Mechanistic analysis of RNA polymerase III regulation by the retinoblastoma protein

Christopher G.C.Larminie¹, Carol A.Cairns¹,
Renu Mital², Klaus Martin³,
Tony Kouzarides³, Stephen P.Jackson³ and
Robert J.White^{1,3,4}

¹Institute of Biomedical and Life Sciences, Division of Biochemistry and Molecular Biology, Davidson Building, University of Glasgow, Glasgow, G12 8QQ, UK, ²Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA and ³Wellcome/CRC Institute, Tennis Court Road, Cambridge CB2 1QR and Departments of Zoology and Pathology, University of Cambridge, Cambridge, UK

⁴Corresponding author

The tumour suppressor protein RB restricts cellular growth. This may involve inhibiting the synthesis of tRNA and 5S rRNA by RNA polymerase (pol) III. We have shown previously that RB can repress pol III transcription when overexpressed either *in vitro* or *in vivo*. We also demonstrated that pol III activity is elevated substantially in primary fibroblasts from RB-deficient mice. Here we address the molecular mechanism of this regulation. RB is shown to repress all types of pol III promoter. It can do this even if added after transcription complex assembly. Functional assays demonstrate that RB targets specifically the general pol III factor TFIIB. A physical interaction between TFIIB and RB is indicated by fractionation, pull-down and immunoprecipitation data. We show that TFIIB activity is elevated in primary fibroblasts from RB-deficient mice. TFIIB is a multisubunit complex that includes the TATA-binding protein (TBP) and a TFIIB-related factor called BRF. We show that RB itself contains regions of homology to both TBP and BRF and propose a model in which RB disrupts TFIIB by mimicking these two components.

Keywords: RB/RNA polymerase III/TBP/TFIIB/transcription

Introduction

The retinoblastoma protein (RB) is a 105 kDa nuclear phosphoprotein that is encoded by an important tumour suppressor gene (reviewed by Weinberg, 1995; Whyte, 1995). In normal cells, RB is involved in constraining growth and proliferation; in its absence, the ability of cells to shut down these functions is compromised (Weinberg, 1995; Whyte, 1995). RB is mutated in a variety of tumours, including retinoblastomas, small-cell lung carcinomas, sarcomas and bladder carcinomas (Weinberg, 1995). In many other human malignancies, the function of RB is lost due to the disruption of upstream control pathways (Pines, 1995; Weinberg, 1995). It is therefore of considerable importance to understand fully the ways in which RB is able to influence cellular activity.

We have demonstrated recently a novel function for RB in controlling RNA polymerase (pol III) transcription (White *et al.*, 1996). Overexpression of RB represses pol III templates in transfected cells, whereas control pol II promoters are unaffected. Furthermore, purified recombinant RB inhibits pol III transcription when added to a system reconstituted with fractionated factors. These experiments demonstrate that RB can repress pol III when it is overexpressed. To determine whether endogenous levels of RB can control pol III under physiological circumstances, we investigated the expression of class III genes in cells that have lost RB function (White *et al.*, 1996). When two human osteosarcoma lines were compared, RB-deficient SAOS2 cells were found to have a more active pol III transcription apparatus than RB-positive U2OS cells. In addition, primary fibroblasts from RB-knockout mice were shown to have much higher pol III activity than primary fibroblasts from wild-type mice. No general increase in pol II activity was seen when SAOS2 cells were compared with U2OS cells or when *Rb*^{-/-} fibroblasts were compared with *Rb*^{+/+} fibroblasts. These experiments established that endogenous RB plays an important role in suppressing pol III transcription.

The minimal region of RB that is required for growth suppression comprises amino acid residues 379–928 (Qin *et al.*, 1992). The same sequences are sufficient to inhibit pol III transcription (White *et al.*, 1996). Various deletions that remove segments from within this region abolish the ability of RB to regulate pol III (White *et al.*, 1996). Several naturally occurring mutations that prevent RB from functioning as a tumour suppressor also inhibit its ability to repress pol III (White *et al.*, 1996). This raises the possibility that regulation of pol III contributes to the tumour suppressive activity of RB (Nasmyth, 1996; White, 1997).

The viral oncoproteins SV40 large T antigen and adenovirus E1A can bind to RB and neutralize its function (Whyte, 1995). Both T antigen and E1A can also activate pol III transcription (Loeken *et al.*, 1988; Patel and Jones, 1990; White *et al.*, 1996). One way in which they may achieve this is by overcoming the repression of pol III transcription by RB (White *et al.*, 1996). The ability of these oncoproteins to activate pol III by relieving the physiological constraint that is normally provided by RB may contribute to their transforming capability.

Transcription by pol III involves at least two general factors, named TFIIB and TFIIC (reviewed by Willis, 1993; White, 1994; Geiduschek and Kassavetis, 1995). TFIIB is a multisubunit complex that contains the TATA-binding protein TBP (Hernandez, 1993; Rigby, 1993). One of the TBP-associated factors (TAFs) in TFIIB is structurally and functionally related to the pol II general factor TFIIB (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Lopez-de-Leon *et al.*, 1992; Khoo *et al.*,

1994; Wang and Roeder, 1995; Mital *et al.*, 1996). This TAF has been variously named TDS4, PCF4, BRF and TFIIB90, but we shall refer to it as BRF. Although yeast TFIIB has been reconstituted from recombinant components (Kassavetis *et al.*, 1995; Roberts *et al.*, 1996; Ruth *et al.*, 1996), the mammalian factor is much less well characterized (reviewed by Hernandez, 1993; Rigby, 1993). This partly reflects a tendency for TFIIB to dissociate during purification (Lobo *et al.*, 1992; Taggart *et al.*, 1992; Chiang *et al.*, 1993; Teichmann and Seifart, 1995). Several groups have identified polypeptides as candidate TAFs for human TFIIB (Lobo *et al.*, 1992; Taggart *et al.*, 1992; Chiang *et al.*, 1993; Teichmann and Seifart, 1995; Wang and Roeder, 1995; Mital *et al.*, 1996), but there is little consensus and BRF is the only subunit which has been cloned and had its function established categorically (Wang and Roeder, 1995; Mital *et al.*, 1996). Most pol III templates lack a TATA box and so are not recognized directly by TFIIB; in these cases, TFIIB is recruited via protein-protein interactions with promoter-bound TFIIC (reviewed by Willis, 1993; White, 1994; Geiduschek and Kassavetis, 1995).

In the current work, we have investigated the mechanistic basis of pol III regulation by RB. We demonstrate that RB is able to disrupt the activity of a preassembled pol III transcription complex. We also show that it can inhibit expression from all types of pol III promoter. Functional studies implicate TFIIB as a specific target for repression by RB. In support of this, we find that a population of RB molecules consistently co-fractionates with cellular TFIIB. Furthermore, immunoprecipitation and pull-down experiments demonstrate an association between RB and TFIIB. RB itself contains a region of homology to TBP that is followed by a region of homology to BRF. We present a model in which RB inactivates TFIIB by mimicking TBP and BRF in order to disrupt TFIIB.

Results

RB can disrupt the activity of a preformed pol III transcription complex

Fully formed pol III transcription complexes are extremely stable and support multiple rounds of transcription without dissociating from the template (Lassar *et al.*, 1983; reviewed by White, 1994). We tested whether RB is able to repress transcription from a preinitiation complex that had already assembled on the adenovirus VA₁ gene (Figure 1). Initially, we used template commitment assays to establish that, under the conditions of our experiments, a stable transcription complex had assembled on VA₁ following a 15 min incubation. Figure 1A demonstrates that 15 min is sufficient for VA₁ to recruit factors and thereby exclude the expression of a second template that was added subsequently (lanes 4 and 5). However, pre-emptive complex formation did not require the presence of TFIIB (lanes 5 and 7). We therefore carried out additional assays to confirm that TFIIB is recruited to the complex within the 15 min period. In these experiments (Figure 1B), VA₁ was again preincubated with a full set of factors but was then challenged by a second template that had been preincubated separately with TFIIC in the absence of TFIIB. The 15 min preincubation was found

to be sufficient to allow VA₁ to recruit TFIIB into a stable complex and thereby exclude expression of the second gene.

Having established that these conditions allow the stable assembly of TFIIC and TFIIB onto the VA₁ promoter, we then tested whether RB is able to disrupt the preformed complex (Figure 1C). The pol III factors were mixed with either glutathione S-transferase (GST) or a GST fusion protein containing residues 379–928 of RB. The recombinant proteins were added either 15 min prior to the addition of VA₁ DNA (Figure 1C, lanes 1 and 2), simultaneously with the addition of VA₁ (lanes 3 and 4) or 15 min after the factors were mixed with VA₁ DNA (lanes 5 and 6). Nucleotides were then added to allow transcription. RB was found to repress transcription to a similar extent whether it was added before, during or after initiation complex assembly.

RB represses all types of pol III promoter

Three distinct types of promoter structure are utilized by pol III, and this diversity reflects clear differences in transcription factor requirements: type 1 promoters have internal A and C blocks and are unique to 5S rRNA genes; type 2 promoters have internal A and B blocks and are utilized by most pol III templates, including tRNA and VA genes; type 3 promoters are located entirely upstream of the transcription unit, include a TATA box, and are found in U6 and 7SK genes (reviewed by Willis, 1993; White 1994; Geiduschek and Kassavetis, 1995). We tested the ability of RB to regulate transcription directed by each of these promoter types. Increasing quantities of RB(379–928) were added to reactions containing 5S (type 1), VA (type 2) or U6 (type 3) templates (Figure 2). As controls, we added an equal amount of GST (Figure 2B) or a GST fusion protein containing residues 612–711 of RB (Figures 2A and C). We have demonstrated previously that GST–RB(612–711) is unable to regulate VA₁ transcription (White *et al.*, 1996). With each template, transcription was strongly repressed by the GST–RB(379–928) protein, whereas an equal amount of the control protein had little or no effect. We conclude that RB is effective in repressing transcription from all categories of pol III promoter.

RB inactivates TFIIB specifically

Since RB can regulate each of these promoter types, it seemed likely that it acts upon a general component of the pol III transcription apparatus that is utilized by all of these templates. We therefore investigated its ability to inactivate the pol III enzyme and the general pol III factors TFIIB and TFIIC. If RB represses transcription by inhibiting a specific component, then it should be possible to restore expression in the presence of RB by adding more of that component. We carried out add-back experiments to test whether this is the case.

VA₁ transcription was reconstituted using partially purified factors (Figure 3A). Recombinant RB repressed expression in this system, as previously (Figure 3A, lanes 1 and 2). Inclusion of additional pol III or TFIIC fractions did not restore transcription (lanes 3 and 4). In contrast, a fraction containing partially purified TFIIB produced a substantial reversal in the level of inhibition (lane 5). Figure 3B demonstrates that repression by RB can be reversed completely in a dose-dependent manner using

Fig. 1. RB inactivates pol III transcription whether added before, during or after initiation complex assembly. **(A)** Template 1 (500 ng), as indicated, was preincubated for 15 min at 30°C with either a full set of fractionated factors (all lanes except 5 and 7) or with 2 μ l of CHep-1.0 alone (lanes 5 and 7). Template 2 (500 ng) was then added, together with 1 μ l each of 0.38M-TFIIB and 0.48M-TFIIB in the case of lanes 5 and 7. Nucleotides were added immediately to initiate transcription. The VA template was pVA_I and the tRNA template was Mcet1. The short transcript that runs just below the tRNA in lanes 2–5 is derived from pVA_I. **(B)** Template 1 (500 ng), as indicated, was preincubated for 15 min at 30°C with a full set of fractionated factors. Template 2 (500 ng) was preincubated separately with 2 μ l of CHep-1.0. The reactions were then mixed and nucleotides were added immediately to initiate transcription. The VA template was pVA_I and the B2 template was pAG38. **(C)** Fractionated factors were preincubated for 15 min at 30°C before the addition of pVA_I (250 ng); after a further 15 min at 30°C, nucleotides were added and transcription was allowed to proceed. Reactions were supplemented with 250 ng of GST or GST–RB(379–928), as indicated, which were added at the indicated times.

Fig. 2. RB represses transcription of 5S, VA_I and U6 genes. **(A)** Transcription of pHu5S3.1 (250 ng) using nuclear extract (10 μ g) preincubated (15 min at 30°C) with no addition (lanes 1 and 5) or with 100, 200 or 300 ng of GST–RB(379–928) (lanes 2, 3 and 4, respectively) or 100, 200 or 300 ng of GST–RB(612–711) (lanes 6, 7 and 8, respectively). **(B)** Transcription of pVA_I (250 ng) using fractionated factors preincubated (15 min at 30°C) with no addition (lanes 1 and 5) or with 75, 150 or 300 ng of GST–RB(379–928) (lanes 2, 3 and 4, respectively) or 75, 150 or 300 ng of GST (lanes 6, 7 and 8, respectively). **(C)** Transcription of pU6/Hae/RA.2 (500 ng) using nuclear extract (10 μ g) preincubated (15 min at 30°C) with no addition (lanes 1 and 5) or with 100, 200 or 300 ng of GST–RB(379–928) (lanes 2, 3 and 4, respectively) or 100, 200 or 300 ng of GST–RB(612–711) (lanes 6, 7 and 8, respectively).

the TFIIB fraction (lanes 1–5). In contrast, the same fraction has little or no stimulatory effect when added in the absence of recombinant RB (lanes 6–9). This is because TFIIC is limiting and TFIIB is in relative excess in the reconstituted system, as shown by the ability of the TFIIC fraction to increase transcription (lanes 10–12).

Thus, TFIIB activity is in excess in the absence of recombinant RB, but becomes limiting when RB is added. This demonstrates that RB is able to bring about a specific decrease in TFIIB activity such that it becomes limiting for transcription. The fact that a TFIIB fraction alone is sufficient to overcome repression indicates that the

Fig. 3. (A) TFIIIB relieves repression by recombinant RB whereas TFIIIC and pol III do not. Transcription of pVA₁ (250 ng) using fractionated factors preincubated (15 min, 30°C) with 250 ng of GST (lane 1) or GST-RB(379–928) (lanes 2–5) and 3 µl of A25(1.0) fraction containing pol III (lane 3), CHep-1.0 fraction containing TFIIIC (lane 4) or A25(0.15) fraction containing TFIIIB (lane 5). (B) TFIIIB is limiting for transcription in the presence of recombinant RB, whereas in the absence of recombinant RB TFIIIC is limiting and TFIIIB is in excess. Transcription of pVA₁ (250 ng) using fractionated factors preincubated (15 min, 30°C) with 250 ng of GST (lanes 1 and 6–12) or GST-RB(379–928) (lanes 2–5) and 1 µl (lanes 3 and 7), 2 µl (lanes 4 and 8) or 4 µl (lanes 5 and 9) of A25(0.15) fraction containing TFIIIB or 1 µl (lane 10), 2 µl (lane 11) or 4 µl (lane 12) of CHep-1.0 fraction containing TFIIIC.

activities of TFIIIC and pol III are not compromised by RB. We conclude that RB inhibits TFIIIB specifically.

A TBP-associated component of TFIIIB is inactivated by RB

In order to determine which part of TFIIIB is the target for repression by RB, we carried out add-back experiments using TBP alone or the TAF component of the complex (Figure 4). TFIIIB TAFs (B-TAFs) were affinity-purified by chromatography on a column containing immobilized TBP, as described previously (White and Jackson, 1992; White *et al.*, 1995a). We also prepared a control fraction by the same procedure but using a column without TBP. Whereas the B-TAF fraction can reconstitute pol III transcription in the presence of TBP, TFIIIC and pol III, the control fraction is inactive (White *et al.*, 1995a).

Recombinant RB was used to repress VA₁ transcription in a system reconstituted with fractionated factors (Figure 4). Addition of recombinant TBP made no difference to

Fig. 4. Repression by RB can be relieved using affinity-purified TFIIIB TAFs but not using TBP. Transcription of pVA₁ (250 ng) using fractionated factors preincubated (15 min, 30°C) with 250 ng of GST (lane 1) or GST-RB (lanes 2–9), and 1, 3 or 6 µl of TBP (lanes 3–5, respectively), 3 or 6 µl of B-TAF fraction (lanes 6 and 7, respectively) or 3 or 6 µl of control fraction (lanes 8 and 9, respectively).

the level of inhibition. In contrast, the affinity-purified B-TAF fraction produced a significant, dose-dependent reversal of repression. That this response is due to the presence of TBP-associated factors in the B-TAF fraction is demonstrated by the inability of the control fraction to relieve inhibition. This experiment suggests that a TAF component of TFIIIB is the specific target for repression of pol III transcription by RB.

TFIIIB interacts with immobilized recombinant RB

We carried out pull-down experiments to test for a physical interaction between RB and pol III factors (Figure 5). Crude phosphocellulose (PC) fractions were incubated on a vibrating platform with glutathione-agarose beads carrying immobilized GST or GST-RB, and the unbound material was then assayed for transcription factor activity. When PC-C fractions were treated in this way, we detected no depletion of TFIIIC activity (Figure 5A). In contrast, TFIIIB activity was partially depleted from PC-B fractions using GST-RB (Figure 5B). Depletion under these conditions was relatively inefficient and we were unable to improve it by extending the incubation period because prolonged treatment in this way resulted in a general loss of transcriptional activity. Nevertheless, these results suggest that the RB portion of GST-RB interacts with TFIIIB. We confirmed this by Western blotting the material that remained bound to the beads after extensive washing. Figure 5C shows that both TBP and BRF were detected in association with the GST-RB beads, but were not bound to GST alone. These data provide evidence for a physical association between RB and TFIIIB.

A population of RB molecules co-fractionates with TFIIIB

The results above indicate a physical and functional interaction between TFIIIB and RB when the latter is overexpressed as a recombinant protein. A more important issue is whether these factors interact with each other

Fig. 5. Immobilized RB binds TFIIB but not TFIIC. (A) Transcription of pVA₁ (250 ng) using 2 μ l of PC-B (all lanes) and 2 μ l of PC-C (lane 1), 2 μ l of LDB (lane 2), 4 μ l of supernatant from PC-C that had been preincubated with GST beads (lane 3) or 4 μ l of supernatant from PC-C that had been preincubated with GST-RB beads (lane 4). (B) Transcription of pVA₁ (250 ng) using 2 μ l of CHep-1.0 (all lanes) and 2 μ l of PC-B (lane 1), 2 μ l of LDB (lane 2), 4 μ l of supernatant from PC-B that had been preincubated with GST beads (lane 3) or 4 μ l of supernatant from PC-B that had been preincubated with GST-RB beads (lane 4). (C) Proteins from PC-B that remained bound to GST-RB (lanes 1 and 4) or GST (lanes 2 and 5) beads after extensive washing were resolved on an SDS-7.8% polyacrylamide gel and then analysed by Western immunoblotting with antibodies against TBP (lanes 1–3) or BRF (lanes 4–6). Lane 3 contains 10 ng of recombinant TBP. Lane 6 contains 15 μ l of PC-B.

when they are present at physiological concentrations. To address this, we assayed for the presence of RB in TFIIB fractions by immunoblotting fractionated factors and probing with an anti-RB antibody. Consistent co-purification would be suggestive of a stable interaction between these two factors.

Figure 6A demonstrates that RB is readily detectable in TFIIB fractions that have been purified through phosphocellulose and DEAE-Sephadex (lane 3) or phosphocellulose and a glycerol gradient (lane 4). In contrast, little or no RB was detected in fractions containing TFIIC (lane 1), pol III (lane 2), or the TBP-containing complex SL1 (lane 5). These data support the contention that RB interacts specifically with TFIIB.

To provide an additional test for an association between RB and TFIIB, we examined whether these factors co-fractionate during gradient chromatography on heparin-Sepharose. A phosphocellulose fraction containing RB and TFIIB was applied to a heparin-Sepharose column and the bound proteins were eluted with a linear salt gradient. Individual fractions were assayed for their content of TFIIB (Figure 6B, upper panel) and RB (Figure 6B, lower panel). Neither TFIIB nor RB was detectable before fraction 49; both factors began to elute in fraction 50, peaked in fractions 51 and 52, and then tailed off gradually in the next few fractions. Thus, RB co-fractionates closely with TFIIB on a heparin-Sepharose salt gradient.

These data demonstrate that a population of RB molecules co-purifies with TFIIB on phosphocellulose, DEAE-Sephadex and heparin-Sepharose columns, and on glycerol gradients. We have also found RB in TFIIB fractions that were prepared using Q-Sepharose, Mono Q and Macro-Prep CM resins (data not shown). Such consistent co-fractionation is suggestive of a stable interaction. In contrast, little or no RB was detected in fractions containing pol III, TFIIC or the TBP-containing complex SL1 (Figure 6A). Together, these results suggest that there is a specific physical and functional interaction between endogenous RB and TFIIB.

Endogenous RB associates with TFIIB

We carried out immunoprecipitation assays as an additional test of whether cellular RB associates with TFIIB (Figure 7). Fractions containing TFIIB were immunoprecipitated using a monoclonal antibody that specifically recognizes the hypophosphorylated (active) form of RB. After extensive washing in 200 mM KCl and 0.1% NP-40, the precipitated material was immunoblotted and probed with antibodies against TBP and BRF. We found that both these TFIIB subunits were co-precipitated with RB (Figure 7A, lanes 4 and 8). Neither TBP nor BRF was immunoprecipitated using a control antibody that recognizes Sp1 (Figure 7B, lanes 5 and 10). As an additional test of specificity, we carried out immunoprecipitations with the anti-RB antibody in the presence of blocking agents. A short GST-RB polypeptide containing the region of RB that is recognized by this antibody was able to block the co-precipitation of TBP and BRF (Figure 7B, lanes 3 and 8). In contrast, an equal amount of GST protein did not prevent the immunoprecipitation of these subunits (Figure 7B, lanes 2 and 7). These experiments provide direct evidence that endogenous RB associates with TFIIB at physiological concentrations.

Extracts of cells from RB-knockout mice have elevated TFIIB activity

We have shown previously that endogenous RB represses pol III transcription in primary fibroblasts from embryonic mice (White *et al.*, 1996). If this effect is achieved through the repression of TFIIB, then we would expect to find an increase in TFIIB activity in these cells following disruption of the *Rb* gene. To test this, we prepared extracts from primary cells that had been isolated from either wild-type or RB-knockout mice (Jacks *et al.*, 1992). The RB-negative extracts supported higher rates of pol III transcription than the RB-positive extracts (Figure 8A). As a control for factor recovery, we compared the levels of TFIIC in these extracts and detected little or no difference (Figure 8B). In contrast, when TFIIB activity

Fig. 6. A population of RB molecules co-fractionates with TFIIB. (A) RB is readily detectable in fractions containing TFIIB but not in fractions containing pol III, TFIIC or SL1. Fractionated factors (15 μ l) as indicated were resolved on an SDS–7.8% polyacrylamide gel and then analysed by Western immunoblotting with anti-RB antibody G3-245. The TFIIC fraction in lane 1 was affinity-purified and has a protein concentration of 93 ng/ μ l. The pol III fraction in lane 2 was A25(1.0) and has a protein concentration of 15 ng/ μ l. The TFIIB fraction in lane 3 was prepared by chromatography on phosphocellulose followed by glycerol gradient sedimentation; it has a protein concentration of 200 ng/ μ l. The TFIIB fraction in lane 4 was A25(0.15) and has a protein concentration of 80 ng/ μ l. The SL1 fraction in lane 5 was SS700 and has a protein concentration of 260 ng/ μ l. (B) RB co-fractionates with TFIIB during gradient chromatography on heparin–Sepharose. The upper panel shows TFIIB activity of individual fractions and the lower panel shows the RB content of the same fractions. In each case lane 1 contains buffer, lane 2 contains input material (PC-B), lane 3 contains flow-through, and lanes 4–18 contain fractions 46–60, respectively. TFIIB activity was assayed using 4 μ l of the indicated fraction, 2 μ l of PC-C and 500 ng of pVA_T; after 15 min incubation at 30°C, nucleotides were added to assay transcription. RB content was assayed using 14 μ l of the indicated fractions which were resolved on an SDS–7.8% polyacrylamide gel and then analysed by Western immunoblotting with anti-RB antibody G3-245.

was measured by complementation assays, it was found to be significantly elevated in the RB-negative extracts (Figure 8C). We conclude that TFIIB is a physiological target for repression by RB in mouse embryonic fibroblasts.

Discussion

We have shown previously that endogenous RB plays an important role in suppressing the level of pol III transcription in primary murine fibroblasts (White *et al.*, 1996). In this study, we have addressed the molecular mechanism of this regulation. We have demonstrated that RB is able to disrupt the function of a preformed pol III transcription complex. We have also shown that it can regulate the activity of each type of pol III promoter. This appears to be achieved by the specific inactivation of TFIIB. Since

TFIIB is required for the expression of all class III genes, its targeting by RB provides an explanation for the general increase in pol III transcription that is observed in RB-knockout mice (White *et al.*, 1996).

RB can repress transcription from a preformed pol III initiation complex

The ability of RB to regulate transcription is believed to be restricted to a defined time window during the cell cycle. Thus, RB is active during early G₁ phase, is inactivated at the G₁/S transition, and then remains inactive until the end of mitosis (reviewed by Weinberg, 1995; Whyte, 1995). Since DNA replication erases initiation complexes from class III genes (Wolffe and Brown, 1986), complexes must reform during S and G₂ phases, when RB is believed to be inactive. For RB to repress TFIIB during the subsequent G₁ phase, it may need to regulate transcription complexes that had assembled during the previous cycle. We have confirmed that RB is indeed capable of inactivating a preassembled pol III preinitiation complex. Alternatively, it is possible that the pol III preinitiation complex is disrupted during mitosis; in this case RB would inhibit complexes that reform during the early part of G₁.

RB represses pol III transcription by inactivating TFIIB

We have presented several independent lines of evidence that implicate TFIIB as the component of the pol III transcription apparatus that is targeted by RB. (i) Addition of recombinant RB to reconstituted transcription reactions causes a specific decrease in the activity of TFIIB such that this factor becomes limiting for transcription. Repression by RB is overcome by the addition of more TFIIB, but not by TFIIC or pol III. (ii) Recombinant RB that has been immobilized on beads depletes fractions of TFIIB, but not TFIIC activity. The BRF and TBP subunits of TFIIB were shown to bind to the immobilized RB. (iii) A population of RB molecules co-purifies with TFIIB during a variety of distinct fractionation procedures. In contrast, little or no RB is detected in fractions containing TFIIC, pol III or SL1. Although we cannot exclude the possibility that the co-purification of TFIIB and RB is fortuitous, we consider that this is unlikely given the consistency with which it is observed under a variety of distinct chromatographic conditions. We have also fractionated TFIIB from SAOS2 cells, which contain inactive mutant RB. We find that TFIIB can be readily separated from SAOS2 RB (data not shown), which supports the idea that co-fractionation of TFIIB and RB reflects a functional interaction. In addition, we have examined the fractionation properties of RB that has been expressed in Sf9 cells. This recombinant RB displayed chromatographic behaviour that was distinct from that of TFIIB (data not shown). It is therefore unlikely that the co-fractionation of TFIIB with a population of cellular RB molecules is due to these factors possessing identical chromatographic properties. The most plausible explanation of the co-purification data is that a proportion of endogenous TFIIB exists in association with RB. (iv) TFIIB subunits (TBP and BRF) co-immunoprecipitate with RB. This effect is specific, since it is not seen using control antibodies and it can be blocked using an excess

Fig. 7. TBP and BRF co-immunoprecipitate with RB. (A) Proteins from PC-B that were immunoprecipitated using anti-TBP antibody bound to protein A-Sepharose (lanes 2 and 6), protein A-Sepharose alone (lanes 3 and 7) or anti-RB antibody bound to protein A-Sepharose (lanes 4 and 8) were resolved on an SDS-7.8% polyacrylamide gel and then analysed by Western immunoblotting with antibodies against TBP (lanes 1-4) or BRF (lanes 5-8). Lane 1 contains 10 ng of recombinant TBP. Lane 5 contains 15 μ l of PC-B. (B) Proteins from PC-B that were immunoprecipitated using anti-RB antibody (lanes 2, 3, 7 and 8), anti-TBP antibody (lanes 4 and 9) or anti-Sp1 antibody (lanes 5 and 10) were resolved on an SDS-7.8% polyacrylamide gel and then analysed by Western immunoblotting with antibodies against TBP (lanes 1-5) or BRF (lanes 6-10). The reactions in lanes 2 and 7 contained 1.5 μ g of GST. The reactions in lanes 3 and 8 contained 1.5 μ g of GST-RB(379-611). Lane 1 contains 10 ng of recombinant TBP. Lane 6 contains 15 μ l of PC-B.

Fig. 8. Primary fibroblasts from RB-knockout mice have elevated TFIIIB activity. (A) Transcription of pVA₁ (250 ng) using whole-cell extract (24 μ g) prepared from RB^{+/+} (lanes 1 and 3) or RB^{-/-} (lanes 2 and 4) primary fibroblasts. (B) Band shift assay using 4 ng of radiolabelled B-block oligonucleotide probe, 500 ng of poly(dI-dC), no protein (lane 1), 12.7 μ g of RB^{-/-} extract (lane 2) or 12.7 μ g of RB^{+/+} extract (lanes 3-5) and 200 ng of B-block (lane 4) or MSV (lane 5) oligonucleotide competitor. (C) Transcription of pVA₁ (500 ng) using 4 μ l of PC-C and 5 ng of recombinant TBP (all lanes) and 25 μ g of heat-treated RB^{+/+} extract (lane 1), 25 μ g of heat-treated RB^{-/-} extract (lane 2), no addition (lane 3) or 3 μ l of heat-treated Mono Q-purified TFIIIB (lane 4).

of the epitope. In addition to detecting TBP and BRF in immunoprecipitates from an anti-RB antibody, we have also co-precipitated RB using antibodies against TBP and BRF (data not shown). These data provide direct evidence for an interaction between RB and TFIIIB. We were unable to detect this interaction using a TFIIIB fraction from SAOS2 cells, in which RB is inactive (data not

shown). (v) TFIIIB activity is elevated in extracts prepared from RB-knockout fibroblasts. This provides genetic evidence for a functional interaction between TFIIIB and RB. We consider that these independent lines of evidence together provide a compelling case for believing that RB regulates pol III transcription by targeting the general factor TFIIIB.

Fig. 9. Sequence homology between RB, TBP and BRF. The regions of RB that are related to TBP and BRF are indicated and the alignments are shown underneath. Sequence identities are indicated with black boxes and sequence similarities (using highly stringent criteria) are indicated with grey boxes.

Adenovirus infection

Although E1A can overcome the repressive effects of RB, there is little or no increase in TFIIB activity when HeLa cells are infected by adenovirus (Hoeffler and Roeder, 1985; Yoshinaga *et al.*, 1986). Instead, the activation of pol III transcription that accompanies adenoviral infection is mediated by changes in TFIIC (Hoeffler and Roeder, 1985; Yoshinaga *et al.*, 1986; Sinn *et al.*, 1995). We believe that the explanation for this apparent paradox lies in the fact that RB activity is very low in HeLa cells. Although accurate quantitation is extremely difficult, our best estimate is that 5–10% of TFIIB is associated with RB in extracts made from uninfected asynchronous HeLa cells (data not shown). Even if E1A displaces all of this RB during adenovirus infection, the overall effect on total TFIIB activity is unlikely to be detectable. In order to stimulate pol III transcription significantly, adenovirus must employ an additional mechanism. The activation of TFIIC is the decisive step in stimulating pol III transcription during infection of HeLa cells (Hoeffler and Roeder, 1985; Yoshinaga *et al.*, 1986).

The relatively small proportion of TFIIB that associates with RB in HeLa cells is consistent with the high pol III activity of this line. It is likely that HPV E7 protein that is expressed in these cells may help overcome repression by RB, although we have yet to confirm this for pol III. In other cell types, RB appears to play a much more significant role in repressing pol III. This is particularly obvious in mouse embryo fibroblasts, where deletion of RB causes a 5-fold increase in pol III transcription (White *et al.*, 1996). We have shown previously that E1A can overcome repression by RB under conditions in which

RB activity is high (in those experiments it was added in excess) (White *et al.*, 1996). In contrast to the results in HeLa cells, we would predict that TFIIB activity will increase significantly during adenovirus infection of cells such as fibroblasts that have high RB activity.

RB may bind TFIIB by mimicking TBP and BRF

Our results indicate that TFIIB is subject to repression by RB, but do not determine which of its subunits is targeted. The fact that repression is overcome using affinity-purified TFIIB TAFs but not TBP alone suggests that RB targets one or more of the pol III TAFs. However, the possibility remains that an unidentified intermediate component is required for the association between RB and TFIIB. Proof of direct binding will require interaction studies with purified and separated subunits of TFIIB. Such analyses are hampered at present by the uncertainty concerning the precise composition of human TFIIB.

Much of the function of RB is carried out by a region called the 'pocket' that stretches between residues 379 and 792 (Weinberg, 1995; Whyte, 1995). The pocket is composed of two essential regions, A and B, that are separated by a spacer, the sequence of which is not required for function (Weinberg, 1995; Whyte, 1995). Disruption of either the A domain or the B domain can prevent RB from repressing pol III (White *et al.*, 1996). It has been observed previously that the A domain of the RB pocket bears 21% identity and 38% similarity to a 151 amino acid region from the C-terminus of TBP (Hagemeier *et al.*, 1993). The C-terminal domain of TBP that encompasses this homology is sufficient to support pol III transcription (White and Jackson, 1992). We found

Fig. 10. Model proposing that RB may bind and inactivate TFIIB by mimicking TBP and BRF.

that an extended 230 amino acid region of BRF (residues 96–326) displays 15% identity and 33% similarity to the B domain and C-terminal region (residues 665–910) of RB (Figure 9). This homology was assessed using very stringent criteria for similarity (R/K, E/D, S/T, F/Y and L/I/V/M). Thus, RB bears homology to two of the subunits of the TFIIB complex that it is able to regulate.

Because the level of homology between RB, TBP and BRF is relatively low, one cannot be certain that it is significant. However, we are struck by the fact that adjacent regions of RB bear a resemblance to two polypeptides that interact within TFIIB. This apparent resemblance could provide the key to the mechanism of repression. The adjacent homologous regions may allow RB to present a molecular surface that resembles TBP alongside BRF. This could enable it to associate with another TAF subunit through a similar set of interactions to those that occur in intact TFIIB. We therefore propose a model in which RB is able to bind TFIIB by mimicking the interaction surface that is presented by TBP alongside BRF (Figure 10). Clearly, this does not displace TBP and BRF, since our pull-down and immunoprecipitation assays have shown that these polypeptides remain associated with RB. Additional interactions between subunits may hold the inactive complex together in the presence of RB.

Regulation of TAF activity by RB may provide a common mechanism for the cell cycle control of transcription by pols II and III

We have presented evidence that RB is able to regulate pol III transcription by interacting with the TFIIB complex. This finding is reminiscent of recent data which show that RB can bind to the TAF_{II}250 subunit of the pol II TFIID complex (Shao *et al.*, 1995). Thus, association with class-specific TAFs appears to provide one mechanism by which RB is able to regulate gene expression. In contrast to the situation with TFIID and TFIIB, we detected no RB in fractions containing the pol I-specific TBP-containing complex SL1 (Figure 6A). Instead, RB is reported to control pol I transcription by binding to the class-specific regulator UBF (Cavanaugh *et al.*, 1995).

Both genetic and biochemical data have implicated TAF_{II}250 in regulating the passage through G₁ phase (Sekiguchi *et al.*, 1988, 1991; Hisatake *et al.*, 1993; Ruppert *et al.*, 1993; Wang and Tjian, 1994). It remains to be determined to what extent RB is involved in this effect. We have shown previously that the activity of a TAF component of TFIIB increases significantly when cells progress from early G₁ into S phase (White *et al.*, 1995b). Since RB represses TFIIB and becomes in-

activated at the G₁/S transition, it seems likely that the increase in pol III transcription that accompanies progression into S phase may reflect, at least in part, the release of TFIIB from inhibition by RB. We are currently investigating this possibility. The existing data suggest that a common mechanism for achieving cell cycle control of transcription by pols II and III may involve the regulation of TAF activity by RB.

Materials and methods

Plasmids

The plasmids used in this work have all been described previously (White *et al.*, 1989, 1995a).

Protein fractions

Nuclear extracts were prepared from proliferating HeLa cells by the method of Dignam *et al.* (1983). The extracts were then chromatographed on phosphocellulose according to the method of Segall *et al.* (1980). Direct assay confirmed that PC-B fractions (0.1–0.35 M KCl step) contained TFIIB and pol III, PC-C fractions (0.35–0.6 M KCl step) contained TFIIC and pol III, and PC-D fractions (0.6–1.0 M KCl step) contained SL1.

The 0.38M-TFIIB and 0.48M-TFIIB fractions were prepared from PC-B by Mono Q gradient chromatography, as described by Lobo *et al.* (1992). Fractions were dialysed into LDB buffer (20 mM HEPES-KOH, pH 7.9, 17% glycerol, 100 mM KCl, 12 mM MgCl₂, 0.1 mM EDTA, 2 mM DTT). The 0.38M-TFIIB fraction eluted at ~340 mM KCl and had a protein concentration of 0.45 mg/ml. The 0.48M-TFIIB fraction eluted at ~450 mM KCl and had a protein concentration of 0.11 mg/ml. Western immunoblotting analysis revealed that TBP and BRF are present in the 0.38M-TFIIB but not in the 0.48M-TFIIB (Mital *et al.*, 1996). The A25(0.15) and A25(1.0) fractions were prepared by chromatography of PC-B fractions on DEAE-Sephadex, as previously (White *et al.*, 1995a). A25(0.15) is a 50–150 mM (NH₄)₂SO₄ step fraction; it contains TFIIB and has a protein concentration of 0.08 mg/ml. A25(1.0) is a 0.15–1.0 M (NH₄)₂SO₄ step fraction; it contains pol III and has a protein concentration of 0.015 mg/ml. For glycerol gradient fractionation of TFIIB, PC-B was sedimented for 24 h at 39 000 r.p.m. in an SW40Ti rotor on a 15–50% glycerol gradient in 50 mM Tris-HCl, pH 7.9, 200 mM KCl, 0.5 mM EDTA, 12.5 mM MgCl₂, 1 mM DTT, 0.1 mM PMSF. Fractions were then dialysed into LDB. For heparin gradient chromatography, PC-B was applied to heparin-Sepharose CL-6B in PC buffer (20 mM HEPES-KOH, pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT) containing 100 mM KCl. After extensive washing, the bound protein was eluted using a linear gradient up to 1.2 M KCl. Fractions were dialysed into LDB. Recombinant TBP, affinity-purified TFIIB TAFs (B-TAF), and the corresponding control fraction (CON) were prepared as previously (White *et al.*, 1995a).

The CHep-1.0 fraction was prepared by chromatography of PC-C on heparin-Sepharose CL-6B, as previously (White *et al.*, 1995a). CHep-1.0 contained TFIIC and pol III, and had a protein concentration of 0.6 mg/ml. Affinity-purified TFIIC was prepared as previously (White *et al.*, 1995a) and had a protein concentration of 0.093 mg/ml.

The SS700 fraction was prepared by chromatography of PC-D fractions on S-Sepharose. PC-D was applied to S-Sepharose in AM buffer (20 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 20% glycerol, 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF) plus 100 mM KCl. After washing with this buffer, the column was eluted with AM buffer plus 320 mM KCl to generate the SS320 fraction, and then with AM buffer plus 700 mM KCl to generate the SS700 fraction. Peak fractions were dialysed into LDB. The SS700 fraction contained SL1 and had a protein concentration of 0.26 mg/ml.

Recombinant GST and GST-RB fusion proteins were prepared as previously (White *et al.*, 1996). Significant batch variations were noted in the specific activities of different GST-RB preparations. The reason for this is unknown.

Transcription and band shift assays

Transcription and band shift assays were carried out as described previously (White *et al.*, 1989), except that transcription was for 1 h at 30°C and band-shifts were run in 0.25× TBE. The B-block and MSV oligonucleotides have been described previously (White *et al.*, 1989). Transcription reactions that were reconstituted using fractionated

factors contained 1 µl each of 0.38M-TFIIB (0.45 µg) and 0.48M-TFIIB (0.11 µg) and 2 µl of CHep-1.0 (1.2 µg) fractions.

Pull-down assays

PC-C or PC-B fractions (25 µl) were incubated at 4°C on an orbital shaker with 25 µl of glutathione-agarose beads containing equivalent amounts (as estimated by Coomassie blue staining) of immobilized GST or GST-RB (379–928). After 10 min, the beads were pelleted and 4 µl aliquots of supernatant were removed for transcription assays. The remainder of the samples were incubated at 4°C on the orbital shaker for a further 3 h. Samples were then pelleted, supernatants removed and the beads were washed five times with 700 µl of LDB. The bound material was analysed by Western blotting.

Western blotting

Western immunoblotting was carried out as previously (White *et al.*, 1995a). The antibodies used for immunoblotting were G3-245 (Pharminogen) against RB, SL30 against TBP and α-CSH409 against BRF.

Immunoprecipitation

PC-B fractions (50 µl) were incubated at 4°C for 6 h on an orbital shaker with 25 µl of protein A-Sepharose beads containing prebound antibodies. Samples were then pelleted, supernatants removed and the beads were washed four times with 700 µl of LDB and once with 700 µl of LDB containing 200 mM KCl and 0.1% NP-40. The bound material was analysed by Western blotting. The antibodies used for immunoprecipitation were G99-549 (Pharminogen) against RB, MTBP-6 against TBP and IC6 against Sp1.

Primary embryonic fibroblasts

Early passage primary cells from Rb^{-/-} and Rb^{+/+} mice (Jacks *et al.*, 1992) were grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum. Whole-cell extracts were prepared as previously (White *et al.*, 1995a).

Acknowledgements

We are grateful to James Millner-White for advice concerning sequence homology, Bart Williams and George Mulligan for primary embryonic fibroblasts, Sibylle Mittnacht for Sf9-expressed RB, Jane Flint for antibody MTBP-6 and Nouria Hernandez for antibodies SL30 and α-CSH409. This work was supported by the following grants: SP2314/0101 to R.J.W. and SP2143/0101 to S.P.J. from the Cancer Research Campaign, CO5766 to R.J.W. from the Biotechnology and Biological Sciences Research Council, SCI/180/94/365/G to R.J.W. from the Nuffield Foundation and NIH R01GM38810 to Nouria Hernandez. R.J.W. is a Jenner Fellow of the Lister Institute of Preventive Medicine.

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Received on July 5, 1996; revised on January 7, 1997