Genetic evidence for similar negative regulatory domains in the yeast transcription activators GAL4 and LAC9

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ABSTRACT

The GAL4 protein of *Saccharomyces cerevisiae* and the LAC9 protein of *Kluyveromyces lactis* are transcription activator proteins with similar structure and function. Greatest similarity occurs in the C region near the carboxy terminus, where 16 of 18 amino acids are identical. The function of the C region is unclear. Here we show that the structural similarity is reflected in functional similarity. Single amino acid changes in the C region of GAL4 and LAC9 create a similar phenotype: constitutive gene expression. In *S. cerevisiae* the constitutive phenotype caused by *GAL4* mutants can be abolished by overproduction of GAL80. These results support a model in which the C region of GAL4 and LAC9 constitute similar negative regulatory domains that interact with GAL80 in *S. cerevisiae* and an unidentified GAL80 homolog in *K. lactis*. This protein-protein interaction prevents expression of the galactose operon in the uninduced state.

INTRODUCTION

The yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* each contain a set of genes, the galactose regulon, whose transcription is inducible by galactose. Galactose-inducible transcription in *S. cerevisiae* requires the *GAL4* gene (1) while in *K. lactis* the *LAC9* gene is required (2, 3, 4). The predicted GAL4 and LAC9 proteins, containing 881 and 865 amino acids, respectively, show three regions of amino acid sequence similarity (3, 4). The region of greatest similarity, termed the C region, lies near the carboxy terminus of the proteins and contains 16 out of 18 identical amino acids. Such conservation of amino acids, especially in view of the lack of conservation throughout most of the protein sequence, strongly suggests that the C region of these two proteins has a similar function. What might the function be?

A possible function could be to regulate the activity of the transcription activation domain of GAL4 and LAC9. GAL4 has at least two separate functional domains, one for binding DNA and another for activating transcription. The DNA-binding domain lies within the amino-terminal 74 amino acids (5). This domain enables GAL4 to bind 17 bp DNA sequences, termed upstream activator sequences (UAS_G), that lie in front of each structural gene in the galactose regulon (6, 7, 8, 9). GAL4 appears to have two transcription activation domains, amino acids 148–196 and 768–881 (10). These domains are thought to activate transcription by contacting the transcription machinery of the cell.

Activation of transcription by GAL4 is regulated. When cells are grown in the absence of inducer, galactose, the negative regulatory protein GAL80 binds to GAL4 and prevents activation of transcription (11, 12, 13). Galactose or one of its metabolites dissociates GAL80 from GAL4 thereby allowing GAL4 to activate transcription. During both inducing and uninducing conditions of growth GAL4 is bound to UAS_G (6, 14). These events only pertain to cells growing in the absence of glucose. When glucose is present, GAL4 dissociates from UAS_G and the galactose regulon cannot be induced even if galactose is present in the medium. This phenomenon is called glucose or catabolite repression (15, 16, 17).

Binding of GAL80 to GAL4 has been verified biochemically (18). Genetic experiments show that GAL80 interacts with some portion of the 28 carboxy terminal amino acids of GAL4 (19, 20). The C region is within this 28 amino acid stretch and one transcription domain is nearby or overlapping (10).

Our understanding of the domain structure of LAC9 is based both on the structural and functional similarity of LAC9 and GAL4 and upon direct experimentation. The DNA binding domain of LAC9 lies between amino acids 85 and 228 (21, Halverson, Nandabalan and Dickson, unpublished results). This region shows high amino acid sequence similarity to the 74-amino terminal amino acids of GAL4 (3, 4). The amino acid sequence similarity of LAC9 and GAL4 produces a functional similarity so that both proteins bind to similar UAS_G that have the general form 5'CGG(N11)CCG (22–24). The location of the transcription activation domain(s) in LAC9 has not been determined experimentally, but they should be similar to those in GAL4 since LAC9 can replace GAL4 in *S. cerevisiae* (25).

The transcription activation activity of LAC9 is tightly regulated, and only appears when cells are grown in the presence of inducing sugars, galactose or lactose (26). It has been hypothesized that the product of *LAC10* (27) functions in a manner analogous to GAL80 and regulates the transcription

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activation domain(s) of LAC9 (25). The functional similarity between GAL80 and LAC10 is based entirely upon the constitutive phenotype caused by a mutation in these genes. Salmeron *et al.* (28) have shown that the activity of the LAC9 transcription activation domain can be regulated in *S. cerevisiae* by GAL80, implying that GAL80 recognizes similar structures in GAL4 and LAC9.

To examine the function of the C region of GAL4 and LAC9 we introduced single amino acid changes in this region and examined their effect on expression of the galactose regulon in *S. cerevisiae* and *K. lactis*. The data presented here indicate that the C region constitutes a negative regulatory domain that mediates binding to a negative regulator, GAL80 in *S. cerevisiae* and its *K. lactis* analogue, which remains to be identified.

MATERIALS AND METHODS

Culture conditions

Yeast were grown in minimal medium (Min) containing double strength yeast nitrogen base (without amino acids, Difco) supplemented with adenine sulfate (20 mg/L), arginine (20 mg/L), histidine (20 mg/L), isoleucine (30 mg/L), leucine (20 mg/L), lysine (20 mg/L), methionine (20 mg/L), threonine (200 mg/L), tryptophan (20 mg/L), uracil (20 mg/L), and valine (150 mg/L). Min was supplemented with glycerol (2% w/v; MinGly), glycerol and potassium lactate, pH 5.5 (3%, MinGlyLactate), sorbitol (2%, MinSorb), lactose (2%, MinLac), or galactose (2%, MinGal). Plasmids were maintained in strains by omission of the appropriate supplement(s) from the medium.

Plasmids and strains

S. cerevisiae strains used in this study were: YM2631 (*ura3−52 his3D200 ade2D101 lys2−801 trp1D901 gal4D542 LEU2::GAL1-lacZ* (pRY181) met$^-$; a gift from M. Johnston, Washington University, St. Louis, Mo.). The *gal4, his3, ade2*, and *trp1* alleles are deletions of all coding sequences. *K. lactis* strains used in this study were:JD108 (*his2−2 trp1 ura3−1 lac9::URA3* Ura$^-$). The entire *LAC9* coding region, between the BglII and BstEII sites was replaced using *URA3* (22), and a Ura$^-$ isolate was selected using 5-fluorooratic acid (29).

The low copy-number YCp50 centromere vector (30) was used to introduce *GAL4* and mutant derivatives, YCp50-GAL4 (4), into *S. cerevisiae* strain YM2631. Transformants were selected for the Ura$^+$ phenotype. *GAL80* was introduced into *S. cerevisiae* on the high-copy number vector YEp426 (30) with selection for Lys$^+$ transformants. The YEp426-GAL80 plasmid was constructed by inserting a 3.1 kb HindIII fragment carrying *GAL80* into the unique HindIII site of YEp426.

LAC9 and mutant alleles were integrated into the *K. lactis* genome via homologous recombination at the HpaI site of *ARSIB* using the vector pIB3-LAC9-B (4). Plasmids were integrated in order to have a population of stable transformants with a known number of copies of *LAC9* per cell. Unlike *S. cerevisiae*, there are no stable, low-copy number, centromere-type vectors for *K. lactis* (24). To integrate a plasmid, ten to twenty μg of plasmid DNA, linearized by digestion with HpaI, was transformed into JD108, and Ura$^+$ transformants were selected. Integration at *ARSIB* and copy number were determined by Southern blots using chromosomal DNA (31) digested with BamHI which cleaves the vector once. The radioactive probe for the blots was pRS2 (4) cut with BamHI. The same plasmids and procedures were used to integrate *LAC9* and mutant alleles into the genome of strain YM2631. For these experiments plasmids were linearized at the ApaI site in *URA3*.

Wild-type and mutant *GAL4* alleles were integrated into the *K. lactis* genome via the KpnI site in the *K. lactis urA3* gene. *GAL4* alleles were transferred from YCp50-GAL4 plasmids as 3.7 kb EcoRI-BamHI fragments into pDM37 (pUC18 with a 1.6 kb *K. lactis* DNA fragment carrying *URA3*, a gift from Chiron Corp. Emeryville, CA) cut with EcoRI and BamHI. The resulting plasmids were linearized by digestion with KpnI followed by transformation into JD108. Ura$^+$ transformants were selected and Southern blots were performed to determine if a single or multiple plasmids had integrated at the expected site.

Miscellaneous procedures

β-galactosidase activity was determined on permeabilized whole cells (32) grown to an absorbance (A_{600nm}) of 0.5 to 2.5. One unit of enzyme activity hydrolyzes one nanomole of o-nitrophenyl-β,D-galactoside per min per A_{600nm}. The procedure of Kunkel *et al.* (33) was used to introduce nucleotide changes into *LAC9* and *GAL4*. DNA probes for Southern blots were labeled using the method of Feinberg and Vogelstein (34). Yeast were transformed using the lithium acetate procedure (4).

RESULTS

Phenotypic effect of amino acid changes in the C region of LAC9

If the C region of LAC9 (Figure 1) constitutes a negative regulatory domain that interacts, in the uninduced state, with a regulatory protein to prevent transcription then it should be possible to disrupt this protein-protein interaction by single amino acid changes in the C region. Consequently, the transcription activation domain of LAC9 should function and the galactose regulon should be expressed constitutively. The constitutive phenotype can be evaluated in *K. lactis* by measuring β-galactosidase activity, the product of *LAC4*, a gene whose expression is tightly controlled by LAC9 (Wray *et al.*, 1987).

Single amino acid changes were introduced into the C region of LAC9 by oligonucleotide-directed mutagenesis. Each mutant allele was separately integrated into the chromosome of a *K. lactis* strain deleted for the wild-type *LAC9* allele. As a control, the wild-type *LAC9* allele was integrated in the same manner. Integration at the expected chromosomal location and the presence of a single copy of *LAC9* were verified by Southern blot analysis (data not shown). The data shown in Table 1 demonstrate that amino acid changes Asn849→Tyr, Thr850→Ala, or Phe861→Ser cause an increase in the uninduced level of β-galactosidase activity and thereby produce a constitutive phenotype.

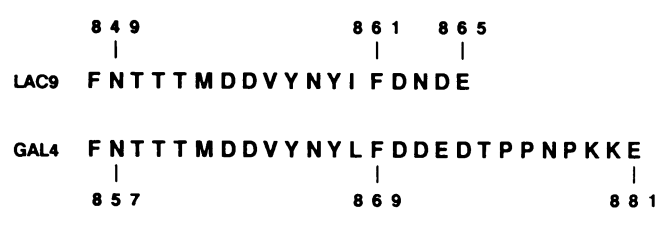

Figure 1. Structure of the LAC9 and GAL4 C regions. Amino acids comprising the C region of LAC9 are shown, residue 848 to the amino terminal residue 865. The C region of GAL4 extends from residue 856 to 872. Residue 881 is the amino terminus of GAL4.

The constitutive phenotype produced by the C region amino acid changes could be due to a reduced affinity of the variant LAC9 proteins for a negative regulatory protein, as we hypothesize, or constitutivity could be due to an increase in the activity of the transcription activation domain. This latter possibility arises because it is not known whether the C region and the transcription activation domain are separate or overlapping. If the C region mutations had affected the transcription activation domain then the resulting variant LAC9 proteins should be more effective activators of the galactose regulon under inducing conditions and this should be reflected in higher levels of β-galactosidase activity. The data presented in Table 1 show that none of the C region variant LAC9 proteins increased the induced level of β-galactosidase activity over that seen in strains making wild-type LAC9. Therefore, the phenotypic effect of C region mutations is most likely due to a reduction in the affinity of LAC9 for a negative regulator.

Phenotypic effect of amino acid changes in the C region of GAL4

The phenotypic effect of amino acid changes in the GAL4 C region was examined using a strain of *S. cerevisiae* deleted for *GAL4* and having a β-galactosidase coding region fused to the GAL4-responsive UAS_G. The amino acid changes made in GAL4 corresponded to the changes made in LAC9. GAL4 mutants were introduced into the host strain on a CEN vector to maintain a low copy number per cell. Two of the three amino acid changes, Thr858→Ala, and Phe869→Ser, resulted in an increased level of β-galactosidase activity in the uninduced state (Table 2). Such constitutive expression of the galactose regulon suggests that the variant GAL4 proteins are not interacting normally with the negative regulator GAL80. The third change, Asn857→Tyr, did not increase the level of β-galactosidase activity under uninduced growth conditions (Table 2), indicating that this change did not affect interaction with GAL80.

To examine the possibility that the constitutive β-galactosidase activity produced by the Thr858→Ala and Phe869→Ser amino acid changes was due to an alteration in the transcription activation domain located near the carboxy terminus of the protein, we measured β-galactosidase activity under inducing conditions. Strains carrying the variant GAL4 proteins gave the same induced level of β-galactosidase activity as wild-type GAL4 (Table 2). We conclude that the three variant GAL4 proteins have normal transcription activation activity.

Strain	LAC9 allele	β-galactosidase Activity Uninduced	Induced
JD156	Asn849→Tyr	130	532
JD157	Asn849→Tyr	149	520
JD160	Asn849→Tyr	114	650
JD158	Thr850→Ala	106	381
JD159	Thr850→Ala	90	312
JD161	Phe861→Ser	73	320
JD147	wild-type	4.0	491
JD148	wild-type	4.9	544
JD149	wild-type	4.6	517
JD108	deletion	3.9	0.64

Table 1. Effect of LAC9 C region amino acid changes on galactose regulon expression in *K. lactis*. Uninduced cultures were grown in MinSorb medium containing 20 μg uracil/ml while induced cultures were grown in the same medium supplemented with 2% galactose. Values represent the average of two separate experiments and the deviation from the average was less than ± 20%.

In an attempt to show that the phenotypic effect of C region amino acid changes was due to small, localized distortions in the GAL80 negative regulatory domain of GAL4 rather than to large, extended distortions in structure we introduced conservative amino acid changes that might not cause any phenotypic effects. The conservative changes Asn857→Asp and Phe869→Tyr produced no detectable effect on β-galactosidase levels indicating that the GAL4 variant proteins were functioning normally (Table 2). The data for the Asn857→Tyr change (Table 2) also supports the idea that amino acid changes in the C region do not result in an extended area of distortion in the conformation of GAL4.

Additional support for the idea that the GAL4 C region forms the GAL80 interaction domain was gathered by examining the effect of GAL80 overproduction. If changes in C region amino acids decrease the affinity of GAL4 for GAL80 then it should be possible to overcome the consequences of decreased affinity by increasing the intracellular concentration of GAL80 (35). Overproduction of GAL80 was achieved by putting *GAL80* on

GAL4 allele	β-galactosidase Activity Uninduced	Induced	GAL80
Asn857→Tyr	0.33 ± 0.3	173 ± 26	ND
Asn857→Tyr	0.17 ± 0.2	180 ± 13	ND
Asn857→Asp	0.35 ± 0.2	186 ± 30	ND
Thr858→Ala	119 ± 21	180 ± 9	16 ± 8
Thr858→Ala	186 ± 9	192 ± 18	25 ± 5
Phe869→Ser	209 ± 36	188 ± 62	218 ± 30
Phe869→Ser	352 ± 32	187 ± 28	214 ± 30
Phe869→Tyr	0.53 ± 0.6	215 ± 17	ND
wild-type	0.33 ± 0.1	148 ± 9	1.2 ± 0.4
wild-type	0.22 ± 0.1	186 ± 15	1.1 ± 1.3
wild-type	ND	183 ± 43	4.1 ± 2.1
wild-type	ND	186 ± 37	3.7 ± 2.0
none	0.2 ± 0.0	8.7 ± 0.9	0.16 ± 0.2

Table 2. Effect of GAL4 C region amino acid changes on galactose regulon expression in *S. cerevisiae*. Strain YM2631 was transformed with YCp50 carrying the indicated GAL4 allele. Cells were grown in MinGlyLactate without uracil for uninduced conditions and in the same medium supplemented with 2% galactose for induced conditions. For the column marked GAL80 the strain carried YEp426-GAL80 in addition to the indicated GAL4 allele and the culture medium was MinGlyLactate lacking uracil and lysine. The values represent the average of three or four separate assays ± the standard deviation of the mean. Each line represents data for an individual strain. ND: not determined.

Strain	GAL4 allele		β-galactosidase Activity Uninduced	Induced
JD163	wild-type	(2)	0.58	86
JD164	wild-type	(1)	1.1	81
JD175	Asn857→Tyr	(2)	2.1	103
JD176	Asn857→Tyr	(2)	2.2	83
JD167	Thr858→Ala	(1)	8.9	90
JD169	Thr858→Ala	(1)	6.5	110
JD172	Phe869→Ser	(1)	6.4	83
JD173	Phe869→Ser	(1)	6.3	70

Table 3. Effect of GAL4 C region amino acid changes on expression of the galactose regulon in *K. lactis*. Strain JD108 was transformed with linearized plasmid DNA, Ura^+ transformants were analysed by Southern blotting for the expected chromosomal integration event and for the number of copies of the vector integrated (shown in parentheses). Uninduced cultures were grown in MinGly and induced cultures in MinLac. The values for uninduced cultures are the average of four samples taken on the same culture at different times. The values for the induced cultures are the average of two time points. All values deviated less than ±20%.

a high-copy number vector. The strain carrying the Thr858→Ala change responded to overproduction of GAL80 (Table 2).

The phenotypic effect of GAL4 C region amino acid changes was also examined in *K. lactis*. Both the Thr858→Ala and Phe869→Ser amino acid changes resulted in increased β-galactosidase activity for uninduced cells (Table 3) and thus created a constitutive phenotype. The Asn857→Tyr change, which did not produce a β-galactosidase constitutive phenotype in *S. cerevisiae*, produced a slight, two to three-fold, constitutive phenotype in *K. lactis* (Table 3). Under induced growth conditions these C region amino acid changes behaved just like wild-type GAL4. We conclude from the data presented in Tables 2 and 3 that amino acids Thr858 and Phe 869 are part of a GAL4 domain that interacts with a negative regulator, GAL80 in *S. cerevisiae* and an unidentified GAL80 homolog in *K. lactis*.

DISCUSSION

Data presented in this paper support the hypothesis that the highly conserved region of amino acids near the carboxy terminus of LAC9 and GAL4, which we termed the C region, forms part of a negative regulatory domain. Our studies extend previous work showing that some or all of the 28 (19) or 30 (20) carboxyl-terminal amino acids of GAL4 form a domain that interacts with GAL80 and that some part of LAC9 can interact with GAL80 (28). We propose that the C region of GAL4 interacts with the negative regulator GAL80 to prevent the transcription activation domains of GAL4 from functioning in the uninduced state. In *K. lactis* the C region of LAC9 interacts with an unidentified homolog of GAL80 to prevent LAC9 from activating transcription in the absence of inducer. Support for these hypotheses was obtained by showing that specific amino acid changes in the C region of LAC9 and GAL4 created a constitutive phenotype in *K. lactis* and *S. cerevisiae*, respectively, as would be predicted by our model. Our hypothesis was further strengthened because GAL4 C region mutants also gave a constitutive phenotype in *K. lactis* (Table 3). We were unable to do the reciprocal experiment and demonstrate that LAC9 C region mutants caused a constitutive phenotype in *S. cerevisiae* because wild-type LAC9 itself gave a constitutive phenotype in *S. cerevisiae* (4, 28). Further support for our hypothesis was obtained by showing that the constitutive phenotype caused by GAL4 C region variant Thr858→Ala could be reversed by overproduction of GAL80 (Table 2).

The rational for determining the structure-function relationship of the C region rests on the assumption that single amino acid changes produce domain-specific, localized pertubations in conformation rather than large, indirect effects on adjacent domains. Although only a few amino acid residues were changed, the data indicated that this assumption held for the C region because amino acid changes at some but not at nearby residues produced a constitutive phenotype. Furthermore, at a particular residue only amino acid replacements that changed the chemical nature of the residue produced a constitutive phenotype (Table 2).

An alternative explanation for the constitutive phenotype would argue that C region amino acid changes increased the activity of the transcription activation domain. Based upon current ideas of how the GAL4 transcription domains work (36), we would predict that if this explanation were true then the C region variant proteins should give a higher induced level of β-galactosidase activity than wild-type GAL4. Since the C region variant proteins did not activate transcription any better than wild-type GAL4 in the induced state it seems unlikely that the constitutive phenotype is due to changes in the transcription activation domains of GAL4 or LAC9.

Another alternative explanation for the constitutive phenotype would argue that the C region amino acid changes resulted in a higher steady state level of GAL4 or LAC9. If this were true there should also be a higher induced level of β-galactosidase activity in strains having the variant proteins since increasing the level of GAL4 in *S. cerevisiae* (37) or in *K. lactis* (25) and LAC9 in *K. lactis* (Dickson, unpublished results) increased the level of β-galactosidase activity under inducing conditions.

A striking characteristic of the C region is its high similarity in both GAL4 and LAC9. There are only three regions, representing about one-third of all amino acid residues, in these two proteins showing amino acid sequence similarity and of these three the C region is the most similar, 88% identity (3, 4). Such conservation of amino acids argues strongly for functional similarity. Our data support a functional similarity since identical amino acid changes in the C region of GAL4 and LAC9 yield a constitutive phenotype and since GAL4 C region mutants give a constitutive phenotype in both *S. cerevisiae* and *K. lactis*. While the molecular interaction between the C region of GAL4 and GAL80 and between the C region of LAC9 and the *K. lactis* GAL80 homolog are similar, they are not identical since the Asn849→Tyr change in LAC9 gave a constitutive phenotype (Table 1) while the same change in GAL4 (Asn857→Tyr, Table 2) did not. In this regard, our data do not rule out the possibility that other regions of GAL4 and LAC9 are part of the GAL80 interaction domain.

It follows from our data and other similarities between the galactose operons in *S. cerevisiae* and *K. lactis* (24) that *K. lactis* must have a homolog of GAL80. The most likely candidate is *LAC10* (27) because mutations in this gene give a constitutive phenotype for galactose operon expression. However, an unidentified gene is a possibility. The data of Salmeron *et al.* (1989) also supports the existence of a *K. lactis* GAL80 homolog. These authors showed that transcription activation by LAC9 could be reduced in *S. cerevisiae* by overproduction of GAL80. We were unable to reproduce this result but our lack of success is probably due to overproduction of LAC9 caused by differences in strains and vectors. Efforts to clone the *K. lactis* GAL80 homolog and demonstrate a direct physical interaction with the C region of LAC9 and GAL4 are in progress.

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ADDENDUM

Salmeron *et al.* (Genetics, **125**, 21–27, 1990) came to the same conclusion we came to regarding the function of the C-region of *GAL4*.

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