An E2F-binding site mediates the activation of the proliferative isoform of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase by phosphatidylinositol 3-kinase

Silvia FERNÁNDEZ DE MATTOS*†, Eric W.-F. LAM† and Albert TAULER*¹

*Departament de Bioquímica i Biologia Molecular-Divisió IV, Facultat de Farmàcia, Universitat de Barcelona, Av. Diagonal 643, E-08028 Barcelona, Catalunya, Spain, and

†Cancer Research-UK Labs and Section of Cancer Cell Biology, Imperial College School of Medicine at Hammersmith Hospital, Du Cane Road, London W12 0NN, U.K.

In the present study, we demonstrate that E2F is implicated in the regulation of the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF2K/Fru-2,6-BPase) during cell division. The expression of this enzyme is induced during the G₁/S transition of the cell cycle. We identified and monitored the E2F-pocket protein complexes that bind to the E2F site of the F-type promoter during cell-cycle entry, and we analysed their contribution to the phosphatidylinositol 3-kinase (PI 3-kinase)-mediated regulation of the promoter. We found that the predominant E2F complex bound to the F-type promoter in unstimulated/quiescent cells contains E2F4, DP1 and p130 proteins. In serum-stimulated (S-phase) cells, the composition of the complex switched to E2F1/4, DP1 and p107, together with cyclin A and cyclin-dependent kinase 2. Treatment with the PI 3-kinase specific inhibitor LY 294002 prevented the formation of

the S-phase complex, suggesting that activation of the PI 3-kinase pathway is essential for the formation of this complex. Further supporting this idea, we obtained results showing that treatment of cycling NIH 3T3 cells with either wortmannin or LY 294002 induces the accumulation of the transcriptionally repressive p130–E2F4–DP1 complex. Using the Rat-1 ER–E2F1 cell line where E2F1 activity can be conditionally induced, we demonstrated that E2F activity is involved in the *in vivo* transcriptional regulation of the F-type 6PF2K/Fru-2,6-BPase gene. Taken together, our results show that the F-type 6PF2K/Fru-2,6-BPase is a genuine E2F-regulated gene, and that its regulation by the PI 3-kinase pathway is at least partially mediated through the E2F transcription factor.

Key words: cell cycle, E2F, glycolysis.

INTRODUCTION

Over seven decades ago, biochemical studies by Warburg demonstrated that most human and animal tumours show altered metabolic profiles and display high rates of glucose uptake and glycolysis. This original observation has been widely confirmed. The glucose uptake *in vivo* of several clinical tumours is about an order of magnitude higher than that of normal tissue [1]. Although these metabolic changes are not the fundamental cause of cancer, they are essential for providing the energy and the biosynthetic precursors that cells require for entering the cell cycle.

Growth factors and transforming oncogenes activate the glycolytic flux by modulating the activity of several key enzymes of this metabolic route. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF2K/Fru-2,6-BPase) is a glycolytic enzyme whose expression is induced during the G₁/S transition of the cell cycle [2]. This enzyme catalyses both the synthesis and degradation of Fru-2,6-P₂, a potent stimulator of 6-phosphofructo-1-kinase activity. This enzyme is one of the key regulators of the glycolytic pathway, and is in part responsible for the high glucose uptake observed in proliferative and cancer cells. Changes in Fru-2,6-P₂ concentration have been shown to have an immediate effect on cell-cycle progression and survival [3,4].

Four different genes encoding distinct 6PF2K/Fru-2,6-BPase isoenzymes have been described, and their expression is tissue and cell-type-specific (reviewed in [5]). The F-type isoenzyme encoded by gene A is predominantly functional in proliferative tissues and can be activated transcriptionally by growth factors

or oncogenic transformation. The mitogen-activated protein kinase and phosphatidylinositol 3-kinase (PI 3-kinase) pathways have recently been implicated in the regulation of this isoform during the G₁/S transition of the cell cycle [2,6]. However, the specific transcription factors responsible for this regulation are still unknown. Binding sites for the oncogenic transcription factors E2F and AP-1, located in the F-type promoter, have been suggested to be involved in the regulation of this isoform during cell proliferation [7,8]. Mutagenic analysis showed that loss of any of these sites impairs the promoter response to growth factors. However, only the E2F-binding site seems to be essential for the early response of the promoter.

The E2F transcription factors function as heterodimeric complexes with the DP family of proteins. To date, six distinct E2F (*E2F1–6*) and two DP (*DP1–2*) genes have been cloned and characterized [9]. E2F proteins regulate the activity of promoters containing E2F-binding sites, including genes encoding S-phase regulatory proteins as well as regulators of cell-cycle progression ([10]; but see [10a]). E2F activity is negatively regulated by interaction with the retinoblastoma family of pocket proteins (pRb, p107 and p130); these proteins alter the function of E2F by direct binding, which leads to repression of its transactivation activity [11]. The ability of pocket proteins to bind to E2F is regulated by their cell cycle-dependent phosphorylation [12]. Pocket proteins are hypophosphorylated during G₀ and early G₁, and these forms bind and inhibit E2F-dependent transcriptional activity. Mitogenic growth factors induce sequential activation of the cell cycle-dependent kinase complexes, which in turn phosphorylate pocket proteins and cause them

Abbreviations used: BrdUrd, bromodeoxyuridine; cdk2, cyclin-dependent kinase 2; DCS, donor calf serum; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FCS, foetal calf serum; 4-OHT, 4-hydroxytamoxifen; 6PF2K/Fru-2,6-BPase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B.

¹ To whom correspondence should be addressed (e-mail tauler@farmacia.far.ub.es).

to release E2F activity [13]. Despite a wealth of knowledge on the proliferative function of the PI 3-kinase pathway, little is known about its connection with the E2F family of transcription factors. To determine how the activation of PI 3-kinase pathway could regulate the transcriptional activity of F-type 6PF2K/*Fru-2,6-BPase* promoter, we have identified the cell cycle-specific complexes that bind to the E2F site of the F-type promoter, and we have analysed their contribution to the PI 3-kinase-mediated regulation of the promoter.

Our results show that the transcription factor E2F is a target of PI 3-K/protein kinase B (PKB) in the regulation of 6PF2K/*Fru-2,6-BPase*. The present study also demonstrates clearly that the F-type 6PF2K/*Fru-2,6-BPase* gene is an *in vivo* target of the E2F transcription factor, in particular E2F1.

EXPERIMENTAL

Materials

Foetal calf serum (FCS), donor calf serum (DCS), Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin were purchased from Biological Industries (Kibbutz Beit Haemek, Israel). T4 polynucleotide kinase was obtained from New England Biolabs (Hitchin, Herts., U.K.), T3 RNA polymerase was from Promega (Chilworth, Southampton, U.K.) and RNase A was from Boehringer Mannheim (Lewes, East Sussex, U.K.). The Random Primer DNA-labelling kit was from Biological Industries. Hybond-N membranes were from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). Epidermal growth factor (EGF) and 4-hydroxytamoxifen (4-OHT) were from Sigma. LY 294002 was from Calbiochem (La Jolla, CA, U.S.A.). ExpressHyb Hybridization Solution was from Clontech Laboratories (UK Limited, Basingstoke, U.K.). [γ - 32 P]ATP and [α - 32 P]dCTP (3000 mCi/mmol) were from Amersham Pharmacia. Other materials and chemicals were of the highest quality available.

Cell cultures

Cells were grown in DMEM supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin, 10% (v/v) FCS for Rat-1 and Rat-1 ER-E2F1 cells and 10% (v/v) DCS for NIH 3T3 cells, in a 5% CO₂ humidified atmosphere at 37 °C. For starvation conditions, cells were grown in DMEM–0.5% FCS for 48 h, and induced with fresh 10% serum, EGF (25 ng/ml) or 4-OHT (300 nM). When necessary, cells were preincubated for 30 min in the presence of LY 294002 at the indicated concentrations (Calbiochem, U.K.). Cycling NIH 3T3 cells were treated with 25 μ M LY 294002 or 100 nM wortmannin (Calbiochem, U.K.) for 16 h.

Extraction of DNA-binding proteins

DNA-binding protein extracts from NIH 3T3 cells were prepared essentially as described in [14]. Briefly, cells were trypsinized and spun at 1000 rev./min for 10 min at 4 °C. The cell pellet was washed with ice-cold PBS, and cells were then resuspended in 400 μ l of cold buffer A [10 mM Hepes/KOH (pH 7.9) 1.5 mM MgCl₂/10 mM KCl/0.5 mM dithiothreitol/0.2 mM PMSF] by gently flicking the tube. The cells were allowed to swell on ice for 10 min. After a brief vortex, samples were centrifuged for 2 min at 4 °C, and the supernatant fraction was discarded. The pellet was resuspended in 30–40 μ l of cold buffer C [20 mM Hepes/KOH (pH 7.9)/25% glycerol/420 mM NaCl/1.5 mM MgCl₂/0.2 mM EDTA/0.5 mM dithiothreitol/0.2 mM PMSF] and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min at 4 °C. The

supernatant fraction contains the DNA-binding proteins. Protein yield was quantified by Bradford analysis (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.).

Electrophoretic mobility-shift and supershift analyses of E2F DNA-binding complexes

E2F gel retardation assays were performed essentially as described in [15], using a double-stranded oligonucleotide (5'-GATCTCGCTTAGGCGGGAGACGG-3') containing the E2F-binding site localized at the first exon of F-type gene as a probe [7]. The DNA probe was radiolabelled using T4 polynucleotide kinase. The DNA-binding protein extract (20 μ g) was incubated with 1–2 ng of 32 P-labelled oligonucleotide probe in a total volume of 20 μ l at 30 °C for 10 min. The reactions were electrophoresed on 4% polyacrylamide gels in 0.33 $\times$ Tris-buffered EDTA at 4 °C. Gels were then dried and exposed to X-ray films. Supershift assays were performed by adding 1 μ l of concentrated antibodies before the addition of the probe. Rabbit anti-pRB2/p130 and anti-cyclin A antisera were kindly provided by Dr A. Giordano (Philadelphia, U.S.A.) and Dr J. Pines (Cambridge, U.K.) respectively. Anti-pRb mouse monoclonal antibody 21C9 [16] was a gift from Dr Sybille Mittnacht (London, U.K.). Anti-DP1, -E2F1, -E2F4 rabbit antisera were raised against peptides corresponding to unique C-terminal regions of the respective human proteins and have been described previously [17]. The antibodies raised against E2F2 (L-20), E2F3 (N-20) and E2F5 (MH5 and E-19) were acquired from Santa Cruz Biotechnology. Anti-cyclin-dependent kinase 2 (cdk2) antibody was kindly provided by Dr O. Bachs (Barcelona, Spain).

Cell-cycle analysis

Cell-cycle analysis was performed by combined propidium iodide (PI) and bromodeoxyuridine (BrdUrd) staining. Before collection, fibroblasts were incubated for 30 min with 10 μ M BrdUrd (Sigma). Cells were trypsinized, collected by centrifugation and resuspended in PBS, before fixing in 90% ethanol. The fixed cells were incubated first with 2 M HCl, then with 0.5% Triton X-100 for 30 min at room temperature, and then with FITC-conjugated anti-BrdUrd antibodies (BD Biosciences, U.K.) at 1:3 dilution for 30 min, with PBS washes between each treatment. The cells were incubated with 5 μ g/ml propidium iodide (Sigma), 0.1 mg/ml RNase A (Sigma), 0.1% Nonidet P40 and 0.1% trisodium citrate for 30 min before analysis using a Becton Dickinson FACSsort analyser. The cell-cycle profile was analysed using the Cell Quest software.

RNA isolation and analysis

Total RNA was extracted from Rat-1 cells by the LiCl/urea method [18]. The RNA was dissolved in sterile water, and the concentration and purity of each sample were assessed by measuring the absorbance *A* at 260 nm and by the *A*₂₆₀/*A*₂₈₀ ratio respectively. The abundance of 6PF2K/*Fru-2,6-BPase* mRNA was measured by Northern-blot analysis using ExpressHyb solution according to the manufacturer's instructions (Clontech), and a 1 kb *Eco*RI fragment isolated from a pKB plasmid [19] was used as a probe. The integrity of the RNA was verified by observing the rRNA bands in ethidium bromide-stained gel under UV irradiation. The level of mRNA was evaluated by densitometric scanning of the autoradiogram.

RNase protection assay

RNase protection assay was essentially performed as described by Cifuentes et al. [20]. To detect the presence of L-type mRNA,

an *Eco*RI digested fragment obtained from p2K500 [21] was cloned into Bluescript (+) plasmid and linearized with *Ava*II. Complementary RNA was synthesized with T3 RNA polymerase in the presence of 50 μ Ci of [α - 32 P]UTP. The synthesized riboprobe was 390 bases long, of which 319 bases are complementary to the first, second and third exons encoding the hepatic isoenzyme-specific transcript. This probe is also complementary to a 192-base region, shared by both the L- and F-type isoenzymes, corresponding to the second and third exons of the gene. To detect the presence of F-type mRNA, a PCR probe was made from a genomic 6PF2K/Fru-2,6-BPase fragment [22] with the following oligonucleotides: forward 1aF 5'-AAAGGCATTGC-CGCCCCGGAAGTG (which is located 5' upstream from exon 1aF), reverse 1aF 5'-TGTAATACGACTCACTATACAGTTA-CTCTTCCCA AGTTTTGG (which is located at the 3' of exon 1aF). The underlined nucleotide sequence contains the T7 RNA polymerase-binding motif. The synthesized riboprobe was 292 bases long, of which 218 bases are complementary to the 1aF exon of F-type mRNA. Samples of 10 μ g of total RNA were incubated at 90 °C for 10 min with a molar excess of the purified riboprobe. The tubes were cooled slowly to 42 °C, and maintained at this temperature overnight. A mixture of RNase T1 and RNase A was used to degrade the single-stranded unhybridized probe. After digestion, the protected fragments were collected by centrifugation at 4 °C in the presence of guanidine thiocyanate and propan-2-ol, and subjected to electrophoresis on 6% polyacrylamide/8 M urea DNA-sequencing gels.

RESULTS

Analysis of nuclear protein binding to the E2F site of the F-type 6PF2K/Fru-2,6-BPase promoter

To investigate the functional E2F complexes associated with the E2F-binding site of the F-type promoter in quiescent or mitogen-

Figure 1 Electrophoretic mobility-shift analysis of E2F oligonucleotide in quiescent NIH 3T3 fibroblasts

NIH 3T3 were plated in 150 mm culture dishes (3×10^6 cells/dish) and serum-starved for 48 h with 0.5% FCS-containing medium. DNA-binding protein extracts were prepared and used for gel mobility-shift experiments as described in the Experimental section. The positions of the complexes are indicated as A–D. In the competition experiment (lanes 2–4), increasing amounts of non-labelled E2F probes ($5 \times$, $10 \times$ and $50 \times$ excess) were included in the binding reaction mixture.

Figure 2 Effects of LY 294002, EGF and serum on the E2F DNA-binding complexes of the 6PF2K/Fru-2,6-BPase promoter

(A) NIH 3T3 fibroblasts serum-starved for 48 h were pretreated for 30 min with the indicated concentrations of LY 294002 and then stimulated with EGF (25 ng/ml) for 20 h. The carrier for the inhibitor (DMSO) was added as a control. DNA-binding protein extracts were obtained from the treated cells and the extracts were then used for gel mobility-shift experiments using the E2F probe as described in the Experimental section. The lane marked by '-' corresponds to the control in which the mobility shift was performed without cell extract. Results from one representative experiment out of three for each condition are shown. (B) NIH 3T3 cells arrested at G_0 by serum deprivation were stimulated to re-enter the cell cycle with 10% DCS. Cells harvested at indicated times were labelled with BrdUrd (shown as BrdU in the Figure), fixed, stained with propidium iodide and FITC-conjugated anti-BrdUrd antibodies, and processed for FACS analysis. (C) Serum arrested and re-stimulated NIH 3T3 cells (0, 12, 16, 20, 24 and 28 h after serum induction) as well as cycling NIH 3T3 fibroblasts were harvested for mobility-shift analysis as described.

Figure 3 Antibody supershift analysis of E2F DNA-binding complexes in quiescent and serum-stimulated NIH 3T3 fibroblasts

DNA-binding protein extracts from 48 h serum-starved NIH 3T3 fibroblasts (no serum) and from NIH 3T3 cells re-stimulated with 10% DCS for 20 h after serum deprivation (serum) were used for antibody supershift analysis as described in the Experimental section. (A) Anti-p107, anti-pRb or anti-p130 (1 μ l) was added to the reaction tubes before the addition of the probe. (B) Anti-E2F1 (original batch), anti-DP1 or anti-E2F4 (1 μ l) was added to the reaction tubes before the addition of the probe. Notably, anti-E2F1 did not shift any of the original E2F complexes. (C) Anti-cyclin A or anti-cdk2 (1 μ l) was added to the reaction tubes before the addition of the probe. *, non-specific band. Results from representative experiments are shown in Figure 3.

stimulated cells, we performed mobility-shift analysis on DNA-binding proteins from cell extracts. The oligonucleotide probe used in these experiments contains the consensus E2F-binding region found in the F-type promoter. To analyse the complexes in quiescent cells, NIH 3T3 cells were serum-starved for 48 h, nuclear extracts were then obtained and the binding of proteins to the promoter was assessed (Figure 1). Mobility-shift analysis revealed at least four species of DNA-binding complexes, presumably containing E2F (complexes A–D). To determine whether the observed DNA–protein complexes were specific for the E2F site, a competition assay was performed (Figure 1). In this assay, the unlabelled DNA fragment (unlabelled probe) was used as a specific competitor, and increasing amounts of the unlabelled probe were added to the binding reaction mixture. As it can be observed, excess unlabelled oligonucleotide efficiently competed with complexes A and B, whereas complexes C and D were unaffected. This suggested that complexes A and B were specific for the E2F site of the probe. We then focused on the characterization and functional analysis of the E2F-specific DNA–protein complexes A and B found on the F-type promoter.

Effect of LY 294002 on the E2F DNA-binding complexes bound to the F-type 6PF2K/Fru-2,6-BPase promoter

We have demonstrated previously that EGF regulates the expression of the 6PF2K/Fru-2,6-BPase gene through the PI 3-kinase/PKB pathway [6]. To investigate whether the transcription factor E2F is a target of PI 3-kinase/PKB in the regulation of 6PF2K/Fru-2,6-BPase by EGF, we analysed the effect of the PI 3-kinase inhibitor LY 294002 on the formation of the E2F complexes by electrophoretic mobility-shift analysis. To this end, quiescent NIH 3T3 fibroblasts either untreated or pretreated with LY 294002 for 30 min were stimulated with EGF for 20 h. DNA-binding protein extracts were prepared from these cells and used for gel shift assay (Figure 2A). Consistent with earlier results, the E2F complexes A and B were detected in the quiescent NIH 3T3 fibroblasts. Interestingly, following EGF stimulation, complex A disappeared and gave way to a slower migrating complex A'. This effect was completely abrogated by treatment with the specific PI 3-kinase inhibitor LY 294002. Complex B was unaffected by either the EGF or LY 294002 treatment. This result demonstrates that the activation of the PI 3-kinase pathway is essential for the formation of the 'active' complex A'.

Figure 4 Mobility and supershift analyses of E2F DNA-binding complexes in cycling and wortmannin- or LY 294002-treated NIH 3T3 fibroblasts

(A) Cycling NIH 3T3 cells untreated or treated with LY 294002 or wortmannin for 16 h were harvested, lysed and used for mobility-shift analysis. (B) Lysates derived from cycling NIH 3T3 cells were used for supershift analysis by anti-pRB, p107, p130, cyclin A, DP1, E2F1 (batch 2), E2F4, E2F2, E2F3 and E2F5 antibodies. (C) Lysates from wortmannin-treated NIH 3T3 cells were used for supershift analysis as described.

Activation of NIH 3T3 cells with 10% DCS for 20 h also induced similar changes in E2F complexes to those observed with EGF stimulation (Figures 2B and 2C).

Identification of E2F DNA-binding complexes by antibody supershift assay

The E2F–DP heterodimers can form ‘free’ E2F DNA-binding complexes by themselves or with the pRb family of pocket proteins to produce ‘complexed’ E2Fs. To study the presence of the pocket proteins in the DNA-binding protein complexes formed on the E2F site of the *6PF2K/Fru-2,6-BPase* promoter, antibody supershift experiments were performed on both quiescent and serum-stimulated cell extracts using anti-p107, anti-pRB and anti-p130 antibodies (Figure 3A). As shown in Figure 3(A),

the anti-p130 antibody efficiently shifted the majority of complex A in quiescent cells, whereas the anti-p107 antibody only shifted part of the complex. In contrast, in serum-stimulated cell extracts, the anti-p130 antibody did not shift complex A', whereas this complex was completely shifted by addition of the anti-p107 antibody. Consistent with previous studies [15,23], no supershift was detected with the anti-pRB antibody in these murine fibroblasts. These results suggest a difference in the composition of the E2F complexes regarding the cell-cycle state, with p130 as the predominant pocket protein in G_0 cells, being substituted by p107 when cells enter the G_1/S phase after EGF stimulation.

To characterize further the DNA–protein complexes observed on the E2F site of the F-type promoter, we next analysed for the presence of distinct members of the E2F family of transcription factors. Antibodies with specific function in supershift assays were available for E2F1, E2F4 and DP1 (Figure 3B). The results obtained revealed that anti-E2F4 and anti-DP1 antibodies shifted complexes A and B in quiescent cells and complexes A' and B in serum-stimulated cells, indicating that E2F4 and DP1 are present in these complexes. Although a new complex could be detected in the presence of the anti-E2F1 antibody, the complexes A, A' and B remained unshifted in the presence of this antibody. This indicates that the complex observed in the presence of this anti-E2F1 is not the result of a supershift. The fact that E2F4 and DP1 antibodies shifted both complexes in quiescent and serum-stimulated cell extracts suggested that E2F4 and DP1 are the predominant E2F family members present under these conditions.

It has been demonstrated that p130 and p107 can stably associate with the cyclin A/cdk2 complex, and these interactions do not prevent the pocket proteins from binding to E2F [24]. To assess whether the slower migratory rate of complex A' was also due to the presence of cyclin A/cdk2, supershift analyses were performed with cell extracts from quiescent and serum-stimulated cells using cyclin A and cdk2 antibodies (Figure 3C). The result showed that complex A' in stimulated cells was efficiently shifted by anti-cyclin A antibody, whereas complex A in the quiescent cells was not affected. It is noteworthy that the introduction of the anti-cyclin A antibody also gave rise to a minor non-specific band (*), which is not a result of supershifted complex A. The anti-cdk2 antibody had no effect on complex A, and a shift was detected for complex A'. These results led us to conclude that the active A' E2F complex in S-phase cells contains cyclin A and cdk2, and that these proteins are absent in complex A when cells are forced to quiescence.

To confirm further these findings, we treated cycling NIH 3T3 cells with another specific PI 3-kinase inhibitor, wortmannin, which functions differently from LY 294002. Treatment with wortmannin caused the accumulation of E2F complexes similar to those observed in the LY 294002-treated cells (Figure 4A). Supershift assays were performed to identify the E2F complexes before and after wortmannin treatment. These supershift analyses showed that in cycling NIH 3T3 cells, the majority of the slower migrating ‘complexed’ E2Fs contain p107, E2F4, DP1 and cyclin A, whereas the ‘free’ E2F complexes consist largely of DP1 binding to either E2F1 (more mobile complex: B') or E2F4 (less mobile complex B) (Figure 4B). It is notable that the more mobile ‘free’ E2F complex (B'), consisting of DP1 and E2F1, is not present at high levels in the EGF-stimulated cells and in the serum-induced ‘S-phase’ fibroblasts. This is likely to be the result of the high levels of E2F1 in the cycling NIH 3T3 cells and reflects the fact that a great number of the EGF or serum-stimulated (20 h) NIH 3T3 cells are at late G_1 and early S phase, where the E2F1–DP1 level is low (Figures 2B and 2C). The supershift results also demonstrated that wortmannin caused the

Figure 5 Effect of 4-OHT on 6PF2K/Fru-2,6-BPase mRNA levels in the Rat-1 ER-E2F1 cell line

(A) Total RNA extracted from Rat-1 cells, rat liver (L) and rat muscle (M) was hybridized with the hepatic probe and digested with RNase as described in the Experimental section. The resulting RNA duplexes were analysed after electrophoresis in acrylamide/urea gel. (B) Total RNA from Rat-1 cell and rat liver (L) was hybridized with the F-type specific probe and processed as described above. (C) Rat-1 ER-E2F1 cells were seeded (1.5×10^6 cells/100 mm dish) and serum-starved 1 day later for 48 h with 0.5% FCS-containing medium. Then, cells were induced with 10% serum, or treated with 300 nM 4-OHT for the indicated times. Total RNA (15 μ g lane) was extracted from cultured cells and analysed by Northern-blot analysis as described in the Experimental section (a representative Northern-blot analysis is shown). The intensity of the autoradiographic signal was quantified by densitometric scanning and corrected for the activation by 4-OHT in the parental Rat-1 cell line. Values are expressed as a ratio to the value of untreated cells, which was taken as 1 arbitrary unit. Data are means for three independent experiments.

accumulation of a species of 'complexed' E2F, containing predominantly p130, E2F4 and DP1, and that the 'free' E2F complex found in the wortmannin-treated NIH 3T3 cells was made up largely of E2F4 and DP1 (Figure 4C). Collectively, these results indicate that inhibition of the PI 3-kinase activity by either wortmannin or LY 294002 induces the accumulation of the E2F complexes containing p130, E2F4 and DP1. This E2F complex has previously been shown to regulate E2F-dependent transcription from E2F-regulated genes negatively [25–27].

Effect of E2F1 on 6PF2K/Fru-2,6-BPase mRNA levels

To investigate further the role of E2F transcription factors in F-type promoter regulation, we next analysed whether changes in E2F activity can influence transcription of the 6PF2K/Fru-2,6-BPase gene in its chromosomal context. From the different isoforms of 6PF2K/Fru-2,6-BPase encoded by gene A, the Rat-1 cell line expresses only the F-type isoform, as demonstrated by RNase protection assays (Figures 5A and 5B). In this experiment,

no 319-base protection band was obtained in Rat-1 cells using a specific riboprobe containing the L-type exon. In contrast, a single band of 218 bases was observed in Rat-1 cells using a specific riboprobe encoding the F-exon (Figure 5B). These results allowed us to use a Rat-1 cell line that expresses E2F1 fused to the oestrogen receptor ligand-binding domain (ER-E2F1) [28] to investigate regulation of the F-type promoter. The Rat-1 ER-E2F1 cells contain a modified version of the oestrogen receptor that responds only to 4-OHT. In the absence of 4-OHT, the fusion protein is localized in the cytoplasm, whereas after addition of 4-OHT most of the fusion protein is translocated to the nucleus [29]. This inducible system allows the transactivation of an E2F site-containing promoter in the absence of *de novo* protein synthesis. Rat-1 ER-E2F1 cells were serum-starved for 48 h, before 4-OHT was added to activate the ER-E2F1 fusion. Total RNA was extracted from the 4-OHT-treated cells at different times and Northern-blotted for 6PF2K/Fru-2,6-BPase expression. As shown in Figure 5(C), the abundance of 6PF2K/Fru-2,6-BPase mRNA increases after 4-OHT addition in the ER-E2F1 cells. The level of activation after 16 h of 4-OHT treatment was similar to that obtained with serum addition. As an internal control, the same experiment was performed in the parental Rat-1 cell line. In this case, 4-OHT treatment did not induce any change in the expression of the 6PF2K/Fru-2,6-BPase mRNA (results not shown). These results suggest that activation of the E2F1 protein in quiescent fibroblasts can transactivate the endogenous gene promoter to the same extent as serum induction, and it confirms the importance of the E2F transcription factor in the regulation of the 6PF2K/Fru-2,6-BPase F-type promoter activity and thus its gene expression.

DISCUSSION

The progression of cells from G_1 into S and through S phase requires co-ordinated expression of a set of genes whose products are required for cell-cycle control and DNA synthesis. In this scenario, the transcription factor E2F controls cell proliferation by regulating the expression of genes encoding cell-cycle regulatory proteins, including cyclin E, cyclin A, B-myb, E2F1, cdc2 and c-myc, as well as proteins involved in DNA synthesis, such as dihydrofolate reductase, thymidine kinase and thymidylate synthetase [15,30–35]; but see [32a]. Protein synthesis is the major energy-consuming process in G_1 and S phases of the cell cycle. A direct link between ATP production and regulation of protein synthesis has been reported recently. It has been shown that the intracellular concentration of ATP can directly regulate protein synthesis by modulating the mammalian target of rapamycin activity [36]. However, it is not clear how energy production can be integrated with the cell cycle. An E2F-binding site has been identified on the promoter of the proliferative isoform of the glycolytic enzyme 6PF2K/Fru-2,6-BPase [7]. This suggests that E2F might be involved in the regulation of the ATP content of the cell during cell division by controlling the expression of the glycolytic enzymes, such as 6PF2K/Fru-2,6-BPase.

Previous studies of our group demonstrate that the PI 3-kinase pathway is necessary for the expression of 6PF2K/Fru-2,6-BPase during cell proliferation [6]. In the present study, we have identified and characterized the E2F-pocket protein complexes that bind to the E2F site of the F-type promoter during cell-cycle entry, and we have also analysed their contribution to the PI 3-kinase-mediated regulation of the promoter. Our results led us to propose that the predominant E2F complex bound to the F-type promoter in unstimulated/quiescent cells largely consists of

E2F4, DP1 and p130 proteins, as well as a low level of p107. In the serum-stimulated (S-phase) cells, the complex switches to E2F4, DP1 and p107, together with cyclin A and cdk2. Inhibition of the PI 3-kinase pathway prevents the formation of this S-phase complex, since in LY 294002-pretreated serum-stimulated cells the composition of the E2F complex is the same as in the unstimulated cells. Similarly, treatment of cycling NIH 3T3 cells with either LY 294002 or wortmannin induced the accumulation of the transcriptionally repressive E2F4–DP1–p130 complex. A previous study has demonstrated that the E2F site located on the F-type *6PF2K/Fru-2,6-BPase* promoter is essential for its trans-activational activity, and deletion of the E2F-binding site completely abrogated its activity [7]. Based on this finding, our present results strongly suggest that the switching of the E2F4–DP1–p130 to the E2F4–DP1–p107–cyclin A–cdk2 complex on the E2F site of the F-type *6PF2K/Fru-2,6-BPase* promoter is essential for the transcriptional activation during the G₁/S transition of the cell cycle. These results agree with recent findings which show that p130 is the principal pRb family member associated with growth-related genes in G₀ and early G₁, when these genes are transcriptionally inactive [37]. The binding of p130 to these promoters could be responsible for the recruitment of histone deacetylase-1 that is required for transcriptional repression [38]. The results obtained in the present study also demonstrate the involvement of p107 in the formation of an S-phase-specific E2F–p107–cyclin A–cdk2 complex in response to cell-cycle entry. Consistent with our results, in Saos-2 cells, the number of p107-containing E2F complexes associated with interchromatin and heterochromatin is higher in S-phase compared with G₀/G₁; in contrast, the level of E2F/p130 complexes is higher in arrested cells [39]. In primary T-cells, E2F4 has also been reported to switch from p130 to p107 in response to cell-cycle re-entry [40]. *p107* has been demonstrated to be an E2F-responsive gene [41], and its expression is negatively regulated by the p130–E2F complex; this could explain the accumulation of this protein as part of the E2F complex as cells traverse from G₀ into G₁ and S. Interestingly, genomic footprinting experiments with the *B-myb*, *cyclin A* and *cdc2* gene promoters have shown that the E2F-binding sites on these promoters are occupied in G₀ and early G₁, when these promoters are repressed by the p130/p107–E2F complexes, but are largely unoccupied during the G₁/S transition. These results demonstrate that these E2F-regulated genes are primarily controlled by derepression through the E2F site and that the S-phase-specific E2F–p107–cyclin A–cdk2 complexes might not actually *in vivo* exist on these E2F sites [42–44]. Consistent with the latter idea, chromatin immunoprecipitation experiments performed on T98G human glioblastoma cells also prove the absence of binding of p107 protein to the E2F-responsive promoters during the G₁/S transition, even though mobility-shift analysis indicates the presence of E2F–p107–cdk2 complexes in late G₁ and S in these cells [37].

Notably, we cannot detect the presence of pRb-containing E2F complexes on the E2F site of the F-type promoter in the NIH 3T3 cells in our mobility-shift experiments. This is not surprising considering previous studies that suggest the absence of pRb in E2F-regulated promoters such as E2F1, cyclin A, *B-myb* or p107 [37]. Previous mobility-shift studies using NIH 3T3 cells have also showed the lack of pRb-containing complex on E2F sites of other promoters [15,23]. These findings indicate that the types of E2F–pocket protein complexes detected in the present study could be specific for NIH 3T3 and that, in general, these E2F–pocket protein complexes can be species- and cell type-dependent. For instance, E2F–pRb has been shown to be the predominant E2F complex present in G₀ phase in primary human B lymphocytes [45], whereas E2F–p130 is the predomi-

nant E2F complex found in quiescent mouse B cells. Therefore the absence of pRb binding to these promoters does not exclude a role of this protein in the regulation of the F-type promoter. The ability of p130 and pRb to switch roles is highlighted by mouse gene deletion studies, demonstrating that the pRB family of three pocket proteins have overlapping roles and can functionally substitute for one another to repress E2F-dependent gene transcription [46].

Individual E2F–DP heterodimers associate specifically with distinct members of the retinoblastoma family of pocket proteins. Previous studies have shown that E2F4-containing E2F–DP heterodimers preferentially complex with p107 and p130 [47]. In agreement with this, our results demonstrated that E2F4 is present in the E2F complex of the F-type *6PF2K/Fru-2,6-BPase* in both unstimulated and stimulated cells. By recruiting p130 and p107, the E2F4-containing complex may function as a transcriptional repressor of the gene expression. Moreover, E2F4 together with E2F1 was also detected in the ‘free’ E2F–DP complex, presumably transcriptionally active. However, the presence of other members of the E2F family cannot be excluded.

We have reported previously that PKB mediates the transcriptional regulation of F-type promoter by the PI 3-kinase pathway [6]. The present results demonstrate that inhibition of the PI 3-kinase pathway and thus PKB activity by LY 294002 blocks the switch of the transcriptionally repressive E2F–p130 to the S-phase-specific complex containing E2F4, DP1, p107, cyclin A and cdk2. Taken together, these results suggest that PKB mediates the transcriptional activity of F-type promoter at least partially by modulating the DNA-binding complexes formed on the E2F site. These changes in E2F complexes can be explained by the effects of PKB on several components of the cell-cycle machinery. PKB has previously been shown to phosphorylate and inactivate GSK3 β , which can in turn lead to the stabilization of cyclin D1 [48]. Moreover, PKB can repress the transcription of the cdk inhibitor p27^{Kip1} by negatively regulating the forkhead family of transcription factors [49]. Furthermore, PKB can also directly phosphorylate p21^{Cip1}, rendering it to relocate to the cytoplasm and thus suppressing its cdk-inhibiting activity [50]. Thus, the PI 3-kinase/PKB signalling pathway can modulate E2F transcriptional activity via its action on cellular levels of cyclins and their inhibitors, which in turn modulate pocket protein activity. The possibility that PKB could also affect other components of the characterized complexes may also be considered. For example, very recently [51], the forkhead transcription factor FKHR-L1 has been shown to induce the expression of p130 and, consequently, p130–E2F complex accumulation and G₀ arrest.

Darville et al. [7] demonstrated that the F-type promoter can be activated by E2F using transient and stable transfection assays. However, this approach is not always reliable and results obtained are often not conclusive, as several genes, including *cdc2* and *c-myc*, which were originally defined as E2F-regulated genes using this experimental approach, have been proved to be cell-cycle-controlled but not E2F-regulated genes [28,33]. As a result of this, it was important for us to confirm the ability of E2F to induce the expression of the endogenous F-type isoform, since the accessibility of DNA-binding sites for the transcription factors is largely regulated by chromatin structure [52]. In the present study, using the Rat-1 ER–E2F1 cell line, we confirmed and extended the work of Darville et al. [7]. We demonstrate for the first time that the transcription factor E2F is involved in the transcriptional regulation of the F-type *6PF2K/Fru-2,6-BPase* gene *in vivo*. We show that activation of E2F1–ER in response to 4-OHT induces the expression of *6PF2K/Fru-2,6-BPase* mRNA, suggesting that the mere translocation of the E2F1 protein to the

nucleus can transactivate the endogenous promoter. Although we used the inducible E2F1–ER system for the present study, it is likely that the *6PF2K/Fru-2,6-BPase* gene is not only the direct transcriptional target of E2F1 but also under the transcriptional control of other members of the E2F family of transcription factors. In support of this notion, our mobility-shift analysis suggests that the *6PF2K/Fru-2,6-BPase* gene is also transcriptionally repressed through the E2F sites by the p130–E2F4–DP1 complex at G₀ and early G₁ and its expression is regulated by a derepression mechanism.

In summary, we have characterized the E2F–pocket protein complexes that bind to the E2F site of the F-type promoter during the cell-cycle entry, and we have analysed their contribution to the PI 3-kinase pathway-mediated regulation of the promoter. We provide evidence showing that the F-type gene is under transcriptional control by E2F during the cell cycle and show definitively that *6PF2K/Fru-2,6-BPase* is an E2F-regulated gene. This study identifies a potential mechanism by which glycolytic pathway/glucose metabolism can be coupled to the cell-cycle machinery and also suggests a new role for E2F in cell-cycle control-by regulating the energy content of the cell.

We would like to thank Manel Joaquin and Joan Gil for continued interest in this work, Lolita Banerji for her invaluable help with the bandshift experiments and Priam Villalonga for a critical reading of the manuscript. We also thank K. Helin for the gift of the Rat-1 ER–E2F1 cell line. S.F. de M. is a recipient of a predoctoral fellowship from the Universitat de Barcelona. The work is supported by the Ministerio de Ciencia y Tecnología (BMC 2001-3809).

REFERENCES

- Gatenby, R. A. (1995) The potential role of transformation induced metabolic changes in tumour–host interaction. *Cancer Res.* **55**, 4151–4156
- Joaquin, M., Salvado, C., Bellosillo, B., Lange, A. J., Gil, J. and Tauler, A. (1997) Effect of growth factors on the expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in Rat-1 fibroblasts. *J. Biol. Chem.* **272**, 2846–2851
- Pérez, J. X., Roig, T., Manzano, A., Dalmau, M., Boada, J., Ventura, F., Rosa, J. L., Bermudez, J. and Bartrons, R. (2000) Overexpression of fructose 2,6-bisphosphatase decreases glycolysis and delays cell cycle progression. *Am. J. Physiol. Cell Physiol.* **279**, C1359–C1365
- Durante, P., Gueuning, M. A., Darville, M. I., Hue, L. and Rousseau, G. G. (1999) Apoptosis induced by growth factor withdrawal in fibroblasts overproducing fructose 2,6-bisphosphate. *FEBS Lett.* **448**, 239–243
- Okar, D. A., Manzano, A., Navarro-Sabate, A., Riera, L., Bartrons, R. and Lange, A. J. (2001) PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. *Trends Biochem. Sci.* **26**, 30–35
- Fernández de Mattos, S., de los Pinos, E., Joaquin, M. and Tauler, A. (2000) Activation of phosphatidylinositol 3-kinase is required for transcriptional activity of F-type 6-phosphofructo-2 kinase/fructose-2,6-bisphosphatase: assessment of the role of protein kinase B and p70 S6 kinase. *Biochem. J.* **349**, 59–65
- Darville, M. I., Antoine, I. V., Mertens-Strijthagen, J. R., Dupriez, V. J. and Rousseau, G. G. (1995) An E2F-dependent late serum-response promoter in a gene that controls glycolysis. *Oncogene* **11**, 1509–1517
- Joaquin, M., Salvado, C., Fernández de Mattos, S. and Tauler, A. (2002) An intronic AP-1 sequence mediates the transcriptional activation of the F-type 6-phosphofructo-2 kinase/fructose-2,6-bisphosphatase by serum. *Biochim. Biophys. Acta* **1574**, 131–136
- Dyson, N. (1998) The regulation of E2F by pRB-family proteins. *Genes Dev.* **12**, 2245–2262
- DeGregori, J., Kowalik, T. and Nevins, J. R. (1995) Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. *Mol. Cell. Biol.* **15**, 4215–4224
- Erratum. *Mol. Cell. Biol.* (1995) **15**, 5846–5847
- Helin, K., Harlow, E. and Fattaey, A. (1993) Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. *Mol. Cell. Biol.* **13**, 6501–6508
- Qian, Y., Luckey, C., Horton, L., Esser, M. and Templeton, D. J. (1992) Biological function of the retinoblastoma protein requires distinct domains for hyperphosphorylation and transcription factor binding. *Mol. Cell. Biol.* **12**, 5363–5372
- Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E. and Sherr, C. J. (1993) Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D dependent kinase CDK4. *Genes Dev.* **7**, 331–342
- Andrews, N. C. and Faller, D. V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.* **19**, 2499
- Lam, E. W.-F. and Watson, R. J. (1993) An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. *EMBO J.* **12**, 2705–2713
- Belbrahem, A., Godden-Kent, D. and Mittnacht, S. (1996) Regulation and activity of the retinoblastoma protein family in growth factor-deprived and TGF β -treated keratinocytes. *Exp. Cell Res.* **225**, 286–293
- Lam, E. W., Choi, M. S., van der Sman, J., Burbidge, S. A. and Klaus, G. G. (1998) Modulation of E2F activity via signaling through surface IgM and CD40 receptors in WEHI-231 B lymphoma cells. *J. Biol. Chem.* **273**, 10051–10057
- Auffray, C. and Rougeon, F. (1980) Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* **107**, 303–314
- Tauler, A., Rosenberg, A. H., Colosia, A., Studier, F. W. and Pilkis, S. J. (1988) Expression of the bisphosphatase domain of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6642–6646
- Cifuentes, M. E., Espinet, C., Lange, A. J., Pilkis, S. J. and Hod, Y. (1991) Hormonal modulation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene expression in rat hepatoma cells. *J. Biol. Chem.* **266**, 1557–1563
- Colosia, A. D., Marker, A. J., Lange, A. J., el-Maghrabi, M. R., Granner, D. K., Tauler, A., Pilkis, J. and Pilkis, S. J. (1988) Induction of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase mRNA by refeeding and insulin. *J. Biol. Chem.* **263**, 18669–18677
- Lange, A. J., Espinet, C., Hall, R., el-Maghrabi, M. R., Vargas, A. M., Miksicek, R. J., Granner, D. K. and Pilkis, S. J. (1992) Regulation of gene expression of rat skeletal muscle/liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Isolation and characterization of a glucocorticoid response element in the first intron of the gene. *J. Biol. Chem.* **267**, 15673–15680
- Voorhoeve, P. M., Watson, R. J., Farlie, P. G., Bernards, R. and Lam, E. W.-F. (1999) Rapid dephosphorylation of p107 following UV irradiation. *Oncogene* **18**, 679–688
- Devoto, S. H., Mudryj, M., Pines, J., Hunter, T. and Nevins, J. R. (1992) A cyclin A–protein kinase complex possesses sequence specific DNA binding activity: p33cdk2 is a component of the E2F cyclin A complex. *Cell* **68**, 167–176
- Rayman, J. B., Takahashi, Y., Indjeian, V. B., Dannenberg, J. H., Catchpole, S., Watson, R. J., te Riele, H. and Dynlacht, B. D. (2002) E2F mediates cell cycle-dependent transcriptional repression *in vivo* by recruitment of an HDAC1/mSin3B corepressor complex. *Genes Dev.* **16**, 933–947
- Furukawa, Y., Iwase, S., Kikuchi, J., Nakamura, M., Yamada, H. and Matsuda, M. (1999) Transcriptional repression of the E2F-1 gene by interferon- α is mediated through induction of E2F-4/pRB and E2F-4/p130 complexes. *Oncogene* **18**, 2003–2014
- Le Cam, L., Polanowska, J., Fabbriozzi, E., Olivier, M., Phillips, A., Ng Eaton, E., Classon, M., Geng, Y. and Sardet, C. (1999) Timing of cyclin E gene expression depends on the regulated association of a bipartite repressor element with a novel E2F complex. *EMBO J.* **18**, 1878–1890
- Vigo, E., Muller, H., Prosperini, E., Hateboer, G., Cartwright, P., Moroni, M. C. and Helin, K. (1999) CDC25A phosphatase is a target of E2F and is required for efficient E2F induced S phase. *Mol. Cell. Biol.* **19**, 6379–6395
- Koziczak, M., Muller, H., Helin, K. and Nagamine, Y. (2001) E2F1-mediated transcriptional inhibition of the plasminogen activator inhibitor type 1 gene. *Eur. J. Biochem.* **268**, 4969–4978
- Ohtani, K., DeGregori, J. and Nevins, J. R. (1995) Regulation of the cyclin E gene by transcription factor E2F1. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 12146–12150
- Geng, Y., Eaton, E. N., Picon, M., Roberts, J. M., Lundberg, A. S., Gifford, A., Sardet, C. and Weinberg, R. A. (1996) Regulation of cyclin E transcription by E2Fs and retinoblastoma protein. *Oncogene* **12**, 1173–1180
- Neuman, E., Flemington, E. K., Sellers, W. R. and Kaelin, Jr, W. G. (1994) Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. *Mol. Cell. Biol.* **14**, 6607–6615
- Erratum. *Mol. Cell. Biol.* (1995) **15**, 4460.
- Dalton, S. (1992) Cell cycle regulation of the human cdc2 gene. *EMBO J.* **11**, 1797–1804
- Roussel, M. F., Davis, J. N., Cleveland, J. L., Ghysdael, J. and Hiebert, S. W. (1994) Dual control of myc expression through a single DNA binding site targeted by ets family proteins and E2F-1. *Oncogene* **9**, 405–415
- Wade, M., Kowalik, T. F., Mudryj, M., Huang, E. S. and Azizkhan, J. C. (1992) E2F mediates dihydrofolate reductase promoter activation and multiprotein complex formation in human cytomegalovirus infection. *Mol. Cell. Biol.* **12**, 4364–4374
- Dennis, P. B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S. C. and Thomas, G. (2001) Mammalian TOR: a homeostatic ATP sensor. *Science* **294**, 1102–1105
- Takahashi, Y., Rayman, J. B. and Dynlacht, B. D. (2000) Analysis of promoter binding by the E2F and pRB families *in vivo*: distinct E2F proteins mediate activation and repression. *Genes Dev.* **14**, 804–816

- 38 Stiegler, P., De Luca, A., Bagella, L. and Giordano, A. (1998) The COOH-terminal region of pRb2/p130 binds to histone deacetylase 1 (HDAC1), enhancing transcriptional repression of the E2F-dependent cyclin A promoter. *Cancer Res.* **58**, 5049–5052
- 39 Zini, N., Trimarchi, C., Claudio, P. P., Stiegler, P., Marinelli, F., Maltarello, M. C., La Sala, D., De Falco, G., Russo, G., Ammirati, G. et al. (2001) pRb2/p130 and p107 control cell growth by multiple strategies and in association with different compartments within the nucleus. *J. Cell. Physiol.* **189**, 34–44
- 40 Moberg, K., Starz, M. A. and Lees, J. A. (1996) E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. *Mol. Cell. Biol.* **16**, 1436–1449
- 41 Ren, B., Cam, H., Takahashi, Y., Volkert, T., Terragni, J., Young, R. A. and Dynlacht, B. D. (2002) E2F integrates cell cycle progression with DNA repair, replication, and G₂/M checkpoints. *Genes Dev.* **16**, 245–256
- 42 Tommasi, S. and Pfeifer, G. P. (1995) *In vivo* structure of the human cdc2 promoter: release of a p130–E2F-4 complex from sequences immediately upstream of the transcription initiation site coincides with induction of cdc2 expression. *Mol. Cell. Biol.* **15**, 6901–6913
- 43 Huet, X., Rech, J., Plet, A., Vie, A. and Blanchard, J. M. (1996) Cyclin A expression is under negative transcriptional control during the cell cycle. *Mol. Cell. Biol.* **16**, 3789–3798
- 44 Zwicker, J., Liu, N., Engeland, K., Lucibello, F. C. and Muller, R. (1996) Cell cycle regulation of E2F site occupation *in vivo*. *Science* **271**, 1595–1597
- 45 van der Sman, J., Thomas, N. S. and Lam, E. W.-F. (1999) Modulation of E2F complexes during G0 to S phase transition in human primary B-lymphocytes. *J. Biol. Chem.* **274**, 12009–12016
- 46 Herrera, R. E., Sah, V. P., Williams, B. O., Makela, T. P., Weinberg, R. A. and Jacks, T. (1996) Altered cell cycle kinetics, gene expression, and G1 restriction point regulation in Rb-deficient fibroblasts. *Mol. Cell. Biol.* **16**, 2402–2407
- 47 Beijersbergen, R. L., Kerkhoven, R. M., Zhu, L., Carlee, L., Voorhoeve, P. M. and Bernards, R. (1994) E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 *in vivo*. *Genes Dev.* **8**, 2680–2690
- 48 Diehl, J. A., Cheng, M., Roussel, M. F. and Sherr, C. J. (1998) Glycogen synthase kinase-3 β regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.* **12**, 3499–3511
- 49 Medema, R. H., Kops, G. J. P. L., Bos, J. L. and Burgering, B. M. T. (2000) AFX-like Forkhead transcription factors mediate cell cycle regulation by Ras and PKB via p27. *Nature (London)* **404**, 782–787
- 50 Zhou, B. P., Liao, Y., Xia, W., Spohn, B., Lee, M. H. and Hung, M. C. (2001) Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat. Cell Biol.* **3**, 245–252
- 51 Kops, G. J., Medema, R. H., Glassford, J., Essers, M. A., Dijkers, P. F., Coffey, P. J., Lam, E. W. and Burgering, B. M. (2002) Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. *Mol. Cell. Biol.* **22**, 2025–2036
- 52 Pazin, M. J. and Kadonaga, J. T. (1997) What's up and down with histone deacetylation and transcription? *Cell* **89**, 325–328

Received 19 April 2002/16 July 2002; accepted 24 July 2002

Published as BJ Immediate Publication 24 July 2002, DOI 10.1042/BJ20020622