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Adenovirus E1a prevents the retinoblastoma gene product from repressing the activity of a cellular transcription factor

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The retinoblastoma (Rb) gene product forms a complex with the cellular transcription factor DRTF1, a property assumed to be important for mediating negative growth control because certain viral oncogenes, such as adenovirus E1a, prevent this interaction and mutant Rb alleles, which have lost the capacity to regulate growth, encode proteins that fail to associate with DRTF1. In this study, we show that the wild-type Rb protein can specifically repress transcription from promoters driven by DRTF1 whereas a naturally occurring mutant Rb protein cannot. Furthermore, Rb-mediated transcriptional repression can be overridden by adenovirus E1a; this requires regions in E1a necessary for cellular transformation. The Rb protein therefore acts *in trans* to repress the transcriptional activity of DRTF1 whereas adenovirus E1a prevents this interaction and thus maintains DRTF1 in a constitutively active state. The Rb protein and adenovirus E1a therefore have opposite effects on the activity of a common molecular target. Transcriptional repression mediated by the Rb protein and inactivation of repression by the E1a protein are likely to play an important role in mediating their biological effects.

Key words: E1a/repression/retinoblastoma gene product/transcription

Introduction

The retinoblastoma (Rb) gene product negatively regulates cellular proliferation by controlling progression through the G_1 phase of the cell cycle (Goodrich *et al.*, 1991). This effect is believed to be mediated by un- or under phosphorylated Rb protein which predominates during G_1 (Buchkovich *et al.*, 1989; DeCaprio *et al.*, 1989). Certain viral oncogenes, such as adenovirus E1a and SV40 large T antigen, sequester the Rb protein through regions which are necessary for cellular transformation and thus for overcoming normal growth control (DeCaprio *et al.*, 1988; Whyte *et al.*, 1988; Dyson *et al.*, 1989) and, furthermore, the Rb gene is frequently mutated in tumour cells (Hu *et al.*, 1990; Huang *et al.*, 1990). It is, therefore, the generally held view that both sequestration by viral oncogenes and inactivation by mutation overcome the negative regulation that is normally imposed on the cell cycle by the wild-type Rb protein.

Recently, a number of potential targets for the Rb protein have been defined (Bagchi *et al.*, 1991; Bandara and La Thangue, 1991; Chellapan *et al.*, 1991; Chittenden *et al.*, 1991; Defeo-Jones *et al.*, 1991). A particularly good candidate is the cellular transcription factor DRTF1, initially defined in F9 embryonal carcinoma (EC) stem cells as a differentiation-regulated transcription factor (La Thangue and Rigby, 1987), which binds to a DNA sequence (the E2F binding site; La Thangue *et al.*, 1990) which occurs in the transcriptional control regions of a number of cellular genes that encode proteins involved in cell cycle progression (Blake and Azizkhan, 1989; Pearson *et al.*, 1991). Importantly, the Rb protein forms a stable complex with DRTF1 which can be dissociated *in vitro* by viral oncogenes that bind the Rb protein (Bandara and La Thangue, 1991). This releases transcriptionally active DRTF1 which, presumably, is then able to activate genes and thus contribute to the uncontrolled proliferation characteristic of cellular transformation. Moreover, the protein products of all naturally occurring mutant Rb alleles so far studied fail to bind stably to DRTF1 (Bandara *et al.*, 1991; L.R.Bandara and N.B.La Thangue, unpublished data), further underscoring the potential importance of the Rb protein–DRTF1 interaction in regulating normal cell cycle events. These observations, together with others (Chellapan *et al.*, 1991; Chittenden *et al.*, 1991), suggest that the Rb protein is a transcription factor. However, it differs from many other conventional transcription factors because its DNA binding specificity is provided by an unrelated and independently acting transcription factor. It should be noted that the HeLa cell transcription factor E2F has similar properties to DRTF1 (Bagchi *et al.*, 1990; Chellapan *et al.*, 1991) although the exact relationship of DRTF1 to E2F remains to be determined.

In this study, we show that the wild-type Rb protein can specifically repress transcription of a promoter driven by DRTF1 whereas a naturally occurring mutant Rb protein that fails to bind to DRTF1 *in vitro*, does not affect transcription. Adenovirus E1a can override Rb-mediated transcriptional repression but a mutant E1a protein that does not bind the Rb protein cannot. The Rb protein is therefore a repressor of transcription, an effect mediated through its ability to form a complex with and modulate the activity of DRTF1. Transcriptional repression exerted by the Rb protein and inactivation of this repression by E1a are likely to be important in regulating cellular proliferation.

Results

The DRTF1 binding site functions as an upstream activating sequence in F9 EC stem cells

In F9 EC cells, which have a cellular E1a-like activity (Imperiale *et al.*, 1984), DRTF1 exists for the most part in the uncomplexed form. DRTF1, which binds efficiently to the distal E2F motif in the adenovirus E2A promoter, is down-regulated as F9 EC stem cells differentiate to parietal endoderm-like cells (F9 PE) (La Thangue *et al.*, 1990). To determine if this motif, and hence DRTF1, activates

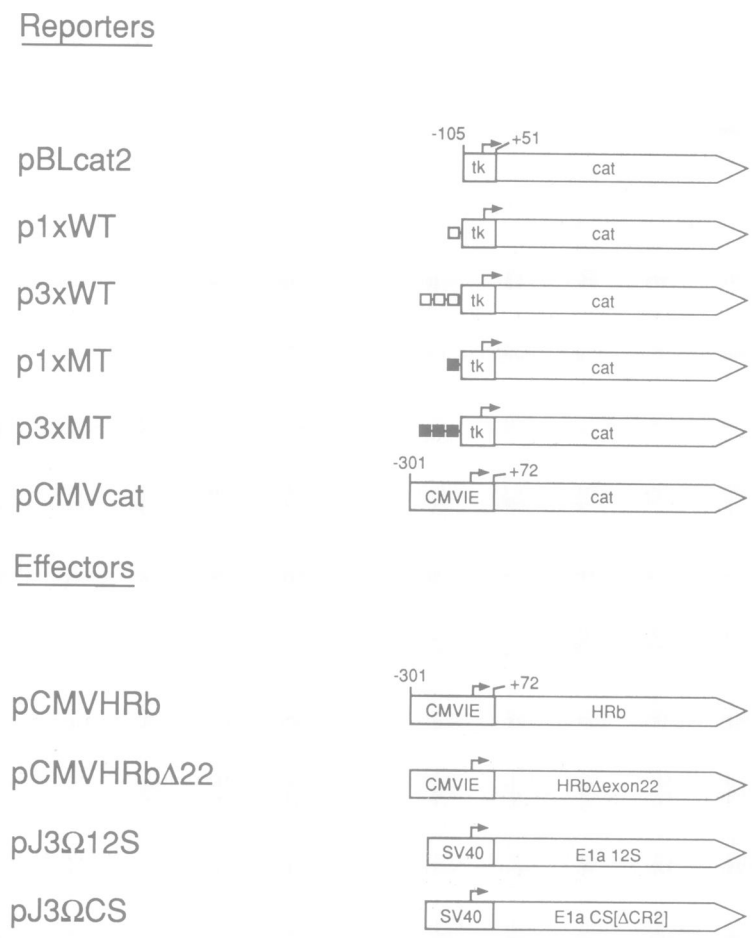

Fig. 1. Schematic representation of constructs. Open and solid boxes denote wild-type and mutant DRTF1 binding sites, respectively.

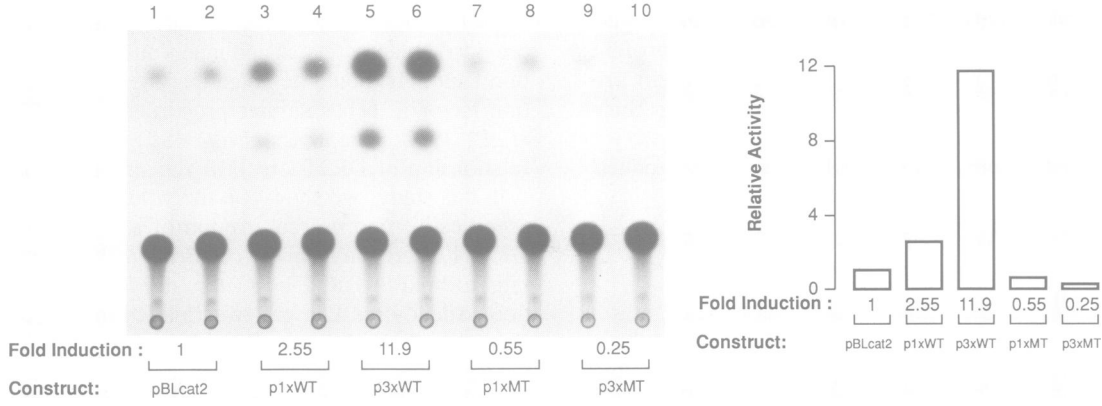

Fig. 2. DRTF1 is an upstream activator in F9 EC cells. The indicated reporter constructs (20 μg) were transfected into F9 EC cells. CAT activities are shown as fold induction relative to the minimal promoter construct pBLcat2. The values are the mean of four determinations from two separate experiments. A relative activity of 1 denotes 2.3% acetylation.

transcription *in vivo*, we prepared a panel of constructs in which transcription from the minimal herpes simplex virus thymidine kinase (tk) promoter in the context of the reporter construct pBLcat2 (Luckow and Schütz, 1987) was driven by either one or three wild-type E2F motifs positioned immediately upstream of the tk sequences (Figure 1; p1×WT and 3×WT, respectively); two control templates containing either one or three mutant binding sites (Figure 1; p1×MT and p3×MT respectively) were also prepared. The wild-type DRTF1 binding site contained the distal E2F site in E2A, encompassing nucleotides −71 to −50, a site that has previously been shown to be a high affinity site for DRTF1 (La Thangue *et al.*, 1990). A sequence where nucleotides −62 to −60 (CGC), which are critical for binding DRTF1 (La Thangue *et al.*, 1990) are altered served as a negative control in p1×MT and p3×MT.

Both p1×WT and p3×WT had greater transcriptional activity relative to pBLcat2 (Figure 2; 2.5- and 12-fold, respectively) and the control reporters p1×MT and p3×MT (Figure 2; 4.6-fold, p1×WT relative to p1×MT; 48-fold, p3×WT relative to p3×MT). This E2F motif, therefore, functions as an upstream activating sequence in F9 EC stem cells indicating that DRTF1 activates transcription *in vivo*. Together with our earlier data we conclude that *in vivo*, uncomplexed DRTF1 is a positively acting sequence specific transcription factor.

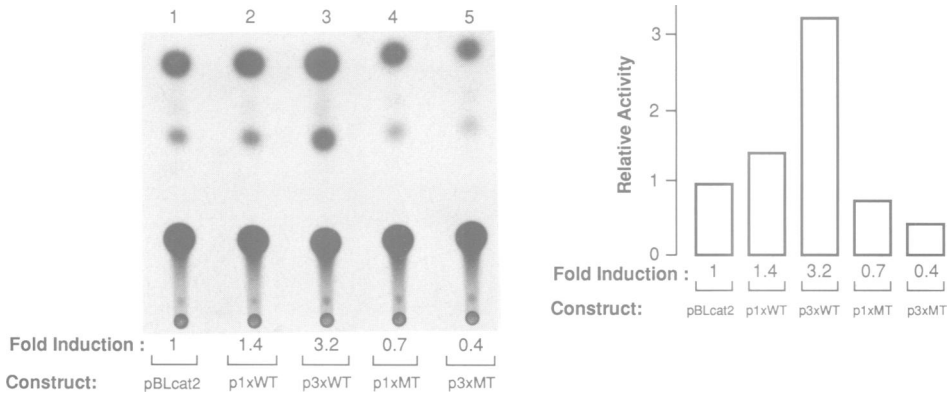

Fig. 3. DRTF1 is an upstream activator in SAOS-2 cells. SAOS-2 cells were transfected with 20 μg of the indicated reporter constructs. All CAT activities are expressed relative to pBLcat2. The values are the mean of two separate experiments. A relative activity of 1 denotes 5.3% acetylation.

The Rb gene product represses the transcriptional activity of DRTF1

Because the Rb gene product binds both *in vitro* and *in vivo* to DRTF1 (Bandara and La Thangue, 1991; Bandara *et al.*, 1991) and since it is known to negatively regulate cellular proliferation, we reasoned that one potential mechanism to explain its biological properties may be an ability to repress the activity of cellular transcription factors such as DRTF1. In order to test this hypothesis we assayed the effect of co-expressing the wild-type Rb gene product with the DRTF1-dependent reporter p3×WT. We did, however, choose to perform this experiment in a different cell type for two reasons. Firstly, F9 EC stem cells have an endogenous cellular E1a-like activity (Imperiale *et al.*, 1984; La Thangue and Rigby, 1987) which may inactivate the co-expressed Rb protein in a manner analogous to viral E1a and, secondly, they synthesize wild-type Rb protein (data not shown) which could obscure the activity of the co-expressed Rb protein. For these reasons we used SAOS-2 cells (Shew *et al.*, 1990) which, importantly, contain a naturally occurring mutant Rb allele that encodes a protein, with a large C-terminal truncation, localized in the cytoplasm. This truncated Rb protein fails to bind to adenovirus E1a (Hu *et al.*, 1990) and DRTF1 (see later) and was thus unlikely to interfere with the activity of the co-expressed Rb protein.

Both p1×WT and p3×WT had greater transcriptional activity than p1×MT and p3×MT (Figure 3; 2-fold, p1×WT relative to p1×MT; 8-fold, p3×WT relative to p3×MT) indicating that a DRTF1 binding site can act as an upstream activating sequence in SAOS-2 cells. We next assessed the effect of the wild-type Rb protein supplied *in trans* on the transcriptional activity of p3×WT by co-transfecting an expression vector in which the wild-type Rb coding sequence is driven by the CMV enhancer and promoter (pCMVHRb; Figure 1); expression of the transfected Rb gene product was confirmed using an antibody that reacts only with the wild-type protein in SAOS-2 cells (data not shown). The wild-type Rb protein significantly reduced the activity of p3×WT to a level that was similar to the constitutive activity of p3×MT (Figure 4; compare tracks 3 with 4, 5-fold reduction) indicating that the wild-type Rb protein inactivates the upstream activation in p3×WT.

The specificity of this effect was assessed in two ways. First, we asked if the wild-type Rb protein could affect the activity of the control reporter constructs that contain mutant DRTF1 binding sites. The basal activity of p3×MT, which contains three copies of the mutant DRTF1 binding site (Figure 1), was not significantly affected when co-transfected with pCMVHRb (Figure 4; compare tracks 6 with 7). Next, we assessed the effect of the wild-type Rb protein in pCMVcat in which transcription is driven by the constitutively strong CMV enhancer and promoter. Likewise, co-transfection of pCMVHRb failed to affect significantly the activity of pCMVcat (Figure 4; compare tracks 9 with 10). We conclude, therefore, that the ability of the Rb protein to inactivate transcription is specific and mediated through wild-type E2F sites by repressing the activity of DRTF1.

Secondly, we studied the effect of co-expressing a mutant Rb protein. The mutant RbΔ22 protein is encoded by a naturally occurring mutant Rb allele that lacks the amino acid coding information present in exon 22 (Hu *et al.*, 1990) which, we show later in this study, fails to bind to DRTF1 in SAOS-2 cells. Thus, if the Rb protein has to bind to DRTF1 in order to effect transcriptional repression then co-expression of RbΔ22 should have little or no effect. This prediction was confirmed by providing RbΔ22 *in trans* by co-transfecting pCMVHRbΔ22 with p3×WT, when the activity of p3×WT was not significantly affected (Figure 4; compare tracks 3 with 4); again, the synthesis of the RbΔ22 protein was confirmed with a monoclonal antibody (data not shown).

We conclude from these studies that the Rb protein acts *in trans* to repress the transcriptional activity of DRTF1 and thus prevents transcriptional activation. The Rb protein is therefore a transcriptional repressor, an effect mediated by its ability to modulate the activity of DRTF1.

Adenovirus E1a overcomes Rb-mediated transcriptional repression

The Rb protein is sequestered from DRTF1 by the adenovirus E1a protein and we have previously suggested that this leads to an increase in the transcriptional competence of DRTF1 (Bandara and La Thangue, 1991). We tested this idea by studying the effect of E1a 12S on the transcriptional activity of Rb-repressed DRTF1 in SAOS-2 cells. First, we assessed the effect of E1a 12S alone on the activity of p3×WT. On its own, E1a 12S increased the activity of p3×WT 3-fold (Figure 5; compare tracks 3 and 4 with 1

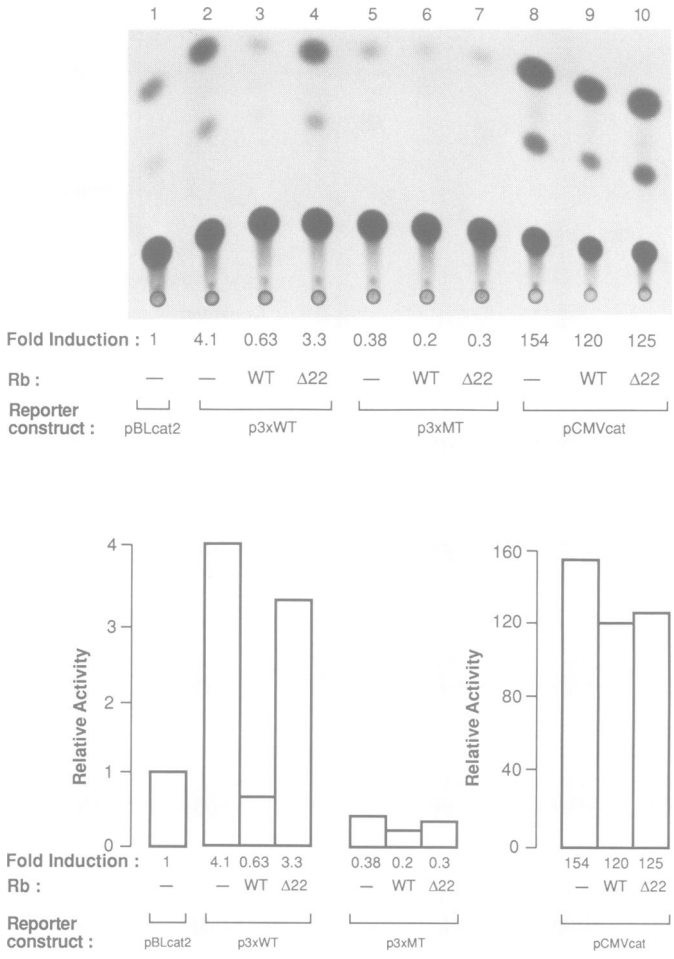

Fig. 4. The Rb gene product represses *trans*-activation by DRTF1. The Rb expression vectors were co-transfected with the indicated reporter constructs into SAOS-2 cells. The amounts of the reporter constructs used were as follows: 5 μg of pBLcat2, p3×WT and p3×MT, and 0.5 μg of pCMVcat. The level of the Rb expression vectors was at 0.6 M excess over the reporter constructs. The amount of extract used in the assay was adjusted to obtain a signal in the linear range although the final values shown in the bar chart relate to the total activity. Values are the mean of at least two determinations from different experiments. A relative activity of 1 denotes 1.5% acetylation. Note that the wild-type but not the mutant Rb protein represses the transcriptional activity of DRTF1.

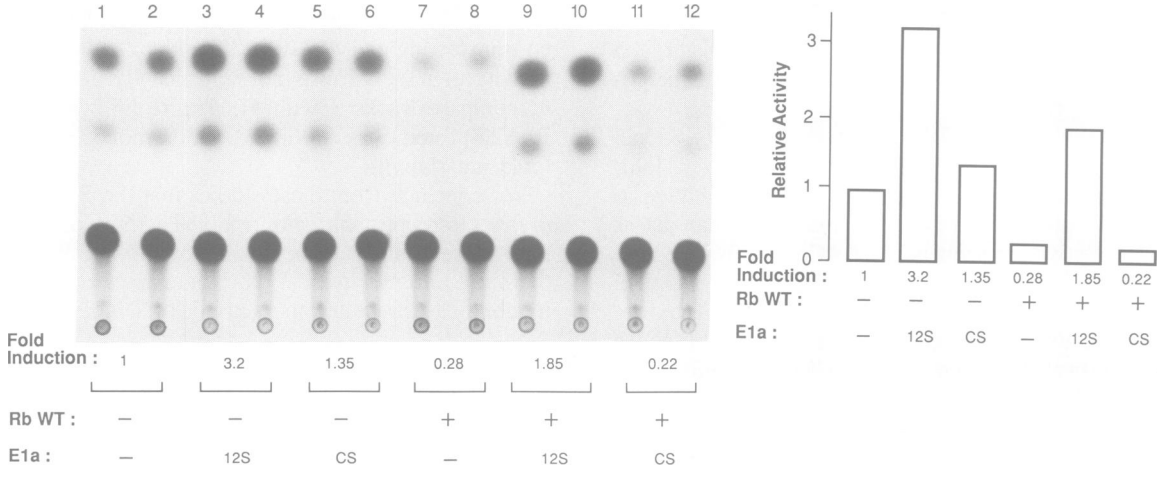

Fig. 5. Adenovirus E1a overcomes Rb-mediated transcriptional repression. SAOS-2 cells were transfected with p3×WT (5 μg) alone or together with the indicated expression vectors. The Rb and E1a expression vectors were at 0.25 and 1.2 M excess over p3×WT, respectively. CAT activities were corrected for transfection efficiency and expressed relative to p3×WT. Values shown are the mean of two separate experiments. A relative activity of 1 denotes 7.6% acetylation. Note that the wild-type but not mutant E1a overcomes the repression mediated by the Rb protein.

and 2) whereas an E1a mutant lacking conserved region 2, CS (which lacks amino acid residues 120−133), which fails to bind several cellular proteins including the Rb protein (Bandara and La Thangue, 1991), had no significant effect (Figure 5; compare tracks 5 and 6 with 1 and 2). Although the mutant Rb protein synthesized in SAOS-2 cells cannot bind to DRTF1 (see later), the Rb-related protein p107 can (Cao *et al.*, 1992; Shirodkar *et al.*, 1992; L.R.Bandara, J.P.Adamczewski, T.Hunt and N.B.La Thangue, submitted). It is likely, therefore, that the activation mediated by E1a 12S in SAOS-2 cells is mediated by sequestration of p107 from DRTF1, thus increasing the levels of transcriptionally active protein. This idea is consistent with the lack of effect by E1a CS (Figure 5; compare tracks 1 and 2 with 5 and 6), which cannot bind p107 (Whyte *et al.*, 1989).

As with the Rb protein-mediated repression, transcriptional activation by E1a 12S was specifically mediated through DRTF1 since the wild-type reporter p3×WT, but not the control mutant reporter p3×MT, was induced by E1a 12S (Figure 5; compare tracks 1 and 2 with 3 and 4 and Figure 6; compare tracks 3 and 6). Transcriptional repression by the Rb protein and activation by E1a, therefore, occur through the same motif.

We next asked if the E1a 12S protein could overcome the Rb-mediated repression of DRTF1 and thus co-transfected both pCMVHRb and pJ3Ω12S with p3×WT. Alone, the Rb protein repressed p3×WT (Figure 5; compare tracks 1 and 2 with 7 and 8). Indeed, the E1a 12S protein was able to overcome this repression because when it was co-expressed with the Rb protein, there was a significant increase in the activity of p3×WT (Figure 5; compare tracks 7 and 8 with 9 and 10). In contrast, CS, which cannot bind the Rb protein, failed to alleviate the repression (Figure 5; compare tracks 9 and 10 with 11 and 12). These data indicate that the E1a protein is able to overcome the repression imposed on DRTF1 by the Rb protein and that sequences in E1a necessary for Rb binding are required for this effect.

Although E1a could overcome this transcriptional repression, co-expression of the Rb protein with E1a decreased the level of E1a-dependent activation (3.2- to 1.85-fold induction; Figure 5). One possible explanation for this effect is that there was not enough E1a protein available to affect all the DRTF1 complexes that were dissociated in the absence of co-expressed Rb protein and thus some DRTF1 could remain transcriptionally inactive. We tested this idea by determining if *trans*-activation of p3×WT by E1a 12S was influenced by the level of Rb protein. For this, increasing amounts of the Rb expression vector were co-transfected into the cells together with a constant amount of the E1a 12S expression vector. As expected, in the absence of the Rb protein, E1a 12S activated p3×WT efficiently (Figure 7; compare tracks 2 with 3) and in the absence of E1a 12S the Rb protein efficiently repressed (Figure 7; compare track 2 with 7). However, as the amount of Rb protein was increased there was a concommitant decrease in *trans*-activation of p3×WT by E1a 12S. We believe that the most likely explanation for this is that the amount of active E1a protein becomes limiting as the concentration of the Rb protein increases, and thus more DRTF1 remains complexed and therefore transcriptionally inactive.

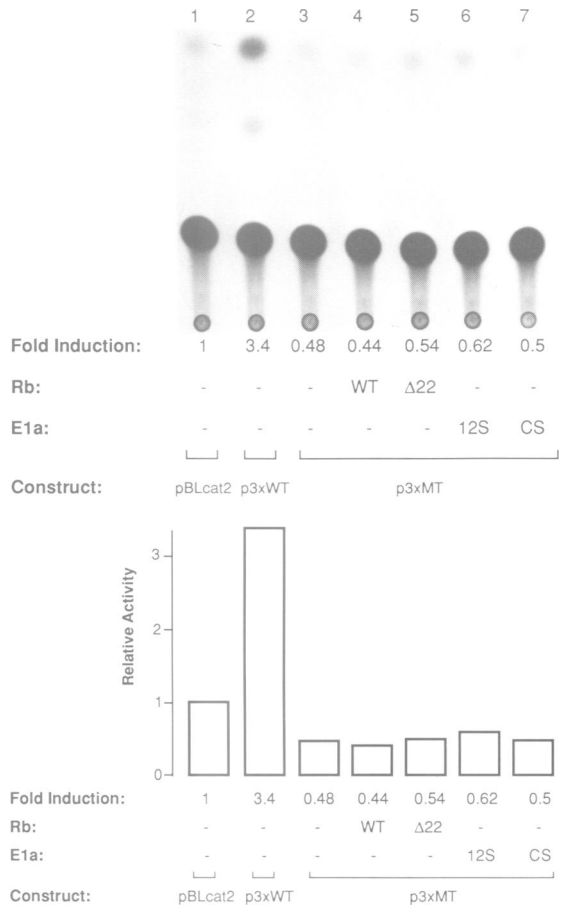

Fig. 6. Adenovirus E1a 12S activates through a wild-type DRTF1 binding site. SAOS-2 cells were transfected with 5 μg of the reporter constructs shown. Where indicated, the Rb and E1a expression vectors were at 0.6 and 1.2 M excess, respectively, over the reporter construct. The values shown are the mean of two separate experiments. A relative activity of 1 denotes 0.5% acetylation. Note that wild-type E1a has negligible effect on the mutant construct.

The wild-type Rb protein but not Δ22 binds to DRTF1 in SAOS-2 cells

We confirmed that the wild-type Rb protein was able to bind to DRTF1 in SAOS-2 cells by studying their binding properties in gel retardation assays. Typically, DRTF1 exists as three types of complexes, referred to as DRTF1a,b and c (La Thangue *et al.*, 1990) and previously we have shown that affinity purified DRTF1b and c are transcriptionally active *in vitro* (Shivji and La Thangue, 1991). In SAOS-2 cell extracts, DRTF1 complexes could be detected (Figure 8; track 2) and were shown to be specific by competing with either wild-type (Figure 8; tracks 3 and 4) or mutant (Figure 8; track 5) binding sites (exactly the same motifs that were incorporated into p3×WT and p3×MT constructs, respectively); however, the distinction between DRTF1b and c was not clear and they will therefore be collectively referred to as DRTF1b. The complexed form of DRTF1, DRTF1a, was clearly resolved in SAOS-2 cell extracts, which, as we discussed earlier and confirm in this experiment, did not contain the Rb protein. These cells express a mutant Rb allele lacking exons 21−27 (Bártek

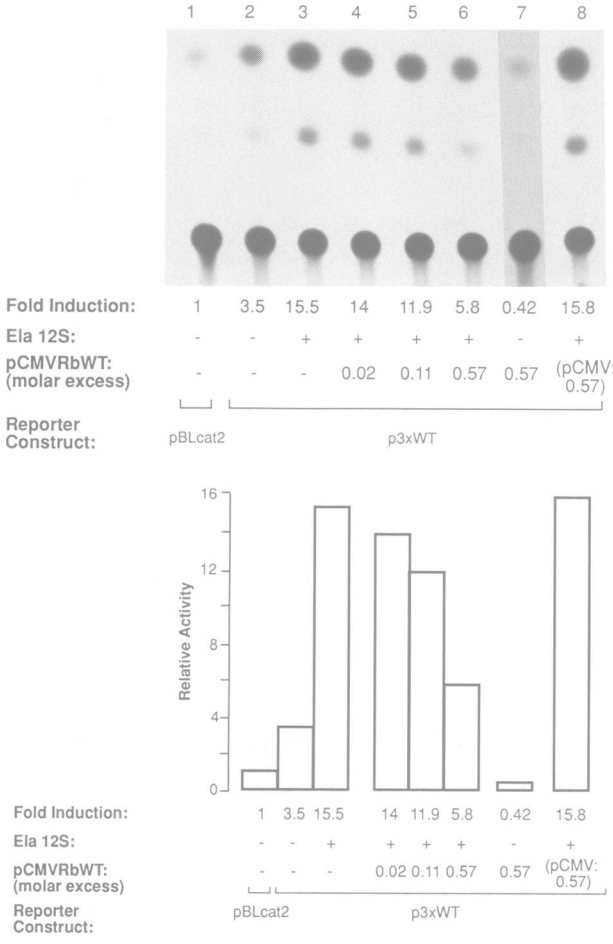

Fig. 7. The Rb protein prevents activation by E1a 12S. SAOS-2 cells were transfected with 5 μg of reporter constructs. Where indicated, 0.6 M excess of the E1a 12S expression vector was co-transfected. The amount of the Rb expression vector is indicated. The expression vector pCMV contained an unrelated coding sequence and served as a control for promoter competition at the highest concentration of Rb expression vector. The values shown are the mean of two separate experiments. A relative activity of 1 denotes 1% acetylation. All data shown in this figure were obtained from the same experiment.

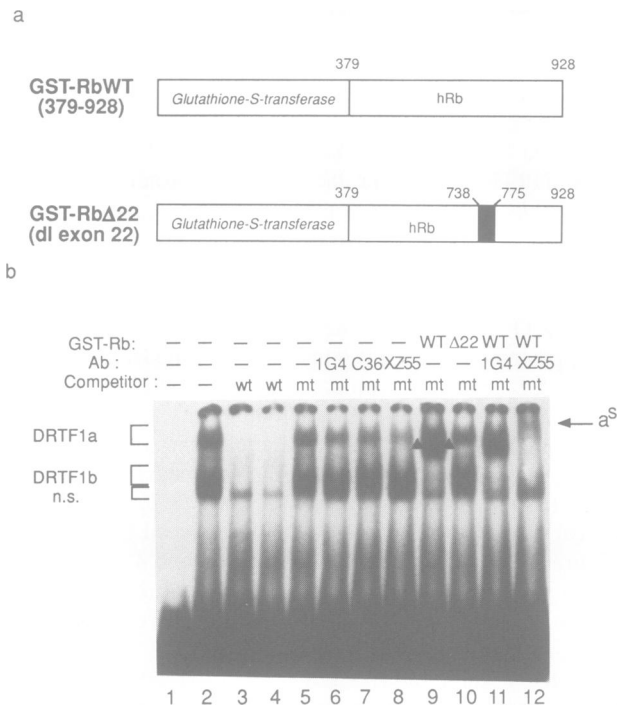

Fig. 8. The Rb mutant cannot bind to DRTF1 in SAOS-2 cells. (**a**) Diagram of GST-fusion proteins. The solid box denotes a deletion of exon 22. (**b**) *In vitro* reconstitution of DRTF1a in SAOS-2 cells. Whole cell extracts were assessed for DNA binding activities by gel retardation using a wild-type DRTF1 binding site. The specificity of the binding activities was confirmed by competing with the wild-type (33 and 66 M excess; tracks 3 and 4, respectively) or the mutant (200 M excess, track 5) DRTF1 binding sites. Monoclonal antibodies C36 and XZ55 react with different regions of the Rb protein and have the same isotype as the control antibody IG4. Tracks 9 and 10 have equivalent amounts of the wild-type and the mutant Rb fusion proteins (estimated by gel electrophoresis). The Rb-induced complex in tracks 9 and 11 is indicated by ▲. The super-shifted DRTF1a complex in track 12 is indicated by a^s. n.s. denotes a non-specific complex. The probe alone is shown in track 1.

et al., 1992) but containing the C36 epitope (Whyte *et al.*, 1988). Since C36 did not have any effect on this DRTF1a complex (Figure 8, compare track 7 with 6) we conclude that the mutant Rb protein made in SAOS-2 cells is not in this complex. The SAOS-2 DRTF1a complex may contain the Rb-related protein, p107, which is expressed in SAOS-2 cells (Cao *et al.*, 1992; Shirodkar *et al.*, 1992) and can bind to DRTFl (L.R.Bandara, J.P.Adamczewski, T.Hunt and N.B.La Thangue, submitted). The wild-type and RbΔ22 coding sequences assayed *in vivo* were expressed as GST fusion proteins containing amino acid residues 379–928 (GST–Rb and GST–RbΔ22), affinity purified (see Materials and methods) and added to the SAOS-2 cell extract. The wild-type fusion protein, GST–Rb, but not the mutant GST–RbΔ22, efficiently assembled with free DRTF1b causing a slower migrating complex (Figure 8, compare track 9 with 10; GST–Rb induced complex indicated by▲. That the GST–Rb induced complex did in fact contain the added GST fusion protein was confirmed by assaying the effect of a monoclonal antibody, XZ55, that recognizes an epitope within GST–Rb (Hu *et al.*, 1991). Antibody XZ55, but not a control antibody, caused a slower migrating complex indicating that GST–Rb was present in the induced complex (Figure 8, compare track 11 with 12). We conclude from this experiment that the coding information in exon 22 is necessary for complex formation between the Rb protein and DRTF1.

The combined conclusion from these data is that by forming a complex with DRTF1, the Rb protein is able to repress its transcriptional activity. Adenovirus E1a protein overcomes this by sequestering the Rb protein, yielding the free and transcriptionally active transcription factor. Adenovirus E1a and the Rb protein, therefore, have opposite effects on the activity of a common cellular target.

Discussion

The Rb gene product represses the transcriptional activity of DRTF1

Several important conclusions can be drawn from the data presented in this study. First, the wild-type Rb protein represses transcription, an effect mediated through its ability to complex with and hence modulate the activity of DRTF1. Secondly, a mutant Rb protein that fails to bind to DRTF1 cannot repress transcription. Thirdly, the adenovirus E1a protein overcomes transcriptional repression by sequestering and inactivating the Rb protein.

We believe that repression of DRTF1 by the Rb protein may help explain the well-documented negative growth regulating properties of the wild-type Rb protein. The un- or underphosphorylated form of the Rb protein is believed to negatively regulate cell cycle progression by controlling progression through G_1 into S phase (Goodrich *et al.*, 1991). Because DRTF1 binding sites occur in the transcriptional control regions of genes that encode proteins involved in cell cycle progression [some, for example DHFR and DNA polymerase α, are required for DNA synthesis and replication (Blake and Azizkhan, 1989; Pearson *et al.*, 1991)] it is possible that repression of these genes during G_1 would prevent cells beginning DNA synthesis and hence progressing through S phase. This mechanism could also account for the loss-of-function that has been assumed to occur upon mutation of the Rb gene in tumour cells, since these mutants are usually unable to bind to DRTF1 (Bandara *et al.*, 1991; and this study). Our analysis indicates that one such mutant encodes a protein that cannot repress transcription, and thus according to this model would be unable to limit the synthesis of important cell cycle molecules. This, we suggest, would help the proliferation rate of such cells to remain unrestrained.

Adenovirus E1a prevents transcriptional repression by the Rb protein

It was documented some time ago that the oncogenes of several DNA tumour viruses, such as adenovirus E1a, SV40 large T antigen and the HPV E7 protein, sequester the Rb protein through regions in these viral proteins that are necessary to transform tissue culture cells (Lillie *et al.*, 1987; DeCaprio *et al.*, 1988; Whyte *et al.*, 1988; Dyson *et al.*, 1989). It was reasoned, therefore, that this would inactivate the Rb protein and thus prevent it performing its normal cellular function. A molecular insight into a potential mechanism of action of these viral oncogenes was suggested when it was shown that the Rb protein is in a complex with DRTF1 from which it can be sequestered by the action of these oncogenes (Bandara and La Thangue, 1991; L.R.Bandara and N.B.La Thangue, unpublished data). The Rb protein was therefore predicted to exert its effects at the transcriptional level, and furthermore its activity was likely to be modulated by these viral oncogenes. We have now established that this is indeed the case because adenovirus E1a could override the repression imposed on DRTF1 by the wild-type Rb protein. Adenovirus E1a (and presumably large T antigen and E7) and the Rb protein therefore have opposite effects on DRTF1. The E1a protein is able to usurp the repression imposed on DRTF1 by sequestering the Rb protein and thus in effect converts DRTF1 from being a repressed to an active transcription factor. For the reasons discussed above, this process is likely to lead to the constitutive production of proteins that are required for cell cycle progression.

A mechanism for coupling cell cycle events to transcription

It is likely that complex formation between the Rb protein and DRTF1 is necessary for the transcriptional repression described here. A clear prediction would therefore be that this interaction is regulated during the cell cycle, and perhaps that it should occur predominantly during G_1 when the Rb protein is known to exert its biological effect. In support of this idea, the related transcription factor E2F is apparently subject to this type of behaviour during cell cycle progression (Chellapan *et al.*, 1991; Shirodkar *et al.*, 1992).

But how can cell cycle events be coupled to transcription? We believe that a potential mechanism for this lies in our observation that cyclin A can stably associate with the DRTF1 transcription factor complex (Bandara *et al.*, 1991; L.R.Bandara, J.P.Adamczewski, T.Hunt and N.B.La Thangue, submitted). Cyclin A binds to and regulates the activity of the mitotic kinase catalytic subunit $p34^{cdc2}$ (Draetta *et al.*, 1989; Minshull *et al.*, 1990) and the related kinase subunit $p33^{cdk2}$ (Pines and Hunter, 1990; Tsai *et al.*, 1991). Several lines of evidence now suggest that cyclin A has a role in enabling progression through S phase (Girard *et al.*, 1991) and that its association with $p33^{cdk2}$ is involved in this process (Fang and Newport, 1991). Indeed, $p33^{cdk2}$ assembles with the DRTF1 transcription factor complex in a cyclin A-dependent fashion (L.R.Bandara, J.P. Adamczewski, T.Hunt and N.B.La Thangue, submitted) thus defining a potential mechanism for coupling cell cycle events to transcription. Although we do not wish to imply that $p33^{cdk2}$ is the kinase catalytic subunit responsible for regulating the binding of the Rb protein to DRTF1, it is nevertheless possible that it or a related catalytic subunit does phosphorylate the Rb protein because the Rb protein shows a pattern of phosphorylation *in vivo* that is reminiscent of the activity of a *cdc2*-like kinase (Lees *et al.*, 1991; Lin *et al.*, 1991).

It is also possible that mechanisms that do not directly involve *cdc2*-like kinases regulate the Rb protein–DRFT1 interaction. For example, cellular molecules that act in a mechanistically analogous fashion to adenovirus E1a could conceivably exist in cells, as has previously been suggested to be the case in F9 EC cells (Imperiale *et al.*, 1984; La Thangue and Rigby, 1987). Whatever the nature of this mechanism, Rb protein-dependent transcriptional repression is likely to be of fundamental importance in controlling cell cycle events.

Materials and methods

Cell culture

F9 EC and SAOS-2 cells were maintained as adherent monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum. Cells were re-plated at a density of $\sim 5 \times 10^4$ per ml (F9 EC) or 1×10^5 per ml (SAOS-2) every 3 and 4 days, respectively.

Whole cell microextracts

SAOS-2 cells grown on 10 cm tissue culture plates to near confluency were washed with PBSA, scraped and resuspended in a maximum volume of 80 μl of extraction buffer (Schöler *et al.*, 1989). Following cell disruption by consecutive freeze–thawing (three times), extracts were cleared by centrifugation. Up to 4 μl of whole cell extract was used in gel retardation assays.

Plasmids

DRTF1-responsive reporter constructs were all derived from pBLcat2 (Luckow and Schütz, 1987) where the bacterial CAT gene expression is driven by the minimal HSV tk promoter (−105 to +51). Wild-type or mutant DRTF1 binding sites were cloned into the *Bam*HI site directly upstream of the tk promoter. The wild-type oligonucleotide was taken from the −71 to −50 region of the adenovirus E2A promoter and the mutant oligonucleotide is mutated in nucleotides −62 and −60 (La Thangue *et al.*, 1990). pCMVcat has previously been described (La Thangue and Rigby, 1988). Rb expression vectors were generated by replacing the coding sequences in pCMVcat with a *Bam*HI fragment comprising the wild-type human Rb cDNA from pJ3ΩHRb (Bernards *et al.*, 1989) or a mutant Rb cDNA deleted in exon 22 from pSVΔ22 (Hu *et al.*, 1990). Rb protein

expression was confirmed by immunostaining transfected cells with the monoclonal antibody IF8 (Bártek *et al.*, 1992). Wild-type (pJ3Ω12S) and mutant (pJ3ΩCS) E1a expression vectors were derived from Sp12S and SpCS, respectively (Bandara and La Thangue, 1991). A *Bgl*II linker was inserted at both ends of the cDNA and the latter was subcloned into the *Bam*HI site of pJ3ΩHRb replacing the Rb cDNA.

Transient transfection

Approximately 5–6 h prior to transfection, F9 and SAOS-2 cells were re-plated at a density of 1×10^6 or 2×10^6 per 10 cm tissue-culture dish, respectively. Cells were transfected by the calcium phosphate co-precipitate procedure of Gorman *et al.* (1982) and exposed to the precipitate for ~15 h. After transfection the cells were washed twice with DMEM, re-fed with fresh medium and harvested after a further 24 h. The amounts of each construct used per transfection are detailed in the figure legends. For each transfection, pBluescript SK was included to maintain a total of either 20 or 25 μg of DNA. All SAOS-2 transfections included an internal control (pCMV-βgal; Tassios and La Thangue, 1990) and extracts were assayed for CAT activity after normalizing for β-galactosidase activity. For F9 EC transfections, 50 μg protein extract was used. CAT activity was determined as previously described (Gorman *et al.*, 1982). TLC plates were scanned and quantified by a phosphorImager (Molecular Dynamics Ltd, UK).

Gel retardation assays

Gel retardation assays were performed as previously described (Bandara and La Thangue, 1991). Whole cell microextracts were assayed in a buffer containing 122 mM NaCl, in 50 mM Tris–HCl (pH 7.9), 0.2 mM EDTA, 1 mM DTT and 15% glycerol. The wild-type and the mutant binding sites were identical to those described for CAT constructs.

Antibodies and fusion proteins

Monoclonal antibodies used for *in vitro* studies have been described before (Bandara and La Thangue, 1991; Hu *et al.*, 1991). The purification of GST–Rb fusion proteins which contain human Rb coding sequences (residues 379–928), has been previously described (Bandara *et al.*, 1991). The purity and the relative concentration of these proteins were assessed by SDS–PAGE.

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