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An alternative pathway for gene regulation by Myc

Karen Peukert, Peter Staller[1], Andreas Schneider[1], Gordon Carmichael[2], Frank Hänel[3] and Martin Eilers[1,2]

Hans-Knöll-Institut für Naturstoff-Forschung, Department of Cell and Molecular Biology, Beutenbergstrasse 11, 07745 Jena, [1]Zentrum für Molekulare Biologie Heidelberg, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany and [2]Department of Microbiology, University of Connecticut Health Center, Farmington, CT 06030-3205, USA

[3]Corresponding authors

K.Peukert and P.Staller contributed equally to this work

The c-Myc protein activates transcription as part of a heteromeric complex with Max. However, Myc-transformed cells are characterized by loss of expression of several genes, suggesting that Myc may also repress gene expression. Two-hybrid cloning identifies a novel POZ domain Zn finger protein (Miz-1; Myc-interacting Zn finger protein-1) that specifically interacts with Myc, but not with Max or USF. Miz-1 binds to start sites of the adenovirus major late and cyclin D1 promoters and activates transcription from both promoters. Miz-1 has a potent growth arrest function. Binding of Myc to Miz-1 requires the helix–loop–helix domain of Myc and a short amphipathic helix located in the carboxy-terminus of Miz-1. Expression of Myc inhibits transactivation, overcomes Miz-1-induced growth arrest and renders Miz-1 insoluble *in vivo*. These processes depend on Myc and Miz-1 association and on the integrity of the POZ domain of Miz-1, suggesting that Myc binding activates a latent inhibitory function of this domain. Fusion of a nuclear localization signal induces efficient nuclear transport of Miz-1 and impairs the ability of Myc to overcome transcriptional activation and growth arrest by Miz-1. Our data suggest a model for how gene repression by Myc may occur *in vivo*.

Keywords: gene repression/Miz-1/Myc/POZ domain

Introduction

c-*myc* was identified as the cellular homologue of the transforming oncogene of the avian retroviruses MC29, OK10, MH2 and FH3; it encodes a central regulator of mammalian cell proliferation and apoptosis (for review, see Henriksson and Lüscher, 1996).

Myc protein is a helix–loop–helix/leucine zipper (HLH/LZ) protein that binds specifically to DNA and recognizes CAC(A/G)TG elements (Blackwell *et al.*, 1990; Prendergast and Ziff, 1991). *In vivo*, Myc forms heterodimers with a second HLH/LZ protein termed Max (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991). Binding to Max is a prerequisite for transformation by Myc (Amati *et al.*, 1993b). Further proteins that compete with Myc for dimerization with Max have been identified and termed Mad, Mnt or Mxi proteins (Ayer *et al.*, 1993; Zervos *et al.*, 1993; Hurlin *et al.*, 1996). The Myc–Max complex is a potent activator of transcription due to activation domains localized in the amino-terminus of Myc (Kato *et al.*, 1990; Kretzner *et al.*, 1992). Several genes have been identified that are regulated by Myc–Max complexes *in vivo* (for review, see Henriksson and Lüscher, 1996).

Cells transformed by constitutive expression of Myc are characterized by the loss of expression of numerous genes, suggesting that Myc may also exert a negative function on gene expression. Genes that are repressed in Myc-transformed cells encode, for example, cell surface proteins involved in cell adhesion (e.g. Judware and Culp, 1995) and interaction of cells with the immune system (e.g. Bernards *et al.*, 1986; Versteeg *et al.*, 1988; Inghirami *et al.*, 1990), and cell cycle regulators like c/EBP-α (Freytag and Geddes, 1992), cyclin D1 (Philipp *et al.*, 1994) and c-*myc* itself (Penn *et al.*, 1990). The findings suggest that gene repression contributes significantly to the phenotype of Myc-transformed cells.

Loss of expression of certain genes may be an indirect consequence of the transformed state and thus be an indirect consequence of transcriptional activation by the Myc–Max complex. However, several observations indicate that this is not the case. First, for both the adenovirus major late (AdML) and the c/EBP-α promoter, specific sequence determinants have been identified that are required for repression by Myc. In both cases, these elements are localized close to the start site of transcription and differ from the E-box elements recognized by Myc–Max complexes (Li *et al.*, 1994). Second, an analysis of mutant alleles of Myc for repression of cyclin D1 expression *in vivo* identified alleles of Myc that were capable of repression, but not transactivation; some of these alleles mapped to the leucine zipper of Myc, suggesting that interactions of this domain were critical for transactivation by Myc (via interaction with Max), but not transrepression (Philipp *et al.*, 1994). Third, detailed time course experiments revealed that repression of the endogenous c-*myc* gene after induction of conditional alleles of Myc is an early response and precedes the appearance of the transformed phenotype (Facchini *et al.*, 1997).

These observations prompted us to search for proteins that may mediate gene repression by Myc. We now report the identification of one such protein by two-hybrid cloning. We call this protein Miz-1 (for Myc-interacting zinc finger protein). Miz-1 is a zinc finger/POZ (BTB) domain protein (Bardwell and Treisman, 1994; Albagli *et al.*, 1995; Chen *et al.*, 1995). In contrast to other POZ domain proteins, Miz-1 is a soluble protein that binds to and transactivates the AdML and cyclin D1 promoters.

Association with Myc induces nuclear sequestration and renders Miz-1 insoluble, characteristic features of POZ domain proteins; a mutant of Miz-1 that lacks the POZ domain is largely unaffected by Myc. The data suggest a model in which Myc inhibits gene transcription by inducing the inhibitory functions of the Miz-1 POZ domain.

Results

Previous work had shown that the integrity of the HLH domain of Myc was critical for gene repression by Myc in stable cell lines (Philipp *et al.*, 1994) and in transient transfection assays (A.Schneider, unpublished; see below). To identify novel proteins that interact with the carboxy-terminus of Myc, a DNA fragment encoding the basic region and the HLH/LZ domain (amino acids 355–439 of human Myc) was fused in-frame to the DNA-binding domain of GAL4 (amino acids 1–147) and used as a bait in a two-hybrid screen (Fields and Song, 1989). A total of 2×10^5 independent transformants of a HeLa cell cDNA library tagged with the GAL4 activation domain were screened. One clone that conferred β-galactosidase activity was characterized further (Figure 1A). No interaction was detected between the protein encoded by this clone and either the DNA-binding domain of GAL4 alone or a GAL4–BCY-1 chimera used as a negative control. Interaction with Myc was abolished by deletion of the HLH domain (Δ370–412) in Myc, but not by the insertion of four amino acids between the HLH domain and the leucine zipper (In412) or by the deletion of the entire leucine zipper (Δ412–434). A specific interaction was also detected with N-Myc, but not with Max or USF, two HLH proteins closely related to Myc (Figure 1B). We concluded that we had identified a protein that specifically interacts with the HLH domain of both c- and N-Myc.

Full-length cDNA molecules were isolated using a 5′-RACE protocol and sequenced; they encode a protein of 803 amino acids with a predicted mol. wt of 87 970 Da (Figure 1C). Sequencing revealed that the clone we had isolated encoded a zinc finger protein with 13 zinc fingers, 12 of which are immediately clustered in the carboxy-terminal half of the protein. We will refer to this protein as Miz-1 (Myc-interacting zinc finger protein). At its amino-terminus, Miz-1 carries a BTB/POZ domain, which has been identified as a negative regulatory domain (see below) (Bardwell and Treisman, 1994). A closely related murine cDNA has been isolated (Schulz *et al.*, 1995); the encoded mouse and human proteins are 92% identical over their entire length. The clone obtained in the two-hybrid screens encodes amino acids 269–803 of the full-length Miz-1 protein. A series of deletion mutants was used to localize further the domain of Miz-1 that interacts with Myc (Figure 1D). The results define two regions flanking the central 12 zinc fingers of Miz-1 that are required for interaction with Myc, as deletion of either amino acids 269–308 or 637–718 of Miz-1 abolishes interaction in the two-hybrid assay.

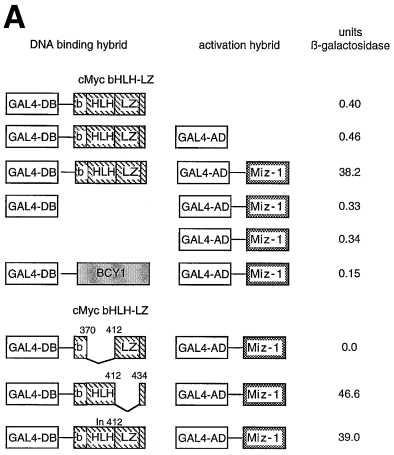
Structure prediction programs predict that amino acids located between zinc fingers 12 and 13 of Miz-1 have a high propensity to form an amphipathic α-helix (see Figure 1E) (Lupas *et al.*, 1991). As the HLH motif domain has a similar structure (Ferré D'Amaré *et al.*, 1993), it seemed conceivable that both domains interact with each other. To test this prediction, we deleted amino acids 641–715 of Miz-1; this deletion abolished interaction with Myc in the two-hybrid assay (Figure 1E). A smaller deletion of amino acids 683–715 strongly inhibited association of Miz-1 with Myc. To test whether these amino acids interact with Myc as part of a helical structure, we constructed two further mutants of Miz-1: one in which five hydrophobic amino acids within the putative helical segment were replaced either by prolines or by a glycine (L695P, I699P, A702P, V703G, V706P; designated '4 Pro') and a second mutant in which Ile699 was replaced by proline and Ser700 by glycine (I699P, S700G; designated '1 Pro'). The '4 Pro' mutant of Miz-1 did not interact with Myc in the two-hybrid assay; interaction between the '1 Pro' mutant of Miz-1 and Myc was reduced >10-fold relative to wild-type Miz-1 (Figure 1E). Taken together, the data show that amino acids located between zinc fingers 12 and 13 of Miz-1 are required for interaction with Myc in the two-hybrid assay and suggest that they interact as part of an α-helix.

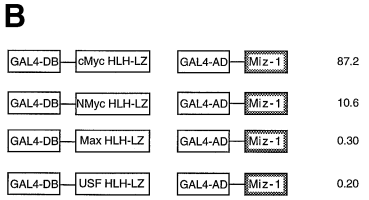
Two experiments were designed to confirm the specific association between Miz-1 and Myc. First, amino acids 269–803 of Miz-1 were fused to glutathione-*S*-transferase (GST) and the GST–Miz-1 fusion protein was purified and incubated with *in vitro* synthesized, radiolabelled Myc protein (Figure 2A). Myc associated with GST–Miz-1 but not GST; a mutant allele of Myc lacking the HLH domain bound significantly less well to GST–Miz-1, although a low level of residual binding was detectable *in vitro*. Radiolabelled Max interacted neither with GST–Miz-1 nor with GST.

To show further that Myc and Miz-1 interact *in vivo*, a polyclonal antibody was raised against a His-tagged protein encompassing amino acids 269–803 of Miz-1. This antibody specifically recognized recombinant Miz-1 in *Escherichia coli* extracts (data not shown), full-length Miz-1 after expression in HeLa cells (Figure 6A) and a protein of the expected molecular weight in extracts of HeLa cells (see Figure 7A). HeLa cells were co-transfected with expression vectors encoding either Myc or full-length Miz-1. Lysates were prepared by sonication in buffer containing non-ionic detergents, clarified by centrifugation and pre-absorption to protein G–Sepharose and precipitated with either pre-immune or anti-Miz-1 antibody. Precipitates were washed extensively, separated by SDS–PAGE, blotted and probed with a monoclonal antibody directed against human Myc. Myc was detected in anti-Miz-1, but not control immunoprecipitates (Figure 2B). In the inverse experiment, polyclonal antibodies against Miz-1 detected the protein in precipitates with an anti-Myc monoclonal antibody, but not in control precipitates with the same amount of an irrelevant control antibody (Figure 2B). We concluded that Myc and Miz-1 associate *in vivo*. In these experiments, ~2% of the transfected Miz-1 was found complexed with Myc; however, the experiment shown in Figure 6A demonstrates that the low salt conditions required for immune precipitation strongly favour solubilization of free Miz-1, as the Miz–Myc complex is poorly soluble in low salt buffers. Data shown in Figure 6 further suggest that most Miz-1 protein expressed in cells associates with Myc under the experimental conditions. The low abundance of both proteins and the inability to detect Miz-1 by labelling with [^{35}S]-

methionine or [^{32}P]phosphate in non-transfected cells have so far precluded attempts to demonstrate association of the endogenous proteins.

The murine homologue of Miz-1 has been identified independently in a screen for proteins that bind to the major start site of the TATA-less polyoma virus major late promoter (L.Rapp and G.Carmichael, submitted). To test whether Miz-1 interacts with related sequences at the





C

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GGGCTTCTCTGTGACTGCACCTTTGTGGTGGACGGTGTTCACTTTAAGGCTCATAAAGCA 40
Gly Leu Leu Cys Asp Cys Thr Phe Val Val Asp Gly Val His Phe Lys Ala His Lys Ala

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Val Leu Ala Ala Cys Ser Glu Tyr Phe Lys Met Leu Phe Val Asp Gln Lys Asp Val Val

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His Leu Asp Ile Ser Asn Ala Ala Gly Leu Gly Gln Met Leu Glu Phe Met Tyr Thr Ala

AAGCTGAGCCTGAGCCCTGAGAACGTGGATGATGTGCTGGCCGTGGCCACTTTCCTCCAA 100
Lys Leu Ser Leu Ser Pro Glu Asn Val Asp Asp Val Leu Ala Val Ala Thr Phe Leu Gln

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Pro Gly Gly Asn Ala Glu Ala Leu Ala Thr Glu Gly Gly Asp Lys Arg Ala Lys Glu Glu

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GACAACATCCGCCCACACAAGTGCAGCGTGTGCAGCAAGGCCTTCGTGAACGTGGGGGAC 600
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GGGGCTGAGGGCCAGCCCGCACTGGCAGAGACCTCCCCTACAGCTCCTGAATGTCCCCCG 800
Gly Ala Glu Gly Gln Pro Ala Leu Ala Glu Thr Ser Pro Thr Ala Pro Glu Cys Pro Pro

CCTGCCGAGTGA 803
Pro Ala Glu •
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start site of the AdML promoter, electrophoretic mobility shift experiments were performed with purified recombinant Miz-1 and ^{32}P-labelled oligonucleotides spanning the polyoma virus major late promoter start site (Figure 3A). Binding of recombinant Miz-1 was easily detectable; supershifting with specific antibodies confirmed that the shifted band contained Miz-1 protein. Binding of Miz-1 was specific as it was competed by a 100-fold excess of an oligonucleotide spanning the polyoma major late promoter transcription start site, but not by a mutated, non-functional derivative (L.Rapp and G.Carmichael, submitted). Binding was also competed by a similar molar excess of oligonucleotides derived from the AdML start site, but not by a mutated derivative (for sequences, see Materials and methods). In similar experiments, a specific interaction of Miz-1 with the major start site of the human cyclin D1 promoter was detected (see below). No interaction of Miz-1 was detected with the E-box element of the prothymosin-α intron, which is a target for transactivation by Myc (Desbarats *et al.*, 1996).

To test whether Miz-1 affects expression of the AdML promoter, transient transfection experiments were performed in which increasing amounts of cytomegalovirus (CMV)-Miz-1 plasmid were co-transfected with an AdML reporter plasmid into HeLa cells (Figure 3B). Sequences contained in this reporter span from –45 to +65 nucleotides relative to the major start site and do not encompass the E-box element located 60 nucleotides upstream of the major start site. As a control for transfection efficiency, a CMV-lacZ plasmid was co-transfected. Expression of Miz-1 transactivated the AdML promoter, and maximal activation was 10-fold relative to the CMV-lacZ control. Similar results were obtained in QT6 cells (not shown). A derivative of the AdML promoter which contains a mutated start site (kind gift of B.Roeder) was poorly transactivated by Miz-1, suggesting that binding of Miz-1 to the AdML promoter is required for activation (Figure 3B). Also, a mutant of Miz-1 that lacked zinc fingers 1–12 (Δ309–636) activated the AdML promoter inefficiently, further suggesting that DNA binding by Miz-1 is required for full activation (Figure 3C).

We wondered whether Myc affected transactivation by Miz-1 and repeated these experiments in the presence of a constant amount of a CMV-Myc expression vector. Expression of Myc did not interfere with the basal activity of the AdML promoter in HeLa cells (Figure 4A) nor with expression of Miz-1 (data not shown). However, expression of Myc significantly impaired transactivation of the AdML promoter by Miz-1 (Figure 4A).

Two controls showed that complex formation between Miz-1 and Myc was required for inhibition of transactivation. First, a mutant allele of Myc that lacks the HLH domain and failed to interact with Miz-1 also failed to inhibit Miz-1 function although it was expressed at the same level as the wild-type protein (Figure 4A). Transactivation by Miz-1 was abolished by expression of In412Myc, a mutant of Myc that fails to bind Max and to transform cells yet interacts with Miz-1 (see Figure 1A) and is capable of repression *in vivo* (Philipp *et al.*, 1994); a complete deletion of the leucine zipper of Myc (Δ412–434) had a partial effect on inhibition (data not shown). Second, transactivation of the AdML promoter by a deletion mutant of Miz-1 (Δ641–715) that failed to interact with Myc in the two-hybrid assay was resistant to inhibition by Myc (Figure 4B). The data show that complex formation between Miz-1 and Myc is required for inhibition of transactivation by Miz-1.

To test whether binding of Myc to Miz-1 was not only necessary, but also sufficient for inhibition, we expressed the GAL4–Myc(355–439) fusion protein used as a bait in

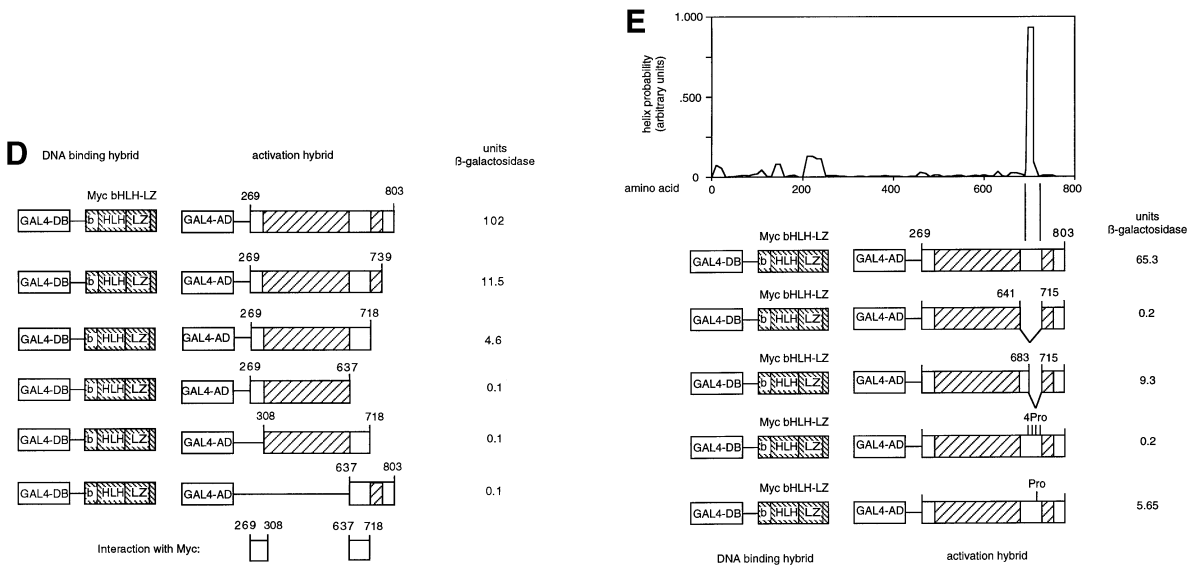

Fig. 1. Isolation and sequence of Miz-1. (**A**) Miz-1 interacts with the HLH domain of Myc in a yeast two-hybrid system. The left column shows the different GAL4 baits that were used, the middle column shows the Miz-1 clone that was recovered. It corresponds to amino acids 269–803 of the human Miz-1 sequence. The right column indicates the specific galactosidase activity of each transformed strain. (**B**) Miz-1 interacts specifically with c-Myc and N-Myc, but not with Max or USF. (**C**) Sequence of the full-length Miz-1 cDNA. Arrows indicate the POZ/BTB domain located at the amino-terminus; the predicted zinc fingers are numbered and underlined. (**D**) Myc interaction domains in the carboxy-terminus of Miz-1. The numbers indicate the amino acids of Miz-1 retained in each construct. The stripes indicate the cluster of 12 zinc fingers and the isolated thirteenth zinc finger, respectively. (**E**) A putative α-helix in Miz-1 is required for interaction with Myc. Top: prediction of coiled-coil elements in Miz-1 (Lupas *et al.*, 1991). Bottom: two-hybrid assays with the indicated mutant alleles of Miz-1. Mutations are described in the text.

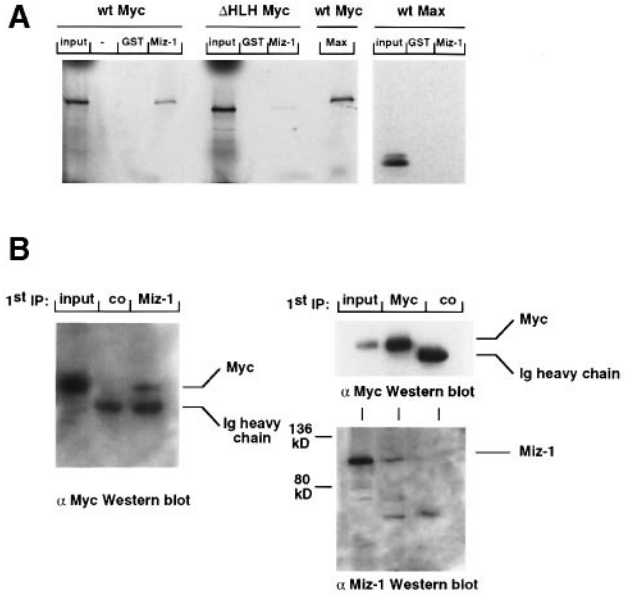

Fig. 2. *In vitro* and *in vivo* interaction between Miz-1 and Myc. (**A**) Miz-1 and Myc specifically associate via the HLH domain *in vitro*. ^{35}S-labelled, *in vitro*-synthesized Myc, MycΔHLH and Max proteins were incubated with GST alone or with equal amounts of a GST–Miz-1(269–803) fusion protein. Beads were washed extensively and the recovered material separated by SDS–PAGE and visualized by fluorography. The 'input' corresponds to 20% of the loaded material. (**B**) *In vivo* interaction. Miz-1 and Myc were expressed by transient transfection in HeLa cells. Lysates were prepared and immunoprecipitations carried out with the indicated antibodies. Left: shown is an α-Myc Western blot of immunoprecipitates with either pre-immune or anti-Miz-1 antibodies. Right, top: shown is an anti-Myc Western blot of immunoprecipitates with either a control or an anti-Myc antibody. Bottom: the same gel was re-probed with an anti-Miz-1 antibody. The 'input' corresponds to 10% of the material present in the lysates.

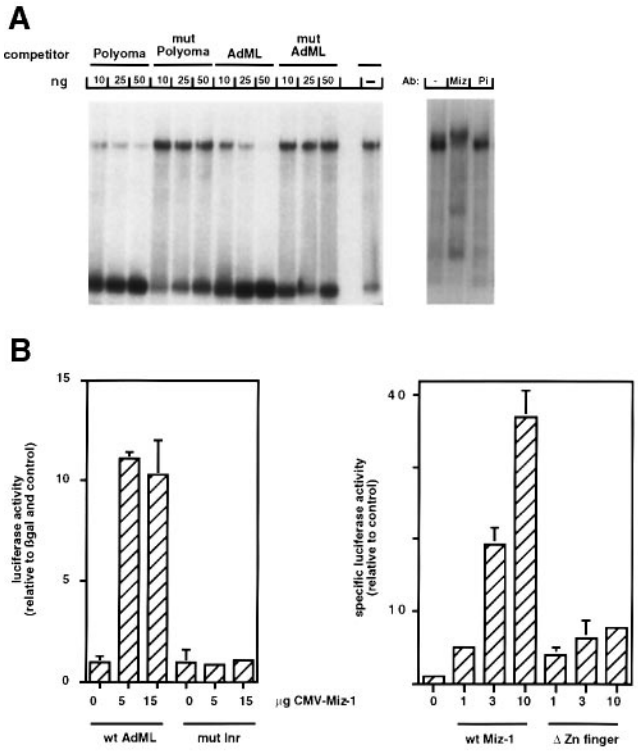

Fig. 3. Miz-1 binds to and activates the AdML core promoter. (**A**) EMSA containing recombinant, purified Miz-1 protein and ^{32}P-labelled oligonucleotides spanning the major start sites of the polyoma virus late promoter. Left: shown is competition by either polyoma late promoter oligonucleotides, a mutated derivative of the promoter, oligonucleotides spanning the start site of the AdML promoter or a mutated derivative. Right: shown are shifts obtained after incubation of recombinant Miz-1 with either pre-immune (Pi) or anti-Miz-1 antibody. (**B**) Transactivation of the AdML promoter by Miz-1. Shown are the results of transient transfection experiments with the indicated amount of a CMV-Miz-1 expression plasmid and a reporter with either the wild-type AdML core promoter or a mutated derivative driving a luciferase reporter gene. The results are plotted relative to a co-transfected CMV-lacZ expression plasmid. (**C**) Deletion of zinc fingers (1–12) of Miz-1 inhibits its ability to transactivate the AdML promoter. The results show the specific luciferase activity relative to a control.

the two-hybrid assay (Figure 4C). GAL4–Myc(355–439) efficiently inhibited activation by wild-type Miz-1. Thus, the domain of Myc that interacts with Miz-1 is both necessary and sufficient to inhibit Miz-1 function. The GAL4–Myc(355–439) protein lacks the transactivating domain of Myc, precluding the possibility that inhibition of transactivation is due to squelching of the transactivation domain of Miz-1. To support this notion further, we fused the potent transactivation domain of the viral transactivator VP16 to Miz-1. VP16–Miz-1 chimeras strongly transactivated the AdML promoter and transactivation was sensitive to inhibition by GAL–Myc (Figure 4B). The data show that Myc does not inhibit Miz-1 function by squelching its transactivation domain.

Surprisingly, both Miz-1ΔPOZ and a VP16–Miz-1ΔPOZ chimera that lack the amino-terminal POZ domain of Miz-1 were poorly inhibited by expression of GAL–Myc (Figure 4C), although transactivation by either protein in the absence of Myc was less potent than that of the corresponding wild-type allele of Miz-1. The POZ domain is not required for interaction with Myc either in the two-hybrid assay or *in vitro*. Thus, association between Miz-1 and Myc is required, but not sufficient for full inhibition of Miz-1 function. In addition, inhibition of Miz-1 function by Myc requires the integrity of the amino-terminal POZ domain of Miz-1.

In vivo, cells transformed by Myc contain high amounts of both USF and Max, HLH/LZ proteins that are closely related to Myc (Littlewood *et al.*, 1992). In contrast to Myc, USF activates transcription of the AdML, c/EBPα and cyclin D1 promoters (Phelps *et al.*, 1988; Du *et al.*, 1993; Li *et al.*, 1994; Philipp *et al.*, 1994). To test whether USF affected transactivation by Miz-1, transient transfection experiments were performed as before with a CMV-USF expression vector. Both USF and Miz-1 transactivated the AdML promoter; together, there was an additive effect on the AdML promoter and no sign of either mutual inhibition or synergistic activation could be detected (Figure 4D). This is consistent with the lack of interaction between USF and Miz-1 in the yeast two-hybrid assay (Figure 1B). Similarly, Max failed to interact with Miz-1 in two-hybrid assays. By itself, Max did not affect expression of the AdML promoter and did not affect transactivation of the AdML promoter by Miz-1 (Figure 4D). We concluded from these experiments that interaction with and inhibition of Miz-1 is specific for Myc.

Both Max and Miz-1 interact with the HLH domain of Myc; thus, Max might compete with Miz-1 for complex formation with Myc. Two experiments were set up to

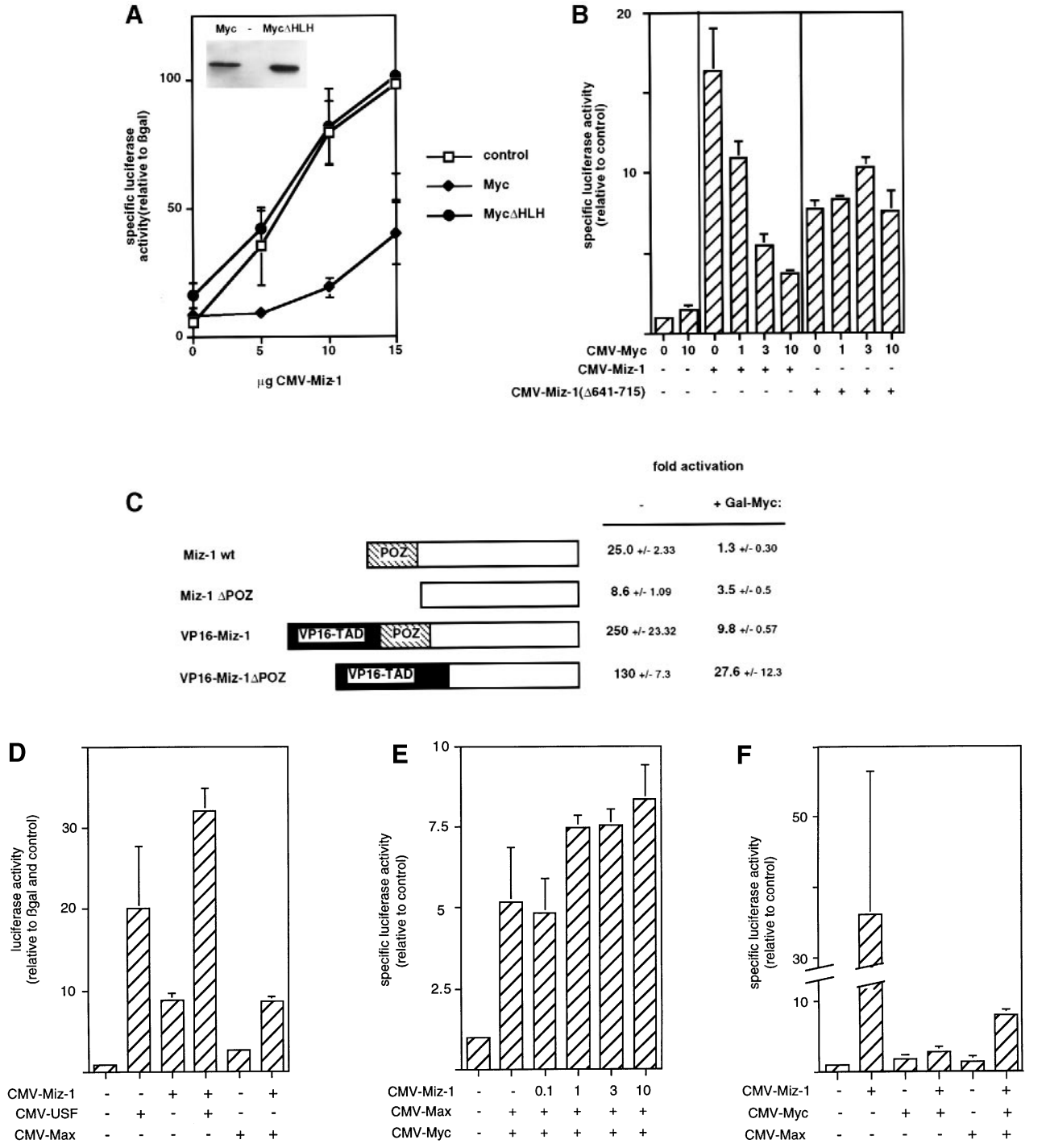

Fig. 4. Association with Myc inhibits transactivation by Miz-1. (**A**) HeLa cells were transfected with increasing amounts of a CMV-Miz-1 expression vector in the presence of a constant amount of either CMV-Myc or CMV-MycΔHLH expression vectors (5 μg each) as indicated. The insert shows a Western blot documenting expression of either wild-type Myc or MycΔHLH. (**B**) A mutant of Miz-1 that fails to interact with Myc in the two-hybrid assay is resistant to inhibition by Myc. Shown is the fold activation relative to control by either wtMiz1 or Miz-1(Δ641–715) of an AdML reporter plasmid in the presence of increasing amounts of CMV-Myc. (**C**) Inhibition of different alleles of Miz-1 by a GAL–Myc(355–439) fusion protein. Shown is the fold activation relative to control of a co-transfected AdML reporter plasmid (as in A) with the indicated combination of effector plasmids. (**D**) Neither USF nor Max inhibit transactivation by Miz-1. HeLa cells were transfected as above. The graph shows the fold activation relative to control and a co-transfected CMV-βgal standard. (**E**) Ectopic expression of Miz-1 does not inhibit activation of prothymosin-α by Myc–Max heterodimers. The graph shows the fold activation relative to control of a prothymosin-α reporter by expression of Myc and Max in the presence of increasing amounts of Miz-1. (**F**) Expression of Max partly relieves Myc-mediated inhibition of Miz-1 function. In the presence of Max and Myc, Miz-1 activated 8.1 ± 0.9-fold versus 2.8 ± 0.8 in the presence of Myc alone.

address this question *in vivo*. First, we tested whether ectopic expression of Miz-1 influenced transactivation by Myc and Max from an E-box–TATA construct; to do this, we made use of the observation that Myc and Max synergistically activate the prothymosin-α enhancer (Desbarats *et al.*, 1996). We observed a 5-fold activation

of a ProT-Luc reporter plasmid by Myc and Max in the absence of Miz-1; including up to 10 μg of CMV-Miz-1 vector did not affect activation by Myc and Max (Figure 4E). We concluded that either formation of a ternary Myc–Miz-1–Max complex is possible or that the affinity of Miz-1 for Myc is not high enough to disrupt a Myc–Max complex under the experimental conditions. To test the latter possibility, we analysed the effect of Max on the inhibition of Miz-1 function by Myc. We observed that Max partly reversed Myc-mediated inhibition of Miz-1 (Figure 4F), suggesting that Max and Miz-1 may form alternate complexes with Myc *in vivo*. This is also suggested by data on the intracellular localization of Miz-1 (see Figure 6).

In order to identify potential cellular target genes for Miz-1 transactivation, we turned to the cyclin D1 promoter which is repressed by Myc in a Max-independent fashion (Philipp *et al.*, 1994). The cyclin D1 promoter contains four E-boxes upstream of the TATA-less start site and might thus be a target for both activation and repression by Myc (see Figure 5B). Indeed, the response in cyclin D1 mRNA levels to ectopic expression of Myc is strongly affected by the genetic background of a cell (Marhin *et al.*, 1996).

Gel-shift assays showed that Miz-1 specifically interacts with the major start site of the human cyclin D1 promoter (Herber *et al.*, 1994), but not with a minor start site located 80 bp further upstream (Philipp *et al.*, 1994) (Figure 5A). For transient transfections, a cyclin D1 reporter plasmid containing both start sites and the upstream E-box elements was used as reporter (Figure 5B). In the absence of Miz-1, Myc transactivated expression of cyclin D1 at low levels of expression and inhibited at higher expression levels (Figure 5C); similar responses have been reported for the full-length AdML promoter, which also contains both an E-box and an Inr element (Li *et al.*, 1994). Ectopic expression of Miz-1 activated the reporter 4-fold (not shown). Very little activation of the cyclin D1 promoter by Myc was observed in the presence of Miz-1; instead, repression was much more pronounced (Figure 5C). Thus, Miz-1 affects the response of the cyclin D1 promoter to Myc and may be one of the host factors that determine how a cell responds to ectopic expression of Myc.

How does Myc inhibit the function of Miz-1? Repression of Miz-1 transactivation by Myc requires the integrity of the POZ domain, suggesting that inhibition is not due solely to association of both proteins and steric blockage of Miz-1 function by Myc (e.g. by interference with DNA binding). The POZ domain of several transcription factors has been shown to target the protein to discrete subnuclear foci and to inhibit DNA binding of the attached zinc fingers and transcriptional activation *in vivo* (e.g. Bardwell and Treisman, 1994; Dhordain *et al.*, 1995). POZ domain proteins are usually insoluble under conditions that are used to solubilize transcription factors. This is also true for the endogenous proteins, demonstrating that it is not an artefact caused by overexpression (Dhordain *et al.*, 1995); therefore, the POZ domain appears to act as a negative regulatory domain for transcription factor function.

Two experiments were set up to test whether this applies to Miz-1. First, we fractionated transfected HeLa cells

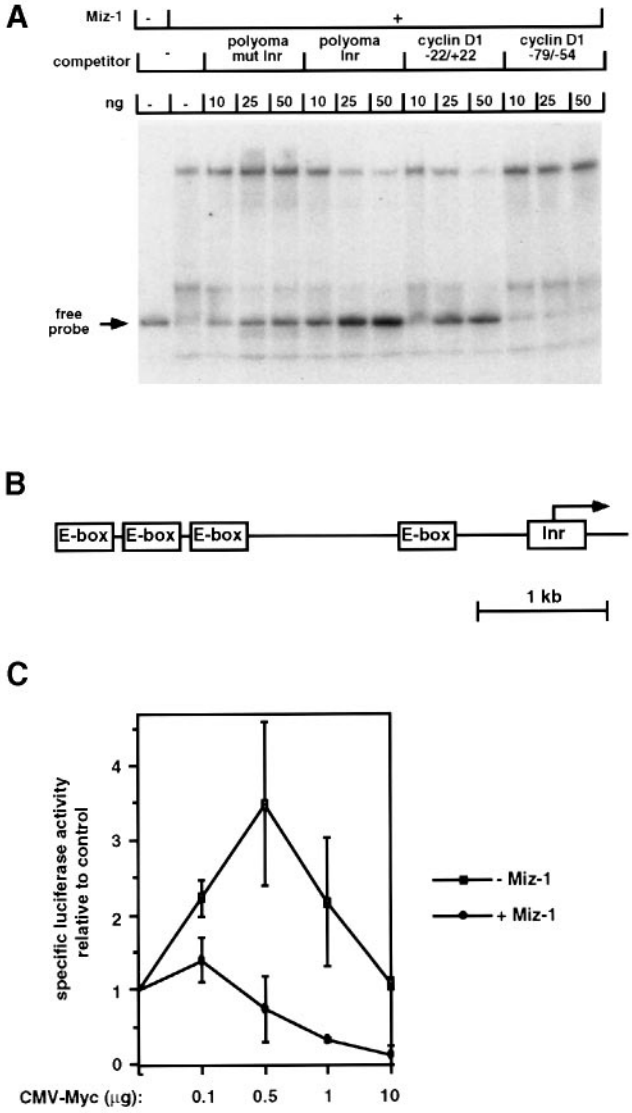

Fig. 5. Co-ordinate regulation of the cyclin D1 promoter by Myc and Miz-1. (**A**) Miz-1 interacts specifically with the major start site of the cyclin D1 promoter. Shown are the results from an electrophoretic shift experiment using oligonucleotides surrounding the major start site of the human cyclin D1 promoter as a probe. Competing oligonucleotides are indicated above each lane. Nucleotides –79/–54 span a minor start site of the human cyclin D1 promoter (Philipp *et al.*, 1994). (**B**) Structure of the cyclin D1 promoter; the positions of the upstream E-boxes and the major start site (Herber *et al.*, 1994) are indicated. (**C**) Miz-1 affects the response of the cyclin D1 promoter to ectopic expression of Myc. Shown are the results from transient transfection assays in HeLa cells with increasing amounts of a CMV-Myc expression vector either in the absence or presence of a constant amount of CMV-Miz-1. Promoter activity in the absence of Myc is arbitrarily set to one.

and found that ~20% of nuclear Miz-1 protein was soluble in buffer containing 200 mM NaCl, and >80% soluble in the presence of 420 mM NaCl (Figure 6A). Second, immunofluorescence of transfected HeLa cells showed a homogeneous staining of Miz-1 within the nuclei, with no sign of aggregation or clustering (Figure 6B). Thus, either the physical properties of the Miz-1 POZ domain differ from those of other POZ domains or its activity is masked within the context of the whole protein.

We wondered whether association with Myc affected

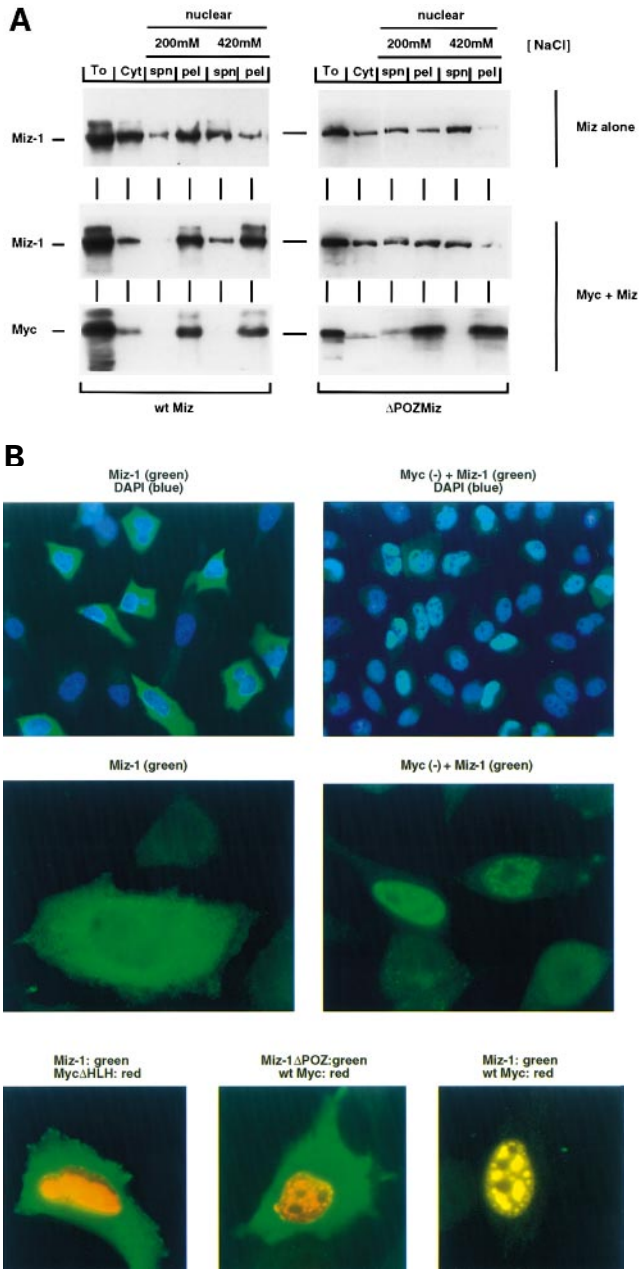

Fig. 6. Myc sequesters Miz-1 *in vivo*. (**A**) HeLa cells expressing Miz-1 or Miz-1ΔPOZ either alone or together with Myc were fractionated into cytosol and nuclei. Nuclei were extracted in the presence of either 200 or 420 mM NaCl as indicated and separated into soluble and insoluble material. Shown are Western blots documenting the distribution of Myc, Miz-1 or Miz-1ΔPOZ in equal aliquots of each fraction. (**B**) Shown are immunofluorescence photomicrographs documenting the localization of wild-type Miz-1 in the absence or presence of co-transfected Myc. Top: cells were counterstained with DAPI to visualize nuclei. Middle: individual cells after expression of Miz-1 either in the presence or absence of Myc. Bottom: shown are immunofluorescence micrographs of representative cells after expression of the indicated proteins. The red colour documents localization of Myc, the green colour documents expression of Miz-1 in the same cell. (**C**) Quantitation of the results. Shown is the percentage of cells with a predominant cytosolic signal for either Miz-1 (left panel) or Myc, Max and GAL–Myc(355–439) as indicated (right panel). The co-transfected expression plasmids are indicated below the panels; the protein stained for is indicated below the horizontal bar.

this behaviour, and thus we repeated fractionation and immunofluorescence in the presence of Myc. Upon co-expression of Myc, Miz-1 protein was completely insoluble at 200 mM and >90% insoluble at 420 mM NaCl (Figure 6A). Also, Miz-1 localized in a number of discrete, subnuclear foci identical to what is observed for other POZ/BTB domain proteins (Figure 6B). Judged by 4′,6′-diamidino-2-phenylindole (DAPI) staining, these foci are free of DNA. Co-staining with antibodies directed against Myc showed co-localization of Myc and Miz-1 within the nucleus (Figure 6B). This ‘sequestration’ of Miz-1 depended on the HLH domain of Myc, demonstrating that Myc needs to interact with Miz-1 to induce sequestration (Figure 6B).

Myc proteins previously have been shown to aggregate easily upon extraction (e.g. Evan and Hancock, 1985); thus Miz-1 might be dragged into such complexes in a non-specific manner. To exclude this possibility, we expressed the Miz-1ΔPOZ mutant either alone or together with Myc. Upon fractionation, Miz-1ΔPOZ was fully soluble at 420 mM salt both in the presence and absence of Myc (Figure 6A); immunofluorescence experiments showed no sign of co-aggregation of Myc and Miz-1ΔPOZ (Figure 6B).

The data show that complex formation with Myc alters the physical properties of Miz-1 *in vivo* and renders the protein insoluble in the nucleus. Like inhibition, sequestration depends on association of Myc with Miz-1 and requires the integrity of the Miz-1 POZ domain. Most probably, therefore, sequestration of Miz-1 by Myc accounts for the functional inhibition of transactivation, and association with Myc induces the normally latent activity of the Miz-1 POZ domain. The data also suggest that most Miz-1 protein associates with Myc in these cells.

It is apparent from the data that only a fraction of Miz-1 was translocated into the nucleus in the absence of Myc,

whereas all Miz-1 was detected in the nucleus in the presence of Myc (Figure 6A and B; a quantitation is shown in C). In the absence of Myc, a significant proportion of cells showed strong cytosolic staining, with only some protein localized in the cell nucleus; this was even more pronounced for a mutant Miz-1 protein lacking the POZ domain. In the presence of Myc, most cells expressing Miz-1 showed an exclusively nuclear staining of Miz-1. Expression of Myc did not affect the subcellular localization of mutants of Miz-1 that lack the POZ domain; indeed, such mutants acted as partial dominant-negative alleles for nuclear import of Myc and led to a retention of Myc protein in the cytosol (Figure 6C). Expression of Max increased the frequency of cytosolic wild-type Miz-1, supporting the notion that Max and Miz-1 compete for binding to Myc (Figure 6C). The data suggest that Myc and Miz-1 proteins are co-imported into the nucleus under the experimental conditions.

Why is nuclear import of Miz-1 inefficient in the absence of Myc? It is possible that this is an artefact due to the high expression levels achieved upon transient transfection. To exclude this possibility, we fractionated non-transfected HeLa cells and determined the distribution of endogenous Miz-1 by Western blotting (Figure 7A). Most of the endogenous Miz-1 co-fractionated with the cytosolic marker protein, β-tubulin, similar to what was found for the transfected protein. A small amount was contained in the nucleus and was insoluble in the presence of 420 mM NaCl, suggesting that it may be sequestered by Myc. Thus both endogenous and transfected proteins show a similar subcellular distribution.

Next, we tested whether Miz-1 contains a nuclear localization signal (NLS). Inspection of the Miz-1 sequence revealed a single weak homology to a consensus NLS, located at amino acids 370–374. However, fusion of amino acids 360–380 to green fluorescent protein failed to target the protein to the nucleus, suggesting that Miz-1 may lack an NLS (data not shown). To test this notion directly, we fused the SV40 large-T NLS to the amino-terminus of Miz-1 and found that the resulting chimera (NLS–Miz-1) was localized exclusively in the nucleus even in the absence of co-expressed Myc (Figure 7B). Most probably, therefore, Miz-1 lacks a functional NLS.

We hypothesized that the lack of an NLS might serve to limit the amount of Miz-1 in the nucleus and might thus lower the amount of Myc required to inhibit Miz-1 *in vivo*. To support this hypothesis, we tested whether Myc was able to inhibit transactivation by NLS–Miz-1 (Figure 7B). As before, ectopic expression of Myc efficiently inhibited transactivation by wild-type Miz-1 protein. In contrast, Myc was inefficient at inhibiting transactivation by NLS–Miz-1 (Figure 7B). Titration experiments showed that this lack of inhibition was not due to a failure of Myc to interact with NLS–Miz-1: when low amounts of Miz-1 or NLS–Miz-1 were used to activate transcription, expression of high amounts of Myc could indeed inhibit transactivation by both Miz-1 and NLS–Miz-1 (Figure 7B).

To gain insight into the biological function of Miz-1, we expressed the protein together with a hygromycin resistance plasmid by transient transfection in both NIH 3T3 and HeLa cells. After selection, the number of resistant colonies was determined (Figure 8A). Expression of Miz-1 strongly inhibited colony formation of both cell lines, suggesting that Miz-1 inhibits cell growth. Similarly, no colonies could be established after infection of either RAT1A (not shown) or Balb/c-3T3 cells (Figure 8E) with a recombinant retrovirus expressing Miz-1.

Suppression of growth may be due to inhibition of cell proliferation, induction of apoptosis or both. Visual inspection of HeLa cells transfected with a Miz-1 vector together with a resistance marker revealed a number of single large cells after selection, suggesting that Miz-1 inhibits proliferation. To demonstrate this formally, we transfected HeLa cells transiently and determined the percentage of cells expressing Miz-1 that progressed into mitosis. To prevent further cell cycle progression, taxol was added for 18 h before harvesting. Expression of either GFP or β-galactosidase did not affect progression into mitosis (Figure 8B and data not shown). Upon expression of Miz-1, the total number of transfected cells was unaltered (not shown); however, few cells expressing Miz-1 progressed into mitosis (Figure 8B). We concluded that Miz-1 inhibits cell cycle progression of HeLa cells.

To determine at which point in the cell cycle arrest occurs, we co-transfected an expression plasmid encoding Miz-1 together with a plasmid encoding the surface protein CD20, which allows the identification of transfected cells in a fluorescence-activated cell sorting (FACS) experiment. Staining of transfected HeLa cells for DNA showed that cells expressing Miz-1 accumulated in the S-phase of the cell cycle, both in the absence and presence of taxol (Figure 8C). Thus, expression of Miz-1 in HeLa cells allows progression into S-phase, but not into mitosis; whether the failure of HeLa cells to arrest in G_1 is due to the presence of the papillomavirus E6 and E7 proteins remains to be determined.

A series of mutants of Miz-1 was used to determine which domains of Miz-1 were required for inhibition. Both Miz-1 and NLS–Miz-1 were equally efficient at inhibiting cell cycle progression, suggesting that arrest by Miz-1 occurs in the cell nucleus (Figure 8B). Deletion of zinc fingers 1–12 abolished cell cycle arrest by Miz-1, demonstrating that binding of Miz-1 to DNA is necessary. Deletion of the POZ domain somewhat diminished, but did not abolish cell cycle arrest; this is similar to the effects of this mutant in transactivation. Taken together, the data are explained most easily by the notion that arrest of cell cycle progression by Miz-1 occurs by transactivation of a set of Miz-1 target genes.

Two experiments were set up to test whether Myc affects cell cycle arrest by Miz-1. First, a number of mutants of Miz-1 that are deficient in interaction with Myc (see Figure 1E) were tested for their ability to arrest proliferation of HeLa cells (which express high levels of Myc) (Figure 8B). All mutants efficiently induced cell cycle arrest, demonstrating that arrest by Miz-1 does not depend on association with Myc. Significantly, all four mutants were up to 10-fold (Δ641–715) more effective than wild-type Miz-1 in arresting HeLa cell proliferation. The data strongly suggest that association with endogenous Myc relieves cell cycle arrest by Miz-1.

Ectopic expression of Myc in HeLa cells efficiently induced apoptosis in the presence of taxol; thus, we could not determine whether Myc overcomes a Miz-1-dependent cell cycle arrest in this assay. Therefore, we performed colony formation assays in NIH 3T3 cells in the presence

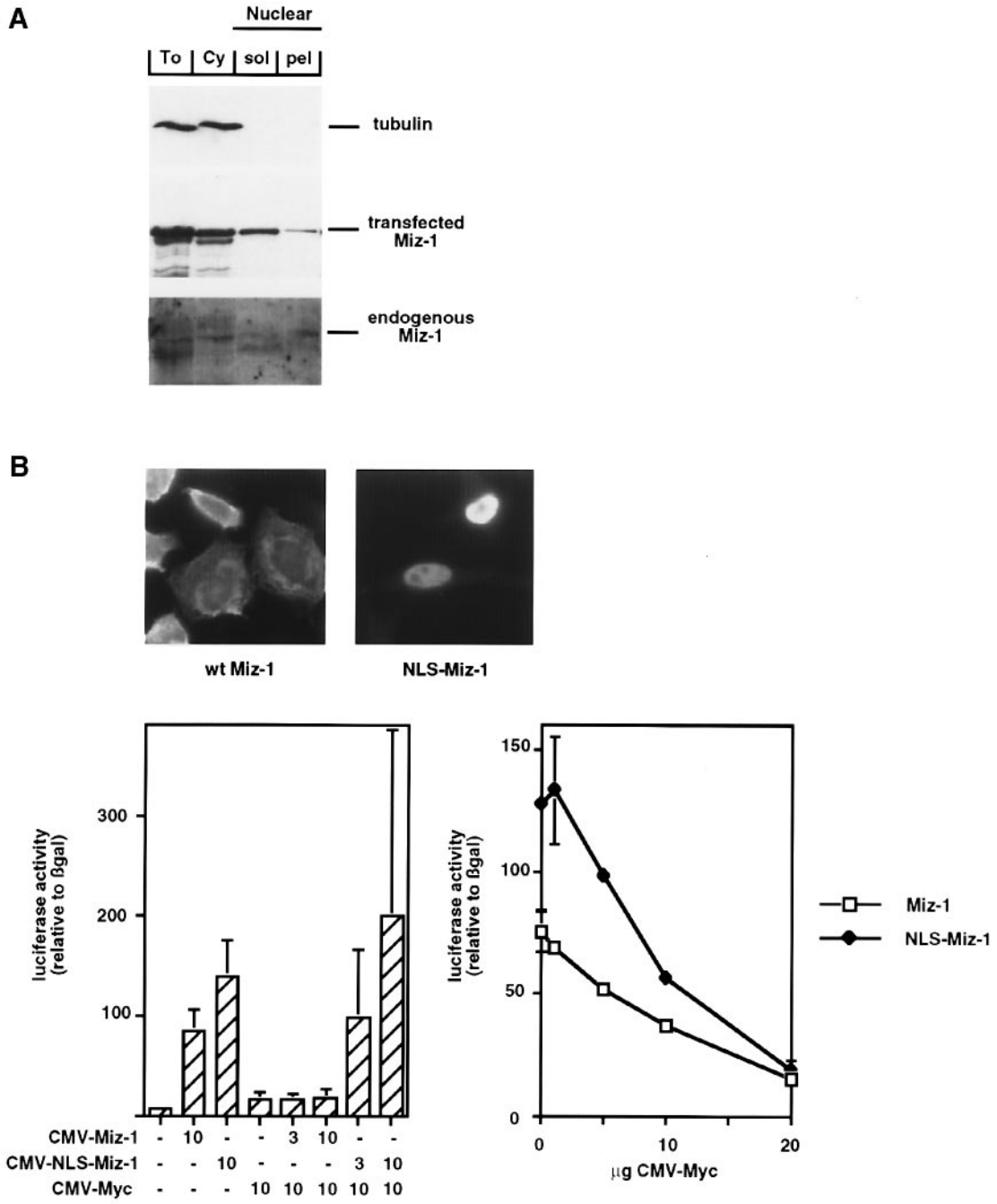

Fig. 7. Fusion of a nuclear localization signal induces efficient nuclear transport of Miz-1. (**A**) HeLa cells were fractionated as in Figure 6. Shown are Western blots documenting the distribution of tubulin, transfected Miz-1 and endogenous Miz-1 from non-transfected cells fractionated in parallel. (**B**) Top: immunofluorescence micrographs documenting the intracellular distribution of Miz-1 and NLS–Miz-1 in the absence of co-expressed Myc. Bottom: Myc effectively inhibits transactivation of the AdML promoter by Miz-1, but not NLS–Miz-1. The left panel shows the luciferase activity in the presence of the indicated amounts of expression plasmids, the right panel shows a titration of the CMV-Myc vector in the presence of a constant amount of either CMV-Miz-1 or CMV-NLS–Miz-1. Shown is the luciferase activity relative to control and a co-transfected β-galactosidase plasmid.

or absence of Myc and observed that ectopic expression of Myc partly relieved growth suppression by Miz-1 (Figure 8D). In this assay, Myc was unable to relieve the arrest induced by either NLS–Miz-1 or Miz-1ΔPOZ, similar to what is observed for transactivation (Figure 8D). Further, mutants of Miz-1 that fail to interact with Myc in the two-hybrid assay were more resistant to rescue by Myc (Figure 8D), although the effects were not as strong as in HeLa cells. The data show that ectopic expression of Myc can partly overcome a cell cycle arrest by Miz-1; they strongly suggest that this is at least in part due to association with Miz-1 and interference with Miz-1-dependent transactivation by Myc.

We were concerned that both arrest by Miz-1 and its relief by Myc might be restricted to the very high expression levels achieved with CMV-derived vectors. Therefore, we infected both RAT1A and Balb/c-3T3 cells with a recombinant retrovirus expressing Miz-1 together

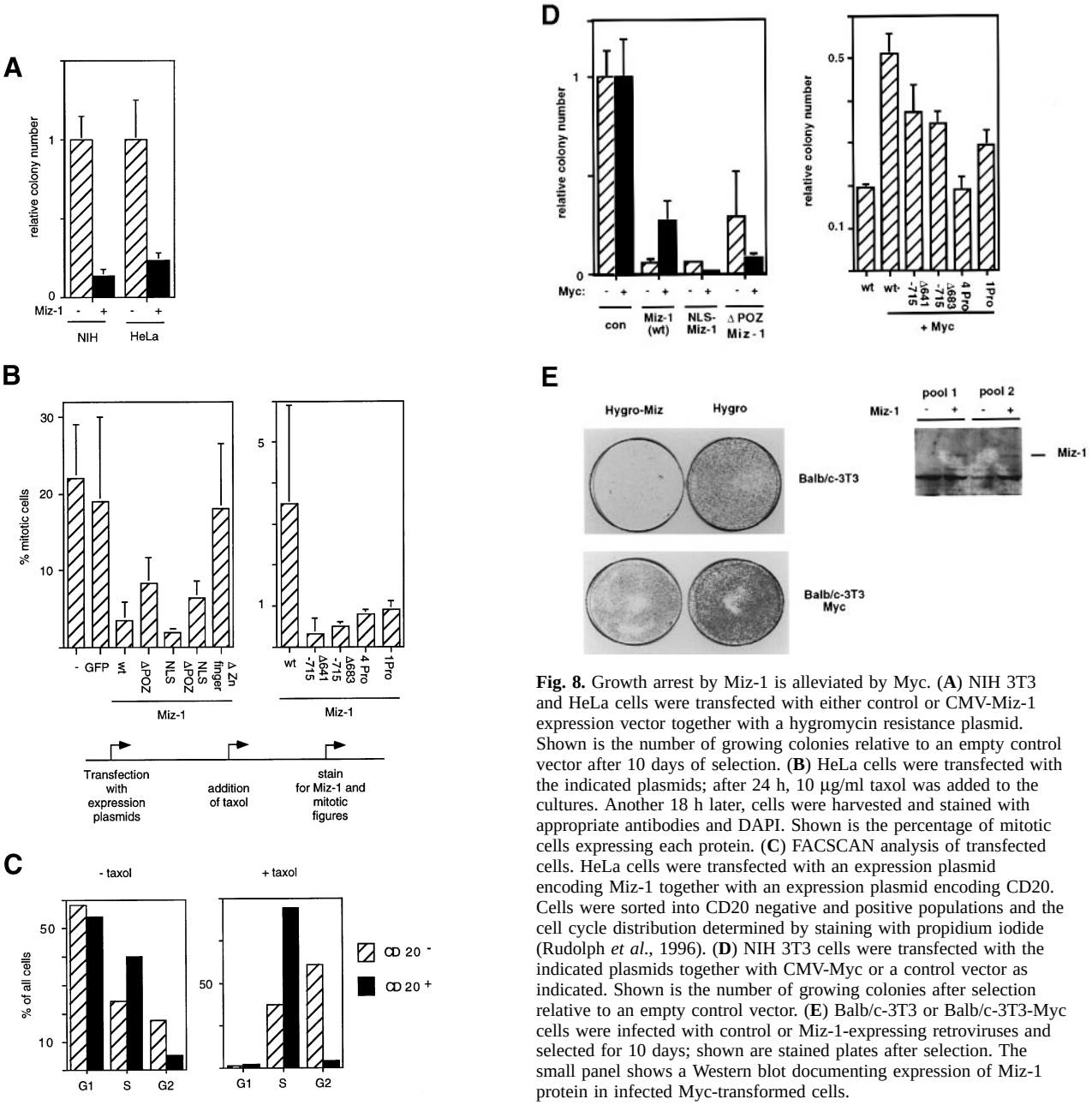

Fig. 8. Growth arrest by Miz-1 is alleviated by Myc. (**A**) NIH 3T3 and HeLa cells were transfected with either control or CMV-Miz-1 expression vector together with a hygromycin resistance plasmid. Shown is the number of growing colonies relative to an empty control vector after 10 days of selection. (**B**) HeLa cells were transfected with the indicated plasmids; after 24 h, 10 μg/ml taxol was added to the cultures. Another 18 h later, cells were harvested and stained with appropriate antibodies and DAPI. Shown is the percentage of mitotic cells expressing each protein. (**C**) FACSCAN analysis of transfected cells. HeLa cells were transfected with an expression plasmid encoding Miz-1 together with an expression plasmid encoding CD20. Cells were sorted into CD20 negative and positive populations and the cell cycle distribution determined by staining with propidium iodide (Rudolph *et al.*, 1996). (**D**) NIH 3T3 cells were transfected with the indicated plasmids together with CMV-Myc or a control vector as indicated. Shown is the number of growing colonies after selection relative to an empty control vector. (**E**) Balb/c-3T3 or Balb/c-3T3-Myc cells were infected with control or Miz-1-expressing retroviruses and selected for 10 days; shown are stained plates after selection. The small panel shows a Western blot documenting expression of Miz-1 protein in infected Myc-transformed cells.

with a hygromycin resistance gene. No resistant colonies were recovered in either cell line, whereas efficient colony formation was observed with empty control vectors (Figure 8E; data for RAT1A cells not shown). In parallel, we infected Balb/c-3T3 cells that previously had been infected with a retrovirus expressing Myc (Jansen-Dürr *et al.*, 1993) and RAT1A-MycER cells (Eilers *et al.*, 1989); expression of either Myc or MycER proteins allowed colony formation in the presence of Miz-1; the resulting colonies, however, grew more slowly than control cells (Figure 8E). Western blotting revealed that the growing cells expressed Miz-1 protein (Figure 8E).

Our data show that Miz-1 exerts a strong growth-suppressive effect in the cell nucleus and that growth arrest by Miz-1 is alleviated by Myc. Alleles of Miz-1 that lack the POZ domain, are constitutively localized in the nucleus or are deficient in interaction with Myc are all more potent than wild-type Miz-1 in arresting the proliferation of HeLa and NIH 3T3 cells; the data strongly suggest that Myc overcomes Miz-1-dependent growth arrest by interfering with the transcriptional activation of a set of Miz-1 target genes.

Discussion

Enhanced expression of c-*myc* is observed in many human tumours and causally contributes to tumorigenesis. One mechanism by which Myc transforms cells has been clearly defined: Myc forms a heteromeric complex with its partner protein Max that binds to and activates transcription from CACGTG sequences (see Introduction). Binding to Max is a prerequisite for transformation by Myc, and at least two of the known target genes of the Myc–Max complex, ODC and cdc25A, have transforming potential by themselves (Auvinen *et al.*, 1992; Amati *et al.*, 1993b; Galaktionov *et al.*, 1995). The findings strongly suggest

that Myc exerts its transforming function at least in part by activating transcription of a critical set of cellular target genes.

A similar argument can be made for gene repression by Myc. Genes that are repressed in Myc-transformed cells are involved in the control of proliferation and differentiation, cell adhesion and recognition of cells by the immune system (see Introduction). Thus, gene repression by Myc can be expected to significantly affect the phenotype of transformed cells. Several proteins have been implicated in gene repression by Myc. For example, Myc interferes with the function of CCAAT-binding transcription factors, potentially by inducing the phosphorylation of these proteins (Yang *et al.*, 1991). Viral Myc has been shown to squelch the transcriptional activation domain of c/EBP, a transcription factor involved in cellular differentiation (Mink *et al.*, 1996). Also, direct interactions have been reported between Myc and either YY-1 or TFII-I, two proteins that can interact with initiator elements and may be part of the basal transcription machinery (Roy *et al.*, 1993; Shrivastava *et al.*, 1993).

We now report the identification of a protein that interacts with the carboxy-terminal HLH domain of Myc. Miz-1 belongs to the BTB/POZ family of zinc finger proteins and interacts with DNA in a sequence-specific manner. Several lines of evidence suggest that Miz-1 is involved in gene repression by Myc *in vivo*. First, a mutational analysis of the carboxy-terminus of Myc has shown that the integrity of the HLH domain is critical for gene repression *in vivo*. In contrast, the requirement for the leucine zipper was much less pronounced. In particular, a mutation (In412) that inserts four amino acids between the HLH domains and the leucine zipper is fully competent for repression *in vivo*, yet fails to interact with Max and is transformation deficient (Philipp *et al.*, 1994). The association between Miz-1 and Myc provides a rationale for these observations, as Miz-1 interacts with the HLH domain, but not the leucine zipper of Myc. Further, these findings provide an explanation as to why repression can be hormone independent in MycER chimeras (Philipp *et al.*, 1994). As the attached hormone-binding domain is thought to act by simple steric hindrance (Picard *et al.*, 1988), the simplest explanation is that it sterically interferes with protein–protein interactions of the leucine zipper, but not in the HLH domain, which extends in a linear way away from the leucine zipper (Ferré D'Amaré *et al.*, 1993).

Second, sequences close to the start site of transcription have been shown to determine the extent to which the AdML and the c/EBPα promoters are repressed by Myc (Li *et al.*, 1994). In addition, a number of genes that are repressed by Myc *in vivo* are encoded by TATA-less promoters with defined start sites, suggesting that they use initiator elements. We have shown that Miz-1 specifically interacts with sequences at the start site of the AdML and the cyclin D1 promoter (Figures 3 and 5). Mutation of these sequences or deletion of the zinc fingers inhibits transcriptional activation of the AdML promoter by Miz-1. Thus, interaction of Myc with Miz-1 can explain the specificity of repression that is observed *in vivo*.

Third, interaction with Miz-1 reflects the specificity seen for gene repression by HLH proteins. Both USF and Max are more abundant than Myc in cells that are transformed by Myc (Berberich and Cole, 1992; Littlewood *et al.*, 1992), and repression by Myc occurs in the presence of an excess of these proteins. Neither USF nor Max repress either the AdML or the cyclin D1 promoters (Li *et al.*, 1994; Philipp *et al.*, 1994). In yeast two-hybrid experiments, both c- and N-Myc, but neither Max nor USF, interact with Miz-1. Thus, the specific interaction of Myc with Miz-1 distinguishes it from other, non-transforming HLH proteins and provides an explanation as to how Myc can repress genes in the presence of an excess of closely related transcription factors.

Fourth, our observations suggest a potential mechanism as to how gene repression by Myc may occur. The POZ domain has been shown to target transcription factors to discrete subnuclear foci *in vivo* and render the proteins insoluble upon extraction (e.g. Dhordain *et al.*, 1995). The POZ domain of the ZID protein inhibits both DNA binding by the zinc fingers and transcriptional activation, potentially by inducing homodimerization of ZID (Bardwell and Treisman, 1994). In the absence of Myc, Miz-1 is soluble and capable of sequence-specific transactivation *in vivo*. However, inhibition of Miz-1 function by Myc requires the integrity of the POZ domain and correlates with the loss of soluble Miz-1, strongly suggesting a model in which association with Myc induces the otherwise latent inhibitory functions of the POZ domain of Miz-1.

In vivo, Miz-1 has a potent growth arrest function in several rodent and human cell lines. The arrest exerted by different alleles of Miz-1 correlates closely with their ability to transactivate transcription. Most likely, therefore, arrest by Miz-1 occurs via the transcriptional activation of a set of growth-inhibitory genes. Although the critical target genes for this arrest function are unknown, it is noteworthy that ectopic expression of cyclin D1 can arrest cells during S-phase of the cell cycle (Pagano *et al.*, 1994), suggesting that cyclin D1 may play a role in the Miz-1-dependent growth arrest. A requirement for Myc function late in the cell cycle (during S- and G2-phase) has been demonstrated in B-cells (Shibuya *et al.*, 1992).

We have not obtained any evidence that arrest of proliferation by Miz-1 occurs via inhibition of transcriptional activation by Myc. In particular, alleles of Miz-1 that fail to interact with Myc in the two-hybrid assay are capable of arrest and are more potent than wild-type Miz-1 in arresting proliferation. These findings place Miz-1 in a growth control pathway downstream of Myc. However, previous work has shown that induction of cell proliferation, like all known biological effects of Myc, requires association with Max. In particular, mutations of Myc that render the leucine zipper of Myc unable to interact with Max are biologically inactive; they can be complemented by corresponding mutations in the leucine zipper of Max (Amati *et al.*, 1993a,b). The data show that association of Myc with Max is required for Myc to be active, and strongly suggest that Max is the only required partner protein for the leucine zipper of Myc.

Our data do not question this concept for a number of reasons. First, Miz-1 interacts with the HLH domain, not the leucine zipper of Myc (Figure 1A). Second, the effects of ectopic expression of Max on the interaction of Miz-1 with Myc *in vivo* are relatively small (see, for example,

Figure 4E). While they suggest that Myc–Max and Myc–Miz-1 form alternative complexes *in vivo*, they are also compatible with the notion that ternary Myc–Max–Miz-1 complexes form but are less stable than binary Myc–Miz-1 complexes under the experimental conditions. A full understanding of the role of Miz-1 in Myc biology will require, therefore, the identification of point mutants of Myc that abolish interaction with Miz-1, but not Max. Experiments to identify such mutations are under way.

Any proposed mechanism in which Myc inhibits another protein's function by association must explain how the relatively small amount of Myc protein present even in a transformed cell can exert a significant negative effect on gene expression *in vivo*. Our data suggest one potential mechanism as to how this may occur. Miz-1 lacks a nuclear import signal and both endogenous and transfected forms of Miz-1 accumulate in the cytosol of cells. Ectopic expression of Myc blocks transcriptional activation and alleviates arrest by wild-type Miz-1; in contrast, a constitutively localized form of Miz-1 is more resistant to Myc for both gene activation and inhibition of cell cycle progression. Thus, limiting the amount of Miz-1 in the nucleus is critical for Myc to inhibit efficiently both transactivation and cell cycle arrest by Miz-1 *in vivo*.

In the presence of high amounts of Myc protein, association with Myc itself provides an NLS to Miz-1. Whether Myc contributes significantly to nuclear import of Miz-1 at physiological concentrations remains to be determined. As Miz-1 arrests proliferation in the nucleus, it seems likely that other proteins interacting with Miz-1 may also provide NLSs and regulate the amount of Miz-1 in the cell nucleus. In this sense, stimulation of nuclear import of Miz-1 by Myc may be reminiscent of that of E2F-4, which also lacks a nuclear import signal. Import of E2F-4 is stimulated by association with either p107 or p130, pocket proteins that negatively regulate E2F-4 function *in vivo* (Lindeman *et al.*, 1997).

In several experiments, we observed that cytosolic Miz-1 associates with microtubuli (see, for example, Figure 7B) and indeed can target a Gal–Myc chimera to microtubuli (data not shown). If mechanisms exist that stabilize association of Miz-1 with microtubuli, it is also conceivable that Miz-1 can be an upstream regulator of Myc function by regulating nuclear import of Myc. *In vivo*, high expression of Miz-1 is observed in brain, in muscle and in several myeloid cell lines (A.Schneider, unpublished). Nuclear import of Myc is blocked in differentiating myeloid and neuronal cells, and both cells accumulate either c- or N-Myc protein in the cytosol (Craig *et al.*, 1993; Wakamatsu *et al.*, 1993). In HeLa and HL6o cells, Myc protein has been shown to accumulate on microtubuli, and the amino-terminus of Myc interacts with tubulin *in vitro* (Alexandrova *et al.*, 1995). Thus, the association of Myc and Miz-1 with microtubuli may be stable in differentiating cells and then serve as a cytosolic anchor for both Miz-1 and Myc.

Finally, we note the recent localization of a fragment of Miz-1 to 1p36.1–1p36.2 (Tommerup and Vissing, 1995). This localization was confirmed using the entire human clone as probe (M.Schwab, personal communication). Loss of heterozygosity (LOH) occurs frequently at this locus in a number of human tumours (Schwab *et al.*, 1996). Among them are both neuroblastoma and colon carcinoma, two tumours in which alterations at *myc* gene loci are well documented and play an important role.

Materials and methods

Two-hybrid experiments

To generate the bait plasmid, the Myc BR/HLH/LZ region (amino acids 355–439) was amplified by PCR using pSP65-cmycIIA (Eilers *et al.*, 1989) as template and inserted into pGBT9 (Clontech). A total of 2×10^5 independent transformants of a human HeLa cDNA library (Matchmaker, Clontech) were screened according to the manufacturer's instructions. The mutant alleles of Myc used have been described (Stone *et al.*, 1987). To generate GAL–USF, a cDNA fragment corresponding to amino acids 193–311 was amplified by PCR using CMV-USF as template (kind gift of B.Roeder) and inserted into pGBT9. GAL–NMYC (amino acids 177–456) was a kind gift of Jörg Schürmann and Manfred Schwab. To generate GAL–Max, a fragment encoding amino acids 38–108 of human Max was amplified using pVZ21Max (kind gift of B.Eisenman) as template.

Structure predictions were carried out using an algorithm described by Lupas *et al.* (1991); the calculation was performed at the ISREC coils server (http://ulrec3.unil.ch/coils/COILS_doc.html).

Reporter and expression plasmids

The AdML –45/+65 wild-type and mutant constructs were kindly provided by Robert G.Roeder as CAT reporter constructs and have been described elsewhere (Du *et al.*, 1993). The AdML sequences were excised and inserted into pXP1 luc (Nordeen, 1988). The wild-type reporter constructs contain a major (–9 to +10: 5′-TTCGTCCTCACTCT-CTTCC-3′) and a minor (+37 to +59: 5′-TTGGGGTGAGTACTCC-CTCTGAA-3′) transcription start site. The mutated reporter constructs contain the following sequences: (–9 to +10: 5′-TTCGACGGCAC-AAACTTCC-3′), (–39 to +59: 5′-TTGGGCTGACTGCTCCGGCA-CTT-3′). The CMV-Myc, CMV-MycΔHLH, CMV-USF and CMV-Max constructs have been described (Philipp *et al.*, 1994; Desbarats *et al.*, 1996). To generate CMV-Miz-1 wt, CMV-Miz-1ΔPOZ, CMV-NLS–Miz-1 and CMV-NLS–ΔPOZ-Miz-1, PCR was used to amplify either full-length Miz-1 or a ΔPOZ derivative (amino acids 105–803); the corresponding fragments were inserted into the *Eco*RI and *Xba*I sites of pUHD 10.1.

To generate recombinant retroviruses, Miz-1 cDNA was inserted into pbabe-hygro (Morgenstern and Land, 1990); generation of viruses and infection of cells were carried out according to Pear *et al.* (1993). Transient transfection experiments were performed as described previously (Desbarats *et al.*, 1996).

Gel-shift experiments

To express recombinant Miz-1 protein, a cDNA fragment encoding amino acids 269–803 was cloned into a bacterial expression vector (pRSET) that supplies an N-terminal cassette of six histidines; the fusion protein was purified by affinity chromatography on an Ni^{2+}–Sepharose column (Qiagen, Hilden, Germany). Binding was carried out for 10 min at room temperature in 50 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 10 mM EDTA, 10 mM $ZnCl_2$, 100 mM Tris pH 7.5.

Oligonucleotides were used as follows: polyoma, 5′-TTGACATTTT-CTATTTTAAGAGTCGGGAGGAAAATTA-3′; polyoma mut, 5′-TTG-ACATTTAGTCGGGAGGAAAATTA-3′; cyclin D1 –22/+22, 5′-AGT-TTTGGTGAAGTTGCAAAGTCCTGGAGCCTCCAGAGGGCTGT-3′; cyclin D1 –79/–54, 5′-CCTCCCGCTCCCATTCTCTGCCGGG-3′; AdML, 5′-GATCCGGCGCGTTCGTCCTCACTCTCTTCCGCATCG-CTGTCT-3′; and AdML mut, 5′-GGCGCGTTCGTCCTCACTCTCTC-CCGCATCGCTGTCTG-3′.

Protein-binding assays

In vitro. Transcription/translation was carried out in reticulocyte lysate (Promega) according to the manufacturer's protocol. Twenty μg of affinity-purified GST fusion protein was bound to 100 μl of glutathione–agarose (100 μg/ml) (Sigma) in the presence of 20 mM HEPES pH 7.8, 100 mM KCl, 5 mM $MgCl_2$. To assay for specific interactions, 10 μl of [^{35}S]methionine-labelled *in vitro*-translated protein was added and incubated for 90 min at 4°C. The beads were washed four times in 20 mM HEPES pH 7.8, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.5% NP-40. Bound proteins were analysed by SDS–gel electrophoresis and fluorography.

In vivo. After transient transfection, cells were lysed by sonication in 20 mM HEPES-KOH; 150 mM KCl, 0.5% NP-40; 0.5 mM EDTA, pH 7.4 in the presence of protease inhibitors. Lysates were clarified by sonication and subjected to immunoprecipitation as described (Rudolph *et al.*, 1996).

Cell fractionation and immunocytochemistry

Immunofluorescence was carried out as described previously (Rudolph *et al.*, 1996) with the following antibodies (all diluted 1:50): monoclonal anti-Myc 9E10 antibody, polyclonal anti-Max antibody (kind gift of B.Lüscher), monoclonal anti-β-tubulin antibody (Boehringer Mannheim) or polyclonal anti-GAL4 antibody (kind gift of Achim Leutz). Miz-1 antiserum was generated by immunization of rabbits with recombinant Miz-1 protein. DNA was stained with 100 μg/ml DAPI.

Cells were fractionated by lysis in buffer containing 0.5% NP-40 and nuclei isolated by low speed centrifugation. Soluble and insoluble nuclear material after salt extraction was separated by centrifugation at 100 000 *g*.

Pictures were taken using a Leica immunofluorescence microscope equipped with a Photometrics CH250 CCD camera. Picture analysis, colour assignment and superposition of different pictures were done electronically using IPlab spectrum software on a Macintosh computer.

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References

Albagli,O., Dhordain,P., Deweindt,C., Lecocq,G. and Leprince,D. (1995) The BTB/POZ domain: a new protein–protein interaction motif common to DNA- and actin-binding proteins. *Cell Growth Differ.*, **6**, 1193–1198.

Alexandrova,N., Niklinski,J., Bliskovsky,V., Otterson,G.A., Blake,M., Kaye,F.J. and Zajac Kaye,M. (1995) The N-terminal domain of c-Myc associates with alpha-tubulin and microtubules *in vivo* and *in vitro*. *Mol. Cell. Biol.*, **15**, 5188–5195.

Amati,B., Brooks,M.W., Levy,N., Littlewood,T.D., Evan,G.I. and Land,H. (1993a) Oncogenic activity of the c-Myc protein requires dimerization with Max. *Cell*, **72**, 233–245.

Amati,B., Littlewood,T.D., Evan,G.I. and Land,H. (1993b) The c-Myc protein induces cell cycle progression and apoptosis through dimerization with Max. *EMBO J.*, **13**, 5083–5087.

Auvinen,M., Paasinen,A., Andersson,L.C. and Holtta,E. (1992) Ornithine decarboxylase activity is critical for cell transformation. *Nature*, **360**, 355–358.

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.

Bardwell.V.J. and Treisman,R. (1994) The POZ domain: a conserved protein–protein interaction motif. *Genes Dev.*, **8**, 1664–1677.

Berberich,S.J. and Cole,M.D. (1992) Casein kinase II inhibits the DNA-binding activity of Max homodimers but not Myc/Max heterodimers. *Genes Dev.*, **6**, 166–176.

Bernards,R., Dessain,S.K. and Weinberg,R.A. (1986) N-myc amplification causes down-modulation of MHC class 1 antigen expression in neuroblastoma. *Cell*, **47**, 667–674.

Blackwell,T.K., Kretzner,L., Blackwood,E.M., Eisenman,R.N. and Weintraub,H. (1990) Sequence-specific DNA binding by the c-myc protein. *Science*, **250**, 1149–1151.

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.

Chen,W., Zollman,S., Couderc.J.L. and Laski,F.A. (1995) The BTB domain of bric a brac mediates dimerization *in vitro*. *Mol. Cell. Biol.*, **15**, 3424–3429.

Craig,R.W., Buchan,H.L., Civin,C.I. and Kastan,M.B. (1993) Altered cytoplasmic/nuclear distribution of the c-myc protein in differentiating ML-1 human myeloid leukemia cells. *Cell Growth Differ.*, **4**, 349–357.

Desbarats,L., Gaubatz,S. and Eilers,M. (1996) Discrimination between different E-box binding proteins at an endogenous target gene of Myc. *Genes Dev.*, **10**, 447–460.

Dhordain,P. *et al*. (1995) The BTB/POZ domain targets the LAZ3/BCL6 oncoprotein to nuclear dots and mediates homomerization *in vivo*. *Oncogene*, **11**, 2689–2697.

Du,H., Roy,A.L. and Roeder,R.G. (1993) Human transcription factor USF stimulates transcription through the initiator elements of the HIV-1 and the Ad-ML promoters. *EMBO J.*, **12**, 501–511.

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.

Evan,G.I. and Hancock,D.C. (1985) Studies on the interaction of the human c-myc protein with cell nuclei: p62 c-myc as a member of a discrete subset of nuclear proteins. *Cell*, **43**, 253–261.

Facchini,L.M., Chen,S., Marhinb,W., Lear,J.N. and Penn,L.Z. (1997) The Myc negative autoregulation mechanism requires Myc–Max association and involves the c-myc P2 minimal promoter. *Mol. Cell. Biol.*, **17**, 100–114.

Ferré D'Amaré,A.R., Prendergast,G.C., Ziff,E.B. and Burley,S.K. (1993) Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain. *Nature*, **363**, 38–45.

Fields,S. and Song,O. (1989) A novel genetic system to detect protein–protein interactions. *Nature*, **340**, 245–246.

Freytag,S.O. and Geddes,T.J. (1992) Reciprocal regulation of adipogenesis by Myc and C/EBP alpha. *Science*, **256**, 379–382.

Galaktionov,K., Lee,A.K., Eckstein,J., Draetta,G., Meckler,J., Loda,M. and Beach,D. (1995) CDC25 phosphatases as potential human oncogenes. *Science*, **269**, 1575–1577.

Henriksson,M. and Lüscher,B. (1996) Proteins of the Myc network: essential regulators of cell growth and differentiation. *Cancer Res.*, **68**, 109–182.

Herber,B., Truss,M., Beato,M. and Muller,R. (1994) Inducible regulatory elements in the human cyclin D1 promoter. *Oncogene*, **9**, 1295–1304.

Hurlin,P.J., Queva,C., Koskinen,P.J., Steingrimsson,E., Ayer,D.E., Copeland,N.G., Jenkins,N.A. and Eisenman,R.N. (1996) Mad3 and Mad4: novel Max-interacting transcriptional repressors that suppress c-myc dependent transformation and are expressed during neural and epidermal differentiation. *EMBO J.*, **15**, 2030–2038.

Inghirami,G., Grignani,F., Sternas,L., Lombardi,L., Knowles,D.M. and Dalla-Favera,R. (1990) Down-regulation of LFA-1 adhesion receptors by c-*myc* oncogene in human B lymphoblastoid cells. *Science*, **250**, 682–686.

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.

Judware,R. and Culp,L.A. (1995) Over-expression of transfected N-*myc* oncogene in human SKNSH neuroblastoma cells down-regulates expression of beta 1 integrin subunit. *Oncogene*, **11**, 2599–25607.

Kato,G.J., Barrett,J., Villa-Garcia,M. and Dang,C.V. (1990) An amino-terminal c-myc domain required for neoplastic transformation activates transcription. *Mol. Cell. Biol.*, **10**, 5914–5920.

Kretzner,L. Blackwood,E.M. and Eisenman,R.N. (1992) Myc and Max proteins possess distinct transcriptional activities. *Nature*, **359**, 426–429.

Li,L., Nerlov,C., Prendergast,G., MacGregor,D. and Ziff,E.B. (1994) c-Myc represses transcription *in vivo* by a novel mechanism dependent on the initiator element and Myc box II. *EMBO J.*, **13**, 4070–4079.

Lindeman,G.J., Gaubatz,S., Livingston,D.M. and Ginsberg,D. (1997) The subcellular localization of E2F-4 is cell cycle dependent. *Proc. Natl Acad. Sci.USA*, **94**, 5095–5100.

Littlewood,T.D., Amati,B., Land,H. and Evan,G.I. (1992) Max and c-Myc/Max DNA-binding activities in cell extracts. *Oncogene*, **7**, 1783–1792.

Lupas,A., Van Dyke,M. and Stock,J. (1991) Predicting coiled coils from protein sequences. *Science*, **252**, 1162–1164.

Marhin,W.W., Hei,Y.-J., Chen,S., Jiang,Z., Gallie,B., Phillips,R.A. and Penn,L.Z. (1996) Loss of Rb and Myc activation co-operate to suppress cyclin D1 and contribute to transformation. *Oncogene*, **12**, 43–55.

Mink,S., Mutschler,B., Weiskirchen,R., Bister,K. and Klempnauer,K.H. (1996) A novel function for Myc: inhibition of c/EBP-dependent gene activation. *Proc. Natl Acad. Sci. USA*, **93**, 6635–6640.

Morgenstern,J.P. and Land,H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.*, **18**, 3587–3596.

Nordeen,S.K. (1988) Luciferase reporter gene vectors for analysis of promotors and enhancers. *BioTechniques*, **6**, 454–457.

Pagano,M., Theodoras,A.M., Tam,S.W. and Draetta,G.F. (1994) Cyclin D1-mediated inhibition of repair and replicative DNA synthesis in human fibroblasts. *Genes Dev.*, **8**, 1627–1639.

Pear,W.S., Nolan,G.P., Scott,M.L. and Baltimore,D. (1993) Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl Acad. Sci. USA*, **90**, 8392–8396.

Penn,L.J.Z., Brooks,M.W., Laufer,E.M. and Land,H. (1990) Negative autoregulation of c-myc transcription. *EMBO J.*, **9**, 113–121.

Phelps,W.C., Yee,C.L., Münger,K. and Howley,P.M. (1988) The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. *Cell*, **53**, 539–547.

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.

Picard,D., Salser,S.J. and Yamamoto,K.R. (1988) A movable and regulatable inactivation function within the steroid binding domain of the glucocorticoid receptor. *Cell*, **54**, 1073–1080.

Prendergast,G.C. and Ziff,E.B.(1991) Methylation-sensitive sequence-specific DNA binding by the c-myc basic region. *Science*, **251**, 186–189.

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.

Roy,A.L., Carruthers,C., Gutjahr,T. and Roeder,R.G. (1993) Direct role for Myc in transcription initiation mediated by interactions with TFII-I. *Nature*, **365**, 359–361.

Rudolph,B., Zwicker,J., Saffrich,R., Henglein,B., Müller,R., Ansorge,W. and Eilers,M. (1996) Activation of cyclin dependent kinases by Myc mediates transcriptional activation of cyclin A, but not apoptosis. *EMBO J.*, **15**, 3065–3076.

Schulz,C.T., Hopwood,B., Rathjen,P.D. and Wells,J.R. (1995) An unusual arrangement of 13 zinc fingers in the vertebrate gene Z13. *Biochem. J.*, **311**, 219–224.

Schwab,M., Praml,C. and Amler,L.C. (1996) Genomic instability in 1p and human malignancies. *Genes Chromosomes Cancer*, **16**, 211–229.

Shibuya,H., Yoneyama,M., Ninomiya-Tsuji,J., Matsumoto,K. and Taniguchi,T. (1992) IL-2 and EGF receptors stimulate the hematopoietic cell cycle via different signaling pathways: demonstration of a novel role for *c-myc*. *Cell*, **70**, 57–67.

Shrivastava,A., Saleque,S., Kalpana,G.V., Artandi,S., Goff,S.P. and Calame,K. (1993) Inhibition of transcriptional regulator Yin-Yang-1 by association with c-Myc. *Science*, **262**, 1889–1892.

Stone,J., de Lange,T., Ramsay,G., Jakobovits,E., Bishop,J.M., Varmus,H. and Lee,B. (1987) Definition of regions in human c-*myc* that are involved in transformation and nuclear localization. *Mol. Cell. Biol.*, **7**, 1697–1709.

Tommerup,N. and Vissing,H. (1995) Isolation and fine mapping of 16 novel human zinc finger-encoding cDNAs identify putative candidate genes for developmental and malignant disorders. *Genomics*, **27**, 259–264.

Versteeg,R., Noordermeer,I.A., Kruese-Wolters,M., Ruiter,D.J. and Schrier,P.I. (1988) c-*myc* down-regulates class HLA expression in human melanomas. *EMBO J.*, **7**, 1023–1029.

Wakamatsu,Y., Watanabe,Y., Shimono,A. and Kondoh,H. (1993) Transition of localization of the N-Myc protein from nucleus to cytoplasm in differentiating neurons. *Neuron*, **10**, 1–9.

Yang,B.S., Geddes,T.J., Pogulis,R.J., de Crombrugghe,B. and Freytag,S.O. (1991) Transcriptional suppression of cellular gene expression by c-Myc. *Mol. Cell. Biol.*, **11**, 2291–2295.

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..

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