Formation of α-Pal/Max Heterodimers Synergistically Activates the eIF2-α Promoter*

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The transcription factor α-Pal recognizes two tandem palindromic repeats within the promoter of eukaryotic translation initiation factor 2-α (eIF2-α). Whereas both binding sites have the same “core domain” sequence (CGCATGCCG), they differ with respect to their flanking sequences. Of the two sites, the 5’-cap proximal site has a higher binding affinity for α-Pal than does the 5’-cap distal site (Jacob, W. F., Silverman, T. A., Cohen, R. B., and Safer, B. (1989) J. Biol. Chem. 264, 20372–20384). The well characterized transcription factor Max binds to sequences that are remarkably similar to the core domain that α-Pal recognizes. To date, all of the Max heterodimer partners lack DNA binding domains and are thus dependent on Max interacting with DNA. Here we report that the two α-Pal sites have very different binding activities with respect to the E-box-binding protein Max. The 5’-cap distal or low α-Pal affinity site binds both α-Pal and Max. Furthermore, both heterodimers and homodimers of each of these proteins bind to this site. In contrast to the low affinity site, the high affinity site does not bind Max as a homodimer. This is the first documented case where Max heterodimerizes with a transcription factor that has affinity for DNA independent of Max.

The eukaryotic translation initiation factor eIF2 is composed of three subunits, α, β, and γ (1). Formation of the eIF2-GTP-tRNA\text{met} ternary complex is the first step in translation initiation and is tightly regulated. The amount of eIF2-α, and its activity, is controlled at transcriptional, translational, and posttranslational levels (2). Inhibition of protein synthesis via posttranslational modification of eIF2-α by the hemin-regulated protein kinase has been demonstrated to play an important role in regulating protein synthesis in reticulocytes (3, 4). The double-stranded RNA activated eIF2-α kinase, PKR, inhibits protein synthesis in response to viral infection (5). A diverse collection of viral families has evolved unique proteins that inactivate PKR, allowing protein synthesis to proceed unabated (6–8). It has also been shown that PKR acts as a tumor suppressor (9, 10). Analogous protein kinases that act on eIF2-α have been described in both plants and yeast (11, 12). This suggests an essential role for eIF2-α in protein biosynthesis and cell growth.

The TATAA-less promoter of eIF2-α has multiple transcriptional start sites (13). Within this GC rich region are several Sp1 sites and two unusual direct repeat palindromic sequences (CGCATGCCG) that are located just upstream of the transcriptional start site cluster (13). Our laboratory has cloned and characterized a transcription factor, named α-Pal, that binds to these palindromic sequences (13). Scarpulla and Gopalakrishnan (14) have cloned an identical factor termed NRF-1. α-Pal is a putative bZip protein with strong homology to P3A2 and ewg (15). Whereas both α-Pal binding sites have the same “core domain” sequence, they differ with respect to their flanking sequences (Fig. 1A). Of the two sites, the 5’-cap proximal site has a higher binding affinity for α-Pal (K_d\text{app} = 3 \times 10^{-9} \text{ M}) than does the second 5’-cap site (K_d\text{app} = 4.2 \times 10^{-8} \text{ M}), as measured by Scatchard analysis (13).

The well characterized transcription factor Max binds to several closely related sequences, termed E-boxes. The canonical consensus E-box sequence is either CACGTG or CATGTG. Additionally, several noncanonical binding sites (CA NN TG) have been reported (16–18). Although Max homodimers have the ability to recognize and bind to these cis-elements, they lack a transcriptional activation domain and thus act as transcriptional inhibitors. In order to serve as a transcriptional activator, Max must heterodimerize with a bZip protein that has an activation domain. Max heterodimerizes with a myriad of transcription factors, such as c-Myc, n-Myc, l-Myc, Mad-1, Mad-2, Mxi-1, Mnt-1, and Mnt-2 (19–22). To date, all of the Max heterodimerizing partners lack DNA binding domains and are thus dependent on Max interacting with DNA. Whether Max homodimerizes or heterodimerizes is dependent upon the phosphorylation state of Max and the bio-availability of a binding partner (23, 24). For example, in many cells, Max is constitutively expressed and c-Myc is transiently expressed under conditions that favor growth. Thus, in quiescent cells Max tends to be found as homodimers that bind to E-box elements and inhibit transcription. Upon growth activation, Max is phosphorylated by casein kinase II, which negatively affects homodimerization. Concurrently, c-Myc is translated, which causes it to readily heterodimerize with Max. c-Myc then can bind to and activate transcription of E-box-regulated genes (19, 21, 23, 24).

Here, we report that the two α-Pal sites have very different binding activities with respect to the E-box-binding protein Max. The 5’-cap proximal, or high affinity α-Pal site, has affinity for both α-Pal and Max. Furthermore, both heterodimers and homodimers of each of these proteins bind to this site. Heterodimerization of Max and α-Pal is dependent on post-translational modification of either Max or α-Pal. In contrast to the high affinity site, the low affinity site binds Max as a homodimer. This is the first documented case in which Max heterodimerizes with a transcription factor that has affinity for DNA independent of Max. The implications of α-Pal/Max heterodimerization and homodimer competition at the eIF2-α promoter are discussed.
MATERIALS AND METHODS

Cells—Human 293 cells, obtained from ATCC, were grown with 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 4.5 g/liter glucose and 10% horse serum.

Transfections—Twenty-four hours prior to transfection, subconfluent flasks of 293 cells were plated (approximately 40% confluent) in 60-mm plates. The next day the cells were washed with fresh medium and transfected with 2 μg of plasmid DNA/plate (1 μg of pCD2 enhancer, with insert (see below) and 1 μg of p-green fluorescent protein) using SuperFeck reagent (Qiagen) in accordance with the manufacturer’s protocol. Forty-eight hours posttransfection, the cells were examined for green fluorescent protein expression. If greater than 40% of target cells expressed green fluorescent protein, the monolayers were harvested via lysis with luciferase lysis buffer (Promega), and luciferase activity was measured via luminometry.

Immune Precipitations—Immune precipitations were performed as described previously (25).

Oligonucleotide Probes—DNA encoding the high affinity, low affinity or both low and high affinity sites were generated on an oligonucleotide synthesizer (Applied Biosystems Inc.).

The transcriptional start site is delineated with an arrow.


displaced by cloning the open reading frame of

Motif as a Dimer—To determine whether α-Pal binds to DNA as a monomer or as a dimer, EMSA was performed with combinations of full-length and truncated α-Pal. The full-length and truncated α-Pal cDNAs were in vitro transcribed and then translated separately or together. The N-terminal truncated protein contained both the bZIP domain and the putative activation domain. The proteins were then used in EMSAs using 32P-labeled high affinity site containing probes (Fig. 1). When the full-length construct was used, only a single band was detected by EMSA (Fig. 2, lane 2). When only truncated histidine-tagged α-Pal was used, a single band with higher mobility relative to full-length α-Pal was detected (Fig. 2, lane 3). When the truncated α-Pal was mixed with full-length α-Pal, a band of intermediate mobility was also detected (Fig. 2, lane 4). Taken together, these results demonstrate that α-Pal binds as a homodimer to the high affinity site. Bacterially derived, histidine-tagged α-Pal bound to probes that contained the high affinity site, the low affinity site or both sites (data not shown). Because the bacterially derived α-Pal reacted with the probe, no eukaryotic posttranslational modification is necessary for homodimerization or DNA binding activity.

Max Binds to the Low α-Pal Affinity Site—The sequence of both the high and low α-Pal affinity sites contains an internal motif that resembles an E-box (Fig. 1B). Combined with the recent reports that Max binds to other canonical and noncanonical sequences in addition to the classic E-box, we investigated whether the Max protein binds to either of the α-Pal binding sites. Sequence analysis of the 9-base pair tandem direct repeat α-Pal binding sites found in the promoter region of the eIF2-α gene revealed identical internal E-box related motifs (Fig. 1B, boldface). To determine whether these nonconsensus sites could bind Max, EMSA of Max and α-Pal binding was performed with 32P-labeled either high or low affinity sites in their native context. The coupled transcription/translation system was used to generate α-Pal and Max.Binding of α-Pal occurred preferentially to the high affinity site (Fig. 3). Binding of α-Pal to the low affinity site was less efficient. In contrast, Max bound with high efficiency only to the probe containing the low affinity site (Fig. 3). This dramatic alteration in binding efficiency and specificity correlates with a single base difference in the sequence flanking the identical E-box motifs (Fig. 1B).

1 The abbreviations used are: EMSA, electrophoretic mobility shift assay; TNT, transcribed and translated.

FIG. 1. Sequence of EMSA probes. A, the sequences of the low and high α-Pal affinity sites overlap. The canonical binding sequence of the low and high α-Pal affinity sites are underlined. The sequence of the 126-base pair probe contains both the high and low affinity α-Pal site. The transcriptional start site is delineated with an arrow. B, comparison of the low and high α-Pal affinity sites with other E-box sequences (see Fig. 9).
radiolabeled probe or radiolabeled α-Pal were qualitatively identical. Both binding reactions generated a single retarded mobility band (Fig. 4, ω-Pal). Because the specific activity of the 35S-labeled protein was approximately one-fifth that of the 32P-labeled probe, so were the relative intensities of the shift bands.

When this technique was tried with the E-box binding factor Max, stable binding of 35S[Met-Max to the high affinity probe could not be demonstrated, even though it contains a nonconsensus E-box sequence. Because the low affinity α-Pal probe also contained an E-box sequence, but in a different context, the binding experiment was repeated using the low affinity α-Pal probe. Binding of 35S[Met to unlabeled low affinity probe could not be demonstrated. It is likely that this is the result of Max having only two methionine residues per polypeptide whereas α-Pal has eleven. When both Max and α-Pal were incubated together with the low affinity probe, a strong shift band of lower mobility was formed. An identical experiment with the exception that 32P radiolabeled probe was used with the 35S[Met-labeled Max strongly suggested that binding occurred between the low affinity probe and an α-Pal/Max heterodimer (data not shown).

α-Pal and Max Form Heterodimers—When 35S[methionine-labeled human 293 cell extracts were used in immune precipitation reactions, both α-Pal and Max could be specifically isolated with their respective antisera (Fig. 5). When the deoxycholate wash buffer was replaced with 0.4% Nonidet P-40 in phosphate-buffered saline, α-Pal/Max heterodimers could be co-immune precipitated with either anti-Max or anti-α-Pal serum (Fig. 5). Preimmune serum was without effect. This provides additional evidence that native α-Pal and native Max exist as heterodimers.

α-Pal and Max Binding Activity Is Enhanced in α-Pal/Max Heterodimers—At physiologic concentrations (10–15 M), neither α-Pal nor Max can form stable complexes with the high or low affinity probe, respectively (Fig. 6). However, if identical amounts of α-Pal and Max are first preincubated together, a strong shift band is formed with either high or low affinity probe. Because this strong band can be supershifted with anti-Pal (Fig. 6, lane 5) or anti-Max (lane 6), it is most likely to be an α-Pal/Max heterodimer. Preimmune serum did not affect the α-Pal/Max heterodimer. It is likely, therefore, that the native form of α-Pal is an α-Pal/Max heterodimer. A repeat of this experiment using His-Max and His-α-Pal purified from bacteria resulted in no detection of α-Pal/Max heterodimers (results not shown). This suggests that α-Pal has to be posttranslationally modified for Max heterodimerization. Another possibility is that the histidine tags on both proteins inhibited dimerization. The latter is unlikely because His-tagged α-Pal and His-tagged Max can homodimerize.

Alternative Secondary Structures Affect Binding Activities—Direct repeats of DNA binding sequences can theoretically lead to the formation of alternative “slip” and stem-loop structures that may affect their recognition by proteins (see Fig. 9) (26, 27). The 126-mer probe of the eIF2 promoter region contains sufficient flanking sequence as well as the tandem repeat binding sequences for α-Pal to allow such structures to form. We therefore compared the binding of α-Pal and Max to either the 126-mer probe, which contains both the high and low affinity α-Pal binding sites in a tandem array, or an equivalent mix of the individual high and low affinity binding sites probes (Fig. 7). Saturating amounts of α-Pal and the 126-mer probe generated a single low mobility shift band, indicating that both the high and low affinity binding sites were filled (Fig. 3, lanes 1 and 2); binding of Max was not seen (Fig. 3, lanes 1 and 2). In contrast, when the high and low affinity binding sites were
provided on separate 30-mer probes, both bound α-Pal, whereas Max bound to only the low affinity sequence (Fig. 3, lanes 6 and 7). When both α-Pal and Max were provided simultaneously, the 126-mer probe did not form any additional complexes; in contrast, the high and the low 30-mer probes formed complexes with α-Pal and Max, respectively (Fig. 3, lanes 3 and 8). Cold competitors for the high and low affinity binding sequences confirmed binding specificity (Fig. 3, lanes 4, 5, 9, and 10). It is likely, therefore, that binding site accessibility plays an important role in α-Pal and Max binding to the eIF2-α promoter.

Luciferase Assays Suggest That the α-Pal Affinity Sites Act Synergistically in Vivo—Each of the α-Pal affinity sites was
cloned in its native orientation into the SmaI site of the promoterless luciferase expression vector pGL2-enh, individually or together. Forty-eight hours posttransfection, human 293 cells were lysed and assayed for luciferase activity via luminometry. When compared with the parental vector, the pGL2-enh vector encoding the low α-Pal affinity site led to a >3-fold increase in luciferase expression (Fig. 8). However, insertion of the high α-Pal affinity site without the low affinity site repressed luciferase activity by more than 90% (Fig. 8). When both the low and high α-Pal affinity sites were inserted in the

**FIG. 7.** Alternative structures of the probe affect the accessibility of Max to its low affinity binding site. Binding of either α-Pal or Max to their respective high or low affinity binding sites with 30-base pair probes can occur without interference by its noncognate ligand (compare sixth and seventh lanes with eighth lane). In contrast, if the two sites are directly linked, binding of Max to the same molar equivalent of the high and low affinity binding sites is blocked by the presence of α-Pal (compare third and seventh lanes). 0.5 μl of α-Pal, 0.5 μl of Max, 4 × 10^5 cpm of 30-mer probes, and 8 × 10^5 cpm of the 126 probe were used per assay, as indicated in the figure.

**FIG. 8.** Luciferase assays suggest that the two α-Pal affinity sites act synergistically in vivo. Human 293 cells were transiently transfected with luciferase expression vector, pGL2-enhance, that either contained no insert, sequence identical to the low affinity α-Pal probe, sequence identical to the high α-Pal affinity probe, or sequence that contained 48 base pairs that encoded both the low and high affinity α-Pal sites that reflect the actual gene sequence (see under “Materials and Methods”).
vector, luciferase activity increased over 12-fold (Fig. 8). Because the vector that contained both α-Pal affinity sites increased reporter gene activity by 12-fold, whereas the low affinity site increased reporter gene activity 3-fold, this suggests that the two sites act synergistically as cis-promoter elements within the cell.

**DISCUSSION**

The transcription factor Max plays a pivotal role in controlling gene expression by its ability to dimerize with several different bZIP proteins. Whereas Max has a DNA binding domain, it lacks a transcriptional activation domain. Max homodimers bind to promoter regions and act as transcriptional repressors. When Max dimerizes with bZIP proteins that have transcriptional activating domains, such as c-Myc, then binding leads to an increase in transcriptional activity of the target gene (19, 21). When some cells undergo differentiation, the Max heterodimerization partner switches from c-Myc to Mad or Maxi, leading to transcriptional repression (19–22).

Here we report that Max binds to a cis-element in the eIF2-α promoter that was previously thought to bind only the transcription factor α-Pal. In EMSA, Max bound as homodimers to the low affinity α-Pal site. Because the histidine-tagged Max protein was derived from *Escherichia coli*, no posttranslational modification was needed for binding activity. Under these conditions, Max failed to bind to the high affinity α-Pal site. Bacterially derived α-Pal bound to both the high and low affinity sites without the apparent need for modification.

The most intriguing data presented here were the (seemingly contradictory) results of the EMSA and luciferase data. When a probe that contains both the low and high affinity α-Pal sites was used in an EMSA and α-Pal was titrated, the high affinity site was utilized exclusively, with little or no binding to the low site, until the high affinity site was filled. There was no evidence of cooperativity between the two sites.

In contrast to the EMSA data, the luciferase results argue that the two α-Pal affinity sites act synergistically with one another. There are at least three explanations that may clarify this incongruity. In the EMSA, we used probes that were generated on an automated oligonucleotide synthesizer, whereas in the luciferase assays, we used supercoiled plasmids. This difference in the three-dimensional structures of the target DNA could account for the paradoxical difference seen in these assays. The luciferase data are probably more relevant because the assay is in vivo, and supercoiled plasmid DNA is more similar to chromatin than oligonucleotides are. The biggest problem with this explanation is that supercoiled DNA should be much more sterically constrained than the relaxed oligonucleotides. Thus, one might expect that both sites could be occupied on oligonucleotides, while being more constrained when the DNA is supercoiled.

A second explanation for the differences between the luciferase and EMSA data is that there are one or more proteins in addition to α-Pal and Max that are responsible for the effect. It is well known that Max binds to several other transcription factors, such as c-Myc, Mad, and Maxi. Also, various posttranslational modifications could be responsible for the effect seen.

A third possible explanation for these phenomena is that the two α-Pal sites fold onto one another, generating one or more of the structures depicted in Fig. 9. Clearly, in an EMSA, either of these conformations would restrict α-Pal binding to a single site per probe. It is also feasible that such a structure in the luciferase assay could lead to the synergistic results reported above. Because the high affinity α-Pal site construct acted as a repressing cis-element and the low affinity site construct acted as positive element, one of the structures in Fig. 9 may eliminate the repressing element while creating a hybrid, stronger acting positive cis-element. Structural studies to detect single stranded regions and hairpin loops are ongoing in our laboratory.

The heterodimerization of Max and α-Pal reported here is Promethean because it is the first reported dimerization of Max with a transcription factor that has affinity for DNA in the absence of Max. This type of dimerization expands the repertoire of target sequences for Max. Whereas homodimers of Max bind to the low affinity α-Pal site, Max requires α-Pal for binding to the high affinity site. This creates some intriguing possibilities for gene regulation. Max is constitutively expressed in many cell types throughout the cell cycle and is thought to be regulated via CKII kinase. Studies are ongoing in our laboratory to characterize the levels and localization of α-Pal in various cells. One might hypothesize that in the absence of α-Pal, the low affinity α-Pal site would be occupied by Max homodimers (or other Max containing heterodimers). In this case, the low affinity α-Pal site would act as a negative cis-acting element. Although in the luciferase assays reported above the low affinity α-Pal site acted as a positive cis-element, it should be noted that this assay was done with actively dividing, serum-stimulated cells. It is feasible that when α-Pal
is available, Max homodimers would be displaced by either α-Pal homodimers or α-Pal/Max heterodimers, leading to transcriptional activation. Furthermore, α-Pal/Max heterodimers might recruit different transcription factors from α-Pal homodimers. This model may explain why the eIF2-α promoter has two different α-Pal affinity sites.

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REFERENCES
1. Merrick, W. C. (1994) Biochimie 76, 822–830
2. Hershey, J. W. B. (1991) Ann. Rev. Biochem. 60, 717–755
3. Hershey, J. W. B. (1989) J. Biol. Chem. 264, 20821–20826
4. Chen, J. J., London, I. M. (1995) Trends Biochem. Sci. 20, 105–108
5. Wek, R. C. (1994) Trends Biochem. Sci. 19, 491–496
6. Katze, M. G. (1995) Trends Microbiol. 3, 75–78
7. Samuel, C. E. (1998) Curr. Top. Microbiol. Immunol. 233, 125–145
8. Jagus, R., Gray, M. M. (1994) Biochimie 76, 779–791
9. Clemens, M. J. (1997) Int. J. Biochem. Cell Biol. 29, 945–949
10. Samuel, C. E., Kohen, K. L., George, C. X., Ortega, L. G., Rende-Fournier, R., and Tanaka, H. (1997) Int. J. Hematol. 65, 227–237
11. Langland, J. O., Langland, L., Zeman, C., Saha, D., Roth, D. A. Plant J. 12 393–400
12. Romano, P. R., Garcia-Barrio, M. T., Zhang, X., Wang, Q., Taylor, D. R., Zhang, F., Herring, C., Mathews, M. B., Qin, J., and Hinnebusch, A. G. (1998) Mol. Cell. Biol. 18, 2282–2297
13. Jacob, W. F., Silverman, T. A., Cohen, R. B., and Safer, B. (1989) J. Biol. Chem. 264, 20372–20384
14. Gopalakrishnan, L., and Scarpulla, R. C. (1995) J. Biol. Chem. 270, 18019–18025
15. Efiok, B. J. S., Chiorini, J. A., and Safer, B. (1994) J. Biol. Chem. 269, 18921–18930
16. Blackwell, T. K., Huang, J., Ma, A., Kretzner, L., Alt, F. W., Eisenman, R. N., and Weintraub, H. (1993) Mol. Cell Biol. 13, 5216–5224
17. Papoulas, O., Williams, N. G., and Kingston, R. E. (1992) J. Biol. Chem. 267, 10470–10480
18. Walhout, A. J. M., Vliet, P. C. van der, Timmers, H. Th. M. (1998) Biochim. Biophys. Acta 1397, 189–201
19. Bernards, R. (1995) Curr. Biol. 5, 859–861
20. Hurlin, P. J., Ayer, D. E., Grandori, C., and Eisenman, R. N. (1994) Cold Spring Harb. Symp. Quant. Biol. 59, 109–116
21. Ryan, K. M., and Birnie, G. D. (1996) Biochem. J. 314, 713–721
22. Hurlin, P. J., Queva, C., and Eisenman, R. N. (1997) Genes Dev. 11, 44–58
23. Desharats, L., Schneider, A., Muller, D., Burgin, A., and Eilers, M. (1996) Experientia 52, 1123–1129
24. Bousset, K., Oelgeschlager, M. H. H., Henrikssohn, M. Schreek, S., Burkhardt, H., Litchfield, D. W., Luscher-Firzlaff, J. M., and Luscher, B. (1994) Cell. Mol. Biol. Res. 40, 501–511
25. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K. (1987) Current Protocols in Molecular Biology: A Laboratory Manual, pp. 1057–1058, John Wiley & Sons, Inc., NY
26. Kato, M., Matsunaga, K., and Shimizu, N. (1998) Biochem. Biophys. Res. Commun. 246, 532–534
27. Mace, H. A., Pelham, H. R., Travers, A. A. (1983) Nature 304, 555–557
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