Oncogene (1998) 17, 867–876

http://www.stockton-press.co.uk/onc

The E2F-family proteins induce distinct cell cycle regulatory factors in p16-arrested, U343 astrocytoma cells

Peter B Dirks[1,2], James T Rutka[1,2], Sherri Lynn Hubbard[1], Soma Mondal[2] and Paul A Hamel[2]

[1]Divisions of Neurosurgery and Surgical Research, Hospital for Sick Children, University of Toronto; and [2]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada

We previously demonstrated that P16^{Ink4a} (p16) expression in p16-deficient U343 astrocytoma cells causes a G_1 cell cycle arrest, profound changes in cytoskeletal proteins and alterations in expression and activity of the pRB and E2F family proteins. We examine here the effects of expressing wild type or mutant versions of the downstream targets of p16 in U343 astrocytomas. We first attempted to block proliferation of U343 cells using the dominant mutant of pRB, Δp34. Expression of this mutant in the human osteosarcoma, SAOS-2, potently blocked proliferation but did not affect the cell cycle of U343 cells. We next showed that expression of E2F-1, E2F-2, E2F-3 and E2F-4 are each able to overcome this p16-dependent cell cycle arrest but exhibit distinct biological activities. Adenoviral-mediated expression of E2F-1, E2F-2, E2F-3, or E2F-4 overcame the p16-dependent cell cycle block and induced alterations in cell morphology. E2F-5, only in conjunction with DP1, promoted cell cycle progression. For both E2F-1 and E2F-2, but not E2F-3 or E2F-5/DP1, cell cycle re-entry was associated with almost quantitative cell death. Only small numbers of dying cells were observed in E2F-4-expressing cultures. Expression of the different E2F's altered the expression of distinct sets of cell cycle regulatory proteins. E2F-1 induced endogenous E2F-4 expression and also caused an increase in pRB, p107 and cyclin E levels. Expression of E2F-4 caused a weak increase in E2F-1 levels but also strongly induced pRB, p107, p130 and cyclin E. However, E2F-1 and E2F-4 clearly regulate expression of distinct genes, demonstrated when E2F-4 caused a threefold increase in the levels of cdk2 whereas E2F-1 failed to increase in this cyclin dependent kinase. Similarly, expression of E2F-1 or E2F-2 were shown to have distinct effects on the expression of cdk2, cyclin E and pRB despite both of these closely related E2F-family members potently inducing cell death. Thus, E2F-1, E2F-2, E2F-3 and E2F-4 are able to overcome the p16-dependent proliferative block in U343 astrocytoma cells. While overcoming this cell cycle block, each of the E2F's uniquely affect the expression of a number of cell cycle regulatory proteins and have distinct abilities to promote cell death.

Keywords: E2F-family; p16; astrocytomas; cell cycle; apoptosis

Correspondence: PA Hamel
Received 15 September 1997; revised 27 March 1998; accepted 27 March 1998

Introduction

Unregulated proliferation of cancer cells is associated with significant alterations in the expression and activity of the molecular cell cycle machinery. A growth regulatory pathway implicated in determining passage through the G_1 restriction point has been defined which is comprised of the cyclin-dependent kinase inhibitor (CKI) p16^{Ink4a} (p16), cyclin D, cdk4 and the product of the *RBI* gene, pRB (for reviews see Sherr, 1996; Sidle *et al*., 1996; Weinberg, 1995). Alterations in the expression or activity of at least one member of this pathway has been documented in the majority of human cancers.

An important target of this pathway is the product of the *RBI* gene, pRB (Friend *et al*., 1986). Many studies have now demonstrated that a functional pRB protein is critical for the growth inhibitory activity of p16 (Lukas *et al*., 1995a,b; Medema *et al*., 1995; Parry *et al*., 1995; Ueki *et al*., 1996; Yeager *et al*., 1995). In the absence of pRB, expression of p16 has little apparent effect on the cell cycle profile of cells. Additionally, pRB itself exhibits growth regulatory properties (Huang *et al*., 1988; Lee *et al*., 1988a,b). So, for example, when expressed in the human osteosarcoma cell line, SAOS-2, pRB elicits a potent block to proliferation and induces morphological changes (Hinds *et al*., 1992). However, while some cells are very sensitive to pRB activity, a number of other pRB-deficient cell lines appear to be refractory to the effects of this negative regulator of proliferation (for example see Muncaster *et al*., 1992). One of the best characterized of these are the human cervical carcinoma cell line, C33A (Zhu *et al*., 1993). While being defective for functional pRB, ectopic expression of wild type pRB in this line failed to block proliferation. In addition, expression of a dominant mutant of pRB, Δp34 (Hamel *et al*., 1992a,b), which is constitutively active due to mutations in many of its phosphorylation sites, also fails to block the C33A cell cycle (Sellers *et al*., 1995). These latter data suggest that, for at least some cell types, inhibition of the downstream portion of the p16-cyclin D/cdk4-pRB-E2F pathway is not sufficient to elicit a cell cycle block.

Another important target of this pathway is the family of transcription factors collectively known as E2F. The E2F family currently consists of five members, E2F-1 (Helin *et al*., 1992; Kaelin *et al*., 1992), E2F-2 (Ivey-Hoyle *et al*., 1993; Lees *et al*., 1993), E2F-3 (Lees *et al*., 1993), E2F-4 (Beijersbergen *et al*., 1994; Ginsberg *et al*., 1994; Sardet *et al*., 1995) and E2F-5 (Buck *et al*., 1995; Hijmans *et al*., 1995; Sardet *et al*., 1995). When heterodimerized with DP1

(Bandara *et al*., 1994; Helin *et al*., 1993; Krek *et al*., 1993) or DP2 (Wu *et al*., 1995; Zhang and Chellapan, 1995), the E2F's efficiently recognize the DNA consensus sequence TTT/(C/G)(C/G)CGC (Wade *et al*., 1995; Yee *et al*., 1989) in the promoters of genes whose expression are usually regulated in a cell cycle-dependent manner. These genes include dihydrofolate reductase, thymidine kinase, DNA polymerase-α, thymidylate synthetase, B-*myb*, *cdc2*, cyclin A, cyclin E, c-*myc* and the E2F's themselves (Botz *et al*., 1996; DeGregori *et al*., 1995a, 1997; Farnham and Schimke, 1986; Hamel *et al*., 1992b; Hiebert *et al*., 1989, 1991; Hurford *et al*., 1997; Karlseder *et al*., 1996; Shan *et al*., 1994; Shimizu *et al*., 1995; Slansky *et al*., 1993; Thalmeier *et al*., 1989; Zhu *et al*., 1995). pRB and p107 also have E2F binding sites within their promoters, and it is thought that these pocket proteins may autoregulate their own expression through these sites (Gill *et al*., 1994; Shan *et al*., 1994; Zhu *et al*., 1995).

Several studies have demonstrated that unscheduled expression or overexpression of some of the E2F-2 family proteins in growth arrested cells can lead to cell cycle re-entry, suggesting that restraint of E2F activity is the critical endpoint of a variety of different negative growth signals. In serum starved cells, E2F-1 overexpression can induce S-phase entry (Johnson *et al*., 1993, 1994b; Shan *et al*., 1994). Deregulated E2F-1 expression can overcome TGFβ or γ-irradiation-induced growth arrest as well as $p21^{Cip1/WAF1}$, $p27^{Kip1}$ and $p16^{Ink4a}$ mediated arrest (DeGregori *et al*., 1995b; Schwarz *et al*., 1995). E2F-1, E2F-2 and E2F-3 overcame p16-induced cell cycle arrest of Rat 1 cells which constitutively express Bcl-2 (Lukas *et al*., 1996). However, E2F-4 and E2F-5 expression in the same system failed to overcome the block unless they were co-expressed with DP1. Together with the apparent restricted tissue specificity of expression of some of the E2Fs (Dagnino *et al*., 1997a,b) and pRB-family proteins (Jiang *et al*., 1997), these data imply a complex cell cycle regulatory pathway where the specific outcomes may vary in different tissues and at different points during development.

We have been studying the effects of restoration of expression of the CKI $p16^{Ink4a}$ (p16) in the p16-deficient astrocytoma cell line, U343 MG-a (U343). p16 expression in this cell line causes a G_1 cell cycle arrest associated with an alteration in expression and activity of the pRB- and E2F-family members (Dirks *et al*., 1997). This growth arrest is also associated with alteration in cell phenotype. Since pRB and the E2Fs are downstream targets of the pathway regulated by p16, we wished to determine the effect of overexpression of the dominant pRB mutant, Δp34, on the proliferation of U343 cells and determine the effect of expressing the E2F-family proteins in these p16 growth arrested astrocytoma cells. We demonstrate here that the expression of Δp34 has no apparent effect on the proliferation of U343 cells and that expression of the E2F family of transcription factors in the p16-arrested cells differ in their abilities to promote cell cycle progression and cell death. Additionally, the E2F's distinctly alter the expression of specific cell cycle regulatory factors in these U343 astrocytoma cells.

Results

Expression of pRB in U343 cells does not alter their cell cycle

We first wished to determine if expression of a dominant mutant of pRB, Δp34, would cause cell cycle arrest of U343 astrocytomas, analogous to the effect of expressing p16 in these same cells (Figure 1). Thus, both human SAOS-2 osteosarcoma cells and U343 astrocytomas were infected with adenovirus expressing either β-galactosidase or the Δp34 pRB dominant mutant (Hamel *et al*., 1992a,b). The Western blot probed with the α-HA antibody, 12CA5, demonstrates that the HA-tagged, Δp34 mutant is expressed in both SAOS-2 cells and U343 cells. The levels of expression in the U343 cells are consistently lower than are seen for SAOS-2 by Western analysis. Staining of U343 cells revealed, however, that most of the cells in the culture were expressing the Δp34 protein (data not shown). Expression of Δp34 in SAOS-2 cells resulted in the expected changes in morphology, cells becoming flat with abundant cytoplasm, while little effect was observed for the U343 cells. The failure of pRB to arrest U343 cells was consistent with its failure to repress E2F-1 levels. As the lower panel in Figure 1 illustrates, Δp34 expression in SAOS-2 resulted in quantitative repression of E2F-1 levels. While expression of p16 in U343 cells also represses E2F-1 expression (Dirks *et al*., 1997), we saw no changes in E2F-1 levels in cells infected with the Δp34-expressing virus relative to the control βgal virus. As expected, no changes in the cell cycle profile of these cells were seen using FACS analysis (data not shown). Thus, despite being an important down stream target of p16 activity and a potent inhibitor of SAOS-2 proliferation, ectopic expression of Δp34 in the p16-deficient U343 astrocytoma cells has little effect on their cell cycle.

E2F factors overcome p16-dependent G_1 arrest

We next examined whether expression of the different E2F-family proteins could overcome the 16-dependent cell cycle block in these cells. Induction of p16 in U343

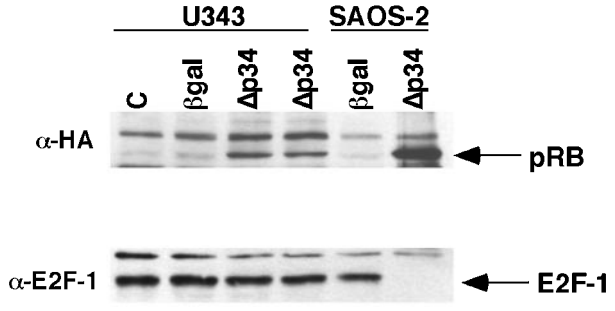

Figure 1 Expression of the pRB mutant, Δp34, in U343 or SAOS-2 cells. Exponentially growing U343 astrocytomas or SAOS-2 osteosarcomas were infected with adenoviruses expressing β-galactosidase (β-gal) or the HA-tagged, dominant pRB mutant, Δp34. Cells were harvested after 3 days and probed for the expression of Δp34 (upper panel) using the anti-HA antibody, 12CA5, or for expression of E2F-1 (lower panel). Expression of Δp34 or E2F-1 in uninfected U343 cells is indicated (C)

cells in the presence of serum results in a reversible G_1 arrest by 72 h (Dirks *et al*., 1997). Associated with p16 expression in these cells is a decrease in expression of endogenous E2F-1, pRB and p107, but no change in the expression of E2F-4.

Figure 2 and Table 1 demonstrates that 48 h following infection of p16-arrested astrocytomas with adenovirus expressing E2F-1, E2F-2, E2F-3, or E2F-4, cells had progressed into the S and G_2/M phases of the cell cycle. Only E2F-5 does not affect the cell cycle profile of these cells, although co-expression with DP1 with E2F-5 does induce cell cycle re-entry. No effect on the p16-imposed, G_1 block is evident in cells infected with the control adenovirus.

P16 expression in U343 cells results in marked cellular enlargement and cell flattening associated with reorganization of the cytoskeleton (Dirks *et al*., 1997). As Figure 3 shows, expression of the different E2F's significantly alters this flat cell phenotype. E2F-1, E2F-2, E2F-3 or E2F-4 decreases astrocytoma cell size whereas infection with the E2F-5 expressing virus causes cells to further increase in size and form a completely confluent monolayer of cells. This altered morphology is not evident when E2F-5 is co-expressed with DP1, this latter infection having little observable effect on cell morphology and being similar to the morphology of cells following infection with the control virus.

As evident in Figure 3 and suggested by the pre-G_1 peak present in the FACS analysis in Figure 2, expression of some of the E2F's in p16-arrested U343 cells causes cell death with characteristics consistent with apoptosis. Specifically, E2F-1 and E2F-2 causes death of the majority of cells by 72 h post-infection.

Table 1 Percentage of U343 cells in different cell cycle fractions

Adenovirus	*% G_1*	*% S*	*% G_2/M*
None	95	2	3
CMV	95	2	3
E2F-1	82	8	10
E2F-2	66	10	24
E2F-3	82	15	3
E2F-4	66	24	10
E2F-5	95	2	3
E2F-5/DP1	74	23	3

The percentage of cells in G_1, S and the G_2/M phases of the cell cycle in p16-mediated growth arrested U343 cells or p16-expressing U343 cells 48 h following infection with the control or E2F-expressing adenoviruses are indicated

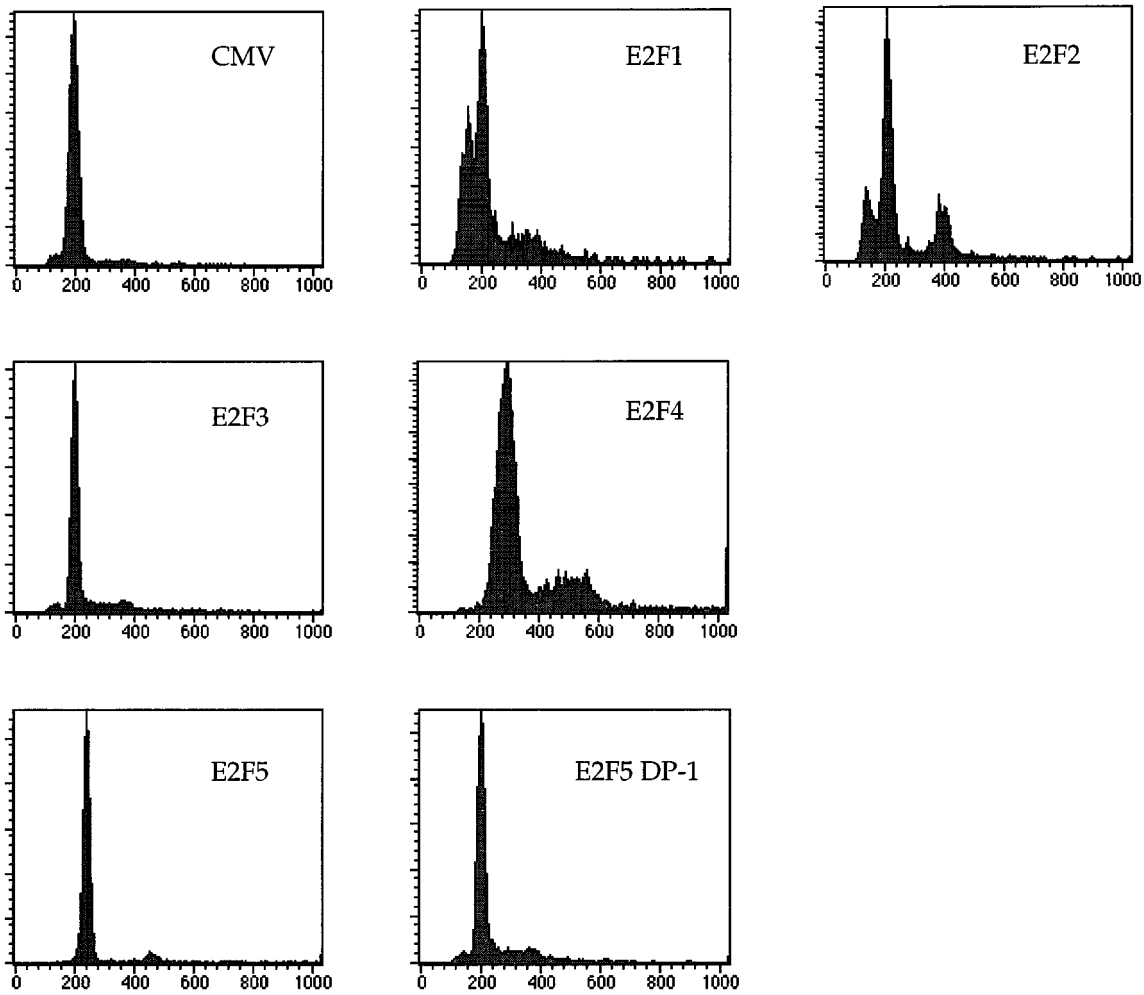

Figure 2 FACS-analysis of p16-expressing E2F-infected astrocytoma cells. p16-induced U343 astrocytoma cells expressing E2F-1, E2F-2, E2F-3, or E2F-4 all overcome the p16-dependent G_1 arrest, causing the cells to enter into the S and G_2/M phases of the cell cycle. Expression of E2F-5 does not affect the cell cycle profile of these cells, although co-expression with DP1 with E2F-5 does induce cell cycle re-entry. No effect on the p16-imposed, G_1 block is evident in cells infected with the control adenovirus (CMV)

For these E2F's, cells become rounded, detach from the culture dish, acquire large intracytoplasmic vacuoles, and show cytoplasmic blebbing and loss of a distinct nucleus (data not shown). In contrast, E2F-3 expression causes little cell death despite cells entering the cell cycle. Expression of E2F-4 only weakly induces cell death, greater than 90% of the cells still being adherent 72 h after infection. As expected from the FACS analysis, expression of E2F-5 alone or in combination with expression of DP1, has no apparent effect on cell survival. Thus, these data demonstrate that while E2F-1, E2F-2, E2F-3 or E2F-4 are all able to overcome the p16-dependent cell cycle arrest, only expression of E2F-1 and E2F-2 results in potent cell death in these U343 cells.

E2F infection alters expression of other E2Fs and pRB family members

We next examined the levels of expression and subcellular localization of the ectopically expressed E2F's following infection of the p16-arrested U343 cells (Figures 4 and 5). As expected, adenoviral infection of each of the E2F's greatly increases the levels of the specific E2F used (Figure 4). Longer exposures of these same Western blots reveal, however, that expression of some of the E2F's alters the level of expression of other E2F-family members. These changes were more carefully examined by fractionating the cells into cytoplasmic and nuclear components and assaying them for E2F expression (Figure 5). For example, infection with the E2F-1 expressing adenovirus results in increased levels of E2F-1 in both the nuclear and cytoplasmic compartments. On longer exposures of this same blot, however, it is evident that ectopic expression of E2F-2 and E2F-4 also induce E2F-1 expression. The induced E2F-1 protein in all these cases is nuclear. E2F-4 levels are also affected by expression of distinct E2F-family members. E2F-4 is present in the p16-arrested U343 cells exclusively in the nuclear compartment. Infection with the E2F-4-expressing adenovirus greatly increases E2F-4 levels in both the cytoplasm and the nucleus. However, while ectopic expression of E2F-2 failed to have an appreciable effect on E2F-4 expression, infection with the E2F-1 virus increases the endogenous E2F-4 almost five-fold. Thus, while both E2F-1 and E2F-2 drive these p16-arrested astrocytomas into the cell cycle leading to cell death, these two E2F-family proteins distinctly affect the expression of at least one E2F-family member.

Since expression of some of the E2F-family members in the p16-growth arrested U343 cells alters the

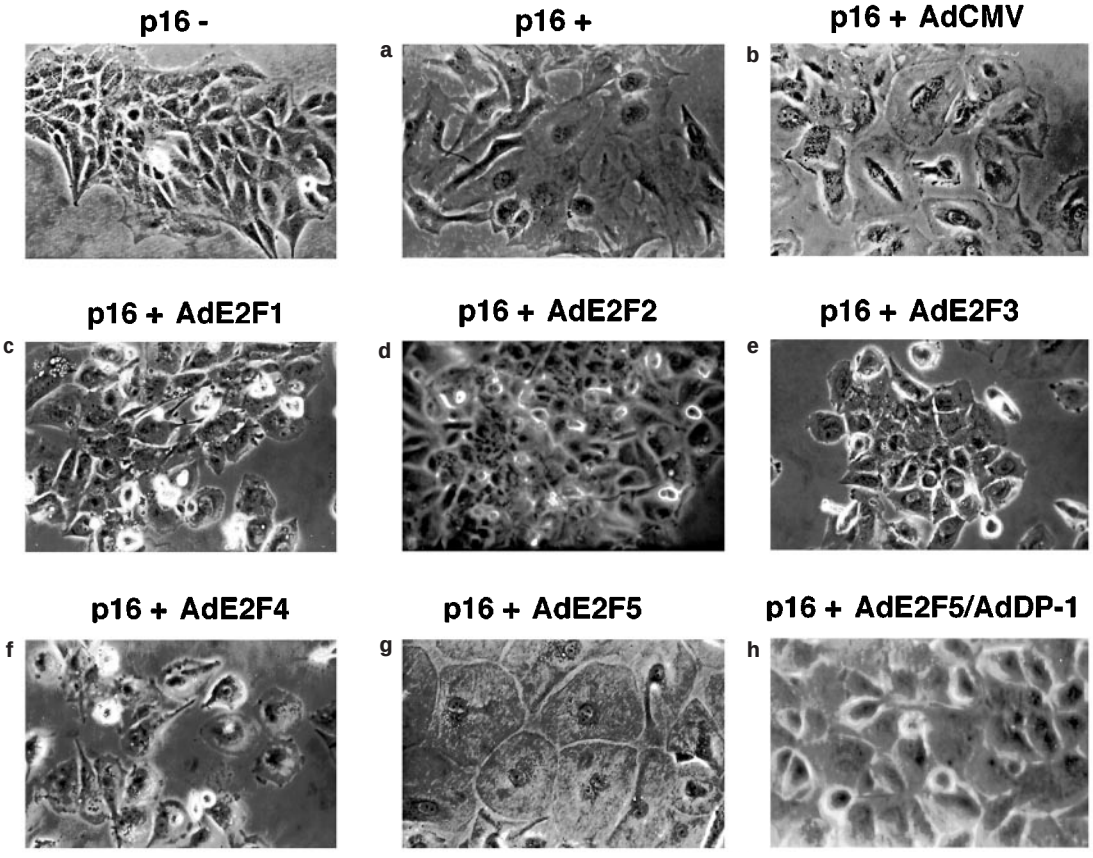

Figure 3 Morphology of p16-expressing E2F-infected astrocytoma cells. Growth arrested U343 cells expressing p16 from the tetracycline operator for 5 days (**a**) were infected with a control adenovirus (**b**) or virus expressing E2F-1 (**c**), E2F-2 (**d**), E2F-3 (**e**), E2F-4 (**f**), E2F-5 (**g**) or E2F-5 and DP1 together (**h**). By 48 h following infection, E2F-1 and E2F-2 potently decreased the cell size and caused a large amount of cell death. E2F-3 and E2F-4 expression also altered the p16-dependent flat cell phenotype, E2F-4 expression causing some cell death and E2F-3 having little apparent effect on cell viability. Infection with the E2F-5 expressing virus resulted in the most distinctive phenotype, cells further increasing in size, forming a completely confluent layer of cells, each with an enormous cytoplasm devoid of vacuoles. Co-expression of E2F-5 with DP1 had little observable effect on cell morphology as did the infection with the control virus

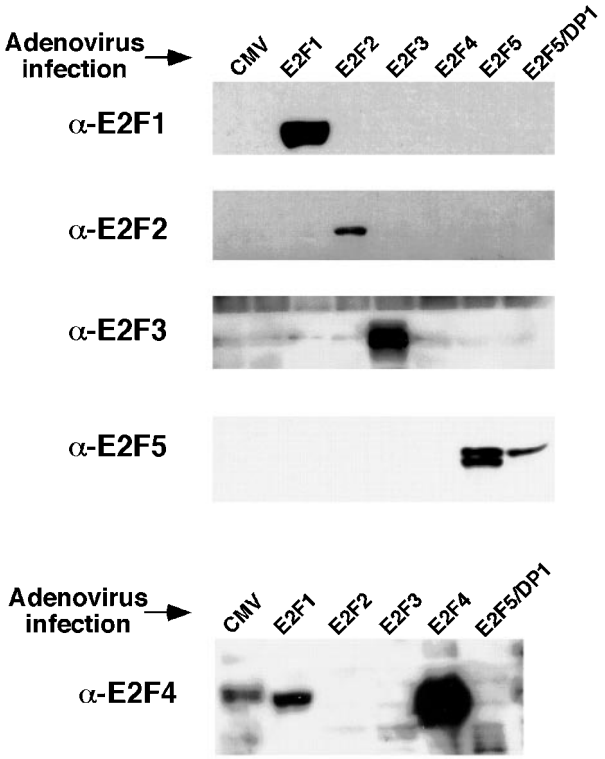

Figure 4 E2F protein expression in infected U343 cells. p16-arrested U343 cells were infected with control adenovirus (CMV) or viruses expressing E2F-1, E2F-2, E2F-3, E2F-4, E2F-5 or E2F-5 together with DP1. Whole cell lysates were prepared and the expression of each of the E2F's in each infection assessed by Western analysis using antibodies specific to the individual E2F-family members. As expected, infection of each of the E2F's resulted in significant increases in the expression of that particular E2F. Interesting, for E2F-5, co-expression with DP1 resulted in a significant change in its mobility, the majority of E2F-5 in the latter case present in the slower migrating form

expression of a number of the endogenous E2F's, we next determined whether E2F expression influences expression of other cell cycle regulatory proteins. Thus, as depicted in Figure 6, the steady-state levels of cdk2, cyclin E, c-*myc* were determined by Western analysis. Cdk2 (Figure 6a) migrates as a single species in the p16-arrested cells infected with the empty virus (CMV lane). All of the E2F-family proteins which induced cell cycle progression result in the appearance of the faster migrating, hyperphosphorylated, active form of cdk2. However, expression of two specific, E2F's, E2F-2 and E2F-4, also increase the overall level of cdk2 in these cells (an increase in cdk2 levels is also seen for E2F5/DP1). The kinase activity associated with cdk2 was determined following immunoprecipitation with an α-cdk2 antibody (Figure 6b). As expected the amount of cdk2 immunoprecipitated from the cells infected with the different E2F's reflected the relative levels of cdk2 observed by Western analysis (Figure 6a). However, only cells infected with the E2F-4-expressing virus show a significant, large increase in cdk2-associated kinase activity (Figure 6b), while cdk2-associated kinase activity in E2F-1 and E2F-2 infected cells are increased only moderately. The alterations in cdk2-associated kinase activities could be somewhat correlated with the changes in cyclin E levels in these cells, the levels of this latter cell cycle regulatory protein also being distinctly altered by the different E2F's (Figure 6a). Specifically, all of the E2F's which cause cell cycle progression increase cyclin E levels. However, E2F-1, E2F-2 and E2F-4 expression results in a greater than 15-fold greater increase in cyclin E levels relative to its increase seen for E2F-3 and E2F-5/DP1.

We also determined if the cell cycle progression mediated by expression of the E2F-family members might depend on c-*myc* induction. As Figure 6a clearly demonstrates, however, the E2F's which overcome p16-dependent cell cycle arrest, including those which induce cell death, do so independent of c-*myc* induction.

We next examined the expression of the pRB-family proteins following E2F-family protein expression (Figure 7). In the case of pRB, E2F-1 and E2F-4 induce pRB expression. Only a very weak signal for pRB is detectable following infection with the viruses expressing E2F-2, E2F-3 or E2F-5/DP1. The levels for pRB in the latter cases are identical to those observed in mock infection of these p16-arrested cells. p107 levels are only weakly altered following E2F-1, E2F-2, and E2F-3 expression, although a slightly greater increase in p107 was reproducibly observed following E2F-4 expression relative to the CMV controls. p130 exhibits the most varied effects following E2F expression. Only E2F-4 significantly increases p130, the p130 in these cells found in both the hypo- and hyperphosphorylated forms. In contrast to E2F-4, expression of E2F-2 and E2F-3, both of which cause cell cycle reentry, result in repression of p130 levels while E2F-1 expression has little effect on the level or phosphorylation state of p130.

Taken together, we have demonstrated that the different E2F's exhibit distinct biological activities when expressed in astrocytomas growth arrested due to expression of the cyclin-dependent kinase inhibitor, p16. These activities include their ability to induce cell death and to specifically alter the expression of distinct cell cycle regulatory proteins.

Discussion

The E2F-family proteins are important targets of the pRB-family of transcriptional repressors. Deregulated E2F activity overcomes cell cycle arrest imposed by a variety of signals including that of CKI's (DeGregori *et al*., 1995b; Lukas *et al*., 1996). Additionally, deregulation of E2F activity has been associated with cellular transformation (Johnson *et al*., 1994a; Singh *et al*., 1994; Xu *et al*., 1995). As we demonstrate here, the E2F's exhibit distinct activities when overcoming p16-mediated G_1 cell cycle arrest. These distinct activities include their ability to cause cell death, alter cell morphology and alter the expression of distinct cell cycle regulatory proteins.

Our data demonstrate that deregulated expression of E2F-1, E2F-2, E2F-3, or E2F-4 overcome a p16-dependent G_1 cell cycle arrest in U343 astrocytomas. E2F-5 overcomes the p16 block only when co-expressed with DP1, presumably due to the nuclear localization of E2F-5 only in the context of added DP1. These data are in contrast to a previous report in

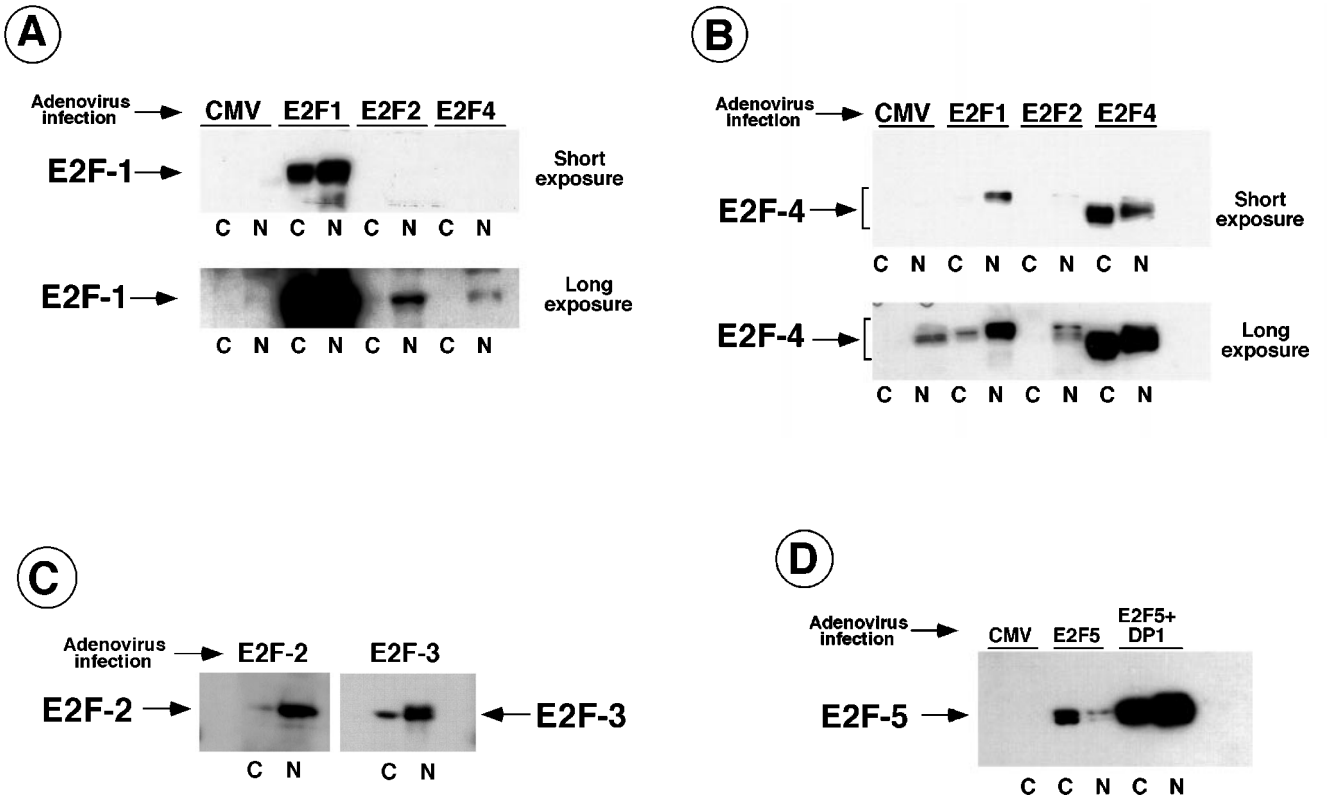

Figure 5 Cellular compartmentalization of the E2F's in infected U343 cells. The cellular location of the various E2F's was determined by Western analysis. (**a**) Expression of E2F-1 in nuclear (N) or cytoplasmic (C) fractions following infection of p16-arrested U343 cells with control virus (CMV) or virus expressing E2F-1, E2F-2 or E2F-4. Blots were exposed for either 10 s (short exposure) or 5 min (long exposure). (**b**) Same as (**a**) but blots were probed for expression of E2F-4. (**c**) Compartmentalization of E2F-2 or E2F-3 following infection of viruses expressing E2F-2 or E2F-3, respectively. (**d**) Expression of E2F-5 in cells infected with control virus (CMV), virus expressing E2F-5 or in cells infected with both E2F-5 and DP1-expressing viruses

which forced expression of E2F-1 but not E2F-4 could overcome a p16-induced cell cycle block (Lukas *et al*., 1996). In this latter case, E2F-1 or E2F-4, expressed under the control of the tetracycline operator (Gossen and Bujard, 1992) or from microinjected expression plasmids, were expressed in Rat-1 cells, programmed to express Bcl-2, or R12 cells, respectively. One apparent reason for the failure of E2F-4 to overcome the p16-dependent cell cycle block in these Rat-1 cells was its failure to efficiently translocate to the nucleus following its synthesis. In the infection protocol we employed, E2F-4 was present at significant levels in the cytoplasm. However, at least as much E2F-4 was present in the nuclear compartment of the infected U343 astrocytomas as in the cytoplasmic fraction. It is not evident why E2F-4 compartmentalization differs in these two cell lines. Potentially, higher levels of E2F-4 are achieved in our infection protocol, these levels being sufficiently different to cause at least some E2F-4 to translocate to the nucleus in our study. Alternatively, the strong proliferative block provided by p16 in these astrocytomas may generate a strong signal for the nuclear compartmentalization of E2F-4. The latter possibility would be consistent with the previously described, cell cycle dependent compartmentalization of this particular E2F-family member (Lindeman *et al*., 1997).

Additionally, our data, using the p16-arrested U343 cells, differ significantly from the results obtained using the same E2F-expressing viruses used to drive serum starved REF52 cells into cycle (DeGregori *et al*., 1997). In the latter system, E2F-1, E2F-2, E2F-3 and E2F-4 all overcame the block to REF52 proliferation imposed by serum starvation. However, only E2F-1 appeared to induce apoptosis in these cells, E2F-2 having no apparent effect on cell survival in this system. Differences in E2F regulation of a number of cell cycle regulatory proteins, specifically cdk2 and cyclin E, were also evident compared to the results obtained for p16-arrested U343 astrocytomas. The basis for the distinct effects of expressing the E2F's in either the p16-arrested U343 cells (this paper) and the serum starved REF52 cells (DeGregori *et al*., 1997) is not apparent. Indeed, with the exception of the potent induction of apoptosis by E2F-1 in most cell culture systems, the different E2F's appear to have somewhat distinct biological activities depending on the cell cycle status, the nature of the cell cycle block and the type of cells employed for the experiment (see Lukas *et al*., 1996 for example).

All of the E2F-family members which overcame the cell cycle block imposed by p16 expression caused induction of cyclin E levels. However, three specific E2F's, E2F-1, E2F-2 and E2F-4, increased cyclin E levels greater than ten times higher than cyclin E induction due to E2F-3 or E2F-5/DP1 expression. We believe that the difference in the levels of cyclin E reflects whether the specific E2F-family members directly or indirectly induce cyclin E expression. E2F's causing only modest increases in cyclin E levels would do so indirectly as a consequence of driving cells

through the cell cycle. In contrast, E2F's which strongly induce cyclin E expression are predicted to directly activate cyclin E promoter activity.

Unlike the effect on cyclin E expression, E2F-induced cell cycle progression had no observable effect on c-*myc* expression. The c-*myc* promoter has been shown to be a potential target of pRB-mediated repression (Hamel *et al*., 1992b; Hiebert *et al*., 1989). This repression was dependent on the presence of an intact E2F-binding site in the P2 promoter region. Our data and those published previously (DeGregori *et al*., 1995a,b) support the notion that c-*myc* induction does not depend on E2F transcriptional activity. Rather, these data suggest that the role of E2F in controlling c-*myc* expression may be to repress c-*myc* expression as cells exit the cell cycle. During cell cycle re-entry, c-*myc* induction is independent of E2F activity and would be mediated through other elements in the c-*myc* P2 promoter region.

While the E2F's did not affect the expression of c-*myc*, expression of some E2F-family members altered the levels of other cell cycle regulatory factors, including the E2F's themselves. For example, E2F-1, but not E2F-2, specifically increased levels of E2F-4. In a reciprocal manner, E2F-4, as well as E2F-2, induced the expression of E2F-1. The pRB-family proteins were also differentially affected by ectopic E2F expression. Both E2F-1 and E2F-4 significantly increased pRB

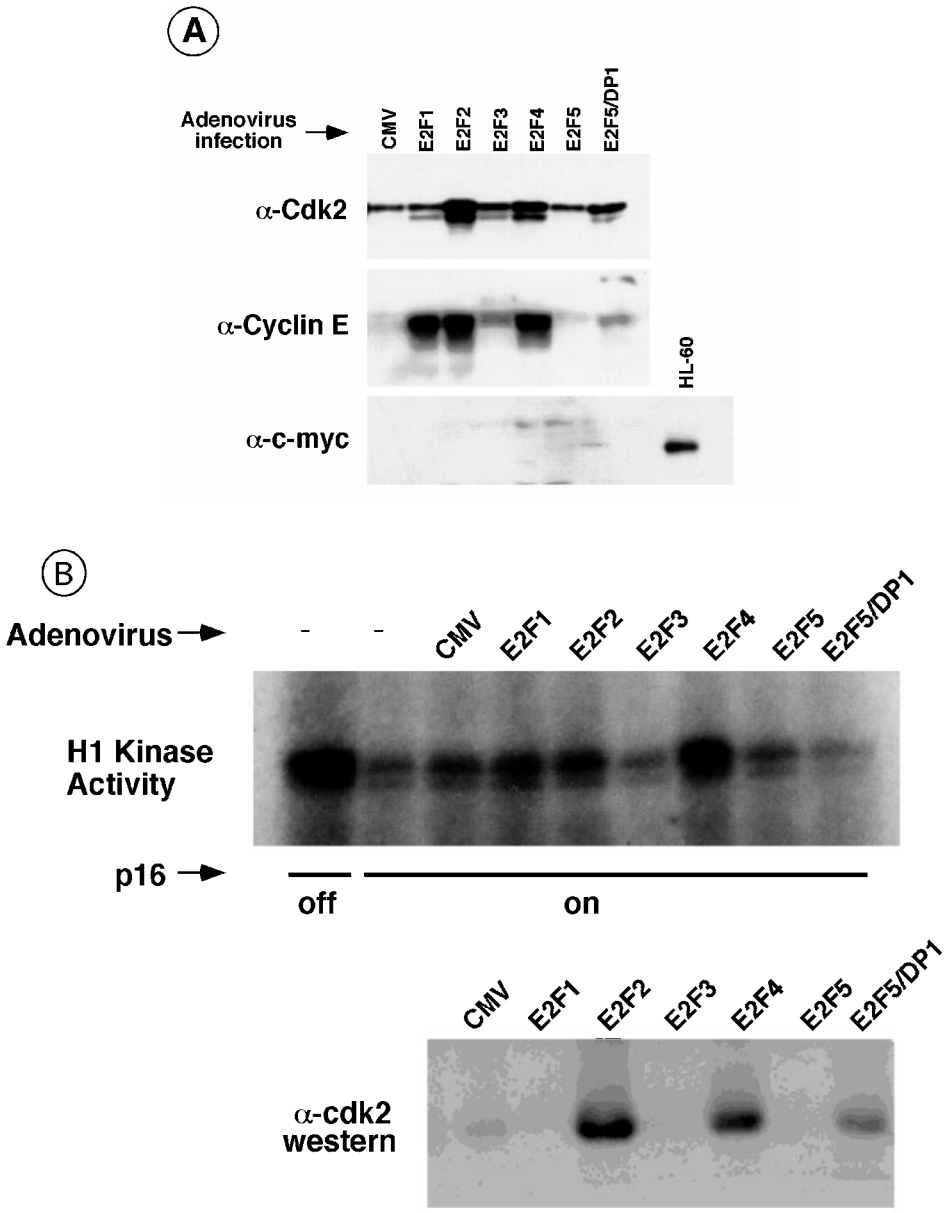

Figure 6 Effect of E2F expression on the levels of cyclin E, cdk2 and c-*myc*. (**a**) Expression levels of the cdk2 (upper panel), cyclin E (middle panel) or c-*myc* (lower panel) were determined by Western analysis of whole cell lysates following infection of the control (CMV) or E2F-expressing adenoviruses. In the case of c-*myc*, HL-60 cells were used as a positive control for c-*myc* expression. (**b**) Kinase activity (upper panel) associated with cdk2 in cells infected with control (CMV) or E2F-expressing viruses using histone HI as a substrate. Cdk2-associated kinase activity was also determined for U343 cells where p16, under the control of the *tetO* was repressed (off) and in cells where p16 was induced (on). The amount of cdk2 isolated for kinase assay was determined by Western analysis of a fraction of the immunoprecipitated material (lower panel). Longer exposures of this blot show low levels of cdk2 present in the CMV, E2F-1 and E2F-3 lanes (data not shown). The levels of cdk2 isolated by immunoprecipitation reflect their relative levels determined by Western analysis (see Figure 6a)

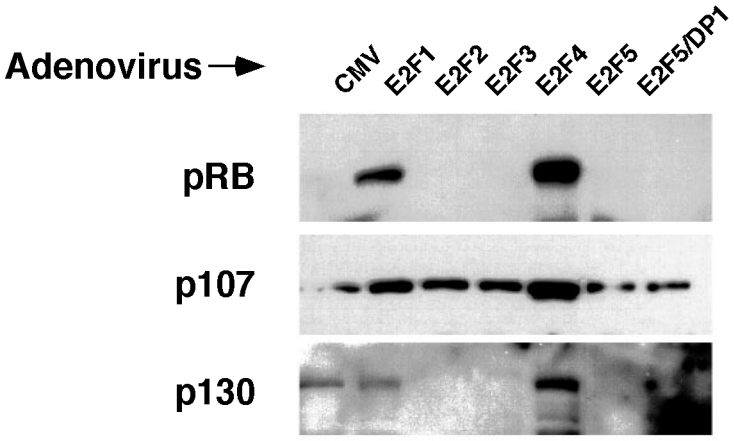

Figure 7 Effect of E2F expression on pRB-family proteins. The levels of pRB (upper panel), p107 (middle panel) and p130 (lower panel) were determined by Western analysis of whole cell lysates in p16-arrested cells following infection with control (CMV) or the E2F-expressing viruses indicated

levels. In contrast, E2F-4 specifically increased p130 expression while E2F-2 and E2F-3 repressed the levels of p130 (data not shown). Since all of these E2F's are capable of driving cells into the cell cycle, we conclude that each of these E2F-family proteins are involved in the transcriptional regulation of distinct targets. We predict, then, that the recognition of E2F-binding sites by the different E2F-family proteins will depend on the context of that binding site in a particular promoter region. Recent data have also demonstrated that the different pRB-family proteins participate in the regulation of distinct E2F-responsive genes (Hurford *et al*., 1997). Taken together with their developmentally regulated patterns of expression (Dagnino *et al*., 1997a,b; Jiang *et al*., 1997), these experiments predict that the distinct members of the pRB- and E2F-families may not necessarily be redundant and have, in fact, tissue-specific roles and/or cell cycle-specific activities.

It was somewhat surprising that ectopic expression of the dominant pRB mutant, Δp34 (Hamel *et al*., 1992a,b), had no observable consequences on the proliferative potential of these cells. Our previous data demonstrated that p16 expression had profound effects on the phosphorylation state of pRB and the levels of pRB and E2F-1 expression (Dirks *et al*., 1997). We hypothesize, therefore, that while pRB and E2F-1 are important downstream targets of p16 activity, there may exist additional downstream targets in pathways parallel to pRB-E2F-1 which are also regulated by p16. Inhibition of both (all) these pathways is required for cell cycle arrest. The existence of these pathways is supported by the recent observations that a number of cellular factors outside of the p16-cyclin D/cdk4-pRB-E2F-1 pathway can bind to specific members of this pathway. So, for example, the estrogen receptor has recently been shown to bind to cyclin D1 in a cdk4-independent manner (Neuman *et al*., 1997; Zwijsen *et al*., 1996, 1997). Additional cyclin D1-associated factors have also been described (Hirai and Sherr, 1996). Given the differences in susceptibly of different cell lines to the effects of pRB expression, we expect that some of these pathways may be tissue or cell specific.

Materials and methods

Cell culture

The malignant astrocytoma cell line U343MG-a (U343), reconstituted with $p16^{Ink4a}$ (p16) under the control of the tetracycline repressor system (Gossen and Bujard, 1992), has been described elsewhere (Dirks *et al*., 1997). For p16 induction, $1-2\times10^5$ cells were plated in 6 cm dishes. The following day, medium containing 10% serum and 4 μg/mL tetracycline (p16 repressed) was replaced with medium containing 10% serum without tetracycline (p16 induced).

Infection of cells with adenoviruses

U343 astrocytoma cells were growth arrested following induction of p16 for 5 days as previously described (Dirks *et al*., 1997). Cells were then infected with adenovirus expressing either E2F-1, E2F-2, E2F-3, E2F-4 or E2F-5, under the control of the cytomegaloviral (CMV) promoter (viruses a kind gift of J DeGregori, G Leone and J Nevins (DeGregori *et al*., 1997)). An adenovirus containing only the CMV promoter was used as a control and, in the case of E2F-5, co-infection with a virus expressing the E2F heterodimeric partner, DP1, was also performed. Cells were infected with 100 p.f.u./cell and were maintained in 10% serum containing medium without tetracycline (p16 induced). Adenoviruses expressing either βgalactosidase or the HA-tagged, pRB mutant, Δp34, were kindly provided by J Leiden and have been described previously (Wang *et al*., 1993). Human osteosarcoma SAOS-2 cells or U343 cells were infected with these two viruses as described for the adenoviruses expressing the E2F's with the exception that a multiplicity of infection of ten was used. Expression of the Δp34-HA mutant was determined by Western analysis using the anti-HA antibody 12CA5 as we have previously described (Hamel *et al*., 1992a,b).

Flow cytometric analysis

Determination of the proportion of cells present at different stages of the cell cycle was performed by FACS analysis as we have described previously (Kiess *et al*., 1995a). The percentage of cells in different phases of the

cell cycle calculated using Cell Fit software (Becton-Dickinson, San Diego, CA, USA).

Antibodies

Antibodies to cdk2 (SC-163), cdk4 (SC-260), cyclin E (SC-198), p107 (SC-318), p130 (SC-317), E2F-1 (SC-193), E2F2 (SC-633), E2F3 (SC-878), E2F-4 (SC-866x) and E2F-5 (SC-999) were obtained from Santa Cruz Biotech. Inc. (Santa Cruz, CA) and to pRB (14001A) and p16 (15126E) from Pharmingen (Richmond, CA). The monoclonal antibody to c-*myc* was provided by Dr L Penn (The Ontario Cancer Institute, Toronto, ON).

Western blots and kinase assays

Total cell lysates (120 mM NaCl, 0.5% NP-40, 50 mM TrisCl pH 8.0, 10–30 μg) or fractionated nuclear and cytoplasmic lysates were made as previously described (Kiess *et al*., 1995a) and were subjected to SDS–PAGE. Proteins were transferred to polyvinylidene difluoride (Immobilon P) membranes by semi-dry transfer. Blots were rehydrated prior to immunodetection, and then were blocked in 5% skim milk in PBS/0.1% Tween 20 at room temperature for 1 h. Primary and secondary antibody incubations were performed in blocking solution at room temperature for 1 h. Primary antibodies were diluted 1 : 1000 and the goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies were used at 1 : 5000–1 : 8000 dilutions. Detection was performed using the enhanced chemiluminescence system (ECL, Amersham, Oakville, ON) according to the manufacturer's instructions. For kinase assay, cdk2 was immunoprecipitated from 200 μg U343 cell lysate using the α-cdk2 antibody (SC-163) as we have performed previously (Kiess *et al*., 1995b and references therein). A portion of this immunoprecipitate was used to determine by Western analysis the amount of cdk2 isolated. The remainder was used in kinase assays with Histone HI as substrate, as we have described previously (Kiess *et al*., 1995b).

Acknowledgements
This work was supported in part through a grant from the MRC to JTR, through a grant to PAH from the National Cancer Institute of Canada with support from the Canadian Cancer Society, a grant to PAH from the Medical Research Council of Canada (MT-14342) and through funds from The Research Institute, The Hospital for Sick Children to JTR. PBD is supported by an NCIC research fellowship with funds provided by the Canadian Cancer Society as well as a research fellowship from the MRC.

References

Bandara LR, Lam EW, Sorensen TS, Zamanian M, Girling R and La Thangue NB. (1994). *EMBO J.,* **13,** 3104–3114.
Beijersbergen RL, Kerkhoven R, Zhu L, Carlee L, Voorhoeve PM and Bernards R. (1994). *Genes Dev.,* **8,** 2680–2690.
Botz J, Zarfass-Thome K, Spitkovsky D, Delius H, Vogt B. Eilers M, Hatzigeorgiou A and Jansen-Durr P. (1996). *Mol. Cell. Biol.,* **16,** 3401–3409.
Buck V., Allen EK, Sørensen T, Bybee A, Hijmans EM, Voorhoeve PM, Bernards R and La Thangue NB. (1995). *Oncogene,* **11,** 31–38.
Dagnino L, Fry CJ, Bartley SM, Farnham P, Gallie BL and Phillips RA. (1997a). *Mech. Dev.,* **66,** 13–25.
Dagnino L, Fry CJ, Bartley SM, Farnham P, Gallie BL and Phillips RA. (1997b). *Cell Growth Diff.,* **8,** 553–564.
DeGregori J, Kowalik T and Nevins J. (1995a). *Mol. Cell. Biol.,* **15,** 4215–4224.
DeGregori J, Leone G, Miron A, Jakoi L and Nevins JR. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 7245–7250.
DeGregori J, Leone G, Ohtani K, Miron A and Nevins JR. (1995b). *Genes Dev.,* **9,** 2873–2887.
Dirks PD, Patel K, Hubbard SL, Ackerley C, Hamel PA and Rutka JT. (1997). *Oncogene,* **15,** 2037–2048.
Farnham PJ and Schimke RT. (1986). *Mol. Cell. Biol.,* **6,** 2392–2401.
Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM and Dryja TP. (1986). *Nature,* **323,** 643–646.
Gill RM, Hamel PA, Jiang Z, Zacksenhaus E, Gallie BL and Phillips RA. (1994). *Cell Growth Diff.,* **5,** 467–474.
Ginsberg D, Vairo G, Chittenden T, Xiao Z-X, Xu G, Wydner KL, DeCaprio JA, Lawrence JB and Livingston DM. (1994). *Genes & Dev.,* **8,** 2665–2679.
Gossen M and Bujard H. (1992). *Proc. Natl. Acad. Sci. USA,* **89,** 5547–5551.
Hamel PA, Gill M, Phillips RA and Gallie BL. (1992a). *Oncogene,* **7,** 693–701.
Hamel PA, Gill RM, Phillips RA and Gallie BL. (1992b). *Mol. Cell. Biol.,* **12,** 3431–3438.
Helin K, Lees JA, Vidal M, Dyson N, Harlow E and Fattaey A. (1992). *Cell,* **70,** 337–350.
Helin K, Wu C, Fattaey AR, Lees JA, Dynlacht BD, Ngwu C and Harlow E. (1993). *Genes & Dev.,* **7,** 1850–1861.
Hiebert SW, Blake M, Azizkhan J and Nevins JR. (1991). *J. Virol.,* **65,** 3547–3552.
Hiebert SW, Lipp M and Nevins JR. (1989). *Proc. Natl. Acad. Sci. USA,* **86,** 3594–3598.
Hijmans EM, Voorhoeve PM, Beijersbergen RL, Van 'T Veer LJ and Bernards R. (1995). *Mol. Cell. Biol.,* **15,** 3082–3089.
Hinds PW, Mittnacht S, Dulic V, Arnold A, Reed SI and Weinberg RA. (1992). *Cell,* **70,** 993–1006.
Hirai H and Sherr CJ. (1996). *Mol. Cell. Biol.,* **16,** 6457–6467.
Huang HJ, Yee JK, Shew JY, Chen PL, Bookstein R, Friedmann T, Lee EY and Lee WH. (1988). *Science,* **242,** 1563–1566.
Hurford Jr, RK, Cobrinik D, Lee M-H and Dyson N. (1997). *Genes & Dev.,* **11,** 1447–1463.
Ivey-Hoyle M, Conroy R, Huber H, Goodhart PJ, Oliff A and Heimbrook DC. (1993). *MCB,* **13,** 7802–7812.
Jiang Z, Zacksenhaus E, Gallie BL and Phillips RA. (1997). *Oncogene,* **14,** 1789–1797.
Johnson DG, Cress WD, Jakoi L and Nevins JR. (1994a). *Proc. Natl. Acad. Sci. USA,* **91,** 12823–12827.
Johnson DG, Ohtani K and Nevins JR. (1994b) *Genes Dev.,* **8,** 1514–1525.
Johnson DG, Schwarz JK, Cress WD and Nevins JR. (1993). *Nature,* **365,** 349–352.
Kaelin Jr, WG, Krek W, Sellers WR, DeCaprio JA, Ajchenbaum R, Fuchs CS, Chittenden T, Li Y, Farnham PJ, Blanar MA, Livingston DM and Flemington EK. (1992). *Cell,* **70,** 351–364.
Karlseder J, Rotheneder H and Wintersberger E. (1996). *Mol. Cell. Biol.,* **16,** 1659–1667.
Kiess M, Gill RM and Hamel PA. (1995a). *Cell Growth & Differ.,* **6,** 1287–1298.
Kiess M, Gill RM and Hamel PA. (1995b). *Oncogene,* **10,** 159–166.
Krek W, Livingston DM and Shirodkar S. (1993). *Science,* **262,** 1557–1560.

Lee H-JS, Yee J-K, Shew J-Y, Chen P-K, Bookstein R, Friedmann T, Lee EY-HP and Lee W-H. (1988a). *Science,* **242,** 1563 – 1566.

Lee WH, Bookstein R and Lee EY. (1988b). *J. Cell. Biochem.,* **38,** 213 – 227.

Lees JA, Saito M, Vidal M, Valentine M, Look T, Harlow E, Dyson N and Helin K. (1993). *Mol. Cell. Biol.,* **13,** 7813 – 7825.

Lindeman GJ, Gaubatz S, Livingston DM and Ginsberg D. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 5095 – 5100.

Lukas J, Aagaard L, Strauss M and Bartek J. (1995a). *Cancer Res.,* **55,** 4818 – 4823.

Lukas J, Otzen Peterson B, Holm K, Bartek J and Helin K. (1996). *Mol. Cell. Biol.,* **16,** 1047 – 1057.

Lukas J, Parry D, Aagaard L, Mann DJ, Bartkova J, Strauss M, Peters G and Bartek J. (1995b). *Nature,* **375,** 503 – 506.

Medema RH, Herrera RE, Lam F and Weinberg RA. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 6289 – 6293.

Muncaster M, Cohen B, Phillips RA and Gallie BL. (1992). *Cancer Res.,* **52,** 654 – 661.

Neuman E, Ladha MH, Lin N, Upton TN, Miller SJ, DiRenzo J, Pastell RG, Hinds PW, Dowdy SF, Brown M and Ewen M. (1997). *Mol. Cell. Biol.,* **17,** 5338 – 5347.

Parry D, Bates S, Mann DJ and Peters G. (1995). *Embo J.,* **14,** 503 – 511.

Sardet C, Vidal M, Cobrinik D, Geng Y, Onufryk C, Chen A and Weinberg RA. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 2403 – 2407.

Schwarz JK, Bassing CH, Kovesdi I, Datto MB, Blazing M, George S, Wang X-F and Nevins JR. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 483 – 487.

Sellers WR, Rodgers JW and Kaelin Jr, WG. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 11544 – 11548.

Shan B, Chang C-Y, Jones D and Lee W-H. (1994). *Mol. Cell. Biol.,* **14,** 299 – 309.

Sherr CJ. (1996). *Science,* **274,** 1672 – 1677.

Shimizu M, Ichikawa E, Inoue U, Nakamura T, Nakajima T, Nojima H, Okayama H and Oda K. (1995). *Mol. Cell. Biol.,* **15,** 2882 – 2892.

Sidle A, Palaty C, Dirks P, Wiggan O, Kiess M, Gill RM, Wong AK and Hamel PA. (1996). *Crit. Rev. Biochem. Mol. Biol.,* **31,** 237 – 271.

Singh P, Wong SH and Hong W. (1994). *EMBO J.,* **13,** 3329 – 3338.

Slansky JE, Li Y, Kaelin WG and Farnham PJ. (1993). *Mol. Cell. Biol.,* **12,** 5620 – 5631.

Thalmeier K, Synovzik H, Mertz R, Winnacker EL and Lipp M. (1989). *Genes Dev.,* **3,** 527 – 536.

Ueki K, Ono Y, Henson JW, Efird JT, von Deimling A and Louis DN. (1996). *Cancer Res.,* **56,** 150 – 153.

Wade M, Blake MC, Jambou RC, Helin K, Harlow E and Azizkhan JC. (1995). *J. Biol. Chem.,* **270,** 9783 – 9791.

Wang C, Petryniak B, Thompson CB, Kaelin WG and Leiden JM. (1993). *Science,* **260,** 1330 – 1335.

Weinberg RA. (1995). *Cell,* **81,** 323 – 330.

Wu C-L, Zukerberg LR, Ngwu C, Harlow E and Lees JA. (1995). *Mol. Cell. Biol.,* **15,** 2536 – 2546.

Xu G, Livingston DM and Krek W. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 1357 – 1361.

Yeager T, Stadler W, Belair C, Puthenveettil J, Olopade O and Reznikoff C. (1995). *Cancer Res.,* **55,** 493 – 497.

Yee AS, Raychaudhuri P, Jakoi L and Nevins JR. (1989). *Mol. Cell. Biol.,* **9,** 578 – 585.

Zhang Y and Chellapan SP. (1995). *Oncogene,* **10,** 2085 – 2093.

Zhu L, van den Heuvel S, Helin K, Fattaey A, Ewen M, Livingston D, Dyson N and Harlow E. (1993). *Genes & Dev.,* **7,** 1111 – 1125.

Zhu L, Zhu L, Xie E and Chang L-S. (1995). *Mol. Cell. Biol.,* **15,** 3552 – 3562.

Zwijsen RM, Wientjens E, Klompmaker R, van der Sman J, Bernards R and Michalides RJ. (1997). *Cell,* **88,** 405 – 415.

Zwijsen RML, Klopmaker R, Wientjens EBHGM, Kristel PMP, Van Der Burg B and Michalides RJAM. (1996). *Mol. Cell. Biol.,* **16,** 2554 – 2560.