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# Impact of Nonnatural Amino Acid Mutagenesis on the in Vivo Function and Binding Modes of a Transcriptional Activator

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Given the central role of protein–protein interactions in biological functions, the development of methods for the discovery and characterization of such interactions has been of paramount importance in the scientific community.[1] Ideally these methods are compatible with live-cell studies so that protein–protein interactions can be investigated in their native context. Toward that end, recent advances in nonsense suppression technology have enabled the site-specific incorporation of amino acids bearing moieties for photo-activatable cross-linking reactions into proteins in bacteria, yeast, and human cells.[2] However, there have been few applications of this technology in eukaryotes, perhaps due to challenges in obtaining efficient incorporation of the nonnatural amino acids as well as limited knowledge about the impact of these changes on structure and function.[3] Here we demonstrate an enhanced tRNA/synthetase system for efficient incorporation in vivo (*S. cerevisiae*) of the photoactivatable amino acid *p*-benzoyl-L-phenylalanine (pBpa) throughout the transcriptional activation domain of the prototypical eukaryotic transcriptional activator Gal4. The pBpa-containing proteins maintain the key functions of Gal4, suggesting that this nonnatural amino acid has minimal impact on a critical binding surface within the protein. In vivo cross-linking with these mutants captured a key binding partner of Gal4, Gal80, and defined the binding interface of this complex beyond that predicted by conventional approaches. More broadly, this strategy should be suitable for the characterization and discovery of transcription factor binding partners in the native cellular environment.

The multipartner binding profile and intrinsically disordered nature of the transcriptional activation domains (TADs) of amphipathic activators produce significant roadblocks in studies of the function and structure of these essential proteins.[4,5] The most well-studied member of this family, Gal4, is a yeast transcriptional activator that regulates the GAL genes, whose products are required for galactose catabolism.[6,7] As a result, Gal4 is highly

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sensitive to the nutrient environment, such that only under galactose growth conditions does it function as an activator. The environmental sensitivity of Gal4 is largely regulated via a strong (nanomolar $K_D$) interaction between the repressor Gal80 and a portion of the carboxy-terminal transcriptional activation domain of Gal4.[7–9] Conventional mutagenesis and structural studies suggest that the middle region of the transcriptional activation domain (amino acids 851–871) comprises the binding site for Gal80, yet the Gal4•Gal80 complex impacts the function of the entire activation domain (840–881) (Figure 1).[9–11] We hypothesized that in vivo cross-linking would provide molecular-level detail regarding this important regulatory interaction. In addition to the interaction with Gal80, the Gal4 TAD engages in a set of interactions with coactivators in the transcriptional machinery to stimulate transcription under restrictive growth conditions (galactose).[5,8–14] Thus, the impact of pBpa mutations on the binding modes and function of Gal4 can be assessed by examining transcriptional activity under restrictive and permissive growth conditions.

As has been detailed previously, the nonsense suppression strategy typically utilizes the amber stop codon to encode the nonnatural amino acid.[2,15,16] Thus, to investigate the effect of *p*-benzoyl-L-phenylalanine incorporation on the binding and function of the Gal4 TAD, 10 individual Gal4 mutants were created in which amber stop codons (TAG) replaced the natural codons at specified positions within this sequence; in addition to the sequence encoding the mutant Gal4 TAD, each plasmid bears the sequence for the LexA DNA binding domain (Figure 2a). Six of the positions within Gal4(840–881) (W840, Y846, F849, F856, Y867, F869) are hydrophobic aromatic residues at which replacement with pBpa might be expected to have minimal impact. In contrast to this were positions T852, T859, and D871, all of which are polar residues. These plasmids were introduced into *S. cerevisiae* along with a plasmid encoding a mutant *E. coli* tRNA/synthetase pair ($tRNA^{Tyr}_{CUA}$/TyrRS) for pBpa as previously reported.16 However, very low yields of full-length protein were observed by Western blot analysis, likely due to low expression levels of the mature tRNA (Figure 2b). To address this, a yeast polIII promoter (SNR52) was inserted upstream of the *E. coli* $tRNA^{Tyr}_{CUA}$ which enables the efficient expression of functional *E. coli* $tRNA^{Tyr}_{CUA}$ in yeast.[17] We first examined the Phe849pBpa mutant and observed that expression increased from undetectable to >20% of unmodified LexA+ Gal4(840–881) (Figure 2b and c). Significantly, read-through in the absence of pBpa was minimal, consistent with previous reports.[16]

Next, we examined the functional impact of incorporating pBpa into LexA+Gal4(840–881) using quantitative *β*-galactosidase assays. Truncation of protein synthesis at residue 849 would result in a protein with little transcriptional activity;[12] thus activity observed in yeast grown without pBpa would be an indicator of read-through.[18] Consistent with the Western blot of Figure 2c, little transcriptional activity was observed in yeast grown without pBpa, with a >10-fold activity increase in the presence of pBpa (Figure 2d). More importantly, these results suggest that incorporation of pBpa does not substantially impact the ability of Gal4 to participate in essential interactions with the transcriptional machinery, at least in the context of residue 849. The interaction of the Gal4 TAD with Gal80 is tighter and more specific than the Gal4•transcriptional machinery interactions. To assess if this interaction was impacted, the transcriptional activity of the Phe849pBpa mutant was assessed in

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noninducing conditions (Figure 2d). Under glucose or raffinose growth conditions, little transcriptional activity was observed, indicating that the mutant retained its ability to interact with Gal80 and thus its environmental sensitivity.

Extending this study to the broader range of mutations throughout the Gal4 TAD, use of the enhanced tRNA/synthetase pair resulted in improved incorporation at all positions, although the efficiency varied (Figure 3a). In addition, all maintained their responsiveness to growth conditions (Supporting Figure S1), suggesting that the Gal4•Gal80 binding interaction is intact. To test this, live yeast cells expressing the pBpa mutants were irradiated at 365 nm to create direct Gal4–protein cross-links (Figure 3b). Upon lysis and Western blot analysis we observed that all Gal4 mutants formed several cross-linked products, consistent with the multipartner binding model proposed for their function.[14] More specifically, all formed a cross-linked product with a molecular weight of ~80 kDa, consistent with a LexA +Gal4•Gal80 product (Figure 3b, red box). This was confirmed by the introduction of a c-Myc-tagged version of Gal80 into yeast and visualization of the cross-linked products with a c-Myc antibody (Figure 3c). Similarly, incorporation of pBpa into Gal80 (residue 245) leads to cross-linking with LexA+Gal4 (Supporting Figure S3).

Although all mutants formed cross-links with Gal80, the extent varied with position. Seven of the positions lie within the previously identified binding site for Gal80 (852, 856, 859, 861, 867, 869, and 871).[9] Most striking is the Phe856pBpa mutant in which little cross-linked product is observed despite considerable evidence that this residue makes key contacts with Gal80 (Figure 1a).[9,11] This is not due to attenuated binding affinity, as the pBpa-containing TAD exhibits a $K_D$ for Gal80 nearly identical ($0.7 \pm 0.2$ $\mu$M) to that of the native sequence ($1.2 \pm 0.1$ $\mu$M) (Supporting Figure S2).[19] Similarly, the enhanced cross-linking observed with the Thr852pBpa and Tyr867pBpa mutants is not due to an altered affinity for Gal80 (Thr852pBpa: $0.8 \pm 0.3$ $\mu$M; Tyr867pBpa: $0.8 \pm 0.2$ $\mu$M). Somewhat surprising was that positions beyond the previously defined Gal80 binding sequence (residues 851–871) also exhibited effective cross-linking. At the amino terminus of the sequence, for example, the Phe849pBpa and Tyr846pBpa mutants both produce strong bands corresponding to the Gal4•Gal80 cross-linked product (Figure 3b). To probe this further, we examined pBpa mutations at positions 875(Pro) and 879(Lys), and upon irradiation of yeast bearing these mutants, cross-linking with Gal80 was observed (Supporting Figure S4). Cross-linking efficiency was attenuated with the Lys879pBpa mutant, similar to the Trp840pBpa mutant, and this may indicate limited binding interactions at the extreme amino-and carboxy-terminus of the Gal4 TAD.[20] This cross-linking pattern reveals an extended binding interface between Gal80 and Gal4 not previously identified through conventional deletion and mutagenesis experiments, highlighting the higher resolution information accessible by this approach.

In conclusion, we have described the facile incorporation of the nonnatural and photoactivatable amino acid pBpa into a transcription factor (Gal4) in vivo using a modified tRNA/synthetase system. Importantly, the function and binding interactions of Gal4 remain largely intact with this substitution as all mutant proteins retained their ability to activate transcription and to respond to environmental cues. Further, cross-linking in vivo enabled capture of a key masking protein, Gal80, and characterization of the binding interface with

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this protein. The improved expression yields observed with this system will be particularly valuable for identifying novel and functionally relevant binding partners of transcription factors that often have high turnover and are present in low abundance inside the cell.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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18. In absence of pBpa, two outcomes are observed: incorporation of a natural amino acid produces full-length protein with an undefined mutation (‘read-through’) or, more commonly, the amber codon is interpreted as a ‘stop’ and a truncated protein is produced. The absolute and relative yields of these two products varies from mutant to mutant.

19. In this instance, the benzophenone moiety may be poorly positioned for cross-linking, particularly as the efficiency of benzophenone cross-linking is well-known to vary with amino acid identity. Wittelsberger A, Thomas BE, Mierke DF, Rosenblatt M. FEBS Lett. 2006; 580:1872–6. [PubMed: 16516210]

20. An additional contributor to attenuated cross-linking of the Trp840pBpa mutant may be the reduced expression level of this construct (Fig 3a).



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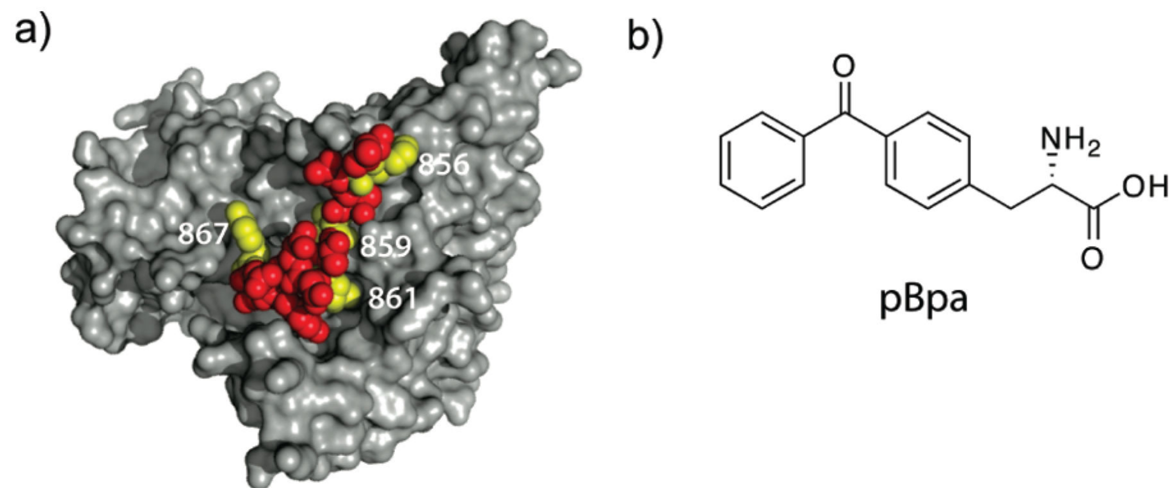


**Figure 1.**

(a) Structure of a short region of the Gal4 TAD (red and gold) bound to the inhibitor protein Gal80 (gray). Four of the amino acids replaced with pBpa in this study are highlighted in gold. Labels correspond to *S. cerevisiae* numbering. PDB ID 3E1K.[11] (b) Structure of *p*-benzoyl-L-phenylalanine (pBpa).

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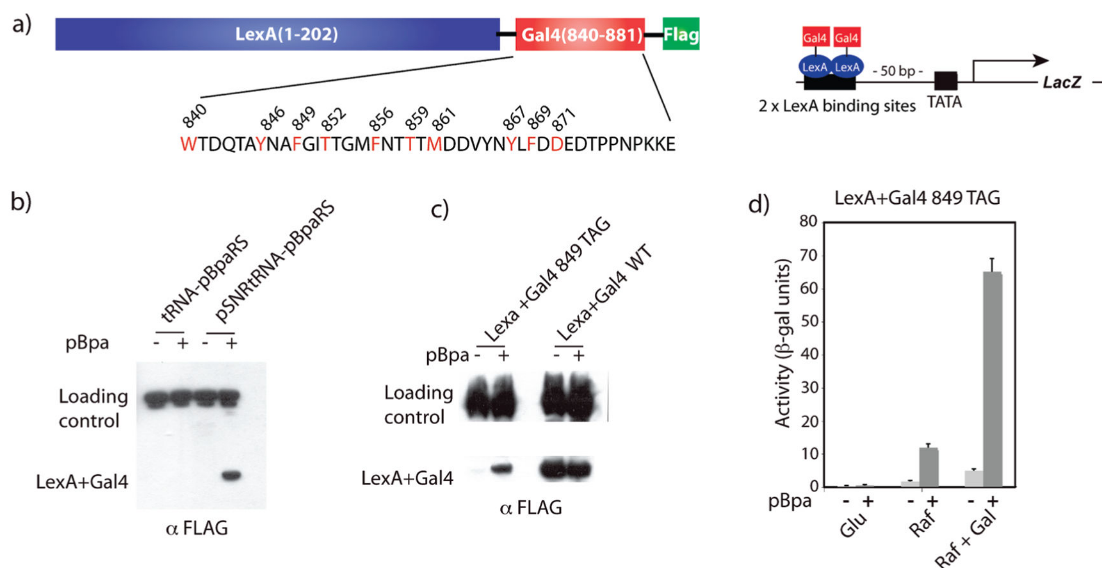


**Figure 2.**

In vivo incorporation of pBpa into FLAG-tagged LexA+Gal4 Phe849pBpa. (a) To assess the impact of pBpa on the function and binding modes of the Gal4 TAD, plasmids encoding the DNA binding domain of LexA fused to Gal4(840–881) as well as a FLAG tag were constructed. The LexA DBD was utilized to exclusively examine transcriptional activation at the two unique LexA binding sites upstream of the LacZ reporter. Positions at which pBpa mutagenesis was carried out are indicated in red. (b) Yeast cells bearing plasmids encoding LexA+Gal4 849 TAG and the pBpa-specific tRNA/synthetase pair expressed by ptRNA-pBpaRS or pSNRtRNA-pBpaRS were grown in the presence or absence of 2 mM pBpa and analyzed by Western blot (α-FLAG). (c) Yeast cells bearing plasmids encoding LexA+Gal4 849 TAG or LexA+Gal4 WT and the pBpa-specific tRNA/synthetase pair pSNRtRNA-pBpaRS were analyzed as described in (b). (d) *β*-Galactosidase assays were used to measure the activity of the LexA+Gal4 849 TAG mutant in the presence or absence of 2 mM pBpa under permissive or restrictive conditions. Each activity is the average of values from at least three independent experiments with the indicated error (SDOM). See Supporting Information for additional details.

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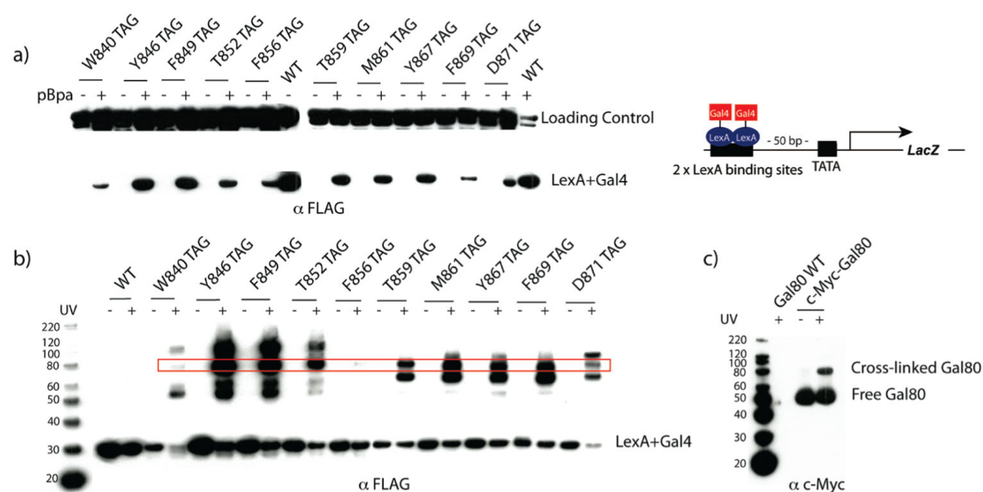


**Figure 3.**

Incorporation of pBpa and in vivo cross-linking of pBpa-containing Gal4 mutants. (a) Yeast cells expressing LexA+Gal4 amber mutants and the pBpa-specific tRNA/synthetase pair pSNRtRNA-pBpaRS in the presence or absence of 2 mM pBpa were analyzed by Western blot (α-FLAG). (b) Live yeast cells expressing LexA+Gal4 mutants in media containing 2 mM pBpa were exposed to 365 nm light, and the cell lysates were immunoprecipitated (α-LexA) and analyzed by Western blot (α-FLAG). The band at 30 kDa corresponds to full length LexA+Gal4. The band at 80 kDa (red box) is at the molecular weight of a LexA +Gal4•Gal80 cross-linked product. WT LexA+Gal4 sample was diluted 4-fold prior to loading. (c) Verification of Gal80. Yeast strains bearing a plasmid expressing c-Myc Gal80 and LexA+Gal4 849 TAG were grown in media containing 2 mM pBpa and irradiated with UV light. Subsequently, the lysate was probed using a Western blot with a c-Myc antibody. A band at 80 kDa corresponding to a cross-link between LexA+Gal4 and c-Myc Gal80 only occurs when the yeast cells contain c-Myc Gal80 and are irradiated with UV light. See Supporting Information for the experimental details.