



Transcriptional regulation by the carboxyl terminus of c-Myb depends upon both the Myb DNA-binding domain and the DNA recognition site

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The c-Myb protein binds to DNA, can regulate transcription, and is required for normal hematopoiesis in vertebrates. Either amino- or carboxy-terminal truncation of this protein is required for efficient oncogenic activation. Previous studies have shown that the carboxyl terminus of c-Myb that is deleted in v-Myb contains negative regulatory domains. We now demonstrate that specific mutations within this carboxyl terminus result in greater transcriptional activation than truncation of the entire carboxyl terminus. Furthermore, this increased transcriptional activation depends upon the presence of the highly conserved Myb DNA-binding domain and is also dependent upon the nature of the Myb-binding sites within the target promoter. In a similar fashion, an activating mutation within the heptad leucine repeat region of c-Myb that is also present in v-Myb functions only in conjunction with the Myb DNA-binding domain and with particular Myb-binding sites. These results suggest a model in which multiple domains of the c-Myb protein are highly interdependent for transcriptional regulation. These interactions are promoter-specific and are not well modeled by heterologous fusion proteins.

Keywords: c-Myb; DNA-binding; v-Myb

Introduction

The *c-myb* gene plays a critical role in the development of a normal hematopoietic system in vertebrates (Mucenski *et al.*, 1991). The *v-myb* oncogenes of both AMV and the E26 acute leukemia viruses both encode internal portions of the c-Myb protein that are truncated at both their amino and carboxyl termini (reviewed in Ganter and Lipsick, 1998). Both c-Myb and v-Myb can function as DNA-binding transcription factors (reviewed in Ness, 1996). The c-Myb proteins are highly conserved among vertebrates and contain an amino-terminal DNA-binding domain comprised of three imperfect fifty-two amino acid Myb repeats, a central transcriptional activation domain adjacent to a heptad leucine repeat, and a carboxy-terminal negative regulatory region (Klempnauer and Sippel, 1987; Sakura *et al.*, 1989) (Figure 1). Fusion proteins in which the DNA-binding domain of v-Myb or c-Myb was replaced by that of the yeast transcription factor GAL4, were used to map a central transcriptional

activation domain (Kalkbrenner *et al.*, 1990; Weston and Bishop, 1989). However, this activation domain is not sufficient for transcriptional activation in the context of the native v-Myb protein (Ibanez and Lipsick, 1990).

A c-Myb negative regulatory region was identified by demonstrating that truncation of the carboxyl terminus can increase transcriptional activation, both in GAL4-c-Myb fusions and in the native protein (Hu *et al.*, 1991; Kalkbrenner *et al.*, 1990; Sakura *et al.*, 1989). This negative regulatory activity was mapped to two non-contiguous regions of chicken c-Myb (PS and BN) using a series of small carboxy-terminal deletions in a GAL4-c-Myb fusion protein (Dubendorff *et al.*, 1992). Interestingly, a region of the carboxyl terminus which lies between these two negative regulatory domains has been highly conserved among the c-Myb proteins of vertebrates, the related A-Myb and B-Myb proteins of vertebrates, and the Myb proteins of sea urchins and *Drosophila*, but this region has no known function (Ganter and Lipsick, 1998). When full-length c-Myb and carboxy-terminal truncations were assayed for transcriptional activation in budding yeast, no negative regulation was observed (Chen and Lipsick, 1993; Seneca *et al.*, 1993). These results imply that additional animal cell-specific proteins may be required for negative regulation by the carboxyl terminus.

Recent work has characterized more precisely the Myb sequences that are associated with both positive and negative control of transcriptional activation function. Amino-terminal truncation into the DNA-binding domain reduces the affinity of c-Myb for DNA both *in vitro* and *in vivo*, possibly correlating with the high transformation activity of such mutants (Dini and Lipsick, 1993). In contrast, a negatively charged region amino-terminal to the Myb repeats appears to inhibit both DNA-binding and transcriptional activation. In addition, oncogenic amino terminal truncations permit transcriptional inhibition by cyclin D in a CDK-independent fashion (Ganter *et al.*, 1998). A heptad leucine repeat region that contains a putative 'leucine zipper' has been proposed to function in negative regulation, possibly by promoting c-Myb dimer formation and/or binding to other proteins (Kaneishi *et al.*, 1992; Nomura *et al.*, 1993; Tavner *et al.*, 1998). However, the region containing this heptad leucine repeat, but not the leucines themselves, was shown to be required for both transcriptional activation and transformation by v-Myb (Fu and Lipsick, 1996). Transcriptional activation therefore appears not to be encoded merely within a single central domain of v-Myb, but instead lies within several subdomains throughout the protein (Chen *et al.*, 1995; Fu and Lipsick, 1996). An additional transcriptional activation domain has been mapped

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within the carboxyl terminus when c-Myb is assayed in budding yeast (Seneca *et al.*, 1993).

DNA binding appears to be the only function located within a contiguous domain, but this also may be negatively regulated by carboxy-terminal sequences (Ramsay *et al.*, 1991; Tanaka *et al.*, 1997). Studies using phage display or yeast two-hybrid assays have also suggested that the DNA-binding domain and the carboxyl terminus of c-Myb may physically interact with one another (Dash *et al.*, 1996; Keiwitz and Wolfes, 1997). A protein called p100 has been proposed to competitively inhibit this interaction, thereby regulating c-Myb function (Dash *et al.*, 1996). p100 was identified because of a sequence similarity to the 'EVES' motif within the carboxyl terminus of c-Myb which was required for interaction with the DNA-binding domain in yeast two-hybrid

assays. Transient infection of a p100 expression vector inhibited transcriptional activation by c-Myb. Interestingly, the same protein was previously identified as a co-activator for the EBNA 2 protein of the Epstein-Barr virus (Tong *et al.*, 1995).

Much of this work paints an increasingly complex picture of the domain organization of c-Myb. In order to examine the function and cooperation of c-Myb domains, we have continued our investigation of the carboxy-terminal region by now using deletion mutants in the context of the Myb DNA-binding domain. In addition, we have constructed and tested a new series of linker insertion mutants within the central activation domain, heptad leucine repeat region and the negative regulatory domains of the carboxyl terminus of c-Myb including the 'EVES' sequence. Our analysis implies that the regulatory function of the carboxyl terminus is highly dependent upon both the Myb DNA-binding domain and the presence of particular Myb-binding sites within the target promoter. These results are reminiscent of recent models of glucocorticoid receptor function in which the DNA itself has been proposed to be an allosteric effector of the protein (Lefstin and Yamamoto, 1998).

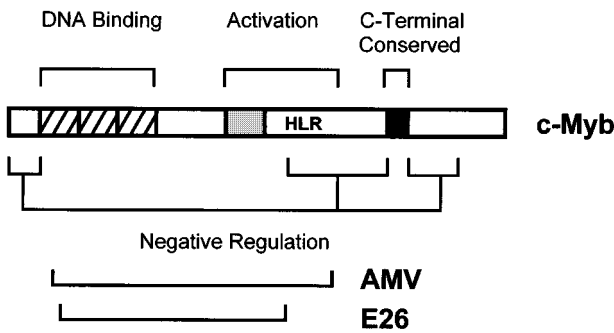


Figure 1 Regulatory regions within the c-Myb protein. The schematic diagram shows a linear representation of the c-Myb protein with the DNA-binding, central transcriptional activation and conserved carboxy-terminal domains as indicated. The three Myb repeats of the DNA-binding domain are diagonally hatched. An acidic central transcriptional activation domain that had been mapped using GAL4-fusion proteins is shaded gray. A carboxy-terminal region that is highly conserved among c-Myb, A-Myb, B-Myb, sea urchin and *Drosophila* Myb is shaded black. The heptad leucine repeat region is indicated by HLR. The brackets below indicate the portion of the c-Myb protein that is encoded by oncogenes of the AMV and E26 leukemia viruses

Results

The carboxyl-terminus of c-Myb is required for maximal transcriptional activation

In order to investigate the role of the central acidic and heptad leucine repeat regions in transcriptional activation by c-Myb, we generated two linker insertion mutations in c-Myb similar to those originally constructed in v-Myb (Lane *et al.*, 1990). The 859 mutation inserted two additional amino acids (Gly-Pro) within the central v-Myb transcriptional activation domain (corresponding to an insertion after residue 304 in chicken c-Myb, 304GP) and abolished its activity. In contrast, the 1114 mutation which

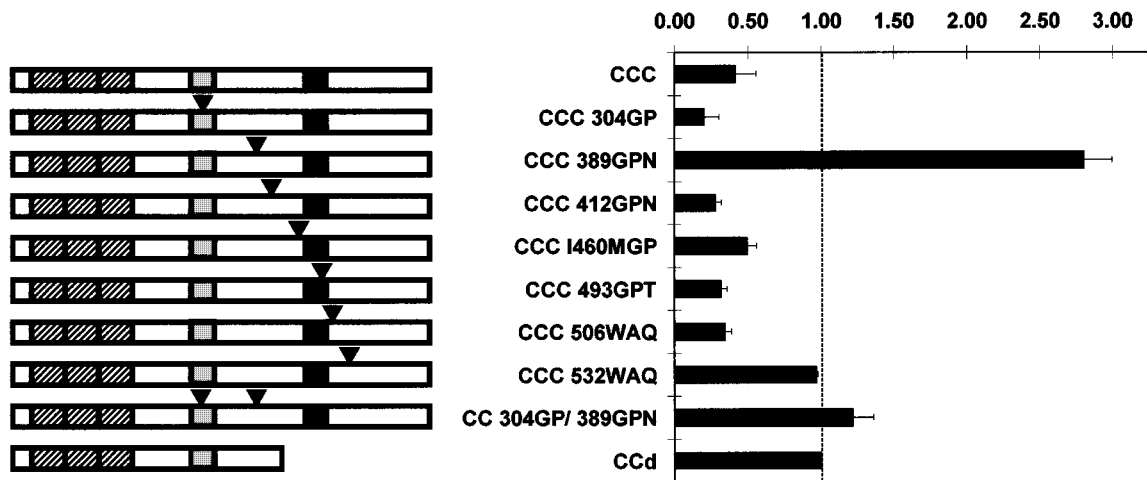


Figure 2 Transcriptional activation by C-terminal linker insertion mutants of c-Myb. A series of linker insertion mutants of c-Myb were assayed for transcriptional activation using a reporter gene in which five Myb-binding sites from the *mim-1* promoter were placed upstream of a simple TATA box. A similar reporter with five mutant Myb-binding sites showed no activation. The domains of c-Myb are indicated as described in Figure 1. The arrowheads represent the sites of two or three amino acid in-frame insertions in c-Myb that are presented in more detail in Figure 3. Each c-Myb mutant was assayed at least three times. CCC is the full-length chicken c-Myb protein. Data are presented relative to CCd that was arbitrarily assigned a value of 1.00. Bars indicate mean values and average deviation of the mean

inserted three amino acids (Gly-Pro-Asn) within the heptad leucine repeat of v-Myb (corresponding to an insertion after residue 389 in chicken c-Myb, 389GPN) increased the transcriptional activation activity of that protein. In addition, we constructed a double linker insertion in c-Myb containing both the 304GP and 389GPN mutations.

In the context of full-length c-Myb, the 304GP mutation decreased transcriptional activation from a reporter containing five strong Myb-binding sites from the *mim-1* promoter and a simple TATA box by approximately 50% (Figure 2). This result implies that the central acidic domain that is conserved in all c-Myb and A-Myb proteins of vertebrates, but not in B-Myb proteins of vertebrates or Myb proteins of invertebrates, is required for full transcriptional activation by c-Myb. In contrast, the 389GPN mutation greatly increased transcriptional activation of full-length c-Myb by approximately sixfold (Figure 2). This was significantly greater than the twofold increase caused by deletion of the entire carboxyl terminus of c-Myb that is missing in v-Myb (CCd). The presence of both the 304GP and the 389GPN mutations in full-length c-Myb resulted in transcriptional activation at levels similar to that of CCd. Similar levels of all of the different Myb proteins were detected by immunoblotting (data not shown). These results imply that alteration of the heptad leucine repeat region can reveal a latent transcriptional activation that requires the presence of both the central acidic domain and the intact carboxyl terminus of c-Myb for maximal activity. However, the central acidic domain is not absolutely required for transcriptional activation in the presence of the 389GPN mutation in the heptad leucine repeat region as shown by activity of the double mutant 304GP/389GPN.

To further investigate the function of the carboxyl terminus of c-Myb in transcriptional activation, we also generated a new series of linker insertion mutations at c-Myb amino acids 412GPN, I460MGP, 493GPT, 506WAQ and 532WAQ (Figures 2 and 3). Of the five additional linker insertions in the new series, only the one designated as 532WAQ showed higher activity than c-Myb itself (Figure 2). However, it was only as active as the carboxy-terminally truncated control, CCd, and did not exhibit the much higher activity seen with mutant 389GPN. Linker insertion 532WAQ is of particular interest because it occurs within the BN negative regulatory domain adjacent to a MAPK kinase phosphorylation site (Aziz *et al.*, 1993, 1995). In addition this mutation disrupts the 'EVES' motif that has been reported to interact with the Myb DNA-binding domain in yeast two-hybrid assays (Dash *et al.*, 1996). Our results are consistent with a model in which this region of c-Myb can inhibit transcriptional activation by c-Myb. However, these data also imply that p100 alone is unlikely to be responsible for negatively regulating the full activity of c-Myb that can be revealed by alterations in the heptad leucine repeat region such as the 389GPN mutation.

Deletion of two negative regulatory domains in the carboxyl terminus activates c-Myb more than simple truncation

We previously used deletion mutations in a GAL4-c-Myb fusion protein (GAL4-CC) to map negative

regulation of transcriptional activation via a reporter gene containing five GAL4-binding sites (Dubendorff *et al.*, 1992). A GAL4 fusion containing the entire carboxyl terminus of c-Myb was transcriptionally inactive, whereas a deletion similar to that found in v-Myb was quite active. We then attempted to map the negative regulatory domain and found that a series of adjacent, single small deletions within the carboxyl

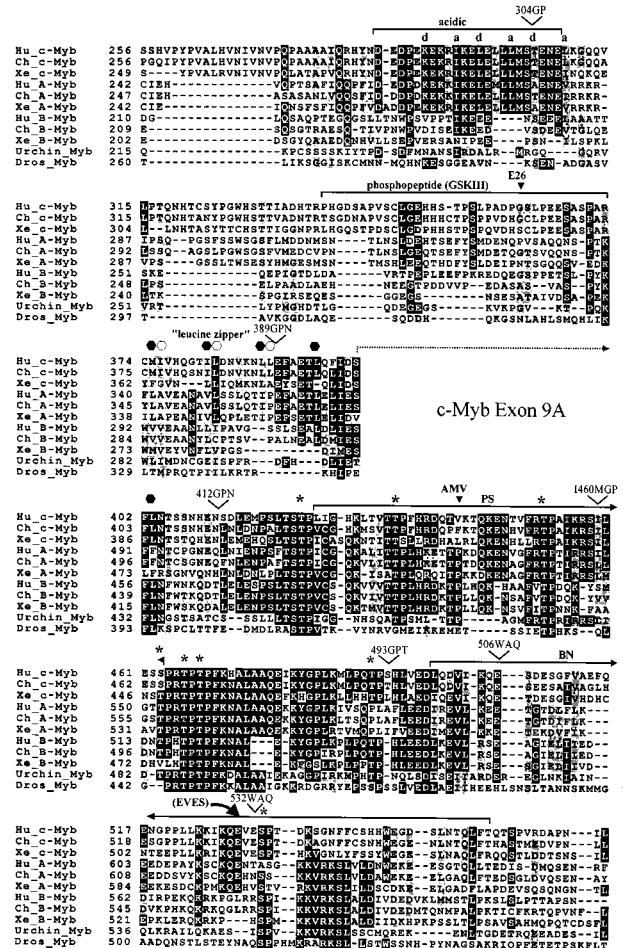


Figure 3 Alignment and location of mutations in the carboxyl terminus of c-Myb. Myb protein sequences were aligned using the CLUSTALW implementation of the Parsimony After Progressive Alignment (PAPA) method (Feng and Doolittle, 1990; Thompson *et al.*, 1994) with some additional adjustments based on blocks identified with the MACAW local alignment program (Schuler *et al.*, 1991). Shading was then performed using the BOXSHADE program. Black shading indicates identical residues at a given position; gray shading indicates similarity. Hu=human; Ch=chicken; Xe=Xenopus; Urchin=sea urchin; Dros=Drosophila. Residues similar to those in alternatively spliced Exon 9A of c-Myb are not shown. AMV=carboxy-terminal truncation of v-Myb of AMV; the *a* and *d* positions in a predicted helical wheel are indicated above the alignment of the central activation domain sequences; hexagons=the leucines of two heptad leucine repeats also referred to as the 'leucine zipper'; asterisks=highly conserved putative phosphorylation sites for proline-directed kinases; PS and BN=deletions in c-Myb that together activate transcription; EVES=sequence that mediates interaction with the DNA-binding domain in yeast two-hybrid assays and that also contains a serine that is phosphorylated in c-Myb *in vivo* and by MAPK *in vitro*; arrows indicate positions of residues introduced by the linker insertion mutants described in this paper. All sequences were obtained from the Genbank resource at NCBI

terminus showed little or no activity compared with the highly active carboxy-terminal truncation, GAL4-Cd. However, the PS/BN double deletion (aa 425–464 and 499–558) within the carboxyl terminus showed approximately 60–70% of the activity of the carboxy-terminally truncated fusion protein, confirming the negative regulatory activity of these two domains. We obtained similar results with LexA-c-Myb fusion proteins, and in this case we also showed that the carboxyl terminus of c-Myb inhibited transcriptional activation, but not nuclear transport or DNA binding.

Studies of v-Myb showed that mutations within the DNA-binding domain may affect transcriptional activation without diminishing DNA binding (Grasser *et al.*, 1992; Lane *et al.*, 1990). Furthermore, mapping of transcriptional activation domains of native v-Myb gave quite different results than studies of GAL4-v-Myb fusion proteins (Chen *et al.*, 1995; Fu and Lipsick, 1996; Ibanez and Lipsick, 1990). Therefore, we wished to determine the effect of deleting the two previously defined carboxy-terminal negative regulatory domains of c-Myb in the presence of the highly conserved Myb DNA-binding domain.

The transcriptional activation of full-length c-Myb (CCC), a complete carboxy-terminal deletion similar to that of v-Myb (CCd), a variant of c-Myb containing two smaller deletions of the negative regulatory domains (CCC PS/BN), and the linker insertion within the heptad leucine repeat (389GPN) were compared (Figure 4). We initially used the same

reporter gene described above that contains five tandem copies of the strongest (type A) Myb-binding site from the *mim-1* promoter (Ness *et al.*, 1989), a simple TATA box from the adenovirus E1b gene (Lillie and Green, 1989) and the luciferase coding region (EW5-LUC). The PS/BN double deletion mutant of c-Myb was remarkable in that it activated transcription to a much greater degree than a complete deletion of the carboxyl terminus. This heightened activity of the CCC PS/BN mutant was similar to that of the 389GPN mutant that altered the heptad leucine repeat region. This heightened activity was not observed with deletion of either the PS or BN region alone (Figure 4 and data not shown). These results suggest that both of these mutant proteins may be disrupting a similar intrinsic inhibitory conformation of full-length c-Myb.

Transcriptional regulation by the carboxyl terminus of c-Myb is dependent upon the DNA to which the protein is bound

We had previously noted that negative regulation by the carboxyl terminus of c-Myb seemed to vary depending upon the promoter being tested. In particular, a promoter containing nine Myb-binding sites from chicken genomic DNA (KHK) placed upstream of the same TATA box was minimally activated by full-length c-Myb, but was markedly activated by v-Myb or a similar carboxy-terminal truncation of c-Myb (Dubendorff *et al.*, 1992). In contrast, a promoter containing five copies of the

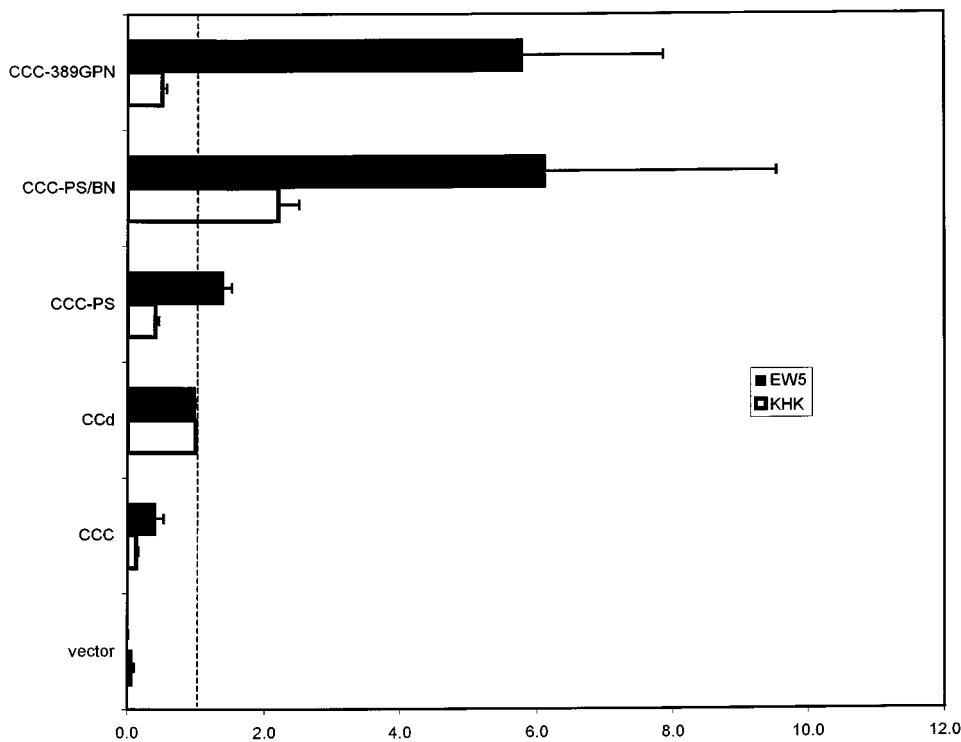


Figure 4 Transcriptional regulation by the C-terminus of c-Myb depends upon the specific promoter. A series of deletion mutants of c-Myb were assayed for transcriptional activation using either a reporter gene in which give Myb-binding sites (EW5) were placed upstream of a simple TATA box and a luciferase cDNA, or a reporter gene in which fragments of chicken genomic DNA containing nine binding sites for v-Myb (KHK) were placed upstream of the same TATA box and luciferase cDNA. A similar reporter with five mutant Myb-binding sites showed no activation. Each c-Myb mutant was assayed at least three times. CCC is the full-length chicken c-Myb protein. Data are presented relative to CCd which was arbitrarily assigned a value of 1.00. Bars indicate mean values and average deviation from the mean

strong Myb-binding site from the *mim-1* promoter upstream of the same TATA box could be activated by both v-Myb and c-Myb. We therefore wished to test whether the 'superactivation' caused by the 389GPN and CCC PS/BN mutations was dependent upon the promoter (Figure 4). Consistent with our previous report using CAT reporter genes, full-length CCC barely activated the KHK-LUC reporter gene whereas the truncated CCd protein activated the same reporter reasonably well. The CCC PS/BN double deletion within the carboxyl terminus was more active on KHK-LUC than a simple truncation, similar to results with the EW5-reporter gene. However, the 389GPN linker insertion within the heptad leucine repeat did not activate the KHK-LUC reporter as well as CCd, whereas 389GPN was much more active than CCd on the EW5-LUC reporter. Similar levels of each Myb protein were produced with either reporter indicating that differential protein stability cannot

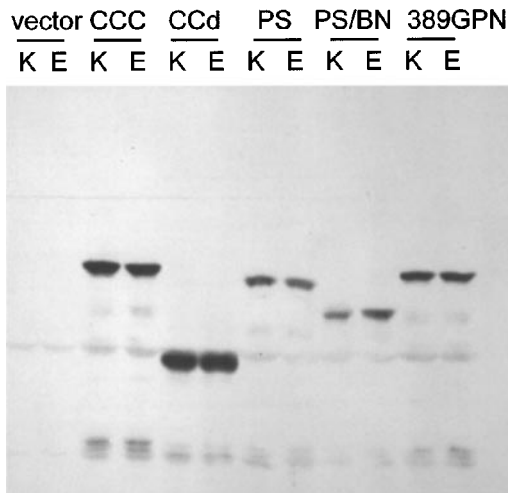


Figure 5 Differential activation of promoters by C-terminal mutants of c-Myb does not depend upon differential protein production. Extracts of the same cells used for luciferase assays with the KHK (K) or the EW5 (E) promoters in Figure 4 were resolved by SDS-PAGE. The Myb proteins were then detected by immunoblotting using anti-Myb monoclonal antibodies. The relative mobility of molecular weight standards is indicated on the right

account for the observed differences in transcriptional activation (Figure 5). These data demonstrate that transcriptional regulation by the carboxyl terminus of c-Myb is quite dependent upon the DNA to which the protein is bound.

Transcription regulation by the carboxyl terminus of c-Myb depends specifically upon the Myb DNA-binding domain

We had previously shown that a specific double deletion within the carboxyl terminus of c-Myb (PS/BN) could activate a GAL4-c-Myb fusion protein approximately 60–70% as well as a simple carboxy-terminal truncation (Dubendorff *et al.*, 1992). However, in the context of the full-length protein this double deletion was much more active than a simple carboxy-terminal truncation (Figure 4). These results suggested that the presence of the Myb DNA-binding domain was required for this increased activation. We therefore wished to determine whether the increased transcriptional activation by the 389GPN linker insertion mutant within the heptad leucine repeat also required the presence of the Myb DNA-binding domain. For this purpose, a series of GAL4-Myb fusion proteins were produced and tested for their ability to activate a reporter gene containing GAL4-binding sites upstream of the same minimal TATA box (Figure 6). As previously reported, a fusion protein containing the full-length carboxyl terminus of c-Myb (GAL4-CC) was inactive, whereas a carboxy-terminal truncation similar to that of v-Myb (GAL4-Cd) was quite active. A fusion protein containing the PS/BN double deletion within the carboxyl terminus (GAL4-CC PS/BN) was almost as active as a complete truncation. However, the linker insertion mutant within the heptad leucine repeat region was completely inactive without the Myb DNA-binding domain (GAL4-CC 389GPN). Similar levels of each GAL4 Myb protein were produced, indicating that differential protein stability cannot account for the observed differences in transcriptional activation (Figure 7). In fact, the most active protein (GAL4-CCd) was actually the least abundant. These results imply that relief of negative regulation by disruption of the heptad leucine

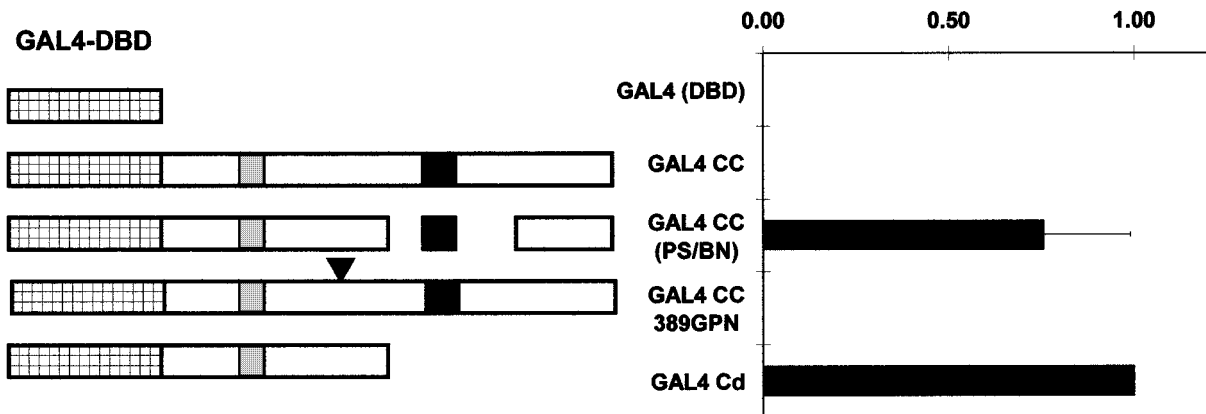


Figure 6 Lack of activation by GAL4-c-Myb fusion protein with a heptad leucine repeat disruption. Four different GAL4-c-Myb fusion proteins were assayed for transcriptional activation using a reporter in which five GAL4-binding sites were placed upstream of the same simple TATA box and a luciferase cDNA. Each activator was assayed at least three times. Data are presented relative to GAL4-Cd which was arbitrarily assigned a value of 1.00. Bars indicate mean values and average deviation from the mean

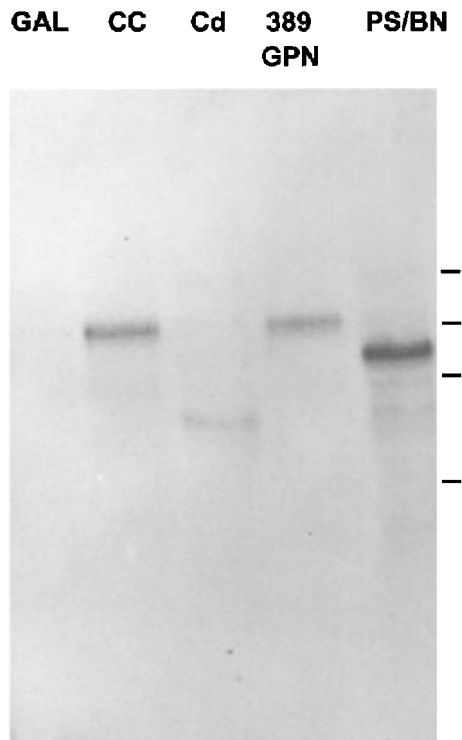


Figure 7 Differential activation by GAL4-c-Myb fusion proteins does not depend upon differential protein production. An anti-Myb immunoblot of extracts of cells used for luciferase assays. Extracts of the same cells used for luciferase assays with the KHK (K) or the EW5 (E) promoters in Figure 6 were resolved by SDS-PAGE. The Myb proteins were then detected by immunoblotting using anti-Myb monoclonal antibodies. The relative mobility of molecular weight standards is indicated on the right

repeat region requires the specific presence of the Myb DNA-binding domain, as does 'superactivation' the double deletion within the carboxyl terminal negative regulatory domain.

Discussion

A variety of experiments have suggested that eukaryotic transcription factors are composed of separable DNA-binding and activation domains (Ptashne, 1988). This conclusion is based largely upon the fusion of functional domains of heterologous proteins, often from different species. In this study we have examined the role of different regions of the c-Myb protein in transcriptional regulation. Initial studies with deletion mutants demonstrated that c-Myb had three functional domains, an amino-terminal DNA binding domain, a central transcriptional activation domain, and a carboxy-terminal negative regulatory domain (Sakura *et al.*, 1989). Fusions of v-Myb and c-Myb with the DNA-binding domains of GAL4 and LexA were also used to define a central transcriptional activation domain (Kalkbrenner *et al.*, 1990; Weston and Bishop, 1989). However, more detailed studies have shown that in the case of v-Myb, the activation domain identified by GAL4 fusions is not sufficient for transcriptional activation or oncogenic transformation in the context of the native protein (Chen *et al.*, 1995;

Fu and Lipsick, 1996; Ibanez and Lipsick, 1990). Furthermore, mutants within the DNA-binding domain of v-Myb have been identified that still bind DNA, but no longer can activate transcription (Grasser *et al.*, 1992; Lane *et al.*, 1990). These results have suggested that the interplay among the different domains of v-Myb and c-Myb is more complicated than suggested by the standard independent-domain model of transcriptional activators.

The failure of the disruption of the heptad leucine repeat to activate transcription in GAL4-fusion proteins suggest that rather than being a negative regulator itself, mutations in this region of the protein may regulate the conformation and interaction of other domains of the c-Myb protein. In this regard, we have also observed that the presence of the alternatively spliced exon 9A sequences that also interrupt this heptad leucine repeat region can cause increased transcriptional activation by c-Myb proteins (Woo *et al.*, 1998).

Inspection of the sequence of the central transcriptional activation domain that is conserved among all known c-Myb and A-Myb proteins reveals the presence of another hydrophobic heptad repeat with predicted alpha-helical content. In fact, this region scores much higher using the COILS prediction program than that in the more carboxy-terminal heptad leucine repeat (Lupas *et al.*, 1991). Furthermore, a Gly-Pro insertion in this region greatly diminishes transcriptional activation by both v-Myb and c-Myb. These results suggest that the predicted amphipathic helix in this region is likely to be important for the function of the central activation domain. In this region of c-Myb and A-Myb, the predicted α positions of the heptad helical wheel are occupied by Ile, Leu, and Leu, and the predicted d positions are occupied by Lys, Leu, and Thr or Ala (Figure 3). This observation suggests a model in which the previously described heptad leucine repeat region near the carboxyl-terminus of v-Myb interacts with a similar motif within the central transcriptional activation domain (Figure 8). In support of such a model, we note that both of these regions are conserved in all known c-Myb and A-Myb proteins, but not in the B-Myb or invertebrate Myb proteins. In addition, we have previously reported that the same 1114 linker insertion (389GPN in c-Myb) can increase transcriptional activation by the v-Myb protein itself (Lane *et al.*, 1990). The central activation domains of c-Myb and A-Myb have been shown to bind to the transcriptional coactivator CBP (Dai *et al.*, 1996; Facchinetti *et al.*, 1997; Oelgeschlager *et al.*, 1996). Therefore, an interesting possibility is that the previously described heptad leucine repeat of c-Myb may limit the access of CBP to the central activation domain.

The 'superactivation' by mutants of c-Myb with an insertion in the heptad leucine repeat or with two small deletions within the carboxyl terminus also suggests that an additional transcriptional activation domain may be present within the carboxyl terminus of c-Myb. Consistent with this hypothesis, a linker insertion mutation within the central activation domain (304GP) greatly reduces overall transcriptional activation by c-Myb, but does not abolish the ability of the linker insertion mutation within the heptad leucine repeat to increase the residual transcriptional activation

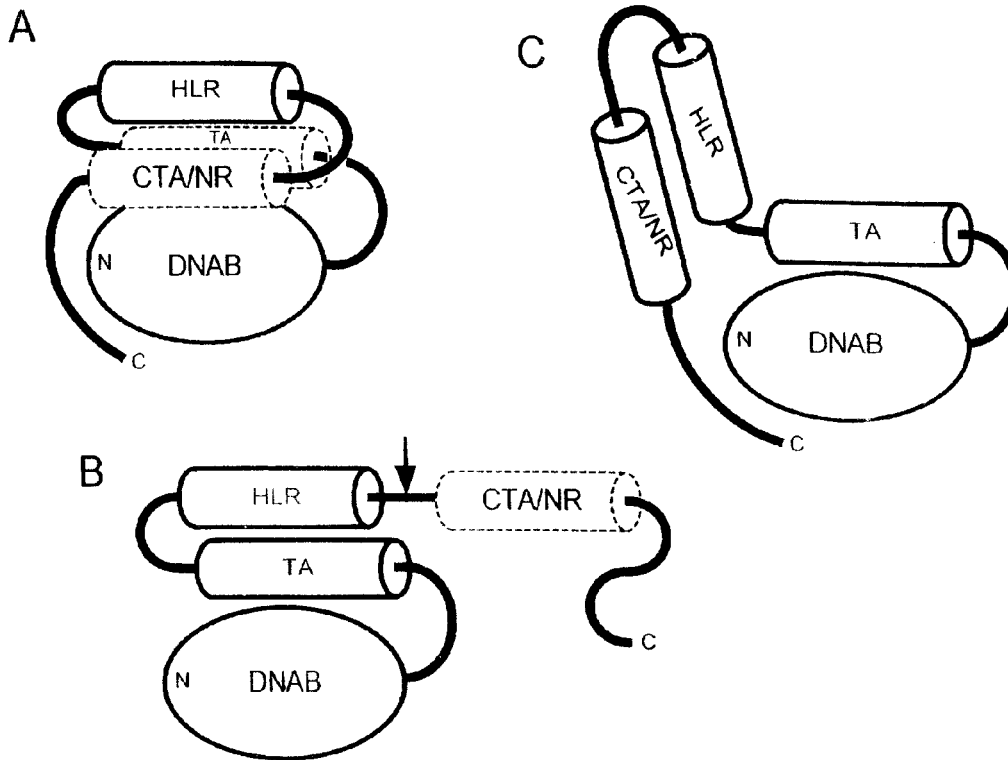


Figure 8 Model for the activation and superactivation of the c-Myb protein. The Myb DNA-binding domain (DNAB) and potential α -helical regions in the central transcriptional activation domain (TA), heptad leucine repeat (HLR), and carboxy-terminal activation/negative regulatory regions (CTA/NR), are represented by the ellipse and the cylinders, respectively. (a) shows the c-Myb protein in its inactive state in which the central and carboxy-terminal activation domains are masked (dashed cylinders). (b) shows c-Myb in its activated state, in which only the central transcriptional activation domain is functional (solid cylinder). This situation includes proteins such as v-Myb that have truncated carboxyl termini (arrow). In (c), the 'open' superactivated state is produced either by disruption of the HLR-TA interaction (linker insertion 389GPN in the HLR), or by disruption of the CTA/NR-TA interaction by specific deletions in the carboxyl terminus (dPS/BN). In this case the specific interaction of the Myb DNA-binding repeats near the amino terminus (N) with a sequence of sequences in the carboxyl terminus (C) is required to maintain the highly active conformation. Thus, superactivation is not possible with the GAL4-c-Myb fusion proteins

function (304GP/389GPN). To address this question we have made GAL4 fusions with the carboxyl terminus of c-Myb that is absent in v-Myb. These proteins do not activate transcription either in animal cells or in yeast (data not shown). In addition, we have also constructed a deletion mutant of c-Myb that lacks both the central activation and heptad leucine repeat regions, but retains the Myb DNA-binding domain and the carboxyl terminus. This protein also does not activate transcription in animal cells (DM Wang and JS Lipsick, unpublished). These results imply that a 'cryptic' transcriptional activation within the carboxyl terminus of c-Myb requires specific interactions with both the Myb DNA-binding domain and central regions of the c-Myb protein (Figure 8). A candidate for such a 'cryptic' transcriptional activation domain is the very highly conserved, threonine/proline-rich sequence within the carboxyl terminus of c-Myb, A-Myb, B-Myb, sea urchin Myb, and *Drosophila* Myb that as yet has not been assigned any function. This region has been reported to augment but not be sufficient for transcriptional activation by B-Myb (Nakagoshi *et al.*, 1993).

In this paper we have found that either disruption of the heptad leucine repeat or the introduction of two small deletions within the carboxyl terminus of c-Myb can increase transcriptional activation significantly more than a simple carboxyl-terminal truncation.

Presumably these mutations mimic physiologic protein-protein interactions or post-translational modifications that serve a similar function *in vivo*. Interestingly, both the Myb DNA-binding domain and specific promoter DNAs are required for this 'superactivation'. In this regard, the regulation of c-Myb appears to be similar to that of the glucocorticoid receptor in which DNA itself can be viewed as an allosteric effector of transcriptional activation (Lefstin and Yamamoto, 1998).

Materials and methods

Plasmid constructions

DNA restriction and modifying enzymes were purchased from New England Biolabs (Beverly, MA, USA). Recombinant DNA manipulations were carried out using standard techniques (Ausubel *et al.*, 1989). Retroviral constructs that express c-Myb and c-Myb truncations have been described (Dini and Lipsick, 1993; Fu and Lipsick, 1997; Grasser *et al.*, 1991). Single and double truncations of c-Myb are named accordingly to the sequences present (C) or deleted (d) in a fashion similar to v-Myb. Thus, the c-Myb proteins deleted of amino-terminal, carboxy-terminal, or both sequences missing in v-Myb are referred to as dCC, CCd or dCd, respectively.

Deletions within the carboxyl terminus of c-Myb were constructed by blunt-end ligation of the appropriately

restricted and Klenow-treated DNA where such treatment would give in-frame deletions, or by the use of single-stranded DNA adaptors as described previously (Dubendorff *et al.*, 1992). The resulting deletions within the carboxyl terminus were transferred from the pSG424 plasmid expressing them as GAL4 fusion proteins to the retroviral construct that expresses them as native c-Myb derivatives through a pSP73 intermediate containing the chicken *c-myb* gene (pSP73CCC; Dini and Lipsick, 1993). Briefly, *Xma*I–*Bam*HI fragments from pSG424 derivatives carrying each carboxy-terminal deletion were used to replace the wild type fragment of pSP73CCC. The entire coding sequence for each c-Myb mutant was then transferred to the retroviral vector on a *Cl*aI fragment. Carboxy-terminal deletions are named according to the restriction sites used to make the deletion (Dubendorff *et al.*, 1992). All of the mutant proteins migrated with the expected relative mobilities on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

The v-Myb 1114 linker insertion (389GPN in c-Myb) was transferred from v-Myb into c-Myb on a *Sal*I–*Msc*I fragment, and from c-Myb into GAL4-c-Myb using the *Xma*I–*Bam*HI fragment described above. The 304GP and 1460MGP mutations were made by ligating a kanamycin selection cassette flanked by *Apa*I linkers into plasmid DNA that had been linearized at *Hinc*II and *Ssp*I sites, respectively (Lane *et al.*, 1990). Construction of additional linker insertion mutations was by a modification of this method in order to target the insertions to blunt-ended *Hinf*I sites within a specific region of the plasmid DNA (Dubendorff, 1997). All mutant proteins encoded by these linker insertion mutants exhibited SDS–PAGE mobilities indistinguishable from that of wild-type c-Myb.

Reporter plasmids bearing five type A Myb-binding sites derived from the *mim-1* promoter sequence (EW5), nine Myb-binding sites from chicken genomic DNA (KHK), or five GAL4-binding sites were constructed by cloning the appropriate DNA fragments into polyA-E1b-Luc or E1b-CAT, which contain a synthetic adenovirus E1b TATA sequence driving luciferase or CAT gene expression, respectively (Fu and Lipsick, 1996; Lillie and Green, 1989).

Cells and media

Quail QT6 fibroblasts were grown in Dulbecco's modified essential medium (DMEM) supplemented with 5% fetal calf serum, 4.5 g of glucose per liter, nonessential amino acids, L-glutamine, sodium pyruvate, 100 µg of streptomycin per ml, and 100 U of penicillin per ml in a humidified 10% CO₂/90% air incubator at 37°C.

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DNA transfections, immunoblotting and assays for β -galactosidase, luciferase and CAT activity

Transient transfections into QT6 quail fibroblasts were performed using a modification of the calcium phosphate procedure of Chen and Okayama as described previously (Chen and Okayama, 1987; Ibanez and Lipsick, 1990). Transfections included 2–5 µg of activator plasmid, 1 µg reporter DNA, 0.5 µg of a plasmid expressing β -galactosidase (β -gal) from the CMV promoter as an internal control for transfection efficiency, and sufficient yeast tRNA to bring the total amount of nucleic acids to 10 µg per 10 cm diameter plate of cells.

Half of the cells from each transfection were solubilized by boiling for 4 min in SDS–PAGE loading buffer. Volumes of each sample normalized for β -gal activity were then subjected to SDS–PAGE through 10% gels and the proteins transfected to nitrocellulose (BA85; Schleicher & Schuell, Keen, NH, USA). Transiently expressed Myb proteins were detected using monoclonal anti-Myb-2.2, 2.7 or 5E11 antibodies (Evan *et al.*, 1984; Sleeman, 1993). Blots were developed using rabbit anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Promega, Madison, WI, USA) and BCIP/NBT.

Cell extracts were prepared from the other half of each transfection and β -gal activity and luciferase activities were determined as previously described (Ausubel *et al.*, 1989; Sambrook *et al.*, 1989). The phase extraction method was employed to determine the CAT activity in normalized volumes of extract as previously described (Seed and Sheen, 1988). Appropriate dilutions were assayed in those cases where the level of CAT activity indicated that substrate was limiting. A background value was obtained by extracting CAT assay mix to which no cell extract had been added. Activities shown in the figures have been corrected for background and represent the averages of at least three experiments.

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