The EMBO Journal vol.15 no.21 pp.5917–5927, 1996

Dual mechanisms of repression of E2F1 activity by the retinoblastoma gene product

Eldad Zacksenhaus[1,2,3], Zhe Jiang[1], Robert A.Phillips[1,4] and Brenda L.Gallie[1,4]

[1]Division of Immunology and Cancer Research, Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8 and [4]Department of Molecular and Medical Genetics, University of Toronto, Canada M5G 1L6

[2]Present address: Oncology Research Laboratories, The Toronto Hospital, 67 College Street, Toronto, Ontario, Canada M5G 2M1

[3]Corresponding author

The retinoblastoma gene product, pRb, negatively regulates cell proliferation by modulating the activity of the transcription factor E2F1 that controls expression of S-phase genes. To dissect transcriptional regulation of E2F1 by pRb, we developed a means to control the subcellular localization of pRb by exchanging its constitutive nuclear localization signal (NLS) with an inducible nuclear targeting domain from the glucocorticoid receptor (GR). In co-transfection experiments in hormone-free media, pRb$^{\Delta NLS}$–GR sequestered E2F1 in the cytoplasm; addition of steroid hormones induced co-translocation of pRb$^{\Delta NLS}$–GR and E2F1 to the nucleus. A pRb allele lacking a NLS, pRb$^{\Delta NLS}$, also sequestered E2F1 in the cytoplasm. Both nuclear and cytoplasmic pRb$^{\Delta NLS}$–GR repressed transcription from a simple, E2F1-activated, promoter equally well. pRb$^{\Delta NLS}$–GR exerted differential effects on complex promoters containing an activator and E2F sites that acted as either positive or negative elements. We propose a dual mechanism of transcriptional repression by pRb which allows tight control of E2F1-responsive genes: a pRb–E2F1 repressor unit is assembled off DNA to pre-empt transcriptional activation by E2F1; recruitment of this repressor unit to cognate binding sites on promoters allows silencing of adjacent promoter elements.

Keywords: E2F/nuclear transport/pRb/retinoblastoma/transcriptional repression

Introduction

In mammalian cells, the decision of whether to undergo DNA synthesis and cell duplication, or to stop proliferation and terminally differentiate, is made late in G_1 at the restriction (R) point (Pardee, 1990). The retinoblastoma gene product, pRb, is thought to play a key role in regulating the transition through the R point in response to mitogenic and differentiation signals (Zacksenhaus *et al.*, 1993a; Wang *et al.*, 1994; Weinberg, 1995). Accordingly, overexpression of pRb can induce growth arrest at G_1 only prior to the R point (Goodrich *et al.*, 1991). Moreover, the time during the cell cycle when pRb is inactivated by phosphorylation coincides with the R point (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989), and various proliferation and differentiation signals converge to control the activity of pRb by regulating the cyclin-dependent kinases (CDKs) which phosphorylate and inactivate pRb (Sherr and Roberts, 1995). The transforming proteins of several DNA viruses, including adenovirus E1a (Whyte *et al.*, 1988), SV40 large T (DeCaprio *et al.*, 1988) and papillomavirus E7 (Dyson *et al.*, 1989) bind and inactivate pRb, apparently as a prerequisite for bypassing cellular control over the R point.

Heterozygous germline mutations in the retinoblastoma gene, *RB1*, predispose to retinoblastoma in infants and, to a lesser extent, osteosarcoma in the second decade of life. Somatic mutations in *RB1* are prevalent in a wide spectrum of tumors including lung, breast, prostate and bladder (Gallie, 1994). Other tumors with apparently normal *RB1* frequently have activated cyclin D1 or CDKs, or mutated CDK inhibitors, rendering pRb hyper-phosphorylated and non-functional (reviewed by Weinberg, 1995).

pRb is a member of a family of proteins, including p107 (Ewen *et al.*, 1991) and p130 (Hannon *et al.*, 1993; Li *et al.*, 1993) that share a region—termed the pocket domain—through which they interact with various nuclear factors. pRb appears to exert its effect on cell growth and differentiation through interaction with transcription factors (Defeo-Jones *et al.*, 1991; Huang *et al.*, 1991; Kim *et al.*, 1992; Hagemeier *et al.*, 1993a; Wang *et al.*, 1993), modulators of chromatin conformation (Dunaief *et al.*, 1994; Singh *et al.*, 1995), proto-oncogenes (Welch and Wang, 1993; Xiao *et al.*, 1995) and differentiation factors (Gu *et al.*, 1993; Schneider *et al.*, 1994). The most well-characterized partners for the pocket proteins are members of the E2F-DP family of heterodimeric transcription factors (Bandara *et al.*, 1994; Helin and Harlow, 1994). pRb binds preferentially with E2F1, 2 and 3; p107 with E2F4; and p130 with E2F4 and 5 (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Hijmans *et al.*, 1995; Sardet *et al.*, 1995; Vairo *et al.*, 1995). The interaction of pRb with E2F1 alone or DP1 alone is weak, whereas interaction with the E2F1/DP1 heterodimer is highly stable *in vitro* (Helin and Harlow, 1994). Phosphorylation of either pRb (Chellappan *et al.*, 1991) or E2F1 (Fagan *et al.*, 1994) prevents formation of the pRb–E2F/DP ternary complex. pRb blocks transcriptional activation by binding a region in E2F which interacts with the general transcription factor, TBP (Hagemeier *et al.*, 1993b).

Through its interaction with E2F1, pRb controls expression of genes involved in G_1/S transition and DNA synthesis (Degregori *et al.*, 1995). In co-transfection experiments, pRb represses transcription of E2F-responsive genes such as the adenovirus E2A gene (Arroyo and Raychaudhuri, 1992; Hamel *et al.*, 1992; Hiebert *et al.*, 1992; Zamanian and La Thangue, 1992). While the E2F sites in some promoters such as c-myc are positive

elements, in many cellular promoters, such as B-Myb (Lam *et al.*, 1994), *RB1* (Zacksenhaus *et al.*, 1993c) and E2F1 (Hsiao *et al.*, 1994; Johnson *et al.*, 1994), E2F-binding sites behave as silencers and disruption of E2F binding stimulates transcription. Indeed, Weintraub *et al.* have shown that an E2F-binding site can act as a positive or a negative element depending on the presence of pRb, suggesting that pRb may silence transcription once bound to E2F on DNA (Weintraub *et al.*, 1992, 1995). The temporal events leading to transcriptional repression by pRb are, however, ill-defined. It is unclear whether pRb interacts with E2F on or off the promoter, or whether the sequestration of E2F is linked to silencing by pRb. In this report, we describe a pRb allele with inducible nuclear localization and show that pRb can control the subcellular localization of E2F1, indicating that the intracellular interaction of pRb and E2F1 is remarkably strong. We suggest that a pRb–E2F1/DP1 complex is assembled off DNA, that pRb pre-empts transcriptional activation by E2F1, and that pRb–E2F1/DP1 arrives at an E2F-site on a promoter as a repressor unit composed of a DNA binding domain (provided by E2F1/DP1) and an active repressor domain (provided by pRb). We further examine the effects of having the pRb–E2F1/DP1 complex on or off simple and complex promoters containing positive or negative E2F sites.

Results

Inducible nuclear transport of pRb$^{\Delta NLS}$–GR chimera

The C-terminal region of the glucocorticoid receptor (GR) contains an inducible nuclear localization signal (NLS), and hormone and heat shock protein 90 (HSP90) binding domains that confer hormonal regulation by controlling conformation and subcellular localization (Giguere *et al.*, 1986; Rusconi and Yamamoto, 1987). Amino acid 491 of the GR is located within a constitutive bipartite NLS at the border of the hormone-binding domain (Picard and Yamamoto, 1987). Deletion up to amino acid 515 removes the entire constitutive NLS and results in consistent cytoplasmic localization and hormone-inducible nuclear accumulation (Kang *et al.*, 1994). We made a series of fusion constructs consisting of full-length pRb, and various derivatives, fused in-frame to amino acid 491 or 515 of the GR (Figure 1A). Expression of the pRb–GR plasmids was analyzed by transient transfection into C33A cells, a $RB^{-/-}$ cervical carcinoma cell line (Scheffner *et al.*, 1991). Expression of the different pRB–GR proteins was similar and ~3-fold lower than expression from wild-type pRb plasmids (Figure 1B).

Subcellular localization of the various pRb–GR plasmids was assessed by immunostaining following transient transfection into C33A cells which were maintained in steroid hormone-depleted medium (see Materials and methods). A fusion chimera between native pRb and the GR (pRb^{WT}–GR) was constitutively nuclear, suggesting that the NLS of pRb can override the effect of the GR (Figure 1A and C, panel a). Therefore, we fused the GR to a NLS-deficient pRb, $pRb^{\Delta NLS}$ (Zacksenhaus *et al.*, 1993b; Figure 1A). The resultant chimera, $pRb^{\Delta NLS}$–GR491, was confined to the cytoplasm in the absence of hormone (Figure 1C, panel c); addition of dexamethasone (Dex) induced nuclear localization (Figure 1C, panel d). Nuclear accumulation of $pRb^{\Delta NLS}$–GR491 was detected as early as 10–15 min after addition of hormone and reached a maximum within 30–45 min (data not shown). Judged by immunostaining, the subcellular localization of the constructs with GR515 were similar to those with GR491, though nuclear accumulation of the former in the presence of Dex was slower (Figure 1C and data not shown). The two chimeric proteins $pRb^{\Delta NLS}$–GR491 and $pRb^{\Delta NLS}$–GR515, were both used in the experiments described below with similar results. In further experiments and discussion we have referred only to '$pRb^{\Delta NLS}$–GR'. Fusion proteins containing deletions that disrupted the integrity of the pocket domain, $pRb^{\Delta NLS\Delta Dra}$–GR and $pRb^{NLS\Delta 22}$–GR, were constitutively cytoplasmic, even in the presence of hormone (Figure 1C, panel b).

To confirm the subcellular distribution of $pRb^{\Delta NLS}$–GR, transfected C33A cells were fractionated into nuclear and cytoplasmic components and the presence of immunoreactive pRb was assessed by Western blotting (Figure 1D). Importantly, in the absence of hormone, $pRb^{\Delta NLS}$–GR was present predominantly in the cytoplasmic fraction. Following the addition of hormone, $pRb^{\Delta NLS}$–GR was found both in nuclear and cytoplasmic fractions, although by immunostaining it appeared completely nuclear (compare Figure 1C with D). This discrepancy was due to the fact that phosphorylated species and pocket mutants of pRb leak out of the nucleus during nuclear–cytoplasmic fractionation under low salt conditions (Mittnacht and Weinberg, 1991; Templeton *et al.*, 1991; Figure 1D). $pRb^{\Delta NLS}$–GR does not show a mobility shift when phosphorylated (data not shown), so the proportion of hypo- and hyper-phosphorylated species was not evaluated in these experiments.

pRb$^{\Delta NLS}$–GR co-localizes with nuclear adenovirus E1A and cytoplasmic SV40 large T^{Asn128}

To determine whether in the absence of hormone, cytoplasmic $pRb^{\Delta NLS}$–GR was able to interact with pRb-binding proteins, we analyzed the effect of the viral oncoproteins, E1A and large T, on the subcellular localization of $pRb^{\Delta NLS}$–GR. Co-transfection of E1A caused nuclear localization of $pRb^{\Delta NLS}$–GR in the absence of Dex (Figure 2, panels a and b). SV40 large T^{Asn128} has a mutation that transforms a lysine residue to asparagine in its NLS, rendering the protein cytoplasmic (Lanford and Butel, 1984). When $pRb^{\Delta NLS}$–GR and T^{Asn128} were co-transfected into C33A cells, both proteins were retained in the cytoplasm, even in the presence of Dex (Figure 2, panels c and d). Thus, $pRb^{\Delta NLS}$–GR subcellular localization can be completely overridden by interaction with T^{Asn128} or E1A. These results indicate that the GR motif controls nuclear localization, but not the conformation of $pRb^{\Delta NLS}$–GR.

pRb$^{\Delta NLS}$–GR and pRb$^{\Delta NLS}$ modulate the subcellular localization of E2F1

We next determined the subcellular localization of co-transfected $pRb^{\Delta NLS}$–GR and E2F1. E2F1 transfected into C33A cells maintained in 10% FCS, was localized in the nucleus as previously observed (Qin *et al.*, 1995; also see below). Co-transfection of E2F1 with $pRb^{NLS\Delta 22}$–GR, the latter of which is defective in binding E2F1, did not disturb the nuclear transport of E2F1, shown by double

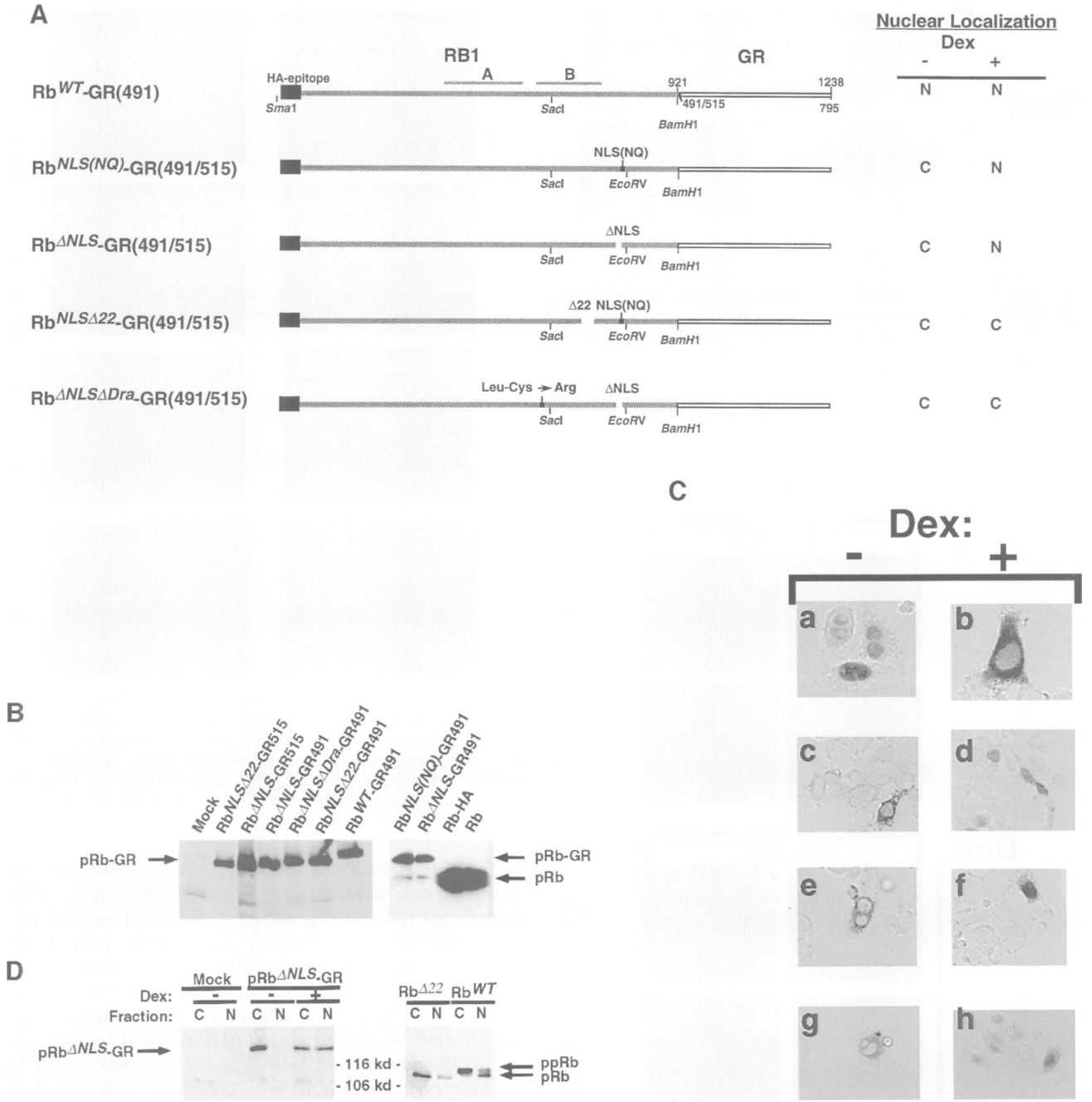

Fig. 1. (**A**) Schematic structure of pRb–GR chimera and several derivatives. The stop codons of pRb and pRb$^{\Delta NLS}$ were converted into *Bam*HI sites and ligated to *Bam*HI sites previously introduced into the glucocorticoid receptor at amino acids 491 or 515 (Giguere *et al.*, 1986). The pRb–GR plasmids are under control of RSV–LTR promoter. The subcellular localization of the pRb–GR derivatives in the presence or absence of Dex, determined as in (C), is indicated. (**B**) Expression of pRb–GR fusion proteins in C33A cells. The indicated expression plasmids were transfected into C33A cells maintained in 10% fetal bovine serum and the transiently expressed proteins were detected by Western blot analysis with anti-pRb antibodies (G3-245, PharMingen). (**C**) Subcellular localization of pRb–GR chimera. C33A cells maintained in 10% charcoal-treated fetal bovine serum and phenol red minus α-MEM, were transfected with the indicated plasmids. The following day, cells were washed once with PBS, and re-fed fresh medium. After an additional 24 h, cells were exposed to 10 μM Dex (+) or ethanol (–). 1 h later, cells were washed twice with cold PBS and immunostained using monoclonal anti-pRb antibodies (G3-245). (a) pRb^{WT}–GR; (b) pRb$^{NLS\Delta 22}$–GR; (c and d), pRb$^{\Delta NLS}$–GR491; (e and f), pRb$^{\Delta NLS}$–GR515; (g and h), pRb$^{NLS(NQ)}$–GR515. (**D**) Subcellular localization of pRb–GR proteins expressed in C33A cells. C33A cells maintained in 10% charcoal-treated fetal bovine serum and phenol red minus α-MEM, either mock-transfected or transfected with pRb$^{\Delta NLS}$–GR, pRb or pRb$^{\Delta 22}$, were fractionated into nuclear and cytoplasmic components and Western blotted, using monoclonal anti-pRb antibodies (G3-245, PharMingen). Cells were treated with 5 μM Dex (+) or ethanol (–) 36 h prior to harvesting the cells, as indicated.

immunofluorescent staining (Figure 3, panels a and b). In 10% FCS, co-expressed E2F1 and pRb$^{\Delta NLS}$–GR were retained predominantly in the cytoplasm (Figure 3, panels c and d). In 10% dialyzed serum without Dex, pRb$^{\Delta NLS}$–GR and E2F1 were exclusively cytoplasmic (Figure 3, panels e and f); addition of Dex induced nuclear accumulation of both proteins with a similar kinetics, suggesting that pRb$^{\Delta NLS}$–GR and E2F1 enter the nucleus as a complex (Figure 3, panels g and h, i and j).

To confirm that the cytoplasmic interaction between

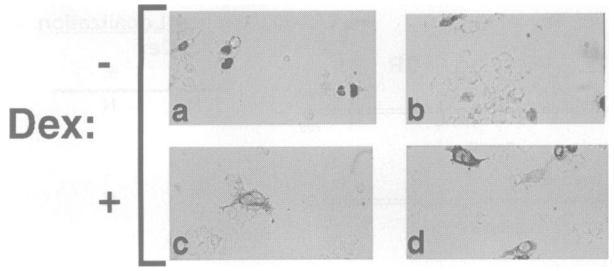

Fig. 2. Adenovirus E1A and SV40 Large T^{Asn128} dictate the subcellular localization of $pRb^{\Delta NLS}$–GR. C33A cells maintained in 10% 10K dialyzed serum in α-MEM without phenol red, were transfected with $pRb^{\Delta NLS}$–GR either alone (not shown), with E1A (a and b) or with T^{Asn128} (c and d). (Panels **a** and **b**) Cells were maintained in the absence of Dex and immunostained with pRb antibody (a) or E1A antibody (b). (Panels **c** and **d**) Dex was added to a final concentration of 10 μM 1 h prior to immunostaining with pRb antibody (c) or Large T antibody (d).

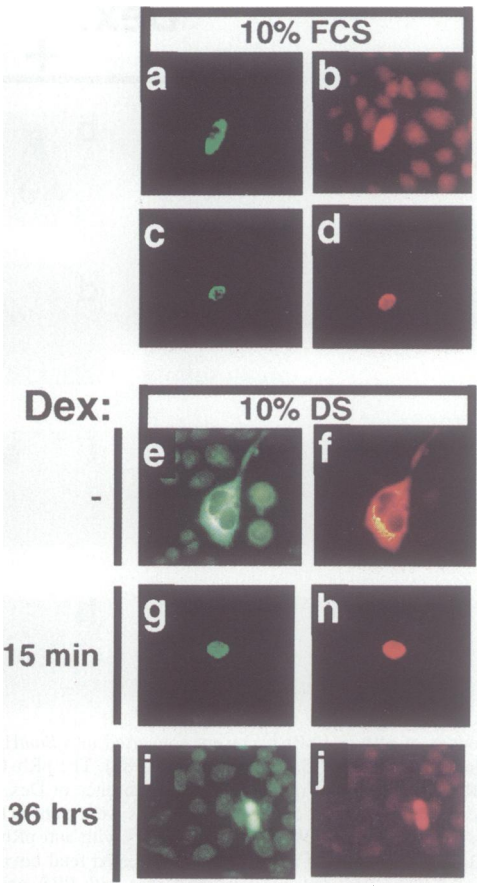

Fig. 3. $pRb^{\Delta NLS}$–GR modulates the subcellular localization of E2F1. C33A cells maintained in α-MEM plus 10% fetal calf serum (FCS) were transfected with pECE–E2F1, pCMV–DP1 (0.5 μg each) plus $pRb^{NLS\Delta 22}$–GR (4 μg) (panels **a** and **b**); E2F1/DP1 plus $pRb^{\Delta NLS}$–GR (panels **c** and **d**). C33A cells maintained in 10% 10K dialyzed serum (DS) in α-MEM without phenol red, were transfected with E2F1/DP1 plus $pRb^{\Delta NLS}$–GR in the absence of Dex (panels **e** and **f**); in the presence of 10 μM Dex for 15 min (panels **g** and **h**); or in the presence of 5 μM Dex for 36 h (panels **i** and **j**). Transfected cells were examined by fluorescent immunostaining with pRb monoclonal antibody (G3-245, PharMingen) and E2F1 rabbit polyclonal antibody (c-20, Santa Cruz). Secondary antibodies used were fluorescein (FITC)-conjugated goat anti-mouse (stains pRb green) and rhodamine-conjugated goat anti-rabbit (stains E2F1 red).

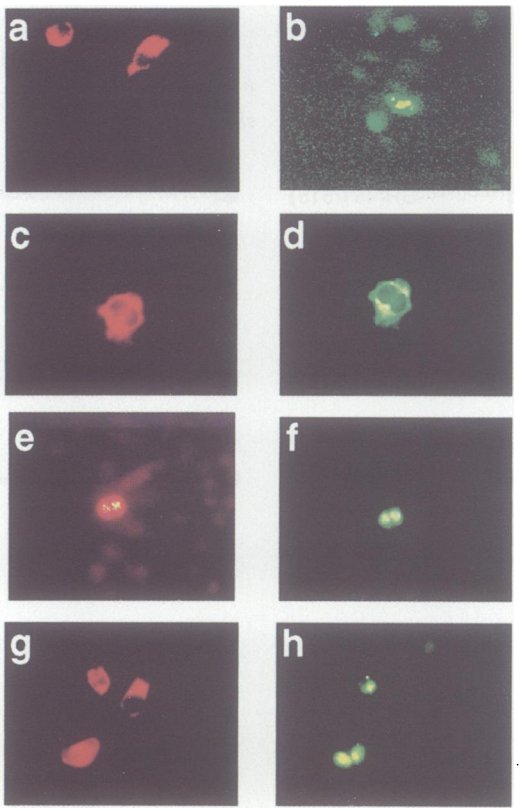

Fig. 4. $pRb^{\Delta NLS}$ controls nuclear transport of E2F1. C33A cells were transfected with the following plasmids and subject to double immunofluorescent staining with rabbit polyclonal antibodies to pRb (red) and monoclonal E2F1 or E1A antibodies (green). (Panel **a**) $pRb^{\Delta NLS}$ alone (4 μg); (**b**) pECE–E2F1, pCMVNDP1 alone (0.5 μg each); (**c** and **d**) $pRb^{\Delta NLS}$ plus E2F1/DP1; (**e** and **f**) $pRb^{\Delta NLS}$ plus E1A; (**g** and **h**) $pRb^{\Delta NLS\Delta Dra}$ plus E1A.

pRb and E2F1 was not a unique property of the pRb–GR fusion protein, we examined the $pRb^{\Delta NLS}$ derivative. When transfected individually, $pRb^{\Delta NLS}$ and E2F1 resided predominantly in the cytoplasm and nucleus, respectively (Figure 4, panels a and b). However, co-expression of E2F1 and $pRb^{\Delta NLS}$ resulted in retention of both in the cytoplasm (Figure 4, panels c and d). In contrast, co-transfection of $pRb^{\Delta NLS}$ with E1A resulted in their co-localization to the nucleus (Figure 4, panels e and f), whereas $pRb^{NLS\Delta Dra}$ (unable to bind E1A) was refractory to the presence of E1A (Figure 4, panels g and h).

Cytoplasmic $pRb^{\Delta NLS}$–GR represses transcription of a simple E2F1-regulated promoter

To determine the effect of subcellular localization of $pRb^{\Delta NLS}$–GR on E2F-dependent transcription, we used a synthetic promoter, $E2F_4$-TA.CAT (formerly $E2F_4$.CAT), that includes a TATA box and four tandem E2F binding sites (Helin *et al.*, 1993) (Figure 5A). The low activity of the $E2F_4$-TA.CAT in C33A cells maintained in hormone-depleted serum was stimulated 4- to 7-fold by co-transfecting 15–30 ng E2F1 and DP1 expression plasmids (Figure 5B). Strikingly, $pRb^{\Delta NLS}$–GR repressed the $E2F_4$-TA.CAT promoter irrespective of the presence or absence of Dex (Figure 5B). To rule out the possibility that the efficient repression in the absence of hormone was due to leakage of overexpressed $pRb^{\Delta NLS}$–GR into the nucleus, the amount of added $pRb^{\Delta NLS}$–GR was titrated. Even when the amount

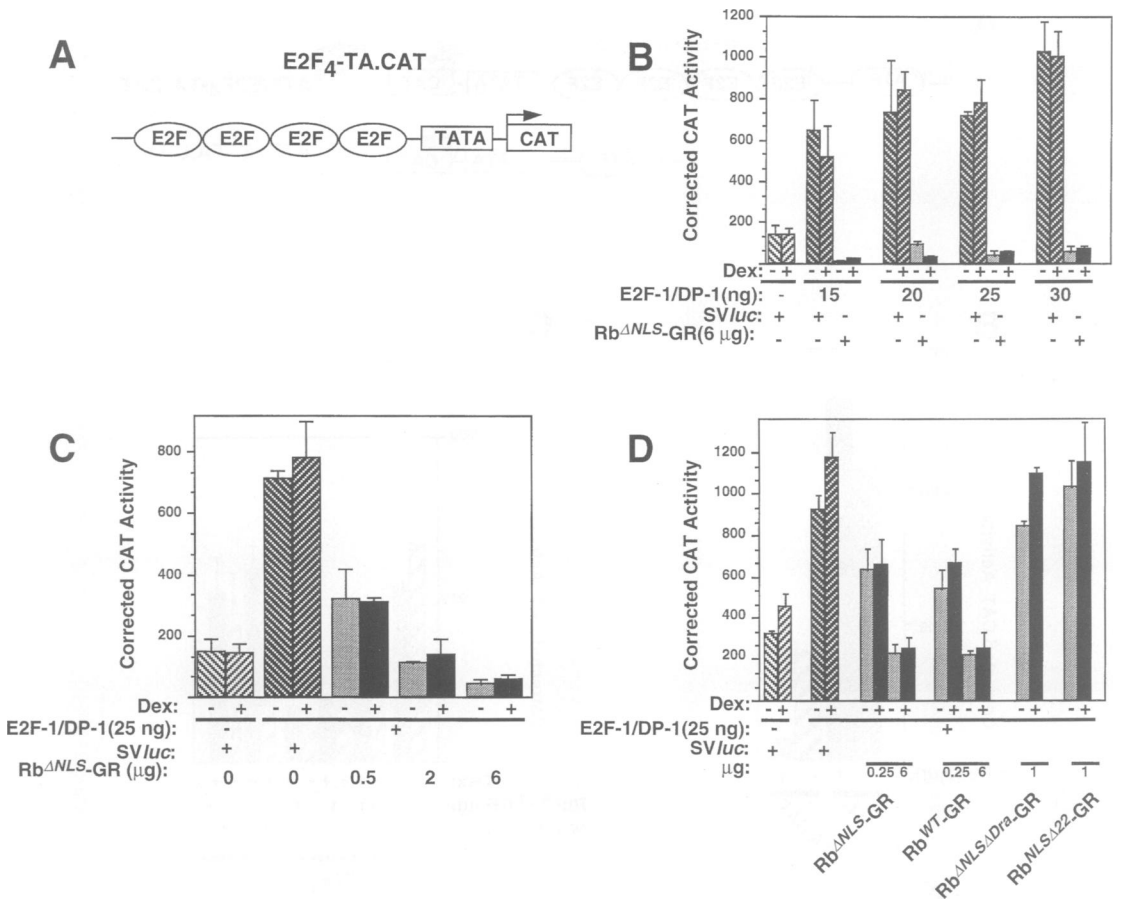

Fig. 5. Constitutive transcriptional repression of a simple promoter, $E2F_4$-TA.CAT, by cytoplasmic $pRb^{\Delta NLS}$–GR. (**A**) Schematic structure of $E2F_4$-TA.CAT (Helin *et al.*, 1993). (**B**) C33A cells maintained in 10% 10K dialyzed serum in α-MEM without phenol red, were transfected with $E2F_4$-TA.CAT (2 μg), $pRb^{\Delta NLS}$–GR (6 μg), and the indicated amounts each of pECE–E2F1 and pCMV–DP1, together with pRSV–GAL that served as internal control (2 μg). pSVluc, containing the luciferase gene under control of SV40 early region, was used as a control or for adjusting the total amount of transfected DNA. 6 h after transfection, the cells were washed once with PBS and re-fed fresh medium with either ethanol as control or Dex (5 μM). CAT activity was measured 36 h later. (**C**) C33A cells were transfected with $E2F_4$-TA.CAT (2 μg), E2F1 and DP1 (25 ng each), pRSV–GAL (2 μg) and increasing amounts of $pRb^{\Delta NLS}$–GR. pSVluc was used as a control or for adjusting the total amount of transfected DNA. (**D**) C33A cells were transfected with $E2F_4$-TA.CAT (2 μg), E2F1 and DP1 (25 ng each), RSV–GAL (2 μg) and the indicated expression plasmids. Arrow bars indicate standard deviations of duplicate (B and C) or triplicate (D) transfections from representative experiments.

of transfected $pRb^{\Delta NLS}$–GR was in the linear range of repression, cytoplasmic $pRb^{\Delta NLS}$–GR repressed $E2F_4$-TA.CAT activity as efficiently as Dex-induced nuclear $pRb^{\Delta NLS}$–GR (Figure 5C). If nuclear leakage accounted for repression in the absence of hormone, we would have observed a reduced efficiency of repression when $pRb^{\Delta NLS}$–GR was titrated. Transcriptional repression by $pRb^{\Delta NLS}$–GR was similar to the constitutively nuclear pRb^{WT}–GR both in the absence or presence of hormone; mutations in the pocket domain, such as $pRb^{\Delta NLS\Delta Dra}$–GR and $pRb^{NLS\Delta 22}$–GR, abrogated transcriptional repression (Figure 5D). We conclude from these data that cytoplasmic $pRb^{\Delta NLS}$–GR efficiently sequesters E2F1 in the cytoplasm, leading to strong repression of simple E2F1-regulated promoters.

Nuclear and cytoplasmic $pRb^{\Delta NLS}$–GR exert differential effects on complex promoters containing positive or negative E2F binding sites

We next sought to determine the effect of subcellular localization of $pRb^{\Delta NLS}$–GR on complex E2F-regulated promoters. We tested two synthetic promoters, ATF/$E2F_4$-TA.CAT (Figure 6A) and $E2F_2$/ATF-TA.CAT (Figure 7A) (formerly E2F/ATF-TA.CAT; Weintraub *et al.*, 1992) which contain identical ATF binding sites but different arrays of E2F sites (Loeken and Brady, 1989) that act as negative (ATF/$E2F_4$-TA.CAT) or positive ($E2F_2$/ATF-TA.CAT) elements with respect to ATF activity.

When placed downstream of an ATF site, the four E2F sites acted as a negative element: ATFΔE-TA.CAT from which the E2F sites were removed gave over twice the activity of ATF/$E2F_4$-TA.CAT (Figure 6B). Co-expression of wild-type pRb repressed ATF/$E2F_4$-TA.CAT but not ATFΔE-TA.CAT (Figure 6B). Both nuclear and cytoplasmic $pRb^{\Delta NLS}$–GR further repressed ATF/$E2F_4$-TA.CAT (Figure 6C). Repression was dependent on the amount of transfected DNA and the integrity of the pocket domain (Figure 6C).

The $E2F_2$/ATF-TA.CAT reporter contains two E2F sites that act as a positive element upstream of an ATF site (Weintraub *et al.*, 1992) (Figure 7A and B). Activity of $E2F_2$/ATF-TA.CAT was 20–40% higher than ATF-TA.CAT in different experiments with different preparations of plasmid DNA. Neither pRb (Weintraub *et al.*, 1992) nor

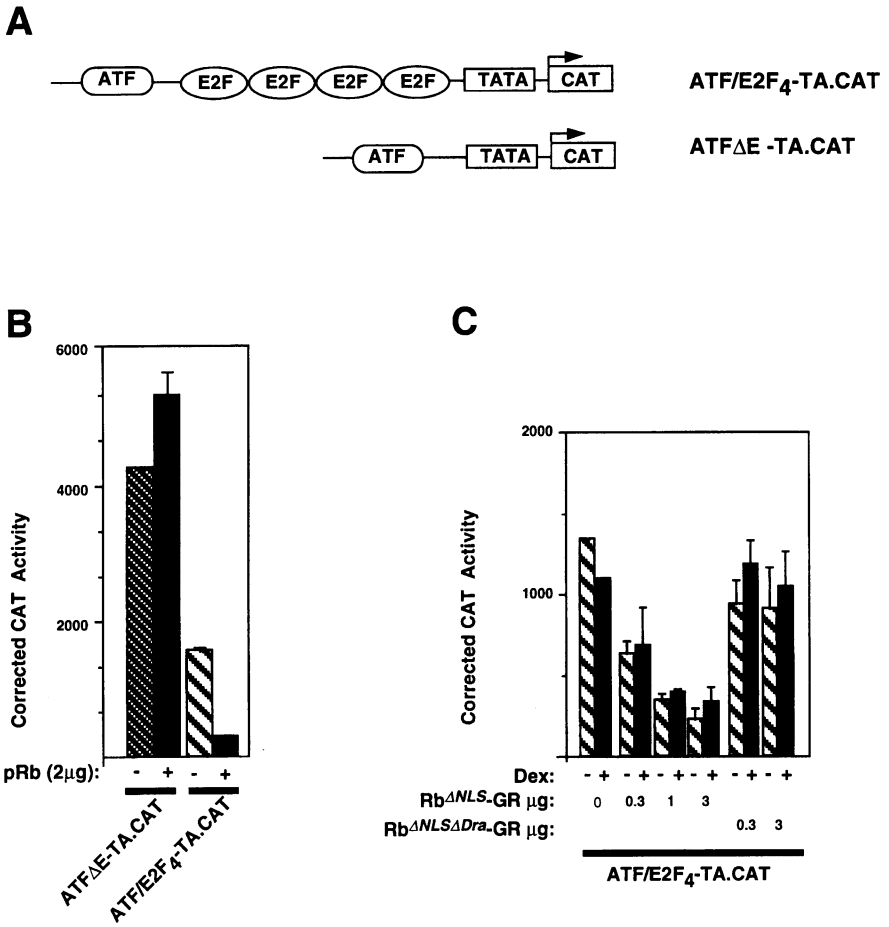

Fig. 6. Constitutive repression by pRb$^{\Delta NLS}$–GR of a complex promoter, ATF/E2F$_4$-TA.CAT, in which the E2F sites reduce transcriptional activity. (**A**) Schematic structures of ATF/E2F$_4$-TA.CAT and ATFΔE-TA.CAT. (**B**) C33A cells maintained in 10% FCII were transfected with ATFΔE-TA.CAT or ATF/E2F$_4$-TA.CAT with or without pRb. (**C**) C33A cells maintained in 10% 10K dialyzed serum in α-MEM without phenol red, were transfected with ATF/E2F$_4$-TA.CAT (2 μg), the indicated amounts of pRb$^{\Delta NLS}$–GR or pRb$^{NLS\Delta Dra}$–GR, together with the internal control, pRSV–GAL (2 μg), and CAT activity was determined in the absence or presence of Dex (5 μM). Arrow bars indicate standard deviations of duplicate transfections of a representative experiment.

pRb$^{\Delta NLS}$–GR (data not shown) repressed ATF-TA.CAT which lacks the E2F sites. Expression of pRb both repressed E2F activity and silenced the adjacent ATF site in E2F$_2$/ATF-TA.CAT (Weintraub *et al.*, 1992) (Figure 7B). Moderate repression of E2F$_2$/ATF-TA.CAT occurred when pRb$^{\Delta NLS}$–GR was in the cytoplasm (Figure 7C); full repression was achieved only when pRb$^{\Delta NLS}$–GR was induced by Dex to localize to the nucleus (Figure 7C). Specifically, under conditions (2 μg) in which cytoplasmic pRb$^{\Delta NLS}$–GR repressed 1.3-fold, nuclear pRb$^{\Delta NLS}$–GR repressed E2F$_2$/ATF-TA.CAT promoter activity 5-fold (Figure 7C). pRb^{WT}–GR and native pRb repressed equally in the absence or presence of Dex, once the amount of transfected DNA was adjusted to correct for expression levels (Figures 1B and 7C), whereas the pocket mutant, pRb$^{NLS\Delta 22}$–GR, had no effect on E2F$_2$/ATF-TA.CAT.

Discussion

A critical decision in the life of a cell is whether to proliferate or differentiate in response to internal and external cues. Many regulatory pathways are in place to ensure that genes participating in DNA synthesis or differentiation are not expressed prior to this decision. In the absence of such tight regulation, abnormal proliferation or cell death may ensue. Accumulating evidence indicates that through its interaction with E2F1, pRb regulates the R point by blocking the activity of genes required for DNA synthesis and cell cycle progression. Herein, we described a model for the temporal events that lead to transcriptional repression by pRb. In contrast to the current view that E2F1 is a simply an activator which is blocked by pRb, our results suggest that E2F1 is part of a repressor unit, composed of a DNA binding domain (E2F1/DP1) and a repressor (pRb), which is assembled independent of and prior to binding of E2F to DNA. If E2F arrived at promoters unguarded by pRb, inappropriate activation of S-phase genes with potential deleterious consequences could result. Our results suggest that this scenario would not happen as long as unphosphorylated pRb is available to interact with E2F prior to DNA binding. This mechanism of regulation is made possible by the relative excess of pRb over E2F in a cell and the high intracellular interaction between pRb and E2F1, documented in this study. This model for transcriptional repression by pRb allows tight regulation of transcription of S-phase genes, the loss of which may be detrimental to normal cell growth and differentiation.

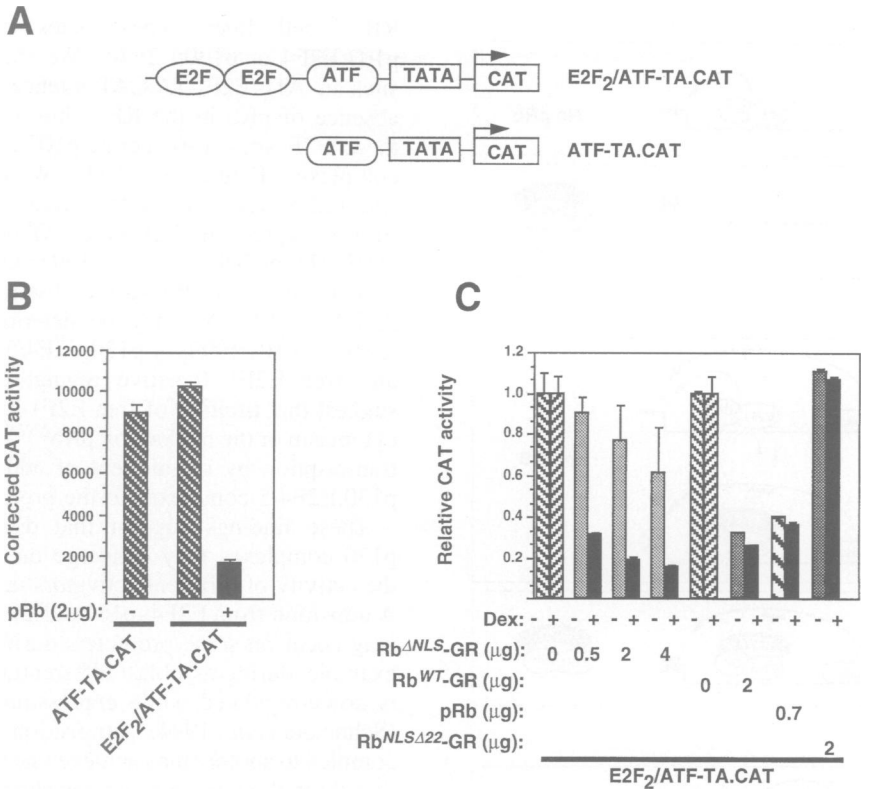

Fig. 7. Dex-inducible transcriptional repression by $pRb^{\Delta NLS}$–GR of a complex promoter, $E2F_2$/ATF-TA.CAT, in which the E2F sites promote transcriptional activity. (**A**) Schematic structures of $E2F_2$/ATF-TA.CAT and ATF-TA.CAT (Weintraub *et al.*, 1992). (**B**) C33A cells maintained in 10% FCII were transfected with $E2F_2$/ATF-TA.CAT or ATF-TA.CAT with or without pRb. (**C**) C33A cells maintained in 10% 10K dialyzed serum in α-MEM without phenol red, were transfected with $E2F_2$/ATF-TA.CAT (2 μg), the indicated amounts of $pRb^{\Delta NLS}$–GR, pRb^{WT}–GR, pRb or $pRb^{NLS\Delta 22}$–GR, together with the internal control, pRSV–GAL (2 μg), and CAT activity was determined in the absence or presence of Dex (5 μM) as described in the legend to Figure 2. pSVluc was used as a control or for adjusting the total amount of transfected DNA. CAT values were normalized for the respective $E2F_2$/ATF-TA.CAT activities in the presence or absence of Dex. Arrow bars indicate standard deviations of triplicate transfections.

The pRb–E2F1/DP1 repressor unit

It has been unknown whether pRb interacts with E2F1 already bound to a promoter, hence repressing ongoing transcription, or whether pRb–E2F interaction can occur prior to DNA binding, in which case pRb might pre-empt transcriptional regulation by E2F1. *In vitro* studies have not been informative in this regard since pRb forms a stable complex with E2F1 and DP1 both on and off E2F binding sites, revealed by co-immunoprecipitation and band-shift experiments (Chellappan *et al.*, 1991; Helin *et al.*, 1993). Our strategy of controlling nuclear localization of pRb allowed us to document the intracellular interaction of pRb and E2F1 in the absence of DNA binding. We showed that $pRb^{\Delta NLS}$–GR can modulate the subcellular localization of E2F1 and that $pRb^{\Delta NLS}$, a NLS-deficient pRb, also sequestered E2F1 in the cytoplasm in co-transfection experiments. These results strongly suggest that prior to the R point, E2F is complexed with pRb, allowing the latter to pre-empt transcription activation by E2F and further use E2F as a docking site to access E2F1-responsive genes and actively repress transcription. The pRb–E2F1/DP1 complex may be regarded as a repressor unit composed of a DNA binding domain (provided by the E2F1/DP heterodimer) and an active repressor domain (provided by pRb). One implication is that disruption of any component of the pRb–E2F/DP repressor unit could abrogate transcriptional silencing by pRb. This is in accord with knock-out experiments, published after the submission of this paper, showing that E2F1 acts as a tumor suppressor when homozygously deleted in the mouse (Field *et al.*, 1996; Yamasaki *et al.*, 1996).

Our results also raise the possibility that pRb may control the subcellular localization of E2F under physiological conditions. Using co-transfection experiments, nuclear co-transport of complex proteins has been implicated previously for nucleoplasmin (Dingwall *et al.*, 1982), progesterone (Guiochon-Mantel *et al.*, 1989), histone H2A (Moreland *et al.*, 1987), and MYC:MAX (Makela *et al.*, 1992). However, because available antibodies cannot detect endogenous E2F1 by immunostaining and because of possible contamination during nuclear/cytoplasmic fractionation, proving co-translocation of pRb and E2F *in vivo* is not presently possible.

Mechanisms of transcriptional repression by pRb

Using $pRb^{\Delta NLS}$–GR, we tested the effect of subcellular localization of the pRb–E2F repressor unit on three types of promoter. Our results demonstrate a dual mechanism of transcriptional repression by pRb: (i) pre-emptive transcriptional repression, achieved by sequestering E2F1 off DNA; (ii) transcriptional silencing, achieved by recruitment of the pre-assembled pRb–E2F1/DP1 complex to the promoter.

A simple E2F-regulated promoter, $E2F_4$-TA.CAT, stimu-

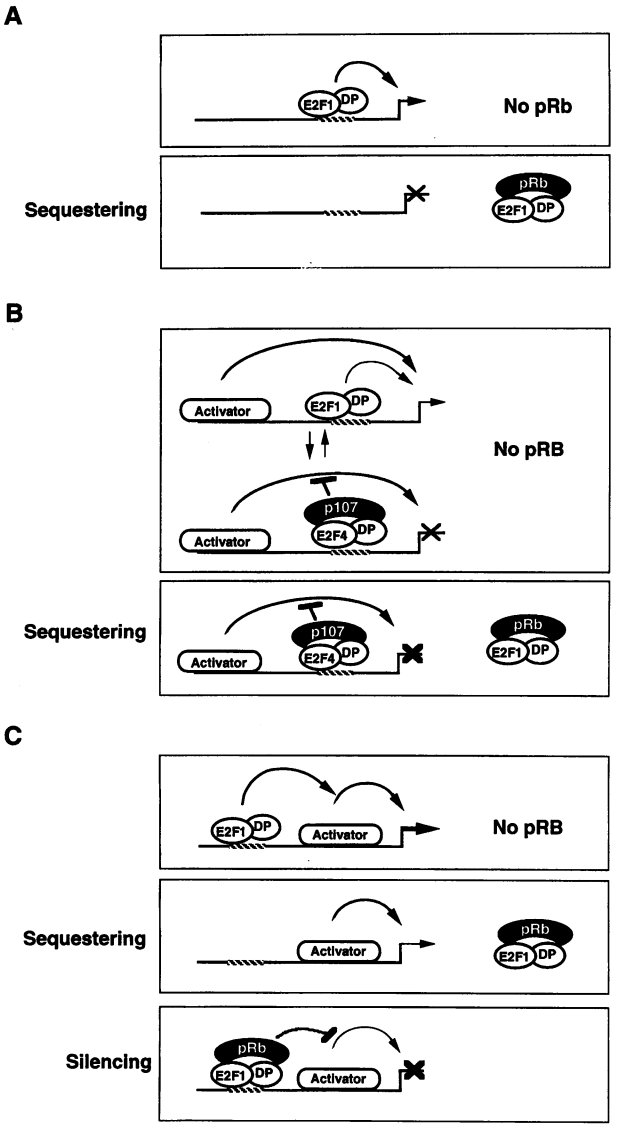

Fig. 8. Dual mechanisms of transcriptional repression by pRb: sequestration and silencing. (**A**) A simple E2F1-activated promoter is repressed by sequestration of E2F1/DP1 by pRb. Similar repression of promoter activity is achieved whether the pRb–E2F1/DP repressor unit is on or off the E2F site. (**B**) A complex promoter with a negative E2F element recruits either free E2F (activation) or p107:E2F4 (or p130:E2F4/5)(repression). Sequestration of E2F1 allows more p107:E2F4 (or p130:E2F4/5) to access and silence the promoter. (**C**) A complex promoter with a positive E2F element preferentially recruits free E2F. Sequestration of E2F1 by pRb only neutralizes its activity. Recruitment of the pRb–E2F1/DP repressor unit to the promoter is required for transcriptional silencing.

lated by co-expressed E2F1/DP1, was repressed by pRb$^{\Delta NLS}$–GR irrespective of subcellular localization (Figures 5 and 8A). We demonstrated that repression by cytoplasmic pRb$^{\Delta NLS}$–GR correlated with its ability to sequester E2F1 in the cytoplasm in the absence of hormone (Figure 3).

In many complex cellular promoters, E2F binding sites are negative elements, the disruption of which leads to 2- to 3-fold enhancement of promoter activity in unsynchronized cells (Zacksenhaus *et al.*, 1993c; Hsiao *et al.*, 1994). For example, the E2F sites in the B-Myb and *RB1* promoters act as negative elements both in normal and in RB$^{-/-}$ cell lines. These sites preferentially recruit p107:E2F4 or p130:E2F4/5. We show that the four E2F sites in ATF/E2F$_4$-TA.CAT silence the ATF site in the absence of pRb in the RB$^{-/-}$ line C33A, suggesting that these E2F sites also recruit p107:E2F4 or p130:E2F4/5 complexes (Figures 6 and 8B). We note that E2F1, E2F4 and E2F5 were shown to activate and pRb, p107 and p130 to repress the E2F$_4$-TA.CAT promoter (Helin *et al.*, 1993; Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Hijmans *et al.*, 1995). In the absence of pRb, activity of ATF/E2F$_4$-TA.CAT may be determined by the relative levels of p107:E2F4 or p130:E2F4/5 (negative regulation) and free E2F1 (positive regulation) (Figure 8B). We suggest that titration of free E2F1 by sequestration in the cytoplasm or the nucleus by pRb$^{\Delta NLS}$–GR further represses transcription by recruitment of additional p107:E2F4 or p130:E2F4/5 complexes to the promoter (Figure 8B).

These findings suggest that distinct E2F–pRb/p107/p130 complexes may converge on E2F sites to regulate the activity of different activators in a complex promoter. A transition from E2F4–p107 to E2F1/2/3–pRb complexes may occur on some promoters during differentiation. For example, during myoblast differentiation, p107 expression is down-regulated while expression of pRb is sustained (Schneider *et al.*, 1994). A transition from one E2F binding complex to another may achieve fine transcriptional regulation through a single promoter element.

In other complex promoters, E2F binding sites act as positive elements, the disruption of which leads to loss of promoter activity. Such promoters preferentially recruit free E2Fs, rather than p107:E2F4 or p130:E2F4/5 complexes. On such a promoter, E2F$_2$/ATF-TA.CAT, the full repressive effect of pRb$^{\Delta NLS}$–GR was only achieved when the fusion protein was induced to enter the nucleus (Figures 7 and 8C). In the absence of hormone, cytoplasmic pRb$^{\Delta NLS}$–GR may only sequester E2F1, neutralizing the positive effect of the E2F sites, but not silencing the downstream ATF site.

Why some E2F binding sites serve as positive elements and others as negative elements is still unclear. The E2F sites in the synthetic reporters ATF/E2F$_4$-TA.CAT and E2F$_2$/ATF-TA.CAT have opposing effects (Figures 6 and 7). A swapping analysis between ATF/E2F$_4$-TA.CAT and E2F$_2$/ATF-TA.CAT may reveal whether the differential activity of these E2F sites is related to the relative position of the E2F and ATF elements, the number of E2F sites, the junction DNA sequence between the E2F elements and vector DNA, or the TATA boxes.

Materials and methods

Cell culture and transfection

The RB$^{-/-}$ C33A cervical carcinoma and Saos-2 osteosarcoma cell lines (obtained from the American Culture Collection) and NIH 3T3 mouse fibroblasts were cultured in α-MEM medium (GIBCO-BRL), supplemented with 10% fetal calf serum or fetal clone II (Hyclone).

To deplete the medium of steroid hormones, the C33A cells were initially maintained in conventional steroid-free charcoal-treated fetal bovine serum (CBI) and phenol red minus α-MEM (GIBCO-BRL). After testing several media/sera and synthetic growth factors, we found 10% dialyzed serum (10 000 mol. wt cut-off, Sigma) to be optimal; there was no difference in the subcellular distribution of pRb$^{\Delta NLS}$–GR, but the transfection efficiency of C33A cells was ~10-fold better than in charcoal-treated serum (data not shown). Plasmid DNA was purified on Qiagen columns. Cells were plated in 60 mm dishes and transfected

by the calcium phosphate method the following day at ~50% confluence. For immunostaining, coverslips were sterilized by immersing in 75% ethanol and placed in 60 mm dishes prior to plating the cells. At 6–8 h after transfection, the cells were washed once with PBS, and re-fed fresh medium. Dexamethasone (Sigma), dissolved in ethanol, or pure ethanol as control, was added at 5 μM for 36 h or at 10 μM for 1 h or less. Assays were performed 2 days after transfection.

Plasmids

pRb–GR: the stop codon of the mouse *RB1* gene was converted into a *Bam*H1 site by PCR with an antisense oligo 5′-AGGGCCCTGAGGGATCCG-CTTTTCCTTCTT (*Bam*HI site underlined) and a primer that overlaps the *Sac*I site in *RB1*. The template was either wild-type *RB1* or $RB1^{\Delta NLS}$ (Zacksenhaus *et al.*, 1993b). The PCR product was gel-purified, digested with *Sac*I and *Bam*HI and ligated into pECE–HA–RB1 pre-digested with *Sac*I, located in *RB1*, and *Bam*HI, located in the SV40 polyadenylation site in pECE. Clones isolated at this stage from the two plasmids were sequenced on both strands to verify the integrity of the DNA sequence. Next, the stop-to-*Bam*HI modified *RB1* was transferred as a *Hin*dIII (blunted)–*Bam*HI fragment into a *Kpn*I (blunted), *Bam*HI-digested RSV–hGH clone I491, kindly provided by V.Giguere (Giguere *et al.*, 1986). Additional $pRb^{\Delta NLS}$–GR derivatives were generated by exchanging *Sac*I–*Eco*RV fragments between $pRb^{\Delta NLS}$–GR and $RB1^{NLS(NQ)}$ (Zacksenhaus *et al.*, 1993b), $RB1NLS^{(NQ)\Delta 22}$ (Zacksenhaus *et al.*, 1993b) and $RB1^{\Delta NLS\Delta Dra}$. The latter, $RB1^{\Delta NLS\Delta Dra}$, was generated by cutting $RB1^{\Delta NLS}$ with *Dra*III, blunting with Klenow, followed by re-ligation. The $pRb^{\Delta NLS}$–GR515 series was constructed by replacing a $BamHI^{491}$–*Xho*I fragment that contains the C-terminus GR and the polyadenylation site from $pRb^{\Delta NLS}$–GR491 with a $BamHI^{515}$–*Xho*I fragment from RSV–hGH clone I515 (Giguere *et al.*, 1986).

ATF/$E2F_4$.CAT: two oligos (5′-TCGAGCCCGTGACGTCACCCGC) specifying the fibronectin ATF element (Weintraub *et al.*, 1992) flanked by *Xho*I sites were subcloned into the unique *Xho*I site in $E2F_4$.CAT (Helin *et al.*, 1993). Clones containing the ATF site were identified by digestion with *Aat*II (which cuts the vector and the ATF oligo releasing a 332 bp fragment) and orientation of the ATF site was determined by sequencing several clones. ATFΔE.CAT was derived from ATF/$E2F_4$.CAT by digestion with *Pst*I and *Xba*I, which flank the four E2F sites, blunt-ending with Klenow and re-ligation. All constructs were verified by restriction digest and/or Western blots, and PCR products were sequenced to identify possible PCR artifacts.

Immunostaining

Immunostaining using HRP-conjugated secondary antibody followed by DAB/H_2O_2 reaction was as described (Zacksenhaus *et al.*, 1993b). Immunofluorescent labeling was performed at room temperature on cells grown and transfected on coverslips in a 60 mm dish. About 36 h after transfection, cells were washed once with PBS, fixed with cold (–20°C) methanol for 30 min, washed three times with PBS for a total of 15 min, and treated with blocking solution [BS; 1% BSA, w/v (Sigma), 2% normal goat serum (Vector), in PBS] for 1 h. The BS was replaced with a mixture of mono- and polyclonal antibodies diluted in BS and incubation proceeded for 1 h. Monoclonal antibodies were diluted 1:100 to 1:150. Polyclonal anti-pRb and anti-E2F1 were diluted 1:2000 and 1:1000, respectively. Excess antibodies were washed off with three changes of PBS for a total of 20–30 min. In the dark, a mixture of fluorescent anti-mouse and anti-rabbit secondary antibodies was diluted in BS (1:200) and applied for 1 h. After three washes in PBS for 20 min, the coverslips were mounted on slides using anti-fader mounting medium (Kirkegaard and Perry Laboratories, Cat#71-00-16), observed by fluorescence microscopy and photographed with a MC80 camera (Zeiss, Germany).

The monoclonal anti-pRb (G3-245) recognizes an epitope in the N-terminus of pRb (aa 300–380, PharMingen). The rabbit polyclonal anti pRb (c-15) and anti-E2F1 (c-20) and a monoclonal anti-E2F1 (KH95) were from Santa Cruz. The monoclonal anti adenovirus 2 E1A (AB-1) was from Oncogene Science; anti-large T (419) was a gift from E.Harlow. HRP-conjugated goat anti-mouse and anti-rabbit secondary antibodies were from Bio-Rad. Rhodamine-conjugated goat anti-rabbit and fluorescein (FITC)-conjugated goat anti-mouse antibodies were purchased from Jackson Immunoresearch Laboratories, Inc.

Immunoblots

Nuclear–cytoplasmic fractionation was performed as described by Templeton *et al.* (1991), except that instead of using Dounce homogenizer, the swollen cells were disrupted by 20 rapid passes through a Pasteur pipette. The extent of cellular disruption and the appearance of isolated nuclei were monitored microscopically.

CAT assays

C33A cells were transfected with the indicated reporter and effector plasmids together with RSV–βGAL as internal control. pSVluc, containing SV40 early region and luciferase gene, was used as a stuffer or control plasmid. Assays for chloramphenicol acetyl transferase (CAT) and β-galactosidase activities were as described previously (Zacksenhaus *et al.*, 1993c) except that instead of freeze–thaw, cells were lysed in 100 μl Reporter lysis buffer (Promega). Each CAT assay was performed at least three times using duplicate or triplicate dishes as noted.

Acknowledgements

We thank Drs V.Giguere for the provision of pRSV–hGHI491 and pRSV–hGHI515, J.Butel for large $T^{\Delta NLS}$, W.Kaelin and P.Hamel for pECE–E2F1, S.Shirodkar for pCMV–DP1, E.Harlow for large T antibody and $E2F_4$.CAT, S.Weintraub for E2F/ATF-TA.CAT and ATF-TA.CAT, R.Bremner for pSVluc, S.Egan, L.Penn and D.Bentley for critical comments on the manuscript, O.Jin for sequencing one of the PCR products, and Ms G.Fidalgo for assistance in preparation of plasmid DNA. E.Z. was supported in part by an NCI post-doctoral fellowship. This work was supported by the National Cancer Institute of Canada with funds from the Terry Fox Run; the Medical Research Council of Canada; the Canadian Genetic Disease Network; the Retinoblastoma Family Association; and the Royal Arch Masons of Canada.

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Received on November 27, 1995; revised on July 11, 1996