The EMBO Journal vol.14 no.19 pp.4814–4826, 1995

Identification of a Myc-dependent step during the formation of active G_1 cyclin–cdk complexes

Philipp Steiner, Angelika Philipp, Jiri Lukas[1], Dee Godden-Kent[2], Michele Pagano[3], Sybille Mittnacht[2], Jiri Bartek[1] and Martin Eilers[4]

Zentrum für Molekulare Biologie Heidelberg (ZMBH), Im Neuenheimer Feld 282, 69120 Heidelberg, Germany, [1]Danish Cancer Society, Strandboulevarden 49, 2100 Copenhagen, Denmark, [2]Institute of Cancer Research, Chester Beatty Laboratories, Fulham Road, London SW3 6JB, UK and [3]Mitotix Inc., 1 Kendall Square, Cambridge, MA 02139, USA

[4]Corresponding author

Activation of conditional alleles of Myc can induce proliferation in quiescent cells. We now report that induction of Myc in density-arrested fibroblasts triggers rapid hyperphosphorylation of the retinoblastoma protein and activation of both cyclin D1- and cyclin E-associated kinase activities in the absence of significant changes in the amounts of cyclin–cdk complexes. Kinase activation by Myc is blocked by inhibitors of transcription and requires intact DNA binding and heterodimerization domains of Myc. Activation of cyclin E–cdk2 kinase in serum-starved cells occurs in two steps. The first is induced by Myc and involves the release of a 120 kDa cyclin E–cdk2 complex from a 250 kDa inactive complex that is present in starved cells. This is necessary, but not sufficient, to generate full kinase activity, as cdc25 phosphatase activity is limiting in the absence of external growth factors. *In vivo* cdc25 activity can be supplied by the addition of growth factors. *In vitro* recombinant cdc25a strongly activates the 120 kDa, but only poorly activates the 250 kDa cyclin E–cdk2 complex. Our data show that two distinct signals, one of which is supplied by Myc, are necessary for consecutive steps during growth factor-induced formation of active cyclin E–cdk2 complexes in G_0-arrested rodent fibroblasts.

Keywords: cell cycle/cyclin D1/cyclin E/Myc/retinoblastoma protein

Introduction

c-*myc* was identified as the cellular homologue of the transforming oncogene of four different avian retroviruses (for a review see Meichle *et al.*, 1992). The gene encodes a transcription factor of the helix–loop–helix/leucine zipper family that forms a heterodimer with a partner protein termed Max (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991; Blackwood *et al.*, 1992). The complex binds specifically to CAC(G/A)TG sequences and activates transcription from such sites (for a review see Amati and Land, 1994). Further proteins, termed Mad and Mxi, (Ayer *et al.*, 1993; Zervos *et al.*, 1993) have been identified that heterodimerize with Max, but not Myc; complexes between these proteins and Max repress transcription (Ayer *et al.*, 1995; Schreiber-Agus *et al.*, 1995).

In mammalian cells several independent lines of evidence have implicated c-*myc* in the control of cell proliferation. Expression of c-*myc* is tightly regulated and closely parallels the proliferative status of cells (Westin *et al.*, 1982a,b; Kelly *et al.*, 1983; Kelly and Siebenlist, 1986). Cells that express c-*myc* from constitutive promoters fail to arrest in response to growth factor deprivation in culture or to physiological controls in an intact animal (Keath *et al.*, 1984; Langdon *et al.*, 1986; Eilers *et al.*, 1991; Evan *et al.*, 1992). Conversely, inhibition of c-*myc* expression inhibits cell proliferation and leads to an accumulation of cells in the G_1 phase of the cell cycle (Kaczmarek *et al.*, 1986; Heikkila *et al.*, 1987; Prochownik *et al.*, 1988); also, overexpression of Max inhibits cell proliferation (W.Gu *et al.*, 1993). Homozygous deletions of c-*myc* are lethal during early embryogenesis (Davis *et al.*, 1993). Taken together these data show that c-*myc* exerts an essential regulatory function in cell proliferation. They strongly suggest that controls which normally restrict progression through the G_1 phase of the cell cycle are overridden in the presence of deregulated c-*myc*. Several genes have been identified that are regulated by Myc *in vivo* (Eilers *et al.*, 1991; Benvenisty *et al.*, 1992; Bello-Fernandez *et al.*, 1993; Reisman *et al.*, 1993; Gaubatz *et al.*, 1994), however, with the possible exception of ornithine decarboxylase, none of them are candidates for important positively acting growth regulatory molecules.

Progress through the mammalian cell cycle is regulated by a set of kinases, termed cyclin-dependent kinases (cdks) and their regulatory subunits, the cyclins (for a review see Sherr, 1994). During the G_1 phase of the cell cycle both cyclin D1–cdk4 (or cdk6) and cyclin E–cdk2 complexes are active. They cooperate to phosphorylate and thereby inactivate the retinoblastoma protein (pRb) (Hatakeyama *et al.*, 1994), which in its hypophosphorylated form inhibits progression through the G_1 phase of the cell cycle (for a review see Weinberg, 1995). These cyclin–cdk complexes are therefore possible candidates that might mediate the mitogenic activities of Myc.

Expression of genes encoding cyclins and cdks is often low in quiescent cells and regulated in a cell cycle- or growth factor-dependent manner (for a review see Müller *et al.*, 1993). Transcriptional activation of cyclin or cdk genes might, therefore, be one mechanism by which Myc exerts its mitogenic effect (Jansen-Dürr *et al.*, 1993; Jiang *et al.*, 1993; Hanson *et al.*, 1994; Hoang *et al.*, 1994). Induction of conditional alleles of Myc in density-arrested or growth factor-deprived rat fibroblasts leads to a strong induction of cyclin A mRNA. In contrast, of the genes encoding cyclins E, D1 and D2 and cdk2 and cdk4 only cyclin E is moderately up-regulated by Myc (Jansen-Dürr

et al., 1993; Solomon *et al.*, 1995). However, cyclin E protein is detectable in quiescent cells and up-regulation of cyclin E mRNA seems to follow, not to precede, hyperphosphorylation of pRb in rat fibroblasts after induction of Myc (Jansen-Dürr *et al.*, 1993; this paper). Taken together these data raise the possibility that enhancing expression of cyclin and/or cdk genes may only be one of several mechanisms by which Myc acts to facilitate progression through the early cell cycle.

Activation of cdks is a multi-step process in which synthesis of the subunits, assembly and catalytic activity are regulated by multiple different regulatory processes (for reviews see Sherr, 1994; Morgan, 1995). The specific targets, if they exist, of Myc within this process have not been identified. In order to address this issue we have now analysed the activity of both cyclin E- and cyclin D1-dependent kinases in rat fibroblast cells (RAT1) that carry a conditional allele of Myc, MycER (Eilers *et al.*, 1989), and find that activation of Myc induces both cyclin E- and cyclin D1-dependent kinases. Our data define specific steps in the activation of either kinase that depend on the function of the c-*myc* proto-oncogene.

Results

To analyse effects of Myc on the phosphorylation status of pRb we prepared extracts from density-arrested RAT1A-MycER cells before and at several time points after addition of hydroxytamoxifen (HO-TAM), a synthetic oestrogen analogue that is an agonist for the MycER protein (Eilers *et al.*, 1989; Philipp *et al.*, 1994) (Figure 1A). About 80% of pRb was present in a faster migrating, hypophosphorylated form before activation of Myc. In contrast, only a slower migrating, hyperphosphorylated form of pRb was observed 4 h after activation of Myc and at later time points. These data demonstrate that induction of Myc triggers hyperphosphorylation of pRb.

Under these conditions cyclin E mRNA increases about 3-fold in response to activation of MycER within 24 h, whereas the expression of cyclins D1 and D2 and of cdk2 and cdk4 is unaffected by Myc (Jansen-Dürr *et al.*, 1993; Solomon *et al.*, 1995; P.Steiner and M.Eilers, unpublished observations). We tested whether changes in cyclin or cdk expression were responsible for triggering hyperphosphorylation of pRb. The same extracts were probed with specific antibodies, which showed that cyclins E and D1 and cdk2 and cdk4 were easily detectable in extracts from quiescent cells and that no significant changes were observed in the amounts of any of the proteins during the time of pRb hyperphosphorylation (Figure 1A). In addition, Myc does not appear to activate a hitherto unknown protein kinase, as dominant negative alleles of cdk2 (B.Rudolph and M.Eilers, in preparation) and antibodies against cyclin D1 (see below) inhibited cell cycle progression in response to activation of Myc.

Both cyclin D1- and cyclin E-associated kinases have been suggested to cooperate in phosphorylation of pRb (Hatakeyama *et al.*, 1994). To demonstrate directly that activation of Myc induced cyclin E-dependent kinase activity cyclin E immunoprecipitates prepared from extracts of density-arrested RAT1A-MycER cells before and at several time points after induction of Myc were subjected to kinase assays using histone H1 as substrate (Figure 1B). Cyclin E-dependent kinase activity was barely detectable in immunoprecipitates from quiescent cells; activation of Myc led to a 5-fold increase during the first 6 h and a further increase at later time points. To test whether Myc affected the assembly of cyclin E–cdk2 complexes cyclin E was immunoprecipitated from density-arrested Rat1A cells before and 6 h after induction of Myc. Immunoprecipitates were separated on SDS–PAGE and probed with antibodies directed against cdk2. Similar amounts of cdk2 were found complexed with cyclin E before and after activation of Myc (Figure 1C). Addition of ionic detergent (RIPA) abolished co-precipitation of cdk2 with cyclin E antibodies. No cdk2 protein was detectable in immunoprecipitates with a control antiserum, demonstrating the specificity of the observed interaction. Quantitation of a time course showed that the amount of cyclin E–cdk2 complex varied <2-fold during the experiment (Figure 1D). Thus Myc triggers activation of pre-assembled cyclin E–cdk2 complexes. Phosphorylation of cdk2 at Thr160 results in a faster migrating form of cdk2 (Gu *et al.*, 1992) and is a prerequisite for cdk2 kinase activity (Fisher and Morgan, 1994). However, Western blotting of cdk2 contained in cyclin E immunoprecipitates revealed that it migrated as a doublet before and after activation of Myc (Figure 1E), demonstrating that modification at Thr160 is apparently not the target of Myc induction of cyclin E–cdk2 kinase activity.

Activation of cyclin E–cdk2 kinase might reflect a direct interaction of Myc with components of this complex. Alternatively, Myc might induce the transcription of a gene(s) that participates in this activation. In support of the latter hypothesis we first showed that activation of cyclin E-dependent kinase by Myc was sensitive to inhibitors of transcription, even though incubation of RAT1A-MycER cells in the presence of actinomycin D for several hours did not alter the amount of MycER and cdk2 proteins and even induced cyclin E protein (Figure 2A).

Secondly, we established a number of RAT1A cell lines that express different alleles of Myc as oestrogen-inducible chimeras. Controls showed that all alleles were expressed at similar levels (Figure 2C). From these cell lines extracts were prepared from density-arrested cells before and 12 h after addition of HO-TAM and both the amount of cyclin E protein and of cyclin E-dependent kinase activity was determined (Figure 2B). Similar to what was found for wild-type c-*myc*, we observed that activation of none of the mutated chimeras affected the amount of cyclin E protein. The results also showed that mutations that affect either dimerization with Max (In412 and Δ370–412), transcriptional regulation (Δ128–143) or DNA binding of the Myc–Max complex (In370) abolished activation of cyclin E-dependent kinase. In contrast, mutations that disrupt the integrity of Myc box I or of a domain previously implicated in gene repression by Myc did not impair activation of cyclin E-dependent kinase by Myc (Philipp *et al.*, 1994). These data strongly suggest that activation of cyclin E-dependent kinase by Myc depends on the transcriptional activation of a target gene(s) by Myc–Max heterodimers.

The fact that ectopic expression of Myc reduces expression levels of cyclin D1 mRNA in proliferating fibroblasts prompted us to test whether ectopic expression of Myc might relieve the requirement for cyclin D1-associated

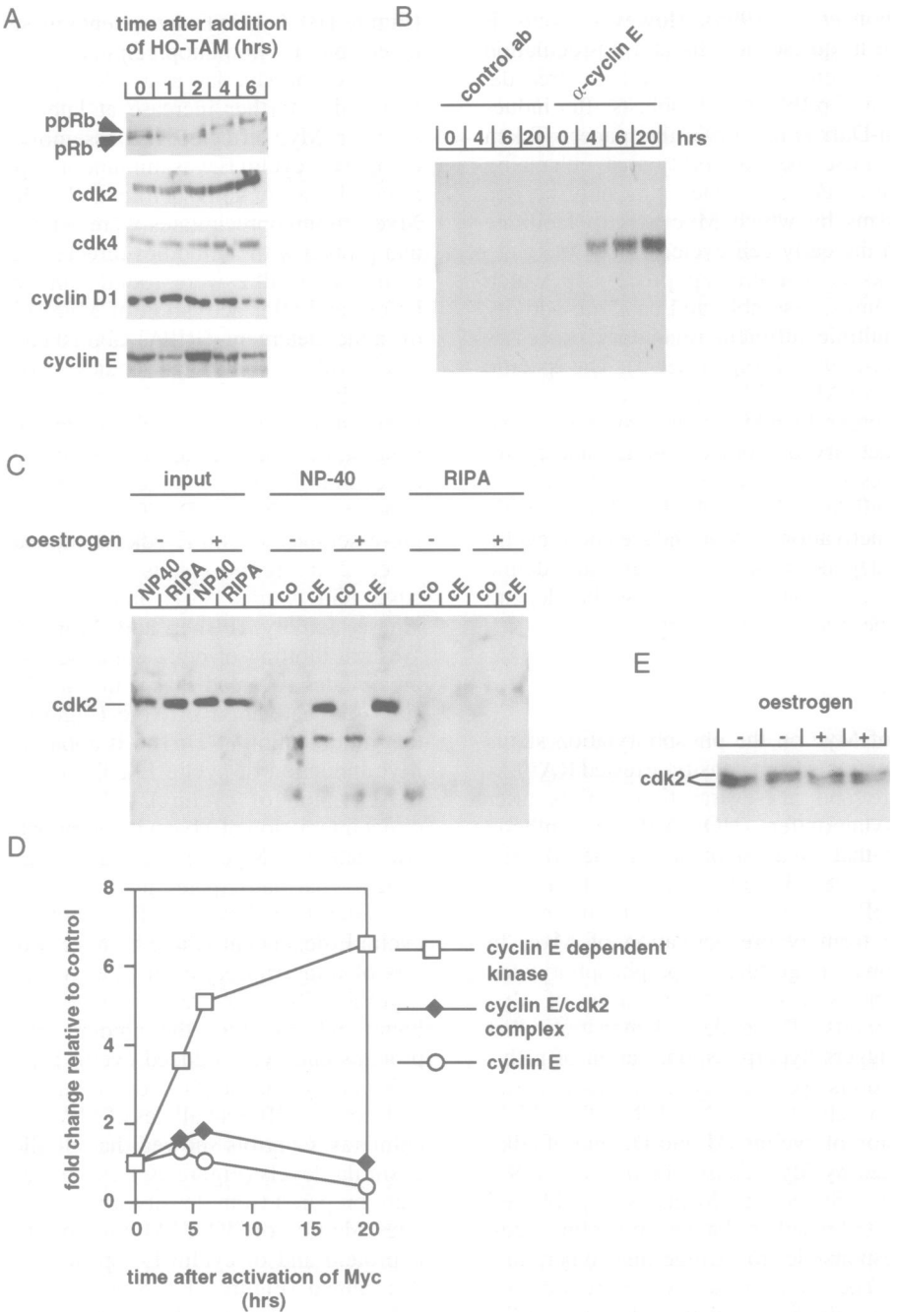

Fig. 1. Activation of Myc triggers hyperphosphorylation of pRb and activation of cyclin E–cdk2 kinase without affecting the amounts of cyclin E–cdk complexes. (**A**) RAT1A-MycER cells were grown to confluence and cells were harvested at the indicated times after addition of HO-TAM (250 nM). Shown are Western blots of cell extracts probed with antibodies directed against pRb, cdk2, cdk4, cyclin D1 and cyclin E. All extracts contained equal amounts of MycER protein (not shown). (**B**) Activation of cyclin E-dependent histone H1 kinase. RAT1A-MycER cells were grown to confluence, HO-TAM was added and cells were harvested after the indicated times; cell extracts were precipitated with either anti-cyclin E or control antibody. Shown is an autoradiogram of histone H1 phosphorylation by the immunoprecipitates. (**C**) Cdk2 and cyclin E are associated in density-arrested cells. Shown is an anti-cdk2 Western blot of total cellular extracts (input) and of anti-cyclin E (cE) and control (co)-immunoprecipitates prepared either in the presence of non-ionic (NP-40) or ionic (RIPA) detergents. RAT1-MycER cells were grown to confluence and either treated with ethanol (–) or with β-oestradiol (250 nM) (+) for 6 h. (**D**) Quantitation of a time course in RAT1A-MycER cells. Shown are the amounts of cyclin E protein, cyclin E–cdk2 complex and cyclin E-dependent histone H1 kinase activity in extracts from density-arrested RAT1A-MycER cells at various points after stimulation with HO-TAM. (**E**) Activation of Myc does not affect modification of cdk2 at Thr160. Cell extracts prepared before and after activation of Myc by β-oestradiol were precipitated with anti-cyclin E antibody, separated on SDS–PAGE and probed with anti-cdk2 antibody as in (C). The faster migrating species results from modification of cdk2 at Thr160 (Gu *et al.*, 1992). Shown are samples from duplicate experiments.

kinase (Jansen-Dürr *et al.*, 1993; Jiang *et al.*, 1993; Lovec *et al.*, 1994). Anti-cyclin D1 antibodies were electroporated into control or Myc-transformed Balb/c-3T3 cells arrested in mitosis by treatment with nocodazole. After electroporation cells were replated and the number of cells entering S phase measured as a function of time (Figure 3A). Electroporation of anti-cyclin D1 antibodies led to a decrease in cells entering S phase in both control and

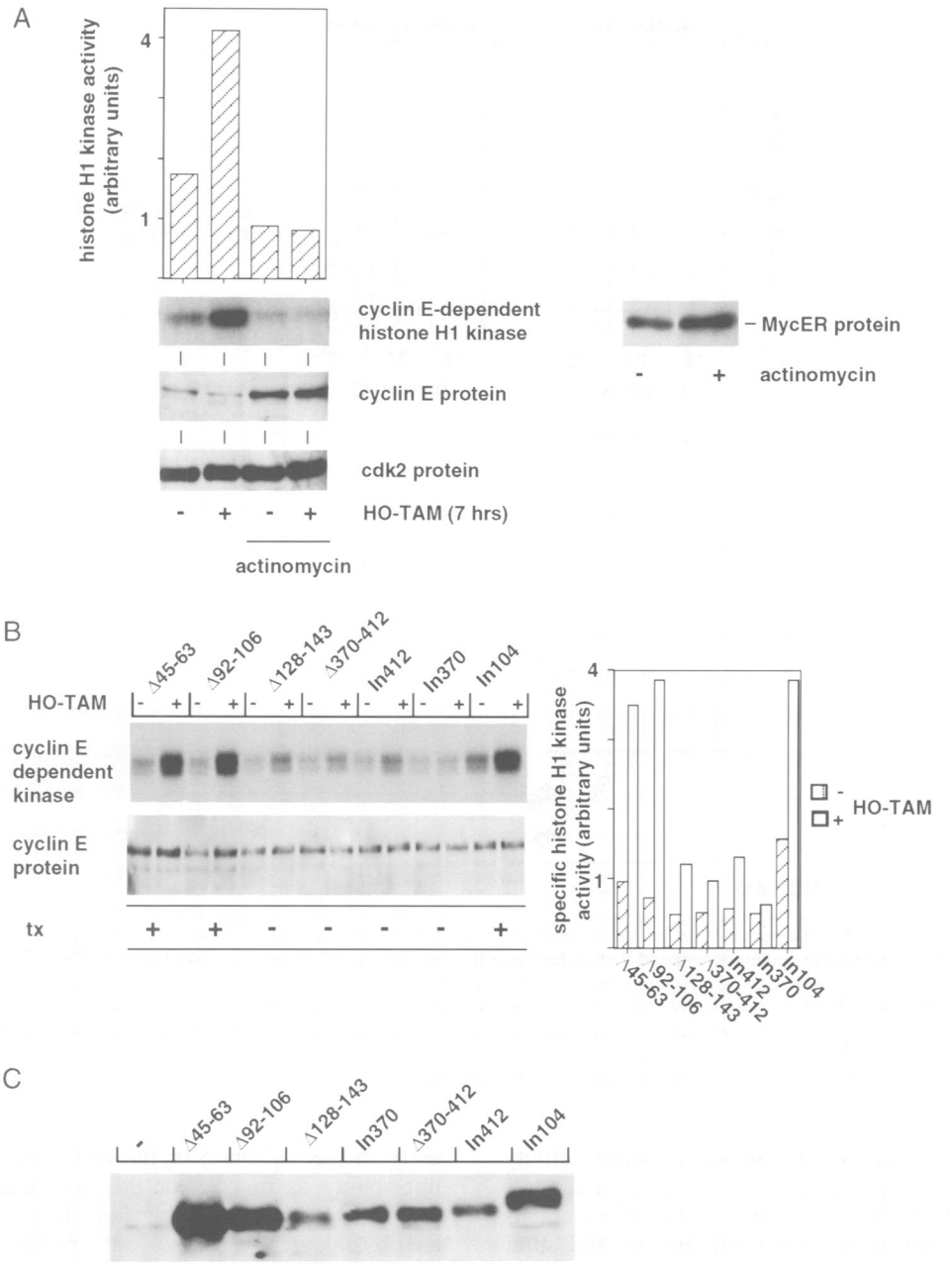

Fig. 2. Activation of cyclin E-dependent kinase by Myc requires transcription and depends on dimerization and DNA binding domains of Myc. (**A**) Actinomycin D blocks induction of cyclin E-dependent kinase by Myc. RAT1A-MycER cells were grown to confluence before either ethanol (–) or HO-TAM (+) were added. Where indicated, actinomycin D (5 μg/ml final concentration) was added at the same time. Extracts were prepared 7 h later. The top panel shows a quantitation of cyclin E-dependent histone H1 kinase activity in these extracts. The panels below show an autoradiogram of cyclin E-dependent histone H1 kinase activity and Western blots probed with anti-cyclin E, anti-cdk2 and anti-human Myc antibodies respectively. (**B**) Activation of cyclin E-dependent kinase activity requires intact dimerization and DNA binding domains of Myc. The upper panel shows an autoradiogram of cyclin E-dependent histone H1 kinase activity before and after stimulation with HO-TAM in extracts of confluent RAT1A cells carrying different alleles of Myc as inducible (ER) chimeras. The lower panel shows a Western blot of cyclin E protein in the same extracts. tx denotes whether the allele causes morphological transformation of RAT1A cells (Philipp *et al.*, 1994). The panel on the right shows a quantitation of the results. (**C**) Western blot documenting the expression of different MycER alleles. Extracts shown in (B) were probed with a polyclonal antibody directed against the oestrogen binding domain of the human oestrogen receptor (a kind gift of S.Robbins, UCSF).

Myc-transformed cells. In parallel experiments anti-cyclin D1 antibodies had no effect on G_1/S progression in SV40-transformed cells (Lukas *et al.*, 1994a; not shown). To confirm that cyclin D1 kinase activity was required for cell cycle induction by Myc anti-cyclin D1 antibodies were microinjected into arrested RAT1-MycER cells and found to significantly inhibit Myc-induced entry into S phase (Figure 3B). The data show that, unlike nuclear oncogenes of DNA tumour viruses, constitutive expression of Myc does not relieve the requirement for cyclin D1 function.

To test whether Myc also affected the activity of cyclin D1-dependent kinase, extracts were prepared from density-arrested cells before and 12 h after activation of Myc. Our results paralleled those for cyclin E–cdk2 kinase: we found that induction of Myc in arrested cells led to a

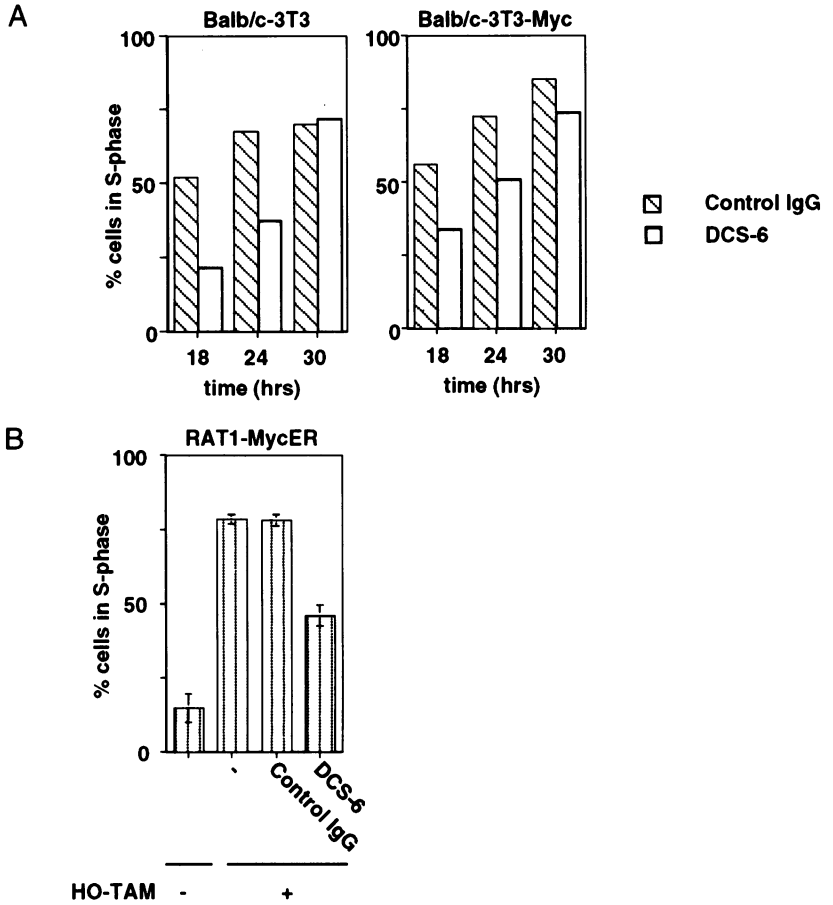

Fig. 3. Ectopic expression of Myc does not relieve the requirement for cyclin D1 protein. (**A**) Mitotic cells of the indicated cell lines were isolated by a nocodazole shake-off technique and electroporated with either control or anti-cyclin D1 monoclonal antibodies (DCS-6, 6 mg/ml). Cells were replated and the number of cells incorporating [^{3}H]thymidine determined after the indicated times. The panel indicates the percentage of positive cells at each time point; results from a representative experiment are shown. (**B**) Cyclin D1 is required for Myc-induced cell cycle entry. RAT1-MycER cells were starved in 0.1% FCS for 60 h and then injected with either control IgG or anti-cyclin D1 (DCS-6, 6 mg/ml) monoclonal antibodies. HO-TAM (250 nM final) was added and the percentage of cells incorporating BrdU determined 12 h later. For each condition >400 successfully injected cells from several independent experiments were evaluated.

5-fold increase in cyclin D1-associated kinase activity (Figure 4A). Immunoprecipitations from parallel cultures labelled with [^{35}S]methionine showed that similar amounts of cdk4 associated with cyclin D1 before and after activation of Myc, demonstrating that the assembly of cyclin D1 and cdk4 occurred essentially independently of Myc in these cells (Figure 4B). Time course experiments revealed that cyclin D1-dependent kinase activity increased relatively slowly after induction of Myc; half-maximal activation was observed 10 h after addition of hormone (in contrast to 4–5 h for cyclin E-dependent kinase) (Figure 4C). (The difference in basal activity between density-arrested and serum-starved cells reflects the higher amounts of cyclin D1 protein present in density-arrested cells.) Indeed, cyclin D1 kinase may well be rate limiting in serum-starved cells, as entry into S phase after stimulation by Myc often occurred significantly later than serum-induced cell cycle entry and coincided with the increase in cyclin D1-associated kinase (data not shown). These data strongly suggest that the increase in cyclin D1 protein that occurs in response to addition of growth factors (Won *et al.*, 1992; Herber *et al.*, 1994), but not to activation of Myc (Solomon *et al.*, 1995), contributes significantly to the increase in cyclin D1-dependent kinase early in the G_1 phase of the cell cycle. Because of the uncertainty as to whether the slow induction of cyclin D1-dependent kinase after induction of Myc reflected a physiologically significant process, it was not investigated further.

To gain insight into the molecular mechanisms underlying activation of cyclin E–cdk2 kinase by Myc, lysates were prepared from density-arrested RAT1A-MycER cells before and after activation of Myc and were separated on a gel filtration column. The amounts of cyclin E protein and of cyclin E-associated kinase activity were determined for each fraction (Figure 5A). Two peaks of cyclin E protein were observed in arrested RAT1A-MycER cells, one with a molecular weight of 250 kDa, the other of 120 kDa. About 50% of cyclin E contained in the latter peak had a retarded mobility due to phosphorylation of the protein. This was demonstrated by its sensitivity to treatment with calf intestine phosphatase (Figure 5B). Similarly, two distinct complexes of cyclin E could be distinguished after fractionation of uninduced cell lysates on either Mono S or Mono Q ion exchange columns (data not shown). Induction of Myc led to the complete disappearance of the large molecular weight peak; instead, all cyclin E protein then migrated as part of the 120 kDa

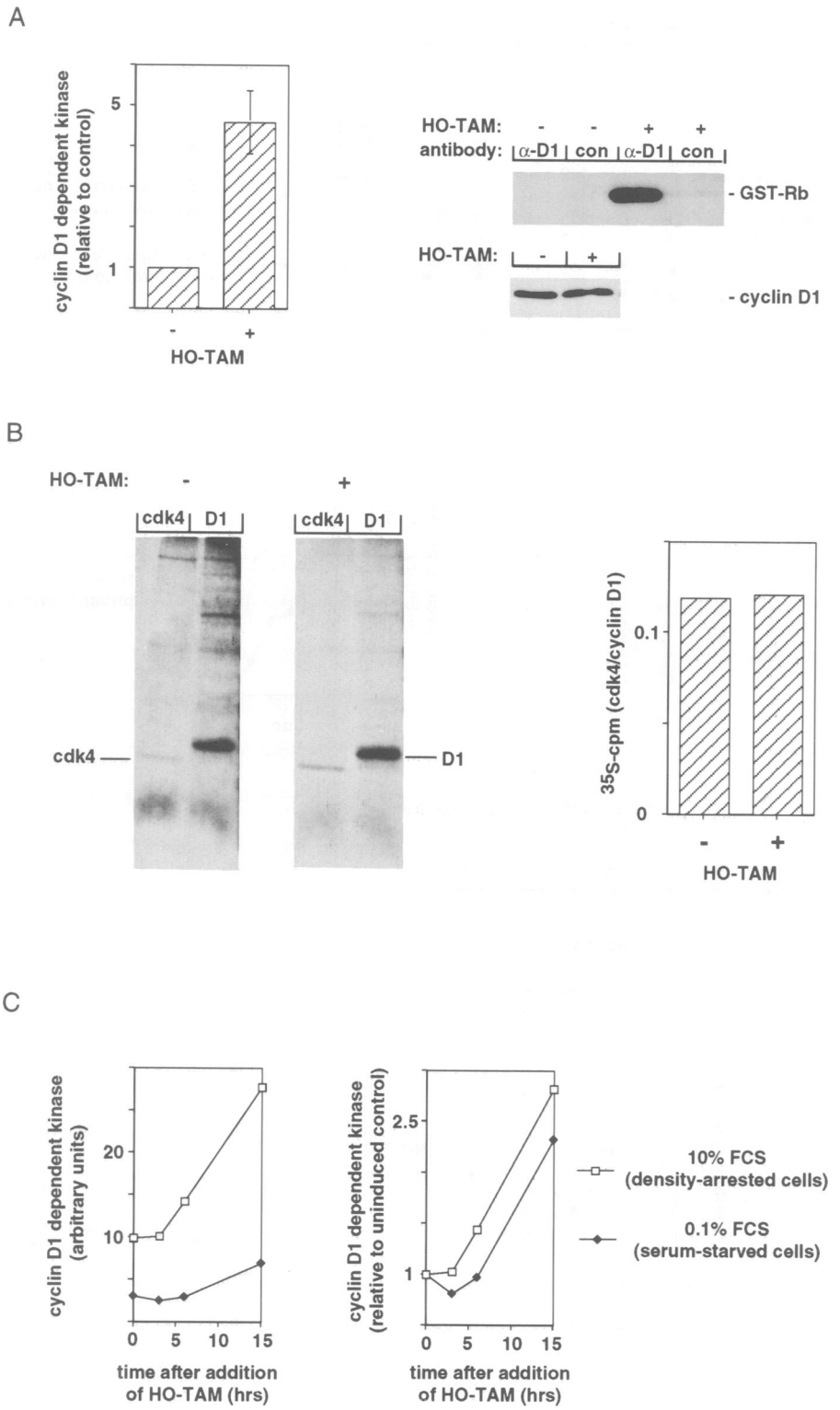

Fig. 4. Induction of Myc triggers activation of cyclin D1-associated kinase. (**A**) Extracts were prepared from confluent RAT1A-MycER cells 12 h after treatment with HO-TAM or ethanol and cyclin D1-dependent kinase activity determined using a GST–pRb fusion protein as substrate. (Left) Quantitation of cyclin D1-associated kinase activity from three independent experiments. (Right) Autoradiogram of cyclin D1-associated kinase activity towards pRb and Western blot documenting the expression of cyclin D1 from one representative experiment. (**B**) Activation of Myc does not affect the association between cyclin D1 and cdk4. RAT1A-MycER cells were grown to confluence, treated with ethanol or HO-TAM overnight (250 nM) and labelled with [^{35}S]methionine and [^{35}S]cysteine (Promix; Amersham). Cyclin D1 was immunoprecipitated in the presence of non-ionic detergents; immunoprecipitates were dissociated, divided in two parts and reprecipitated with antibodies against cyclin D1 and cdk4. (Left) Autoradiograms of the reprecipitates from control or HO-TAM induced cells. (Right) The ratio of cdk4 to cyclin D1 in the precipitates before and after activation of Myc. (**C**) Quantitation of a time course in Rat1-MycER cells. (Left) Shown is the amount of cyclin D1-dependent kinase activity in extracts from either density-arrested or serum-starved Rat1-MycER cells at various points after stimulation with HO-TAM. (Right) Data were replotted relative to uninduced control. Cyclin D1 protein levels did not increase during the time course of Myc activation (not shown; Solomon *et al.*, 1995).

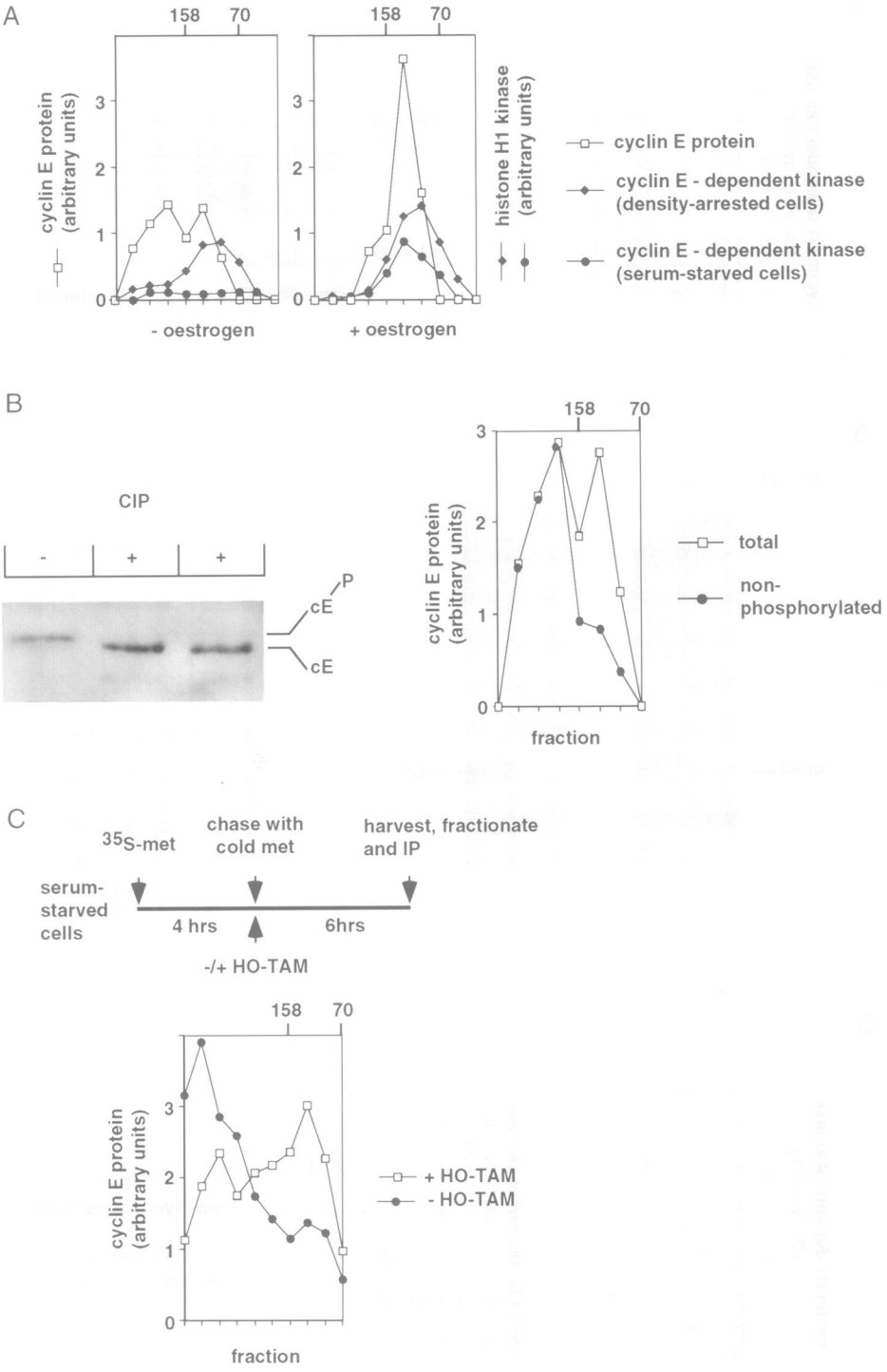

Fig. 5. Activation of Myc triggers the release of cyclin E from catalytically inactive, large complexes. (**A**) Shown are profiles from gel filtration columns documenting the distribution of cyclin E protein and cyclin E-dependent kinase activity before and after activation of Myc in density-arrested or serum-starved cells. Distribution of cyclin E protein was identical under both conditions and is shown for serum-starved cells only. The position of molecular weight markers is shown at the top of the diagram. (**B**) Cyclin E present in the lower molecular weight complex is phosphorylated. (Right) Shown is the profile of a gel filtration column documenting the distribution of hypo- and hyperphosphorylated cyclin E protein from density-arrested Rat1A-MycER cells. (Left) Cyclin E protein contained in the lower molecular weight complex of oestrogen-treated Rat1A-MycER cells was incubated with 50 U calf intestine phosphatase (CIP) (Boehringer) and analysed by Western blotting. (**C**) Induction of Myc triggers the release of a 120 kDa cyclin E complex from a 250 kDa precursor. Serum-starved RAT1-MycER cells were labelled for 4 h in the presence of [^{35}S]methionine and [^{35}S]cysteine (Promix; Amersham). They were refed with fresh medium containing an excess of non-radioactive methionine and cysteine either with or without 250 nM HO-TAM. Cell lysates were prepared 6 h later, separated by gel filtration as before, immunoprecipitated with an anti-cyclin E antibody and separated by SDS–PAGE. Shown is the amount of labelled cyclin E protein present in each fraction as determined from the autoradiogram.

complex. All cyclin E-dependent kinase activity fractionated with the 120 kDa complex, even the residual activity found in density-arrested cells (Figure 5A). No kinase activity could be detected in fractions containing the 250 kDa complex. Activation of Myc triggered a significant increase in cyclin E-associated kinase activity and this activity fractionated with a molecular weight of 120 kDa.

We wondered whether Myc caused the new synthesis of a low molecular weight complex or triggered the transition from a pre-existing 250 to a 120 kDa complex. Pulse labelling experiments revealed that all newly synthesized cyclin E co-fractionated with the 250 kDa complex, irrespective of whether Myc was active or not (data not shown). Significantly, pulse–chase experiments in which HO-TAM was added only during the chase revealed that Myc triggered the transition from a preformed 250 to a 120 kDa complex (Figure 5C).

Recently both cyclin E and cyclin A complexed with cdk2 have been found associated with inhibitors of cyclin-dependent kinases, like p27 and p21, in arrested cells (Y.Gu *et al.*, 1993; Nourse *et al.*, 1994; Polyak *et al.*, 1994a). Therefore, we wondered whether association with a kinase inhibitor might be involved in the transition between the 250 and 120 kDa complexes. High amounts of p27, but not of p21, protein (data not shown) were found in serum-starved RAT1-MycER cells. Activation of Myc caused a >5-fold decrease in p27 protein, but not mRNA levels; indeed, p27 was undetectable after prolonged stimulation of Myc (Figure 6A). Time course experiments revealed that an initial decrease in p27 protein was observed within 2 h after stimulation with oestrogen (Figure 6B). Immunoprecipitations using antiserum directed against p27 revealed that cyclin E was associated with p27 in arrested cells (Figure 6C). Thus the alteration in molecular weight of cyclin E–cdk2 complexes and activation of cyclin E-dependent kinase correlate with a loss of total p27 protein from cell lysates. Immunoprecipitates from ^{35}S-labelled Rat1-MycER cell extracts did not reveal any additional components of cyclin E-containing complexes in either starved or hormone-induced fibroblasts (see Discussion).

The experiments described so far were carried out either in density-arrested or serum-starved cells. We wondered whether the presence of serum growth factors affected the results of our experiments and therefore directly compared the effects of activation of Myc either in the absence or presence of serum. Activation of Myc also caused an increase in both cyclin D1- and cyclin E-dependent kinase activity in serum-starved RAT1-MycER fibroblasts. However, overall kinase activities were significantly lower than in density-arrested cells, both before and after activation of Myc (Figures 4C and 7A). To identify the reason for this difference gel filtration was performed as described above using lysates from serum-starved cells before and after activation of Myc. The distribution of cyclin E protein in serum-starved cells was identical to that in density-arrested cells and activation of Myc triggered an identical redistribution of the protein (not shown). However, in contrast to density-arrested cells, only low kinase activity was observed in the 120 kDa complex of starved and oestrogen-induced cells (Figure 5A).

Kinase activity of cdk2 is inhibited by phosphorylation at Thr14 and Tyr15 (Gu *et al.*, 1992; Sebastian *et al.*, 1993). Dephosphorylation at these sites has been suggested to be catalysed by a mammalian homologue of the yeast cdc25 phosphatase, cdc25a (Hoffmann *et al.*, 1994). Recently cdc25a has been shown to form a complex with and be activated by the growth factor-inducible protein kinase raf (Galaktionov *et al.*, 1995), prompting us to test whether a cdc25-like activity might be limiting in serum-starved cells. Similar to observations made in other experimental systems (Gu *et al.*, 1992; Sebastian *et al.*, 1993), treatment of cyclin E immunoprecipitates from serum-starved cells with recombinant cdc25a stimulated kinase activity, both before and after activation of Myc (Figure 7B). However, activation of Myc sensitized cyclin E-dependent kinase to the action of cdc25a (Figure 7C). Treatment of sizing column fractions from serum-starved cells revealed that the 120 kDa complex was significantly more susceptible to activation by cdc25a than the 250 kDa complex (Figure 7D), suggesting that activation of Myc was a prerequisite for further activation of cyclin E–cdk2 kinase by cdc25a. Thus activation of Myc generated an intermediate form of cyclin E–cdk2 kinase which depended on a cdc25-like activity for full activation. *In vivo* addition of serum stimulated cyclin E-dependent kinase activity, even in the presence of oestrogen (Figure 7C, left panel). Treatment with cdc25a revealed that this was largely due to supplying cdc25 activity, since addition of serum completely abolished the Myc-induced increase in susceptibility to cdc25a (Figure 7C, right panel).

Discussion

In this communication we show that activation of Myc in quiescent rat fibroblasts can lead to an activation of both cyclin E- and cyclin D1-dependent kinases without significant changes in the amount of cyclin–cdk complexes. For cyclin E–cdk2 activation by Myc correlates with the release from an inhibitory precursor complex that contains as one component the p27 kinase inhibitor. Finally, we show that in the absence of serum growth factors activation of either cyclin D1- or cyclin E-associated kinases by Myc is partial; in the case of cyclin E this is largely due to lack of a cdc25-like activity that can be supplied either *in vitro* or *in vivo* by addition of growth factors.

Similarly to the oncogenes of DNA tumour viruses, ectopic expression of oncogenes from RNA tumour viruses often facilitates progression through the G_1 phase of the cell cycle and reduces the growth factor requirements for cell proliferation. Proteins encoded by cellular proto-oncogenes act as part of a cascade that transmits signals from the cell surface to the nucleus; some proto-oncogenes (c-*myc*, c-*jun*, c-*fos*) encode transcription factors that are the end-point of such signalling cascades (for a review see Herrlich and Ponta, 1989). Activation of these 'nuclear' proto-oncogenes is often required for cells to proliferate in response to external growth factors (Heikkila *et al.*, 1987; Prochownik *et al.*, 1988). Conversely, deregulated expression of nuclear proto-oncogenes can allow cells to proliferate in the absence of such factors (Keath *et al.*, 1984; Castellazzi *et al.*, 1991; Eilers *et al.*, 1991; Nakabeppu *et al.*, 1993).

Nuclear oncogenes from DNA tumour viruses bind and sequester two cellular tumour suppressor genes, *p53* and *pRb*; mutants that are deficient in either function often

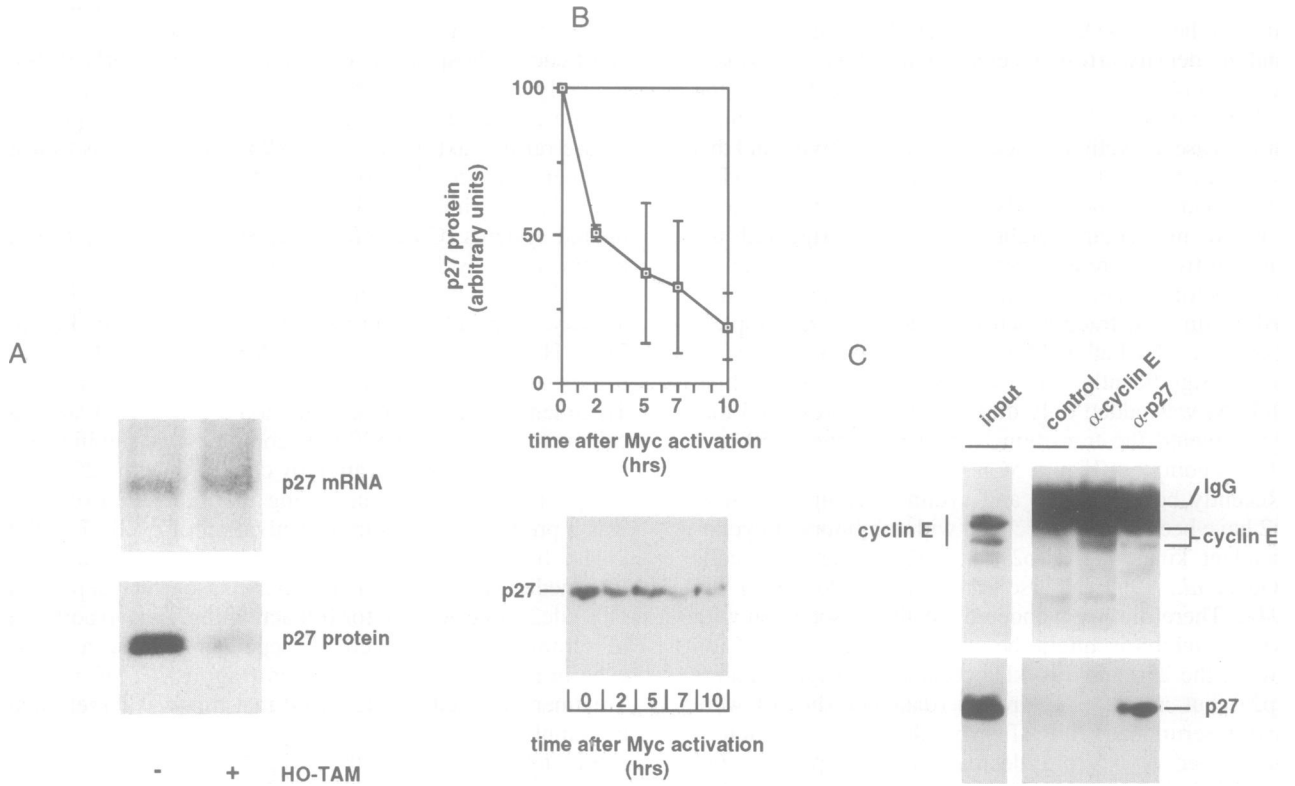

Fig. 6. Activation of Myc triggers loss of p27 protein. (**A**) Northern and Western blots documenting the disappearance of p27 protein, but not mRNA, in total cell extracts of serum-starved RAT1-MycER cells after addition of HO-TAM (15 h). (**B**) Time course of loss of p27 protein in serum-starved RAT1-MycER cells after stimulation of Myc with oestrogen (250 nM). Shown are average values from three independent experiments (top panel) and a representative Western blot documenting the amount of p27 protein in total cell extracts. (**C**) Cyclin E is associated with p27 in serum-starved RAT1-MycER cells. Lysates from serum-starved cells were precipitated with the indicated antibodies. The immunoprecipitates, together with an aliquot of the lysate (input), were separated by SDS–PAGE and probed with antibodies directed against cyclin E (upper panel) and p27 (lower panel). Non-phosphorylated cyclin E protein is detectable with anti-cyclin E, as well as anti-p27, but not in control (anti-Mad) immunoprecipitates. Phosphorylated cyclin E present in the extract is masked by the heavy chain of the first antibody used in the immunoprecipitations.

show a reduced capacity to induce cellular proliferation (for a review see Weinberg, 1995). Inactivation of these two suppressor genes appears, therefore, to contribute to tumorigenesis by DNA tumour viruses. Similarly, Myc protein has been suggested to bind directly to the retinoblastoma protein (Rustgi *et al.*, 1991). However, antibodies against cyclin D1 inhibit proliferation in Myc-transformed cells, strongly suggesting that cyclin D1-dependent kinases are required for G_1 progression in these cells. As cyclin D1-associated kinase is not required for cell cycle progression in cells that are functionally deficient for pRb (Lukas *et al.*, 1994a), these data strongly suggest that Myc does not sequester pRb protein. This report (Figure 1A) shows that activation of Myc induces hyperphosphorylation of pRb. Further experiments show that microinjection of expression plasmids encoding either dominant negative alleles of cdk2 (Van den Heuvel and Harlow, 1993) or the p16, p21 and p27 (Xiong *et al.*, 1993; El-Deiry *et al.*, 1994; Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994) cdk inhibitors inhibit G_1 progression in response to activation of Myc, supporting the notion that activation of cdks is central to induction of cell proliferation and inactivation of pRb by Myc (B.Rudolph and M.Eilers, in preparation).

Alternatively, nuclear oncogenes might facilitate progression through the cell cycle by directly or indirectly enhancing the transcription of cyclin or cdk genes. For example, c-Jun protein has been shown to bind and transactivate the promoter of the cyclin D1 gene in transient transfection assays (Herber *et al.*, 1994). As elevated levels of cyclin D1 facilitate progression through the G_1 phase of the cell cycle (Quelle *et al.*, 1993), these observations suggest a possible model to explain the cell cycle effects of c-Jun.

Activation of conditional alleles of Myc in quiescent cells leads to a moderate induction of cyclin E mRNA levels and a strong induction of cyclin A (Jansen-Dürr *et al.*, 1993). Expression levels of cdk2 and cdk4 are unaffected by Myc in RAT1 cells (Figure 1A). While these data do not rule out the possibility that transcriptional activation of cyclin E by Myc might contribute to the mitogenic effects of Myc, cyclin E protein is present in substantial amounts in both quiescent RAT1 and RAT1A cells and the amount of cyclin E protein remains constant during the time during which cyclin E-dependent kinase activity is activated and pRb hyperphosphorylation occurs *in vivo*.

Conflicting results have been published regarding regulation of cyclin D1 by Myc. In particular, Daksis and co-workers have claimed a strong induction of cyclin D1 mRNA in RAT1-MycER cells after induction of Myc (Daksis *et al.*, 1994), whereas other reports show no effect in resting cells and a repression of cyclin D1 by Myc in

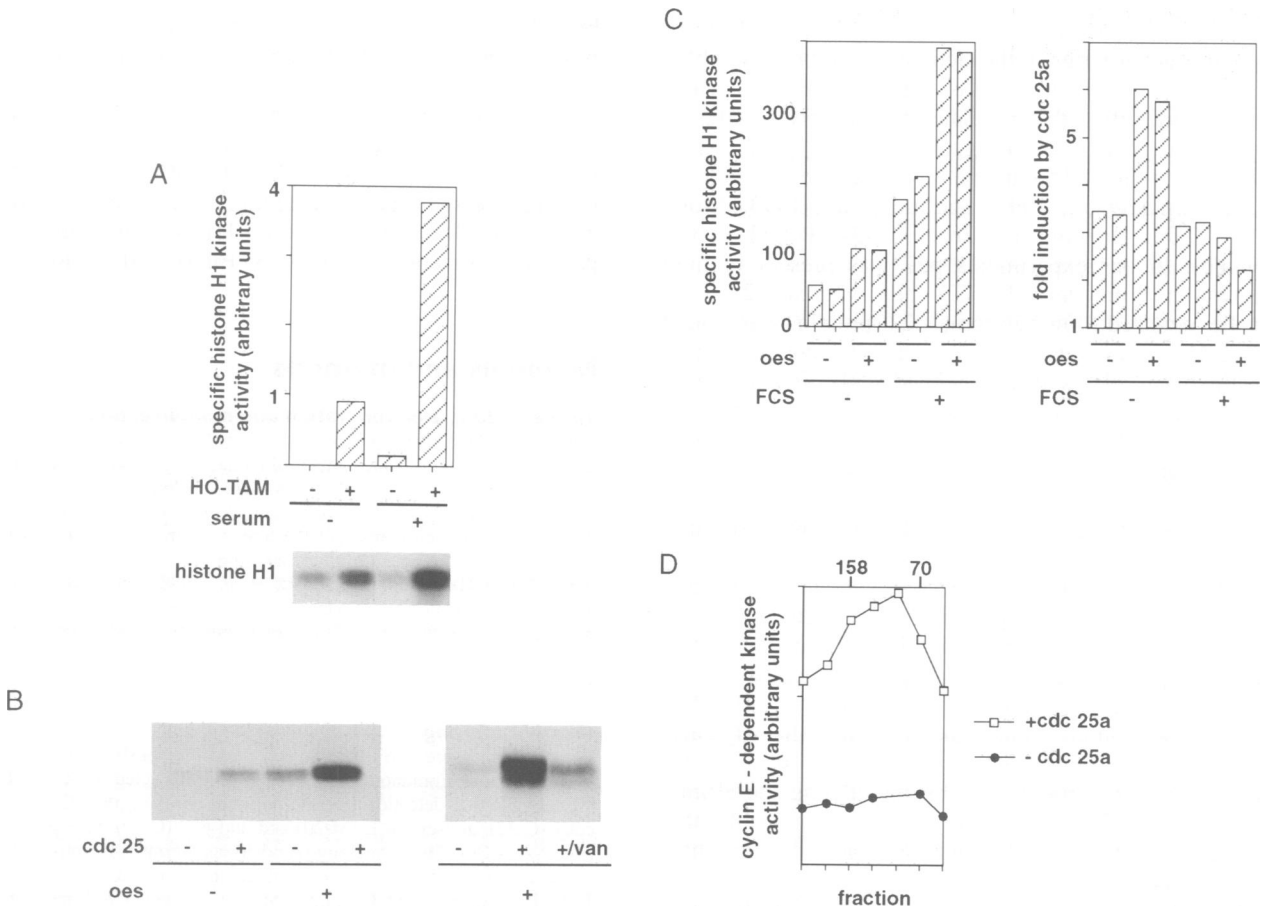

Fig. 7. Coordinate regulation of cyclin E–cdk2 kinase by Myc and serum growth factors. (**A**) Myc and serum growth factors synergize to activate cyclin E–cdk2 kinase. Shown is an autoradiogram and a quantitation of cyclin E-dependent kinase activity in serum-starved (–) and density-arrested (+) RAT1-MycER cells before and after addition of HO-TAM. (**B**) Cdc25 activity is limiting for full activation of cyclin E–cdk2 kinase in serum-starved cells both before and after activation of Myc. (Left) Results of cyclin E immune complex kinase assays from RAT1-MycER cells; where indicated precipitates were treated with recombinant GST–cdc25a fusion protein before the kinase assays. (Right) A similar assay demonstrating that activation by cdc25a is sensitive to inhibition by phosphatase inhibitors (10 mM vanadate, 50 mM NaF and 100 mM β-glycerophosphate). (**C**) Addition of growth factors supplies cdc25 activity *in vivo*. Serum-starved RAT1-MycER cells were stimulated with oestrogen (oes) and/or fetal calf serum (FCS) as indicated. Extracts were prepared 12 h later. Shown are the results from duplicate samples. (Left) The specific cyclin E-dependent kinase activity (without treatment with cdc25) in each sample. (Right) The fold increase by which kinase activity in each sample could be superinduced by treatment with cdc25a *in vitro*. (**D**) The 120 kDa cyclin E–cdk2 complex is significantly more susceptible to cdc25a than the 250 kDa complex. Fractions from the gradient shown in Figure 5A, left panel, from serum-starved cells were assayed for cyclin E-dependent kinase activity with or without treatment with GST–cdc25a *in vitro*.

proliferating cells (Jansen-Dürr *et al.*, 1993; Lovec *et al.*, 1994; Philipp *et al.*, 1994). Work submitted in parallel (Solomon *et al.*, 1995) now clearly reveals that the apparent induction of cyclin D1 mRNA is an artefact resulting from an oestrogen-activated transcriptional activation domain (Berry *et al.*, 1990) in the oestrogen receptor part of the MycER chimera. This is supported by observations that, first, addition of HO-TAM, which does not activate this domain, allows association of MycER with Max and induces cell proliferation in serum-starved RAT1-MycER cells without affecting cyclin D1 mRNA levels. Second, RAT1 cells expressing a mutant MycER with a hormone binding domain that still binds HO-TAM but no longer binds oestrogen (Danielian *et al.*, 1993; Littlewood *et al.*, 1995) are driven into the cell cycle in response to HO-TAM, but fail to up-regulate cyclin D1 mRNA. Finally, RAT1 cells in which wild-type human c-Myc expression can be induced also progress into the cell cycle without increased expression of cyclin D1 mRNA. Taken together these data show that up-regulation of cyclin D1 mRNA does not contribute to Myc-mediated cell cycle entry in RAT1 cells.

Assembly of functional cyclin–cdk complexes is a multistep process in which synthesis, assembly and activation of the subunits are independent events, all of which have been reported to be regulated during the G_0/G_1 progression of serum-stimulated rodent fibroblasts (Morgan, 1995). Assembly of both cyclin E–cdk2 and cyclin D1–cdk4 complexes is controlled by a cdk activating kinase, CAK. CAK phosphorylates homologous threonine residues on both cdk2 and cdk4 (Matsuoka *et al.*, 1994) and is itself a cyclin-dependent kinase (Fisher and Morgan, 1994). Activation of Myc did not alter the apparent phosphorylation of either bulk cdk2 or cdk2 contained in cyclin E immunoprecipitates at Thr160 (Figure 1E) and both cyclin E–cdk2 and cyclin D1–cdk4 complexes were assembled before activation of Myc in quiescent RAT1 cells (Figures 1C and 4B). Therefore, induction of CAK

activity does not appear to be the target for activation of cyclin E–cdk2 complexes by Myc in RAT1 cells. Instead, Myc appears to trigger the activation of pre-assembled cyclin–cdk complexes. A class of inhibitory molecules has been identified that regulates the activity of cdks in response to growth arrest signals, like TGF-β or cAMP, or in response to DNA damage or senescence (Y.Gu *et al.*, 1993; Harper *et al.*, 1993; Serrano *et al.*, 1993; Xiong *et al.*, 1993; Hannon and Beach, 1994; Polyak *et al.*, 1994a,b). In our experiments p27 was present in high amounts in serum-starved RAT1 cells, whereas p21 could not be detected. Furthermore, p27 protein was found associated with cyclin E in arrested cells and loss of p27 protein from cell lysates correlated with dissociation of the 250 kDa complex, suggesting that the dissociation we observe is at least in part due to loss of p27 protein. Indeed, parallel work carried out by Mittnacht and co-workers shows that addition of recombinant p27 *in vitro* converts the 120 kDa complex back to its large molecular weight precursor (Godden-Kent *et al.*, submitted), strongly arguing for a causal role of p27 protein in this process. Recent work shows that p27 protein is degraded via the ubiquitin pathway (Pagano *et al.*, 1995). Our data might, therefore, point to a role of ubiquitin-mediated protein degradation in the induction of cell proliferation by Myc. However, we cannot exclude the possibility that Myc also affects dissociation of inhibitors by other mechanisms than loss of p27 protein, e.g. by modulating the affinity of cyclin E–cdk2 complexes for inhibitor proteins. Further work is required to resolve this issue and to reveal how Myc affects loss of p27 protein.

Our data identify a second step, dephosphorylation of cdk2 at Tyr15 and/or Thr14, that is required for full activation of cyclin E–cdk2 complexes in starved rat fibroblasts. Dephosphorylation at these sites has been analysed in HeLa cells, where no change has been found in the bulk cdk2 population during the G_1/S transition (Gu *et al.*, 1992), however, it has not been looked at during the G_0/S transition in rodent fibroblasts. Our data suggest that dephosphorylation of cdk2 occurs in a growth factor-dependent, but Myc-independent, pathway, potentially via cdc25a (Hoffmann *et al.*, 1994; Jinno *et al.*, 1994). As cdc25a has recently been shown to bind to a downstream effector of Ras, c-raf (Galaktionov *et al.*, 1995), it is tempting to speculate that this pathway reflects the serum requirement we observe. While induction of Myc can be sufficient to induce cell cycle progression in established rodent cell lines, several experiments have previously documented a requirement for co-operating stimuli for sustained proliferation, inhibition of apoptosis and transformation of primary rodent fibroblasts (Armelin *et al.*, 1984; Land *et al.*, 1986; Evan *et al.*, 1992); our data show that such complementary signals may target discrete, non-overlapping steps in the formation of active cyclin–cdk complexes.

Finally, our data show that Myc function contributes to activation of both cyclin E- and cyclin D1-dependent kinases, precluding a simple classification of Myc into either the 'cyclin D1 pathway' or the 'cyclin E pathway'. Such a classification is further complicated by the finding that ectopic expression of cyclin D1 induces expression of high levels of c-*myc* mRNA (Jiang *et al.*, 1993), potentially via an E2F binding site in the c-*myc* promoter (Oswald *et al.*, 1994). This suggests that one mechanism by which ectopic expression of cyclin D1 facilitates progression through the G_1 phase (Quelle *et al.*, 1993) is by elevating c-*myc* expression (and thereby potentially also activating cyclin E-dependent kinase). In support of this idea co-transformation assays of primary rat fibroblasts with cyclin D1 and activated alleles of *ras* are sensitive to inhibition by Mad, showing that even if it exerts similar biological effects to Myc, cyclin D1 does not simply bypass Myc function (K.Haas, M.Eilers and T.Möröy, in preparation).

Materials and methods

Tissue culture, electroporation and microinjection

Cell culture of RAT1-MycER cells was carried out as described previously (Eilers *et al.*, 1991). Mutated alleles of human c-*myc* have been described previously (Philipp *et al.*, 1994); they were subcloned into the retroviral expression vector pMV-7 (Kirschmeier *et al.*, 1988) as chimeras with the hormone binding domain of the human oestrogen receptor (Kumar *et al.*, 1986). Recombinant retroviruses were generated using the packaging cell line GP+E-86 (Markowitz *et al.*, 1988) and used to infect RAT1A cells. Colonies resistant to 800 μg/ml G418 (Calbiochem) were pooled for further analysis. Electroporation and microinjection experiments were carried out as described previously (Lukas *et al.*, 1994a).

Western blotting

Western blots were performed as described previously (Philipp *et al.*, 1994) and the immunolabelled bands were detected with the ECL Western blotting detection system (Amersham). Anti-pRb (C-15), anti-cdk4 (C-22), anti-cyclin E (M-20) and anti-p27 (C-19) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-cdk2 and anti-cyclin D1 antibodies have been described before (Baldin *et al.*, 1993; Pagano *et al.*, 1993). Human Myc was detected with monoclonal antibody 9E10 (Evan *et al.*, 1985).

Immunoprecipitations and immune complex kinase assays

Immune complex kinase assays for cyclin D1 were carried out according to Matsushime *et al.* (1994) using monoclonal antibody DCS-11(Lukas *et al.*, 1994a,b) and a GST–pRb fragment (Meyerson and Harlow, 1994) as substrate. The anti-cdk4 (C-22) antibody was used for re-immunoprecipitations. Cyclin E immunoprecipitations and analysis of cyclin E-dependent kinase were performed according to Dulic *et al.* (1992) using the anti-cyclin E (M-20) antibody. Treatment of cyclin E immunoprecipitates with recombinant GST–cdc25a (a kind gift of Ingrid Hoffmann, EMBL) was carried out according to Hoffmann *et al.* (1994). The anti-p27 (C-19) antibody was used to precipitate p27-containing complexes according to Dulic *et al.* (1992).

Gel filtration

Total cell lysates were prepared in a 0.5% Triton X-100, 150 mM NaCl, 50 mM HEPES, pH 7.4, buffer containing 20 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 mM dithiothreitol, 10 mM β-glycerophosphate, 0.25 mM vanadate and 10 mM NaF and fractionated on a Superose 12 column connected to a FPLC system (both Pharmacia).

Northern blotting

Total RNA was extracted as described before (Jansen-Dürr *et al.*, 1993) and the expression of p27 mRNA was monitored with a labelled, RT-PCR-generated 590 bp fragment based on the published sequence (Polyak *et al.*, 1994b).

Acknowledgements

Work in M.E.'s laboratory is supported by grants from the Bundesministerium für Forschung und Technologie (grant BCT0381-5) and the Human Frontier Science Program (to M.E. and M.P.). We thank Annette Ruppert for excellent technical assistance, Ingrid Hoffmann for recombinant GST–cdc25a and Stefan Gaubatz for RAT1A cell lines carrying different MycER alleles.

References

Amati,B. and Land,H. (1994) Myc–Max–Mad: a transcription factor network controlling cell cycle progression, differentiation and death. *Curr. Opin. Genet. Dev.*, **4**, 102–108.

Armelin,H.A., Armelin,M.C.S., Kelly,K., Stewart,T., Leder,P., Cochran, B.H. and Stiles,C.D. (1984) Functional role for c-*myc* in mitogenic response to platelet-derived growth factor. *Nature*, **310**, 655–660.

Ayer,D.E., Kretzner,L. and Eisenman,R.N. (1993) Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. *Cell*, **72**, 211–222.

Ayer,D.E., Lawrence,Q.A. and Eisenman,R.N. (1995) Mad–Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. *Cell*, **80**, 767–776.

Baldin,V., Lukas,J., Marcote,M.J., Pagano,M. and Draetta,G. (1993) Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev.*, **7**, 812–821.

Bello-Fernandez,C., Packham,G. and Cleveland,J.L. (1993) The ornithine decarboxylase gene is a transcriptional target of c-MYC. *Proc. Natl Acad. Sci. USA*, **90**, 7804–7808.

Benvenisty,N., Leder,A., Kuo,A. and Leder,P. (1992) An embryonically expressed gene is a target for c-Myc regulation via the c-Myc-binding sequence. *Genes Dev.*, **6**, 2513–2524.

Berry,M., Metzger,D. and Chambon,P. (1990) Role of the two activating domains of the oestrogen receptor in the cell-type and promotor-context dependent agonistic activity of the anti-oestrogen 4-hydroxy-tamoxifen. *EMBO J.*, **9**, 2811–2818.

Blackwood,E.M. and Eisenman,R.N. (1991) Max: a helix–loop–helix zipper protein that forms a sequence-specific DNA-binding complex with myc. *Science*, **251**, 1211–1217.

Blackwood,E.M., Lüscher,B. and Eisenman,R.N. (1992) Myc and Max associate *in vivo*. *Genes Dev.*, **6**, 71–80.

Castellazzi,M., Spyrou,G., LaVista,N., Dangy,J.-P., Piu,F., Yaniv,M. and Brun,G. (1991) Overexpression of c-*jun*, *junB*, or *junD* affects cell growth differently. *Proc. Natl Acad. Sci. USA*, **88**, 8890–8894.

Daksis,J.I., Lu,R.Y., Facchini,L.M., Marhin,W.W. and Penn,L.J.Z. (1994) Myc induces cyclin D1 expression in the absence of *de novo* protein synthesis and links mitogen-stimulated signal transduction to the cell cycle. *Oncogene*, **9**, 3635–3645.

Danielian,P.S., White,R., Hoare,S.A., Fawell,S.E. and Parker,M.G. (1993) Identification of residues in the estrogen receptor that confer differential sensitivity to estrogen and hydroxytamoxifen. *Mol. Endocrinol.*, **7**, 232–240.

Davis,A.C., Wims,M., Spotts,G.D., Hann,S.R. and Bradley,A. (1993) A null c-*myc* mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. *Genes Dev.*, **7**, 671–682.

Dulic,V., Lees,E. and Reed,S.I. (1992) Association of human cyclin E with a periodic G1-phase protein kinase. *Science*, **257**, 1958–1961.

Eilers,M., Picard,D., Yamamoto,K. and Bishop,J.M. (1989) Chimaeras between the MYC oncoprotein and steroid receptors cause hormone-dependent transformation of cells. *Nature*, **340**, 66–68.

Eilers,M., Schirm,S. and Bishop,J.M. (1991) The MYC protein activates transcription of the α-prothymosin gene. *EMBO J.*, **10**, 133–141.

El-Deiry,W.S. *et al.* (1994) WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res*, **54**, 1169–74.

Evan,G.I., Lewis,G.K., Ramsay,G. and Bishop,J.M. (1985) Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. *Mol. Cell. Biol.*, **5**, 3610–3616.

Evan,G.I., Wyllie,A.H., Gilbert,C.S., Littlewood,T.D., Land,H., Brooks,M., Waters,C.M., Penn,L.Z. and Hancock,D.C. (1992) Induction of apoptosis in fibroblasts by c-myc protein. *Cell*, **69**, 119–128.

Fisher,R.P. and Morgan,D.O. (1994) A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. *Cell*, **78**, 713–724.

Galaktionov,K., Jessus,C. and Beach,D. (1995) Raf1 interaction with cdc25 phosphatase ties mitogenic signal transduction to cell cycle activation. *Genes Dev.*, **9**, 1046–1058.

Gaubatz,S., Meichle,A. and Eilers,M. (1994) An E-box element localized in the first intron mediates regulation of the prothymosin α gene by c-myc. *Mol. Cell. Biol.*, **14**, 3853–3862.

Gu,W., Cechova,K., Tassi,V. and Dalla-Favera,R. (1993) Opposite regulation of gene transcription and cell proliferation by c-Myc and Max. *Proc. Natl Acad. Sci. USA*, **90**, 2935–2939.

Gu,Y., Rosenblatt,J. and Morgan,D.O. (1992) Cell cycle regulation of CDK2 activity by phosphorylation of the Thr160 and Tyr15. *EMBO J.*, **11**, 3995–4005.

Gu,Y., Turek,C.W. and Morgan,D.O. (1993) Inhibition of CDK2 activity *in vivo* by an associated 20K regulatory subunit. *Nature*, **366**, 707–710.

Hannon,G.J. and Beach,D. (1994) $p15^{INK4b}$ is a potential effector of cell cycle arrest mediated by TGF-β. *Nature*, **371**, 257–261.

Hanson,K.D., Shichiri,M., Follansbee,M.R. and Sedivy,J.M. (1994) Effects of c-*myc* expression on cell cycle progression. *Mol. Cell. Biol.*, **14**, 5748–5755.

Harper,J.W., Adami,G.R., Wei,N., Keyomarsi,K. and Elledge,S.J. (1993) The p21 cdk-interaction protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, **75**, 805–816.

Hatakeyama,M., Brill,J.A., Fink,G.R. and Weinberg,R.A. (1994) Collaboration of G_1 cyclins in the functional inactivation of the retinoblastoma protein. *Genes Dev.*, **8**, 1759–1771.

Heikkila,R., Schwab,G., Wickstrom,E., Loke,S.L., Pluznik,D.H., Watt,R. and Neckers,L.M. (1987) A c-*myc* antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G0 to G1. *Nature*, **328**, 445–449.

Herber,B., Truss,M., Beato,M. and Müller,R. (1994) Inducible regulatory elements in the human cyclin D1 promoter. *Oncogene*, **9**, 1295–304.

Herrlich,P. and Ponta,H. (1989) Nuclear oncogenes convert extracellular stimuli into changes in the genetic program. *Trends Genet.*, **5**, 112–116.

Hoang,A.T., Cohen,K.J., Barrett,J.F., Bergstrom,D.A. and Dang,C.V. (1994) Participation of cyclin A in Myc-induced apoptosis. *Proc. Natl Acad. Sci. USA*, **91**, 6875–6879.

Hoffmann,I., Draetta,G. and Karsenti,E. (1994) Activation of the phosphatase activity of human cdc25A by a cdk2–cyclin E dependent phosphorylation at the G_1/S transition. *EMBO J.*, **13**, 4302–4310.

Jansen-Dürr,P., Meichle,A., Steiner,P., Pagano,M., Finke,K., Botz,J., Wessbecher,J., Draetta,G. and Eilers,M. (1993) Differential modulation of cyclin gene expression by *MYC*. *Proc. Natl Acad. Sci. USA*, **90**, 3685–3689.

Jiang,W., Kahn,S.M., Zhou,P., Zhang,Y.-J., Cacace,A.M., Infante,A.S., Doi,S., Santella,R.M. and Weinstein,I.B. (1993) Overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth control, cell cycle progression and gene expression. *Oncogene*, **8**, 3447–3457.

Jinno,S., Suto,K., Nagata,A., Igarashi,M., Kanaoka,Y., Nojima,H. and Okayama,H. (1994) Cdc25A is a novel phosphatase functioning early in the cell cycle. *EMBO J.*, **13**, 1549–1556.

Kaczmarek,L., Miller,M.R., Hammond,R.A. and Mercer,W.E. (1986) A microinjected monoclonal antibody against human DNA polymerase-A inhibits DNA replication in human, hamster and mouse cell lines. *J. Biol. Chem.*, **261**, 10802–10807.

Keath,E.J., Caimi,P.G. and Cole,M.D. (1984) Fibroblast lines expressing activated c-*myc* oncogenes are tumorigenic in nude mice and syngenic animals. *Cell*, **39**, 339–348.

Kelly,K. and Siebenlist,U. (1986) The regulation and expression of c-myc in normal and malignant cells. *Annu. Rev. Immunol.*, **4**, 317–338.

Kelly,K., Cochran,B.H., Stiles,C.D. and Leder,P. (1983) Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. *Cell*, **35**, 603–610.

Kirschmeier,P.T., Housey,G.M., Johnson,M.D., Perkins,A.S. and Weinstein,B. (1988) Construction and characterization of a retroviral vector demonstrating efficient expression of cloned cDNA sequences. *DNA*, **7**, 219–225.

Kumar,V., Green,S., Staub,A. and Chambon,P. (1986) Localisation of the oestradiol-binding and putative DNA-binding domains of the human oestrogen receptor. *EMBO J.*, **5**, 2231–2236.

Land,H., Chen,A.C., Morgenstern,J.P., Parada,L.F. and Weinberg,R.A. (1986) Behavior of *myc* and *ras* oncogenes in transformation of rat embryo fibroblasts. *Mol. Cell. Biol.*, **6**, 1917–1925.

Langdon,W.Y., Harris,A.W., Cory,S. and Adams,J.M. (1986) The c-*myc* oncogene perturbs B lymphocyte development in Eμ-*myc* transgenic mice. *Cell*, **47**, 11–18.

Littlewood,T.D., Hancock,D.C., Danielian,P.S., Parker,M.G. and Evan,G.I. (1995) A modified oestrogen receptor ligand binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res.*, **23**, 1686–1690.

Lovec,H., Sewing,A., Lucibello,F.C., Müller,R. and Möröy,T. (1994) Oncogenic activity of cyclin D1 revealed through cooperation with Ha-ras: link between cell cycle control and malignant transformation. *Oncogene*, **9**, 323–326.

Lukas,J., Müller,H., Bartkova,J., Spitkovksy,D., Kjerulff,A.A., Jansen-Dürr,P., Strauss,M. and Bartek,J. (1994a) DNA tumor virus oncoproteins and retinoblastoma gene mutations share the ability to relieve the cell's requirement for cyclin D1 function in G1. *J. Cell Biol.*, **125**, 625–638.

Lukas,J., Pagano,M., Staskova,Z., Draetta,G. and Bartek,J. (1994b)

Cyclin D1 protein oscillates and is essential for cell cycle progression in human tumor cell lines. *Oncogene*, **9**, 707–718.

Markowitz,D., Goff,S. and Bank,A. (1988) A safe packaging line for gene transfer: separating viral genes on two different plasmids. *J. Virol.*, **62**, 1120–1124.

Matsuoka,M., Kato,J.-Y., Fisher,R.P., Morgan,D.O. and Sherr,C.J. (1994) Activation of cyclin-dependent kinase-4 (CDK4) by mouse MO15-associated kinase. *Mol. Cell. Biol.*, **14**, 7265–7275.

Matsushime,H., Quelle,D.E., Shurtleff,S.A., Shibuya,M., Sherr,C.J. and Kato,J.-Y. (1994) D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.*, **14**, 2066–2076.

Meichle,A., Philipp,A. and Eilers,M. (1992) The functions of Myc proteins. *Biochim. Biophys. Acta*, **1114**, 129–146.

Meyerson,M. and Harlow,E. (1994) Identification of G_1 kinase activity for cdk6, a novel cyclin D partner. *Mol. Cell. Biol.*, **14**, 2077–2086.

Morgan,D. (1995) Principles of CDK regulation. *Nature*, **374**, 131–134.

Müller,R., Mumberg,D. and Lucibello,F.C. (1993) Signals and genes in the control of cell-cycle progression. *Biochim. Biophys. Acta*, **1155**, 151–179.

Nakabeppu,Y., Oda,S. and Sekiguchi,M. (1993) Proliferative activation of quiescent rat-1A cells by ΔFosB. *Mol. Cell. Biol.*, **13**, 4157–4166.

Nourse,J., Firpo,E., Flanagan,W.M., Coats,S., Polyak,K., Lee,M.-H., Massague,J., Crabtree,G.R. and Roberts,J.M. (1994) Interleukin-2-mediated elimination of the $p27^{Kip1}$ cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature*, **372**, 570–573.

Oswald,F., Lovec,H., Möröy,T. and Lipp,M. (1994) E2F-dependent regulation of human *MYC*: *trans*-activation by cyclins D1 and A overrides tumour suppressor protein functions. *Oncogene*, **9**, 2029–2036.

Pagano,M., Pepperkok,R., Lukas,J., Baldin,V., Ansorge,W., Bartek,J. and Draetta,G. (1993) Regulation of the cell cycle by the cdk2 protein kinase in cultured human fibroblasts. *J. Cell Biol.*, **121**, 101–111.

Pagano,M., Tam,S.W., Theodoras,A.M., Del Sal,G., Yew,P.R., Draetta,G.F. and Rolfe,M. (1995) p27 levels are cell cycle-regulated by the ubiquitin-proteasome pathway. *Science*, **269**, 682–685.

Philipp,A., Schneider,A., Väsrik,I., Finke,K., Xiong,Y., Beach,D., Alitalo,K. and Eilers,M. (1994) Repression of cyclin D1: a novel function of MYC. *Mol. Cell. Biol.*, **14**, 4032–4043.

Polyak,K., Kato,J., Solomon,M.J., Sherr,C.J., Massague,J., Roberts,J.M. and Koff,A. (1994a) $p27^{Kip1}$, a cyclin–Cdk inhibitor, links transforming growth factor-β and contact inhibition to cell cycle arrest. *Genes Dev.*, **8**, 9–22.

Polyak,K., Lee,M.-H., Erdjument-Bromage,H., Koff,A., Roberts,J.M., Tempst,P. and Massague,J. (1994b) Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell*, **78**, 59–66.

Prendergast,G.C., Lawe,D. and Ziff,E.B. (1991) Association of myn, the murine homolog of max, with c-myc stimulates methylation-sensitive DNA binding and ras cotransformation. *Cell*, **65**, 395–407.

Prochownik,E.V., Kukowska,J. and Rodgers,C. (1988) c-myc antisense transcripts accelerate differentiation and inhibit G1 progression in murine erythroleukemia cells. *Mol. Cell. Biol.*, **8**, 3683–3695.

Quelle,D.E., Ashmun,R.A., Shurtleff,S.A., Kato,J.Y., Bar-Sagi,D., Roussel,M.F. and Sherr,C.J. (1993) Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. *Genes Dev.*, **7**, 1559–1571.

Reisman,D., Elkind,N.B., Roy,B., Beamon,J. and Rotter,V. (1993) c-Myc *trans*-activates the *p53* promoter through a required downstream CACGTG motif. *Cell Growth Differentiat.*, **4**, 57–65.

Rustgi,A.K., Dyson,N. and Bernards,R. (1991) Amino-terminal domains of c-*myc* and N-*myc* proteins mediate binding to the retinoblastoma gene product. *Nature*, **352**, 541–544.

Schreiber-Agus,N., Chin,L., Chen,K., Torres,R., Rao,G., Guida,P., Skoultchi,A.I. and DePinho,R.A. (1995) An amino-terminal domain of Mxi1 mediates anti-Myc oncogenic activity and interacts with a homolog of the yeast transcriptional repressor SIN1. *Cell*, **80**, 777–786.

Sebastian,B., Kakizuka,A. and Hunter,T. (1993) Cdc25M2 activation of cyclin-dependent kinases by dephosphorylation of threonine-14 and tyrosine-15. *Proc. Natl Acad. Sci. USA*, **90**, 3521–3524.

Serrano,M., Hannon,G.J. and Beach,D. (1993) A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*, **366**, 704–707.

Sherr,C.J. (1994) G1 phase progression: cycling on cue. *Cell*, **79**, 551–555.

Solomon,D., Philipp,A., Land,H. and Eilers,M. (1995) Expression of cyclin D1 mRNA is not upregulated by Myc in rat fibroblasts. *Oncogene*, in press.

Toyoshima,H. and Hunter,T. (1994) p27, a novel inhibitor of G1 cyclin–Cdk protein kinase activity, is related to p21. *Cell*, **78**, 67–74.

Van den Heuvel,S. and Harlow,E. (1993) Distinct roles for cyclin-dependent kinases in cell cycle control. *Science*, **262**, 2050–2053.

Weinberg,R.A. (1995) The retinoblastoma protein and cell cycle control. *Cell*, **81**, 323–330.

Westin,E.H., Gallo,R.C., Arya,S.K., Eva,A., Souza,L.M., Baluda,M.A., Aaronson,S.A. and Wong-Staal,F. (1982a) Differential expression of the *amv* gene in human hematopoietic cells. *Proc. Natl Acad. Sci. USA*, **79**, 2194–2198.

Westin,E.H. *et al.* (1982b) Expression of cellular homologues of retroviral *onc* genes in human hematopoietic cells. *Proc. Natl Acad. Sci. USA*, **79**, 2490–2494.

Won,K.-A., Xiong,Y., Beach,D. and Gilman,M.Z. (1992) Growth-regulated expression of D-type cyclin genes in human diploid fibroblasts. *Proc. Natl Acad. Sci. USA*, **89**, 9910–9914.

Xiong,Y., Hannon,G.J., Zhang,H., Casso,D., Kobayashi,R. and Beach,D. (1993) p21 is a universal inhibitor of cyclin kinases. *Nature*, **366**, 701–710.

Zervos,A.S., Gyuris,J. and Brent,R. (1993) Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. *Cell*, **72**, 223–232.

Received on June 13, 1995; revised on July 18, 1995