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Phil. Trans. R. Soc. Lond. B 1993 **340**, 333-336
doi: 10.1098/rstb.1993.0075

Transcriptional inhibition by the retinoblastoma protein

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SUMMARY

The retinoblastoma protein, pRB, appears to play a key role in coordinating the regulation of cell cycle position and transcriptional events. pRB undergoes specific cell-cycle-dependent phosphorylation, being underphosphorylated in G1 and heavily phosphorylated in S, G2, and M. The underphosphorylated form is able to interact with the E2F transcription factor. Recently, we have cloned a cDNA for E2F-1. By using this clone and a series of non-pRB binding mutants, we have been able to show that the binding of pRB to E2F-1 causes inhibition of E2F-mediated transactivation. pRB's inhibition of E2F-mediated transcription would be lost by mutation in the retinoblastoma gene in human tumours, by pRB's interaction with DNA tumour virus oncoproteins, or by phosphorylation during the cell cycle.

1. INTRODUCTION

Mutations of both alleles of the retinoblastoma gene are characteristically found in retinoblastomas, a childhood tumour of the retina (reviewed in Weinberg 1992). As a consequence of the two mutations, these tumours fail to express a functional retinoblastoma gene product. The development of these two mutations provide the two rate limiting steps for the tumorigenesis in the retina, and the study of these changes has provided the paradigm for all tumour suppressor genes. Mutations that inactivate the protein products of tumour suppressor genes promote cell growth. Thus tumour suppressor genes produce proteins whose role is to inhibit some step in cell division, the loss of which leads to tumorigenesis. There now are a small group of genes that have been identified as tumour suppressors, including the retinoblastoma gene, p53 (Rotter *et al.* 1993), the Wilm's tumour gene (WT1; Haber *et al.* 1992), neurofibromatosis type 1 (NF1; Rey & Hall 1993), and adenomatous polyposis coli (APC; Hedrick *et al.* 1993).

The identification of the locus whose loss was responsible for the genesis of retinoblastomas led to the molecular cloning of this gene and the identification of its protein product, now known as pRB. This cloning provided the reagents to show that the loss of pRB is not restricted to retinoblastomas but occurs frequently in many human tumours. Tumours that often show loss of functional pRB include osteosarcomas, lung carcinomas, bladder carcinomas, cervical carcinomas, and many soft tissue sarcomas. Thus pRB appears to play an important regulatory role in many different human tissues.

pRB is also an important target in the life cycle of small DNA tumour viruses. Viruses such as adenoviruses, polyomaviruses, and papillomaviruses encode potent oncoproteins that physically associate with pRB (DeCaprio *et al.* 1988; Whyte *et al.* 1988; Dyson *et al.* 1989). For adenoviruses the oncoproteins that bind pRB are encoded by the E1A gene; for polyomaviruses they are the large T antigens; and for the papillomaviruses, the E7 proteins. For each of these proteins the regions that are needed for interaction with pRB are essential for virus-mediated transformation (reviewed in Helin & Harlow 1993). The interaction of the viral proteins with this tumour suppressor gene is thought to inactivate pRB and thus to mimic the loss of pRB seen in human tumours. Therefore, although they use different mechanisms, both viral transformation and many human tumorigenic events depend on the inactivation of pRB.

The first clues to the possible biochemical function of pRB came from viral transactivation studies(Bagchi *et al.* 1991; Bandara & La Thangue 1991; Chellappan *et al.* 1991; Chittenden *et al.* 1991). Again work from adenoviruses provided the systems that allowed this development. An early viral gene, known as E2, is transcriptionally activated by the E1A protein shortly after viral infection. E2F was identified as a cellular transcription factor that bound to a sequence within the E2 promoter. Its activity is greatly enhanced in the presence of E1A and the E2F-binding sequences are important for the activation of the E2 promoter. Recently, the purification of E2F demonstrated that it was bound by pRB. Following the expression of the E1A proteins, this complex was dissociated and E2F-mediated transcription was activated. These results suggested the first model of pRB–E2F regulation. It was suggested that the pRB–E2F complex is transcriptionally inactive and E1A's ability to bind to pRB releases 'free' E2F, which is now active for transcrip-

Phil. Trans. R. Soc. Lond. B (1993) **340,** 333–336
Printed in Great Britain

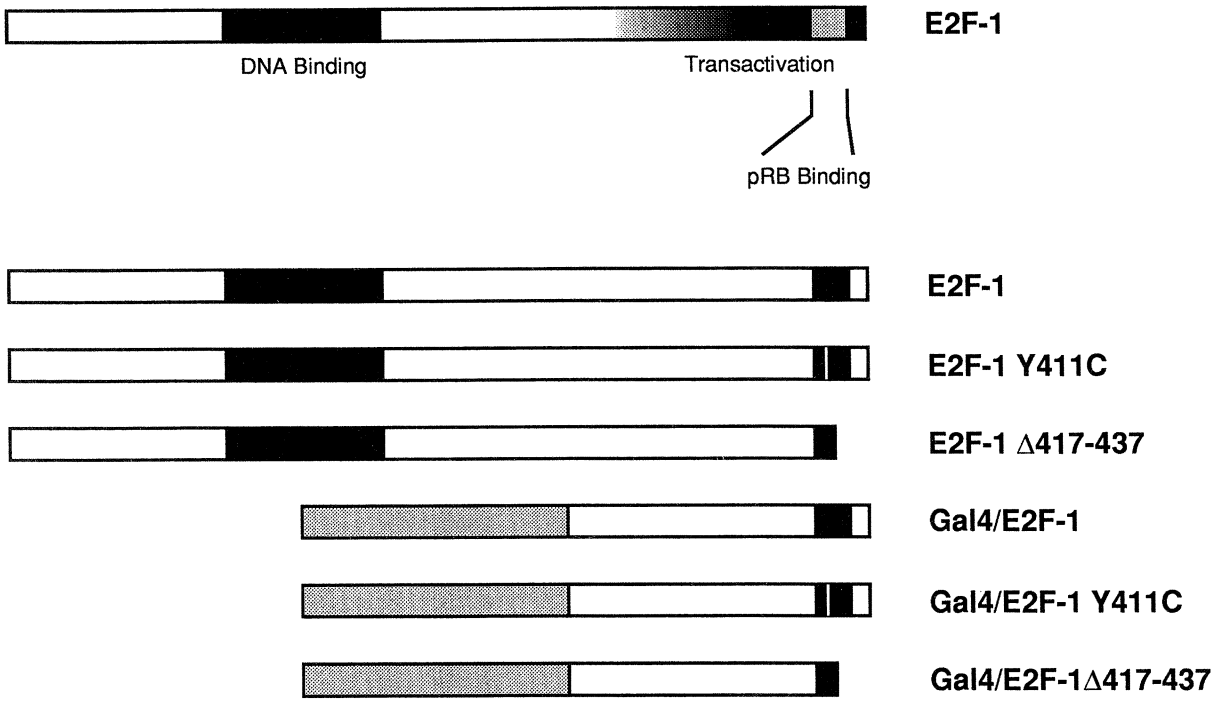

Figure 1. Structure of E2F-1 and E2F-1 mutants. The top bar shows the location of various functional domains of the wild-type E2F-1 protein as determined previously (Helin *et al.* 1992; Kaelin *et al.* 1992). The lower six bars show the structure of E2F proteins used for transactivation experiments. E2F-1Y411C has the tyrosine at residue 411 changed to a cysteine. E2F-1Δ417–437 has a deletion that removes the carboxy-terminal residues from 416 on. The grey bars represent the DNA-binding domain of the *S. cerevisiae* GAL4 protein, amino acids 1 to 147, which has been ligated to the carboxy-terminus of E2F-1 at residue 248.

tion. In this current work, we test this model and show that E2F-mediated transcription is inhibited by the binding of pRB.

2. MATERIALS AND METHODS

The methods used in this manuscript have been described in Helin *et al.*, (1992) and Helin *et al.* (1993). Briefly, two types of transfections were performed. They varied by the types of reporter constructs that were used. One used the adenovirus E2 promoter as a target of E2F-mediated transcription. The other reporter constructs relied on synthetic GAL4 binding sites. In both cases the constructs drove expression of bacterial chloramphenicol acetyl transferase (CAT). Transactivation was measured in all cases as the level of CAT enzyme activity as compared to a reference plasmid containing the Rous sarcoma virus long terminal repeat driving expression of luciferase.

Both transactivation experiments used various wild-type and mutant E2F constructs engineered with either the E2F DNA-binding domain or an GAL4 DNA-binding domain. The structures of the mutant constructs are shown in figure 1.

All transfections were done with human osteosarcoma Saos-2 cells.

3. RESULTS AND DISCUSSION

The recent cloning of a cDNA for the E2F has allowed us to directly test pRB's role in regulating E2F-mediated transcription (Helin *et al.* 1992; Kaelin *et al.* 1992; Shan *et al.* 1992). This cDNA was isolated by the ability of pRB to interact directly with E2F *in vitro*. Several criteria were used to determine that this clone was at least one example of authentic E2F. First, in an *in vitro* binding assay, the protein encoded by this cDNA bound to the appropriate regions of pRB, and this binding could be competed by the viral oncoproteins or peptides representing the viral sequences needed for binding to pRB. Second, a bacterially produced protein encoded by this cDNA was able to bind to E2F DNA-binding sites in a manner that was indistinguishable from authentic cellular E2F. Third, antibodies raised against the encoded protein could immunoprecipitate a protein of 60 kDa from mammalian cells, a size consistent with that reported previously for E2F. Moreover, these immunoprecipitations showed a complex with the underphosphorylated forms of pRB. Fourth, in transactivation experiments the expression of this protein could stimulate transcription from E2F-reporter constructs. Together these data demonstrate that this clone is an authentic E2F, and we now refer to this clone and its protein product as E2F-1. A more extensive discussion of this work is found in Helin *et al.* (1992).

The E2F-1 clone was also used to map various structural domains on this protein. Figure 1 shows a summary of these data, originally reported in Helin *et al.* (1992). The DNA binding domain is found between amino acids 89 and 191, while the transacti-

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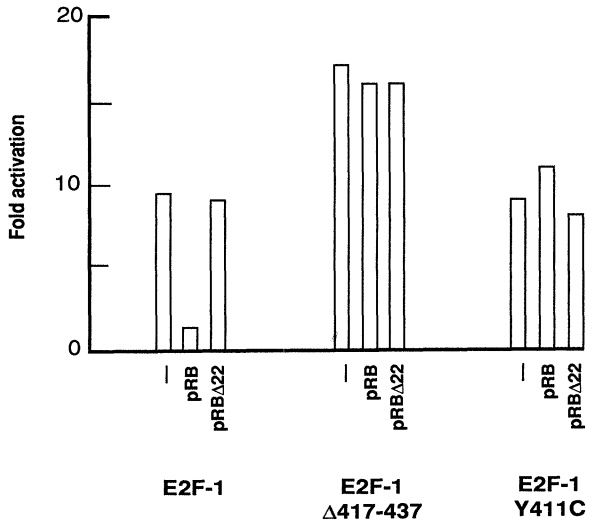

Figure 2. Inhibition of E2F transactivation by the retinoblastoma protein. CAT-reporter constructs with two E2F DNA binding sites were co-transfected with either wild-type (pRB) or mutant (pRBΔ22) retinoblastoma expression constructs. The relative levels of CAT were determined and are shown with the three E2F-1 constructs described in figure 1.

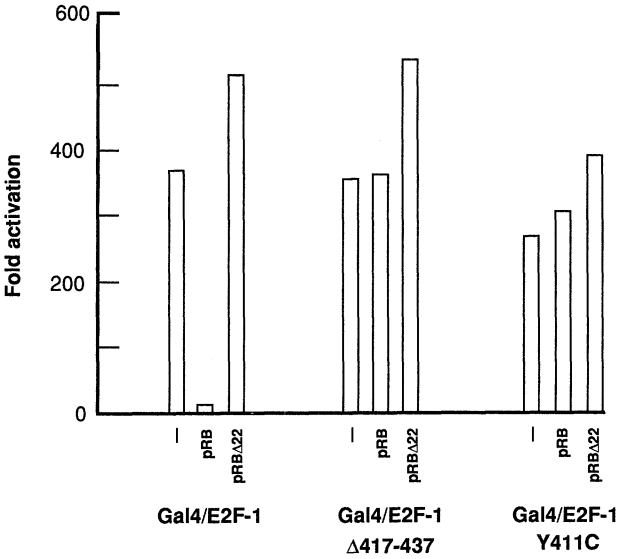

Figure 3. Inhibition of GAL4/E2F transactivation by the retinoblastoma protein. CAT-reporter constructs with five GAL4 DNA-binding sites were co-transfected with either wild-type (pRB) or mutant (pRBΔ22) retinoblastoma expression constructs. The relative levels of CAT were determined and are shown with the three GAL4/E2F-1 constructs described in figure 1.

vation domain was found in the carboxy-terminus. Surprisingly, the region required for binding to pRB was localized to an 18 amino acid stretch found near the carboxy-terminus and within the transactivation domain. Mutagenesis showed that this region is both necessary and sufficient for binding pRB.

One of the important uses of this cDNA has been to determine the effects of the interaction of pRB. To test the ability of pRB to regulate E2F, we tested the ability of exogenously expressed pRB to repress E2F-mediated transcription in two experimental formats. First, we exploited our original observations of transactivation seen with the expression of exogenous E2F. In this assay an E2F-reporter construct is co-transfected with an E2F-expression vector into pRB-deficient cells. This transfection was repeated in the presence of increasing concentrations of a pRB-expression plasmid.

As shown in figure 2, pRB was able to inhibit E2F transactivation in this assay. To establish the specificity of this inhibition, mutations in pRB and E2F that produced proteins that failed to interact with each other were tested. For the retinoblastoma protein, we chose a naturally occurring mutant isolated from a human small cell lung carcinomas. This mutant allele contains a point mutation that results in a splicing defect and yields a deletion of the amino acid sequences encoded by exon 22. The pRB protein encoded by the exon 22 deletion mutant has been shown to be inactive in all of the available assays for pRB function. For mutations in E2F, we constructed two mutations within the pRB-binding region. These two mutations, E2FΔ417 and E2FY411C, were chosen because the resulting proteins cannot bind to pRB *in vitro*. The structures of these mutations are shown in figure 1. As shown in figure 2, both of these mutations were still capable of activating transcription from E2F-regulated promoters to near wild-type levels, yet neither was affected by the presence of pRB. Similarly, the mutant pRB was not able to repress E2F-mediated transcription. These data with the mutant forms of pRB and E2F argue that the physical interaction between pRB and E2F is essential for pRB's role in transcriptional repression.

In the second transcriptional assay, fusion proteins that replace the E2F DNA-binding domain with the DNA binding sequences of the yeast GAL4 transcriptional activator were constructed. These constructs were then tested for their ability to activate transcription from a reporter construct containing GAL4 DNA-binding sites (Lillie & Green 1989). This strategy measures the ability of the exogenously added GAL4/E2F fusion proteins to stimulate transcription without the interference of the endogenous E2F. For controls, all of the mutations described above were included in these assays. As before, we found that wild-type but not mutant pRB could inhibit E2F-mediated transcription and that E2F mutants that cannot interact with pRB are resistant to its inhibitory action while still being competent for transactivation (figure 3).

Taken together these data argue that pRB can inhibit transactivation by E2F. This transcriptional inhibition is dependent on the ability of pRB to interact with E2F, as mutations in either pRB or E2F that block this interaction show no inhibition. The results imply that any naturally occurring mutation that inhibits the interaction of pRB and E2F would stimulate E2F transactivation at least at times in the cell cycle when pRB is regulating E2F. At least the first naturally occurring mutations of pRB that we have tested are consistent with this conclusion, as they

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fail to bind to E2F and also do not act as transcriptional inhibitors.

These results identify direct binding of pRB as one mechanism that is important for regulation of E2F. They imply that any other mechanism that will block the binding of pRB and E2F will also block inhibition of transcription. One well characterized mechanism that is likely to alleviate pRB binding is phosphorylation. pRB undergoes extensive phosphorylation beginning in mid-to-late G1 and continuing until after mitosis. Only the underphosphorylated forms of pRB bind to E2F, suggesting that pRB's inhibition of E2F's action will be confined to the times in which they are bound. Therefore, the kinases and phosphatases that regulate pRB modification would be upstream regulators of this transcriptional regulation. At present the kinases that phosphorylate pRB *in vivo* have not been identified, although the most likely candidates are the cyclin-dependent kinases. Several of the kinases from this class can recapitulate the *in vivo* phosphorylation pattern of pRB and the sites that are modified fit the consensus for these kinases.

One of the major remaining questions is which genes are regulated by the pRB–E2F complexes? This is still a difficult issue to resolve. Several genes whose products have important roles in growth regulation have been shown to contain E2F binding sites in their promoters. These include c-myc, N-myc, DNA polymerase-α, dihydrofolate reductase, thymidine kinase, thymidylate synthase, and cdc2. In some cases, for example c-myc (Hiebert *et al.* 1989; Thalmeier *et al.* 1989), DHFR (Blake & Azizkhan 1989; Means *et al.* 1992), and cdc2 (Dalton 1992), the E2F sites have been shown to be important for regulation, but it is still not clear whether the pRB–E2F complexes regulate these sites. However, the direct link between pRB and E2F-mediated transcription will provide one of the needed experimental systems to advance these studies.

This work was made possible from grants from the National Institutes of Health (U.S.A.) and institutional support from Massachusetts General Hospital to E.H. K.H. is supported by a fellowship from the Danish Medical Research Council, and E.H. is an American Cancer Society Research Professor.

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