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The retinoblastoma protein binds E2F residues required for activation *in vivo* and TBP binding *in vitro*

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ABSTRACT

The retinoblastoma (RB) tumour suppressor protein is capable of repressing the activity of promoters containing DNA binding sites for the transcription factor E2F. Recently a protein which binds RB and possesses the DNA binding characteristics of E2F has been cloned. Here we show that the E2F activation domain is the target for RB-induced repression. RB can silence the 57 residue E2F activation domain but cannot effectively repress an E2F mutant which has reduced RB binding capacity. Extensive mutagenesis of E2F shows residues involved in RB binding are required for transcription activation. Mutations which affect both functions most dramatically lie within the minimal RB binding region. A further subset of sensitive residues lies within a new repeat motif E/DF XX L X P which flanks the minimum RB binding site. These data show that RB can mask E2F residues involved in the activation process, possibly by mimicking a component of the transcriptional machinery. Consistent with this model, we find that the TATA box binding protein TBP can bind to the E2F activation domain *in vitro* in a manner indistinguishable from that of RB.

INTRODUCTION

The retinoblastoma (RB) protein is a negative regulator of cell proliferation. Mutations within the RB gene have been found in a subset of human tumours and introduction of the RB gene into RB negative tumour cells results in growth arrest (for review see ref. 1). Oncogene products of DNA tumour viruses, such as the adenovirus E1A, the SV40 TAg and the papillomavirus E7 proteins, can bind to RB via domains required for cell transformation (2, 3, 4). This has led to the suggestion that disruption of normal RB function is a prerequisite to the transforming phenotype of these viral oncoproteins. Detailed structure/function analysis of RB has revealed that two non-contiguous regions (domain A and domain B) within the RB C-terminus are necessary for the binding of E1A and TAg (5, 6). These two domains (A and B), separated by a 'spacer' a sequence, define the RB 'pocket' a which represents the minimum binding site for these viral oncoproteins.

The precise role of RB in the cellular events leading to cell proliferation is still unclear but increasing evidence suggests that RB functions, at least partly, by modulating the function of transcription factors (for review see ref. 7). Sequences within the RB pocket have been shown to bind a number of cellular proteins (8). Although most of the ones that have been cloned are as yet uncharacterised (9, 10), several RB interacting proteins are transcription factors; these include E2F (11, 12), MyoD (13), Elf-1 (14), PU.1 (15), c-myc (16) and ATF-2 (17). So far three of these factors, E2F, MyoD and Elf-1 have been shown to interact with RB both *in vitro* and *in vivo*. A common feature of all of these interactions seem to be the requirement for some part of the RB pocket domain. The presence of sequence similarity to TBP and TFIIB in the RB pocket, may explain the ability of RB to bind several different regulatory transcription factors (15).

The interaction between RB and the transcription factor E2F is by far the most characterised. The first indication of such an association came from the revelation that RB could be found in a complex of proteins containing E2F DNA binding activity (18, 19, 20). The E2F recognition site [originally identified in the promoter of the adenovirus E2 gene (21)] can be found in the promoter of several cellular genes (for review see ref. 22). The ability of the E1A protein to stimulate transcription of promoters containing E2F sites (presumably by competing for RB binding) prompted the suggestion that RB is a repressor of E2F activity (23). In keeping with this hypothesis, RB can repress the activity of promoters containing E2F recognition sites (24, 25, 26).

A protein with properties of E2F has recently been cloned (11, 12). The E2F protein can bind to the E2F site and can contact the RB protein via a domain containing transcriptional activation functions. Here we show that interactions within the E2F activation domain are sufficient for RB-induced repression. Extensive structure/function analysis reveals that RB binds to residues which are required for transcription activation and that *in vitro* the TBP protein can bind the same residues. These data give rise to a model whereby RB represses E2F activity by

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mimicking the binding specificity of a protein required for transcriptional activation.

MATERIALS AND METHODS

Transient transfections

RB negative SAOS2 cells were maintained as monolayers in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% foetal calf serum. Cells were split 1:3 every three days. Approximately 5×10^6 cells were transfected with 4 μg chloramphenicol acetyltransferase (CAT) reporter constructs and 1 μg of effector DNA by calcium phosphate co-precipitation. Cells were harvested 30 h post transfection and extracts were used for CAT assays and Western blots. CAT assays were carried out as previously described (27). Western blots were probed with an antibody against the GAL4 binding domain (gift of M. Ptashne) to make sure each of the proteins was expressed at equivalent levels. Each transfection was repeated a minimum of three times.

GST-pull-down assay

Five hundred nanograms of the GST fusion proteins on beads were preincubated with bovine serum albumin (final concentration, 1 mg/ml) at room temperature (RT) for 5 min and then rocked for 1 h at RT with either 2−5 μl of *in vitro* translated test protein or 1−5 ng of ^{32}P-labelled *E. coli* expressed protein in 200 μl of Z′ buffer (25 mM Hepes, pH 7.5; 12.5 mM $MgCl_2$; 20% glycerol; 0.1% NP-40; 150 mM KCl; 20 μM $ZnSO_4$). The beads were then washed three times in 1.5 ml of NETN buffer (150 mM NaCl; 1 mM EDTA; 0.5% NP-40; 20 mM Tris-HCl, pH 8.0), pelleted at 500×g for 30 s and boiled in SDS-PAGE sample buffer. Bound proteins were resolved by SDS-PAGE and subjected to autoradiography. The binding reaction was quantitatively analysed on a phosphoimager.

Generation of *in vitro* translated proteins

10−1000 ng of phagemid vector DNA was *in vitro* transcribed and translated with a TNT-kit (Promega) according to the manufacturer's instructions. During the translation, proteins were labelled with [^{35}S]-methionine. Proteins were found to be unstable after prolonged storage at −20°C or repeated thawing and therefore prepared freshly for every experiment.

Preparation of GST fusion proteins

GST fusion protein expression and purification were as previously described (28). Briefly, a 40 ml overnight culture of *E. coli* (XA-90) containing pGEX fusions was diluted 1:10 in 2×YT medium containing ampicillin (100 μg/ml). After incubation for 1 h at 37°C, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and incubation was continued for a further 5 h. For fusion protein recovery, bacterial cultures were pelleted by centrifugation at 3,000×g for 5 min at 4°C and resuspended in 9 ml of MTPBS buffer (140 mM NaCl, 16 mM Na_2HPO4, 4 mM HaH_2PO_4, pH 7.3) containing 1 mM phenylmethylsulfonyl fluoride. The bacterial pellet was then lysed on ice by mild sonication, and 1 ml of MTPBS containing 10% Triton X-100 was added before centriguation at 10,000×g for 5 min at 4°C. The supernatant was rocked gently for 15 min at 4°C with 300 μl of glutathione-Sepharose beads (Promega) which had been washed previously three times in MTPBS. After pelleting at 500×g the beads were resuspended 1:1 (vol/vol) in MTPBS. For analysis of bound fusion protein, the beads were boiled in 4×sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer and loaded onto SDS-polyacrylamide gels. Proteins were visualised by Coomassie blue stain. Fusion proteins were stored at −20°C in 10% glycerol.

Generation of ^{32}P-labelled GST fusion protein

^{32}P-labelling of a GST-TBP fusion protein and subsequent cleavage of the labelled protein were carried out exactly as described (11).

Recombinant DNAs

To create the series of GAL-E2F fusion constructs various E2F DNA fragments were cloned in-frame with the GAL4 DNA binding domain present in the plasmid pHKG (CH and TK unpublished). Plasmids containing sequences for E2F amino acid residues 380−437, 396−437, 396−417 and 417−437 were cloned by using naturally occurring restriction sites. Using oligonucleotide directed mutagenesis specific restriction sites were introduced at E2F amino acid positions 359 (Eco RV), 407 (Eco RI) and 426 (Sal I). These restriction sites were used in combination with naturally occurring sites to generate hybrid GAL4 proteins containing E2F sequences 359−437, 407−437, 359−407, 380−426, 396−426, 426−437, 407−426. *In vitro* mutagenesis was also applied to generate the point mutations within the E2F activation domain.

To create the series of GST-E2F fusion proteins E2F DNA coding sequences were excised from the corresponding GAL-E2F constructs and cloned in-frame with the glutathione S-transferase gene using the pGEX vector system (Pharmacia). pGEX-RB (379−928) and pGEX-2TK-TBP were kindly provided by W.Kaelin.

For *in vitro* translation of the RB protein (residues 379−928) the coding regions were subcloned into the SP-6 expression vector pING 14 (S. Ingles, unpublished). This vector utilises a

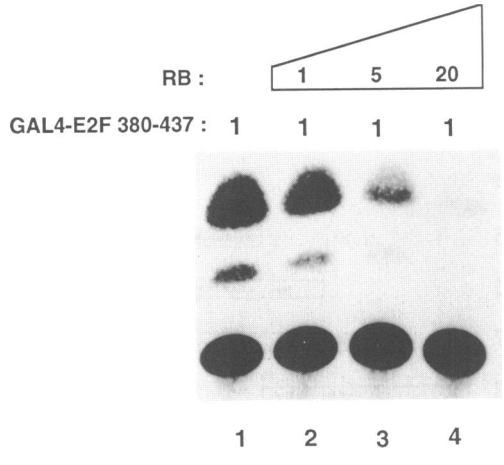

Figure 1. The E2F activation domain is sufficient for RB-induced repression. 1μg of a plasmid expressing the E2F activation domain linked to the GAL4 DNA binding domain (DBD) (pHKGE2F 380−437) was cotransfected with 4 μg of the target plasmid 1×GAL4-E1B-CAT into SAOS-2 cells (lane 1). In lanes 2, 3 and 4 increasing concentrations (1, 5 and 20 μg) of a plasmid expressing the RB pocket region (pHKRB 379−928) was transfected in addition to the plasmids in lane 1. All transfections were made up to 25 μg with pHK. Extracts from these transfections were then used for CAT assays.

methionine codon and a perfect Kozak consensus immediately downstream of the SP6 promoter for translation initiation.

RESULTS

RB silences the E2F activation domain

RB can repress the activity of promoters bearing E2F sites. These sites have the potential to bind several different proteins such as E2F, DP1 (29) and possibly an E2F/DP1 heterodimer. Since RB can bind to the E2F via a region required for activation we wanted to know if the E2F activation domain is sufficient for RBrepression or whether other parts of the E2F protein are required. To address this question we linked the E2F activation domain to a heterologous DNA binding domain (that of GAL4) and asked whether RB can repress its activation functions. In Figure 1 we can see that the GE2F 380−437 chimaera is a very potent activator in SAOS-2 cells which lack functional RB protein. The GAL4 DNA binding domain has negligible activation potential in these cells (27). If we cotransfect increasing concentrations of a vector expressing the RB pocket domain (residues 379−928) we find that the activity of the GE2F 380−437 fusion is quantitatively silenced. This repression is not due to effects on basal transcription since RB does not effect basal expression induced by the GAL4-DNA binding domain. In addition RB does not repress a potent activation domain of the Fos protein which does not bind RB *in vitro* (data not shown). These results indicate that the E2F activation domain is the target for RB repression and that this region of E2F is sufficient for the repressive effect of RB.

The RB binding region co-operates with a repeated motif to activate transcription

Having established that the target for RB repression is the activation domain of E2F, we wanted to investigate the mechanism of this down-regulation. One possibility is that RB binds to residues required for transcriptional activation and masks their potential. We therefore set out to establish whether residues required for RB binding were involved in mediating activation.

We noticed that the E2F activation domain contains a directly repeated motif with the consensus E/DF XX L X P (Figure 2A and B). Three copies of this motif are present in the domain of

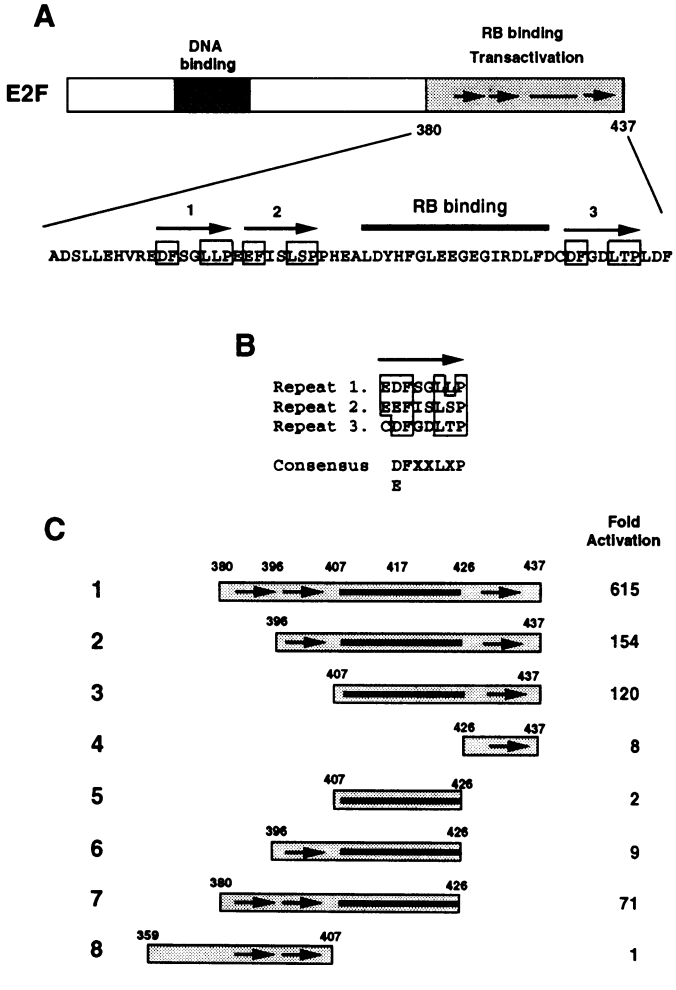

Figure 2. **(A)** The E2F activation domain contains a directly repeated motif. Three copies of a seven residue motif (arrows) are present within the E2F activation domain. These repeats flank the minimum RB binding region (solid line) defined by Helin *et al.* (12). **(B)** Alignment of repeats 1, 2 and 3. **(C)** The repeats co-operate with the minimum RB binding region to activate transcription. 1μg of various deletions of the E2F activation domain linked to the GAL4 DBD were cotransfected with 4 μg of a 1×GAL4-E1B-CAT reporter plasmid into SAOS-2 cells. Extracts from each transfection were used in CAT assays. Values next to the diagrammatic representation of the E2F sequences linked to the GAL4 DBD represent fold activation relative to the activity of the GAL4 DBD.

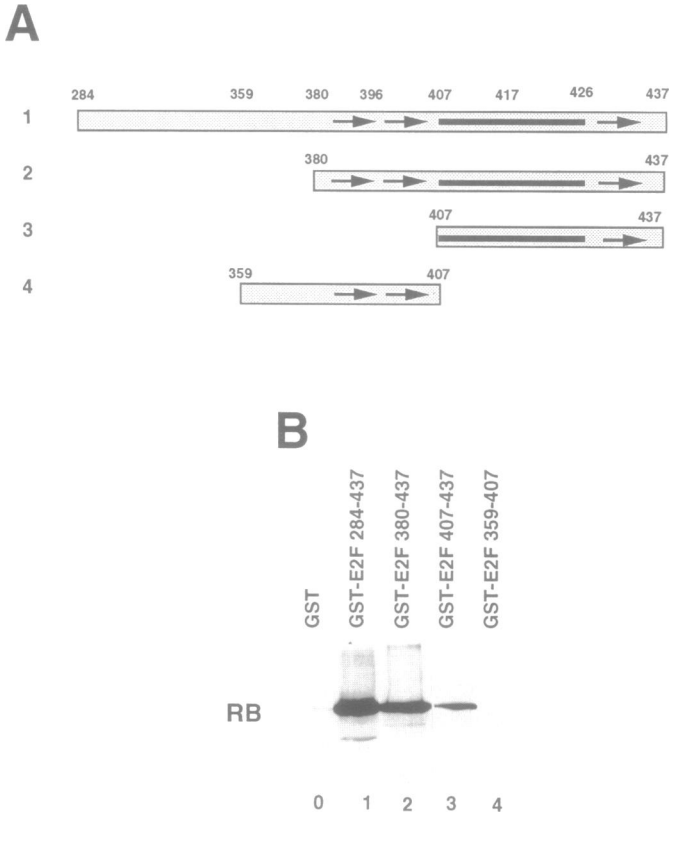

Figure 3. The region of the repeats contributes to full RB binding capacity. Various segments of the E2F activation domain were expressed in bacteria as fusions with the GST protein. These fusions were then incubated with radiolabelled *in vitro* translated RB 379−928 and subjected to a GST-pull-down assay. **(A)** Schematic representation of the E2F sequences linked to GST. **(B)** Results of the GST-pull-down, using GST as a negative control.

E2F (380–437) which is down-regulated by RB. The previously defined binding site for the RB protein (residues 409–426, ref. 12) lies between these repeats. Repeats 1 and 2 directly precede the RB binding region whereas repeat 3 directly follows it. To establish the relationship of the RB binding region and these three repeats in mediating activation, we carried out a detailed deletion analysis of this region. We fused various segments of the E2F C-terminus to the GAL4 DNA binding domain and assayed for their ability to activate transcription from a promoter bearing a single GAL4 binding site. The results shown in Figure 2C indicate that the previously defined RB binding region (407–426) does not activate transcription independently (line 5) compared to the entire E2F activation domain (380–437, line 1). The same is true for the repeats: sequences containing repeats 1 plus 2 (359–407, line 8) or repeat 3 (426–437, line 4) are essentially inactive. However, in the presence of the minimum RB binding region, repeats 1 plus 2, or repeat 3, can activate transcription synergistically (380–426, line 7 and 407–437, line 3). Repeat 2 does not contribute substantially to this synergism in the absence of repeat 1 (396–437, line 2 and 396–426, line 6) which may be an indication that repeats 1 and 2 act as a unit. These data suggest that the repeats contribute to the activity of the E2F activation domain and that they synergise with some component of the RB binding region.

Analysis of RB binding capacity indicates that the repeats may contribute to RB binding affinity as well as transcriptional activation (Figure 3). In a GST-pull-down assay, a domain of E2F which contains the minimum RB binding region (GST-E2F 407–437 lane 3) binds *in vitro* translated RB less efficiently than larger E2F domains which contain the repeats (GST-E2F 284–437 lane 1 and GST-E2F 380–437 lane 2). However, these repeats are unable to bind RB independently (GST-E2F 359–407 lane 4) suggesting that the repeats augment the binding of RB to the minimum binding region without providing independent RB binding capacity.

RB binds residues required for transcriptional activation

The deletion analysis in Figure 2 indicates that the minimum RB binding site is not an independent activation domain but can co-operate with the repeated motif to activate transcription. This still leaves open the possibility that RB does not directly contact residues required for transcriptional activation. To establish whether there is a correlation between the residues required for activation and the residues required for RB binding, nine point mutations were introduced into the E2F activation domain (Figure 4A). Three of these mutations (YF, EEE, DD) are within the minimum RB binding region, five mutations (DF1, EF, SP, DF2, TP) involve residues conserved within repeat 1, 2 or 3 and one

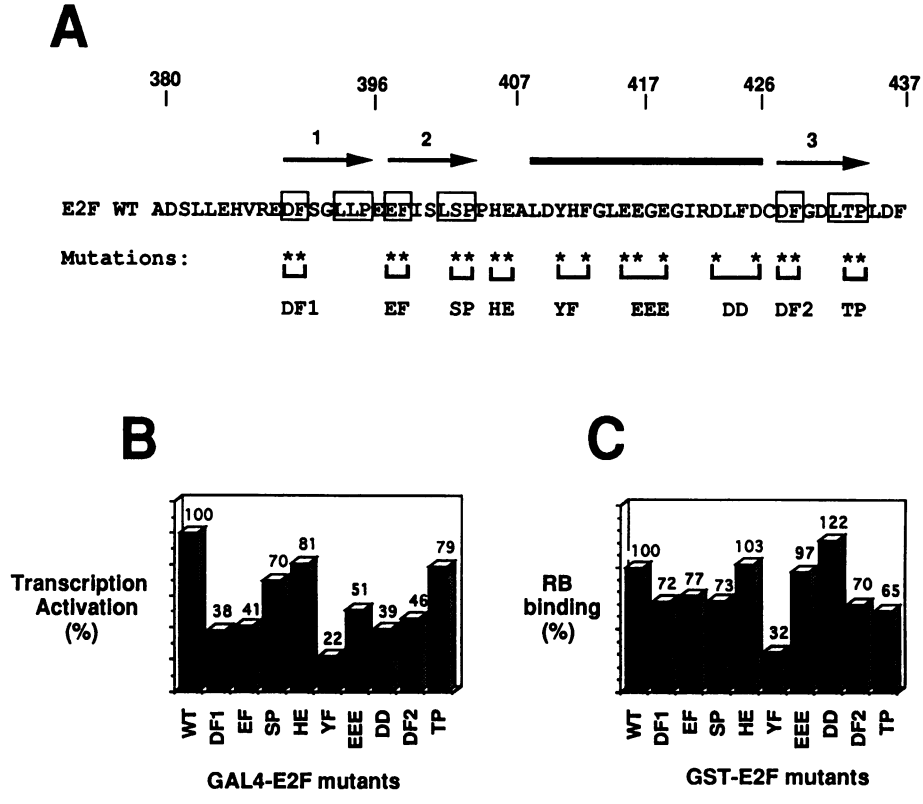

Figure 4. E2F residues required for RB binding are involved in transcriptional activation. **(A)** Position of point mutations with the E2F activation domain. All changes (to alanine) are shown by an asterisk. The repeats (1, 2 and 3) are shown by arrows, with conserved residues boxed and the minimum RB binding region defined by Helin *et al.* (12) is shown with a black line. **(B)** Each of the point mutants shown in A was introduced into the plasmid pHKG E2F 380–437 and assayed for its ability to activate the expression of a 1×GAL4-E1B-CAT reporter plasmid in SAOS-2 cells. Each of the mutants was assayed at least four times and the results were quantified by a phosphoimager. The values given are relative to the WT GAL4-E2F 380–437. **(C)** Each of the point mutations shown in A was introduced into pGEX-E2F 380–437 and expressed in bacteria as a fusion with GST. These GST-E2F mutants were tested for their ability to bind RB 379–928 in a GST-pull-down assay. The binding capacity of each mutant was assessed in four separate experiments. The amount of RB bound in each experiment was quantified by a phosphoimager and the average binding capacity of each mutant is shown relative to the WT GST-E2F 380–437.

mutation (HE) lies in the small intervening sequence between repeat 2 and the minimum RB binding region. All changes were to alanine. These mutations were introduced in GAL4-E2F 380–437 background and tested for their ability to activate a promoter bearing a single GAL4 DNA binding site (Figure 4B). In parallel, these mutations were introduced into GST-E2F 380–437 and assayed for RB binding capacity in a GST-pull-down assay (Figure 4C).

The results of the transactivation studies (Figure 4B) indicate that each of the mutations has an effect on transcriptional activation at varying degrees. The YF mutation, which has the most severe effect on activity, lies in the minimum RB binding region whereas the mutation with the least effect on activity (HE) lies between repeat 2 and the minimum RB binding region. It is also interesting to note that mutagenesis of similar positions within the different repeats has a similar effect. For example the DF1, EF and DF2 mutations (which affect the first two residues of repeat 1, 2 and 3 respectively) show similar reduction in activity (38–46% of WT); similarly, the SP and TP mutations (affecting the last two positions of repeat 2 and 3) show a comparable effect (70 and 79% of WT). These data argue that each of the repeats may function in a similar fashion in inducing transcription. It is worth noting that the reduction in activation functions observed with these mutants was much less apparent when a reporter with 5 GAL4 DNA binding sites was used, suggesting that multiple activators on the promoter may mask the effect of a specific mutation (data not shown).

Analysing these mutants for RB binding reveals that the YF mutation (which falls within the minimal RB binding region) has the most severe effect on RB binding (Figure 4C). This mutation is also the one which has the most dramatic effect in transcriptional activation (Figure 4B). This correlation suggests that RB binds to residues which are important for transcriptional activation. All other mutations which affect RB binding (DF1, EF, SP, DD and TP) involve residues in repeats 1, 2 or 3. These five mutations have less effect on RB binding than the YF mutation but show a similar level of reduction (65–77% of WT).

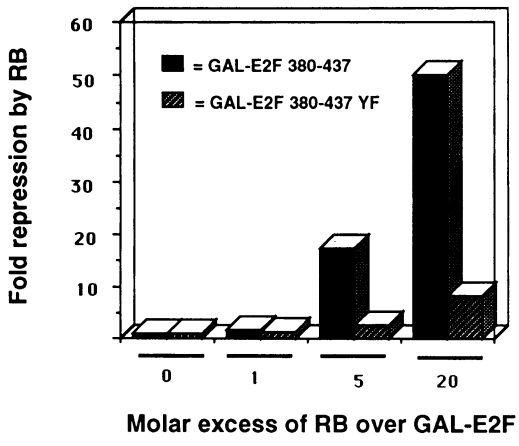

Figure 5. The E2F YF mutant is less responsive to RB repression. Plasmids expressing pHKG E2F 380–437 or pHKF E2F 380–437 YF (1 μg) were assayed for their ability to activate a 1×GAL4-E1B-CAT reporter (4 μg) in SAOS-2 cells, either in the absence or in the presence of different amounts (1, 5, 20 μg) of a plasmid expressing RB 379–928. In all cases transfected DNA was made up to 25 μg with pHK. The histogram shows the fold repression by RB.

These data support the conclusions of the deletion analysis (Figure 3) which shows that sequences outside the minimum RB binding region are required for high affinity RB binding. Finally, three mutations (HE, EEE and DD) show no significant reduction in RB binding ability. Since these mutations have an effect on transcriptional activation, it appears that RB does not bind all residues involved in the activation process. However, it is worth noting that a region containing the EEE and DD residues (407–426) has no independent transactivating capacity and will only activate in co-operation with regions containing the repeats. This argues that RB need not necessarily bind all residues involved in activation to silence the E2F activation domain.

Since RB binds to residues required for transcriptional activation, one model for repression would be that RB simply masks the residues which are important for E2F-induced activation. A prediction of such a model is that prevention of RB binding to E2F would also relieve RB-induced repression. To test such a model we assessed the ability of RB to repress the activation functions of an E2F mutant (GAL4-E2F YF) which has severely reduced RB binding capacity, but still retains a significant amount of transactivating capacity. This mutant was transfected into RB negative SAOS-2 cells either in the absence of exogenous RB or in the presence of increasing concentrations of a plasmid expressing RB 379–928. As seen in Figure 5, E2F 380–437 YF shows greatly reduced responsiveness to RB repression when compared to E2F 380–437. As expected, a slight RB repressive effect is still observed on YF mutant since this protein still retains residual RB binding capacity. These data are consistent with the interpretation that RB binding to E2F is necessary for repression.

RB binds residues required for TBP binding

Since RB binds to residues required for transcriptional activation we considered the possibility that RB binding may preclude the interaction with a protein required for activation. For several reasons, a good candidate for such a protein is the TATA-box binding protein, TBP: first, TBP has a pivotal role in the formation of the preinitiation complex, second, TBP has been shown to contact other activation domains, such as that of E1A (30, 31), Zta (30), PU.1 (15), p53 (33, 34) and c-myc (35), and third, the C-terminues of TBP shows sequence similarity to RB (15). To address whether E2F can form a complex with TBP we asked whether E2F would bind to a GST-TBP fusion in a pull-down assay. Figure 6A shows that *in vitro* translated E2F can bind to a GST-TBP fusion as well as to a GST-RB fusion, but will not bind a GST-Vimentin control. The interaction between E2F and TBP is direct, since a bacterially expressed GST-E2F can bind to a bacterially expressed radiolabelled TBP protein (Figure 6B). In this direct interaction assay, GST-E2F shows a similar affinity for TBP as does GST-E1A. Deletion analysis of E2F shows that TBP can bind to the E2F activation domain in a manner indistinguishable from that of RB: sequences containing the minimum RB binding region also bind TBP, whereas sequences containing repeats 1 and 2 augment, but do not allow, TBP binding (compare Figure 6C and Figure 3). Analysis of the E2F point mutations (shown in Figure 4A) further supports the view that RB and TBP require similar residues for their interaction with E2F (compare Figure 6D and Figure 4C). As observed for RB, the YF mutation substantially reduces TBP binding; mutation of residues with the repeats (DF1, EF, SP, DF2 and TP) has an intermediate effect whereas mutants HE, EEE and DD do not reduce TBP binding. Indeed two of these

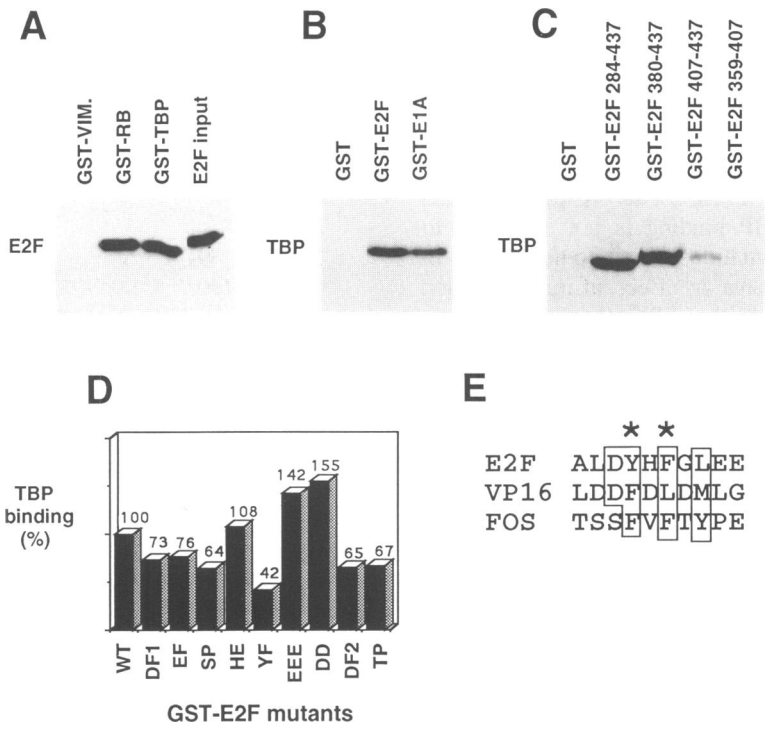

Figure 6. E2F interacts with TBP *in vitro*. **(A)** GST-TBP, GST-RB or a control GST-Vimentin, were incubated with *in vitro* translated radiolabelled E2F and subjected to GST-pull-down. **(B)** TBP binds E2F directly. Bacterially-expressed GST-E2F, GST-E1A or GST were incubated with bacterially expressed, ^{32}P-labelled TBP protein and subjected to GST-pull-down. **(C)** Various GST-E2F deletions were incubated with ^{35}S-labelled *in vitro* translated TBP and subjected to GST-pull-down. **(D)** GST-E2F point mutants were subjected to a pull down after incubation with *in vitro* translated TBP. This was repeated four times and the average amount of radiolabelled TBP bound by each mutant (quantified by a phosphoimager) is shown. **(E)** Alignment of sequences within E2F, VP16 and c-Fos required for the binding of TBP. The asterisks indicate the position of crucial hydrophobic residues (Y_{411} plus F_{413} in E2F, F_{442} in VP16 and F_{341} plus F_{343} in c-Fos) which severely affect TBP binding when mutated.

latter mutants, EEE and DD, appear to increase the ability of TBP to bind E2F which may be indicative of a conformational change allowing better access to TBP. A similar increase in binding to RB is evident with the DD mutant. This remarkable correlation in the residues affecting RB and TBP binding within the E2F activation domain raises the possibility that RB represses E2F activity by precluding the binding to TBP. The fact that the residues needed for TBP binding are also needed for transcriptional activation strongly supports the view that TBP binding is relevant to the transactivating functions of E2F.

DISCUSSION

In this paper we have investigated the mechanism by which RB can repress transcription. We show that the E2F activation domain is the target for RB repression. Detailed structure/function analysis of this domain indicates that RB binds to sequences which are essential for the activation functions of E2F. A point mutation which most severely affects RB binding (YF) is also the one that has the most deleterious effect on transcriptional activation. The decreased RB binding capacity of this YF mutant also correlates with a decreased responsive-ness to RB repression, suggesting that RB has to bind in order to repress.

Our point mutational analysis clearly shows that the minimum RB binding region (409−426) contains sequences crucial to transcriptional activation by E2F. However, this region does not have independent transactivating ability. Sequences outside this region, which contain three copies of a repeated motif, provide additional functions which are essential for transcriptional activation. The repeats themselves have negligible activation capacity on their own, suggesting that the repeats and the sequence of the minimum RB binding region activate transcription synergistically. This synergism may be due to the binding of proteins to each domain or it may be that the repeats provide a certain conformation which is required before a protein involved in the activation process can bind to the RB binding region. These models are not mutually exclusive or the only ones possible.

Our results also support the conclusion reached by Helin *et al.* (12) that sequences 409−426 of E2F are necessary and sufficient for RB binding: independent RB binding is only seen when these sequences are present and a point mutation within this sequence shows the most dramatic effect on RB binding. However, sequences outside this region are required for full RB binding capacity. We know that each of the three repeats contributes to RB binding affinity since point mutations in conserved residues affect RB binding. We cannot distinguish, however, whether these repeated residues provide the correct conformation for the binding of RB to E2F or whether they provide low affinity contact points which stabilise RB binding.

The ability of RB to contact residues which are required for transcriptional activation suggests that RB mimics a protein required for activation and precludes its binding to E2F. We present data that TBP may be such a protein. TBP requires the minimum RB binding region to bind to E2F and the YF mutation which severely affects RB binding is also the most effective in reducing TBP binding. Indeed, comparison of all the point mutations within the E2F activation domain indicates that the requirements for RB and TBP binding are virtually

indistinguishable. Since the RB/E2F interaction has been extensively studied and shown to occur *in vivo*, the striking correlation between RB and TBP binding requirements strongly suggests that the TBP/E2F interaction occurs *in vivo*.

The fact that residues required for TBP binding are also required for transcriptional activation indicates that TBP binding is a functionally relevant event with respect to E2F transactivation. However, TBP binding is not sufficient for activation, since three point mutations which do not affect TBP binding (HE, EEE, DD) do have an effect on transactivation. This leaves open the possibility that E2F interacts with other proteins involved in mediating activation such as TAFs, TFIIB or, as yet, uncharacterised adaptors. In principle, RB may also preclude the interaction of these proteins with E2F by steric hindrance, considering the proximity of the highly sensitive YF residues.

The E2F transcription factor does not possess the well established L X C X E motif, characteristic of viral oncoproteins which bind RB. Our mutagenesis suggests that the Y and F residues, which are the most sensitive with respect to RB binding, are part of a new RB binding motif. This is suggested by the fact that adjacent residues, which flank the YF sequence (HE, EEE, DD), do not affect RB binding, even though two of these (EEE and DD) fall within the minimum RB binding region. This makes it unlikely that mutagenesis of Y and F merely disturbs the conformation of the protein and suggests that these residues mediate the contact with RB. Since mutagenesis of the Y and F residues reduces TBP binding also, we compared the sequence of E2F with other TBP binding transcription factors. Figure 6E shows that the sequence surrounding the Y and F residues has similarity to sequences within VP16 and c-Fos which are required for TBP binding. Mutagenesis of bulky hydrophobic residues within these related motifs (F_{442} of VP16 and F_{341} plus F_{343} in c-Fos) affects both TBP binding and transactivation (35, unpublished observations). This TBP binding motif may define a new consensus for RB recognition.

The ability of RB and TBP to recognise similar sequences implies that these two proteins share a structurally related domain. Consistent with this expectation is the presence of sequence similarity between the RB pocket and the conserved C-terminal domain of TBP (15). This relationship suggests that RB may be able to recognise E2F by mimicking the structure of TBP. A further implication of the RB/TBP relationship is that RB may have the potential to bind (and possibly regulate) other transcription factors which contact TBP. This notion is supported by the fact that RB can bind (in addition to E2F) two transcription factors (PU.1 and c-myc) via activation domains which contact TBP (15, Rene Bernards, personal communication).

The results presented here strongly support the view that RB acts as a repressor by blocking E2F residues important for activation. We present evidence suggesting that RB may mimic the binding specificity of TBP thus precluding TBP from contacting the E2F activation domain. Characterisation of the E2F/TBP and E2F/RB interactions in a purified reconstituted *in vitro* transcription system will hopefully establish the validity of the TBP preclusion model for RB repression.

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REFERENCES

1. Weinberg, R. A. (1991) *Science* **254**, 1138–1146.
2. Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A. and Harlow, E. (1988) *Nature* **334**, 124–129.
3. DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J-Y., Huang, C-M., Lee, W-H., Marsilio, E., Paucha, E. and Livingston, D. M. (1988) *Cell* **54**, 275–283.
4. Dyson, N., Howley, P. M., Munger, K. and Harlow, E. (1989) *Science* **243**, 934–937.
5. Huang, S., Wang, N-P, Tseng, B. Y., Lee, W-H. and Lee, E. Y-H. P. (1990) *EMBO J.* **9**, 1815–1822.
6. Kaelin, W. G., Ewen, M. E. and Livingston, D. M. (1990) *Mol. Cell. Biol.* **10**, 3761–3769.
7. Kouzarides, T. (1993) *Trends Cell Biol.* **3**, 211–213.
8. Kaelin, W. G., Pallas, D. C., DeCaprio, J. A., Kaye, F. J. and Livingston, D. M. (1991) *Cell* **64**, 521–532.
9. Defeo-Jones, D., Huang, P. S., Jones, R. E., Haskell, K. M., Vuocolo, G. A., Hanobik, M. G., Huber, H. E. and Oliff, A. (1991) *Nature* **352**, 251–254.
10. Shan, B., Zhu, X., Chen, P-L., Durfee, T., Yang, Y., Sharp, D. and Lee, W-H. (1992) *Mol. Cell. Biol.* **12**, 5620–5631.
11. Kaelin, W. G., Krek, W., Sellers, W. R., DeCaprio, J. A., Ajchenbaum, F., Fuchs, C. S., Chittenden, T., Li, Y., Farnham, P. J., Blanar, M. A., Livingston, D. M. and Flemington, E. K. (1992) *Cell* **70**, 351–364.
12. Helin, K., Lees, J. A., Vidal, M., Dyson, N., Harlow, E. and Fattaey, A. (1992) *Cell* **70**, 337–350.
13. Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V. and Nadal-Ginard, B. (1993) *Cell* **72**, 309–324.
14. Wang, C-Y., Petryniak, B., Thompson, C. B., Kaelin, W. G. and Leiden, J. M. (1993) *Science* **260**, 1330–1335.
15. Hagemeier, C., Bannister, A. J., Cook, A. and Kouzarides, T. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 1580–1584.
16. Rustgi, A. K., Dyson, N. J. and Bernards, R. (1991) *Nature* **352**, 541–544.
17. Kim, S. J., Wagner, S., Liu, F., O'Reilly, M. A., Robbins, P. D. and Green, M. R. (1992) *Nature* **358**, 331–334.
18. Bandara, L. R. and La Thangue, N. B. (1991) *Nature* **351**, 494–497.
19. Bagchi, S., Weinmann, R. and Raychaudhuri, P. (1991) *Cell* **65**, 1063–1072.
20. Chellappan, S., Hiebert, S., Mudryj, M., Horowitz, J. M. and Nevins, J. R. (1991) *Cell* **65**, 1053–1061.
21. Kovesdi, I., Reichel, R. and Nevins, J. R. (1986) *Cell* **45**, 219–228.
22. Nevins, J. R. (1992) *Science* **258**, 424–429.
23. Bagchi, S., Raychaudhuri, P. and Nevins, J. R. (1990) *Cell* **62**, 659–669.
24. Hiebert, S. W., Chellappan, S. P., Horowitz, J. M. and Nevins, J. R. (1992) *Genes Dev.* **6**, 177–185.
25. Weintraub, S. J., Prater, C. A. and Dean, D. C. (1992) *Nature* **358**, 259–261.
26. Hamel, P. A., Gill, R. M., Phillips, R. A. and Gallie, B. L. (1992) *Cell. Biol.* **12**, 3431–3438.
27. Sutherland, J. A., Cook, A., Bannister, A. J. and Kouzarides, T. (1992) *Genes Dev.* **6**, 1810–1819.
28. Smith, D. B. and Johnson, K. S. (1988) *Gene* **67**, 31–40.
29. Girling, R., Partridge, J. F., Bandara, L. R., Burden, N., Totty, N. F., Hsuan, J. J. and La Thangue, N. B. (1993) *Nature* **362**, 83–87.
30. Lee, W. S., Kao, C. C., Bryant, G. O., Liu, X. and Berk, A. J. (1991) *Cell* **67**, 365–376.
31. Horikoshi, N., Maguire, K., Kralli, A., Maldonado, E., Reinberg, D. and Weinmann, R. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5124–5128.
32. Lieberman, P. M. and Berk, A. J. (1991) *Genes Dev.* **5**, 2441–2454.
33. Seto, E., Usheva, A., Zambetti, G. P., Momand, J., Horikoshi, N., Weinmann, R., Levine, A. J. and Shenk, T. (1992) *Proc. Natl. Acad. Sci. USA* 89, 12028–12032.
34. Liu, X, Miller, C. W., Koeffler, P. H. and Berk, A. J. (1993) *Mol. Cell. Biol.* **13**, 3291–3300.
35. Ingles, C. J., Shales, M., Cress, W. D., Triezenberg, S. J. and Greenblatt, J. (1991) *Nature* **351**, 588–590.