observation suggested two possibilities. First, the activated levels of IL-6 from the truncated construct may not be sufficient to observe repression by RBP; in this case, a trivial limitation of detection of RBP repression is possible. Second,

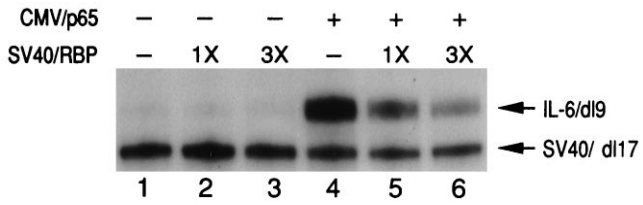

FIG. 3. Results of a transient-expression assay performed with undifferentiated F9 cells and 10 μg of −87/+14/IL-6/dl9 and SV40/dl17 plasmid constructs. The addition of an expression vector for p65 NF-κB (5 μg) and/or SV40/RBP (5 or 15 μg [1× or 3×, respectively]) is indicated above the lanes. The total amount of DNA in each case was adjusted by using an empty SV40 expression vector in lieu of SV40/RBP and with pGem-1 DNA. The levels of IL-6/dl9 and SV40/dl17 mRNAs were scored by using the RNase T_2 protection assay, and arrows indicate the protected fragments expected. Basal IL-6/dl9 mRNA was barely detectable. The levels of p65-activated IL-6/dl9 expression were reduced in the presence of increasing amounts of SV40/RBP, while expression from the internal control SV40/dl17 was unaffected. We have tested the two reported cDNA clones for RBP-2 and RBP-2N in this assay (20); the results were similar in each case. The results shown here and for all subsequent experiments were obtained by using the expression vector for RBP-2.

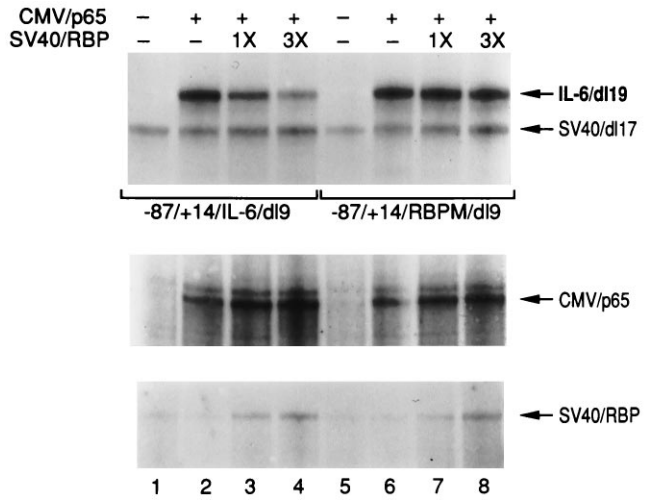

FIG. 4. Comparison of −87/+14/IL-6/dl9 and −87/+14/RBPM/dl9 mRNA levels in transfected F9 cells. Addition of CMV/p65 alone or with SV40/RBP is indicated above the lanes (see the legend to Fig. 3). Aliquots of mRNA were independently scored for levels of IL-6/dl9, SV40/dl17, CMV/p65, and SV40/RBP by using RNase T_2 protection assays and the appropriate antisense probes (see Materials and Methods). Arrows indicate the protected products expected for each mRNA species. The levels of p65-activated IL-6/dl9 expression from wild-type and RBPM plasmids were similar; however, addition of SV40/RBP led to reduced expression from the wild-type construct only. The levels of CMV/p65 and SV40/RBP mRNAs obtained were similar in both cases.

RBP may not target p65 in mediating repression; in this case, RBP may target C/EBP-B alone or coactivation of p65 and C/EBP-β (see below).

In order to determine if the lack of repression from the truncated construct was due to a trivial limitation of detection, a new construct containing two additional ILREs in tandem with the ILRE of the truncated construct was prepared (IL REX3/dl9 [see Materials and Methods]); each ILRE spanned the normal target sites for p65 and RBP (5′-TGGGATTTTC CCA-3′), and they were spaced apart by 10 nt. Similar to the truncated construct, ILREX3/dl9 did not have the C/EBP consensus site. ILREX3/dl9 was expected to give rise to substantially elevated levels of p65-activated transcription relative to that with the truncated or original constructs, which contain a single ILRE (33). As expected, the levels of p65-activated transcription from ILREX3/dl9 were markedly elevated relative to those from the wild-type construct (Fig. 6). However, while the addition of RBP repressed activated transcription from the original construct, there was no detectable effect on activated transcription from ILREX3/dl9. NF-κB-activated IL-6 transcription required the p65 target site within the ILRE, as expected (Fig. 6) (see Materials and Methods). While the levels of activated IL-6 transcription from ILREX3/dl9 were substantially elevated relative to that observed from the truncated construct, in neither case was RBP-mediated repression observed. This result strongly suggested that RBP did not repress p65-activated transcription. Therefore, the ILRE was sufficient for RBP binding but not sufficient for RBP-mediated repression.

RBP represses activation of IL-6 expression by p65 and C/EBP-β. The difference between −87/+14/IL-6/dl9 (activation targeted by RBP) and −74/+14/IL-6/dl9 (activation unaffected by RBP) is the presence of a consensus site for the cellular transcription factor C/EBP-β immediately upstream of the ILRE (Fig. 1). We next tested the effect of RBP on activated IL-6 expression in the presence of p65 and C/EBP-β. In this case, COS-7 cells were examined, since the levels of coactivated transcription achieved were greatest. In contrast to F9 cells, crude extracts prepared from COS-7 cells did not contain RBP-specific binding activity, as reported previously (20). Figure 7A shows the results of a gel retardation assay with a wild-type IL-6 probe and crude extracts prepared from COS-7 cells cotransfected with CMV/p65 and CMV/C/EBP-β or also with CMV/RBP; RBP-specific binding activity can be clearly visualized in the latter case.

In order to optimize coactivation with p65 and C/EBP-β, COS-7 cells were serum stimulated during transient expression (see Materials and Methods). Induction of C/EBP-β activity has been shown to require posttranslational modifications by protein kinases (21, 27); serum stimulation may mediate this induction. Figure 8 shows the results of this experiment and the effects of RBP on basal, p65-activated, and p65/C/EBP-β-activated IL-6 expression. Clearly detectable levels of basal IL-6 expression were observed from the wild-type construct in COS-7 cells (Fig. 8, lane 1). Basal expression was repressed to barely detectable levels upon addition of RBP (Fig. 8, compare lanes 1 and 5). The elevated basal expression here is dependent upon the C/EBP consensus target site (data not shown) and is probably due to endogenous levels of p65 and C/EBP-β. Results described in the previous section showed that p65-activated IL-6 expression is not targeted by RBP. In this case, the residual IL-6 expression untargeted by RBP is not detectable, probably because the activators have not been overexpressed.

Addition of p65 activated IL-6 expression, which was then reduced in the presence of RBP to a residual level (Fig. 8, compare lanes 1, 2, and 3). Expression of CMV/C/EBP-β alone did not activate IL-6 expression from the wild-type construct above the basal level observed (Fig. 8, compare lanes 1 and 4); this result is consistent with previous reports that showed that interaction between p65 and C/EBP-β proteins mediates C/EBP-β-activated transcription (34, 36). However, coexpres-

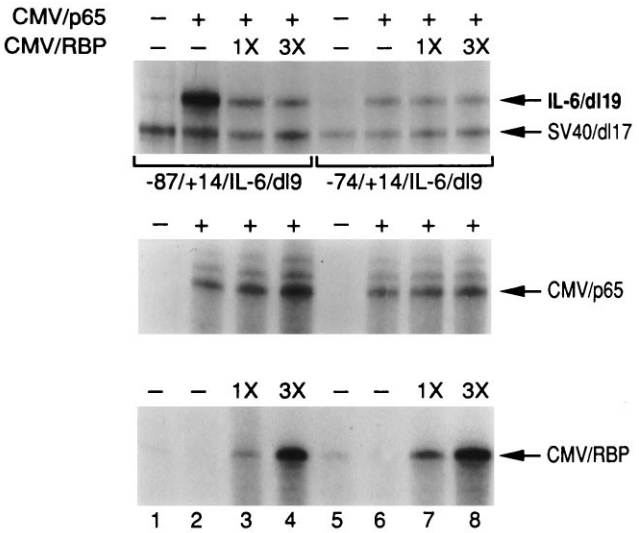

FIG. 5. Comparison of −87/+14/dl9 and −74/+14/dl9 mRNA levels in transfected F9 cells. Addition of CMV/p65 alone or with CMV/RBP is indicated above the lanes. An empty CMV expression vector was used in lieu of CMV/p65 and CMV/RBP. Arrows indicate the protected products expected for IL-6/dl9, SV40/dl17, CMV/p65, and CMV/RBP after RNase T_2 digestion. The levels of mRNA derived from CMV/RBP were considerably greater than those from SV40/RBP used in the previous experiments (5 and 15 μg [1× and 3×, respectively]). The levels of p65-activated IL-6/dl9 from the −87 construct were markedly greater than those from the −74 construct, while RBP led to repressed IL-6/dl9 levels only in the former case.

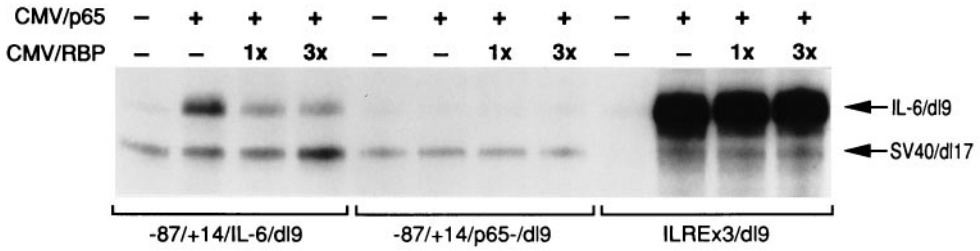

FIG. 6. Comparison of −87/+14/dl9, −87/+14/p65−/dl9, and ILREX3/dl9 in transfected F9 cells. Arrows indicate the protected fragments expected for IL-6/dl9 and SV40/dl17 after RNase T_2 digestion. The addition of CMV/p65 alone or with CMV/RBP is indicated above the lanes (see the legend to Fig. 5). p65-activated IL-6/dl9 mRNA from the p65- construct was undetectable and that from ILREX3 was greatly increased, relative to that from the original −87 construct; RBP repression was observed only in the latter case.

sion of p65 and C/EBP-β gave rise to a further elevation in IL-6 expression, relative to that for p65 alone, as previously reported (30, 37, 42) (Fig. 8, compare lanes 2 and 6), and the addition of RBP repressed activated IL-6 expression to residual levels (compare lanes 6 and 7).

These results show that RBP repressed p65- and C/EBP-β-coactivated IL-6 expression. In both cases of activated IL-6 transcription, that is, with p65 alone or in conjunction with C/EBP-β, residual levels of IL-6 expression were maintained in the presence of RBP. Since the results described in the previous section showed that RBP did not repress p65-activated transcription alone, the residual IL-6 transcription observed in this experiment is consistent with p65 activation untargeted by RBP.

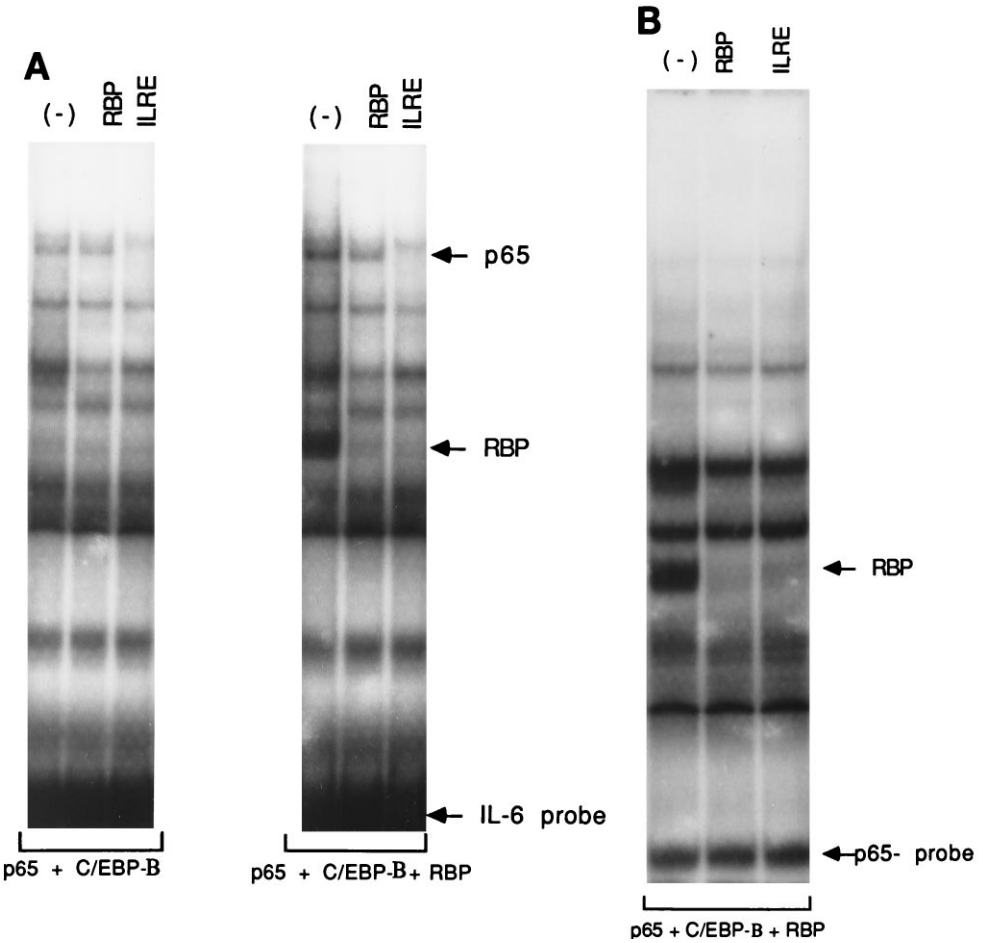

FIG. 7. (A) Gel retardation assay with a radiolabeled probe containing the IL-6 promoter region from nt −87 to −45 and crude extracts prepared from transfected COS-7 cells; the proteins overexpressed during transient expression are shown below the lanes. Specific competitors used are shown above the lanes. Arrows indicate complexes associated with RBP or p65 binding activity. (−), no competitor. (B) Similar analysis with a radiolabeled probe containing the IL-6 promoter from nt −87 to −45 with a mutation in the p65 binding site but with a wild-type RBP target site (p65− probe [see Materials and Methods]). Crude extracts prepared from COS-7 cells transfected with the p65, C/EBP-β, and RBP expression vectors used in the experiment shown in panel A were also used here. While RBP-specific binding activity was apparent, the complex associated with p65 was not detectable.

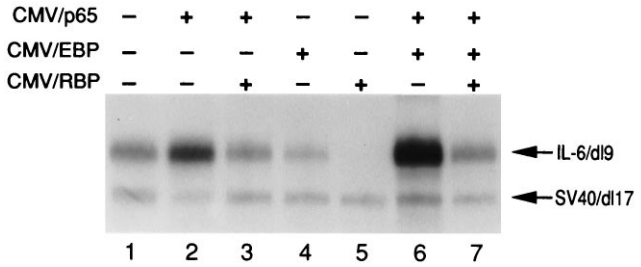

FIG. 8. Results of a transient-expression assay performed in COS-7 cells under conditions of serum stimulation (see Materials and Methods). The addition of CMV/p65 (5 μg), CMV/EBP (5 μg), or CMV/RBP (10 μg) is indicated above the lanes; an empty CMV expression vector was used to maintain equivalent DNA concentrations between samples. Arrows indicate the protected fragments expected for IL-6/dl9 and SV40/dl17 after RNase T_2 digestion. Basal IL-6 expression, relative to the internal control, is elevated in COS-7 cells relative to the case in F9 cells. Addition of CMV/EBP and CMV/p65 elevated IL-6/dl9 expression more than CMV/p65 alone. Basal, p65-activated, and p65/C/EBP-β-activated IL-6 expression were repressed upon addition of CMV/RBP.

RBP does not repress activated IL-6 expression in the absence of p65 binding to the ILRE. Thus far, our results have shown that RBP did not repress IL-6 activation by p65 alone but did repress IL-6 activation by p65 and C/EBP-β. In order to identify the target of RBP, two possibilites were considered. First, RBP may target C/EBP-β alone. Second, RBP may repress synergism between the two activators. In the second case, although RBP did not repress p65 alone, since p65 facilitates C/EBP-β activation, RBP may mediate repression by binding adjacent to p65 at the ILRE.

Both the IL-6 and IL-8 promoters contain closely positioned consensus sites for p65 and C/EBP-β, and in both cases, p65 and C/EBP-β mediated activation in a synergistic manner. Furthermore, C/EBP-β alone activated transcription weakly and only at high concentrations; endogenous levels of NF-κB may contribute to this weak activation. In agreement with previous observations, C/EBP-β activation was undetectable in the absence of coexpressed p65 (Fig. 8). Given these limitations, it was not possible to test if RBP can repress C/EBP-β alone, in the absence of p65, on the wild-type promoter.

On the other hand, studies of the IL-8 promoter showed that interaction between p65 and C/EBP-β can induce expression of a promoter mutant in the NF-κB site (34); in this case, a complex containing both proteins bound to the C/EBP site and activated transcription. Therefore, in order to disassociate C/EBP-β binding to the IL-6 promoter from p65 binding to the ILRE, we employed a construct mutant in the p65 binding site (−87/+14/p65−/dl9). This construct did not exhibit p65 activation (Fig. 6) but did retain the RBP target site; RBP binds to wild-type levels in vitro (Fig. 7B). We then tested the ability of RBP to repress activated IL-6 expression from this construct.

Similar to the wild-type case, addition of C/EBP-β alone did not give rise to detectable IL-6 activation from the p65− construct (data not shown). However, addition of p65 and C/EBP-β did give rise to low but detectable activation; coexpression of RBP did not repress the activated transcription observed (Fig. 9A). The absence of repression was not due to a limitation of detection; addition of RBP clearly repressed the low but detectable IL-6 expression from the wild-type construct obtained without the addition of activators in COS-7 cells (Fig. 8). This result showed that, in the absence of the p65 target site, p65- and C/EBP-β-coactivated IL-6 transcription was not targeted by RBP.

Repression requires proximity between RBP and activators. Results from the previous sections showed that RBP did not repress p65-activated transcription alone (Fig. 5 and 6), that RBP did repress p65/C/EBP-β-activated transcription (Fig. 8), and that binding of p65 to its cognate site was required for repression (Fig. 9A). RBP did not target p65 alone, and yet, p65 binding to the ILRE was required for repression. These results suggested that the close proximity of p65 and RBP binding sites within the ILRE may be important in repression.

In order to test whether the proximity of p65 and RBP binding sites mediated repression, the RBP target site was situated apart from the context of the ILRE. A construct was prepared which contained a mutation in the normal RBP binding site (identical to RBPM, [Fig. 4]) and also a newly positioned RBP site 10 nt downstream of the ILRE and, therefore, distanced from p65 (RBPM/RBP/dl9 [see Materials and Methods]). RBP binds at wild-type levels to this promoter region in vitro (data not shown). The levels of p65/C/EBP-β-activated transcription from RBPM/RBP/dl9 were similar to those from either the wild-type construct (data not shown) or RBPM (Fig. 9B). As expected, RBP did not repress activated IL-6 expression from RBPM; however, RBP also did not repress activated IL-6 expression from RBPM/RBP/dl9 (Fig. 9B). The presence of a viable RBP target site only 10 nt downstream of the p65 binding site did not restore repression in this case. This result showed that repression was mediated by the close proximity of RBP to p65.

The findings of this study emphasized the requirement of the ILRE for RBP repression; both p65 activation through the ILRE and RBP binding within the ILRE are necessary to observe repression. Yet, the ILRE is not sufficient for repression; the C/EBP target site is also required. Taken together, these findings strongly suggest that RBP represses activated IL-6 transcription by binding adjacent to p65 and hindering p65- and C/EBP-β-coactivated IL-6 expression.

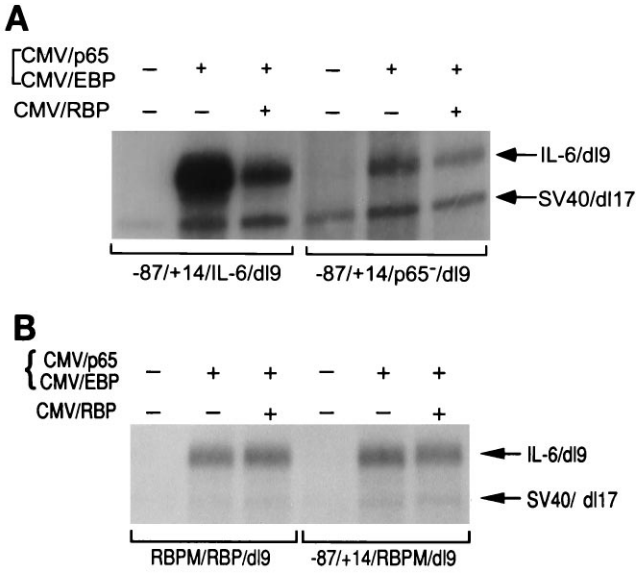

FIG. 9. (A) Comparison of −87/+14/IL-6/dl9 and −87/+14/p65−/dl9 constructs in transfected COS-7 cells under conditions of serum stimulation. Arrows indicate the protected fragments expected for IL-6/dl9 and SV40/dl17 after RNase T_2 digestion. The addition of an expression vector for p65, C/EBP-β, or RBP is indicated above the lanes. Activated IL-6/dl9 expression from the p65− construct was low but detectable; addition of RBP did not repress the activated levels obtained. (B) Similar comparison of −87/RBPM/dl9 and −87/RBPM/RBP/dl9. A wild-type RBP target site placed 10 nt downstream of the ILRE in the RBPM construct did not restore RBP-mediated repression of activated IL-6 expression.

DISCUSSION

The studies presented here document the first case of a mammalian gene that is targeted by RBP: the IL-6 gene. Similar to previous findings with the viral pIX gene, RBP functioned to repress activated IL-6 transcription. This repression was dependent upon RBP binding within the ILRE. While RBP did not repress activation mediated by p65 alone, repression was obtained when coactivation by p65 and C/EBP-β was achieved. C/EBP-β is a poor activator of IL-6 transcription. p65 plays an important role in mediating C/EBP-β activation and, therefore, in optimizing IL-6 expression. RBP repressed this optimal transcription.

While RBP did not target p65 alone, the proximity of p65 and RBP sites within the ILRE facilitated repression. Two experiments addressed this issue. First, an IL-6 construct containing a mutation in the ILRE that disrupted p65 binding, but not RBP binding, was examined for susceptibility to repression. In this case, C/EBP-β and p65 coactivation is mediated by binding to the C/EBP site. Although the levels of activation were low, as expected, RBP repression was not detected. On the other hand, another IL-6 construct containing a mutation that disrupted RBP binding within the ILRE and a wild-type RBP target site 10 nt removed from the p65 binding site gave rise to wild-type levels of C/EBP-β–p65 activation. RBP repression was not detected in this case as well. Binding of p65 and RBP in close proximity, as in the natural IL-6 promoter, was required to obtain RBP repression of coactivation.

RBP may mediate repression by disrupting p65–C/EBP-β interaction (see below), or RBP cobound with p65 may physically impede adjacent C/EBP-β binding to its cognate site. Another possibility is that RBP targets C/EBP-β activation (see below) and that p65 simply mediates C/EBP-β binding to the promoter. In this case, the complex between p65 and C/EBP-β that mediates coactivation at the C/EBP site may differ from the complex that mediates coactivation at the C/EBP/ILRE sites, such that C/EBP-β is no longer accessible to repression.

The p65 activator has been shown to interact with a number of other factors [for example, transcription factor AP1 (35) and accessory factor HMG I(Y) (38)], so that cooperative action between p65 and other factors that may occur at the ILRE may also be targeted by RBP, with subsequent effects on IL-6 expression. In fact, since RBP does not target p65 alone, the biological relevance of RBP repression may be elucidated by understanding cellular conditions during which IL-6 expression at low rather than optimal levels may be important for cellular processes. Alternatively, after IL-6 induction, RBP may play a role in reestablishing basal IL-6 expression; in this case, while NF-κB levels return to those of the uninduced state, RBP may participate in achieving basal IL-6 expression by impeding optimal IL-6 expression in the presence of remaining p65.

Our previous studies established the role of RBP as a transcriptional repressor of the adenovirus pIX gene (7). The pIX promoter is simple, containing only one site for transcriptional activation by the cellular factor SP1 (2). In contrast to that of pIX, the IL-6 promoter is quite complex; in addition to the ILRE and C/EBP binding sites, there are multiple elements upstream that regulate IL-6 expression under certain conditions (29). While the pIX and IL-6 promoters appear disparate, RBP repressed activated transcription from both, and in fact, there are apparent similarities in the position of the RBP target site within each promoter.

In both the pIX and IL-6 promoters, there is a single, natural RBP target site which is immediately adjacent to another transcription factor (TBP in the case of pIX and p65 in the case of IL-6). RBP binding did not impede subsequent binding of the adjacent factor in either case: TBP and RBP cobind the pIX promoter (7), and RBP and p65 cobind the ILRE in vitro (data not shown). As well, in both cases (IL-6 [this study] and pIX [data not shown]), the distancing of RBP binding from the adjacent factor relieves RBP repression. Another similarity between the pIX and IL-6 promoters is that RBP binds between activator(s) and the start site of transcription. In the case of IL-6, however, this does not appear to be important in repression; in distancing the RBP target site from p65, RBP binding was retained between activators and the start site of transcription in this study, and yet, repression was not restored.

Our results suggest that the position of the RBP site adjacent to another transcription factor in the seemingly disparate pIX and IL-6 promoters is not fortuitous but in fact is crucial to RBP repression. RBP may directly impede transcriptional activation by altering protein-protein interactions involving the adjacent transcription factor. This impediment may involve direct interaction with the adjacent factor(s), thereby masking a functional domain or competition for a common cofactor. This possible interaction between RBP and another factor(s) may be mediated by close proximity to the common cofactor, in the case of the pIX gene, or the activator, in the case of the IL-6 gene. For example, RBP may interact with p65 to block p65 interaction with C/EBP-β. We are currently investigating the precise means by which RBP represses transcription of the viral pIX and cellular IL-6 genes.

The important role of RBP in gene regulation in mammalian cells is exemplified by the sequestration of RBP during viral infection. Adenovirus evolved to appropriate RBP to silence pIX gene expression before viral DNA replication. In the case of Epstein-Barr virus, the transcriptional activator EBNA2 interacts with RBP and attains functional RBP DNA binding activity, while masking the RBP repressive domain (17). This study demonstrated the role of RBP in regulating expression from the cellular IL-6 gene, specifically, repression of activated IL-6 transcription. Although IL-6 induction after adenovirus or Epstein-Barr virus infection has not been documented, IL-6 expression is activated by other viral infections (31). It is possible that sequestering of RBP by adenovirus, Epstein-Barr virus, or another virus may lead to unrepressed IL-6 gene expression.

ACKNOWLEDGMENTS

We thank Danny Reinberg for critical reading of the manuscript and Danny Reinberg and Nancy Reich for continued encouragement and support. We thank Ken-Ichi Yamamoto for −45/IL-6/CAT, Céline Gélinas for CMV/p65 and CMV/p50, Kathleen Scotto for CMV/EBP-β, Rodrigo Bravo for the antibody specific to p65 and several fruitful discussions, and Cory Abate for F9 cells.

This research was supported by the National Science Foundation (grant MCB-9407333 to L.D.V.).

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