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Transcription activation by Myc and Max: flanking sequences target activation to a subset of CACGTG motifs *in vivo*

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The Myc oncoprotein has been implicated in control of cell growth, division and differentiation. Although Myc contains a bHLH-LZ motif, it fails to bind DNA alone but can do so by forming heterodimers with an unrelated bHLH-LZ protein, Max. Max homodimers and Myc-Max heterodimers share the ability to bind CACGTG or CATGTG elements. Current models, based on experimentally induced overexpression of Myc and Max in mammalian cells, propose that Max-Max homodimers repress while Myc-Max heterodimers activate transcription through CACGTG binding sites. The interpretation of the results using mammalian cells is complicated by the presence of numerous unrelated CACGTG binding transcription activators and the existence of two alternative Max dimerization partners, Mad and Mxi-1. Thus, the mechanism whereby overexpression of Max leads to transcriptional repression remains to be established. Using a yeast system we show that Max homodimers have the potential to activate transcription through CACGTG motifs. Activation by Max requires DNA binding and amino acids outside the bHLH-LZ domain but is reduced compared with activation by Myc-Max heterodimers. Moreover, transcriptional activation by Myc-Max heterodimers, but not Max-Max homodimers, is strongly inhibited *in vivo* by specific sequences flanking the core CACGTG binding motif, presumably reflecting reduced DNA binding affinity. These results suggest a mechanism for directing the Myc-Max complex to a specific subset of CACGTG-containing target genes.

Key words: binding specificity/Max/Myc/transcription regulation

Introduction

Despite the large body of evidence implicating the Myc oncoprotein in the control of proliferation, differentiation and transformation (Luscher and Eisenman, 1990), little advance was made until recently in understanding its biochemical function. The observation that Myc contains structural motifs common to the bHLH-LZ family of transcription factors (Murre *et al.*, 1989) and can interact specifically with the sequence CACGTG as a heterodimer with an unrelated bHLH-LZ protein, Max (Blackwood and Eisenman, 1991), strongly implied a role for Myc and Max in transcription regulation. Support for this idea came when the N-terminus of Myc was found to activate transcription when fused to a heterologous DNA binding domain (Kato *et al.*, 1990, 1992). Recently, direct evidence for the involvement of Myc and Max in transcription regulation has come both from experiments in yeast, which showed that Myc activates transcription in collaboration with Max (Amati *et al.*, 1992; Crouch *et al.*, 1993), and in mammalian cells, where overexpression of Myc activates transcription from a CACGTG-dependent reporter, presumably as a consequence of heterodimerization with endogenous Max (Kretzner *et al.*, 1992; Reddy *et al.*, 1992; Amin *et al.*, 1993; Gu *et al.*, 1993). In contrast, overexpression of Max in mammalian cells represses transcription (Kretzner *et al.*, 1992; Reddy *et al.*, 1992; Amin *et al.*, 1993; Gu *et al.*, 1993), while in yeast expression of Max from low copy number (LCN) vectors has little discernible effect on transcription (Amati *et al.*, 1993; Crouch *et al.*, 1993).

In mammalian cells most, if not all, Myc is found complexed with Max (Blackwood *et al.*, 1992; Littlewood *et al.*, 1992) and it has been proposed that interaction with Max is necessary for Myc to exert its biological effects. Consistent with this, studies using mutants in the Myc dimerization domain reveal that transformation by Myc requires heterodimerization with Max and that transformation correlates well with the ability of the Myc-Max heterodimer to activate transcription (Amati *et al.*, 1993; Crouch *et al.*, 1993).

Current models propose that transformation by Myc is related to its ability to activate transcription as a heterodimer with Max, while Max-Max homodimers repress transcription (Kato *et al.*, 1992; Kretzner *et al.*, 1992; Mukherjee *et al.*, 1992; Prendergast *et al.*, 1992; Reddy *et al.*, 1992; Amati *et al.*, 1993; Amin *et al.*, 1993; Gu *et al.*, 1993). Elevated levels of Myc found in proliferating cells would therefore increase the ratio of Myc-Max heterodimers to Max-Max homodimers, resulting in increased transcription from target genes and leading to transformation, while overexpression of Max would compete with the Myc-Max complex for DNA binding, and thereby repress transformation (Kato *et al.*, 1992; Makela *et al.*, 1992; Mukherjee *et al.*, 1992; Prendergast *et al.*, 1992; Amati *et al.*, 1993). While this may go some way to describing the molecular events underlying Myc transformation, a number of outstanding questions remain to answered. First, four different Max proteins have been described, p21 and p22 Max, which differ only by the presence of an additional nine amino acids in p22 Max located immediately N-terminal from the Max basic domain (Blackwood and Eisenman, 1991), and two truncated forms of p21 and p22 Max which lack residues C-terminal to the leucine zipper (Makela *et al.*, 1992). The existence of the

different *max* gene products in itself suggests they may serve different functions; however, to date no evidence as to the nature of any functional differences has been forthcoming. Second, Max can heterodimerize with two other bHLH-LZ proteins, Mad (Ayer *et al.*, 1993) and Mxi-1 (Zervos *et al.*, 1993), which, like Myc, require Max to bind DNA. Mad−Max and Mxi-1−Max heterodimers bind the same CACGTG motif recognized by Myc−Max. Like Max, expression of Mad in transient transfection assays results in transcriptional repression (Ayer *et al.*, 1993) while the effects of Mxi-1 on transcription regulation remain to be characterized (Zervos *et al.*, 1993). However, the presence of both Max homo- and heterodimers in cells complicates the interpretation of mammalian transfection experiments; it is not clear, for example, whether the repressive effects of Max overexpression is mediated by Max homodimers or Mad−Max or Mxi-1−Max heterodimers. Moreover, any effect of overexpression of Max, Myc, Mxi-1 or Mad on a CACGTG motif-dependent reporter must be viewed against a background of transcription mediated by other bHLH-LZ proteins including USF (Gregor *et al.*, 1990), TFE3 (Beckmann *et al.*, 1990) and TFEB (D.E.Fisher *et al.*, 1991), all of which are able to bind the same sequences as Max homo- and heterodimers. Thus, studies using mammalian cells to understand the transcriptional regulatory properties of Myc and Max must be viewed in the context of competition between Myc, Mad and Mxi-1 for dimerization with Max and binding to DNA in competition with multiple other factors.

In view of the complexities inherent in using mammalian cells, a yeast expression system has been developed that enables us to examine specifically the regulatory properties of Myc and Max in isolation. The yeast system allows the effects of expression of individual proteins to be assessed against a neutral background and has been used to demonstrate Max-dependent transcription regulation by Myc (Amati *et al.*, 1992; Crouch *et al.*, 1993). In this report we have extended these studies and used yeast to examine more closely the abilities of Max and Myc to activate transcription. The data show that contrary to expectations, Max homodimers have the potential to activate transcription. Transcription activation by Max, although modest in comparison with that mediated by Myc−Max, depends on Max DNA binding, specific CACGTG target sites and amino acids outside the bHLH-LZ domain. Moreover, we also show that sequences flanking the CACGTG binding motif differentially affect DNA binding by Myc and Max, differentiate between the p22 Max−Myc and p21 Max−Myc complexes, and restrict the ability of Myc−Max to bind a subset of CACGTG motifs *in vivo*. In the light of these results we discuss the current models for transcription regulation by Myc and Max.

Results

Max can activate transcription in the absence of Myc

We have taken advantage of the yeast system to examine further transcription regulation by Max and Myc. The expression vectors and reporters used in this report are depicted in Figure 1A. In previous studies using yeast, little or no significant activation was observed when Max proteins were expressed from low copy number (LCN) vectors (Amati *et al.*, 1992; Crouch *et al.*, 1993). In marked contrast, when p22 Max was expressed from a high copy number (HCN) vector, transcriptional activation of the *PHO5* UAS *CYC−lacZ* reporter was readily detected (Figure 1B). The activation obtained by expressing p22 Max from a HCN vector (pKVMax9) was between 30- and 40-fold higher than the background obtained using a control HCN vector (pKV701) alone. In fact, expression of p22 Max from a LCN vector (pRSKVMax9) also resulted in activation significantly above background, particularly if galactose induction of p22 Max expression was allowed to proceed for >12 h. However, activation using the LCN vectors was ~10-fold less than that obtained using the HCN vector and in some experiments was not readily detectable. Thus, previous studies using yeast (Amati *et al.*, 1992; Crouch *et al.*, 1993) may have failed to detect transcription activation by Max proteins largely because LCN vectors were used. Although the absolute level of activation varied from experiment to experiment, results obtained from different yeast colonies within the same experiment were remarkably consistent (Figure 1B) and no activation was observed using control vectors that did not express Max (pKV701 and pRS315KV). As a further control we also expressed CPF1 (CP1, CBF1) from the HCN pKV701 vector; CPF1 is a yeast bHLH protein lacking an activation domain that can bind the same CACGTG sequence as Max (Baker and Masison, 1990; Cai and Davis, 1990; Mellor *et al.*, 1990). In contrast to Max, no activation from the *PHO5* UAS *CYC−lacZ* reporter was observed by expression of CPF1 (data not shown).

To determine whether the increased transcription activation observed using the HCN vector was reflected in increased expression of p22 Max, yeast extracts prepared from Max-expressing cells were used both in a band-shift assay to detect Max-specific DNA binding activity and for Western blotting to determine the levels of Max protein expressed. The results show that Max-specific DNA binding activity is substantially higher (~10-fold) in cells expressing Max from a HCN vector (Figure 1C). Increased Max DNA binding is paralleled by an increased level of p22 Max expression from the HCN vectors, as detected by Western blotting using a polyclonal anti-Max antibody (Figure 1D). Thus, the levels of transcription activation correlate well with the levels of p22 Max protein and Max-specific DNA binding activity present in the cell.

Our observation that Max can activate transcription in the absence of Myc was unexpected since results obtained by transfection of Max expression vectors into mammalian cells suggest that Max may repress transcription (Kretzner *et al.*, 1992; Reddy *et al.*, 1992; Amin *et al.*, 1993; Gu *et al.*, 1993). We therefore performed a number of control experiments (Figure 2) to establish that we were monitoring a direct effect of Max. First, we expressed a derivative of p22 Max in which two conservative amino acid changes were introduced into the Max basic region. This mutant, termed TDN Max9, fails to bind DNA *in vitro* (not shown); it also fails to activate transcription when expressed from a HCN vector in yeast (Figure 2A). Thus, DNA binding is required for transcription activation by Max. Second, we expressed a Max derivative, Mini-Max, which retains the bHLH-LZ domain but which lacks any additional amino acids. This mutant is expressed efficiently in yeast, as detected by Western blotting (not shown), can bind DNA efficiently as a homodimer *in vitro* and can activate transcription if co-

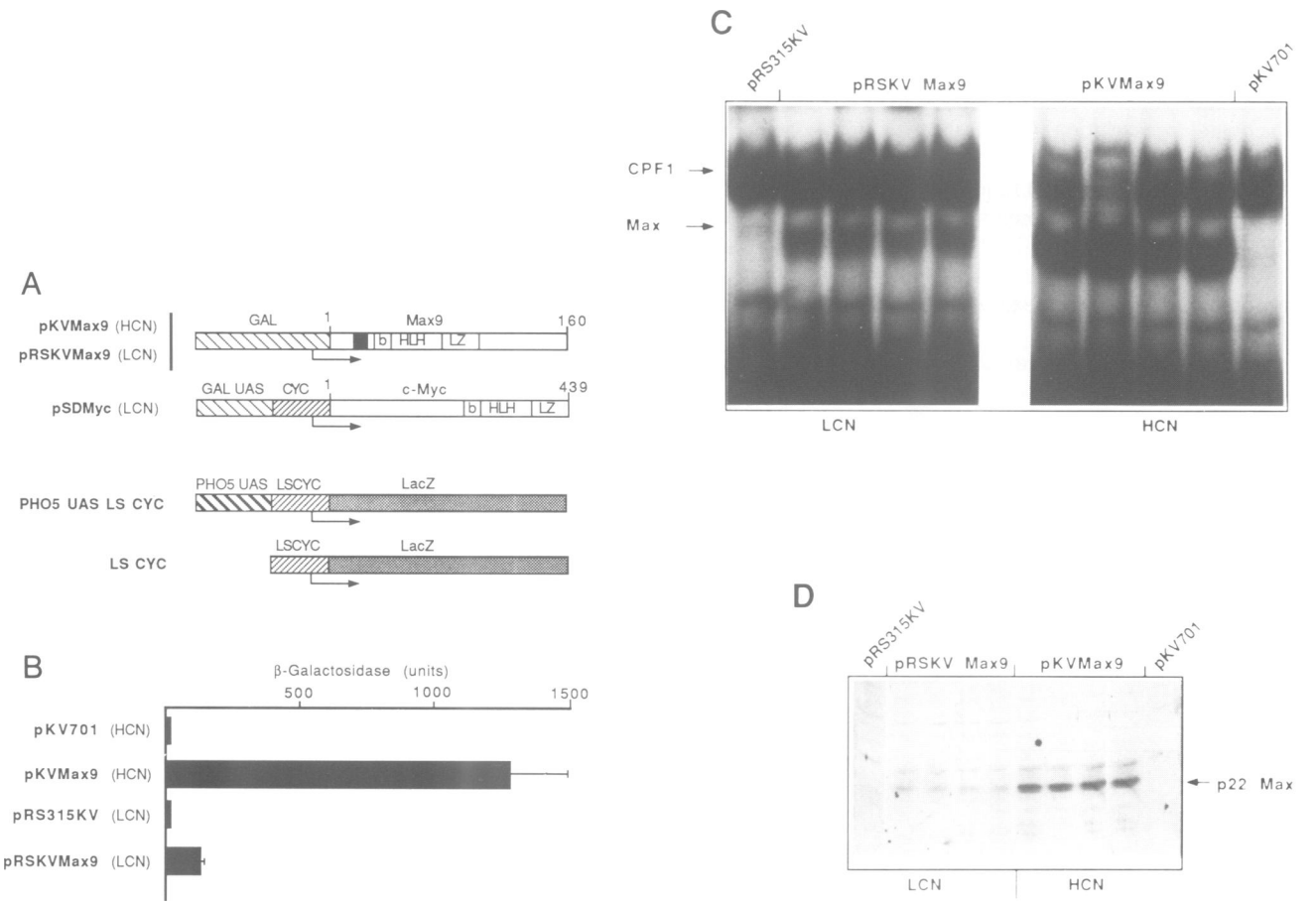

Fig. 1. Max activates transcription. (**A**) Reporters and activators. The Max expression vectors are based on the LCN pRS315, containing a *CEN/ARS* element and *LEU* selectable marker (Sikorski and Hieter, 1989) or HCN pKV701, containing a 2μ origin and *LEU* selectable marker (Cousens *et al.*, 1989). The 160 amino acid p22 Max protein, which contains the nine additional amino acids (filled box) absent from p21 Max, is expressed from the galactose inducible *GAL10* promoter in either the LCN vector, pRSKVMax9 (*CEN/ARS, LEU*), or the HCN vector, pKVMax9 (2μ, *LEU*). In the plasmid, pSDMyc, c-Myc expression is controlled by a galactose inducible promoter comprising the *GAL* UAS upstream from the basal *CYC* promoter in the LCN vector pRS314 (*TRP*) (Amati *et al.*, 1992). The HCN reporters have a *URA3* marker and contain the *lacZ* coding sequences downstream from either the basal LS *CYC* promoter or LS *CYC* downstream from a *PHO5* UAS containing two CACGTG motifs. We have used this reporter previously to demonstrate sequence-specific and Max-dependent transcription activation by Myc (Crouch *et al.*, 1993). The yeast expression vectors and reporters can be introduced in any combination into yeast strain Y700 and maintained independently of growth of the transformed yeast on media containing the appropriate amino acids. (**B**) β-galactosidase expression obtained after co-transformation of yeast with the *PHO5* UAS reporter together with either the HCN or LCN Max expression vectors or control non-expressing vectors. The data shown are the average from independent assays of four different yeast colonies obtained from the same experiment. The result for pKVMax9 is 1265 ± 242 and for pRSKVMax9 156 ± 10. (**C**) Band-shift assay of yeast extracts from the experiment shown in B, using the CACGTG-containing P2 oligonucleotide derived from the *PHO5* UAS as a probe. Each track shown was obtained using 5 μg of extract obtained from yeast derived from independent transformants. Although some variation in the levels of the endogenous CPF1 DNA binding activity is apparent, the relative level of Max DNA binding activity is in all cases substantially higher in the extracts obtained from yeast expressing Max from HCN plasmids. (**D**) Western blot of yeast extracts from the same experiment shown in panels B and C using polyclonal anti-Max antibody as probe.

expressed with Myc *in vivo* (Crouch *et al.*, 1993). However, in contrast to p22 Max, Mini-Max does not activate transcription (Figure 2A), implying that activation by Max does not result from fortuitous dimerization with any yeast transcription factors. Thus, transcription activation by p22 Max requires both DNA binding and amino acids lying outside the bHLH-LZ dimerization domain.

As a further control we wished to rule out unequivocally that the endogenous bHLH transcription activator PHO4 (Ogawa and Oshima, 1990) was in any way involved in Max-dependent transcription activation. Under the high phosphate growth conditions used here, PHO4 is repressed and should not play any role in the transcription activation observed. However, to rule out definitively any involvement of PHO4, these experiments were repeated using a *pho4* disruption strain, Y704 (F.Fisher *et al.*, 1991). p22 Max activated transcription in Y704 as efficiently as in the PHO4-expressing Y700 strain (data not shown), demonstrating that the ability of Max to activate transcription was not mediated indirectly through PHO4.

We also verified that activation by Max required specific sequences and compared the specificity and levels of activation obtained with that observed by co-expressing Myc. Two reporters (see Figure 1 and Crouch *et al.*, 1993) were used: LS *CYC*, containing a modified basal *CYC* promoter and *PHO5* LS *CYC*, which contains two CACGTG elements present in the *PHO5* UAS. The results (Figure 2B) show that activation by both Max and Myc−Max is dependent on the presence of the *PHO5* UAS, and that although the background level of activation from the LS *CYC* reporter was similar for Max and Myc−Max, Myc−Max activated transcription ~6-fold more than Max alone. It is important to stress that although the absolute levels of β-galactosidase activity varied from experiment to experiment (compare β-galactosidase activity obtained using p22 Max in Figure 2A with that in Figure 2B), activation by Max was always less

efficient than that by Myc−Max, up to 15-fold less efficient in some experiments and ~6-fold reduced in the experiment shown.

In summary, these results show that Max has the potential to activate transcription in the absence of Myc and that activation correlates well with the level of protein and DNA binding activity. Activation requires amino acids outside the minimal DNA binding domain and is dependent on both DNA binding and specific target sequences, although activation by Max is modest (~10-fold lower) in comparison with that obtained by co-expressing both Myc and Max.

Activation by Max requires amino acids N- and C-terminal to the bHLH-LZ domain

The results presented so far demonstrate that Max homodimers are able to activate transcription in the absence of Myc. We next determined which regions of Max were required for transcription activation. To this end, a series of Max mutants was constructed, expressed using a HCN vector and their ability to activate an appropriate reporter

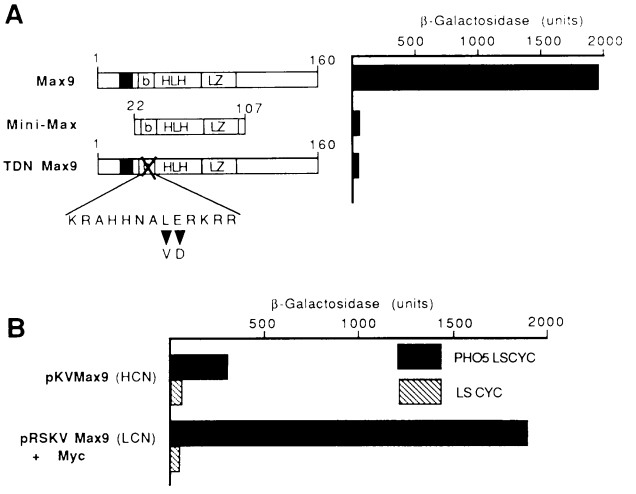

Fig. 2. Transcription activation by Max requires DNA binding and amino acids lying outwith the bHLH-LZ region. (**A**) Yeast were co-transformed with the *PHO5* UAS LS *CYC* reporter and the indicated HCN plasmids expressing p22 Max (pKVMax9) and its derivatives mini-max and TDN Max9, and β-galactosidase levels determined. (**B**) β-galactosidase levels obtained after co-transformation of yeast with the indicated reporters and expression plasmids.

was assessed. All the mutants expressed were able to bind DNA efficiently *in vitro* (data not shown). The results from a typical experiment are shown in Figure 3. As expected, p22 Max activated strongly, at least 40-fold more than the background levels obtained with Mini-Max. p21 Max, a natural variant of p22 Max lacking nine amino acids immediately N-terminal to the basic region, could also activate transcription, although in most experiments the level of activation obtained was around half that seen using p22 Max. Although Mini-Max failed to activate transcription, it was expressed to levels sufficient to allow transcription activation by Myc (Crouch *et al.*, 1993) and similar to those of Max as determined by Western blotting, while band shift assays demonstrated that it bound DNA efficiently when expressed in yeast (data not shown).

The N-terminus of Max is highly acidic and is reminiscent of activation domains found in a number of other transcription factors, prompting us to ask whether this region of Max was involved in activating transcription. Mutant Max9 ΔC107, which retains the N-terminal amino acids of p22 Max but which, like Mini-Max, lacks residues C-terminal to the leucine zipper, also activated transcription efficiently compared with Mini-Max, indicating that the N-terminal region of p22 Max makes a significant contribution to the levels of transcription activation seen. However, since the activation obtained using the ΔC107 mutant was ~3-fold less that obtained with p22 Max, it was possible that residues C-terminal to the leucine zipper could also contribute to the ability of p22 Max to activate transcription. Consistent with this, a mutant ΔC132, containing an intact N-terminus as well as 25 residues C-terminal to the deletion in Mini-Max, activated transcription as efficiently as the wild type (WT) p22 Max protein, >40-fold greater than the background level obtained using Mini-Max and >3-fold better than ΔC107. Activation as efficient as intact p22 Max was also observed using mutants ΔNΔC132 and ΔN12 which contain partial N-terminal deletions as well as 25 and 53 amino acids, respectively, C-terminal to the deletion in Mini-Max. Finally, to confirm that amino acids C-terminal to the leucine zipper were involved in transcription activation, mutant ΔN22, lacking 21 N-terminal residues but containing an intact C-terminus, was assayed. As expected, MaxΔN22 activated transcription efficiently, ~20-fold more than the background obtained using Mini-Max, but ~2-fold less than p22 Max.

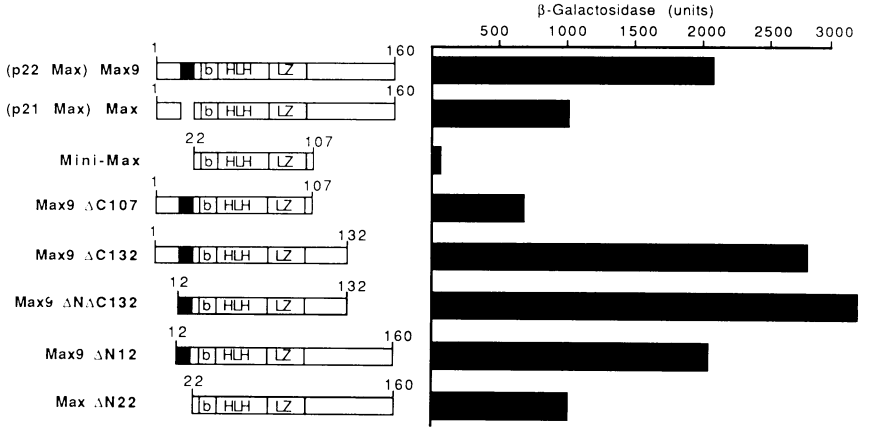

Fig. 3. Efficient transcription activation by Max requires amino acids both N- and C-terminal to the bHLH-LZ domain. The indicated Max WT or mutant proteins were expressed from a HCN vector and activation from the *lacZ* reporter measured.

Taken together these data indicate amino acids both N- and C-terminal to the bHLH-LZ contribute to transcription activation by Max. However, it should be noted that the relative contributions of the N- and C-terminal regions of Max to transcription activation may differ in the intact protein compared with the mutant derivatives used here.

Flanking sequences restrict binding of the Myc–Max heterodimer to a subset of CACGTG motifs in vivo

As both Myc and Max are able to bind the same sequence, CACGTG (Blackwell *et al.*, 1990; Blackwood and Eisenman, 1991; F.Fisher *et al.*, 1991; Halazonetis and Kandil, 1991; Kerkhoff *et al.*, 1991; Prendergast *et al.*, 1991; Prendergast and Ziff, 1991; Berberich and Cole, 1992), the demonstration that both can activate transcription raises the question as to how they maintain their regulatory specificity. Of course, if Max activates transcription less efficiently than the Myc–Max complex this may in itself provide a sufficient degree of regulation. However, other mechanisms may operate and, while differential protein–protein interactions and post-translational modification by casein kinase II are likely to be important for the control of transcription by these proteins, it is also possible that sequences flanking the core CACGTG motif discriminate between DNA binding by Max and the Myc–Max complexes. Indeed, the precise nature of Myc and Max binding specificities is a key issue given the absence of information concerning potential targets for transcriptional regulation by these proteins.

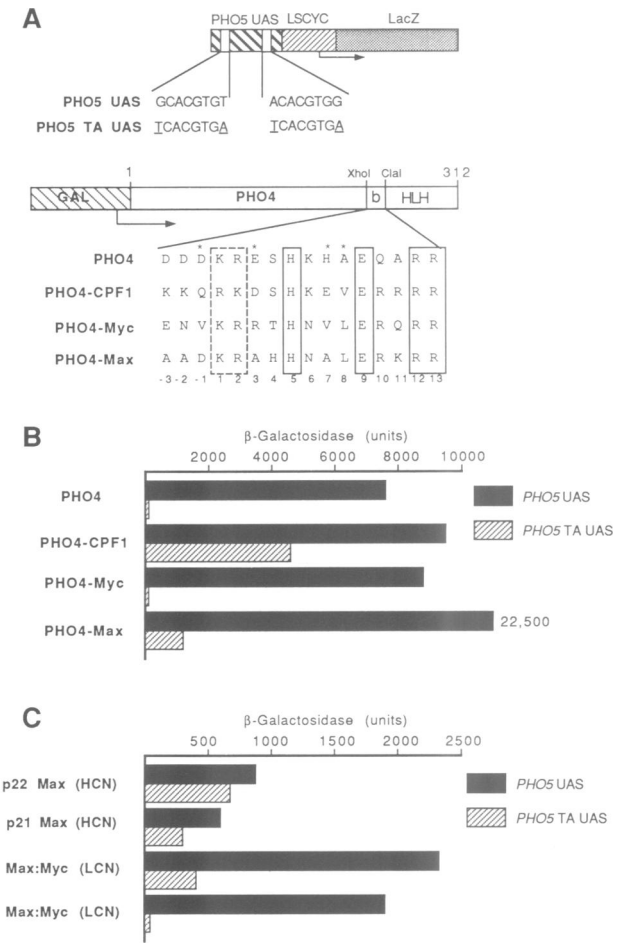

Fig. 4. Max and Myc–Max binding specificities *in vivo*. (**A**) The reporters used contain either a *PHO5* UAS containing two CACGTG motifs or a mutant UAS in which the CACGTG elements are flanked by 5′ T and 3′ A residues. The *PHO4* chimeric plasmids are expressed from a *GAL10* promoter in the pKV701 HCN vector and contain oligonucleotides encoding the relevant amino acids inserted between the *Cla*I and engineered *Xho*I sites flanking the PHO4 basic region. Asterisks indicate amino acids which influence PHO4 DNA binding specificity for the TCACGTGA mutant UAS *in vitro* (Fisher and Goding, 1992). The amino acids across the basic region are numbered as described previously (Fisher and Goding, 1992; F.Fisher *et al.*, 1991) to facilitate comparison between the different basic regions. (**B**) Levels of β-galactosidase activity detected after co-transformation of yeast with the indicated reporters and expression plasmids. (**C**) Transcription activation from the WT or TA-substituted PHO5 UAS by p21 or p22 Max expressed alone from the HCN vector or co-expressed from the LCN vectors with c-Myc.

For two other bHLH proteins a clear discrimination between closely related binding sites has been described. The two yeast bHLH proteins, PHO4 and CPF1, both bind the core sequence CACGTG, but while binding by PHO4 *in vitro* is inhibited by the presence of a T residue immediately 5′ to the CACGTG motif, binding by CPF-1 is not (Fisher and Goding, 1992). Given the degree of similarity between the c-Myc and PHO4 basic regions (Figure 4A and F.Fisher *et al.*, 1991), we asked whether DNA binding by Myc and Max could be similarly affected by sequences flanking the CACGTG recognition sequence.

Since DNA binding specificity is primarily determined by DNA–protein interactions across the basic region and since Myc is unable to bind DNA as a homodimer, we substituted the PHO4 basic region with that from c-Myc. In this chimeric protein, DNA binding specificity is determined by the Myc basic region while PHO4 provides the dimerization interface. We have used this PHO4–Myc chimeric protein previously to demonstrate that the Myc basic region was able to recognize the CACGTG motifs present in the *PHO5* UAS (F.Fisher *et al.*, 1991). As reporter we used either a *PHO5* UAS or a UAS in which the CACGTG motifs were flanked by 5′ T and 3′ A residues. The reporters and activators are depicted in Figure 4A. As expected from our previous work (F.Fisher *et al.*, 1991) both PHO4 and the PHO4–Myc chimera activated the WT UAS efficiently (Figure 4B). In contrast, no activation was observed using the TA-substituted UAS, demonstrating that the Myc basic region, like that of PHO4, could not bind the sequence TCACGTGA *in vivo*. Unlike PHO4, CPF1 can bind TCACGTGA *in vitro* (Fisher and Goding, 1992). As a control, therefore, we expressed a PHO4–CPF1 basic region chimera and asked whether CPF1 specificity *in vitro* was reflected *in vivo*. As expected activation by this construct was only 2-fold less on the TA-substituted UAS than on the WT UAS.

Binding by the Myc basic region was completely inhibited *in vivo* by the presence of a 5′ T and 3′ A residue flanking the CACGTG binding motif. Since the intact Myc protein binds DNA as a heterodimer with Max, it was essential to determine also the sequence specificity of the Max basic region. Using a PHO4–Max basic region chimera activation of the WT *PHO5* UAS was 2- to 3-fold more efficient than that of the WT PHO4 protein, suggesting the PHO4–Max chimera could bind more strongly. More importantly, while activation of the TA-substituted *PHO5* UAS by the PHO4–Myc construct was reduced by at least 180-fold compared with the WT UAS, activation by PHO4–Max was reduced by only 20-fold. Since this *in vivo* assay for DNA

binding is exquisitely sensitive, these results raised the possibility that the binding specificities of Myc and Max with respect to flanking sequences were distinct.

We next asked, therefore, whether the intact p22 or p21 Max proteins could activate transcription from the TA-substituted UAS. To this end, p22 and p21 Max were expressed from HCN vectors (pKVMax9 and pKVMax respectively) and activation from a WT or TA-substituted UAS measured. Compared with the activation obtained on a WT UAS, activation of the mutant UAS by either p21 or p22 Max was reduced by a maximum of 2-fold (Figure 4C). Binding by the intact proteins to the TCACGTGA sequences in the mutant UAS was therefore less sensitive than that of the PHO4−Max chimera, perhaps reflecting a contribution of amino acids outside the basic region to DNA binding specificity.

Since the majority of Myc in cells is complexed with Max (Blackwood *et al.*, 1992; Littlewood *et al.*, 1992), it was important to determine the binding specificity of the heterodimer. Three possibilities were likely: first, the Myc−Max heterodimer could bind CACGTG but not TCACGTGA, indicating that in the heterodimer Myc specificity was dominant; secondly, Max specificity could be dominant, enabling the heterodimer to bind both elements; and thirdly, the Myc−Max complex could bind TCACGTGA but with lower affinity than CACGTG, specificity being determined by both basic regions in the heterodimer. To test these possibilities either p21 or p22 Max was expressed from LCN vectors (pRSKVMax and pRSKVMax9 respectively) together with Myc, and the ability of the resulting Myc−Max heterodimers to activate transcription from the WT or TA-substituted UAS assessed. The results are shown in Figure 4C. As expected both Myc−p22 Max and Myc−p21 Max heterodimers activated transcription efficiently from the WT UAS. In contrast, activation of the TA-substituted UAS by the p22 Max−Myc complex was reduced ~4- to 5-fold in the experiment shown and no more than 7-fold in other experiments, suggesting that both the Myc and Max basic regions are contributing to the binding specificity of the Myc−p22 Max heterodimer. Most striking, however, was the observation that transcription activation by the p21 Max−Myc complex was completely abolished using the TA-substituted UAS as a target, with activation being reduced >30-fold when compared with the WT UAS. These data demonstrate both that Max, Myc−p21 Max and Myc−p22 Max have subtly different binding specificities *in vivo* and that flanking sequences are likely to restrict transcription regulation by the Myc−Max complexes to a subset of CACGTG motifs.

Discussion

Transcription activation by Max

In this paper we demonstrate that Max has the potential to activate transcription, and that activation by Max is dependent on DNA binding to specific target sites and requires amino acid sequences outwith the minimal bHLH-LZ DNA binding domain. This appears to be in direct contrast to several previous reports which suggest that Max homodimers act as a transcriptional repressor (Kretzner *et al.*, 1992; Reddy *et al.*, 1992; Amin *et al.*, 1993; Gu *et al.*, 1993). We believe that the different results are nevertheless easily reconciled.

Previous reports using the yeast system to examine the transcriptional properties of Myc and Max faithfully reproduced Max-dependent transcriptional activation by Myc, but did not detect activation by expression of Max alone (Amati *et al.*, 1992; Crouch *et al.*, 1993). We show here that while the LCN vectors used in previous studies produce sufficient Max to activate transcription in collaboration with Myc, HCN vectors, which lead to higher levels of Max expression and higher levels of Max DNA binding activity, must be used to observe significant transcription activation by Max homodimers. The lower threshold required for activation as a heterodimer with Myc may indicate that either the Myc−Max complex has a higher DNA binding affinity *in vivo* than Max homodimers, or alternatively, a large proportion of Max homodimer DNA binding activity is inhibited, perhaps by post-translational modification. Phosphorylation of Max by casein kinase II, which is also present in yeast, is a candidate mechanism since it inhibits binding by Max homodimers but not by Myc−Max heterodimers (Berberich and Cole, 1992). Increased expression of Max from the HCN vectors would increase proportionally the amount of unmodified Max able to bind DNA and thereby to activate transcription. Irrespective of the difference in levels of activation by Myc and Max our results support the idea that if Max homodimers can bind DNA they will activate transcription.

The interpretation of the results obtained from mammalian cells (Kretzner *et al.*, 1992; Reddy *et al.*, 1992; Amin *et al.*, 1993; Gu *et al.*, 1993) is complicated by the observation that Max can heterodimerize with two other bHLH-LZ proteins, Mad and Mxi-1 (Ayer *et al.*, 1993; Zervos *et al.*, 1993). Under these circumstances it is not possible to know whether the repressive effect of Max overexpression in mammalian cells is mediated by Max homodimers or Mad−Max and Mxi-1−Max heterodimers. The advantage of the yeast system used here is that it has allowed us to examine the effect of Max in the absence of these dimerization partners. As a result we have been able to demonstrate that Max homodimers have the potential to activate transcription. A comparison of the levels of activation obtained by expression of Max and Myc−Max suggests that Max activates transcription weakly in comparison with the Myc−Max complex, an observation supported by the fact that overexpression of Max in yeast can reduce the levels of activation obtained by co-expression of Myc and Max (Amati *et al.*, 1992; our unpublished results). This by itself may be sufficient to explain the results from transient transfection assays in mammalian cells; overexpression of Max could displace the Myc−Max complex from common target sites by competition and, since Max appears to activate transcription less well than Myc−Max, the decreased level of transcription observed would appear as a repressive effect of Max. Consistent with this, the transcriptional repression observed on overexpression of Max in mammalian cells can be reproduced in yeast; co-expression of Max from an HCN vector together with Myc results in activation levels equivalent to those obtained by HCN Max alone and up to 10-fold lower than the activation observed when Myc is co-expressed with LCN Max (our unpublished observations).

In mammalian cells it is not yet clear what proportion of Max is available to bind DNA as a homodimer, given its ability to form heterodimers with Myc, Mad and Mxi-1 and the likely regulation of Max homodimer DNA binding by post-translational mechanisms such as phosphorylation by

casein kinase II. Nevertheless, given the low level of Myc in quiescent cells, the potential regulation of casein kinase II by $p34^{CDC2}$ kinase at mitosis (Litchfield *et al.*, 1992) and its differential subcellular localization during the cell cycle (Gauthier *et al.*, 1991; Yu *et al.*, 1991), it is likely that significant levels of DNA-bound Max homodimers are present in cells at some stages of the cell cycle and that this DNA-bound Max will activate transcription. However, while complexes containing Max but not Myc have been detected in cell extracts (Littlewood *et al.*, 1992), it is not yet clear whether these represent Max homodimers or Max–Mad or Max–Mxi-1 heterodimers.

A role for Max activation

It is clear that expression of Max in yeast can result in sequence-specific transcription activation. We wish to stress, however, that while expression of Max in yeast is clearly specific—no transcription activation is observed by overexpressing either CPF1 or a PHO4 protein containing a deletion that removes its activation domain—these results cannot be taken as formal proof that it plays the same role in mammalian cells. Until transcription activation by Max is observed in mammalian cells, our results must only be taken as indicative. In this respect it is relevant that GAL4–Max chimeras fail to activate transcription in mammalian cells (Kato *et al.*, 1992). However, this may simply reflect a specific conformational requirement for Max to achieve transcription activation that is not obtained using chimeric proteins.

If Myc–Max is required for high level transcription activation of target genes, what role can be envisaged for activation by Max? It seems likely that the genes activated by Myc need to be transcribed at high levels only at the transition from quiescence to proliferation and perhaps also at certain stages in the cell cycle. The ability of Myc to activate transcription appears to be dictated primarily by the levels of Myc expression and by the availability of Max, which may be sequestered by Mad and Mxi-1. However, while the high level of activation of such genes mediated by Myc–Max may not be a permanent requirement, it is possible that some basal level of transcription must be maintained as a housekeeping function even in quiescent cells. Since the level of transcription activation by Max homodimers is likely to be less than that by Myc–Max heterodimers, it seems reasonable to propose that this function could be supplied by Max homodimers. By mediating a low level of transcription activation Max could maintain an open chromatin conformation on Myc-responsive promoters, leaving them primed for a rapid response to increased levels of Myc–Max complex. In contrast, in the absence of activation by Max, complete repression of target genes might well result in formation of a transcriptionally refractive chromatin structure, restricting access by the Myc–Max complex and either preventing transcription activation entirely or delaying the response to increased Myc expression.

Flanking sequences target Myc–Max to a subset of CACGTG motifs in vivo

Little is known of the genes which may be potential targets for regulation by Max and its dimerization partners. Since cells contain multiple other bHLH proteins able to bind, and presumably compete for, the same CACGTG motif recognized by Max and the Myc–Max complex, it is difficult to understand how regulatory specificity is maintained. The potential of the different family members to activate transcription must therefore be differentially regulated, either at the level of DNA binding by heterodimerization, as with Myc, Mxi-1 and Mad, or by post-translational modification, like Max, or at the level of transcription activation by masking or modifying their activation domains. An alternative and complementary possibility is that when multiple members of the bHLH-LZ family able to bind the CACGTG motif are present in a cell at any one time, the many alternative homo- or heterodimers that form may possess subtly distinct DNA binding specificities which would direct them to specific subsets of CACGTG-containing promoters. In this respect our results obtained with Myc and Max may be significant; Myc–p21 Max efficiently activates transcription from the CACGTG motifs in the *PHO5* UAS, but activates at least 30-fold less well if the same elements are flanked by 5′ T and 3′ A residues. In contrast, activation by the Myc–p22 Max complex is reduced by between 4- and 7-fold only, while activation by either Max homodimer is affected by at most 2- to 3-fold. Thus, sequences outside the core 6 bp recognition sequence play a significant role in determining binding specificity of the Myc–Max heterodimer. These results extend significantly the work of Halazonetis and Kandil (1992) who demonstrated that the DNA binding *in vitro* of truncated Myc was influenced by sequences flanking the core 6 bp binding site, and of Prendergast and Ziff (1991) who demonstrated some specificity of the Myc basic region in selection of a subset of E-box motifs *in vitro*. The observation that flanking sequences can restrict binding of bHLH proteins to a subset of CACGTG motifs *in vivo* is novel and highlights the potential for differential gene regulation by these proteins; whereas many genes may contain CACGTG bHLH protein binding sites only a subset will be a target for Myc–Max. Moreover the differences in binding specificity between Max, Myc–p21 Max and Myc–p22 Max suggest that these complexes may recognize distinct but overlapping subsets of target genes.

While this may represent the first demonstration that flanking sequences selectively restrict binding to a core CACGTG motif *in vivo*, it is unlikely that a mechanism to direct different members of the bHLH transcription factor family to subsets of what have previously been accepted as common binding sites, will be restricted to the bHLH proteins used in this study. Indeed, selection and PCR amplification of sequences bound by MyoD and E47 suggests that E47, but not MyoD, binding may also be inhibited by the presence of a T residue 5′ to the CACNTG recognition sequence (Blackwell and Weintraub, 1990). Whether this apparent *in vitro* specificity is reflected *in vivo* remains to be determined.

It is clear that the yeast system employed here will be instrumental in unravelling the complexities of gene regulation by Max and its various dimerization partners and may also prove valuable for examining gene regulation by bHLH proteins in general.

Materials and methods

Expression vectors and reporters

The *PHO5* UAS used in this study has been described previously (F.Fisher *et al.*, 1991) and contains a mutation that changes the P1 element from CACGTT to CACGTG. The LCN Max expression vectors (Crouch *et al.*, 1993) as well as the construction of pSDMyc (Amati *et al.*, 1992) have been described previously. The LS *CYC* reporter has been described

previously (Crouch *et al.*, 1993) and contains multiple point mutations in two CACATG motifs known to bind Myc–Max *in vivo* (Crouch *et al.*, 1993) and Max *in vitro* (unpublished observations). Activation by Max from the *CYC* promoter in the absence of a UAS was between 7- and 10-fold higher than that from LS *CYC* (unpublished observations). Activation by Myc–Max through the *CYC* CACATG elements has been published previously (Crouch *et al.*, 1993).

The HCN Max vectors used contain the same *GAL10* promoter and PGK terminator sequences as the LCN Max expression vector but are based on pKV701 (Cousens *et al.*, 1989). Max deletion mutants were made by PCR using the appropriate primers and deletions confirmed by sequencing. TDN Max9 was constructed in two steps. First, appropriate N- and C-terminal fragments were isolated by PCR using primers which place a *Sal*I restriction site within the basic region and *Bam*HI sites at the N- and C-termini. Each fragment was first cloned as a *Bam*HI–*Sal*I fragment and then reassembled by a triple ligation into pUC as a *Bam*HI fragment. This fragment was subsequently inserted into the unique *Bgl*II cloning site of pKV701. Introduction of the *Sal*I site into the Max basic region created a double conservative amino acid change that abolished Max DNA binding ability.

The PHO4 and PHO4–Myc constructs have been described previously (F.Fisher *et al.*, 1991) as has PHO4–CPF1 (Fisher and Goding, 1992). The PHO4–Max chimera was isolated by insertion of the appropriate oligonucleotide encoding the Max basic region between the unique engineered *Xho*I site and *Cla*I sites flanking the PHO4 basic region. The TA-substituted *PHO5* UAS was made by PCR amplification using mismatched primers that introduced *Bam*HI sites to each end of the UAS and substituted 5′ T and 3′ A residues flanking the two CACGTG motifs. The TA-substituted *PHO5* UAS was then cloned into the unique *Bgl*II site of LS *CYC*.

Yeast strains, transformations and β-galactosidase assays

Yeast strains used were Y700 (*MATα trp1-1 ade2-1 leu2-3 leu2-112 his3-11,15 ura3-52, can 1-100*) and Y704 (F.Fisher *et al.*, 1991), transformations and β-galactosidase assays were performed as described previously (F.Fisher *et al.*, 1991; Crouch *et al.*, 1993). All assays were performed multiple times from different yeast colonies resulting from more than one yeast transformation.

Western blotting and DNA binding assays

For Western blotting and DNA binding band-shift assays, yeast extracts were prepared by glass bead lysis essentially as described by Littlewood *et al.* (1992). For Western blots 10 μg portions of yeast extract were resolved by 15% SDS–PAGE and analysed by Western blotting using a polyclonal rabbit anti-Max antiserum raised against a bacterial GST–Max fusion protein (to be described elsewhere). Reactive proteins were visualized using an alkaline phosphatase-conjugated goat anti-rabbit second antibody (Promega). For DNA binding assays, yeast extract prepared by glass bead lysis was used in band shift assays using the P2 probe (5′-gatccTTGGCACTCACA-CGTGGGACTAGCAg-3′) derived from the *PHO5* UAS (F.Fisher *et al.*, 1991). Similar results have been obtained using the CACGTG-containing CM-1 oligonucleotide (Blackwood and Eisenman, 1991). DNA binding assays were essentially as described (Blackwood and Eisenman, 1991) except that 0.5 μg poly(dI-dC) were included in the reaction together with 5 μg of yeast extract, and incubation was for 30 min at 20°C. Complexes were resolved on a 6% polyacrylamide gel and subsequently dried and autoradiographed.

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