D-type cyclins repress transcriptional activation by the v-Myb but not the c-Myb DNA-binding domain

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The v-Myb DNA-binding domain differs from that of c-Myb mainly by deletion of the first of three repeats. This truncation correlates with efficient oncogenic transformation and a decrease in DNA-binding activity. Here we demonstrate that the D-type cyclins, cyclin D1 and D2 in particular, specifically inhibit transcription when activated through the v-Myb DNA-binding domain, but not the c-Myb DNA-binding domain. Analysis of a cyclin D1 mutant and a dominant-negative CDK4 mutant implied that this repression is independent of complex formation with a CDK partner. Association of cyclin D1 and D2 with the Myb DNA-binding domain could be demonstrated. Increased levels of cyclin D1 and D2 resulted in a stabilization of the Myb proteins, but not in an alteration in binding of the Myb proteins to DNA. These results highlight an unexpected role for cyclin D as a CDK-independent repressor of transcriptional activation by v-Myb but not c-Myb. This differential effect of D-type cyclins on v-Myb and c-Myb might help to explain the mechanism underlying the oncogenic activity of v-Myb, which appears to be a stronger transcriptional activator following the TPA-induced differentiation of transformed monoblasts when cyclin D1 and D2 are down-regulated.

Keywords: D-type cyclins/Myb DNA-binding domain/repeat R1/transcription/v-Myb

Introduction

The *myb* oncogene, which is unique in its hematopoietic specificity of transformation, was first discovered in two independently derived avian retroviruses which cause acute leukemia (for review see Introna *et al.*, 1994). Both AMV and the E26 viruses encode v-Myb proteins that are truncated versions of the cellular c-Myb protein. In addition, the E26 virus expresses a more complex Gag-Myb-Ets fusion protein. The normal *c-myb* proto-oncogene is a regulator of proliferation and differentiation of hematopoietic cells. This gene encodes a 75 kDa nuclear protein that is expressed at high levels in immature hematopoietic cells (Chen, 1980; Klempnauer *et al.*, 1983). Its level of expression decreases dramatically upon spon-

taneous or chemically induced differentiation of these cells (Westin *et al.*, 1982; Gonda and Metcalf, 1984; Duprey and Boettinger, 1985). Furthermore, constitutive expression of exogenous c-Myb in erythroid leukemia cells blocked their differentiation (McClinton *et al.*, 1990), which suggested that down-regulation of c-Myb is a critical step for the progression of differentiation. The AMV *v-myb* oncogene blocks differentiation which results in transformation of immature macrophage precursors (Baluda and Reddy, 1994). On the other hand, forced expression of c-Myb causes the outgrowth of more immature precursor cells that continue to differentiate (Ferraro *et al.*, 1995; Fu and Lipsick, 1997).

In addition, it was shown that c-Myb plays a role in cell growth control and that its expression is associated with cell proliferation in various cell types. In certain cell types, G₁-specific c-Myb mRNA induction was found in continuously cycling cells (Thompson *et al.*, 1986; Catron *et al.*, 1992). The c-Myb mRNA and protein were shown to be induced in late G₁/S phase when resting T lymphocytes are entering from a quiescent stage into the normal cell cycle (Torelli *et al.*, 1985; Reed *et al.*, 1986; Lipsick and Boyle, 1987). In addition, constitutive expression of exogenous c-Myb conferred to a fibroblast cell line (ts13 mutant) the ability to bypass its temperature-sensitive arrest in G₁ phase at the non-permissive temperature (Travali *et al.*, 1991).

The family of Myb proteins is defined by the presence of a Myb domain (repeat), a helix-turn-helix motif of ~50 amino acids (Ogata *et al.*, 1994) which can be present in multiple copies within a single protein. The DNA-binding domain of the Myb family proteins is remarkably conserved in evolution. Genes related to c-Myb have been discovered in *Drosophila*, yeast, plants and the slime mold *Dictyostelium discoideum* (for review, see Lipsick, 1996). The v-Myb DNA-binding domain differs from that of c-Myb mainly by deletion of the first of three repeats and it has been established that similar truncation of the cellular c-Myb protein greatly activates its oncogenic potential, presumably by deregulation of the c-Myb-mediated differentiation/proliferation pathway (Graesser *et al.*, 1991; Dini and Lipsick, 1993). In addition, it has been shown that deletion of the first repeat results in a decrease in DNA-binding activity (Dini and Lipsick, 1993; Tanikawa *et al.*, 1993).

Recently, several additional transcriptional regulators have been identified which contain more distantly related Myb repeats such as the co-repressor N-CoR (Hörlein *et al.*, 1995; Kurokawa *et al.*, 1995), the SWI-SNF component SWI3 (Wang *et al.*, 1996), the ADA component ADA2 (Candau *et al.*, 1996), and the B' subunit of TFIIIB (Kassavetis *et al.*, 1995), a component of the RNA polymerase III initiation complex. The three-dimensional X-ray structure of the yeast telomere-binding protein

RAP1 unexpectedly revealed a Myb-related DNA-binding domain (König *et al.*, 1996) as a protein fold for telomeric DNA recognition. This observation was further supported with the recent identification of two other telomere-binding proteins, the human hTRF protein (Van Steensel and De Lange, 1997) and the *Schizosaccharomyces pombe* Taz1 protein (Cooper *et al.*, 1997), which also contain Myb domains.

The identification of such a wide variety of proteins containing Myb repeats suggests an additional role for the Myb domain in protein–protein interaction, besides its DNA-binding function described so far. The fact that the two related proteins v-Myb and c-Myb, which differ at the N-terminus by one repeat, cause such dramatically different cellular effects led us to investigate their structural differences and their biological/functional relevance in relation to the normal cell cycle. In an effort to address whether the Myb domain could function as a potential protein–protein interaction domain and whether a potential interacting protein might explain the functional differences between c-Myb and v-Myb we concentrated our studies on the cyclins, the positive regulatory subunits of cell division cycle protein kinases.

Cyclins have been identified as positive regulatory proteins that interact with their specific kinase partners termed cyclin-dependent kinases (CDKs). These protein kinases have been shown to be important regulators of major cell cycle transitions in a number of diverse eukaryotic systems. In mammalian cells, ten different cyclin types have been identified so far, the majority of which act at specific stages of the cell cycle. A transient accumulation of cyclin proteins results in activation of their CDK partners and subsequently in phosphorylation of target proteins (Resnitzky and Reed, 1995; Weinberg, 1995). These protein phosphorylation cascades are thought to play a crucial role in the various cell cycle transitions.

Here we report an unexpected relationship between D-type cyclins and the Myb proteins. The identification of a differential effect of D-type cyclins on transcriptional regulation by v-Myb and c-Myb has important implications for our understanding of the mechanism underlying the oncogenic activity of v-Myb.

Results

Cyclin D represses v-Myb- but not c-Myb-specific transcriptional activation

Trans-activation by Myb proteins can be measured by means of a luciferase reporter construct with upstream Myb recognition sites, such as the *mim1-A* site (Ness *et al.*, 1989). Quail QT6 cells provide a convenient system for assaying the ability of both c-Myb and v-Myb proteins to activate transcription from a given promoter since these cells lack both c-Myb and v-Myb. Using this system, we have investigated whether co-expression of different cyclins would result in a differential effect on v-Myb- or c-Myb-specific *trans*-activation. Different cyclins (cyclin A, B1, B2, D1, D2, D3 and E) and the Myb-responsive reporter plasmid EW5(–) were co-transfected with v-Myb (dGE) or c-Myb (CCC) (see Figure 1 for structure of proteins) into quail QT6 cells. Figure 2 shows that co-expression of cyclin A, cyclin E and the B-type cyclins (cyclin B1 and B2) with either v-Myb or c-Myb results

in an increased activation of transcription. Cyclin A superactivated c-Myb ~12-fold, as has been shown before by others for B-Myb (Sala *et al.*, 1997; Ziebold *et al.*, 1997), and also activated v-Myb to a lesser extent (3-fold). Co-expression of cyclin E as well as of the B-type cyclins also resulted in an increased activation by either v-Myb or c-Myb protein, but to a much lesser extent than cyclin A. Interestingly, co-expression of the D-type cyclins resulted in an inhibition of the *trans*-activation of v-Myb, whereas c-Myb *trans*-activation was slightly increased. Cyclin D1 was a particularly effective inhibitor of v-Myb, resulting in a 70% inhibition of transcriptional activity.

Amino-terminal deletion of c-Myb is required for inhibition by cyclin D1

To further map the regions within Myb that confer a differential response to cyclins, different truncated forms of c-Myb (see Figure 1 for structure of proteins) were assayed for *trans*-activation in the presence of cyclin A or cyclin D1. For comparison, relative activity with or without cyclins for each individual construct is presented (Figure 3B). For each construct, *trans*-activation activity without cyclins was assigned to 100% for comparison with samples co-expressed with cyclins (see Figure 3A for activity of different Myb proteins relative to c-Myb in the absence of exogenous cyclins). Again, cyclin A did not show any specific effect for either c-Myb or v-Myb, but rather a general activation of all different types of Myb proteins was observed. In addition, cyclin A also activated the promoter in the vector only control (N-Cla), which may be due to an activation of the general transcription machinery or activation of low levels of endogenous B-Myb.

In contrast, cyclin D1 specifically inhibited *trans*-activation by N-terminally truncated Myb proteins, such as dCC, dCd and dGE, but not by the full-length c-Myb protein (CCC) or the C-terminally truncated Myb version CCd (Figure 1). Expression of the different Myb proteins as well as of cyclin D1 was confirmed by immunoblots (Figure 3C for Myb protein expression; Figure 3D for cyclin D1 expression). Notably, in several independent experiments, the levels of Myb protein in normalized amounts of lysate were higher in the presence of cyclin D1 than in the absence of cyclin D1. Nevertheless, the activity of any tested N-terminally truncated Myb protein was lower upon co-expression of cyclin D1 despite the higher level of Myb protein. To rule out the possibility that the observed differential effect of cyclin D1 on v-Myb and c-Myb *trans*-activation was due to a difference in ratio of cyclin D1 to the Myb proteins studied, we performed a titration experiment. A fixed amount of CCC or dCC (3 µg plasmid DNA) was co-transfected with various amounts of cyclin D1 (1–7 µg plasmid DNA) (data not shown). Co-transfection of 1 µg of the cyclin D1 construct already resulted in an inhibition of dCC transcriptional activation by 82%. Co-transfection of up to 7 µg of the cyclin D1 construct gave similar results, with an inhibition of dCC >95% at 7 µg of cyclin D1. On the other hand, CCC transcriptional activation was consistently activated to an average of 137%, when co-transfected with 1–7 µg of cyclin D1 construct. Importantly, no inhibition of CCC was observed, even at doses of cyclin D1 which exceeded the amount necessary for inhibition of dCC.

Fig. 1. Structure of the different wild-type and mutant Myb proteins used in this study. The c-Myb protein (CCC), v-Myb protein (dGE) and various mutant forms are diagrammed. The dark gray boxes indicate the highly conserved Myb DBD, which is composed of three imperfect direct repeats of ~50 amino acids each. The C-terminal conserved negative regulatory region (NR) and the *trans*-activation domain required for transcriptional activation by the Myb protein are indicated by gray boxes. The vertical bars indicate amino acid substitutions in v-Myb relative to c-Myb. The black box represents the 78-amino acid transcriptional activation domain of the herpes simplex virus VP16 which is fused to various Myb N-termini as diagrammed. Single and double truncations of c-Myb are named according to the sequences present in c-Myb (C) or deleted (d) (Dini and Lipsick, 1993). The c-Myb mutant designated SS(11,12)AA represents site-directed mutagenesis of the CKII phosphorylation sites (Dini and Lipsick, 1993). The c-Myb mutant designated CCC-d9 has its N-terminus deleted and lacks the Myb DBD (Dini and Lipsick, 1993). The c-Myb mutant designated d1-30 has an N-terminal deletion of 30 amino acids just upstream of the Myb DBD (Dini and Lipsick, 1993).

To confirm further that the interaction between Myb and the cyclin D1 maps to the N-terminus, which consists mainly of the Myb DNA-binding domain, different Myb-VP16 fusion proteins (see Figure 1 for protein structure) were tested for *trans*-activation in the presence and absence of cyclin D1. As shown in Figure 4, cyclin D1 inhibited the N-terminally truncated dCVP16 and dVVP16 proteins by an average of 90%. The much weaker inhibition of CCVP16 and CC(d1-30)VP16 by cyclin D1 was similar to the inhibition observed with the negative control N-Cla. Protein expression of the VP16 fusion proteins was confirmed by immunoblotting (results not shown).

Cyclin D1 represses transcriptional activation by v-Myb independently of a CDK partner

The observed difference in the effects of cyclin D1 upon different forms of Myb can be attributed to several possible

mechanisms. Two previously mapped phosphorylation sites (Ser11 and Ser12) (Luscher *et al.*, 1990) can be found at the N-terminus of the c-Myb protein, immediately upstream of the Myb DBD. Both sites are absent in dGE (v-Myb), dCd, and dCC (see Figure 1 for structure of proteins). Therefore, one possible mechanism is that these two sites are positively regulated by the cyclin D-CDK4/6 complex, which could explain the observed activation of c-Myb, but not of v-Myb. We tested this possibility by assaying the mutant CCC SS(11,12)AA, in which both serines at position 11 and 12 were substituted with alanine (Dini and Lipsick, 1993). As shown in Figure 5A, *trans*-activation of CCC SS(11,12)AA was not inhibited by cyclin D1. The Myb protein CCC d1-30, which lacks the first 30 amino acids including Ser11 and Ser12 (Dini and Lipsick, 1993) showed partial inhibition (60%), whereas full inhibition (85%) was only obtained when most of

Fig. 2. Differential effect of D-type cyclins on c-Myb- and v-Myb-responsive transcription. *Trans*-activation by c-Myb (CCC) and v-Myb (dGE) proteins was assayed using the PolyA-EW5(-)Luc reporter construct, which contains a tandem array of five *mim1-A* sites, in the presence of different cyclins. Activity is indicated relative to c-Myb (upper part) or v-Myb (lower part) in the absence of exogenous cyclin, which were assigned a value of 100%. N-Cla represents the negative control (retroviral vector which does not encode any activator protein). Data are averages of two to four experiments and error bars indicate maximum and minimum values of different sets of data points.

repeat R1 was also deleted, as is the case in dCC (Figure 1). These data demonstrate that deletion of c-Myb to the N-terminal equivalent of v-Myb is necessary for full inhibition by cyclin D1 to occur, and that this regulation is not dependent on the N-terminal phosphorylation sites within the c-Myb protein. However, the deletion of the first 30 amino acids of c-Myb which contains a highly acidic region (residues 13–25) also permits partial inhibition (22%) by cyclin D1.

Thus far there is no evidence for additional phosphorylation sites *in vivo* within the N-terminal region of c-Myb which are deleted in v-Myb. Tryptic phosphopeptide mapping identified multiple phosphorylation sites located at amino acids 267–303 within v-Myb and amino acids 338–374 within c-Myb (Boyle *et al.*, 1991). We have shown that mutation of these phosphorylation sites did not affect either *trans*-activation or transformation by v-Myb (Fu and Lipsick, 1996). Furthermore, these sites are not present in the Myb–VP16 fusion proteins which remain sensitive to cyclin D1 (Figure 4). Another phosphorylation site, which was mapped both *in vitro* and *in vivo* (Aziz *et al.*, 1995), is Ser533 located within the C-terminus of c-Myb. This site is also not present in the

v-Myb–VP16 fusion proteins, and therefore cannot be involved in the interactions with cyclin D1 described in this study. Since we could not rule out other potential phosphorylation site(s) within the Myb protein(s) as a target for cyclin D1 regulation, we compared the phosphorylation states of dGE in the presence and absence of cyclin D1 from transfected QT6 cells using 2D gels followed by immunoblot with an anti-Myb antibody (Fu and Lipsick, 1996). No differences in phosphorylation were identified (results not shown), which led us to conclude that phosphorylation by a D1 kinase partner was not the mechanism involved in Myb/cyclin D1 interaction.

To investigate further the requirement of a kinase partner (CDK4 or CDK6) for the observed effects of cyclin D1 on v-Myb activity, a cyclin box mutant of cyclin D1 (cyclin D1-KE) was used (Hinds *et al.*, 1994). This mutant cyclin D1 fails to bind CDKs and as a result is unable to mediate phosphorylation of pocket proteins (Hinds *et al.*, 1994; Beijersbergen *et al.*, 1995). Like wild-type cyclin D1, the cyclin D1-KE mutant inhibited transcriptional activation of v-Myb (Figure 5B). In fact, the inhibition observed with the D1-KE mutant was much greater than with wild-type D1, probably because it cannot be titrated

Fig. 3. Specific action of cyclin A and cyclin D1 on Myb-responsive transcription. **(A)** *Trans*-activation by different Myb proteins. The reporter gene used for this assay is PolyA-EW5(-)Luc, which contains an array of five *mim1-A* sites (Fu and Lipsick, 1996). Activities are shown relative to the full-length c-Myb protein (CCC), which was assigned a value of 100. Data are averages of five experiments. **(B)** *Trans*-activation activity by full-length c-Myb (CCC) and different truncated Myb proteins were tested in the presence of either cyclin A (upper panel) or cyclin D1 (lower panel). The reporter construct used for this assay is PolyA-EW5(-)Luc (Fu and Lipsick, 1996). Activities for each construct are shown relative to *trans*-activation without any exogenous cyclins (gray boxes), which were assigned to 100%. N-Cla represents the negative control (retroviral vector which does not encode any activator protein). Data are averages of two to four experiments and error bars indicate maximum and minimum values of different sets of data points. **(C)** Immunoblot analysis of Myb proteins in the same transfected cell extracts in the presence and absence of either cyclin A or cyclin D1. Normalized volumes of samples assayed in (B) were analyzed by SDS-PAGE, and then Myb proteins were detected with a mixture of anti-Myb-2.2, Myb-2.7 and 5E antibodies as described in Materials and methods. Molecular weight markers are indicated on the left of the gel in kDa. Relative mobilities of transfected Myb proteins are labeled on the right of the gel: 1 = CCC (c-Myb), 2 = dCC, 3 = CCd, 4 = dCd, and 5 = dGE (v-Myb). **(D)** Immunoblot analysis of cyclin D1 in the same transfected cell extracts. Normalized volumes of samples assayed in (B) were analyzed by SDS-PAGE, and then cyclin D1 was detected with a polyclonal anti-D1 antibody as described in Materials and methods. Molecular weight markers are indicated on the left of the gel in kDa.

away by binding to its kinase partner CDK4 or CDK6. This finding supported the notion that the effect of cyclin D1 on v-Myb is independent of its ability to activate a CDK partner. Consistent with this finding, binding of cyclin D1 to a CDK that lacks intrinsic kinase activity, such as the dominant-negative CDK4 mutant (CDK4-DN)

which cannot be activated by cyclin D1 (Ewen *et al.*, 1993), partially relieves the inhibition of wild-type cyclin D1 but not mutant D1-KE on v-Myb. Furthermore, the kinase inhibitor p16^{INK4} (Serrano *et al.*, 1993) did not affect the cyclin D-specific repression of v-Myb. Expression of v-Myb protein, p16^{INK4}, cyclin D1 and cyclin D1-KE was

Fig. 4. Specific inhibition of Myb-VP16-responsive transcription by cyclin D1. *Trans*-activation by different Myb-VP16 fusion proteins was tested in the presence and absence of cyclin D1 and compared with full-length c-Myb (CCC) and N-terminally truncated c-Myb (dCC) protein. The reporter construct used for this assay is PolyA-EW5(-)Luc (Fu and Lipsick, 1996). Activities for each construct are shown relative to *trans*-activation without any exogenous cyclins (gray boxes), which were assigned to 100% for each construct. N-Cla represents the negative control (retroviral vector which does not encode for any activator protein; see Materials and methods). Data are averages of two to four experiments and error bars indicate maximum and minimum values of different sets of data points.

Fig. 5. Amino-terminal deletion of c-Myb is required for inhibition by cyclin D1. **(A)** *Trans*-activation by different N-terminally mutated Myb proteins in comparison with full-length c-Myb (CCC) protein were tested in the presence and absence of cyclin D1. The reporter construct used for this assay is PolyA-EW5(-)Luc (Fu and Lipsick, 1996). Activities for each construct are shown relative to *trans*-activation without any exogenous cyclins (gray boxes), which were assigned to 100% for each construct. N-Cla represents the negative control (retroviral vector which does not encode for any activator protein; see Materials and methods). Data are averages of two to four experiments and error bars indicate maximum and minimum values of different sets of data points. **(B)** The effects of cyclin D1 and its associated kinase activity on v-Myb-responsive transcription. Cyclin D1 (wt and mutant D1-KE) in combination with the catalytically inactive mutant of CDK4 (CDK4-DN) or the kinase inhibitor p16^{INK4} were co-transfected with the Myb-responsive reporter PolyA-EW5(-)Luc (Fu and Lipsick, 1996). The luciferase activities are shown relative to v-Myb (dGE) alone, which was assigned to 100%. Data are averages of two to four experiments and error bars indicate maximum and minimum values of different sets of data points.

confirmed by immunoblots (results not shown). Taken together, these data indicate that cyclin D1 inhibits v-Myb *trans*-activation independently of a CDK partner.

In vivo binding of cyclin D to v-Myb and c-Myb

The observed inhibition of v-Myb activity by D-type cyclins could be either through direct or indirect binding to v-Myb and/or c-Myb proteins. To address this question, we performed immunoprecipitations to study the interaction between cyclin D and v-Myb within BM2 cells which are v-Myb-transformed chicken monoblasts. Figure 6A shows that the endogenous v-Myb protein co-immunoprecipitates with cyclin D2 (lane 4). We were unable to show a co-immunoprecipitation of v-Myb with cyclin D1 from these monoblasts. Considering the strong effect of cyclin D1 on v-Myb *trans*-activation, we attributed this negative result to the anti-D1 antibody not being able to recognize its epitope in the D1-v-Myb complex. A similar finding has been reported before by others (S.Reed, personal communication), in that the immunoprecipitated cyclin D1-CDK4/6 kinase activity was dependent on the anti-D1 antibody used.

To further map the region of interaction between cyclin D2 and the Myb proteins we performed immunoprecipitation studies from transfected QT6 cells (Figure 6B). Full-length c-Myb, v-Myb and an N-terminally deleted form of c-Myb (CCC-d9, entire Myb DBD deleted) were co-transfected with cyclin D2 (Figure 6B). Cyclin D2 was immunoprecipitated and analyzed for Myb binding by Western blot analysis. Co-transfection with c-Myb (CCC) and v-Myb (dGE) resulted in co-immunoprecipitation of either c-Myb or v-Myb with cyclin D2 (Figure 6B, lanes 3 and 6), whereas co-transfection with c-Mybd9 (CCC-d9) did not result in co-immunoprecipitation with cyclin D2 (Figure 6B, lane 9). Because the entire MybDBD is deleted in c-Mybd9, this confirms that the Myb DNA-binding domain is required for interaction with cyclin D2. Since mapping the region of interaction between the Myb proteins and cyclin D2 involved the construct c-Mybd9 (Myb DBD deleted), we were unable to use the Myb 5E antibody which recognizes the Myb DBD and was used for the endogenous immunoprecipitation from BM2 cells (Figure 6A). Therefore, we chose to use the monoclonal Myb2.2 which recognizes the acidic region within the *trans*-activation domain. When the anti-Myb monoclonal antibody (Myb2.2) used for the Western blot was used for immunoprecipitation (conditions for co-immunoprecipitation with cyclin D2), little or no Myb proteins were detected (Figure 6B). However, when the same anti-Myb antibody was used for immunoprecipitation under denaturing conditions, the Myb proteins could be easily detected (Figure 6C). Taken together, these results demonstrate that Ig and Protein G alone do not non-specifically bind to the Myb proteins, but rather that v-Myb and cyclin D2 interact specifically in cells.

Since we were unable to map the region of interaction between the Myb proteins and cyclin D1 with immunoprecipitation studies, we performed a yeast two-hybrid assay between cyclin D1 and different N-terminal forms of Myb, which is summarized in Figure 6D. Cyclin D1 fused to a GAL4 DNA-binding domain (Gal4DBD-D1) was constructed and co-transformed with different Myb proteins into a yeast reporter strain which carries both *HIS3* and

lacZ reporter genes under control of Gal4p-binding site. We found that all of the Myb-VP16 fusion proteins tested do interact with the Gal4DBD-D1 as shown by growth on his⁻ medium and by X-gal assays (see Materials and methods). These two-hybrid data imply that cyclin D1 and the Myb proteins directly interact with each other or that if another protein is required, it is highly conserved from animals to yeast. Neither yeast containing the Gal4DBD-D1 nor the v-Myb-VP16 constructs alone turned blue with X-gal, even after overnight incubation at 37°C. To summarize, cyclin D1 and D2 physically interact both with the N-terminus of the v-Myb and the c-Myb proteins as has been shown with co-immunoprecipitation studies for cyclin D2 and with yeast two-hybrid studies for cyclin D1.

To investigate whether the observed effect of cyclin D1 on v-Myb transcription was due to inhibition of v-Myb binding to DNA, we tested binding of full-length v-Myb protein to its target site in the presence and absence of cyclin D1 or D2 using gel retardation assays. Figure 7 shows that no difference in affinity of the v-Myb protein for the *mim1-A* site was observed whether cyclin D1 or D2 was present or not (in lanes 4–6 increasing amounts of cyclin D1 were added, whereas in lanes 9–11, increasing amounts of cyclin D2 were added to the v-Myb-DNA sample). We also tested binding of different purified, recombinant Myb DNA-binding domains (v-Myb2R DBD, c-Myb2R DBD and c-Myb3R DBD) to their target site in the presence and absence of cyclin D1. No difference in affinity for the *mim1-A* site was observed for any of the Myb DBD proteins whether cyclin D1 was present or not (results not shown). Thus, the observed cyclin D1 and D2 repression of v-Myb transcription is not due to inhibition of binding of the v-Myb protein to its target site in DNA.

Transcriptional activity of v-Myb within BM2 cells is regulated by D-type cyclins

To study the transcriptional activity of endogenous v-Myb protein in the presence and absence of cyclin D, an AMV-transformed chicken monoblast cell line (BM2 cells), which constitutively expresses v-Myb protein (Moscovici *et al.*, 1982), were co-transfected with the Myb-responsive luciferase reporter construct EW5(–). TPA (phorbol ester)-induced differentiation of BM2 cells into macrophages (Pessano *et al.*, 1979; Smarda and Lipsick, 1994) results in down-regulation of cyclin D1 and D2 (Figure 8A, lower panel), whereas the v-Myb protein is still present (Figure 8A, upper panel). Therefore, BM2 cells are an ideal model system to study the effect of D-type cyclins on v-Myb activity *in vivo*. Figure 8B shows that transcriptional activation by endogenous v-Myb is down-regulated in growing BM2 monoblasts when cyclin D1 and D2 are present within the cell. In contrast, in TPA-treated differentiated macrophages which lack cyclin D1 and D2 (Figure 8A), increased v-Myb transcriptional activity can be readily detected. This was further explored by overexpression of either v-Myb or v-Myb-VP16 protein in BM2 cells. Again, transcriptional activity was higher after TPA-induced differentiation into macrophages. To show further that this response is cyclin D-dependent, we co-expressed the different cyclins (D1, D2 and D3) and the cyclin box mutant D1-KE with or without v-Myb within differentiated cells which lack endogenous cyclin D expression. Addition

of any of the D-type cyclins resulted in the repression of v-Myb activity, suggesting that transcriptional activity of v-Myb within BM2 cells is largely regulated by the presence and absence of cyclin D rather than some other effect of TPA. As in QT6 cells, the repression by cyclin D1-KE was even more efficient than the wild-type cyclin D1 (Figure 5B). Interestingly, in BM2 cells, cyclin D2 is the most effective inhibitor of v-Myb rather than cyclin D1, as was observed in QT6 cells (Figure 2). In contrast, c-Myb-VP16 was relatively unaffected by TPA. These

results in BM2 cells are of particular interest, because this *in vivo* system extends our finding from quail QT6 cells to cells that are transformed by v-Myb. In both cases v-Myb transcriptional activity can be inhibited by cyclin D, but proteins with a c-Myb DNA-binding domain are resistant to cyclin D.

Again, to investigate whether the observed difference in transcriptional activity of v-Myb in monoblasts and macrophages was due to inhibition of v-Myb binding to its site by the presence of cyclin D in monoblasts, we

D Bait protein: Gal4DBD-D1

Name	Reporter Gene Activation HIS-selection / β-gal assay	
CC VP16	+	+
CC(d1-30)VP16	+	+
dC VP16	+	+
dV VP16	+	+
dV VP16 (alone)	-	-
Gal4DBD-D1 (alone)	-	-

made extracts of monoblasts and macrophages and tested binding of endogenous v-Myb to the *mim1-A* site. We were unable to see any difference of v-Myb DNA-binding activity in these two extracts, which suggests again that cyclin D action on v-Myb transcription is not due to an inhibition of binding of the v-Myb protein to its target site (results not shown).

Discussion

The present study establishes a new role for D-type cyclins as negative regulators of v-Myb-regulated transcription, but not of c-Myb-regulated transcription. Rather, c-Myb-specific transcription is slightly enhanced in the presence of D-type cyclins. N-terminal deletion of c-Myb to the v-Myb equivalent, was required for maximal repression by D-type cyclins to occur. This effect is specific for D-type cyclins, of which cyclin D1 and D2 have the strongest effect. None of the other cyclins tested (A, B1, B2 and E) showed this repressive phenotype. In fact, all other cyclins tested function as positive regulators of transcription in a more general way and were not v-Myb-specific. Astonishingly, repression of v-Myb transcriptional activation by cyclin D1 was entirely independent of binding to any of its CDK partners. We demonstrated that both v-Myb and c-Myb proteins associate with cyclin D1 and D2 through the Myb DNA-binding domain, but that N-terminal deletion of c-Myb to the v-Myb equivalent is required for maximal inhibition of transcriptional activation to occur. Increased cyclin D1 and D2 resulted in a stabilization of the Myb proteins within the cell, but did not affect binding of the Myb proteins to DNA.

Previous studies have shown that both v-Myb and c-Myb are localized entirely within the cell nucleus, including in the BM2 cell line which expresses high levels of v-Myb, cyclin D1 and D2 (Boyle *et al.*, 1984; Klempnauer *et al.*, 1984). Paradoxically, treatment of BM2 cells with TPA—which abolishes cyclin D expression—was previously reported to inhibit nuclear localization of v-Myb (Klempnauer *et al.*, 1984), whereas we have shown here that TPA treatment of these cells increases transcriptional activation by v-Myb. In our hands, subcellular fractionation has failed to reveal a difference in v-Myb or c-Myb localization with or without TPA (Smarda and Lipsick, 1994). Taken together, our results imply that D-type cyclins inhibit transcriptional activation by v-Myb via a direct interaction which affects transcription, but not nuclear transport or DNA binding.

A related observation has been reported for estrogen receptor (ER)-regulated genes in estrogen-responsive tissue (Zwijsen *et al.*, 1997), where cyclin D1 affects transcriptional activation. In this case cyclin D1 was shown to be a positive regulator of ER-responsive transcription (Zwijsen *et al.*, 1997), whereas our results demonstrate an inhibitory effect of cyclin D1 and D2 on v-Myb-regulated transcription. The observed effect on ER-responsive transcription by cyclin D1 is also independent of binding to a CDK partner (Zwijsen *et al.*, 1997), which suggests a more general role for D-type cyclins as regulators of transcription. Until recently it was thought that cyclin D functions only as a CDK4/6 regulator, to control progression of cells from quiescence (G_0) into the

Fig. 7. Cyclin D does not affect binding of v-Myb to its target site. Analysis of v-Myb-*mim1-A* complexes in response to cyclin D1 and D2. Free DNA is shown in lane 1. Binding reactions with the v-Myb protein are shown in lanes 2–7 and lanes 9–12. Incubation of binding reactions with cyclin D1 are shown in lanes 4–8 (increasing amounts of cyclin D1 were added to reactions 4–6), while lanes 9–13 (increasing amounts of cyclin D2 were added to reactions 9–11) show the binding reactions incubated with cyclin D2. Incubation of binding reactions with an anti-Myb antibody (rabbit polyclonal BP4, Garcia *et al.*, 1991) is shown in lane 3 (α-Myb), with an anti-D1 antibody (mouse monoclonal HD11) in lane 7 (α-D1) and with an anti-D2 antibody (rabbit polyclonal) in lane 12 (α-D2). Myb-specific DNA–protein complexes and free DNA probes are marked on the left of the gel.

Fig. 6. *In vivo* interaction of cyclin D1 and D2 with the N-terminal region of the Myb proteins. (A) Interaction of endogenous cyclin D2 with the v-Myb protein in BM2 cells. Extracts of BM2 cells were immunoprecipitated with the anti-Myb 5E monoclonal antibody (lane 2), a monoclonal anti-β-galactosidase isotype control (lane 3), an anti-D2 polyclonal rabbit serum (lane 4), or a normal rabbit serum control (lane 5). Myb proteins were then detected by immunoblotting with the anti-Myb 5E monoclonal antibody. A sample of the lysate before immunoprecipitation (IP-BM2) was co-electrophoresed (lane 1). (B) *In vivo* interaction between cyclin D2 and various Myb proteins using immunoprecipitation. Cyclin D2 and different Myb proteins were produced by DNA transfection into quail QT6 fibroblasts and immunoprecipitated using either the mouse monoclonal Myb 2.2 antibody (specific against the TA-region of the Myb proteins) (lanes 2, 5, 8 and 11), or a rabbit polyclonal anti-D2 antiserum (lanes 3, 6, 9 and 12). Samples of total cell extract (E) were co-electrophoresed (lanes 1, 4, 7 and 10) and Myb proteins were detected by immunoblotting. (C) Immunoprecipitation of c-Myb and v-Myb using the mouse monoclonal Myb2.2 antibody. Total extracts of transfected QT6 cells (E) or extracts immunoprecipitated with anti-Myb2.2 antibody (specific against the TA-region of the Myb proteins) (lanes 2 and 4) under denaturing conditions (RIPA-buffer) were co-electrophoresed. The Myb proteins were then detected by Western blot analysis using a mixture of anti-Myb2.2 and 2.7 monoclonal antibodies. (D) *In vivo* binding of cyclin D1 to the N-terminal region of the Myb proteins. The structure of the different proteins used in this yeast two-hybrid assay are shown to the left of the table. In the bait protein Gal4DBD–D1, the full-length cyclin D1 protein is fused in frame to the Gal4 DBD. The different Myb target proteins contain a C-terminal VP16 activation domain and different N-terminal Myb domains. All the transformants were plated onto media lacking histidine. Growing transformants were further analyzed for β-gal activity on filters containing the chromogenic substrate X-gal. Neither Gal4DBD–D1 nor the Myb–VP16 protein alone permitted growth on media lacking histidine.

Fig. 8. v-Myb *trans*-activation in BM2 cells is regulated by cyclin D. (A) TPA-induced differentiation of monoblasts into macrophages results in a down-regulation of cyclin D1 and cyclin D2 (lower panel), whereas the v-Myb protein remains present. The upper panel is an immunoblot of BM2 cells with or without TPA treatment using monoclonal anti-Myb 5E antibody. In the lower panel, the same samples were analyzed by immunoblotting with a mixture of anti-D1(mouse monoclonal HD11) and -D2 antibody (polyclonal rabbit antisera). Lanes 6–10 represent TPA-induced samples (macrophages) harvested after 5, 8, 11, 14 and 24 h. Lanes 1–5 represent control samples (monoblasts) harvested at 5, 8, 11, 14 and 24 h after addition of DMSO. (B) The effect of TPA and cyclin D1 on v-Myb-responsive transcriptional activation in BM2 cells. *Trans*-activation of endogenous v-Myb and transfected exogenous v-Myb, v-Myb-VP16 and c-Myb-VP16 fusion proteins were tested with or without TPA induction and in the presence of co-transfected cyclin D1, D2, D3 or the cyclin box mutant protein D1-KE. The reporter construct used for this assay is PolyA-EW5(-)Luc (Fu and Lipsick, 1996). Activities for each construct are shown relative to *trans*-activation activity of v-Myb, v-Myb-VP16 or c-Myb-VP16 in macrophages (+TPA), each of which were assigned to 100%. Data are averages of two to five experiments and error bars indicate maximum and minimum values of different sets of data points.

G₁ phase of the cell cycle. The finding that cyclin D can also function as a CDK-independent negative regulator of v-Myb and as a positive regulator of the estrogen receptor highlights the existence of additional functions for D-type cyclins.

A first link between cyclin D and the Myb proteins has been proposed with the recent identification of DMP1, a Myb-related transcription factor, which was identified in a yeast two-hybrid screen as a cyclin D2-interacting protein (Hirai and Sherr, 1996). Our mammalian *trans*-activation data, yeast two-hybrid data and immunoprecipitation data strongly support the notion that the Myb DBD is the region required for interaction between cyclin D and the Myb proteins. Using either full-length Myb proteins or different Myb-VP16 proteins in *trans*-activation experiments, we have observed a dramatic inhibition of Myb by cyclin D1 upon deletion of repeat R1 of the

Myb DBD. This observation is of particular interest because it shows for the first time that the N-terminal difference between the v-Myb and c-Myb protein, namely repeat R1 of the Myb DBD, is responsible for the differential regulation of transcriptional activity in the presence of an interacting protein.

AMV-transformed chicken monoblasts (BM2 cells), which constitutively express v-Myb protein (Moscovici *et al.*, 1982), can be forced to differentiate into macrophages upon TPA induction (Pessano *et al.*, 1979; Smarda and Lipsick, 1994). At the same time, TPA-induction also results in down-regulation of cyclin D1 and D2 in these BM2 cells. In the present study, we have used these different BM2 cell stages to study transcriptional activity of the endogenous v-Myb protein in its relevant target cells. Based on our QT6 *trans*-activation results described in this study, it was not surprising that a high level of

Fig. 9. Model of Myb oncogenic activity. In normal immature monoblasts the c-Myb protein level is high but is down-regulated upon differentiation into macrophages. Constitutive expression of exogenous c-Myb blocks this differentiation step (Ferraro *et al.*, 1995; Fu and Lipsick, 1997), implying that down-regulation of c-Myb is critical for the cells to differentiate. On the other hand, in v-Myb-transformed monoblasts the v-Myb protein level remains constant, which suggests that its activity may be regulated by other proteins. This regulation is achieved by the presence or absence of cyclin D. Therefore, differentiation of monoblasts into macrophages can only happen if cyclin D is down-regulated.

v-Myb activity could only be detected in TPA-induced macrophages when cyclin D1 and D2 was absent. Even an exogenous v-Myb-VP16 fusion protein was highly active in TPA-induced macrophages and not in monoblasts, which further supports a functional interaction between cyclin D and the Myb protein. Co-expression of different D-type cyclins (D1, D2 and D3) and the cyclin D1-KE mutant in macrophages resulted in a repression of the TPA-induced increase of v-Myb activity upon differentiation. Since the activity of exogenous c-Myb-VP16 fusion protein was not affected by cyclin D (Figure 8B), our current model which is outlined in Figure 9 suggests that v-Myb activity is regulated at least in part by the presence or absence of cyclin D. c-Myb activity seems to be unaffected by the cyclin D status, but is rather determined by its intracellular protein concentration. Therefore, in the process of normal differentiation from monoblasts into macrophages the c-Myb level and activity decreases, while in transformed cells the protein concentration of v-Myb remains stable but its activity is subjected to regulation by cyclin D.

In contrast to the previous view, it was recently shown using different forms of v-Myb proteins, that there is no strong correlation between transcriptional activation and strength of oncogenic transformation (Chen *et al.*, 1995; Engelke *et al.*, 1995b). Our finding that v-Myb has stronger transcriptional activity in differentiated macrophages than in monoblasts, strongly supports and extends this finding. In addition, our data suggest that v-Myb may not only function as a transcriptional activator, but also as a transcriptional repressor depending on the presence of additional factors such as cyclin D. Consistent with this finding, a recent analysis of the Myb binding sites within the N-ras promoter revealed that both c-Myb and v-Myb can function as repressors by significantly reducing the promoter activity (Ganter and Lipsick, 1997). The concept that repression rather than activation can correlate with transformation, such as reported here for v-Myb in BM2

cells, has been described for other oncoproteins. For example, the v-erbA protein has lost the hormone responsiveness of its cellular homologue, but has retained DNA-binding activity, and therefore it has been suggested that transcriptional repression is required for transformation (Fuerstenberg *et al.*, 1992).

To conclude, our data suggest that D-type cyclins, in particular D1 and D2, are involved in the regulation of v-Myb activity in BM2 cells by inhibiting transcriptional activation which in turn prevents monoblasts from differentiating into macrophages. On the other hand, c-Myb activity is not regulated like v-Myb activity, since the presence (monoblasts) or absence (macrophages) of cyclin D has only a minimal effect on c-Myb activity. Rather, its intracellular concentration seems to be the critical factor for differentiation of monoblasts into macrophages (Gonda *et al.*, 1982; Westin *et al.*, 1982). Consistent with this model, the introduction of full-length c-Myb or the c-Myb DNA-binding domain alone can prevent TPA-induced differentiation of v-Myb transformed monoblasts (Smarda and Lipsick, 1994; Engelke *et al.*, 1995a).

Material and methods

Plasmid construction and oligonucleotides

DNA restriction and modifying enzymes were purchased from New England Biolabs (Beverly, MA). Oligonucleotides were made on an Applied Biosystems (Foster City, CA) model 380B synthesizer. Recombinant DNA manipulations were carried out using standard techniques (Sambrook *et al.*, 1989).

The different activator constructs used for transient transfection assays have been described elsewhere: N-Cla, NEO-CCC (c-Myb), N-dCC, N-CCd, N-dCd, N-CCC-d9, N-dGE (v-Myb), N-CCVP16 (c-Myb-VP16), N-dCVP16, N-VVP16 (v-Myb-VP16), N-CC(d1-30) VP16, N-CCC SS(11,12,3)AA and N-CCC d1-30 (Graesser *et al.*, 1991; Dini and Lipsick, 1993).

A yeast-*Escherichia coli* shuttle vector, YEplac181, containing the yeast *LEU2* selectable marker and the 2 μ m origin, was kindly provided by R.Daniel Gietz (Gietz and Sugino, 1988) and was used to construct various Myb expression vectors. In order to construct an expression vector for the Myb proteins under the control of the yeast constitutive *ADHI* promoter, the *HpaI*-*DrdI* insert of the YD (Chen and Lipsick, 1993) plasmid was subcloned into homologous sites of the YEplac18 vector, to obtain the basic vector YL. The constitutive expression vector YLMV for v-Myb-VP16 (dVVP16) was constructed by cloning the insert of YDMV, digested with *HpaI* and *DrdI* (Chen and Lipsick, 1993) into the vector YL, digested with *HpaI* and *DrdI*. The constitutive expression vector YLCCC for c-Myb was constructed by replacing the coding sequence of v-Myb-VP16 of YLMV with the coding sequence of c-Myb from YDCCC. The constitutive expression vector YLCCVP16 for c-Myb-VP16 (CCVP16) was constructed by cloning the VP16-containing insert of N-CCVP16, digested with *SfiI* and *HindIII* into YLCCC/*SfiI*-*HindIII* cut vector. The constitutive expression vector YLCC(d1-30)VP16 was constructed by cloning the N-terminal segment of the insert of N-d1-30CCVP16, digested with *KpnI* and *SphI*, into YLMV/*KpnI*-*SphI* cut vector. The constitutive expression vector YLdC VP16 was constructed in two steps. First, a *BstEII* fragment was exchanged from N-dCd to N-CVP16 (Dini and Lipsick, 1993) to obtain N-dCVP16. This newly obtained construct was then further digested with *KpnI* and *SphI* to clone the N-terminal portion of the insert into the vector YLMV, digested with *KpnI* and *SphI*.

The Gal4DBD-D1 bait plasmid, which contains a C-terminal fusion of the human cyclin D1 gene to the Gal4 DBD, was constructed by subcloning the PCR-amplified gene encoding cyclin D1 into *BamHI* and *PstI* digested pGBT9 vector (obtained from Stan Fields; Bartel *et al.*, 1993). The PCR primers that encompass the D1-encoding region are PCRp-humcyclinD1#3:

5'-AGAATTCTTGGATCCATGGAACACCAGCTCCTG-3'
(upstream primer with internal *BamHI* site)

and PCRp-humcyclinD1#4:

5'-GGAGTACGATGCATTTCAGATGTCCACGTCCTCCG-3'
(downstream primer with internal *Nsi*I site).

The animal cell reporter construct PolyA-EW5(-)Luc, which harbors a simian virus 40 poly(A) site and five *mim1-A* Myb binding sites upstream of an E1b TATA box and the *luciferase* gene has been described elsewhere (Fu and Lipsick, 1996). CMV-driven cyclin expression vectors were kindly provided by Phil Hinds (Hinds *et al.*, 1992, 1994).

Yeast growth and yeast reporter strains

The protocols for growth and maintenance of yeast strains were as described previously (Guthrie and Fink, 1991). The yeast two-hybrid reporter strain HF7c (*MATa ura3-52 his3-200 lys-801 ade2-101 trp1-901 leu2-3, 112 gal4-542 gal80-538 LYS::GAL1-HIS3 URA3::GAL4 17-mers3-CYC1-lacZ*) was obtained from D.Beach and has been described elsewhere (Feilott *et al.*, 1994). Yeast transformation was performed according to the modified lithium acetate method (Ito *et al.*, 1983; Schiestl and Gietz, 1989).

Cell lines/culture, transient DNA transfections, and β -galactosidase and luciferase assays

Quail QT6 fibroblasts. This chemically transformed cell line was grown in Dulbecco's modified essential Eagle's medium (DMEM) supplemented with 5% fetal calf serum, glucose (4.5 g/l), $1\times$ non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, streptomycin (100 μ g/ml) and penicillin (100 U/ml) in a humidified 10% CO₂-90% air incubator at 37°C.

Transient transfections into QT6 quail fibroblasts were performed by a modified calcium phosphate precipitation method (Chen and Okayama, 1987; Ibanez and Lipsick, 1990). Myb-expressing activator plasmids or control vector-only plasmid N-Cla (3 μ g), cyclin-expressing plasmids or control plasmid (vector only) (3 μ g), luciferase reporter plasmid (1 μ g), carrier tRNA (to give a total of 10 μ g of nucleic acid for transfection) and 0.5 μ g of internal control plasmid expressing β -galactosidase from the cytomegalovirus promoter (CMV- β -gal) were co-transfected into $\sim 10^6$ QT6 cells per 10 cm-diameter plate. Two days later, the cells from each transfection plate were scraped in phosphate-buffered saline (PBS). Half of these cells were used for the luciferase assay (de Wet *et al.*, 1987; Ausubel *et al.*, 1989) and β -galactosidase assay (Sambrook *et al.*, 1989); the other half was solubilized by boiling for 3 min in SDS-PAGE loading dyes and subjected to immunoblotting to demonstrate expression of Myb and/or cyclin proteins in transfected cells.

BM2 cells. The AMV-transformed chicken monoblast cell line (BM2) was maintained at 37°C in a humidified incubator containing 10% CO₂. These cells were grown in DMEM supplemented with 5% heat-inactivated chicken serum, 5% fetal calf serum, glucose (4.5 g/l), $1\times$ non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, streptomycin (100 μ g/ml) and penicillin (100 U/ml).

Transient transfections into BM2 cells were performed in the presence of lipofectin (Life Technologies) according to the protocol of the supplier. Basically, Myb-expressing activator plasmids (10 μ g), cyclin-expressing plasmids (10 μ g), luciferase reporter plasmid (5 μ g), carrier tRNA (to give a minimum total of 20 μ g transforming nucleic acid if needed) and 5 μ g of internal control plasmid expressing β -galactosidase from the cytomegalovirus promoter (CMV- β -gal) were co-transfected into $1-2\times 10^7$ BM2 cells per 10 cm-diameter plate. At 4 h after transfection, the cells were diluted into a 16 cm-diameter plate in a total of 40 ml of medium and induced to differentiate with 16 μ l of TPA at a final concentration of 400 ng/ml (or 16 μ l of DMSO in control plate). After 16 h the cells from each transfection plate were scraped in PBS. These cells were used for luciferase assay (Ausubel *et al.*, 1989; de Wet *et al.*, 1987) and β -galactosidase assay (Sambrook *et al.*, 1989).

Western blot analysis

QT6 and BM2 extracts were prepared by lysing cells in SDS loading buffer and boiling the lysate for 3 min. Normalized volumes of QT6 or BM2 lysates (based on internal β -Gal activity for transient transfections) were subjected to SDS-PAGE (10–15% polyacrylamide) and then the proteins were transferred to a nitrocellulose membrane (BA-S 83; Schleicher and Schuell). Immunoblot analysis was performed with anti-Myb [mouse monoclonal antibodies: Myb 5E, Myb2.2 and/or Myb2.7 (Evan *et al.*, 1984; Sleeman, 1993)], anti-cyclin D1 (mouse monoclonal HD11; Santa Cruz Biotechnology), or anti-cyclin D2 (rabbit polyclonal antibody; a gift from D.Parry) antibodies. Immunodetection was performed using an enhanced chemiluminescence system (Pierce).

Immunoprecipitation

BM2 or QT6 cells were washed twice with ice-cold PBS and incubated on ice for 40 min in IP-buffer A (250 mM NaCl/50 mM Na-HEPES, pH 7.0, 1% NP-40) in the presence of protease inhibitors (Boehringer-Mannheim). The extract was incubated on ice for 40 min before centrifugation at 4°C at 12 000 r.p.m. The supernatant was incubated with either anti-Myb antibody (monoclonal α -Myb 5E, specific against the Myb DBD, IgG2a), anti-D2 antiserum (polyclonal rabbit antiserum, a gift from D.Parry), anti- β -galactosidase antibody (monoclonal isotype control antibody from Promega, IgG2a), or preimmune rabbit serum on a rocker at 4°C for 1.5 h in IP-buffer A. After further incubating the antigen-antibody complexes for 45 min on the rocker with Protein G-Sepharose Fast Flow beads (Pharmacia) at 4°C, the complexes were washed three times with ice-cold IP-buffer A. The final complexes bound to Protein G-Sepharose beads were subjected to SDS-PAGE (10% SDS-polyacrylamide) and the proteins further analyzed by immunoblotting (see above).

Yeast two-hybrid assay

The bait molecule pGal4DBD-D1, which lacks transcriptional activity, was transformed together with different Myb-VP16-expressing constructs into the yeast strain HF7c. This yeast reporter host strain carries both a *HIS3* selectable marker and a *lacZ* reporter gene under control of Gal4p-binding sites. After transformation of the reporter strain with the described plasmids, the transformants were plated onto media lacking histidine. Transformants which grew on his⁻ plates, due to interaction of the bait protein with the Myb-VP16 fusion proteins, were further analyzed for β -gal activity. No growth was observed with the Myb-VP16 constructs or Gal4DBD-D1 constructs alone.

Electrophoretic mobility shift assay (EMSA)

The methods used are essentially as described elsewhere for other DNA-binding proteins (Treisman, 1986) and include separating free DNA from protein-complexed DNA on electrophoretic mobility shift gels. A 26-base oligonucleotide *mim1-A* (Ness *et al.*, 1989) probe (5'-TCGACA-CATTATAACGGTTTTTTAGC-3') was used. Double-stranded DNA probe with 4 nt overhangs was 3' end-labeled by end-filling with [α -³²P]dATP and Klenow enzyme (New England Biolabs). Binding reactions were carried out in a final volume of 10 μ l containing 20 mM Tris-Cl pH 7.5, 3 mM MgCl₂, 50 mM NaCl, 15% (v/v) glycerol, and 2 μ g poly [dl-dC], end-labeled DNA fragment and protein sample. Full length v-Myb protein, cyclin D1 and D2 were prepared by *in vitro* transcription/translation (Promega). 6 μ l of full-length v-Myb protein and 2–6 μ l of cyclin D1 or D2 protein were used per reaction. The binding mixture was incubated for 10 min on ice. Where indicated, 4 μ l antibody directed either against v-Myb, cyclin D1 or cyclin D2 was added to the reaction mixture for an additional 10 min. The protein-DNA complexes were separated on a 6% polyacrylamide (29:1 acrylamide:bisacrylamide) gel in 0.25 $\times$ TBE at 10 V/cm for 1 h 45 min, vacuum dried, and subjected to autoradiography.

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