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Transcriptional activation by Myc is under negative control by the transcription factor AP-2

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The Myc protein binds to and transactivates the expression of genes via E-box elements containing a central CAC(G/A)TG sequence. The transcriptional activation function of Myc is required for its ability to induce cell cycle progression, cellular transformation and apoptosis. Here we show that transactivation by Myc is under negative control by the transcription factor AP-2. AP-2 inhibits transactivation by Myc via two distinct mechanisms. First, high affinity binding sites for AP-2 overlap Myc-response elements in two bona fide target genes of Myc, prothymosin-α and ornithine decarboxylase. On these sites, AP-2 competes for binding of either Myc/Max heterodimers or Max/Max homodimers. The second mechanism involves a specific interaction between C-terminal domains of AP-2 and the BR/HLH/LZ domain of Myc, but not Max or Mad. Binding of AP-2 to Myc does not preclude association of Myc with Max, but impairs DNA binding of the Myc/Max complex and inhibits transactivation by Myc even in the absence of an overlapping AP-2 binding site. Taken together, our data suggest that AP-2 acts as a negative regulator of transactivation by Myc.

Key words: AP-2/Myc/ ornithine decarboxylase/prothymosin-α/transcription factor

Introduction

The c-*myc* gene was identified as the cellular homologue of the transforming oncogene of the chicken myelocytomatosis virus, MC29 (Alitalo *et al.*, 1983; Colby *et al.*, 1983; Watson *et al.*, 1983). c-*myc* appears to have a dual function in mammalian cells. First, it acts as a central regulator of cellular proliferation. This is supported by findings that expression of c-*myc* closely parallels cellular proliferation and that inhibition of its function often leads to an arrest of proliferation (Hirning *et al.*, 1991; Davis *et al.*, 1993; Kelly *et al.*, 1983). In addition, the deregulated expression of c-*myc* in tissue culture or in transgenic animals provides a strong mitogenic stimulus and, in culture, is sufficient to cause cell proliferation in the absence of external growth factors (Eilers *et al.*, 1991; Keath *et al.*, 1984; Stewart *et al.*, 1984). Second, c-*myc* appears to be an inducer of apoptotic cell death under conditions of growth factor deprivation or in the presence of wild-type p53 protein (Evan *et al.*, 1992; Hermeking and Eick, 1994). *In vivo*, apoptosis is often accompanied by an elevated expression of c-*myc*, suggesting that c-*myc* may also act as a physiological regulator of apoptotic cell death (Shi *et al.*, 1992).

The protein encoded by c-*myc* is a nuclear phosphoprotein with a very short half-life (Abrams *et al.*, 1982; Donner *et al.*, 1982; Persson and Leder, 1984). Myc protein belongs to the helix−loop−helix/leucine zipper (HLH/LZ) family of transcription factors (Landschulz *et al.*, 1988; Murre *et al.*, 1989) and, like all members of this family, binds specifically to DNA and recognizes E-box sequences with the consensus CAC(G/A)TG (Blackwell *et al.*, 1990, 1993; Kerkhoff *et al.*, 1991; Prendergast and Ziff, 1991). Both in transient transfections and *in vivo*, Myc activates E-box-dependent transcription. Domains necessary for transcriptional activation have been localized to the N-terminus of the protein (Kato *et al.*, 1990; Kretzner *et al.*, 1992; Amin *et al.*, 1993; Gaubatz *et al.*, 1994). The affinity of Myc for DNA is enhanced by heterodimerization with a structurally related protein, Max (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991; Blackwood *et al.*, 1992). Two additional proteins, Mad and Mxi-1, have been identified that heterodimerize with Max, but not Myc (Ayer *et al.*, 1993; Zervos *et al.*, 1993). Max, Mad and Mxi-1 lack transcriptional activating domains and act as repressors of E-box-dependent transcription (Kato *et al.*, 1992; Kretzner *et al.*, 1992; Amin *et al.*, 1993). In contrast to Myc, they are often expressed in quiescent and differentiating cells, suggesting that either homodimers of Max or heterodimers between Max and either Mxi or Mad are predominant in resting cells (Blackwood *et al.*, 1992; Ayer and Eisenman, 1993; Zervos *et al.*, 1993). The contrasting biochemical properties of Myc and its partner proteins, together with their different pattern of expression, suggest a model as to how the growth regulation of Myc-responsive genes may be achieved (Amati and Land, 1994). Both induction of cellular proliferation and apoptosis by Myc depend on its heterodimerization with Max and on intact DNA binding and transcriptional transactivation domains, strongly suggesting that transactivation of target genes by Myc is a prerequisite for its transforming abilities (Stone *et al.*, 1987; Amati *et al.*, 1993).

At least two genes, prothymosin-α and ornithine decarboxylase (ODC), are regulated by Myc *in vivo* via E-box sequences (Eilers *et al.*, 1991; Bello-Fernandez *et al.*, 1993; Wagner *et al.*, 1993). The notion that both are bona fide target genes for Myc is supported by several findings. (i) Transcription of both genes is activated rapidly in response to activation of a conditional allele of Myc

(Eilers *et al.*, 1991; Wagner *et al.*, 1993; Tavtigian *et al.*, 1994). (ii) This induction is independent of protein synthesis and therefore no intermediate protein has to be synthesized for activation by Myc (Eilers *et al.*, 1991; Wagner *et al.*, 1993). (iii) Expression of prothymosin-α closely parallels expression of Myc during differentiation of HL-60 promyelocytic cells and is elevated to supraphysiological levels in tumours that carry amplified Myc (Dosil *et al.*, 1993; Mori *et al.*, 1993; Smith *et al.*, 1993). (iv) Myc-response elements have been identified in the first intron of both genes, and Myc binds to these with high affinity (Bello-Fernandez *et al.*, 1993; Gaubatz *et al.*, 1994). (v) *In vivo*, activation of a hormone-inducible allele of Myc stimulates transcription through these Myc-response elements (Gaubatz *et al.*, 1994). Expression of both prothymosin-α and ODC is required for cell proliferation and both have been implicated in apoptosis, suggesting that they may be important mediators of the physiological changes that occur in response to Myc (Tabor and Tabor, 1984; Sburlati *et al.*, 1991; Furuya *et al.*, 1994; Packham and Cleveland, 1994).

Other genes have been suggested to be targets of transcriptional regulation by Myc (Benvenisty *et al.*, 1992; Reisman *et al.*, 1993). At least in one case (ECA 39), the expression is limited to tumours occurring in response to deregulated expression of c-*myc* in transgenic mice, suggesting that some genes may be specific for transformation by Myc (versus induction of proliferation and apoptosis).

Transcription factors have been suggested to act as part of combinatorial networks. Enhancers and promoters often contain closely spaced or even overlapping binding sites for multiple transcription factors, and proteins binding to these elements engage in multiple co-operative and competitive interactions (Yamamoto *et al.*, 1992). Such interactions have been suggested to: (i) allow the generation of a large number of different outputs from a relatively small number of different factors (Tjian and Maniatis, 1994); (ii) enhance the specificity between closely related members of the same protein family or between very similar DNA sequence elements (Yamamoto *et al.*, 1992); (iii) allow the integration of multiple signals at a given promoter (Diamond *et al.*, 1990; Miner and Yamamoto, 1991); and (iv) allow tissue- or developmental stage-specific interpretation of incoming signals (Ness *et al.*, 1993). Often, sequence elements binding one factor are repeatedly associated with those of a second factor. Little is known about possible interactions between Myc and other transcription factors.

Sequence comparisons between Myc-response elements from the ODC and prothymosin-α gene show an extended sequence homology which is not accounted for by the known specificity of DNA binding of either Myc or Max (Halazonetis and Kandil, 1991; Blackwell *et al.*, 1993). We now report that, in both genes, this homology is due to the presence of high affinity binding sites for the transcription factor AP-2 (Imagawa *et al.*, 1987; Mitchell *et al.*, 1987). We also show that AP-2 acts as an inhibitor of Myc-mediated transactivation, a function that is mediated both by competition of AP-2 with binding of Myc or Max and by a direct protein−protein interaction of AP-2 with the BR/HLH/LZ domain of the Myc protein.

Results

Sequence comparison between Myc-response elements from the ODC and prothymosin-α genes revealed an extended sequence homology (Gaubatz *et al.*, 1994) which could not be accounted for by the known binding specificities of either Myc or Max (Blackwell *et al.*, 1990, 1993; Halazonetis and Kandil, 1991; Prendergast and Ziff, 1991). Comparison of this sequence to a database of binding sequences of known transcription factors showed a striking similarity to the published consensus binding site for the transcription factor AP-2, $GCCN_3GGC$ (Williams and Tjian, 1991a) (see Figure 1A). E-box elements from both rat and human prothymosin-α and from rat, human and hamster ODC genes matched this consensus. Database searches identified a number of similar elements in enhancers or promoters of other genes (Figure 1A). Elements that contain an E-box with an adjacent AP-2 site with slightly different spacing were also found in the promoter of the transforming growth factor β, in the human growth hormone enhancer and in the first intron of the *hsp90* gene. The functional significance of these elements is not known.

To test directly whether AP-2 could bind to the Myc-response element of the rat prothymosin-α gene, we expressed a histidine-tagged allele of AP-2 in *Escherichia coli* and purified it by Ni-affinity chromatography (see Materials and methods). Recombinant AP-2 had a purity of >90%, as judged from Coomassie staining of gels (see Figure 6B). In a gel retardation experiment, this preparation specifically recognized the Myc-response element from the prothymosin-α gene (Figure 1B). The specificity of this interaction was verified using different competitors. First, binding of AP-2 was abolished by competition with a 50-fold molar excess of wild-type oligonucleotide (ProT-wt), but not by competition with a oligonucleotide mutated at three nucleotides in one of the AP-2 half-sites (ProT-mut2; the sequences of the oligonucleotides are shown in Figure 1C). In contrast, mutation of the E-box did not affect competition for binding of AP-2 (ProT-mut4). Also, a synthetic E-box element carrying an optimized binding site for Myc (Halazonetis and Kandil, 1991) did not compete for binding of AP-2 (Figure 1B). For comparison, binding of recombinant Max to ProT-wt is shown in the left hand panel of Figure 1B. Second, we showed directly that mutations in either half-site of the potential AP-2 binding site abolished binding by recombinant AP-2 (data not shown). Similar experiments performed with competitor oligonucleotides encompassing the Myc-response elements from the hamster and human ODC genes revealed that both competed specifically for binding of recombinant AP-2 to a well-characterized binding site from the human metallothionein IIa distal basal level enhancer element (Imagawa *et al.*, 1987) (Met; see Figure 1D).

We wished to compare the relative affinities of AP-2 for the prothymosin-α element and a bona fide AP-2 binding site and used the binding site from the metallothionein enhancer. For these experiments, AP-2 was synthesized in a reticulocyte lysate and incubated with labelled ProT-wt oligonucleotide. Competition was carried out with a 100-, 250- or 500-fold molar excess of ProT-wt or Met oligonucleotide. Specificity of binding was ascertained by

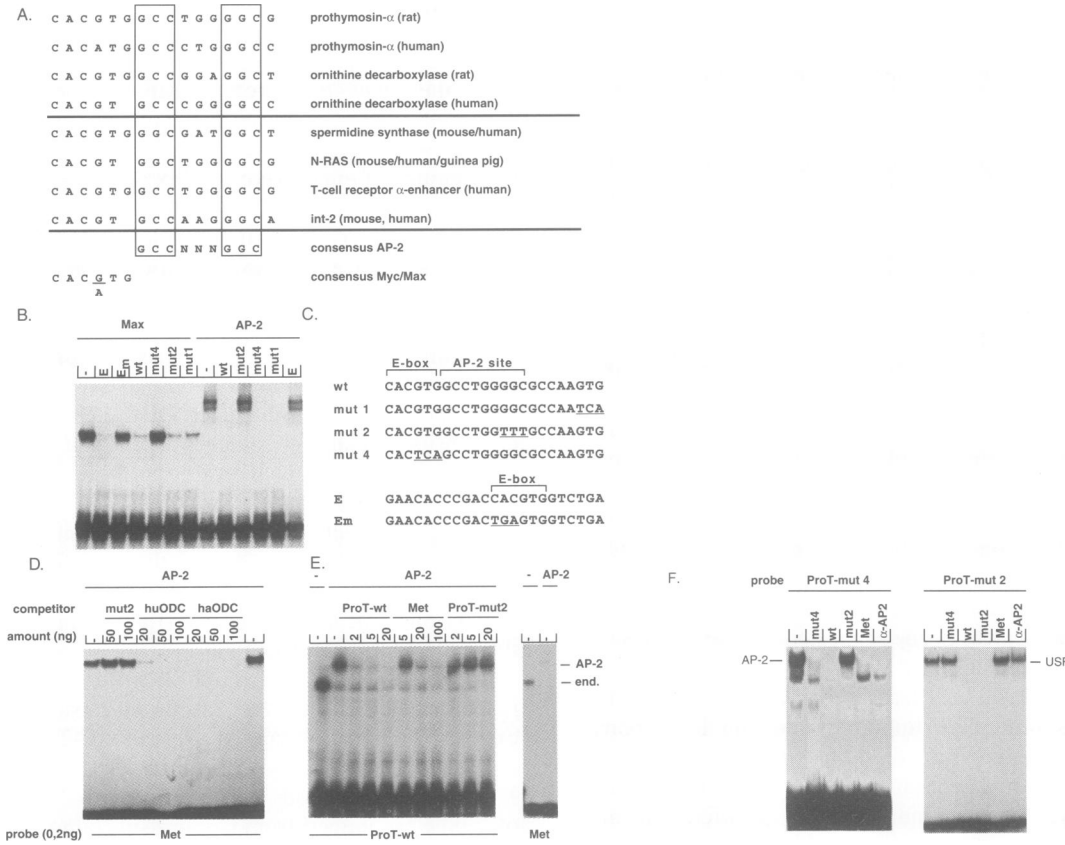


Fig. 1. High-affinity binding sites for AP-2 are present in the Myc-response elements of the prothymosin-α and ODC genes. (**A**) Sequence comparison between the Myc-response elements from prothymosin-α and ODC genes from different organisms (above the bar); also included are similar elements found in enhancers or promoters of other genes. The consensus sequences for Myc/Max binding and AP-2 binding sites are highlighted. (**B**) Binding of recombinant Max and AP-2 to the Myc-response element of the rat prothymosin-α gene. Shown is a gel shift assay with recombinant proteins purified from *E.coli*. Specificity is shown by competition with oligonucleotides at a 50-fold molar excess. The core sequences of the competitor oligonucleotides are shown in (C). E is a synthetic oligonucleotide carrying an optimized Myc-DNA binding site, and E_{mut} is a derivative with a mutated E-box (Halazonetis and Kandil, 1991). (**C**) Core sequence of the competitor oligonucleotides used in the gel shift assays. For the complete sequence of the 36 bp oligonucleotide encompassing the Myc-response element of the rat prothymosin-α gene (ProT-wt) see Materials and methods. (**D**) AP-2 specifically binds to Myc-response elements from the human and hamster ODC genes. Recombinant AP-2 was incubated with an oligonucleotide encompassing the AP-2 site from the metallothionein enhancer (Met) in the presence of a 100-, 250- or 500-fold molar excess of cold oligonucleotide from the human or hamster ODC genes (huODC and haODC, respectively). As a control for specificity, ProT-mut2 was also used as a competitor. (**E**) Relative affinities of AP-2 for the metallothionein and prothymosin-α response elements. AP-2 was synthesized in a reticulocyte lysate and incubated with either ProT-wt or the AP-2 site from the metallothionein promoter (Met). Competitors were added as indicated. end., endogenous binding activity present in some batches of reticulocyte lysate. (**F**) Demonstration of the presence of AP-2 in RAT1A nuclear extracts. RAT1A nuclear extracts were incubated with either radiolabelled ProT-mut4 or ProT-mut2 oligonucleotides, respectively. Addition of competing oligonucleotides and AP-2- specific antibody was performed as indicated.

competition with appropriately mutated oligonucleotides. The reverse experiment with labelled Met oligonucleotide was also performed. The data obtained (Figure 1E) indicate that AP-2 appears to bind with significantly higher affinity to the ProT-wt than to the Met oligonucleotide as (i) binding of AP-2 to ProT-wt was abolished by significantly lower amounts of cold ProT-wt oligonucleotide than Met oligonucleotide and (ii) binding to equimolar amounts of Met oligonucleotide was barely detectable under the conditions of this experiment. Longer exposures revealed that 2 ng of ProT-wt oligonucleotide completely abolished binding of AP-2 to the Met oligonucleotide (data not shown).

AP-2 has been reported to be expressed predominantly in neural crest-derived tissues during mouse embryogenesis (Mitchell *et al.*, 1990) and in embryonic and adult *Xenopus* epidermis during skin differentiation (Snape *et al.*, 1991). To analyse if AP-2 is expressed in cells in which regulation of prothymosin-α and ODC has been reported, we performed gel-shift experiments using nuclear extracts from RAT1A cells (Figure 1F). As probes, we used two oligonucleotides: one specifically mutated in the E-box (ProT-mut4), so that the presence of USF in nuclear extracts would not mask any potential binding by AP-2 and, secondly, an oligonucleotide mutated in the AP-2 binding site, which is bound mainly by USF in extracts from RAT1A cells (ProT-mut2) (A.Schneider, unpublished results). In extracts from RAT1A cells, a predominant band shift was observed when the oligonucleotide ProT-mut4 was used. This band was specifically competed for by either wild-type oligonucleotide (ProT-wt) or an excess of Met oligonucleotide, but not by an oligonucleotide carrying a mutated AP-2 binding site (ProT-mut2) (Figure 1F). In addition, pre-incubation of the nuclear extract with a polyclonal antibody against AP-2 abolished binding. Binding of USF from a nuclear extract was not affected by this antibody (Figure 1F, right panel). These data demonstrate the presence of AP-2 in RAT1A cells.

In addition, Northern and Western blot analysis also demonstrated the presence of AP-2 in RAT1A, HeLa, NIH-3T3 cells and HL-60 cells (data not shown). Taken together, our data show that Myc-response elements from both ODC and prothymosin-α genes contain high affinity AP-2 sites and that the AP-2 protein is present in cells in which regulation of these genes by Myc has been reported.

We then asked whether E-box binding proteins and AP-2 can bind simultaneously to the Myc-response element or whether they bind competitively. To address this question, we first characterized binding of recombinant AP-2, recombinant Max and purified USF by DNase I footprinting analysis. A fragment from the prothymosin-α intron encompassing the Myc-responsive element was end labelled and incubated with increasing amounts of DNase I, either in the absence or presence of either one of the three proteins. The results shown in Figure 2A demonstrate that all proteins lead to specific footprints that were consistent with the results of the gel retardation experiments shown in Figure 1. The region protected by AP-2 from digestion with DNase I extended significantly beyond the actual core binding site and included the central CACGTG sequence of the Myc-response element. Conversely, protection by either USF or Max extended through the GCCTGGGGC sequence recognized by AP-2. Based on these data, we predicted that AP-2 competes with E-box binding proteins for access to this element. To demonstrate this directly, ProT-wt oligonucleotide was pre-incubated with either Max (Figure 2B, left panel) or pre-assembled Myc/Max complexes (Figure 2B, right panel) and subsequently challenged with increasing amounts of recombinant AP-2 under conditions of limiting amounts of radioactive oligonucleotide. In both experiments, AP-2 displaced either Max/Max or Myc/Max complexes. Conversely, if AP-2 was pre-bound, displacement by Myc/Max could not be observed (data not shown). Both the Max/Max complex and the Myc/Max complex were supershifted by addition of anti-Max antibodies (Figure 2C). Similarly, the Myc/Max complex was supershifted by an anti-Myc antibody. In contrast, the complex observed after addition of AP-2 was not affected by either anti-Max or anti-Myc antibodies, even when both proteins were present in the reaction. We conclude that this complex contained only AP-2. Our data show that AP-2 competes for binding to ProT-wt with either Max/Max homodimers or Myc/Max heterodimers.

Transient transfection assays were used to address the role of AP-2 in transactivation by Myc. HeLa cells were transfected with a reporter plasmid carrying a 36 bp sequence encompassing the Myc-response element from the rat prothymosin-α gene in front of a tk81 minimal promoter (Gaubatz *et al.*, 1994) (Figure 3). Co-transfection of a CMV-Myc expression vector caused 20-fold stimulation of reporter gene expression. Increasing amounts of a CMV-AP-2 expression plasmid abolished transactivation by Myc in a dose-dependent manner. Expression of AP-2 alone had little effect on the basal activity of the reporter plasmid. Western blot analysis confirmed that AP-2 did not affect the expression of Myc from the transfected CMV-Myc vector. Similarly, co-transfection of AP-2 together with a CMV-luciferase vector had no effect on luciferase activity (data not shown), suggesting that AP-2 acts specifically as an inhibitor of Myc-mediated trans-

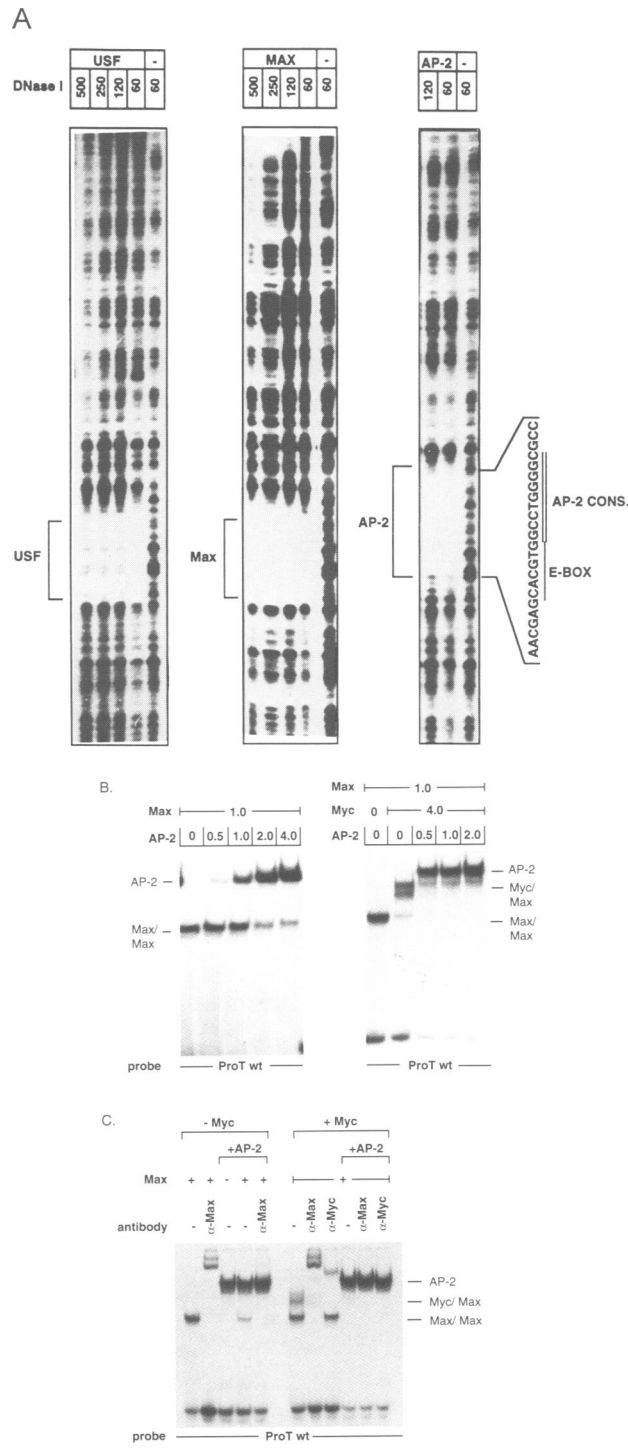


Fig. 2. AP-2 competes for binding with Max/Max complexes and Myc/Max complexes. (**A**) DNase I footprint analysis of the *Xma*I–*Eco*RI fragment of the first intron of the rat prothymosin-α gene. The DNA was end labelled at the *Xma*I site and incubated with increasing concentrations of DNase I (ng/ml) in the presence of recombinant USF, Max or AP-2 as indicated. Alignment of the consensus E-box and the AP-2 binding site is shown on the right. (**B**) Constant amounts of either recombinant Max protein (left panel) or pre-assembled Myc/Max complexes (right panel) were incubated with increasing amounts of recombinant AP-2 before addition of the oligonucleotide probe (ProT-wt). This gel shift assay was performed under conditions where the probe was limiting (50 pg probe). (**C**) Supershift by anti-Max and anti-Myc antibodies. Gel shift assay using 50 pg of the ProT-wt probe and constant amounts of either Max or pre-assembled Myc/Max complexes with or without added AP-2 protein and anti-Max or anti-Myc antibodies as indicated.

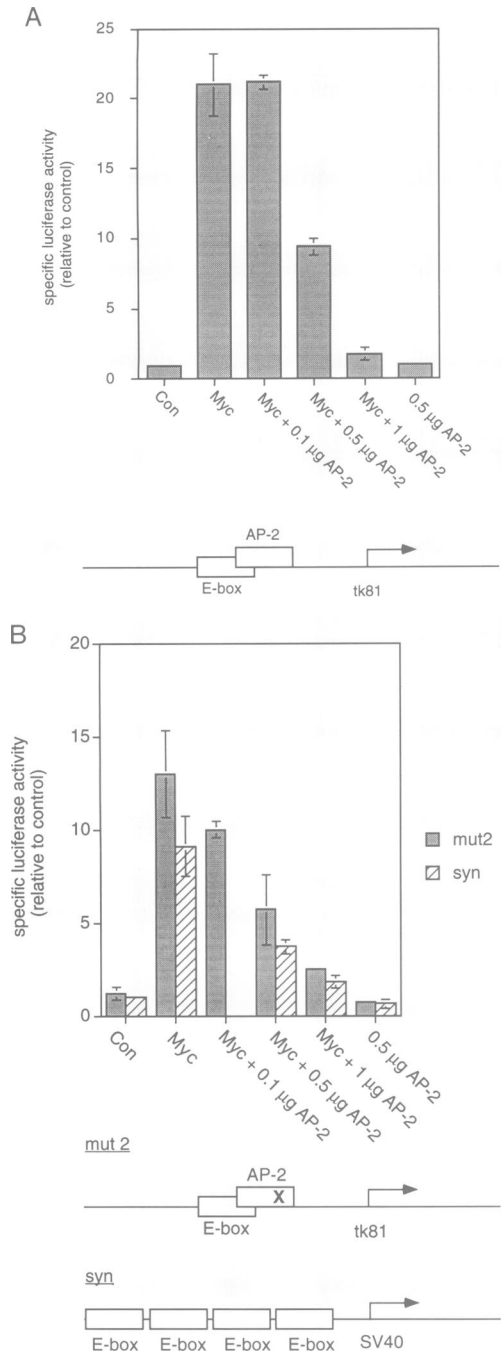


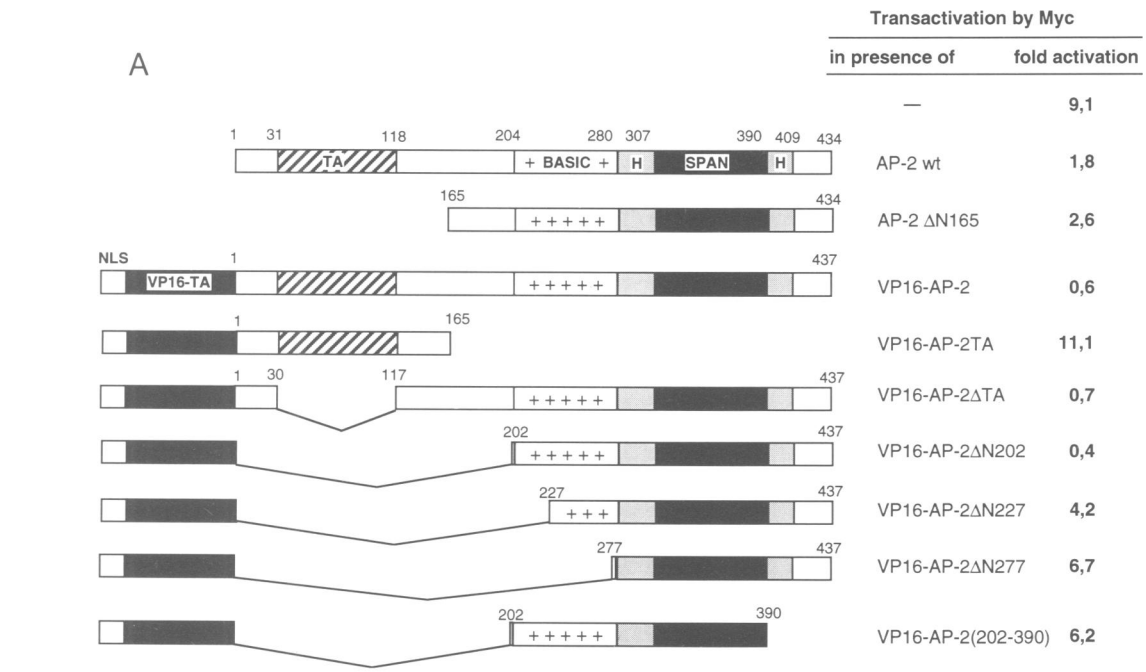
Fig. 3. AP-2 inhibits Myc-mediated transactivation. Transient transfection assays were performed in HeLa cells using a luciferase reporter vector with the wild-type Myc-responsive element from the prothymosin-α gene in front of a minimal TK promoter (**A**), with a mutated version of the Myc- responsive element (ProT-mut2) or a reporter with four synthetic E-box elements in front of a minimal SV40 promoter (syn) (**B**). Myc: co-transfection of 1 µg CMV-Myc expression plasmid. AP-2, co-transfection of CMV-AP-2 expression vector in the amounts indicated; Con, control transfection. CMV-Myc was substituted by 1 µg of empty CMV plasmid.

activation. Taken together, our data show that AP-2 competes for binding of either Myc/Max or Max/Max complexes to the Myc-response element and that AP-2 acts as an inhibitor of Myc-mediated transactivation.

We next tested whether binding of AP-2 to DNA was required for inhibition of Myc-mediated transactivation by AP-2. To address this question, we used a similar reporter construct that carried a 3 bp mutation in one of the half-sites of the AP-2 binding site. The sequence of this reporter corresponds to ProT-mut2 as shown in Figure 1C. Gel retardation experiments demonstrated that this mutation abolished binding of AP-2 without affecting recognition by either Max/Max or Myc/Max complexes (Figure 1B and data not shown). Surprisingly, AP-2 still inhibited transactivation by Myc on this reporter plasmid (Figure 3B). To confirm this observation, we used a reporter plasmid that carried multiple synthetic E-box elements in front of an SV40 minimal promoter (kind gift of George Prendergast) and obtained identical results (Figure 3B). Although we cannot formally exclude that cryptic binding sites for AP-2 affected our results, these data suggest that not only can AP-2 compete for a common binding site, but an additional mechanism must exist by which AP-2 can inhibit Myc function.

In order to identify this mechanism, we analysed which domain of the Myc protein was subject to negative regulation by AP-2 and, further, which domain of the AP-2 protein was required for the repression. A number of deletion mutants of AP-2 were constructed and tested for inhibition of Myc function on a prothymosin-α reporter plasmid lacking the AP-2 binding site. AP-2 deletion mutants were constructed such that the plasmid provided a nuclear localization signal (NLS) and a strong transactivation domain of the viral VP16 protein (Triezenberg *et al.*, 1988). This was done for two reasons. First, the NLS ensured that the chimeras were targeted to the nucleus. Second, the VP16 transactivation domain allowed us to test whether any of the chimeric proteins were targeted to DNA by Myc, even in the absence of a direct DNA binding site for AP-2. Since a reporter construct with a mutated AP-2 site was used in this experiment, expression of the VP16−AP-2 chimeras by themselves did not induce luciferase activity. The data (Figure 4A) show that both full-length AP-2 and a mutant lacking the entire transactivation domain (AP-2ΔN165) inhibited transactivation by Myc. The N-terminal region of AP-2 has been shown to squelch a number of transactivation domains (Kannan *et al.*, 1994), but these data show that this mechanism is not responsible for inhibition of transactivation by Myc (see also Figure 4B). Further deletion analysis showed that the C-terminal region of AP-2 between amino acids 203 and 437 was sufficient to inhibit transactivation by Myc. This region includes potential DNA binding and dimerization domains of the AP-2 protein (Williams and Tjian, 1991b).

Similarly, chimeras between Myc and either the DNA binding domain of the yeast transcription factor GAL4 (Sadowski *et al.*, 1988) or the transactivation domains of the Oct-2 (Annweiler *et al.*, 1994) or VP16 (Triezenberg *et al.*, 1988) proteins were used to identify which domains in Myc were sufficient to be inhibited by AP-2. The ability of the different chimeras to transactivate was also measured in the presence of the N-terminal deletion mutants of AP-2, VP16−AP-2ΔN202 and VP16−AP-2ΔN227. The results are summarized in Figure 4B. Replacing the DNA binding domain of Myc with that of GAL4 almost completely abolished inhibition by AP-2 [Gal4−Myc(1–262)]; conversely, chimeric proteins that carried the transactivation domains of either Oct-2 or VP16 fused to



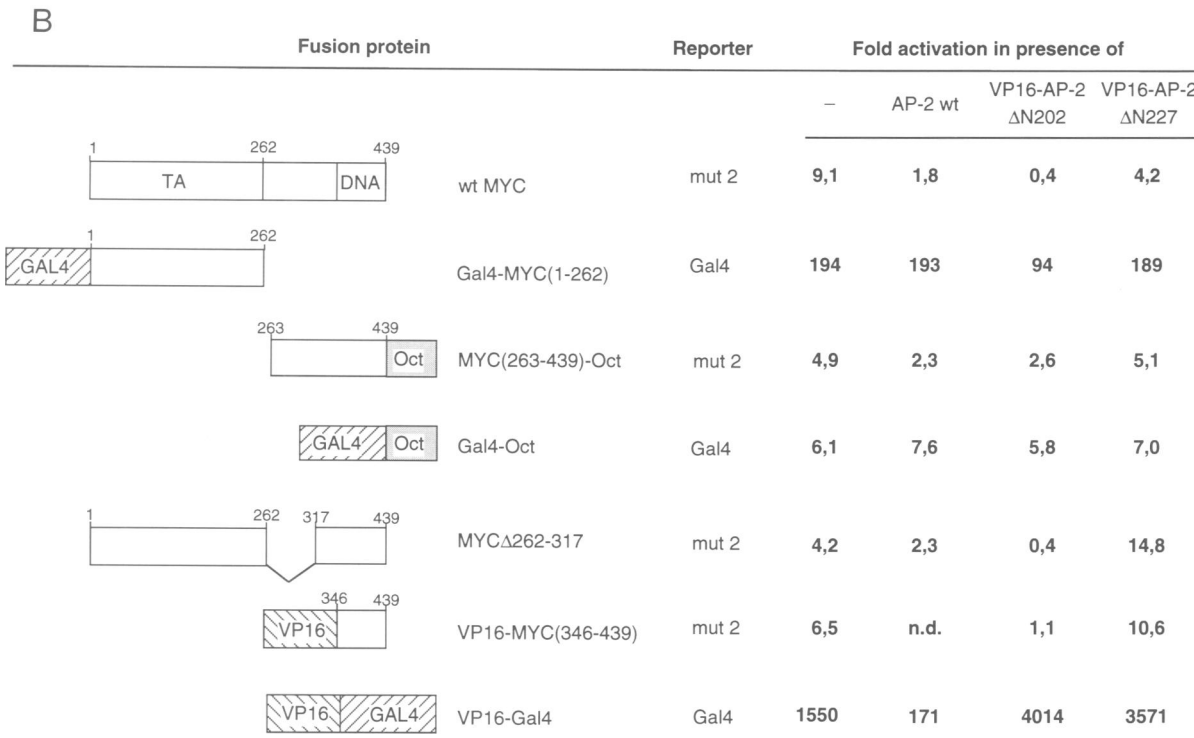


Fig. 4. Domains in the AP-2 protein and Myc protein that are required for repression of Myc-mediated transactivation. Summary of transient transfection assays in HeLa cells using a ProT-mut2 luciferase reporter and a Gal4 luciferase reporter. (**A**) The C-terminus of AP-2 is sufficient for the repression of Myc-mediated transactivation. Shown is the domain structure of the AP-2 protein (Williams and Tjian, 1991a) and of the deletion mutants of AP-2 used in this study. Five μg of the ProT-mut2 luciferase reporter plasmid was co-transfected with 0.5 μg of the indicated CMV-AP-2 expression vector and with either 1 μg CMV-Myc expression vector or 1 μg empty CMV vector. In a control transfection, CMV-AP-2 expression vector was replaced by the empty CMV vector (−). Transactivation by Myc was calculated by dividing the luciferase activity in the presence of CMV-Myc and the respective AP-2 construct by the luciferase activity in the absence of CMV-Myc and normalizing to β-gal activity. TA, transactivation domain of AP-2; NLS, nuclear localization signal; VP16-TA, transactivation domain of VP-16. H-SPAN-H, helix−span−helix motif of AP-2. (**B**) The DNA binding and dimerization domains of Myc are subject to negative regulation by AP-2. Shown is the domain structure of the wild-type Myc protein and of the fusion proteins used in this study. The open bars represent the portion of the Myc protein present in the different fusion proteins. Fusion proteins are aligned to the wild-type Myc protein at the top. Five μg of the ProT-mut2 luciferase reporter plasmid (mut2) or of the Gal4 luciferase reporter plasmid (Gal4) were co-transfected with either 1 μg expression vector or with the respective empty vector. The transactivation that was obtained by the different fusion proteins in the absence or presence of 0.5 μg CMV-AP-2, CMV-VP16−AP-2ΔN202 or CMV-VP-16−AP-2ΔN227 was calculated as in (A) TA, transactivation domain of Myc; DNA, DNA binding domain and dimerization domain of Myc; Gal4, DNA binding domain of Gal4; Oct, C-terminal transactivation domain of Oct-1; VP-16, transactivation domain of VP-16.

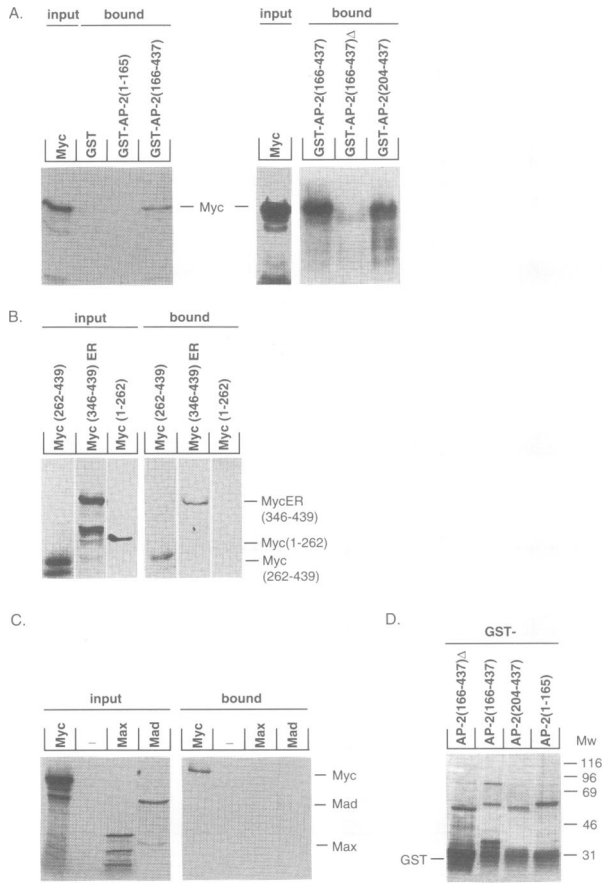


Fig. 5. The C-terminus of Myc, but not Max or Mad, forms a specific complex with the C-terminus of AP-2. (**A**) The indicated GST−AP-2 fusion proteins were incubated with 5 µl [^{35}S]methionine-labelled Myc. Bound proteins were resolved by SDS−PAGE and fluorography. As standard, 2 µl *in vitro* translated Myc was loaded directly on the gel ('input'). (**B**) The indicated C-terminally truncated and two N-terminally truncated alleles of Myc were assayed for interaction with GST−AP-2(166–437) as in (A). (**C**) Max and Mad were transcribed/translated *in vitro* and incubated with GST−AP-2(166–437). Bound proteins (right panel) were resolved as in (A). As a positive control, Myc was included in this experiment. (**D**) SDS−PAGE of GST−AP-2 fusion proteins used for *in vitro* binding assays.

C-terminal regions of Myc [Myc(263–439)−Oct and VP16−Myc(346–439), respectively] were still inhibited by AP-2. Importantly, a chimera that carried only the BR/HLH/LZ domain of Myc fused to VP16 [VP16−Myc(346–439)] was, like the wild-type Myc protein, unable to transactivate in the presence of VP16−AP-2ΔN202, but able to transactivate in the presence of VP16−AP-2ΔN227. Transactivation by a similar VP16−GAL4 chimera was unaffected by either AP-2 derivative. Inhibition was not due to the squelching of the transactivation domain of the VP16−Myc chimera by AP-2, as this required the N-terminus of the AP-2 protein (see inhibition of GAL4−VP16 by full length AP-2). These data show that the DNA binding and dimerization domains of Myc are subject to negative regulation by the C-terminus of AP-2.

We investigated the possibility that this inhibition might be due to protein−protein interactions between the Myc and AP-2 proteins. To test this hypothesis, Myc protein synthesized in a reticulocyte lysate was incubated with equal amounts (Figure 5D) of either glutathione transferase (GST), or GST fusion proteins containing either N-terminal [GST−AP-2(1–165)] or C-terminal [GST−AP-2(166–437)] fragments of AP-2, immobilized on glutathione agarose (Figure 5A, left panel). The results show that Myc specifically interacted with the C-terminal region of AP-2. No interaction occurred with either GST alone or with GST fused to the N-terminus of AP-2. Amino acids 204–437 of AP-2 were sufficient to mediate interaction with Myc [GST−AP-2 (204–437); Figure 5A, right panel]. Internal deletion of amino acids 203–227 within GST−AP-2(166–437) [generating GST−AP-2(166–437Δ)] significantly impaired binding (Figure 5A, right panel). Neither Max or Mad bound to GST−AP-2 in similar experiments (Figure 5C).

One C-terminally truncated Myc(1–262) and two N-terminally truncated alleles of Myc [Myc(262–439) and Myc(345–439)] were synthesized in a reticulocyte lysate to localize the domain of Myc which mediates interaction with AP-2 (Figure 5B). The latter protein was synthesized as a fusion with the hormone binding domain of the human oestrogen receptor (Kumar *et al.*, 1986) as this fragment of Myc does not contain a methionine. The results show that the DNA binding and dimerization domains of Myc are required and sufficient for interaction with AP-2. Taken together, our data show that Myc and AP-2 can form a specific protein−protein complex. Domains of Myc and AP-2 that mediate complex formation are identical to those which mediate inhibition, strongly suggesting that inhibition of Myc- mediated transactivation by AP-2 is, at least in part, due to the formation of this complex.

To assess the functional consequences of AP-2 binding to Myc, we investigated whether AP-2 might compete for binding of Max to Myc or whether AP-2 might interfere with DNA binding of the Myc/Max complex. Two experiments were performed to test these hypotheses. First, *in vitro* binding experiments similar to those described above were carried out after mixing reticulocyte lysates containing Myc and Max proteins. Under these conditions, Max has been shown to associate with Myc *in vitro* (Kato *et al.*, 1992). From these lysates, both Myc and Max could be specifically recovered on GST−AP-2(166–437) (Figure 6A); in the absence of Myc, Max did not associate with GST−AP-2(166–437). We conclude that binding of AP-2 does not preclude association of Myc with Max.

Next we tested whether binding of AP-2 interfered with DNA binding of the Myc/Max complex in the absence of a DNA binding site for AP-2. Full length Myc and Max proteins were expressed in *E.coli* and purified to >90% homogeneity, as judged by Coomassie staining (Figure 6B). Consistent with previous observations, full length Myc protein did not bind to DNA by itself (Figure 6C); in contrast, purified Max bound efficiently to DNA. Pre-incubation of Myc with Max at 42°C (Kato *et al.*, 1992) led to the appearance of a new complex, which bound to DNA specifically (Figure 6C) and reacted with both α-Myc and α-Max antibodies (data not shown). However, since no binding of Myc to GST−AP-2(166–437) was observed at temperatures higher than room temperature (data not shown), we tested whether assembly of the Myc/Max complex could be achieved at temperatures lower than 42°C. We found that, at 15°C, Myc/Max complexes progressively assembled over a time period of

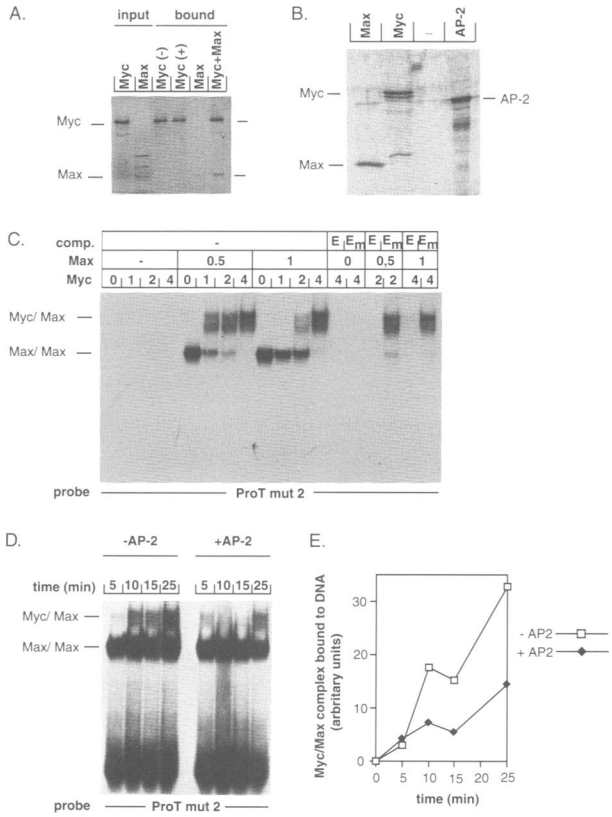


Fig. 6. AP-2 interferes with the DNA binding but not with the formation of the Myc/Max complex. (**A**) *In vitro* binding assays were performed similarly as in Figure 5. Myc and Max were transcribed/translated *in vitro* and incubated with GST−AP-2(166–437) either without (−) or after pre-incubation (+) for 30 min at 30°C. (**B**) SDS−PAGE of purified proteins used in gel shift assays. (**C**) Formation of the Myc/Max complex and binding to the Myc-responsive element of the rat prothymosin-α gene. Gel shift assay using 0.2 ng of the ProT-mut2 probe and purified recombinant proteins. The indicated amounts of Myc and Max were pre-incubated at 42°C for 15 min before addition of the probe in order to allow the assembly of the protein complex. Specificity of the binding is shown by the addition of competing oligonucleotides. E, E-box oligonucleotide; E_m, same oligonucleotide with a mutated E-box (see Figure 1). (**D**) AP-2 inhibits the formation of the Myc/Max complex at 15°C. Myc and Max proteins were pre-incubated for the indicated time period in the absence (−AP-2, left panel) or presence of recombinant AP-2 (+ AP-2, right panel) before addition of 0.2 ng of the ProT-mut2 probe. (**E**) Quantitation of the Myc/Max complexes from the experiment shown in panel D.

30 min (Figure 6D). The addition of stochiometric amounts of recombinant AP-2 to these assembly reactions decreased the speed and extent of complex assembly 2- to 3- fold (Figure 6D). A quantitation of the results is shown in Figure 6E. These data show that binding of AP-2 to Myc does not preclude association of Max with Myc but impairs binding of the Myc/Max complex to DNA in the absence of a DNA binding site for AP-2.

To test whether AP-2 interacts with Myc *in vivo*, a polyclonal antibody was raised in rabbits against recombinant AP-2 protein. HeLa cells were transfected with CMV-Myc and labelled with [^{35}S]methionine. Cells were lysed by mild sonification and immunoprecipitated with pre-immune serum, anti-AP-2 serum, or with a monoclonal antibody against Myc. Figure 7A (left panel) shows that precipitations with the anti-AP-2 antibody contained a protein of the expected size (55 kDa). Comparison of the partial V8 protease digestion pattern of this protein with that of *in vitro* translated AP-2 revealed that this protein was indeed AP-2 (right panel). A small amount of a protein of similar size was found in precipitations with pre-immune antibody; however, no clear V8 pattern could be obtained from this band, suggesting that this protein differed from AP-2. A protein of the same size as AP-2 was found in precipitations with an anti-Myc monoclonal antibody in the presence of non-ionic, but not ionic detergents (Figure 7A, left panel). V8 protease digestion of the excised band showed that this was indeed AP-2 (Figure 7A, right panel). The Myc protein itself was not visible in these immunoprecipitations since it was not efficiently labelled under the conditions of the experiment. For further support that Myc and AP-2 form a complex *in vivo*, lysates from CMV-Myc-transfected HeLa cells were prepared. Immunoprecipitations were performed with either AP-2 antiserum covalently linked to protein A beads or with coupled pre-immune serum. Bound proteins were separated by gel electrophoresis, transferred to a filter and probed with an anti-Myc antibody. Figure 7B shows that Myc protein was present in anti-AP-2 immunoprecipitations, but not in immunoprecipitations using pre-immune serum. Addition of ionic detergent abolished co-fractionation of Myc with AP-2 (Figure 7B; 'RIPA'). The same result was observed using a second rabbit anti-AP-2 serum (not shown). We conclude from these experiments that AP-2 and Myc form a specific complex *in vivo*. To confirm that endogenous Myc and AP-2 proteins are associated *in vivo*, we analysed whether Myc co-purifies with AP-2 on oligonucleotide affinity beads. Nuclear extracts were prepared from non-transfected HeLa cells and incubated with beads to which an oligonucleotide with an AP-2 DNA binding site (from the metallothionein enhancer) was covalently linked. Beads were washed and specifically bound proteins were eluted in 600 mM KCl, separated by gel electrophoresis and transferred onto filters by Western blotting (Figure 7C). Filters were probed with anti-AP-2 or anti-Myc antibody. Comparison with the total amount of protein contained in each fraction (shown at bottom) revealed that AP-2 was enriched >250- fold on the oligonucleotide affinity beads (right panel). Probing the filters with a Myc-specific antibody revealed that Myc co-purified with AP-2 (left panel) and was enriched by a similar fold. Taken together, these data show that Myc forms a specific complex with AP-2 *in vivo*.

Discussion

In this report, we show that Myc-response elements from two target genes of Myc, prothymosin-α and ODC, each contain a high affinity binding site for the transcription factor AP-2 juxtaposed to the E-box. Through these elements, AP-2 inhibits transactivation by Myc by competing for DNA binding by Myc and by directly interacting with the Myc protein, which impairs DNA binding of the Myc/Max complex (Figure 8 gives a schematic summary of the results). Our data define AP-2 as a negative regulator of Myc function.

AP-2 was originally identified as a protein binding specific sequences within the SV40 and metallothionein enhancers (Imagawa *et al.*, 1987; Mitchell *et al.*, 1987).

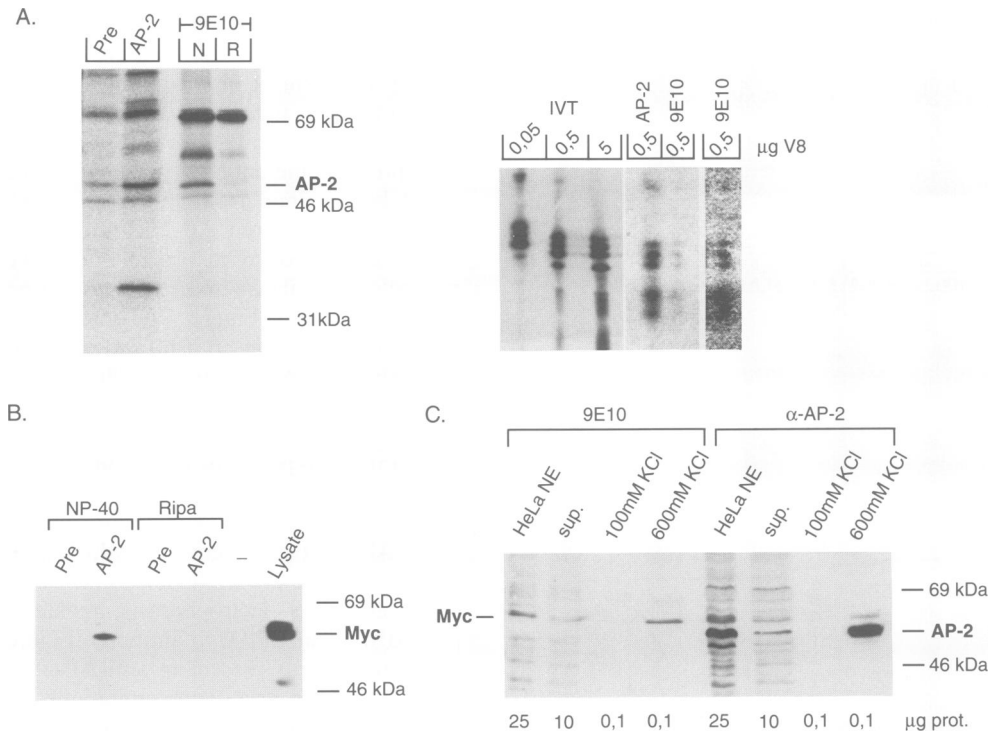


Fig. 7. Binding of Myc to AP-2 *in vivo*. (**A**) The 55 kDa protein that co-precipitates with Myc is AP-2. HeLa cells were transfected with CMV-Myc and labelled with [^{35}S]methionine. Cell lysates were subjected to precipitation with pre-immune serum (Pre), anti-AP-2 serum (AP-2) or monoclonal Myc antibody (9E10). Immunoprecipitations were performed either in a buffer containing 0.2% NP-40 (N) or in RIPA buffer (R). Immune complexes were separated on a 10% polyacrylamide gel. Gels were dried and proteins were detected by autoradiography. From a similar experiment, the 55 kDa protein present in AP-2 and Myc immunoprecipitations was excised from the dried gel and subjected to an in-gel V8 protease digest. For comparison, *in vitro* translated human AP-2 protein (IVT) was similarly subjected to digestion with increasing amounts of V8 protease. The right panel is a longer exposure of the V8 pattern of the 55 kDa protein that co-precipitates with Myc. (**B**) Myc co-precipitates with AP-2. HeLa cells were transfected with CMV-Myc. Cell lysates were immunoprecipitated with pre-immune serum or anti-AP-2 serum coupled to protein A beads. Immunoprecipitation was performed either in a buffer with 0.2% NP-40 or in RIPA. Immune complexes were separated on a 10% polyacrylamide gel. Proteins were transferred on PVDF membrane and probed with a monoclonal anti-Myc antibody (9E10). An aliquot of the HeLa lysate (5% of the input) was loaded directly onto the gel (Lysate). (**C**) Myc and AP-2 co-purify on a AP-2 DNA binding site. HeLa nuclear extracts were incubated with affinity beads to which an oligonucleotide with the AP-2 binding site of the metallothionein enhancer was covalently coupled. Beads were washed three times in 100 mM KCl and bound proteins were eluted in 600 mM KCl. Proteins were separated by SDS–gel electrophoresis, transferred to PVDF and probed with either anti-Myc antibody (9E10) or with anti-AP-2 antiserum. NE, nuclear extract; sup, supernatant of the affinity beads.

In vitro, AP-2 forms homodimers via a helix–span–helix motif that consists of two regions predicted to form amphipathic α-helices separated by a span of 80 amino acids (Williams and Tjian, 1991b). AP-2 has been reported to act as a transcriptional activator in some cells (Williams and Tjian, 1991a). However, transactivation by AP-2 is often low and is restricted to a few cell lines in transient transfection assays (Williams and Tjian, 1991a; Kannan *et al.*, 1994). Also, the proline-rich transactivation domain of AP-2 has been found to be a poor activator if positioned in a distal position, even under conditions where it can activate from a promoter position (Seipel *et al.*, 1992), making it unlikely that AP-2 could activate from this position. Both in the prothymosin-α and in the ODC genes, Myc-response elements are localized in introns up to several kilobase pairs away from the start site of transcription. These data are consistent with the proposed role of AP-2 acting as a inhibitor of Myc-mediated transactivation.

What is the role of AP-2 in gene regulation by Myc? We considered the possibility that Myc might differ from other E-box binding proteins in its interaction with AP-2, and the presence of an adjacent AP-2 site might 'select' between different proteins binding to this E-box (Gaubatz

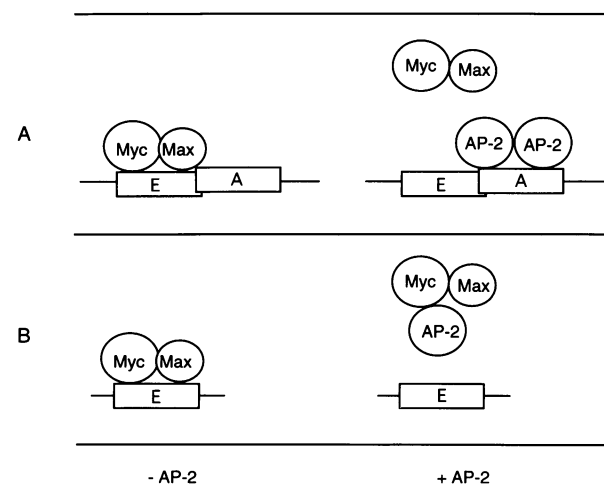


Fig. 8. Summary of the possible interactions between AP-2 and Myc on the prothymosin Myc-response element. AP-2 inhibits transactivation by Myc by a direct competition for DNA binding (**A**) and by formation of a ternary Myc/Max/AP-2 complex which has impaired DNA binding activity (**B**).

et al., 1994). We have tested this hypothesis by stably re-intregrating reporter plasmids that carry either wild-type or mutated alleles of the prothymosin-reporter plasmids into RAT1A-MycER and measuring activation by Myc *in vivo*. No difference between the induction of different reporter plasmids in response to oestrogen was observed (S.Gaubatz and M.Eilers, unpublished). Also, in transient transfection experiments, transactivation by either USF or Myc is not significantly affected by mutations in the distant half-site of the AP-2 site (mutations in the proximal half-site impair transactivation by both, presumably by affecting the residues flanking the E-box; S.Gaubatz and M.Eilers, unpublished). Similarly, the presence of AP-2 sites might distinguish between different Myc-regulated genes and might put a constraint on the regulation of some genes that is not present on others. In this fashion, differential regulation of target genes by Myc might be achieved. However, there is at present no evidence for this notion.

We favour the hypothesis that the interaction of Myc with AP-2 might serve to integrate signals from different signal transduction pathways on a single response element. Several lines of evidence suggest a role for AP-2 during cellular differentiation. First, expression of AP-2 is often up-regulated during retinoic acid- or forskolin-induced differentiation *in vitro* (Lüscher *et al.*, 1989). For example, during HL-60 differentiation, expression of prothymosin-α is down-regulated (Smith *et al.*, 1993). Expression of Myc parallels that of prothymosin-α in these cells. In contrast, AP-2 is expressed constitutively at high levels and further increases during *in vitro* differentiation of HL-60 cells (R.Dosch and M.Eilers, unpublished observation). In keratinocytes, the response of keratin genes to retinoic acid is mediated by AP-2 (Leask *et al.*, 1990, 1991; Stellmach *et al.*, 1991). Second, dominant negative alleles of AP-2 that interfere with DNA binding inhibit differentiation of human teratocarcinoma cells and instead transform these cells (Buettner *et al.*, 1993). Inhibition of Myc transactivation by AP-2 may be one mechanism that contributes to the inhibition of cell proliferation that often accompanies cell differentiation. Our data suggest that, during cellular differentiation, joined E-box/AP-2 elements may 'switch' from an active state, in which they are occupied by Myc/Max heterodimers, to an inactive state in which they are occupied by AP-2 homodimers.

Intriguingly, expression of AP-2 has also been shown to be up-regulated by the expression of N-RAS in human teratocarcinoma cells (Kannan *et al.*, 1994). Although N-RAS-transformed teratocarcinoma cells are tumorigenic, they grow more slowly in culture and acquire a more differentiated phenotype (R.Buettner, unpublished observation). Similarly, expression of RAS causes a stop in proliferation in PC12 cells, secondary rat embryo fibroblasts and Schwann cells, and this can be overcome by the expression of c-*myc* (Land *et al.*, 1986; Ridley *et al.*, 1988). In addition, it is tempting to speculate that SV40 large T antigens might interfere with this signalling pathway: SV40 large T has been shown to form a stable complex with AP-2 and inhibits its binding to DNA (Mitchell *et al.*, 1987). Thereby, expression of SV40 large T might relieve an inhibitory constraint on the function of Myc. As Myc function is required for cell proliferation induced by SV40 large T (Hermeking *et al.*, 1994), binding of AP-2 by SV40 large T might contribute to large T-induced proliferation.

No interaction was found between AP-2 and either Max or Mad, suggesting that both Max/Mad heterodimers are stable in the presence of AP-2. Indeed, in the absence of an AP-2 binding site on DNA, AP-2 had no effect on DNA binding by Max/Max heterodimers *in vitro*. Therefore, in a differentiated cell, the Myc-response element described may exist in alternate states in which it is either occupied by AP-2 or by Max/Max or Mad/Max complexes. We do not understand which rules (except relative abundance and affinity) might govern the occupancy and why different pathways exist. *In situ* hybridization shows that the tissues in which highest levels of AP-2 are found differ from those showing highest levels of expression of Mad mRNA (Mitchell *et al.*, 1990; Kari Alitalo, personal communication). These data suggest that, *in vivo*, alternate pathways may be used by different cells to down-regulate Myc-dependent genes during differentiation.

Materials and methods

Transient transfection experiments

Transient transfection experiments were performed using a standard calcium phosphate protocol as described previously (Sambrook *et al.*, 1989). Briefly, 5 μg reporter plasmid were co-transfected with the indicated amounts of expression vector. The luciferase reporter plasmid with the E-box and AP-2 binding site of the prothymosin-α gene (ProT-wt-tk81luc) has been described (Gaubatz *et al.*, 1994). In ProT-mut2-tk81luc, the AP-2 site is mutated (see Figure 1C). Gal4tkluc is a luciferase reporter with four Gal4 binding sites, syn is a luciferase reporter with four synthetic E-boxes (kind gift of G.Prendergast). In each transfection 0.5 μg Rous sarcoma virus (RSV)-β-gal were co-transfected to normalize for different transfection efficiencies. Results shown represent the average of three independent transfection assays normalized to galactosidase activity. The CMV-Myc expression vector has been described (Philipp *et al.*, 1994). CMV-MycΔ264–317 was generated by transferring the mutant fragment from MLV-MycΔ264–317 (Stone *et al.*, 1987) into CMV-Myc. Expression vectors encoding Gal4–Oct and VP16–Gal4 have been described (Annweiler *et al.*, 1994) and were kindly provided by Dr Thomas Wirth. A vector for the expression of the Myc(263–439)–Oct fusion was obtained by replacing the Gal4 sequences in Gal4–Oct by the sequences encoding for the amino acids 263–439 of human Myc. Gal4–Myc(1–262) was generated by transferring the *Eco*RI–*Cla*I fragment of human c-*myc* (encoding amino acids 1–262) from pSP65cmyc (Eilers *et al.*, 1989) into pSG424 (Sadowski and Ptashne, 1989). To generate VP16–Myc(346–439), polymerase chain reaction (PCR) was used to amplify a fragment encoding amino acids 346–439 of human Myc. The primers used were: 5′ primer: (5′-GGG AAT TCA CCA TGA TCG ATG TTG TTT CTG TGG-3′); 3′ primer (5′-GGC TCG AGG CAC AAG AGT TCC GTA GCT G-3′).

Vectors for the expression of full length murine AP-2 and a chimeric human–mouse N-terminal-deleted AP-2 (ΔN165) were obtained by insertion of AP-2 cDNA into the CMV-based vector pSCT (Huang *et al.*, 1988) and were kindly provided by Marieke Koedood and Pascal Meier. The last three amino acids of AP-2 (434–437) were modified and are followed by an epitope from vesicular stomatitis virus glycoprotein (Kreis, 1986). A CMV-based expression vector for human AP-2 A was obtained by cloning the AP-2 A cDNAs into pCMX-PL1. Deletion mutants of AP-2 (see Figure 4B) were generated by cloning the appropriate human AP-2 A fragment into the expression vector CMV-NLS-VP16 (Zwilling *et al.*, 1995) after amplification by PCR. The primers used were:

VP16–AP-2TA:	5′ primer (5′-GGAGATCTGATGCTTTGGAAA-TTACGGAT-3′) 3′ primer (5′-CGGCTCGAGCTAACCCGGGTC-TTCTACATGCGG-3′).
VP16–AP-2ΔN202:	5′ primer (5′-GGGGTACCCGGCGGCGTGGTG-AACCCCAAC 3′)

VP16−AP-2ΔN227: 5′ primer (5′ GGGGTACCGTACAAGGTCACG-GTGGCGGAA-3′)
VP16−AP-2ΔN277: 5′ primer (5′-GGGGTACCTGCAGGGAGACG-TAAAGCT-3′)
3′ primer (5′-CGGCTCGAGTCACTTTCTGTG-CTTCTCCTC-3′).
VP16−AP-2(202–390): 5′ primer (5′-GGGGTACCCGGCGGCGTGGTG-AACCCCAAC-3′)
3′ primer (5′-CGGCTCGAGTCACGCGGGGCT-GCCGAAGCCGTG-3′).

VP16−AP-2 (expressing the full length human AP-2) was derived from VP16−AP-2TA by insertion of the *Bst*XI−*Xho*I fragment of human AP-2. Finally, an in-frame *Pst*I deletion in VP16−AP-2 generated VP16−AP-2ΔTA.

DNase I footprint analysis

Probes for DNase footprinting were prepared by end-labelling the 350 bp *Xma*I−*Eco*RI fragment from the first intron of the rat prothymosin-α gene. DNA (5 μg) was digested with *Xma*I and end-labelled with 80 μCi [γ-^{32}P]dCTP and 2 U Klenow enzyme for 30 min at 30°C. After heat inactivation for 10 min at 70°C, the DNA was digested with *Eco*RI and isolated by agarose gel electrophoresis. Proteins were incubated with 20 000 c.p.m. end-labelled DNA in 10 mM HEPES, pH 7.8; 6 mM $MgCl_2$; 0.5 mM dithiothreitol (DTT); 10% glycerol; 50 mM KCl; 0.05% NP-40; 10 ng/μl poly(dI)·poly(dC); 0.4 mg/ml bovine serum albumin (BSA); 2% polyvinylethanol for 30 min at 0°C and 1 min at room temperature. Fifty microlitres of DNase I (final concentration: 60–500 ng/ml) in 50 mM NaCl; 10 mM $MgCl_2$; 1 mM $CaCl_2$; 20 mM HEPES, pH 7.8; 10% glycerol was added and incubated for exactly 1 min at room temperature. Free DNA was digested with 30 ng/ml DNase I. The reaction was stopped by addition of 90 μl stop solution (20 mM EDTA, pH 8.0; 1% SDS, 200 mM NaCl, 250 μg/ml tRNA) and 1 μl of proteinase K (20 mg/ml). After 20 min at 37°C, the DNA was extracted twice with equilibrated phenol, twice with chloroform, precipitated and analysed on a 6% polyacrylamide−7 M urea gel. A G + A sequencing reaction was performed according to Sambrook *et al.* (1989) and run on the same gel as a length standard.

Gel shift experiments

Gel shift assays were essentially performed as described previously (Blackwood and Eisenman, 1991). A 36 bp oligonucleotide encompassing the Myc-responsive element of the rat prothymosin-α gene was used: ('ProT-wt': 5′-GCG CAC TTG GCG CCC CAG GCC ACG TGC TCG TTG CGC-3′). The sequence of the mutated derivatives is shown in Figure 1C. Other oligonucleotides used: AP-2 binding site of the human metallothionein II enhancer (Met: 5′-TGA CCG CCC GCG GCC CGT-3′) and an oligonucleotide with an optimized Myc binding site (Halazonetis and Kandil, 1991).

USF (partially purified from HeLa cells) was a kind gift of Vincent Montcollin, Strasbourg. Myc, Max and AP-2 were expressed with an N-terminal cassette of six histidines and purified by affinity chromatography on a Ni^{2+} Sepharose column (Quiagen, Hilden, Germany) according to the manufacturer's instructions. The N-termini of Max, Myc and AP-2 were modified by PCR and cloned into the bacterial expression vector pRSET (Schoepfer, 1993). Bacteria expressing recombinant proteins were lysed in 6 M guanidinium hydrochloride; 0.1 M NaH_2PO_4. Max and AP-2 proteins were eluted with a 0–400 mM imidazole gradient in 5 M urea; 0.1 M Na_2HPO_4, pH 6.5 and dialysed against Dignam buffer D (Imai *et al.*, 1991). Myc protein was renatured while bound on the column using a 5–1 M urea gradient in buffer R (150 mM NaCl, 20% glycerol; 50 mM Tris−HCl, pH 7.4) and eluted with a 0–400 mM imidazole gradient in buffer R.

Transcription/translation of murine AP-2 was carried out in a reticulocyte lysate (Promega, Madison, USA) according to the manufacturer's instructions. Nuclear extracts from RAT1A cells were prepared according to Imai *et al.* (1991).

For antibody experiments, samples were pre-incubated with 1 μl of anti-AP-2 antibody (Santa Cruz, California, USA), 1 μl of pan-Myc antibody or 1 μl of anti-Max antibody (93–1) 15 min on ice before the addition of probe. Myc and Max antibodies were a kind gift of G. Evans.

In vitro protein binding assay

In vitro binding assays were essentially performed as described (Buettner *et al.*, 1993). Twenty micrograms of affinity purified protein was bound to 30 μl glutathione agarose beads (Sigma). To assay for specific interactions, 10 μl [^{35}S]methionine-labelled *in vitro* translated protein was added and incubated for 2 h 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.

Immunoprecipitations

HeLa cells were plated into 150 mm dishes and transfected with 10 μg CMV-Myc using the calcium phosphate method. Twenty four hours after transfection, cells were starved in methionine- free medium supplemented with 10% calf serum for 1 h and labelled for 4 h with 0.5 mCi [^{35}S]methionine in 5 ml of methionine-free medium. The cells were lysed in 3 ml NP-40 lysis buffer [50 mM Tris−HCl, pH 7.5; 150 mM NaCl; 1.5 mM $MgCl_2$; 10% glycerol; 0.2% NP-40; 1 μg/ml aprotinin; 0.5 μg/ml leupeptin; 50 μg/ml phenylmethylsulphonyl fluoride (PMSF)] by sonification. The lysate was pre-cleared with 40 μl protein A−Sepharose beads for 1 h at 4°C. One millilitre of the supernatant was used in immunoprecipitations with 4 μl pre-immune serum, AP-2 antiserum or monoclonal Myc antibody (9E10). After 2 h at 4°C, immune complexes were collected with protein A−Sepharose beads for 1 h at 4°C, washed four times with lysis buffer and separated on a 10% SDS−polyacrylamide gel. Partial proteolytic peptide maps of protein bands excised from SDS−polyacrylamide gels was obtained using V8 protease as described (Harlow and Lane, 1988).

For immunoprecipitations followed by Western blot analysis, lysates from HeLa cells transfected with CMV-Myc were prepared as described above. Pre-cleared lysates were incubated with either 20 μl AP-2 serum coupled to protein A beads or with coupled pre-immune serum. Coupling of antibodies to protein A beads was performed as described (Harlow and Lane, 1988). Immune complexes were washed four times in lysis buffer, separated on a 10% SDS−polyacrylamide gel and transferred onto PVDF membrane. Immunodetection using monoclonal Myc antibody 9E10 was performed as described previously (Jansen-Dürr *et al.*, 1993).

Oligonucleotide affinity purification

An oligonucleotide encompassing the AP-2 binding site of the human metallothionein IIa distal basal level enhancer (sequence: AGGAACTG-ACCGCCCGCGGCCCGTGCAGAG) was trimerized, ligated in pBLCat 2, excised from the vector and concatenated. Ends were filled in with Biotin-21-dUTP (Boehringer Mannheim, Germany), dGTP, dCTP and dATP using Klenow enzyme. Five micrograms of DNA was coupled to 2 mg Streptavidin magnetic beads (Dynal, Hamburg, Germany) according to the manufacturer's conditions. HeLa nuclear extract (400 μg) was incubated with 2 mg oligonucleotide affinity beads in 62.5 mM KCl, 20 mM HEPES pH 7.9, 15% glycerol, 1 mM DTT, 0.1% NP-40 and 0.5 mM PMSF for 20 min at room temperature. Beads were washed three times with the same buffer containing 100 mM KCl and bound proteins were eluted in 600 mM KCl.

Acknowledgements

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