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Max-E47, a Designed Minimalist Protein That Targets the E-Box DNA Site *in Vivo* and *in Vitro*

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Abstract: Max-E47 is a designed hybrid protein comprising the Max DNA-binding basic region and E47 HLH dimerization subdomain. In the yeast one-hybrid system (Y1H), Max-E47 shows strong transcriptional activation from the E-box site, 5′-CACGTG, targeted by the Myc/Max/Mad network of transcription factors; two mutants, Max-E47Y and Max-E47YF, activate more weakly from the E-box in the Y1H. Quantitative fluorescence anisotropy titrations to gain free energies of protein:DNA binding gave low nanomolar K_d values for the native MaxbHLHZ, Max-E47, and the Y and YF mutants binding to the E-box site (14, 15, 9, and 6 nM, respectively), with no detectable binding to a nonspecific control duplex. Because these minimalist, E-box-binding hybrids have no activation domain and no interactions with the c-MycbHLHZ, as shown by the yeast two-hybrid assay, they can potentially serve as dominant-negative inhibitors that suppress activation of E-box-responsive genes targeted by transcription factors including the c-Myc/Max complex. As proof-of-principle, we used our modified Y1H, which allows direct competition between two proteins vying for a DNA target, to show that Max-E47 effectively outcompetes the native MaxbHLHZ for the E-box; weaker competition is observed from the two mutants, consistent with Y1H results. These hybrids provide a minimalist scaffold for further exploration of the relationship between protein structure and DNA-binding function and may have applications as protein therapeutics or biochemical probes capable of targeting the E-box site.

Introduction

The basic-region/helix−loop−helix/leucine zipper (bHLHZ) transcription factor (TF) family includes the ubiquitous Myc/Max/Mad network involved in 50% or more of known cancers and tumors.[1,2] Heterodimeric Myc/Max is a transcriptional activator that binds the Enhancer box (E-box) sequence 5′-CACGTG, thereby regulating the expression of target genes critical for normal cell proliferation and differentiation.[3−6] Myc proteins, which are proto-oncogenic and contain activation domains, do not homodimerize and do not bind to DNA unless partnered with Max, which does not possess an activation domain and serves to regulate Myc activity.[5] In contrast, Max can homodimerize and bind the E-box.[3] Thus, proteins that interfere with Myc/Max dimerization or its recognition of the E-box site may interfere with Myc's disease-promoting activities.

In recent years, use of a dominant-negative (DN) system to inhibit protein function has become increasingly popular. A number of different DN inhibitors of dimeric TFs has been described and mainly divided into two general classes.[7,8] The first class of DN inhibitors binds the same DNA target but lacks an activation domain (AD).[9,10] Such a DN inhibitor binds DNA but fails to activate transcription and thus functions as a competitive inhibitor of the target protein for its cognate binding site.[8,11,12] The Max homodimer belongs to this group of competitive inhibitors, acting as a dose-dependent antagonist of Myc function.[13] The second class of DN inhibitors lacks a DNA-binding domain;[14] such DN inhibitors heterodimerize with target proteins and prevent their DNA binding. For instance, Nasi and co-workers created a c-Myc derivative, Omomyc, capable of homodimerization as well as heterodimerization with

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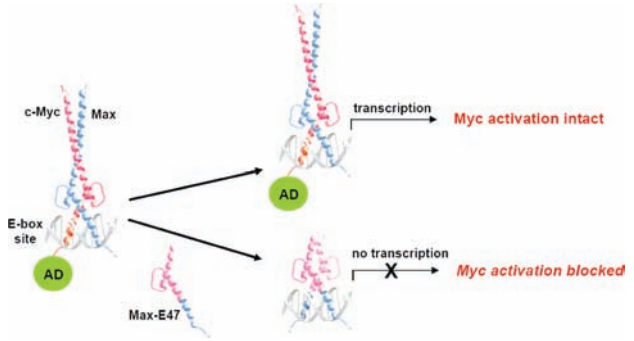

Figure 1. Schematic of the minimalist design strategy. The c-Myc transcriptional activator must heterodimerize with Max in order to bind to the E-box site; c-Myc is proto-oncogenic, so activation at the E-box can lead to disease. Our Max-E47 series of hybrids can effectively compete for binding at the E-box and may serve as dominant negative competitors of native c-Myc/Max, thereby inhibiting activation from the E-box.

c-Myc and Max; Omomyc sequesters c-Myc in complexes with poor DNA-binding ability and prevents heterodimerization with Max.[15] For both classes of DN inhibitors, overexpression is often necessary for efficient inhibitory activity.[8,16]

Owing to the importance of E-box regulation, we applied our minimalist strategy toward design of the first class of DN inhibitors based on the protein α-helix, a straightforward molecular-recognition scaffold that targets the E-box and allows manipulation of gene expression at the level of the protein:DNA recognition event (Figure 1). The Myc/Max network provides an excellent starting point for molecular design, because much experimental data exists including high-resolution structures;[17−19] therefore, it serves as an ideal proof-of-principle to test our minimalist design strategy. Our aim is to generate smaller proteins of simplified structure compared with their native counterparts, while still retaining DNA-binding function. The Max-E47 hybrids comprise 66 amino acids, proteins easily accessible by either chemical synthesis or bacterial expression.

We created the hybrid Max-E47 by fusing the basic region of bHLHZ protein Max and HLH subdomain of bHLH protein E47; hence, we exchanged the DNA-binding regions and dimerization elements between two different protein families toward design of hybrid proteins that target the E-box. These hybrids were assayed for E-box binding function both *in vivo* and *in vitro* by yeast genetic assays and thermodynamic fluorescence anisotropy titrations. The yeast assays demonstrate that the Max-E47 series of hybrids is capable of E-box-responsive reporter gene activation and can compete with native MaxbHLHZ for the E-box target. Strong, specific binding of all three hybrids to the E-box site was measured by fluorescence anisotropy. Hence, the Max-E47 series of hybrids has the potential to serve as the first class of dominant negative inhibitors and inhibit the expression of E-box-responsive genes targeted by transcription factors such as the c-Myc/Max heterodimer.

Results

Two protein mutants were obtained during cloning of the gene encoding Max-E47: Max-E47Y contains a valine to tyrosine

A) native MaxbHLHZ control

basic region ***helix-loop-helix***

ADKRAH**H**NAL**E**RKR**R**-DHIKDSFHSLRDSVPSLQGEKASRAQILDKA

leucine zipper

TEYIQYM-RRKNDTHQQDIDDLKRQNALLEQQVRALEKARSSAQLQT

B) native E47

basic region ***helix-loop-helix***

350 360 370

RERRMANNARERVRV-RDINEAFRELGRMCQMHLKSDKAQTKLLIL

380 390

QQAVQVILGLEQQVRERNLNP

C) Max-E47

ADKRAH**H**NAL**E**RKR**R**-RDINEAFRELGRMCQMHLKSDKAQTKLLIL

QQAVQVILGLEQQVRERNLNP

Max-E47Y

ADKRAH**H**NAL**E**RKR**R**-RDINEAFRELGRMCQMHLKSDKAQTKLLIL

QQAVQ**Y**ILGLEQQVRERNLNP

Max-E47YF

ADKRAH**H**NAL**E**RKR**R**-RDINEAFRELGRMCQMHLKSDKAQTKLLIL

QQAVQ**Y**ILGLEQQ**F**RERNLNP

Figure 2. Sequences of the Max-E47 hybrids. The three highly conserved basic-region residues that make sequence-specific contacts to DNA major groove bases are in bold. The mutated amino acids are in bold and underlined. The numbering follows that used by Ellenberger et al.[20]

mutation at position 385, and Max-E47YF contains an additional valine to phenylalanine mutation at position 393 (Figure 2). Experiments paralleling those for Max-E47 were performed for these fortuitous mutants as well.

The Max-E47 Hybrid Series Targets the E-Box in the Yeast One-Hybrid Assay. We tested our design strategy in the yeast one-hybrid (Y1H) assay with the *HIS3* and *lacZ* reporters. Activation of the *HIS3* reporter was confirmed by growth on medium lacking histidine, whereas activation of the *lacZ* reporter was detected by two colorimetric assays: qualitative X-gal colony-lift filter assay and quantitative *ortho*-nitrophenyl-*β*-galactoside (ONPG) liquid assay.

Max-E47 showed comparably strong transcriptional activation from the E-box as the positive control native MaxbHLHZ: colonies appeared at 2 days on test plates in the *HIS3* assay (Figure 3A). Despite variability in colony numbers between plates, colony sizes were comparable to native MaxbHLHZ. Max-E47Y showed strong colony growth as well: colonies appeared 4 h later. Max-E47YF activated from the E-box more weakly: colonies appeared at 4 days. In comparison, the truncated native MaxbHLH, without leucine zipper, shows no activation from the E-box in any of the yeast assays performed (data not shown). For all transformations, plasmids were extracted from positive transformants, subjected to restriction enzyme digest analysis, and sequenced. Both gel analysis and sequencing confirmed the correct identity of plasmids, which were retransformed into the YM4271[pHisi-1/E-box] strain. The same growth was observed, confirming the initial positive results.

For further confirmation, plasmids were transformed into strain YM4271[pLacZi/E-box], which employs reporter *lacZ*. Both the X-gal colony-lift filter assay and quantitative ONPG liquid assay confirmed the positive results from the *HIS3* selection. In the colony-lift assay, blue color appeared for both Max-E47 and Max-E47Y at 20 min and turned bright blue at

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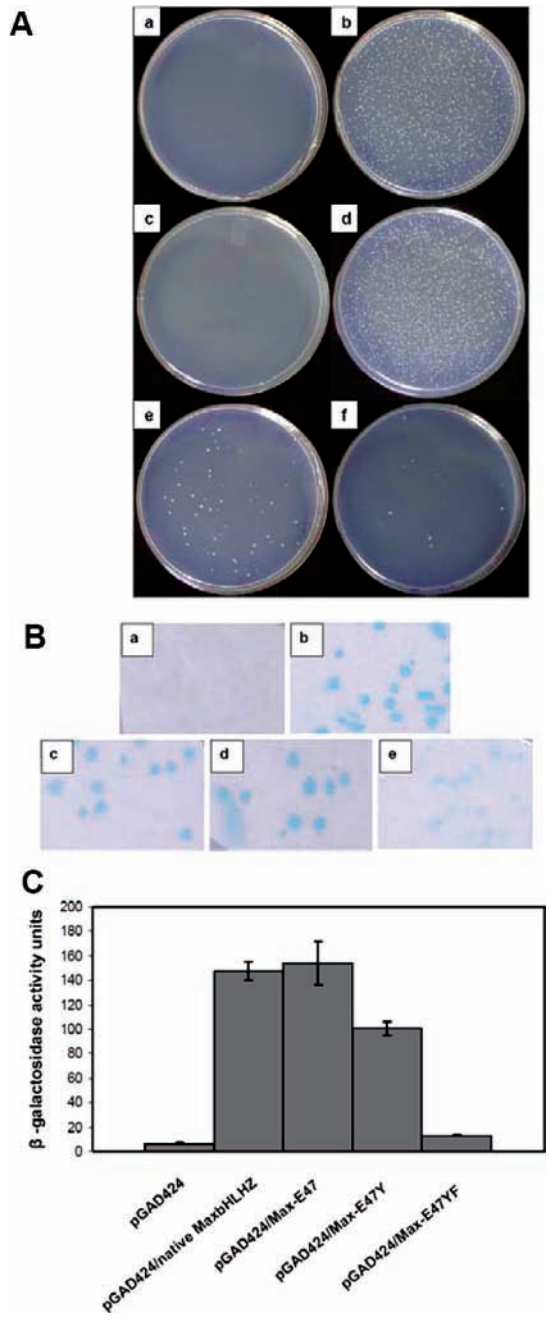

Figure 3. Max-E47 hybrids activate transcription from the E-box (Y1H). (A) The *HIS3* assay of Max-E47 hybrids expressed in pGAD424. SD/−H/−L + 10 mM 3-AT plates were incubated at 30 °C for 6 days: (a) pGAD424 (negative control), clean; (b) pGAD424/native MaxbHLHZ (positive control)—note that the colonies were too crowded to grow larger; same amounts were plated on all plates for comparison; (c) pGAD424/native MaxbHLH; (d) pGAD424/Max-E47: (e) pGAD424/Max-E47Y; (f) pGAD424/Max-E4/YF. (B) The X-gal colony-lift filter assays of Max-E47 hybrids. All of the SD/−U/−L plates were incubated at 30 °C for 4 days before testing. Photos were taken after 2 h incubation: (a) pGAD424 (negative control), very faint blue; (b) pGAD424/native MaxbHLHZ (positive control), vivid blue at 20 min; (c) pGAD424/Max-E47, vivid blue at 20 min; (d) pGAD424/Max-E47Y, vivid blue at 20 min; (e) pGAD424/Max-E47YF, faint blue. (C) Histogram comparing the binding strengths of Max-E47 hybrids to E-box. All values are averages of 9−12 measurements (±SD) from 3−4 separate cell-growth cultures.

2 h, similar to native MaxbHLHZ (Figure 3B). The color for Max-E47YF was much fainter but clearly above background (negative control pGAD424).

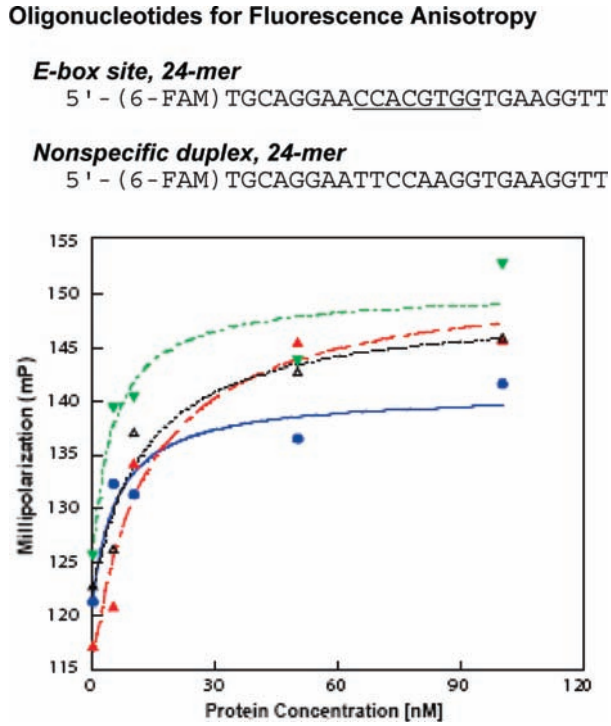

Figure 4. (top) DNA duplexes used in fluorescence anisotropy titrations. "6-FAM" is 6-carboxyfluorescein, and the Max-preferred E-box is underlined (core E-box is CACGTG). (bottom) Representative equilibrium binding isotherms for native MaxHLHZ (●, blue line), Max-E47 (▲, red line), Max-E47Y (▼, green line), and Max-E47YF (△, black line) targeting the E-box. Each isotherm was obtained from an individual titration, and each K_d value is the average of two individual titrations ± SEM.

The ONPG assay quantitatively confirms the trend in transcriptional activation from the E-box of the three hybrids (Figure 3C). The ONPG value for Max-E47 is 153.9 ± 17.7, comparable to native MaxbHLHZ (147.4 ± 7.3). Max-E47Y is somewhat lower at 101.0 ± 5.5 but still gives a high value. Max-E47YF gives a much lower reading of 13.3 ± 0.5; for comparison, the pGAD424 value is 7.0 ± 0.5. The ONPG assay confirms that Max-E47 and Max-E47Y are strongly capable of transcriptional activation from the E-box.

All three Max-E47 hybrids exhibit strong binding affinities to the E-box in in vitro fluorescence anisotropy titrations. Yeast genetic assays measure the E-box-responsive activity of our hybrids in a physiologically relevant, *in vivo* environment. However, these reporter assays rely on indirect means for detection of protein:DNA interactions, and the ONPG assay is not linear or stringently quantitative.[21] Thus, we conducted quantitative fluorescence anisotropy titrations to measure free energies of protein:E-box complexation. Native MaxbHLHZ, Max-E47, Max-E47Y, and Max-E47YF were assayed with fluorescein-labeled 24-mer DNA duplexes (Figure 4); no binding by any protein was detected with the nonspecific DNA control, even at 2 μM monomeric protein concentration (data not shown).

In contrast to the *in vivo* Y1H that shows the E-box-activation trend of Max-E47, Max-E47Y, and Max-E47YF from strongest to weakest, thermodynamic analysis gave comparable low nanomolar K_d values for all four proteins binding to the E-box: native MaxbHLHZ at K_d 14.3 ± 7.9 nM, Max-E47 at 15.3 ± 1.6 nM, Max-E47Y at 8.7 ± 3.3 nM, and Max-E47YF at 6.4

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± 0.5 nM. Hill coefficient analyses of binding isotherms show that all three Max-E47 hybrids have similar values to that of MaxbHLHZ; this indicates that the Max-E47 hybrids, like MaxbHLHZ, likely form dimeric structures for cooperative binding to the E-box site. These dissociation constants compare well with those reported by three different laboratories using electrophoretic mobility shift assay (EMSA),[22] fluorescence anisotropy,[23] or calorimetry[24] for measurement of the MaxbHLHZ domain bound to the E-box, with K_d values in the 1−3 nM range. Thus, by fluorescence anisotropy, strong and specific binding to the E-box was measured by all four proteins, with no pronounced differences in DNA-binding function, as observed in the Y1H.

The Max proteins were difficult to manipulate in quantitative titrations. We suspect protein misfolding and possible formation of soluble aggregates may lead to nonfunctional protein. Therefore, we varied buffers and conditions to find a reliable environment for obtaining quantitative information. Protein misfolding and aggregation was also reported by those groups that measured the binding affinities of the MaxbHLHZ with the E-box,[22−24] as well as in our own studies with other bHLH derivatives.[25] Such intractability appears to be prevalent with the DNA-binding domains of the bHLH superfamily of transcription factors.

In our fluorescence analysis, the buffer system is believed to play an important role in maintaining stably folded protein structure leading to DNA-binding function. However, the chosen *in vitro* conditions may not allow discrimination of fine structural and functional differences among the three hybrids. Additionally, the sequences flanking the E-box site on the FA titration probes are not identical to those in the yeast reporter assays, and these differences likely affect the structure of the DNA ligand targeted by our hybrids. It is also possible that in the yeast system, in which proteins were expressed at low levels, the proteins were properly folded and stable; therefore, differences in DNA-binding activity could be distinguished. A detailed discussion of the discrepancy between *in vivo* and *in vitro* results is provided in the Discussion section.

The Max-E47 Hybrids Effectively Compete with Native MaxbHLHZ for the E-Box Site in the Modified Yeast One-Hybrid System. We have proven that the Max-E47 hybrids can activate transcription from the E-box in the Y1H and that they bind strongly and specifically to the E-box site by quantitative fluorescence analysis. The following results from the modified yeast-one hybrid (MY1H) assay demonstrate that the Max-E47 hybrids can potentially serve as competitive inhibitors of c-Myc/Max binding to the E-box.

Ideally, our yeast system would detect a positive signal from transcriptional activation from the E-box by the c-Myc/Max heterodimer; upon addition of Max-E47, this positive signal would be reduced. However, such a system is complicated, for it would involve protein/protein and protein:DNA interactions between three different proteins nonnative to yeast. A logical alternative is to test only two proteins: whether the Max-E47 hybrids can compete with the MaxbHLHZ homodimer for E-box binding. If the MaxbHLHZ homodimer's ability to activate from the E-box decreases after a Max-E47 hybrid is added, then by extrapolation, this hybrid is also likely to inhibit activation from the E-box site by the c-Myc/Max heterodimer.

In the traditional Y1H, only one protein can be expressed. We developed a MY1H that enables expression of two different proteins from the same plasmid with concomitant detection of transcriptional activation. We demonstrated that our MY1H is an ideal system for testing a second coexpressed protein's ability to inhibit the gene-regulatory activity of the first protein.[26] In the MY1H, plasmid pCETT contains two multiple cloning sites (MCS): the gene cloned into MCSI is expressed as a fusion to the GAL4 AD, while the gene in MCSII has no AD. Both genes are governed by independent truncated *ADH1* promoters, with low expression levels expected. Therefore, by use of pCETT in the MY1H system, the effects of a new protein, or mutant versions of a protein, on activation by a transcription factor can be readily examined.

The MY1H provides the most interesting test of the utility of our minimalist design, because direct competition between a Max-E47 hybrid and the native MaxbHLHZ simultaneously vying for the E-box target site can be assessed. The MaxbHLHZ gene was inserted into MCSI of pCETT; hence, Max is now a transcriptional activator. The genes for the Max-E47 hybrids were cloned into MCSII; hence, the expressed hybrids are repressors. If only AD+MaxbHLHZ is expressed, the homodimer's strong activation from the E-box will be visualized as a positive signal. If a Max-E47 hybrid is coexpressed with AD+MaxbHLHZ, it will compete for the E-box target; hence, MaxbHLHZ:E-box interactions are repressed by a Max-E47 hybrid. Thus, the competitive binding of two proteins targeting the same DNA site can be detected based on the outcome of reporter transcription in the MY1H.

First, we note that all controls in the MY1H functioned properly. Positive control pCETT expressing AD+MaxbHLHZ showed strong activation; in the *HIS3* reporter assay, colonies appeared at 2 days (Figure 5A). In contrast, even with the same transformation efficiency, negative controls pCETT expressing only Max-E47, Max-E47Y, or Max-E47YF gave very small colonies at 6 days (data not shown). Theoretically, there should be no growth, because these hybrids have no AD. However, small colony growth might arise from interactions between the hybrids and endogenous proteins possessing activation domains.

For pCETT/AD+MaxbHLHZ//Max-E47YF (i.e., AD+MaxbHLHZ coexpressed with Max-E47YF), colony growth was strong, and sizes were similar to those of pCETT/AD+MaxbHLHZ, indicating no detectable reduction in transcriptional activation upon expression of Max-E47YF (Figure 5A). (Note that the gene after "/" was inserted into MCSI, and the gene after "//" was inserted into MCSII.) For pCETT/AD+MaxbHLHZ//Max-E47Y, except for a few medium-sized colonies, much smaller colonies were observed. For pCETT/AD+MaxbHLHZ//Max-E47, only tiny colonies can be seen, and the colony number was greatly reduced. These results demonstrate that activation from the E-box by native MaxbHLHZ is strongly inhibited by Max-E47 and Max-E47Y. All plasmids had relatively equal transformation efficiencies (Figure 5A), ruling out the possibility that less growth from coexpression with Max-E47 or Max-E47Y was caused by low transformation efficiencies. In addition, both proteins competing for the DNA target (i.e., AD+MaxbHLHZ and either Max-E47 or Max-E47Y or Max-E47YF) are expected

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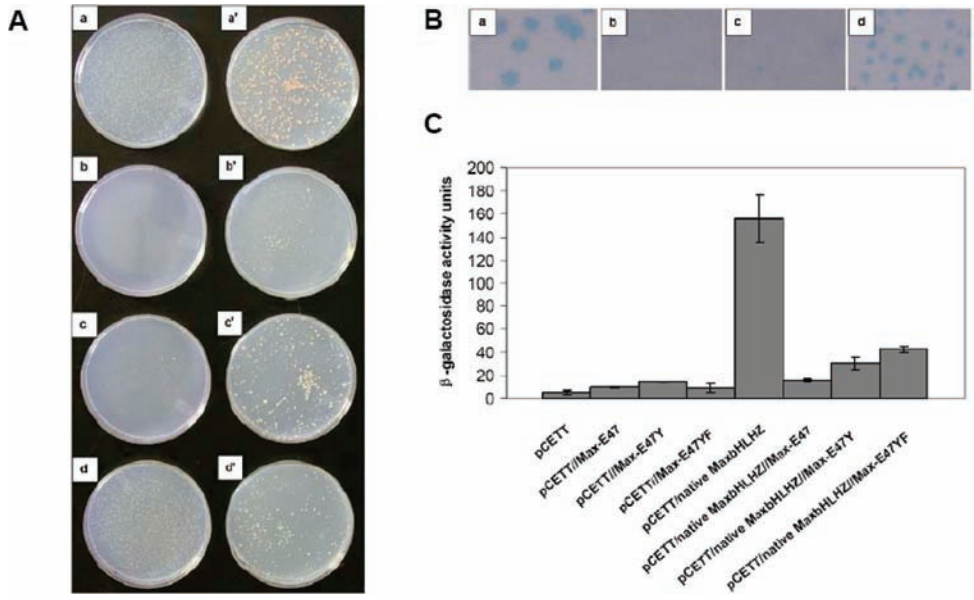


Figure 5. Max-E47 hybrids inhibit native MaxbHLHZ activation from the E-box (MY1H). (A) The *HIS3* assay of the inhibition of native MaxbHLHZ by the Max-E47 hybrids. Plates a−d are transformations plated on SD/−H/−L + 10 mM 3-AT plates, which were incubated at 30 °C for 6 days, while plates a′−d′ are the corresponding SD/-L efficiency plates, which were incubated at 30 °C for 4 days: (a) pCETT/native MaxbHLHZ; (b) pCETT/native MaxbHLHZ//Max-E47; (c) pCETT/native MaxbHLHZ//Max-E47Y; (d) pCETT/native MaxbHLHZ//Max-E47YF. (B) The X-gal colony-lift filter assay. All SD/−U/−L plates were incubated at 30 °C for 4 days. Photos were taken after 2 h incubation: (a) pCETT/native MaxbHLHZ; (b) pCETT/native MaxbHLHZ//Max-E47; (c) pCETT/native MaxbHLHZ//Max-E47Y; (d) pCETT/native MaxbHLHZ//Max-E47YF. (C) Histogram comparing the Max-E47 hybrid inhibition of native MaxbHLHZ activation from the E-box. All values are averages of 9−12 measurements from 3−4 separate cell-growth cultures.

to be produced in comparable amounts in the cells, despite the fact that the levels of protein expression are too low to be detected in the Western blot analysis; this interesting observation indicates that a large excess of Max-E47 (or even Max-E47Y) is not required for efficient inhibition of native MaxbHLHZ binding to the E-box site. More discussion is provided in text below.

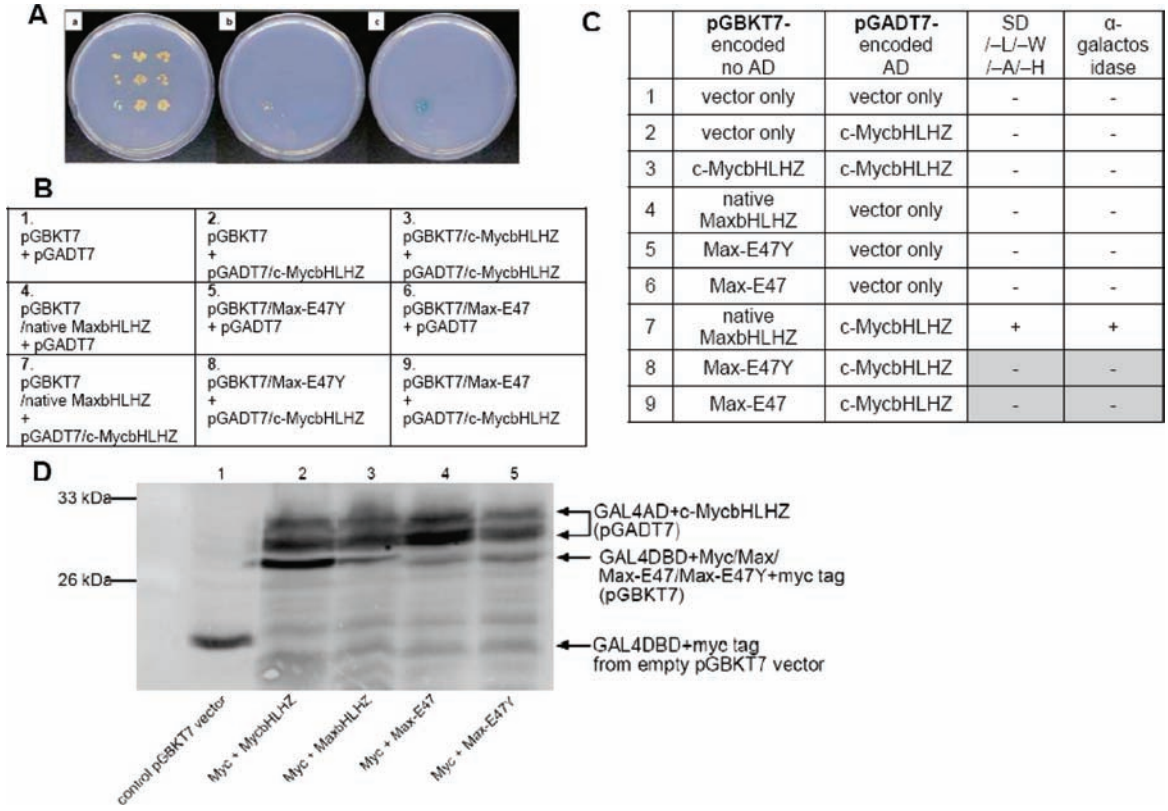
We titrated these transformants on plates containing inhibitor 3-AT (3-aminotriazole) to test their ability to inhibit activation from the E-box by native MaxbHLHZ. Colonies expressing AD+MaxbHLHZ grew well even on 80 mM 3-AT and could only be inhibited on 100 mM 3-AT (all data in this paragraph are shown in Figure S2, Supporting Information). When Max-E47 was coexpressed with AD+MaxbHLHZ, 20 mM 3-AT was enough to inhibit colony growth completely. When Max-E47Y was coexpressed, some colony growth on 30 mM 3-AT was observed, but nothing can be seen on 40 mM 3-AT. When Max-E47YF was coexpressed, cells grew well even on 60 mM 3-AT, and 80 mM 3-AT was required for total inhibition of growth. Therefore, with increased concentrations of 3-AT, the growth of cells transformed with the different hybrids decreased as expected. These results support the conclusion that our Max-E47 hybrids competitively inhibit transcriptional activation by native MaxbHLHZ from the same E-box target. Though not quantitative, these results demonstrate the extent to which the Max-E47 hybrids can inhibit native MaxbHLHZ.

Both the qualitative X-gal colony-lift filter assay and the quantitative ONPG liquid assay confirm the *HIS3* assay results above in the MY1H. In the X-gal assay, pCETT/MaxbHLHZ turned blue rapidly (approximately 20 min) and became bright blue in 2 h (Figure 5B). Transformants coexpressing AD+MaxbHLHZ and Max-E47 or Max-E47Y displayed very faint blue color, suggesting that both hybrids inhibited activation by native MaxbHLHZ from the E-box to a high degree. The transformant containing pCETT/AD+MaxbHLHZ//Max-E47YF showed fairly strong blue color, indicating weak repression, consistent with Max-E47YF being the weakest activator from the E-box.

The ONPG assay quantitatively confirms these results. When only AD+MaxbHLHZ was expressed in pCETT, the ONPG value was 155.8 ± 20.5 (Figure 5C). This value is strongly consistent with that obtained for pGAD424/MaxbHLHZ (147.4 ± 7.3) in the traditional Y1H (Figure 3C), where a different plasmid was used with the same promoter. When Max-E47YF or Max-E47Y was coexpressed, the ONPG values decreased to 42.5 ± 2.6 or 33.4 ± 5.4, respectively. When Max-E47 was coexpressed, the value was even lower at 14.8 ± 0.8; in comparison, background is 5.0 ± 1.8 (pCETT). These quantitative measurements are consistent with the same trend observed in the qualitative *HIS3* and X-gal assays and confirm that Max-E47 is a stronger inhibitor of native MaxbHLHZ activation from the E-box than either Max-E47Y or Max-E47YF.

We emphasize that all of our yeast assays corroborate each other well and repetitively show consistent trends: these include the *HIS3* selection assay and both *lacZ*-based assays (colony-lift and ONPG) in the traditional Y1H, and these same three assays in our MY1H. In the Y1H, Max-E47, Max-E47Y, and Max-E47YF showed E-box-responsive reporter gene activation as listed from strongest to weakest. In the MY1H, Max-E47 and the Y and YF mutants showed the same relative ability to compete with native MaxbHLHZ for occupying the E-box site. Thus, the same ordering of strongest to weakest ability to activate transcription from the E-box in the Y1H and to inhibit native MaxbHLHZ activation from the E-box correlate well between the two yeast systems.

The Max-E47 Hybrids Do Not Interact with the c-MycbHLHZ in the Yeast Two-Hybrid Assay. To further investigate whether the Max-E47 hybrids can serve as competitive inhibitors of c-Myc/Max binding to the E-box, the yeast two-hybrid (Y2H) assay was used to test for protein/protein interactions between the Max-E47 hybrids and the MaxbHLHZ



	pGBKT7-encoded no AD	pGADT7-encoded AD	SD /–L/–W /–A/–H	α-galactosidase
1	vector only	vector only	-	-
2	vector only	c-MycbHLHZ	-	-
3	c-MycbHLHZ	c-MycbHLHZ	-	-
4	native MaxbHLHZ	vector only	-	-
5	Max-E47Y	vector only	-	-
6	Max-E47	vector only	-	-
7	native MaxbHLHZ	c-MycbHLHZ	+	+
8	Max-E47Y	c-MycbHLHZ	-	-
9	Max-E47	c-MycbHLHZ	-	-

Figure 6. Max-E47 hybrids do not interact with c-Myc. (A) Y2H assay. For the same sample, the same cell density was plated on all three plates. The plates were incubated at 30 °C for 6 days: (a) SD/−L/−W plate, colonies appeared at 2 days; (b) SD/−L/−W/−H/−A plate, colonies appeared at 3 days; (c) SD/−L/−W/−H/−A/X-α-gal plate, the blue color developed at 4 days. (B) Sample alignment on each plate in panel A. (C) Summary of Y2H results. The indicated pGBKT7- and pGADT7-encoded proteins were coexpressed in yeast and then tested for adenine and histidine auxotrophy, as well as expression of α-galactosidase after 6 days incubation. The main results are highlighted. (D) Western blot of Y2H. Lane 1, pGADT7 (=GAL4AD) + pGBKT7 (=GAL4DBD); lane 2, pGADT7/c-MycbHLHZ (=GAL4AD+c-MycbHLHZ) + pGBKT7/c-MycbHLHZ (=GAL4DBD+c-MycbHLHZ); lane 3, pGADT7/c-MycbHLHZ (=GAL4AD+c-MycbHLHZ) + pGBKT7/MaxbHLHZ (=GAL4DBD+MaxbHLHZ); lane 4, pGADT7/c-MycbHLHZ (=GAL4AD+c-MycbHLHZ) + pGBKT7/Max-E47 (=GAL4DBD+Max-E47); lane 5, pGADT7/c-MycbHLHZ (=GAL4AD+c-MycbHLHZ) + pGBKT7/Max-E47Y (=GAL4DBD+Max-E47Y). (The HA-tagged Western, Figure S1, which proves comparable c-MycbHLHZ expression levels in all samples, is provided in the Supporting Information.)

or c-MycbHLHZ domain. We chose to examine c-Myc, because it is the most highly characterized of the three Myc isoforms (c-, N-, and L-Myc), including high-resolution structural information.[19]

We initially tried to test the interactions of the Max-E47 series with MaxbHLHZ by expressing the Max-E47 hybrids as GAL4 AD fusions and MaxbHLHZ as the GAL4 DBD (DNA-binding domain) fusion. However, cells transformed with recombinant pGADT7 plasmids containing the Max-E47 hybrid genes or the MaxbHLHZ gene died or grew very slowly. The reason is unclear. However, this phenomenon in the Y2H has been observed by many other researchers, as reviewed by Vidal and Legrain.[27]

In contrast, interactions with the c-MycbHLHZ domain were successfully measured by expressing c-MycbHLHZ as the GAL4 AD fusion, and native MaxbHLHZ and Max-E47 hybrids as GAL4 DBD fusions. Heterodimerization between AD+MycbHLHZ and DBD+MaxbHLHZ functioned as a positive control, because their association should reconstitute a functional transcriptional activator.

The Y2H demonstrated that neither Max-E47 nor Max-E47Y interacts with the c-MycbHLHZ (Figure 6; Max-E47YF was not tested). On SD/−L/−W/−A/−H and SD/−L/−W/−A/−H/X-α-gal plates, the transformant that coexpresses DBD+MaxbHLHZ and AD+MycbHLHZ showed colony growth and blue color as expected, demonstrating that native MaxbHLHZ interacts with c-MycbHLHZ strongly and specifically. In contrast, the transformant that coexpresses DBD+MycbHLHZ and AD+MycbHLHZ resulted in no colony growth and appeared colorless, verifying that c-MycbHLHZ cannot homodimerize. Samples 8 and 9 in Figure 6A show no cell growth or blue color, demonstrating no interaction between Max-E47 or Max-E47Y and c-MycbHLHZ. In this case, the HLH subdomain does not heterodimerize with the HLHZ subdomain. Figure 6C summarizes all test results from the Y2H.

To exclude the possibility of no or low expression, Western blot analysis was performed. Comparable expression levels were observed for all proteins (Figure 6D and Figure S1, Supporting Information). SDS−PAGE analysis affirmed that all lanes in the Western blot were comparably loaded (data not shown). Therefore, the lack of signal in the Y2H cannot be attributed to poor protein expression. These Y2H data show no interaction between Max-E47 (or Max-E47Y) and the c-MycbHLHZ and, by extension, no interaction between Max-E47 (or Max-E47Y) and the c-Myc/Max heterodimer. This result is consistent with no reported protein/protein interactions occurring between the bHLH and bHLHZ families.[28,29]

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Both the Y1H and fluorescence analysis show that the Max-E47, Y, and YF homodimers are capable of binding the E-box. The Y2H demonstrates no protein/protein interactions between the c-MycbHLHZ domain and the Max-E47 series (bHLH). Thus, we conclude that inhibition of transcriptional activation from the E-box in the MY1H is likely due to the Max-E47 hybrids outcompeting native MaxbHLHZ for binding to the E-box site.

Discussion

The Max-E47 Hybrids Inhibit Native MaxbHLHZ in a Dominant-Negative Fashion. The *in vivo* assays in the traditional Y1H (*HIS3* assay, X-gal colony-lift filter assay, and ONPG liquid assay) and the *in vitro* fluorescence analysis showed that Max-E47 activates transcription from the E-box site as strongly as does the MaxbHLHZ. MY1H assays demonstrated that Max-E47 effectively repressed E-box binding by MaxbHLHZ. These results consistently support the conclusion that Max-E47 is a strongly competitive dominant-negative inhibitor of MaxbHLHZ binding to the E-box site. In addition, Y2H assays showed no interaction between Max-E47 and c-MycbHLHZ. Therefore by extension, Max-E47 should be able to serve as a DN inhibitor of the native c-Myc/Max heterodimer that targets the E-box and regulates transcriptional activation.

Moreover, DN proteins are often expressed in excess relative to their targets for efficient inhibition; for example, Vinson and co-workers used a protein:DN inhibitor ratio of 1:15.[8] In our case, although the level of protein expression driven by the truncated *ADH1* promoter is too low to be detected in the Western blot analysis (as discussed in the Yeast Protocols Handbook, Clontech, 2001), both proteins competing for the DNA target are independently expressed from truncated *ADH1* promoters, which are exactly same. In addition, we have demonstrated that in the MY1H system, the expression of the AD-fusion protein from MCSI is not affected by a nonsense control protein expressed from MCSII.[26] Therefore, similar concentrations of both expressed proteins are expected in the cells. We find it highly noteworthy that even under these conditions, Max-E47 (and even Max-E47Y) can efficiently outcompete native MaxbHLHZ for binding to the E-box site. In addition, it is likely that Max-E47 can also inhibit N-Myc/Max and L-Myc/Max heterodimers as well, because these highly conserved Myc isoforms bind to the E-box by heterodimerization with Max.[30,31]

We observed strong correlation between the Y1H and MY1H systems: the strength of activation by the hybrid from the E-box in the Y1H correlated with that hybrid's ability to compete with native MaxbHLHZ for the E-box in the MY1H. The thermodynamic titrations support the conclusion that the hybrids are capable of strong, specific binding to the E-box, but the E-box binding trend so clearly observed in all the yeast assays was not replicated in the fluorescence analysis. It is not uncommon to find that results from *in vivo* and *in vitro* experiments are not consistent (discussed further below), although we emphasize that the fluorescence analysis corroborates our observations in the Y1H and MY1H that all three hybrids effectively target the E-box.

The Max-E47 series was obtained through swapping subdomains of the DNA-binding domains between the bHLHZ and bHLH families. Although subdomain swapping within the same protein family has been successful,[32−35] it was unknown whether subdomain swapping *between* different families would lead to functional hybrids. While this work was in progress, Chapman-Smith and Whitelaw reported a subdomain swap to generate a hybrid comprising the bHLH domain from the Arnt bHLH/PAS protein and the leucine zipper from Max; their hybrid was shown to bind to the E-box by EMSA, but no quantitative binding assessment or *in vivo* work was reported.[36] Our work provides another trial to explore the feasibility of subdomain swapping between different families, and both *in vitro* and *in vivo* results confirm the binding event.

Structural Basis of the Max-E47:E-box Interaction. All of the assays firmly validate that Max-E47 targets the E-box efficiently, thereby demonstrating the successful design of this hybrid protein. The bHLH requires two basic regions to bind DNA, which is achieved by dimerization.[37,38] Therefore, any DNA-binding activity of Max-E47 depends upon dimerized structure via the E47 HLH subdomain, which we chose to use because the E47 HLH strongly homodimerizes. In contrast, most bHLH and bHLHZ proteins do not homodimerize but rather heterodimerize. Thus, both Max and E47 serve comparable roles within their protein families, because they both homodimerize and heterodimerize to regulate partner protein activities.

The unique ability of E47 to homodimerize is tightly correlated with its structure. Ellenberger and co-workers compared their structure of E47 bound to 5′-CACCTG with the native Max bHLHZ:E-box structure;[17] their findings suggest that the HLH subdomain from bHLH proteins, as represented by E47, and the HLH subdomain from bHLHZ proteins, as represented by Max, have distinct structural features.[20] Helix 1 of the E47 HLH is one turn longer than the analogous helix in Max and USF.[17,39] This extra helical turn provides more dimer contact surface between E47 subunits by allowing salt bridge formation between His366 (near the C-terminus of helix 1) and Glu390 (at the C-terminus of helix 2′). The authors observe that this His/Glu pair is present in all E proteins (E47, E12, HEB, and Da) that homodimerize efficiently without a zipper. Another unique feature of E proteins is the triad of glutamines in the HLH. Gln373 participates in hydrogen bonds with the carbonyl oxygen of Gly360 (helix 1) and side chains of Gln364 (helix 1) and Gln381 (helix 2). The authors emphasize that "this network of hydrogen bonds stabilizes the conformation of the loop as well as the orientation of Helices 1 and 2 within each subunit." These distinctive structural features contribute to the markedly enhanced stability of E47 homodimers.[20]

Interestingly, the *in vivo* yeast assays allow the differential activities between the three hybrids to be clearly distinguished.

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The transcriptional activation trend from the E-box is strongly consistent in all the yeast assays, even with different reporters and plasmids. These *in vivo* results can be explained by the E47 structure. Max-E47Y has one mutation, V385Y. According to Ellenberger and co-workers, this valine protrudes from helix 2 and packs against the C-terminus of helix 1.[20] In their comparison of E47 with Max, they note that Val is relatively small, so it allows increased length of α-helical structure of helix 1 in the E47 dimer, and the additional turn permits the His366−Glu390′ salt bridge. However, the larger side chain of tyrosine in Max-E47Y could distort this three-residue extension of helix 1; incidentally, there is a tyrosine at this same position in helix 1 of Max. Therefore, this replacement likely alters the structure of the dimer by interfering with the His366−Glu390′ interchain interaction, thereby affecting DNA-binding activity.

Max-E47YF has the additional V393F mutation. This mutation is only three residues from Glu390 and approximately on the same face of the α-helix. The large aromatic side chain of phenylalanine likely interferes with the His366−Glu390′ interaction as well. Moreover, this mutation occurs at the dimer interface and likely affects formation of the hydrophobic core. Val393 is also very close to the crossover point of helices 2. At this critical junction, a mutation would be expected to affect dimerization ability negatively, consequently lowering DNA-binding activity.[40] Thus, it is not surprising that Max-E47YF's E-box-responsive activity dropped measurably in the yeast assays. The E47 crystal structure has not been deposited in the Protein Data Bank, and therefore, determination of the precise positions of amino acids and their effects on protein structure is difficult to ascertain. This adds more challenge and risk to our design, but our results confirm the basic E47 structure as elucidated.[20]

Differences between *in Vivo* and *in Vitro* Measurements Were Also Observed in Other Designed Systems. That the yeast assays clearly delineate the differences in transcriptional activation capability among the three hybrids can be explained by the structural analysis above. In comparison, the thermodynamic titrations do not discriminate these differences in binding function, although they do affirm that the Max-E47 hybrids are high-affinity, sequence-specific binders of the E-box.

In fact, it is not uncommon that the levels of reporter gene activation by artificial transcription factors measured in cells do not correlate with their DNA-binding affinities measured *in vitro*, as shown by two examples involving artificial zinc-finger TFs. In their design of Zn-finger TFs for regulation of the endogenous human *ERBB-3* gene, Barbas and co-workers found that the six-finger protein pE3Z, with the strong target-site binding affinity of 2 nM, was incapable of altering gene expression; in contrast, the six-finger protein pE3Y, which showed slightly weaker target-site binding affinity than pE3Z, was able to activate gene transcription.[41] The authors offered that this discrepancy may be due to many factors, such as competition with cellular factors that bind to the same site or orientation of the Zn-finger fusion protein with respect to DNA. Similarly, in their study of *VEGF* gene regulation by Zn-finger TFs, Kim and co-workers observed no strong correlation between the levels of gene expression in their Y1H system and the Zn-finger:DNA binding affinities measured *in vitro*.[42] The authors suspected that binding of another protein at the target site or the local chromatin structure may have rendered the target site inaccessible to the Zn-finger TF, which caused inconsistency between the *in vivo* and *in vitro* results. More recently, our group observed that the GAL4 AD fusion of the bHLH domain of bHLH/PAS protein Arnt did not activate E-box-responsive reporter gene expression in the Y1H, while fluorescence anisotropy showed that the same ArntbHLH domain bound to the E-box with $K_d = 40$ nM.[25] Misfolding of the ArntbHLH domain in the yeast cellular environment is the likely reason for its inability to activate reporter gene transcription, as circular dichroism showed little intrinsic structure for the ArntbHLH domain, which also proved to be highly insoluble during fluorescence titrations; likewise, an optimized buffer system was also believed to play an important role in improving and maintaining the protein fold.[25]

Native transcription factors can also show different activities *in vivo* and *in vitro*. Daignan-Fornier and co-workers showed that single-site mutants of the Bas1p DNA-binding domain discriminated between different promoter sequences in yeast but bound equally well to the same promoters when evaluated by EMSA.[43] The authors speculate that the mutations may affect promoter-specific interactions *in vivo*, and note that Bas1p and Bas2p (Pho2p) may need to interact cooperatively in order to activate transcription; the possibility that the mutations affected the concentrations of proteins in the yeast cells was shown unlikely by Western blot analysis, which similarly showed that our Max-E47 series of proteins were also present in comparable concentrations in our Y2H experiments. Mutants of the N-terminal arm of the DNA-binding homeodomain of Bas2 can also distinguish among different promoter sequences in yeast and EMSA; Vershon and co-workers showed that their *in vivo* and *in vitro* binding studies generally correlated except with one mutant, and they suggest that other factors may contribute in the yeast assay.[44] Although these are the closest examples we could find in the literature, the cases above of the designed Zn-finger are TFs are somewhat different from our system; the Zn-finger TFs are targeting different sites in a promoter, whereas ours are different TFs (the Max-E47 series or the Arnt derivatives[25]) targeting the same site. The examples involving Bas proteins may be complicated by additional interactions involved in activating transcription, which should not be an issue in our system. However, these examples demonstrate that *in vivo* and *in vitro* measurements are not always consistent and that the reasons for discrepancy are unclear.

As for Max-E47 and its two mutants, we suspect two main reasons for the *in vivo* and *in vitro* differences in activity. One possibility is the differences in DNA sequences assayed: sequences flanking the E-box site on the FA titration probes were chosen to minimize potential for unintended secondary structure formation, like hairpins, and to minimize resemblence to the E-box sequence (i.e., to minimize binding at fortuitous E-box-like sequences). In comparison, the sequences targeted in the yeast assays comprised four tandem E-box sites cloned upstream of the *HIS3* or *lacZ* reporter genes (multiple target sites are commonly integrated into the genome in yeast reporter assays; the manufacturer, Clontech, recommends three to six

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target sites; see Experimental Section and Supporting Information). Although the basic regions of our hybrids are unlikely to have direct contact with bases outside the E-box, such differences in flanking sequences can critically affect the structure of the DNA ligand recognized by protein; we note that for the Bas proteins and mutants, both *in vitro* and *in vivo* studies on target sites embedded within different promoters had significant effects on binding and transcription activities.[44−46] A second possibility is that the low protein expression levels and the *in vivo* yeast environment provided conditions in which the fine structural differences, which lead to subtle differences in ability to activate transcription from the E-box, among the three hybrids could be distinguished. We emphasize, however, that our Max-E47 hybrids consistently displayed specific E-box-targeting activity both *in vivo* and *in vitro*, and that all our data confirm the conclusion that these hybrids are Class 1 DN inhibitors.

Although the three hybrids bind to the E-box with comparable strengths as measured by fluorescence anisotropy, DNA-binding affinity is only one of many factors that affects reporter activation. In other words, these one or two mutations in Max-E47 may alter protein structure and stability or accessibility of the protein to its DNA target *in vivo*; a strong DNA-binding affinity (thermodynamics) does not necessarily mean that the transcription factor stays on its DNA target long enough to trigger reporter gene activation (kinetics). In addition, interference from endogeneous proteins might also cause the functional discrimination among the three hybrids. It is also possible that in the yeast system, where proteins were expressed at low levels, the proteins were properly folded and stable; therefore, differences in reporter gene activation could be distinguished. However, the *in vitro* conditions of the fluorescence measurements, chosen to maintain protein solubility during the lengthy titration (each data point, after addition of protein aliquot, required overnight incubation to maintain solubility), may not have been optimal for distinguishing differences in DNA-binding function, perhaps by diminishing the fine structural differences among the three hybrids. This fact, again, proves the necessity of performing both *in vitro* and *in vivo* measurements in the study of DNA-binding proteins.

According to the Max bHLHZ:E-box crystal structure, Lys57 in the loop nonspecifically contacts the DNA phosphodiester backbone.[17] Burley and co-workers note that this interaction is significant for the Max:E-box complex, but this interaction does not exist with the E47 loop.[20] However, Max-E47 still targets the E-box as well as does native MaxbHLHZ in the Y1H, MY1H, and quantitative fluorescence analysis, despite loss of this important interaction. Additionally, the Max-E47 hybrid, which lacks a leucine zipper, can target the E-box site as efficiently as does native MaxbHLHZ, which requires its zipper for dimerized structure and DNA-binding function; the truncated MaxbHLH is not functional in the Y1H. Therefore, Max-E47 provides a useful scaffold for further exploration of the relationship between protein structure and DNA-binding function.

Conclusions

These hybrids of Max and E47 are part of our effort to generate minimalist proteins with desired DNA-recognition capabilities from an α-helical molecular recognition scaffold, hence protein-based tools for recognition of desired DNA targets. Our minimalist design strategy provides a launching point for generation of artificial transcription factors based on native proteins that are likely to be easier to express or synthesize than their native counterparts, to be more tractable for high-resolution studies, and to have further applications in fields other than protein design, including drug discovery and functional genomics.[47−49] Already, artificial Zn-finger transcription factors have been reported.[42,50−53]

We chose to apply our minimalist design strategy to the Max/Myc:E-box network, given its broad involvement in normal cellular function, as well as the etiology of cancers and tumors. Although some of Myc's normal cellular activities have recently been found to be independent of Max and cannot be explained by activation from E-box-responsive Myc targets,[54] the short, simplified Max-E47 hybrids may find utility as DN inhibitors of undesirable transcriptional activation from the E-box. Very recently, the designed dominant-negative Omomyc, discussed in the Introduction, was shown to target Myc specifically in a transgenic mouse model of cancer.[55] Omomyc shows the promise of protein-based drugs against disease. Expression of Max-E47 in mammalian cells could provide a bHLH protein capable of interfering with c-Myc's transactivation potential by targeting the E-box DNA site, and these next-generation experiments are being explored. These hybrid proteins may serve as leads for the design of smaller proteins or peptidomimetics with desirable pharmacological properties.

Experimental Section

More experimental details for all procedures are provided in the Supporting Information.

Yeast One-Hybrid System (Y1H). Construction of *HIS3* and *lacZ* Reporter Strains. Four tandem copies of the E-box target sequence (5′-CACGTG) were cloned into the pHISi-1 integrating reporter vector at the *his3−200* locus of *Saccharomyces cerevisiae* YM4271 (Matchmaker one-hybrid system, Clontech, Mountain View, CA). 10 mM 3-AT (3-aminotriazole, Bioshop, Burlington, ON) was sufficient to suppress background due to leaky His3 expression in reporter strain YM4271[pHISi-1/E-box]. Similarly, reporter strain YM4271[pLacZi/E-box] was constructed with four copies of the E-box site upstream of the *lacZ* reporter gene.

Construction of Genes. DNA oligonucleotides were purchased from Operon Biotechnologies (Huntsville, AL). The genes for expression of native MaxbHLHZ (92 aa, residues 22−113[17]), c-MycbHLHZ (87 aa, residues 22−107, numbering from ref 17 according to Ziff and co-workers[56]), native MaxbHLH, and Max-E47 were synthesized in a single PCR reaction.[57] Amplified gene

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inserts were inserted into vector pGAD424 (Matchmaker One-Hybrid System, Clontech). Recombinant plasmids were transformed into *Escherichia coli* strain DH5α and sequenced.

Transformation of Yeast Cells. For the *HIS3* assays, we developed an electroporation protocol based on the methods of Suga and Hatakeyama.[58,59] After electroporation, cells were plated on minimal selective medium (SD, Synthetic Dropout) lacking leucine and histidine with the appropriate amount of 3-AT to suppress background (Matchmaker One-Hybrid System). For the assays using the *lacZ* reporter, plasmids were transformed into the integrating reporter strain YM4271[pLacZ/E-box] by the TRAFO method.[60] Protein:DNA interactions were detected by X-gal colony-lift filter assay and ONPG liquid assay. These protocols are provided in the Yeast Protocols Handbook (Clontech).

Modified Yeast One-Hybrid System (MY1H): Construction of Genes. The genes for expression of native MaxbHLHZ and the hybrids were inserted into vectors pCETT and pCETF.[26]

3-AT Titration Test. One colony was resuspended in 1 mL of sterile H_2O and vortexed vigorously to disperse the cells. For testing of inhibitory activity, 10 μL of cells were pipetted on SD/−L/−H plates containing different concentrations of 3-AT (0−200 mM).

Yeast Two-Hybrid System (Y2H): Construction of Genes. The genes amplified from recombinant pGAD424 plasmids were inserted into vectors pGADT7 (for c-MycbHLHZ) or pGBKT7 (for MaxbHLHZ, Max-E47, and Max-E47y). Cotransformation of the recombinant pGBKT7 and pGADT7 plasmids into strain AH109 was performed by the TRAFO method.[60] After 3−4 days growth, one colony was resuspended in 1 mL of sterile H_2O and vortexed vigorously. A sterile inoculating loop was dipped into the cell dispersion, and cells were spread on SD/−L/−W plates to confirm healthy cell growth and SD/−L/−W/−H/−A and SD/−L/−W/−H/−A/X-α-gal plates for testing.

Western Blot. AH109 cells were transformed with the recombinant pGADT7 and pGBKT7 and grown to exponential phase in YPDA media. Cells were lysed by sonication and separated by SDS−PAGE. Immunodetection was performed with anti-c-Myc or anti-HA antibody (Covance Inc., Princeton, NJ) and visualized by fluorescence on a Molecular Dynamics Storm 840 phosphorimager.

Fluorescence Anisotropy Titrations. The genes for MaxbHLHZ and the hybrids were reconstructed in codons preferred for bacterial expression, cloned into pET-28A(+) (Novagen, Mississauga, ON), expressed from BL21(DE3)pLysS (Stratagene, La Jolla, CA), purified by TALON metal ion affinity chromatography (Clontech) and reversed-phase HPLC (Beckman, Fullerton, CA), and identities were confirmed by ESI-MS (see refs 61, 62 for detailed protocols). The 6-carboxyfluorescein label (6-FAM) was incorporated at the 5′ end of the labeled oligonucleotides (Operon Biotechnologies, Huntsville, AL), and all oligonucleotides were purified by HPLC. Protein concentrations were assessed (Beckman DU 640 UV/vis spectrophotometer) by tyrosine absorbance for MaxbHLHZ, Max-E47Y, and Max-E47YF (absorbance maximum 275−280 nm, e_{275} = 1405 $M^{-1}\cdot cm^{-1}$ per tyrosine) or by measurement at 205 and 280 nm by the method described by Scopes for Max-E47.[63]

Fluorescence was measured on a JY Horiba Fluorolog-3 spectrofluorimeter (University of Toronto). The cell (Starna, Atascadero, CA) contained 1 nM DNA duplex in 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 150 mM NaCl, 2.7 mM KCl, 1 mM EDTA, 800 mM urea, 20% glycerol, 0.1 mg/mL acetylated BSA, and 100 μM bp calf thymus DNA. Several other buffers, including Tris- and HEPES-based buffers, were explored but did not maintain functional protein. The volume change was maintained at <5% of total volume. For each data point, the sample was incubated at 4 °C overnight followed by at least 20 min at room temperature; such extensive incubation was necessary to minimize protein misfolding and aggregation.

Determination of K_d Values. The polarization values were used to calculate apparent dissociation constants using Kaleidagraph 3.6 (Synergy software) and eq 1:[64]

$$P = ((P_{bound} - P_{free})[M]/(K_d + [M])) + P_{free} \quad (1)$$

where K_d corresponds to the apparent monomeric dissociation constant, [M] is the concentration of monomeric protein, P_{free} is the polarization for free DNA, and P_{bound} is the maximum polarization of specifically bound DNA; two independent titrations (R values >0.950) were performed for each K_d value ± SEM (standard error of the mean).

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Supporting Information Available: Detailed experimental protocols for all yeast work, fluorescence spectroscopy, and binding analysis and yeast data, including Y2H Western blot and *HIS3* titration. This material is available free of charge via the Internet at http://pubs.acs.org.

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