RNA Polymerase III Transcription Repressed by Rb through Its Interactions with TFIIIB and TFIIIC2*

Wen-Ming Chu‡, Zengxin Wang§, Robert G. Roeder§, and Carl W. Schmid‡‡

From the §Section of Molecular and Cellular Biology, University of California, Davis, Davis, California 95616, §Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, New York 10021-6399, and ¶Department of Chemistry, University of California, Davis, Davis, California 95616

The retinoblastoma susceptibility gene product (Rb) generally represses RNA polymerase III (Pol III)-directed transcription. This implies that Rb interacts with essential transcription factors. Mutations in either the A or B subdomains in the Rb pocket interfere with Rb-mediated repression of Pol III directed transcription, which indicates that both subdomains are directly involved in this activity. Addition of either purified TFIIIB or purified TFIIIC2 partially relieves Rb-mediated repression and restores activity to nuclear extracts that had been depleted of essential factors by binding to Rb. Pull down and coimmunoprecipitation experiments as well as functional assays indicate that Rb interacts with both TFIIIB and TFIIIC2 and that the A subdomain is primarily required for binding TFIIIB and the B subdomain for binding TFIIIC2. While Rb interacts with both factors, the A subdomain is more important than the B subdomain in directing Rb-mediated repression, and TFIIIB is the principal target of that activity.

Studies examining mechanisms by which Rb suppresses cell growth have focused on its interaction with E2F, a transcription factor that is implicated in the expression of genes required during the S phase of the cell cycle (1). E2F sites are switched by Rb from positive to negative regulators (2). Rb is selectively recruited to promoters through E2F, whereupon it blocks surrounding transcription factors from interaction with the basal transcription machinery (2). Hypophosphorylated Rb binds E2F and blocks progression through the cell cycle; phosphorylation of Rb, modulated by cyclin D, releases this block (3).

In addition to its effects on protein-encoding genes, Rb also represses synthesis of rRNA (4). This suggests that Rb may slow cell growth by targeting additional pathways. Actively growing cells require ongoing synthesis of a variety of small structural RNAs so that Rb, in exerting its control over cell cycle progression (3), might also inhibit the transcription of at least some polymerase (Pol)1 III-dependent templates. In agreement with this suggestion, White et al. (5) reported that Rb represses Pol III-directed transcription. Moreover, this repression is entirely independent of promoter structure. This indicates that Rb must interact with one or more of the common components of the Pol III transcriptional machinery to cause this general repression of Pol III-directed transcription.

Together, these results show that Rb can repress transcription by Pol I, II, and III (2, 4, 5). The basis of this transcriptional versatility is not understood. However, sequence comparisons reveal similarities between the A and the B subdomains of the Rb pocket region to TBP and TFIIIB (6, 7). Possibly, Rb influences Pol II-directed transcription by mimicking these factors (6, 7). The interaction of Rb with activation domains in Pol II factors may preclude interactions with TBP and TFIIIB (2). The participation of TBP in both Pol II- and Pol III-mediated transcription, as well as the similarity of a TFIIIB subunit, TFIIIB90, to TFIIIB, potentially extends this hypothesis to Pol III-directed transcription (5, 8). We investigated the possibility that the A and B subdomains in Rb might mediate repression of Pol III transcription through interactions with Pol III accessory factors. The general mammalian factors include TFIIIC2, TFIIIC1, and TFIIIB (9). TFIIIC2 contains five subunits and binds directly to promoters containing both A and B boxes. TFIIIC1 is less well characterized structurally and stabilizes the binding of TFIIIC2. It also appears to recognize specific sequences in the termination region. TFIIIB, which contains TBP, TFIIIB90, and possibly other subunits, is recruited to the promoter by TFIIIC and facilitates Pol III recruitment.

EXPERIMENTAL PROCEDURES

Transfection and Primer Extension—Primer extension with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) was used to assay transcripts from a basal Alu template, Alu-T, in transiently transfected 293 cells (10). Calcium phosphate was used for transient transfection; RNA was isolated after 48 h in all transient assays (10). For U6 RNA, a marked gene (Su C) was used for transient assays (11), and an annealing temperature of 37 °C was employed as a modification of our standard methods (10).

The following constructs overexpressing Rb were used in transient cotransfection assays. Clone HubAcrp-Rb encodes wild type Rb (12). Clones A-HubAcrp-1 neo-P16 and B-HubAcrp-1-neo-F9 encode Rb extensively mutated in either the A or B subdomain, respectively (12, 13). These substitutions are summarized in Table I. The corresponding products are referred to as Rb extensively mutated in either the A or B subdomains and also as Rb(A) and Rb(B). A luciferase reporter gene was employed to determine transfection efficiency (14).

Cell Culture and Extracts—Human 293 cells were obtained from the American Type Culture Collection. Cell lines 2GR and HGR, which stably express exogenous wild type Rb protein, were derived from 293 and HeLa cells, respectively, by G418 selection after transfection with a construct, HubAcrp-Rb, expressing Rb (12). Overexpression of Rb in these cell lines was verified by Western analysis. Cell lines 293 and 2GR were grown in medium containing 10% newborn calf serum and 50 μg/ml of G418 for 2GR cells. Spinner cultures of HeLa, HGR, and 2GR cells were grown in medium containing 5% calf serum and 50 μg/ml G418 for HGR and 2GR cells.

Factor Purification—TFIIIB was immunopurified from the nuclear
extract prepared from the FLAG-tagged human TBP expressed in HeLa cells, and FLAG-tagged TFIIH90 was expressed in insect Sf9 cells and immunopurified (8, 15). TFIIHC2 was affinity purified using a histidine-tagged variant of TFIIHC2 (24). GST fusion proteins were overexpressed in DH5

| A subdomain (positions 393-572) | B subdomain (positions 646-792) |
|----------------------------------|----------------------------------|
| Wild type                       | Position                         | Rb*A | Wild type | Position | Rb*B |
| L                                 | 506                              | F     |     L     | 649     | E   |
| G                                 | 509                              | W     |     K     | 652     | E   |
| L                                 | 513                              | F     |     K     | 653     | E   |
| L                                 | 519                              | W     |     L     | 657     | E   |
| N                                 | 520                              | Y     |     R     | 661     | W   |
| F                                 | 521                              | D     |     L     | 666     | I   |
| N                                 | 523                              | D     |     L     | 669     | I   |
| L                                 | 524                              | F     |     L     | 670     | I   |
| K                                 | 525                              | E     |     H     | 673     | E   |
| D                                 | 528                              | H     |           |         |     |

FIG. 1. Effect of Rb on Alu RNA expression in vivo. Alu T (15 μg) was cotransfected into 293 cells with Rb-overproducing clones, and the resulting transcripts were assayed for Alu RNA by primer extension. Radiolabeled primer extension products were analyzed by gel electrophoresis, and the position of the 238-nucleotide product corresponding to Pol III-transcribed Alu RNA is indicated. HubAcpr-Rb (15, 10, and 5 μg, respectively, in lanes 4, 6, 7, and 8) produces wild type Rb; B-HubAcpr-1-neo-P9 (15 μg, lane 2) and A-HubAcpr-1-neo P16 (15 μg, lane 3) produce Rb having extensively mutated *B and *A subdomains, respectively. Lanes 1 and 5 are controls. For convenience, concentration ramps are also shown in this and subsequent figures.

**Rb Inhibits Pol III-directed Expression in Vivo—Full-length**

*Alu* transscripts are normally expressed at very low levels so that an active *Alu* template (Alu-T clone) serves as a convenient reporter for Pol III-directed transcription in *vivo* (10). As assayed by primer extension, transient cotransfection of an Rb-overproducing clone greatly decreases the abundance of transcripts derived from a human *Alu* template (Fig. 1, lanes 1 and 5–8). We also observe that transient cotransfection of this Rb-overproducing clone causes a similar decrease in the abundance of transcripts derived from a marked U6 RNA gene (data not shown). The lengths of the primer extension products show that the transcripts result from Pol III-mediated transcription events. Cotransfection with a luciferase reporter controls for transfection and the abundance of endogenous 7SL RNA controls for RNA loading in these experiments (data not shown). Comparison of replicates in the absence (lanes 1 and 5) and in the presence (lanes 4 and 6) of Rb demonstrate the reproducibility of these observations. These initial results extend those of White et al. (5) by showing that Rb also represses Pol III transcriptional activity of an *Alu* template.

**Extensive mutations inactivate either the A or B subdomains of the Rb pocket region** (see “Experimental Procedures,” Table I) largely abolish Rb-mediated repression of *Alu* transcription (Fig. 1, lanes 1–5). We have not determined the level of Rb expression in these transient assays since we confirm these differences in activity by more direct *in vitro* results presented below. However, these initial data suggest that a functional pocket region is required for Rb-mediated repression.
RATIONALE. The A subdomain abolishes Rb mediated cell growth arrest (12). Mutation of the B subdomain interferes with binding to E2F and with Rb's protection against apoptosis (13).

Rb Represses Alu Transcription in Vitro—We tested the effects of purified recombinant Rb upon Pol III-directed transcription in vitro. An Alu template, clone Alu-T, is actively transcribed in nuclear extracts (Fig. 2). Purified GST-Rb(379–928) inhibits this transcription (Fig. 2, compare lane 1 to lanes 2–5 and to lanes 6–9), but high concentrations of GST have little or no effect upon transcription (Fig. 2, lane 10 versus lane 1). Thus, we conclude that Rb inhibits Pol III-directed transcription. Thus, we conclude that Rb inhibits Pol III-directed transcription. Substitution of C with F at position 706 within the B subdomain inhibits Rb's interaction with viral oncogenic proteins (26). Although White et al. (5) reported that this substitution abolishes Rb's repressive activity on VA1 gene transcription, we do not detect a significant difference between the effects of wild type Rb and C706F Rb on Alu transcription (Fig. 2, lanes 2–5 and 6–9). As shown below, an Rb protein having an extensively mutated B subdomain also retains partial repressor activity on VA1 gene transcription, so that our results are internally consistent.

The GST-Rb fusion protein has no effect on Pol II transcription driven by the adenovirus major late promoter (27), providing a negative control for possible nonspecific effects caused by high concentrations of the recombinant protein (data not shown) (5). Thus, Rb specifically represses Pol III transcription. We also find that Rb represses transcription of the 7SK and 7SL RNA genes as well as transcription of tRNA, 5S RNA, and U6 RNA genes (data not shown). These findings both confirm and extend the previous report showing that Rb is a general repressor of Pol III directed transcription (5).

Rb's A and B Subdomains Each Participate in Repressing Pol III Activity—To define regions of Rb that are required for Pol III repression, we tested the ability of mutated Rb-GST fusion proteins to inhibit transcription of the VA1 RNA gene in crude nuclear extracts (Table I, Fig. 3). Transcription of the VA1 RNA gene was repressed by Rb-GST fusion proteins containing either residues 379–928 (lanes 1 and 2 versus lane 5) or residues 393–928 (lanes 12 and 13 versus lane 5). Under these assay conditions, 100–200 ng of GST-Rb(393–928) is sufficient to achieve 50% inhibition (Table II). Extensive mutation of the A subdomain greatly reduces Rb's repressor activity (lane 5 versus lane 11; Table II). The effect of mutating both the A and B subdomains is similar to that of mutating the A subdomain alone (lane 5 versus lanes 6 and 7; Table II). In agreement with the previously discussed results of Fig. 2, the C706F substitution in the B subdomain has little effect on repressor activity (lane 3 and 4 and lanes 1 and 2). In further support of those observations, extensive mutation of the B subdomain causes only a modest decrease in repressor activity (lanes 8 and 9; Table II). Thus, functional assays show that an intact A subdomain is essential for Rb's repressor activity in vitro, but that the B subdomain may also participate in this activity (see "Discussion").

TFIIB and TFIIC2 Overcome Rb-mediated Repression—Functional assays were employed to test which factors restored transcriptional activity to nuclear extracts that had been inhibited with GST-Rb fusion proteins. These extracts were complemented with fractions enriched in Pol III and its accessory factors and tested for transcriptional activity in vitro using an Alu template. In preliminary experiments, crude phosphocellulose fractions were investigated with the following results (data not shown). Addition of the 0.7 M KCl phosphocellulose fraction, P0.7, also wild type GST-Rb(393–928) (500 and 1000 ng in lanes 12 and 13, respectively), which should be compared with the extensively mutated forms of this same protein, GST-Rb(393–928;*A,*B) (500 and 1000 ng in lanes 8 and 9, respectively) or GST-Rb(393–928;*A) (500 and 1000 ng in lanes 10 and 11, respectively). Radiolabeled transcripts were resolved by gel electrophoresis, and the position of VA1 RNA is indicated.

**Table II**

| Protein          | 100 ng | 200 ng | 400 ng |
|------------------|--------|--------|--------|
| GST              | 0.96   |        |        |
| GST-Rb           | 0.68   | 0.23   | 0.14   |
| GST-Rb*A        | 0.79   | 0.66   | 0.47   |
| GST-Rb*B        | 0.71   | 0.46   | 0.19   |
| GST-Rb*A*B      | 0.90   | 0.68   | 0.54   |

**FIG. 2. Effect of Rb on Alu transcription in vitro.** Alu-T is transcribed in vitro using HeLa nuclear extract in the presence of purified wild type GST-Rb(379–928) (0, 0.2, 0.38, 0.6, and 0.8 μg in lanes 1–5, respectively) or mutant GST-Rb(379–928;C707F) (0.2, 0.38, 0.6, and 0.8 μg in lanes 6–9, respectively) or purified GST (1 μg in lane 10). Radiolabeled transcripts are resolved by gel electrophoresis, and the position of Alu RNA is indicated.

**FIG. 3. Effects of A and B subdomains mutations on Adeno VA1 transcription.** The VA1 RNA gene was transcribed in vitro with HeLa nuclear extract (lane 5) or with extract in the presence of six fusion proteins described in Table I. These are wild type GST-Rb(379–928) (250 and 500 ng in lanes 1 and 2, respectively) and mutated GST-Rb(379–928;706F) (250 and 500 ng in lanes 3 and 4, respectively) and also wild type GST-Rb(393–928) (500 and 1000 ng in lanes 12 and 13, respectively), which should be compared with the extensively mutated forms of this same protein, GST-Rb(393–928;*A,*B) (500 and 1000 ng in lanes 8 and 9, respectively) or GST-Rb(393–928;*A) (500 and 1000 ng in lanes 10 and 11, respectively). Radiolabeled transcripts were resolved by gel electrophoresis, and the position of VA1 RNA is indicated.
Rb Represses Pol III Transcription

Fig. 4. A, TFIIIB and TFIIIC2 overcome Rb-mediated repression. Alu-T was transcribed by nuclear extract in the presence of GST-Rb(379–928) (0 ng, lane 1; 200 ng, lane 3; and 400 ng, lane 2 and lanes 4–9) and either purified TFIIIB (20 and 40 ng in lanes 4 and 5, respectively) or purified TFIIIC2 (50 and 100 ng in lanes 6 and 7, respectively), or both (20 and 40 ng of TFIIIB and 50 and 100 ng of TFIIIC2 in lanes 8 and 9, respectively). Radiolabeled transcripts were resolved by gel electrophoresis, and the position of Alu RNA is indicated. B, TFIIIB and TFIIIC2 restore activity to Rb-depleted extracts. The adenovirus VA1 RNA gene was transcribed using nuclear extracts which had been mock-depleted (lanes 1–4) or depleted by binding to Rb-GST fusion protein (lanes 5–9) and then supplemented with purified TFIIIB (40 ng, lane 2), purified TFIIIC2 (50 ng, lane 3), or both TFIIIB (40 ng) and TFIIIC2 (50 ng) (lane 4). Radiolabeled transcripts were resolved by gel electrophoresis and the position of VA1 RNA is indicated.

Fig. 5. A, interaction between Rb pocket subdomains and TFIIIC2. In separate experiments, nuclear extract from HeLa cells (lanes 2–6) or immunopurified TFIIIC2 (lanes 7–11) were incubated with GST-Rb(393–928) (lanes 2 and 8), GST-Rb(393–928;*A) (lanes 3 and 10), GST-Rb(393–928;*B) (lanes 4 and 9), GST-Rb(393–928;*A,*B) (lanes 5 and 12), or GST (lanes 6 and 7). Incubated extracts were bound to glutathione-Sepharose beads for isolation of bound protein and subsequent Western analysis using antibodies against TFIIIC2. Lane 1 shows a positive control using purified recombinant TFIIIC2. The recombinant form of TFIIIC2 has higher molecular weight than endogenous TFIIIC2 due to a 20-residue tag. B, interaction between Rb pocket subdomains and TFIIIB90. Nuclear extract from either HeLa cells (lanes 1–3), or Sf9 cells which overexpress TFIIIB90 (lanes 9–13) or TFIIIB90, which was immunopurified from Sf9 cells (lanes 4–8), was incubated with GST-Rb(379–928;C706F) (lane 1), GST-Rb(379–928) (lane 2), GST (lanes 3, 5, 13, and 9), GST-Rb(393–928) (lanes 5 and 11), GST-Rb(393–928;*A) (lanes 6 and 10), GST-Rb(393–928;*B) (lanes 7 and 9), and GST-Rb(393–928;*A,*B) (lanes 6 and 12), GST (lanes 3, 4, and 13). Incubated extracts were bound to glutathione-Sepharose beads for isolation of bound protein and subsequent Western analysis using antibodies against TFIIIB90. For simplicity, the mobility of TFIIIB90 (lanes 1–3) is aligned with that of the slightly higher molecular weight FLAG-tagged TFIIIB90 (lanes 4–13). TFIIIB90 overexpressed in Sf9 cells is phosphorylated accounting for the doublet observed in these samples.

Effects of Rb on Pol III transcription. These preliminary findings were further tested in more definitive experiments using purified factors.

Addition of either affinity-purified TFIIIB (15) or affinity-purified TFIIIC2 relieves the repression of Alu transcription mediated by addition of GST-Rb to nuclear extract (Fig. 4A, compare lanes 1 and 2 to lanes 4 and 5 and to lanes 6 and 7). This result suggests that Rb targets both of these factors. Addition of both TFIIIB and TFIIIC2 appears somewhat more effective in relieving repression than either factor alone (Fig. 4A, compare lanes 4, 6, and 8, and lanes 5, 7, and 9).

These observations are confirmed by similar functional assays using nuclear extracts which have been partially depleted of Rb-interacting factors by binding to an immobilized GST-Rb (379–928). Western analysis indicates approximately 70% depletion of TFIIIB and TFIIIC2 in these extracts (data not shown). As expected, depleted nuclear extracts are less active than control extracts in transcribing a VA1 RNA gene template (Fig. 4B, lane 1 versus 5). Addition of either TFIIIB (lane 2) or TFIIIC2 (lane 3) alone, or both together (lane 4), partially restores activity to depleted nuclear extracts (Fig. 4B, lanes 2–4). A simple interpretation of these results is that Rb represses transcription by interacting directly with either or both TFIIIB and TFIIIC2.

Rb Binds TFIIIB and TFIIIC2—Pull down experiments using mutant Rb-GST fusion proteins were employed to identify both the factors that bind Rb and the sites in Rb responsible for such binding. As assayed by Western blotting with antibody to the 110-kDa subunit, TFIIIC2 in nuclear extract binds immobilized GST-Rb fusion protein, but not GST (Fig. 5A, lane 2 and 6). Rb’s direct interaction with TFIIIC2 is demonstrated by binding of affinity-purified TFIIIC2 to GST-Rb (Fig. 5A, lane 8 versus 7). Extensive mutation of the B subdomain decreases this interaction (Fig. 5A, lanes 3 and 10 versus lanes 2 and 8), whereas extensive mutation of the A subdomain has much less effect on TFIIIC2 binding (Fig. 5A, lanes 4 and 9 versus lanes 2 and 8).

Similarly, we tested for the binding of TFIIIB to these Rb-GST fusion proteins, by Western blot analysis using an antibody against TFIIIB90. We find that TFIIIB in nuclear extract (Fig. 5B, lanes 1–3) and purified TFIIIB (Fig. 5B, lanes 4–8) bind GST-Rb. Furthermore, Rb directly binds the TFIIIB90 subunit expressed in Sf9 cells (Fig. 5B, lanes 9–12). Extensive mutation of the A subdomain markedly inhibits its interaction with TFIIIB90 (Fig. 5B, lanes 8 and 10), whereas mutation of the B subdomain has little effect (Fig. 5B, lanes 7 and 9). Consistent with this finding, the C706F mutation in the B subdomain has no effect on TFIIIB binding (Fig. 5B, lanes 1 and 2).
Taken together, these results indicate that Rb binds both TFIIIB and TFIIIC2, with the A domain being primarily responsible for TFIIIB binding and the B subdomain primarily being responsible for TFIIIC2 binding.

Since GST pull down experiments are extremely sensitive, coimmunoprecipitation experiments were employed as another test of Rb interactions with both TFIIIB and TFIIIC2. As shown in Fig. 6A, TFIIIC2 coimmunoprecipitates with Rb antibody (lane 2), but not with antibody against La (lane 5) or in a mock immunoprecipitation with protein A (lane 1). A positive control with a GST-Rb pull down shows that GST-Rb(C706F) binds slightly less TFIIIC2 than does wild type Rb (Fig. 6A, lanes 3 and 4). This observation agrees with the previous conclusion that the B subdomain is directly involved in TFIIIC2 binding. TFIIIB also coimmunoprecipitates with Rb as compared with a mock immunoprecipitation, confirming the results from the GST pull down experiments (Fig. 6B, lanes 1 and 2).

Differential Sensitivity of A and B Subdomain Mutants to TFIIIB and TFIIIC2—A simple model postulating competitive equilibrium for the binding of TFIIIB and TFIIIC2 to each other and to Rb explains most of the present observations concerning Rb's repression of Pol III activity (Fig. 7) (see "Discussion"). We imagine a two-site model in which the binding of either TFIIIB or TFIIIC2 to Rb displaces the other. Addition of either factor would shift the equilibrium in favor of the TFIIIB-TFIIIC2-DNA transcription complex and at least partially restore activity. We also observed above that the A subdomain is primarily responsible for binding TFIIIB and the B subdomain is primarily responsible for binding TFIIIC2. Accordingly, this two site model further requires that Rb having an inactive A subdomain would be insensitive to TFIIIB addition and Rb having an inactive B subdomain would be insensitive to TFIIIC2 addition. These predictions are substantially confirmed by functional assays (Fig. 8).

We tested for the ability of purified factors TFIIIB and TFIIIC2 to restore transcriptional activity to extracts which had been inhibited by different Rb proteins having mutated A and B subdomains. Addition of TFIIIC2 almost completely relieves repression by GST-Rb(*A), whereas the addition of TFIIIB has no effect (Fig. 8, lanes 5–10). Conversely, addition of TFIIIC2 has no effect upon repression by GST-Rb(*B), whereas addition of TFIIIB partially relieves repression (Fig. 8, lanes 1–4). In agreement with the previous results, the A subdomain is more important than the B subdomain in directing Rb-mediated repression, and TFIIIB is the principal target of that activity (Fig. 8). However, in this case, addition of both TFIIIB and TFIIIC2 is required to completely relieves repression by GST-Rb(*B) (Fig. 8, lane 4). Similarly, the addition of both TFIIIB and TFIIIC2 is required to completely relieves repression by GST-Rb (Fig. 8, lanes 11–14). The synergistic effects of TFIIIB and TFIIIC2 on Rb fusion proteins having a functional A subdomain provides direct support for this simple thermodynamic model (Fig. 8, lanes 4 and 14). The interaction of TFIIIC2 with the B subdomain can partially repress transcription (Fig. 8, lanes 7–10). Yet this same interaction might modulate repression by displacing TFIIIC2 from its binding to the more important A subdomain. With these qualifications concerning the relative importance of the two subdomains, this competitive two site binding model largely accounts for Rb's activity upon Pol III transcription.

**DISCUSSION**

As previously noted, Rb represses transcription by Pol I, II, and III (1, 4, 5). The discovery that Rb may, in part, control cell cycle progression by regulating Pol I transcription of rRNAs is surprising, as rRNAs are normally extremely abundant and especially long lived (4). Small Pol III directed transcripts, which are often shorter lived and present in limiting amounts,
are involved in all aspects of gene expression. In this context, Rb's universal repression of Pol III-directed transcription potentially provides a more immediate pathway to slow cellular proliferation.

The observation that Rb represses transcription from all known classes of Pol III promoters suggested either that it targets one or more components (Pol III, TFIIIC2, or specific subunits of TFIIIB) that are commonly required for all Pol III genes. Highly purified Pol III failed to re-establish mediated repression (data not shown), suggesting either TFIIIB or TFIIIC or both as likely targets. Purified TFIIIB partially relieves repression by Rb and restores partial activity to nuclear extracts that have been depleted by Rb binding. TFIIIB coimmunoprecipitates with Rb and binds Rb-GST fusion protein, indicating their direct interaction. TFIIIB90 and TBP are sufficient to reconstitute fully functional TFIIIB (8), suggesting that at least one of the two is responsible for the Rb-TFIIIB interaction. Since Rb does not bind TBP (6) and since Rb brings down the recombinant TFIIIB90 from the s9 cell extract, we infer that Rb binds TFIIIB90 within TFIIIB.

The Rb pocket domain is required for repression, but whereas the A subdomain is essential, extensive mutation of the B subdomain results in only a partial loss of activity in vitro. Also, extensive mutation of the A subdomain impairs the interaction between Rb and TFIIIB. It has been proposed that the resemblance of Rb's A subdomain to TBP provides a molecular basis for understanding how this region might interact with TFIIIB (6). However, we do not know if this simple explanation may be extrapolated to the interaction between Rb and TFIIIB90 and whether the B subdomain and TBP simply compete for binding to a common or overlapping site on TFIIIB90. In addition, this interpretation does not explain the roles of either the B subdomain or TFIIIC2.

In assays using the VAI RNA gene, TFIIIC2 partially relieves Rb-mediated repression and partially restores activity to extracts that have been depleted by Rb binding. TFIIIC2 binds GST-Rb fusion protein and coimmunoprecipitates with Rb, supporting the conclusion that this factor also directly interacts with Rb. The B subdomain in Rb is homologous to TFIIIB which, in turn, is homologous to TFIIIB90 (8), suggesting this region as a possible TFIIIC2 binding site. In agreement with this suggestion, the substitution C706F decreases TFIIIC2 binding. Also, while mutation of the B subdomain reduces TFIIIC2 binding, mutation of the A subdomain has no effect on this activity, indicating that TFIIIC2 interacts with the B subdomain. TFIIIC2 contains five subunits and the identity of the subunit which binds Rb remains to be determined. However, as noted, the Rb B subdomain resembles part of TFIIIB (6) and it is also known that the TFIIIB-related TFIIIC interacts with the 102 kDa subunit of TFIIIC2. This raises the intriguing possibility that the Rb B subdomain might interact with the same TFIIIC2 subunit required by TFIIIB90.

Rb's ability to interact independently with both TFIIIB and TFIIIC2 makes it uniquely suited to repress Pol III-directed transcription. A simple model postulating competitive equilibrium between TFIIIB and TFIIIC2 for binding to two exclusive sites on Rb correctly predicts that the addition of either of the two factors would at least partially relieve Rb-mediated repression (Fig. 7). However, we further observe that addition of either of the two purified factors partially restores activity to extracts that had been partially depleted by Rb binding. Results from these depletion experiments can also be reconciled by this model. The presence of a competing, nonlimiting factor would prevent the complete depletion of the other factor from an extract (Fig. 7). Subsequent addition of either factor could promote more efficient use of the other, thereby partially restoring transcriptional activity. An intriguing implication of this simple thermodynamic model is the possibility that Rb might fine tune Pol III transcriptional activity by differentially titrating TFIIIB and TFIIIC2. As one example, the postulated TFIIIC2-Rb B subdomain interaction might modulate the repression caused by the more important binding of TFIIIB which is primarily directed by the A subdomain. Whether this speculation is correct, a two-site model involving the preferential interaction of the A subdomain with TFIIIB and the B subdomain with TFIIIC2 is required to interpret the internally consistent results from the binding studies and functional assays.

We also observe that Rb represses transcription of the U6 RNA gene. The factors which are required for the transcription of the U6 gene are unknown and there is a debate concerning the role of TFIIIB in transcribing this gene (19). Conceivably, an altered form of TFIIIB is responsible for U6 RNA transcription and also subject to Rb-mediated repression (8).

The involvement of small structural RNAs in all aspects of gene expression identifies Pol III-directed transcription as a particularly attractive target for regulating cell proliferation. p53, another tumor suppressor protein, also represses Pol III-directed transcription (29). However, unlike Rb, p53-mediated repression is restricted to just a few classes of Pol III promoters. Pol III-directed transcription increases gradually through G1 phase, reaching a maximum during S and G2, decreasing as cells enter M, and reaching a minimum in late M (30, 31). The cell cycle dependence of Pol III activity follows Rb's phosphorylation cycle. Rb is hypophosphorylated in G1, becomes hyperphosphorylated in S, and is dephosphorylated in G2, as cells enter M, and is dephosphorylated during M phase (32–36). Rb's phosphorylation cycle is under cyclin control and coexpression of cyclin D is sufficient to cause phosphorylation of Rb (37). While we have not directly tested the effects of phosphorylation on Rb's interaction with the Pol III transcriptional machinery, our results provide a plausible mechanism through which Pol III activity could be subordinated to cell cycle regulation. Rb may exert control over cell growth through its ability to subject both Pol I and Pol III to cell cycle regulation (38).

Acknowledgments—We acknowledge Dr. Y.-K. Fung for his generosity in many aspects of this study, and we also thank Dr. Fung and Dr. F.-H. Zhang for providing the HuBAcpr-neo constructs to express Rbp18 and Rbp9. We also appreciate Dr. Don Carlson’s advice for improving our presentation of these results.

3 Z. Wang, Y. J. Hsieth and R. G. Roeder, unpublished results.
REFERENCES

1. Johnson, D. G., Schwarz, J. K., Cress, W. D., and Nevins, J. R. (1993) Nature 365, 349–352
2. Weintraub, S. J., Chow, K. N., Luo, R. X., Zhang, S. H., He, S., and Dean, D. C. (1995) Nature 374, 812–815
3. Weinberg, R. A. (1995) Cell 81, 323–330
4. Cavanagh, A. H., Hempel, W. M., Taylor, L. J., Rogalsky, V., Todorov, G., and Rothblum, L. I. (1995) Nature 374, 177–180
5. White, R. J., Trouche, D., Martin, K., Jackson., S. P., and Kouzarides, T. (1996) Nature 382, 88–90
6. Hagemeier, C., Bannister, A. J., Cook, A., and Kouzarides, T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1580–1584
7. Kouzarides, T. (1995) Trends Cell Biol. 5, 448–451
8. Wang, Z., and Roeder, R. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7026–30
9. Wang, Z., and Roeder, R. G. (1997) Mol. Cell. Biol. 16, 6841–6850
10. Chu, W.-M., Liu, W.-M., and Schmid, C. W. (1993) Nucleic Acids Res. 23, 1750–1757
11. Datta, B., and Weiner, A. (1991) Nature 352, 821–824
12. Fung, Y.-K. T., T’Ang, A., Murphree, A. L., Zhang, F. H., Qiu, W.-R., Wang, S.-W., Shi, X.-H., Lee, L., Driscoll, B., and Wu, K.-J. (1993) Oncogene 8, 2659–2672
13. Haas-Kogan, D. A., Kogan, S. C., Levi, D., Dazin, P., T’Ang, A., Fung, Y. K., Israel, M. A. (1995) EMBO J. 14, 461–72
14. Hickman, M. A., Malone, R. W., Lehman-Bruinsma, K., Shih, T., Knoell, D., Sluska, F. C., Walzem, R., Carlson, D. M., and Powell, J. S. (1994) Hum. Gene Ther. 5, 1477–1483
15. Chiang, C. M., Ge, H., Wang, Z., Hoffmann, A., and Roeder, R. G. (1993) EMBO J. 12, 2749–2762
16. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
17. Taggart, A. K. P., Fisher, T. S., and Pugh, B. F. (1992) Cell 71, 1015–1028
18. Pugh, B. F., and Tjian, R. (1990) Nature 345, 1935–1945
19. Yoon, T.-K., Murphy, S., Bai, L., Wang, Z.-X., and Roeder, R. G. (1995) Mol. Cell. Biol. 15, 2019–2024
20. Bredow, S., Surig, D., Muller, J., Kleinert, H., and Benecke, B. J. (1990) Nucleic Acids Res. 18, 6779–6784
21. Kunik, G., and Peterson, T. (1988) Genes Dev. 2, 196–204
22. Little, R., and Bratten, M. (1989) Genomics 4, 376–383
23. Koski, R. A., and Clarkson, S. G. (1982) J. Biol. Chem. 257, 4514–4521
24. Kaelin, W. G., Jr., Pallas, D. C., DeCaprio, J. A., Kaye, F. J., and Livingston, D. M. (1991) Cell 64, 521–532
25. Nakajima, N., Horikoshi, M., and Roeder, R. G. (1988) Mol. Cell. Biol. 8, 4028–4040
26. Kaye, F. J., Kratzke, R. E., Gerster, J. L., and Horowitz, J. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6922–6926
27. Chesnut, J. D., Stephens, J. H., Dahmus, M. E. (1992) J. Biol. Chem. 267, 10500–10506
28. Yoshinaga, S. T., Boulanger, P. A., and Berk, A. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 84, 3585–3589
29. Chemosnov, I., Chu, W.-M., Botchan, M. R., and Smidt, C. W. (1996) Mol. Cell. Biol. 16, 7084–7098
30. Gottesfeld, J. M., Wolf, V. J., Dang, T., Forbes, D. J., and Hart, P. (1994) Science 263, 81–84
31. White, R. J., Gottlieb, T. M., Downes, C. S., and Jackson, S. P. (1995) Mol. Cell. Biol. 15, 6653–6662
32. Buchkovich, K., Duffy, L. A., and Harlow, E. (1989) Cell 58, 1097–1105
33. DeCaprio, J. A., Ludlow, J. W., Lynch, D., Purukawa, Y., Griffin, J., Pwema-Worms, H., Huang, C.-M., and Livingston, D. M. (1989) Cell 58, 1085–1095
34. Ludlow, J. W., Glendening, C. L., Livingston, D. M., and DeCaprio, J. A. (1993) Mol. Cell. Biol. 13, 367–372
35. Mihara, K., Cao, X., Yen, A., Chandler, S., Driscoll, B., Murphree, A. L., T’Ang, A., and Fung, Y. (1989) Science 246, 1300–1303
36. Qin, X.-Q., Livingston, D. M., Ewen, M., Sellers, W. R., Arany, Z., and Kaelin Jr., W. G. (1995) Mol. Cell. Biol. 15, 742–755
37. Ewen, M. E., Shuss, H. S., Sherr, C. J., Matsushime, H., Kate, J. Y., and Livingston, D. M. (1993) Cell 73, 487–497
38. White, R. J. (1997) Trends Biochem. Sci. 22, 77–80
RNA Polymerase III Transcription Repressed by Rb through Its Interactions with TFIIB and TFIIC2
Wen-Ming Chu, Zengxin Wang, Robert G. Roeder and Carl W. Schmid

J. Biol. Chem. 1997, 272:14755-14761. doi: 10.1074/jbc.272.23.14755

Access the most updated version of this article at http://www.jbc.org/content/272/23/14755

Alerts:
  - When this article is cited
  - When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 17 of which can be accessed free at http://www.jbc.org/content/272/23/14755.full.html#ref-list-1