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Biochemistry

Retinoblastoma protein directly interacts with and activates the transcription factor NF-IL6

(CAAT/enhancer-binding protein/cell division/differentiation/tumor suppressor)

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ABSTRACT **The biological function of the retinoblastoma protein (RB) in the cell division cycle has been extensively documented, but its apparent role in differentiation remains largely unexplored. To investigate how RB is involved in differentiation, the U937 large-cell lymphoma line was induced to differentiate along a monocyte/macrophage lineage. During differentiation RB was found to interact directly through its simian virus 40 large tumor antigen (T antigen)-binding domain with NF-IL6, a member of the CAAT/enhancer-binding protein (C/EBP) family of transcription factors. NF-IL6 utilizes two distinct regions to bind to the hypophosphorylated form of RB *in vitro* and in cells. Wild-type but not mutant RB enhanced both binding activity of NF-IL6 to its cognate DNA sequences *in vitro* and promoter transactivation by NF-IL6 in cells. These findings indicate a novel biochemical function of RB: it activates, by an apparent chaperone-like activity, specific transcription factors important for differentiation. This contrasts with its sequestration and inactivation of other transcription factors, such as E2F-1, which promote progression of the cell cycle. Such disparate mechanisms may help to explain the dual role of RB in cell differentiation and the cell division cycle.**

Fundamental functions of the tumor-suppressor protein RB *in vivo* have been demonstrated in mice in which the *RB* gene is inactivated in the germ line (1, 2). In these animals, many RB-deficient cells, most notably fetal neurons and hematopoietic precursors, fail to exit the cell cycle and continue to divide in aberrant locations. Moreover, some nondividing cells also fail to differentiate terminally and others undergo unscheduled apoptosis (1, 2). These observations and others in tumor cells that have lost expression of functional RB demonstrate that RB is important in cell differentiation and survival as well as in regulating cell cycle progression.

RB is a ubiquitously expressed nuclear protein that exists in different phosphorylated forms in cycling cells (3). The hypophosphorylated form which predominates in G_0 and G_1 phases is thought to be active in regulating progression past a restriction point that allows commitment to S phase and subsequent phases of cell division (4). When hypophosphorylated RB is introduced by microinjection or transfection into cells before commitment to S phase, the cells arrest in G_1 (5, 6). Phosphorylation of RB in mid-G_1 apparently inactivates its function to suppress proliferation. In cells in which RB is inactivated by mutation, regulation of normal cell cycle progression is lost, and malignant growth can ensue. Thus one of the major functions of RB is as a tumor suppressor (4).

The identification of RB-associated proteins has shed considerable light on the molecular pathways involved in the regulation of cell division. One of the cellular proteins with which RB associates is the transcription factor E2F (7). RB is known to sequester and inactivate E2F, which plays a critical role in the G_1/S transition. Since the viral oncoproteins bind to a similar domain of RB as E2F (4, 8), they work, at least in part, by releasing free E2F and promoting deregulated cellular proliferation (9). In normally cycling cells, phosphorylation of RB by cdk kinases also serves to release free E2F, and thereby to allow progression to committed phases of the cell cycle (4).

Some interactions between RB and cellular proteins must occur only in specific differentiated cells, and these transient interactions have been more difficult to discover by simply screening available expression libraries and using the yeast two-hybrid system (9, 10). We have endeavored to explore the potential role of RB in regulating transcription factors important for differentiation. Because of its reported E1A-like activity (11) and well-characterized function in the generation and maintenance of differentiation in hematopoietic cells (12), we investigated NF-IL6 as a candidate for regulation by RB.

MATERIALS AND METHODS

Expression of Glutathione *S*-Transferase (GST) Fusion Proteins. Construction and expression of GST-NF-IL6, GST-NF-IL6~2, and GST-NF-IL6~3 fusion proteins have been described (13). Preparation and expression of RB mutant proteins in the bacterial pET system have also been described (8).

***In Vitro* Binding Assays.** The detailed procedure for the GST pull-down assay has been described (9, 10). Extracts made either from 2×10^6 WR2E3 cells or from bacterial lysates containing RB were used for binding to beads containing 2–3 μg of GST or GST fusion proteins. The far-Western binding assay was performed by using various GST-NF-IL6 fusion proteins (1–2 μg for each lane) separated by SDS/12% PAGE, transferred to Immobilon-P (Millipore), and immunoblotted with RB "sandwiches" consisting of RB, rabbit polyclonal anti-RB antibody 0.47, and alkaline phosphatase-conjugated secondary antibody (8). Bound RB was then visualized with 5-bromo-4-chloro-3-indolyl phosphate toluidinium (BCIP) and nitro blue tetrazolium (NBT) (Promega).

DNA Electrophoretic Mobility-Shift Assay (EMSA). GST fusion proteins, including GST-NF-IL6, GST-RBS, GST-RBH209, and GST-E2F-1, were prepared as described above. E2F100 [the adenovirus 5 E2 promoter sequence, 100 bases upstream of the E2 gene initiation site (11)], which contains sequences with which both NF-IL6 and E2F-1 interact specifically, was labeled with ^{32}P and served as probe. Unlabeled oligodeoxynucleotide (5′-GGACGTCACATTGCACAATCTTAATAA-3′) containing the wild-type (W) recognition sequence of NF-IL6 was used at 100-fold molar excess as a

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Abbreviations: CAT, chloramphenicol acetyltransferase; C/EBP, CAAT/enhancer-binding protein; EMSA, electrophoretic mobility-shift assay; GST, glutathione *S*-transferase; SV40, simian virus 40; T antigen, large tumor antigen.

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competitor, as was a mutant oligodeoxynucleotide (5′-GGACGTCACACTACAAACTCTTAATAA-3′) (M) which cannot be recognized by NF-IL6. The binding reaction mixtures for the NF-IL6/DNA complex contained 10 mM Hepes (pH 7.9), 60 mM KCl, 5 mM Tris (pH 7.0), 0.1 mM EDTA, 10% glycerol, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.2% bovine serum albumin, and 4 ng of ^{32}P-labeled probe. The binding reaction mixtures for the E2F/DNA complexes were as described (8), except that bovine serum albumin (0.2%) was included. Analysis of binding complexes was performed by electrophoresis in a 4.5% polyacrylamide gel in 0.25× TBE (1× is 89 mM Tris/89 mM boric acid/2 mM EDTA) at 4°C.

Transient Transfection and Chloramphenicol Acetyltransferase (CAT) Assays. Plasmids 4X.NF-IL6pA10.CAT and pCMV.NF-IL6~2 were constructed as described (11). The 2.8 kb *RB* cDNA *Bam*HI fragment of p44-2 was inserted in the *Bam*HI site of pCMV.NeoBam to generate pCMV.NeoRB. The equivalent fragment of the RBH209 point mutant (Cys^{706} → Phe; ref. 14) was inserted to generate pCMV.NeoRBH209. Transfections were carried out by conventional calcium phosphate/DNA coprecipitation on 5 × 10^6 WERI-Rb-27 cells. Sixty hours after transfection, the cells were collected and CAT activity was determined (8).

RESULTS

RB and NF-IL6 Interact *in Vitro*. Three forms of NF-IL6 (15)—NF-IL6 (full length), NF-IL6~2 (initiated from aa 24), and NF-IL6~3 (initiated from aa 199)—are expressed in fully differentiated cells by translation from the same mRNA. To examine whether RB specifically interacts with NF-IL6, the cDNAs encoding the three forms of this transcription factor were fused with GST and expressed in bacteria. RB was prepared from RB-reconstituted retinoblastoma cells (WR2E3) (16). As shown in Fig. 1*A*, all three forms of GST-NF-IL6 fusion protein bind to the hypophosphorylated isoform of RB—i.e., the active form that predominates in G_0 and G_1 phases of the cell cycle (4). GST-SV40 T antigen also binds RB, but GST alone does not. To determine which region of RB is required for binding to NF-IL6, various RB deletion mutant proteins were expressed with the pET system (Fig. 1*B*) (8). By passing the bacterial lysates over GST-NF-IL6 or GST-SV40 T antigen fusion protein columns, we determined that the same two RB domains required for binding T antigen (17) are also required for RB to bind to NF-IL6.

Similarly, by using a series of GST-NF-IL6 deletion mutants (13), two distinct domains of NF-IL6 were determined to be important for binding to RB. A large portion of the C-terminal region of NF-IL6, including aa 137–345, could be deleted without abolishing the binding to RB (Fig. 2*A*). Interestingly, a GST-NF-IL6 mutant protein lacking the N-terminal half of NF-IL6 also retained some ability to bind to RB. Thus two distinct regions, one in the N-terminal half and the other in the C-terminal half of the protein, are used by NF-IL6 to interact with RB.

The findings were confirmed by far-Western blotting in which RB was used as a probe to detect GST-NF-IL6 fragments. As shown in Fig. 2*B*, specific proteolytic fragments of the GST fusion proteins (marked by arrows) were detected with strong signals. Only the GST-NF-IL6 deletion mutant missing aa 79–345 (GST-NF-IL6~2B) failed to bind RB. Note that the GST-NF-IL6~3 fusion protein, missing the entire

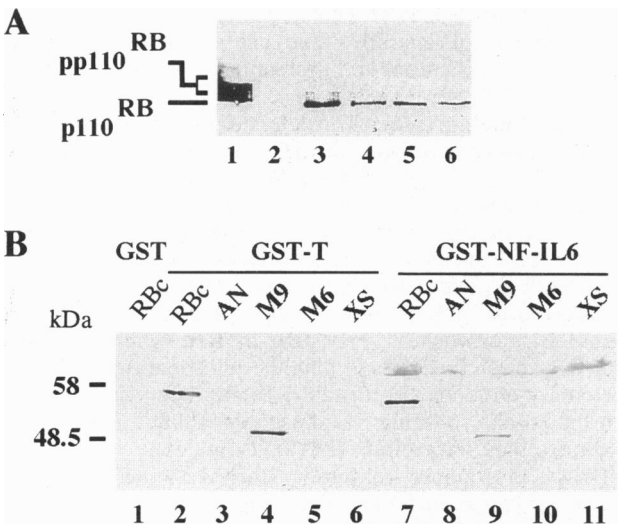

FIG. 1. (*A*) GST, GST-simian virus 40 (SV40) T antigen fusion protein, and GST-NF-IL6 fusion proteins immobilized on glutathione-Sepharose beads were used to bind RB in WR2E3 cell lysates. Lane 1 shows both hyperphosphorylated ($pp110^{RB}$) and hypophosphorylated ($p110^{RB}$) forms of RB immunoprecipitated from the cell lysates with anti-RB antibody 11D7. NF-IL6-3, NF-IL6-2, and NF-IL6 specifically bound hypophosphorylated RB (lanes 4–6, respectively), as did the positive control, SV40 T antigen (lane 3), but not GST alone (lane 2). (*B*) A series of mutant RB proteins including AN (deletion of aa 414–515), M9 (deletion of aa 572–621), M6 (deletion of aa 567–621), and XS (deletion of aa 634–774) were used for binding to either GST-T antigen (lanes 2–6) or GST-NF-IL6 (lanes 7–11) beads and then detected by RB antibody 11D7. Wild-type RB served as control (RBc, lanes 1, 2, and 7).

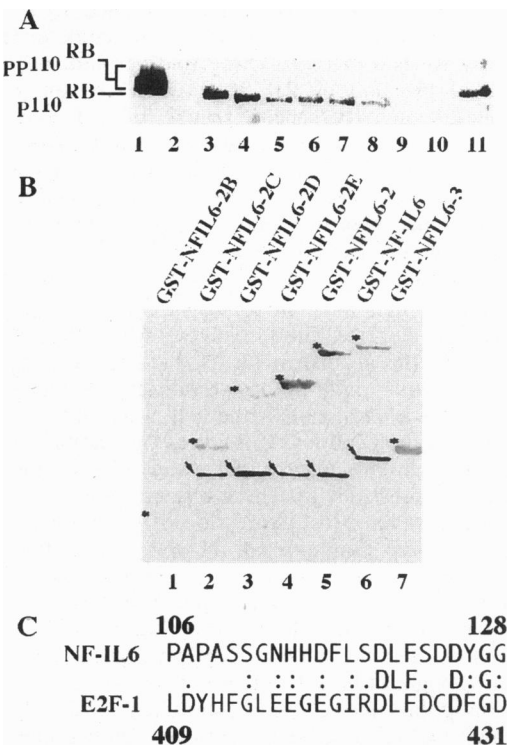

FIG. 2. Determination of regions of NF-IL6 that interact with RB. (*A*) WR2E3 cell lysates were used for binding to beads containing GST alone (lane 2) or each of the fusion proteins GST-T antigen (aa 1–271) (lane 3), GST-NF-IL6 (aa 1–345) (lane 4), GST-NF-IL6-2 (aa 24–345) (lane 5), GST-NF-IL6-2E (aa 24–273) (lane 6), GST-NF-IL6-2D (aa 24–203) (lane 7), GST-NF-IL6-2C (aa 24–136) (lane 8), GST-NF-IL6-2B (aa 24–78) (lane 9), GST-NF-IL6-2A (aa 24–39) (lane 10), and GST-NF-IL6-3 (aa 199–345) (lane 11). Bound RB protein was detected by antibody 11D7. All GST-NF-IL6 fusion proteins except those lacking aa 79–345 (lane 9) or 40–345 (lane 10) retained the ability to bind RB. (*B*) The RB sandwich bound specifically to proteolytic fragments of GST-NF-IL6 (marked by arrows). The bands marked by asterisks represent the intact GST-NF-IL6 fusion proteins which RB sandwiches bind to less strongly. (*C*) The deduced RB-binding sequence of NF-IL6 (aa 106–128) (18) is similar to that of E2F-1 (19). The overall homology between the two sequences is 54%.

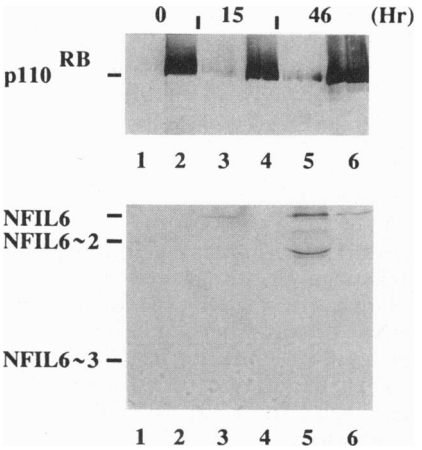

FIG. 3. Interaction of RB with NF-IL6 occurs when U937 cells differentiate along the monocyte/macrophage lineage. Reciprocal immunoprecipitations with 21A1, an anti-NF-IL6 antibody which recognizes the epitopes determined by aa 39–78 (unpublished data) (lanes 1, 3, and 5), or 11D7, an anti-RB antibody (lanes 2, 4, and 6), were performed at various times during differentiation. The upper panel of the Western blot was probed with 11D7 and the lower panel with Z1A1.

N-terminal half of the molecule, was again able to interact with RB. Using two approaches, then, our results show that two portions of NF-IL6, including a portion of the N-terminal half (13), are critical for binding to RB. The region of NF-IL6 from aa 78 to aa 136 contains sequences similar but not identical to those used by E2F-1 to interact with RB (19) (Fig. 2*C*). The exact motif in the C-terminal portion of NF-IL6 used to bind RB is unknown.

RB and NF-IL6 Interact in Differentiating Cells. To explore the interaction between RB and NF-IL6 in cells during differentiation, U937 cells were utilized. These cells can be reliably differentiated over a period of 46 hr into monocyte/macrophage in culture upon treatment with phorbol 12-myristate 13-acetate. They change from large, round, suspended cells with scant cytoplasm, to attached irregularly shaped cells that extend pseudopods (20). The system is useful because differentiation is marked by well-characterized morphologic changes and temporally distinct expression of the three different forms of NF-IL6 (12). During this differentiation process, the hypophosphorylated form of $p110^{RB}$ was immunoprecipitated along with NF-IL6 by an anti-NF-IL6 antibody (Fig. 3). In the reciprocal experiment, an anti-Rb antibody coprecipitated the two functional forms of NF-IL6 (NF-IL6~2 and full-length NF-IL6). The constitutively expressed form (NF-IL6~3) was also coprecipitated. These coimmunoprecipitation results are not due to nonspecific antibody crossreaction: antibodies against RB epitopes did not recognize NF-IL6, and anti-NF-IL6 antibodies did not recognize RB.

RB Activates the Binding of NF-IL6 to DNA. The expression of NF-IL6 and similar members of the CAAT/enhancer-binding protein (C/EBP) family is thought to activate the differentiation process in several cell types (21–23). It would thus be counterproductive for RB to bind and inactivate NF-IL6, as it does E2F-1 in preventing progression of the cell

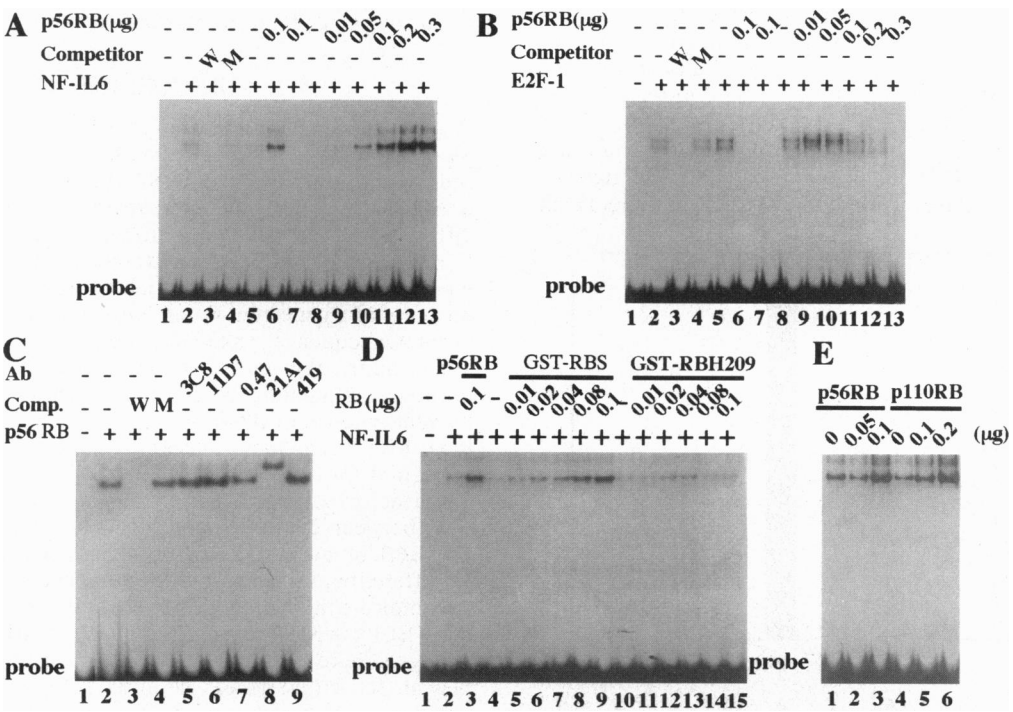

FIG. 4. (*A*) RB enhances NF-IL6 binding to its cognate DNA sequences. GST-NF-IL6 binds to the ^{32}P-labeled E2F100 probe in the EMSA (lane 2). The binding of E2F100 was abolished upon addition of excess of unlabeled wild-type NF-IL6 oligodeoxynucleotide (W, lane 3) but not a mutant oligodeoxynucleotide (M) (lane 4). Increasing concentrations of $p56^{RB}$ enhanced binding to E2F100 at least 10-fold (lanes 6 and 9–13). RB alone did not bind to the probe (lane 7). (*B*) RB inhibits E2F-1 binding to its cognate DNA. Wild-type and mutant E2F-1 oligodeoxynucleotides (8) were used to demonstrate the binding specificity of E2F-1 (lanes 1–4). Upon addition of RB, the binding of E2F-1 to E2F100 was reduced or abolished (lane 6). Increasing concentrations of $p56^{RB}$ progressively diminished binding to E2F100 (lanes 9–13). (*C*) RB is not present in the DNA/NF-IL6 protein complex. Binding of the activated NF-IL6 to the oligodeoxynucleotide could be abolished by competition with wild-type but not mutant NF-IL6 oligodeoxynucleotide (lanes 3 and 4). Various anti-RB antibodies (3C8, 11D7, and 0.47; lanes 5–7) and an unrelated, anti-T antigen antibody (419) (lane 9) failed to bind to these complexes. A supershift was detected when antibody 21A1, which recognizes NF-IL6, was added (lane 8). (*D*) Enhancement of the binding of NF-IL6 to its cognate DNA sequence is specific for wild-type (GST-RBS, lanes 5–9) but not mutated Rb (GST-RBH209, lanes 11–15). (*E*) Full-length $p110^{RB}$ activates DNA binding of NF-IL6 (lanes 5 and 6) in a manner similar to $p56^{RB}$ (lanes 2 and 3). Each reaction mixture contained 50 ng of GST-NF-IL6-2.

cycle. RB may, however, directly activate NF-IL6. To address the influence of RB on the DNA binding activity of NF-IL6, an EMSA designed specifically for NF-IL6 and its cognate DNA sequences was performed in the presence of purified RB. The DNA probe (E2F100) used in this assay contains both NF-IL6 and E2F-1-responsive sequences (11), and forms complexes with either NF-IL6 or E2F-1. As expected, bacterially expressed GST-NF-IL6~2 specifically recognized its appropriate DNA sequence and retarded the migration of labeled DNA probe (Fig. 4*A*). Addition of purified, N-terminally truncated $p56^{RB}$, which still has intact T antigen- and NF-IL6-binding domains (24), however, increased by 10- to 20-fold the binding of NF-IL6 to its recognition sequence, in an RB dose-dependent manner. In contrast, binding of E2F-1 to the same DNA probe was either unaffected or actually decreased by RB (Fig. 4*B*). Thus, RB has opposite effects on the DNA-binding activity of two important transcription factors with which it associates. The molecular mechanism leading to these disparate effects may in part stem from the relative binding affinities of RB for E2F-1 and NF-IL6. E2F-1 binds to RB at least 10 times more strongly than NF-IL6 when measured in the yeast two-hybrid system (ref. 10 and data not shown).

To determine whether RB is present in NF-IL6/DNA complexes, three anti-RB antibodies, 3C8, 11D7, and 0.47, which recognize three different epitopes, were added to the EMSA mixture. None of these antibodies had an effect on the mobility of NF-IL6/DNA complexes, which indicates that RB is not continuously present in the complexes. The presence of NF-IL6 in the complexes, however, was demonstrated by supershift with an anti-NF-IL6 antibody (Fig. 4*C*). The specificities of the antibodies used were confirmed by inability of an anti-T antigen antibody to inhibit complex formation or retard the gel shift (Fig. 4*C*, lane 9). These results suggest that RB does not need continuously to be present in the complex after activating NF-IL6 to bind to DNA. A mutant RB (GST-RBSH209), which harbors a single point mutation ($Cys^{706} \rightarrow Phe$; ref. 14) and fails to bind to either T antigen or NF-IL6, had no effect on the binding of NF-IL6 to target DNA (Fig. 4*D*). This indicates the interaction between RB and NF-IL6 to be an essential step for activating the DNA-binding activity of NF-IL6. Finally, in addition to the N-terminally truncated $p56^{RB}$, full-length $p110^{RB}$, purified from the pET system or from a baculovirus expression system (24, 25), also enhanced the binding of NF-IL6 to specific DNA sequences (Fig. 4*E*). Taken together, these results show that RB, through a direct but transient interaction, enhances the specific DNA-binding activity of NF-IL6.

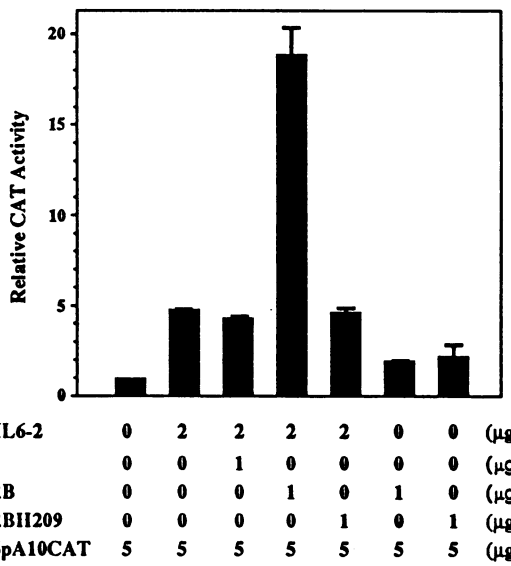

FIG. 5. RB activates NF-IL6 in transcription of a promoter containing NF-IL6 regulatory elements. Human retinoblastoma WERI-27 cells were transfected with one or more of five DNA constructs which express CAT, NF-IL6, wild-type RB, or H209 mutant RB. The amounts of each DNA construct used in the transfections are shown below the histograms. The histograms represent mean CAT activities from three separate transfections. Standard error bars are shown.

RB Enhances Transcriptional Activation by NF-IL6. To explore the functional significance of the interaction between RB and NF-IL6, the effect of RB expression on transcriptional activation by NF-IL6 was examined in transiently transfected cells. Human retinoblastoma WERI-27 cells, which do not express functional RB, were chosen because they provide a background on which the effects of RB expression can be measured. As shown in Fig. 5, NF-IL6~2 alone increased CAT activity to a minor degree (5-fold) compared to vector alone, with (lane 2) or without (lane 3) CMVNeo vector. Such an observation is consistent with previous results showing that expression of NF-IL6 in cells with endogenous RB increases transactivation by NF-IL6 (12). Coexpression of NF-IL6 and RB in RB-null cells, however, synergistically increased CAT activity >18-fold (lane 4). When compared with NF-IL6 expression alone, coexpression of the H209 RB mutant with NF-IL6 (lane 5) had no significant additional effect on basal CAT activity. Expression of RB (either wild-type or the H209 mutant) resulted in nonsignificant, 2-fold enhancement of CAT transcription. These results demonstrate that wild-type RB, in addition to increasing the DNA-binding activity of NF-IL6 *in vitro*, can also enhance the transcriptional activity of NF-IL6 *in vivo*. A mutant RB that does not interact with NF-IL6 fails to enhance specific transcriptional activity.

DISCUSSION

We have demonstrated that wild-type RB interacts, through its SV40 T antigen-binding domains, both *in vitro* and in cultured cells, with the nuclear factor NF-IL6. This interaction occurs in monocyte/macrophage precursors precisely when the cells differentiate and continues in terminally specialized cells. Furthermore, RB directly activates NF-IL6 by enhancing its binding to cognate DNA sequences and by increasing transcription of a gene containing NF-IL6-binding elements in its promoter sequence. Taken together, our observations suggest a heretofore unexplored function for RB: in addition to negatively regulating transcription factors such as E2F to prevent quiescent cells from passing a restriction point in G_1, it positively regulates transcription factor NF-IL6, a factor important for differentiation (26).

Further experiments were performed to study the interactions between RB and another member of the C/EBP family, C/EBPβ, in murine 3T3-L1 fibroblasts, which can be induced to differentiate terminally into adipocytes (22). Preliminary data indicate that RB interacts with and activates DNA binding by C/EBPβ in 3T3-L1 cells, in a manner nearly identical to that presented here for the RB/NF-IL6 interaction. Thus RB may play an active and novel role in the differentiation of several cell types. Although the precise conformational or other change by which RB directly activates these factors remains to be defined, RB does nonetheless fit the definition of a molecular chaperone (27), for it mediates the correct assembly and optimal functional activity of NF-IL6.

Is RB absolutely required for differentiation? The data presented here do not directly address this question. However, the positive correlation between the activity of RB for positively activating NF-IL6 and the differentiation process induced by phorbol ester suggests a potential mechanism by which RB participates in the differentiation. In a similar observation made in cultured cells, RB was reported to interact

with MyoD, a transcription factor important for developmental programs of muscle differentiation. Myoblasts derived from RB-null mouse embryos fail to differentiate terminally in culture, and myotubes can apparently revert to myoblasts when RB is inactivated (28). Although the significance of the interaction between MyoD and RB *in vivo* is still unclear, it is possible that RB is important for myocyte differentiation. Consistent with this view, our preliminary results in $RB^{-/-}$ mouse fibroblasts as another pertinent model system support the observations presented in this report: $RB^{+/+}$ and $RB^{+/-}$ cells can be induced to differentiate into adipocytes by the proper hormone treatment, but similar $RB^{-/-}$ cells cannot unless RB expression is reintroduced by transfection of a wild-type *RB* transgene. These findings clearly suggest that intact, functional RB is crucial for acquisition and maintenance of terminal differentiation of certain cells, especially those for which C/EBP transcription factors play important roles.

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