

The reverse two-hybrid system: a genetic scheme for selection against specific protein/protein interactions

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ABSTRACT

The yeast two-hybrid system is a powerful experimental approach for the characterization of protein/protein interactions. A unique strength of the yeast two-hybrid system is the provision for genetic selection techniques that enable the identification of specific protein/protein interactions. We now report the development of a modified yeast two-hybrid system which enables genetic selection against a specific protein/protein interaction. This reverse two-hybrid system utilizes a yeast strain which is resistant to cycloheximide due to the presence of a mutant *cyh2* gene. This strain also contains the wild-type *CYH2* allele under the transcriptional control of the Gal1 promoter. Expression of the wild-type Gal4 protein is sufficient to restore growth sensitivity to cycloheximide. Growth sensitivity towards cycloheximide is also restored by the coexpression of the avian c-Rel protein and its I κ B α counterpart, p40, as Gal4 fusion proteins. Restoration of growth sensitivity towards cycloheximide requires the association of c-Rel and p40 at the Gal1 promoter and correlates with the ability of the c-Rel/p40 interaction to activate expression from the Gal1 promoter. A genetic selection scheme against specific protein/protein interactions may be a valuable tool for the analysis of protein/protein interactions.

INTRODUCTION

The c-Rel protein is sequestered in the cytoplasm through association with the inhibitor protein, I κ B α (1,2). Cytokine-induced phosphorylation of I κ B α signals ubiquitin-mediated degradation of I κ B α and subsequent nuclear import of c-Rel (3–10). The nuclear c-Rel protein binds DNA and activates expression of multiple target genes, including the gene encoding I κ B α (11–15). Newly synthesized I κ B α is able to enter the nucleus (16–18), and nuclear I κ B α has been suggested to facilitate the displacement of c-Rel from DNA and mediate export of the nuclear c-Rel/I κ B α complex to the cytoplasm (16,18,19). The ability of I κ B α to associate with c-Rel in both the nucleus and the cytoplasm suggests that the nuclear and cytoplasmic c-Rel/I κ B α complexes may have distinct functional and structural properties.

The existence of two c-Rel/I κ B α complexes with distinct functional and structural properties is supported by several lines of evidence. First, the ankyrin repeat domain of I κ B α is sufficient to inhibit nuclear import of c-Rel, while both the ankyrin repeat domain and the PEST domain of I κ B α are required for inhibition of DNA-binding by c-Rel (1,2). Second, while the ankyrin repeat domain of I κ B α is sufficient for association with the wild-type c-Rel protein, both the ankyrin repeat domain and the PEST domain of I κ B α are required for association with mutant c-Rel proteins that are resistant to inhibition of DNA-binding by I κ B α (19,20). Therefore, it can be envisioned that the ankyrin repeat domain is sufficient for the integrity of the cytoplasmic c-Rel/I κ B α complex, while both the ankyrin repeat domain and the PEST domain are required for the integrity of the nuclear c-Rel/I κ B α complex. However, little is known regarding the specific amino acid contacts that define each c-Rel/I κ B α complex.

We have previously utilized the yeast two-hybrid system to identify residues that are required for the formation of c-Rel/I κ B α complexes containing either wild-type or mutant proteins (19). The yeast two-hybrid system is a potent tool for studying protein/protein interactions *in vivo* (21–24). In a commonly utilized version of the *Saccharomyces cerevisiae* two-hybrid system (21), one of the proteins (A) is bound to the Gal4 enhancer region of the Gal1 promoter via its fusion to the Gal4 binding domain (Gal4BD), while the other protein (B) is fused to the Gal4 activation domain (Gal4AD, Fig. 1A). Association of the two fusion proteins (Gal4BD:A and Gal4AD:B) brings the activation domain of Gal4 to the Gal1 promoter. Localization of the Gal4 activation domain to the Gal1 promoter can be used to activate the expression of either a reporter gene such as *lacZ*, or a gene necessary for cell viability such as *HIS3* (21,23).

The use of the *lacZ* reporter gene in the two-hybrid system has allowed for the development of assays for the identification of interacting proteins and for the identification of mutant proteins that are unable to interact with their partner protein (21–24). However, biochemical assays for *lacZ* expression are relatively insensitive and can yield a high frequency of false positive clones. To circumvent the high frequency of false positives when using the *lacZ* gene as a single reporter system, two-hybrid systems in which the use of genes that are required for cell viability such as *HIS3* were developed (22). Such genetic selection schemes have proven to be very effective in the identification of interacting proteins. However, these genetic selection schemes are not able to identify mutant proteins that have lost the ability to associate with their partner protein. Therefore, we asked if the standard

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two-hybrid system could be reversed. That is, can a genetic system be developed in which there is a selection against the association of two proteins? A genetic system which is capable of selecting against the association of two proteins might provide a simple yet powerful approach towards screening pools of randomly generated mutant proteins. Furthermore, such a genetic selection scheme, in conjunction with suitable yeast expression libraries, might facilitate the identification of genes which encode proteins that interfere with a particular protein/protein interaction. This has led to the development of the reverse two-hybrid system.

MATERIALS AND METHODS

Plasmid construction

To construct pCL12, the Gal1 promoter was subcloned as a *SalI*-*SacI* fragment from pRY171 (27) into pRS304 (28) which contains the *TRP1* selectable marker. The PCR primers 5'-GCGC-GCGCTTGTATGCTTCCAGATTAAGAC-3' and 5'-GC-GCGCGAGCTCAAAAACGGTCAAAATGGGTCATAG-3' (introducing a *SacI* site, shown underlined) were used to amplify the 5' *CYH2* sequence from pRS318. The PCR product was subcloned into pCRII (Invitrogen) to construct pCL21. The PCR-derived insert within pCL21 was sequenced to confirm that no PCR-introduced mutations were present. The sequence of the PCR-derived *CYH2* insert within pCL21 matched the published wild type *CYH2* cDNA sequence from codons 1 to 36 (2). The remaining cDNA required to encode the 3' 37-148 codons was subcloned into pCL21 as a *HincII*-*XbaI* fragment from pAS1-CYH2 (22) to construct pCL23. The *SpeI*-*SacI* fragment of pCL23 was subcloned into pCL12 to construct pCL25. Restriction analysis confirmed that the pCL25 construct was correct.

To construct the plasmid encoding Gal4AD:p40-TA, the PCR primers 5'-TCCGAATTCGATACAGCAGTTTACAACAGC-3' and 5'-CTGGAATTCATGGTTATAGCTTTGTAATAT-3' (introducing *EcoRI* sites as shown underlined) were used to amplify the sequence encoding the residues from 540 to 598 of c-Rel from pER31 (19). The *EcoRI*-digested PCR product was cloned into pGal4AD:p40 (19), yielding pGal4AD:p40-TA. The correct orientation of the insert and the absence of PCR-introduced mutations were confirmed by sequence analysis. To construct the plasmid encoding Gal4AD:TA-p40, the PCR primers 5'-TCTCTC-GAGATACAGCAGTTTACAACAGC-3' and 5'-TCCTCGAGA-ATGGTTATAGCTTTGTAATAT-3' (introducing *XhoI* sites as shown underlined) were used to amplify the sequence encoding the residues from 540 to 598 of c-Rel from pER31. The *XhoI*-digested PCR product was subcloned into pGal4AD:p40, yielding pGal4AD:TA-p40. The correct orientation of the insert and the absence of PCR-introduced mutations were confirmed by sequence analysis.

All other plasmids have been previously described (19). All proteins were expressed from the yeast ADH promoter on episomal plasmids. The *HIS3* marker was used to select for the presence of the plasmids expressing the Gal4BD and all the wild-type and mutant Gal4BD:c-Rel proteins and the *LEU2* marker was used to select for the presence of the plasmids expressing the full-length Gal4, Gal4AD, and all wild-type and mutant Gal4AD:p40 proteins.

Selection for *cyh2* yeast strains

The *S.cerevisiae* yeast strain JC981 (α *gal4 gal80 his3 trp1-901 ura3-52 URA3::Gal1* → *lacZ leu2-3,112 cyh2*) was the parental

strain from which CL9 was derived. Standard techniques were used for the selection of *cyh2* yeast strains and transformation procedures (25,26). To select for cycloheximide (CHX)-resistant yeast strains, $\sim 5 \times 10^8$ JC981 cells were plated on YPD (yeast extract, peptone, dextrose) plates containing 10 μ g/ml CHX and incubated at 30°C. CHX-resistant colonies were obtained after 5 days. To ensure that the yeast strains were CHX resistant due to a mutation at the *CYH2* locus (*cyh2*), the CHX-resistant yeast strains were transformed with pRS318 (25) and were plated onto leucine-free synthetic dropout (SD) plates with and without 10 μ g/ml CHX. pRS318 contains the wild type *CYH2* gene driven by its endogenous promoter and restores CHX sensitivity when expressed in mutant *cyh2* yeast (25).

Integration of pCL25 into *cyh2* yeast strains

The pCL25 plasmid, linearized at the unique *HindIII* site within *TRP1* to facilitate integration of the plasmid into the *trp1-901* locus, was transformed into the *cyh2* yeast strains. The transformants were plated onto TRP-free SD plates. To ensure the stable integration of pCL25, the strains were transferred to YPD plates and replica plated onto SD plates lacking tryptophan (TRP). The surviving colonies were replica plated back onto YPD plates and then to TRP-free SD plates. The surviving colonies were also replica plated onto SD plates lacking histidine (HIS), lacking leucine (LEU), lacking uracil (URA), and containing 10 mg/ml CHX to ensure that the correct phenotype had been maintained throughout the manipulation of these strains.

Analysis of c-Rel/p40 interactions

Association between c-Rel and p40 proteins was measured using the standard two-hybrid system essentially as previously described (19) with the following exceptions. The *cyh2* yeast strains were transformed with the indicated plasmids and 3% of the transformation mixture was plated onto HIS-free, LEU-free and TRP-free SD plates that lacked or contained 25 μ g/ml CHX. For analysis of β -galactosidase activity, the transformed yeast were picked from plates lacking CHX into 50 μ l of 100 mM potassium phosphate containing 0.2% Triton X-100, to which was added 0.5 μ l of 1 M dithiothreitol (DTT), 2.5 μ l of 0.1% sodium dodecyl sulfate (SDS) and 7.5 μ l of chloroform. The yeast cells were lysed by vigorous vortexing for 10 s. The chemiluminescent assay used to measure the relative light units produced due to the association of Rel and p40 proteins has been previously described (19). Pictures of the plates were taken 9 days following transformation.

RESULTS

Description of the reverse two-hybrid system

The protein encoded by the *CYH2* gene is responsible for the sensitivity of the yeast to cycloheximide (CHX). Mutation of *CYH2* can produce a CHX-resistant *cyh2* yeast strain. In the reverse two-hybrid system described in this report, a *cyh2* yeast strain containing *CYH2* under the control of the Gal1 promoter is utilized. Because the *CYH2* allele is dominant over the *cyh2* allele, expression of the *CYH2* gene product will confer CHX sensitivity to a CHX-resistant *cyh2* yeast strain (25,30). Thus, localization of Gal4AD to the Gal1 promoter through association of the fusion proteins will activate transcription of *CYH2* and confer sensitivity to CHX. In Figure 1B, the fusion proteins Gal4BD:c-Rel and Gal4AD:p40 are used as an example. Since

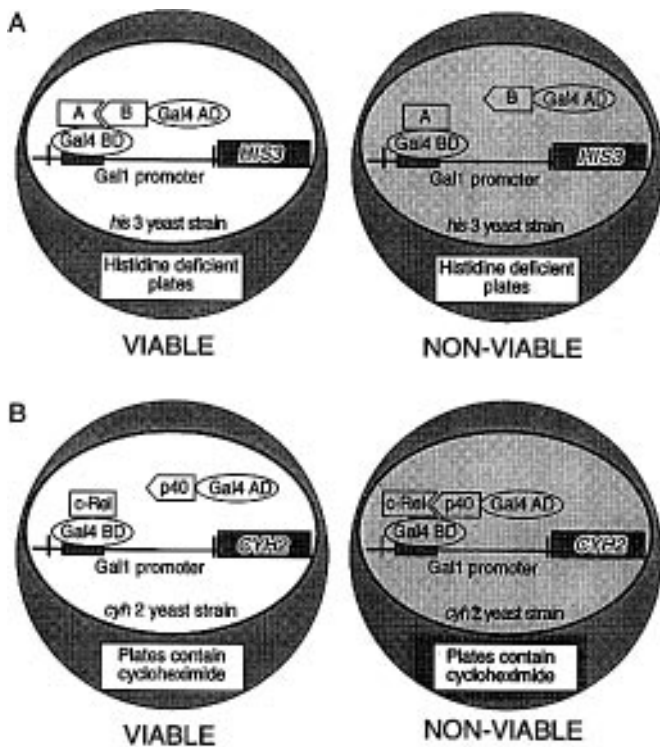


Figure 1. Schematic representation of the standard two-hybrid system and the reverse two-hybrid system. **(A)** The standard Gal4 two-hybrid system is a genetic selection scheme that selects for the association of protein A fused to the DNA-binding domain of Gal4 (Gal4BD:A) and protein B fused to the activation domain of Gal4 (Gal4AD:B). Association of Gal4BD:A and Gal4AD:B results in the localization of the Gal4 activation domain to the Gal1 promoter and in expression of *HIS3*. Survival of the *his3* auxotrophic yeast strain grown on histidine-deficient plates is dependent upon association of Gal4BD:A and Gal4AD:B. **(B)** The reverse Gal4 two-hybrid system is a genetic selection scheme that selects against the association of c-Rel fused to the DNA-binding domain of Gal4 (Gal4BD:c-Rel) and p40 fused to the activation domain of Gal4 (Gal4AD:p40). Association of Gal4BD:c-Rel and Gal4AD:p40 results in the localization of the Gal4 activation domain to the Gal1 promoter and in expression of *CYH2*. Survival of the CHX-resistant *cyh2* yeast strain grown on plates containing CHX is dependent upon the lack of association of Gal4BD:c-Rel and Gal4AD:p40.

the only way for the yeast cell to survive when grown in the presence of CHX is through the absence of *CYH2* expression, the reverse two-hybrid system could provide a selection scheme to identify mutant proteins that are unable to associate.

Yeast strain CL9 supports the reverse two hybrid system and was developed as described in the Materials and Methods section. To demonstrate that fusion proteins associate in CL9 as in another yeast strain that we have previously used, the c-Rel and p40 fusion proteins were expressed in yeast strain CL9. We have previously shown that high levels of β -galactosidase activity are detected when full-length Gal4BD:c-Rel is expressed with Gal4AD in the GGY:171 strain, presumably due to the ability of the C-terminal transactivation domain of c-Rel to activate transcription of *lacZ* (19,31–34). However, activation of *lacZ* expression by Gal4BD:c-Rel-540, which lacks the C-terminal transactivation domain of c-Rel, requires coexpression with Gal4AD:p40 (19). Therefore, we wanted to demonstrate that coexpression of Gal4BD:c-Rel-540 with Gal4AD:p40 was necessary to activate expression of *lacZ* in the seven JC981-derived *cyh2* strains.

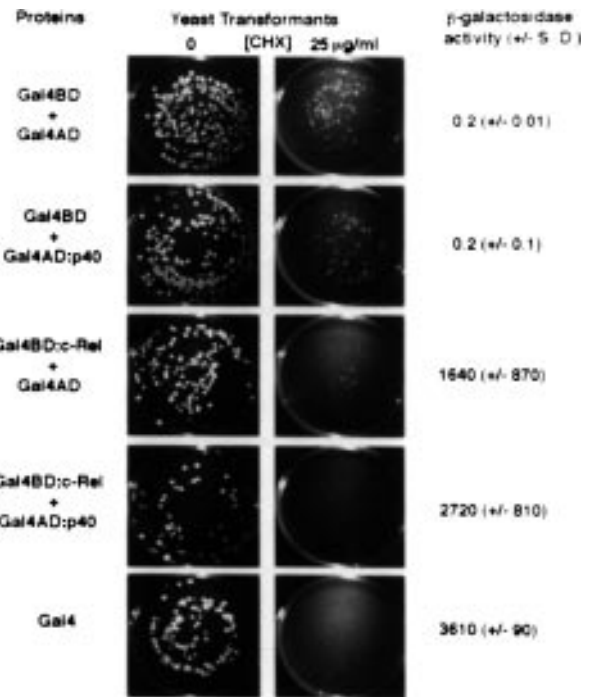


Figure 2. Coexpression of c-Rel and p40 fusion proteins is required to completely inhibit growth in the presence of cycloheximide. The Gal4 protein alone and the indicated combinations of fusion proteins were expressed in the *S.cerevisiae* CHX-resistant *cyh2* yeast strain CL9. Equivalent aliquots of the transformed yeast were plated in the absence and the presence of 25 μ g/ml CHX. Cell lysates obtained from CL9 transformants grown on plates lacking CHX were assayed for levels of β -galactosidase activity by a chemiluminescent assay. β -galactosidase activity is expressed per microgram of protein of yeast lysate. The numbers shown are the average and standard deviations obtained from three independent colonies that were assayed in parallel.

Expression of Gal4BD:c-Rel-540 with Gal4AD and expression of Gal4BD with Gal4AD:p40 did not result in elevated levels of β -galactosidase activity relative to expression of Gal4BD with Gal4AD. Expression of Gal4BD:c-Rel-540 with Gal4AD:p40 resulted in a significant elevation of β -galactosidase activity, while expression of full-length Gal4 resulted in a further elevation of β -galactosidase activity (Figs 2 and 3). Therefore, *lacZ* expression was dependent upon association of the Gal4BD:c-Rel-540 with Gal4AD:p40.

Complete growth inhibition in the reverse two-hybrid system is dependent upon the expression of Gal4AD:p40 with Gal4BD:c-Rel

Any combination of two-hybrid fusion proteins expressed in CL9 resulted in equivalent numbers and sizes of colonies in the absence of CHX (Figs 2, 3, 4 and 5). To determine if the presence of CHX affected colony growth, CL9 was cotransformed with a plasmid expressing Gal4BD and a plasmid expressing Gal4AD. Equivalent aliquots of the transformed yeast were plated in the presence and in the absence of CHX. The presence of CHX did not affect the number of transformants that were obtained, but the overall sizes of the colonies were smaller (Figs 2, 3, 4 and 5).

To determine if the expression of full-length Gal4 was sufficient to confer complete sensitivity to CHX, a plasmid encoding the full-length Gal4 protein was transfected into CL9 and equivalent aliquots of the transformed yeast were plated in the

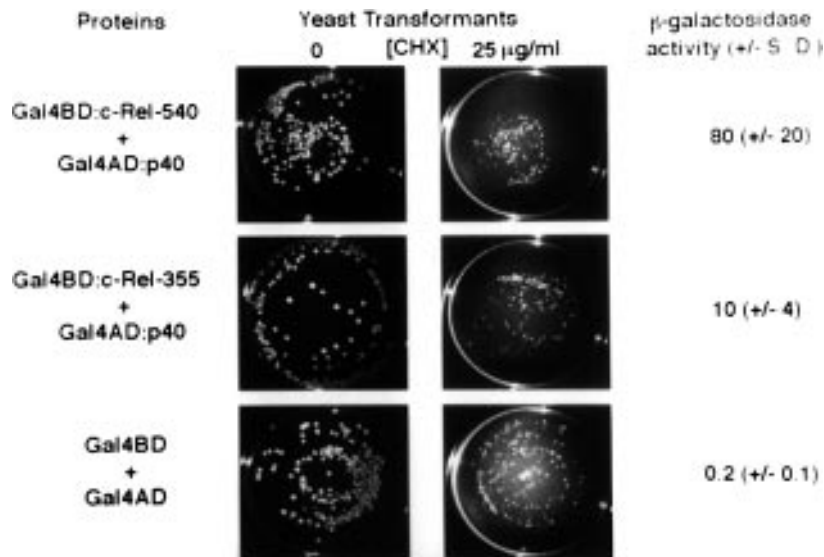


Figure 3. The C-terminal transactivation domain of c-Rel is required for growth inhibition. The indicated combinations of proteins were expressed in the *S.cerevisiae* CHX-resistant *cyh2* yeast strain CL9. Equivalent aliquots of the transformed yeast were plated in the absence and the presence of 25 μ g/ml CHX. Cell lysates obtained from CL9 transformants grown on plates lacking CHX were assayed for levels of β -galactosidase activity by a chemiluminescent assay. β -galactosidase activity is expressed per microgram of protein of yeast lysate. The numbers shown are the average and standard deviations obtained from three independent colonies that were assayed in parallel. The results here and in Figure 4 were obtained from the same experiment.

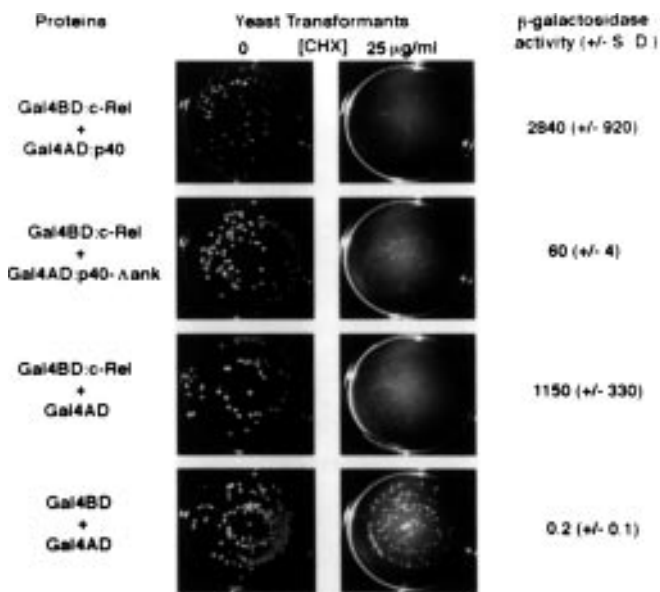


Figure 4. The ankyrin repeat domain of p40 is required for growth inhibition. The indicated combinations of proteins were expressed in the *S.cerevisiae* CHX-resistant *cyh2* yeast strain CL9. Equivalent aliquots of the transformed yeast were plated in the absence and the presence of 25 μ g/ml CHX. Cell lysates obtained from CL9 transformants grown on plates lacking CHX were assayed for levels of β -galactosidase activity by a chemiluminescent assay. β -galactosidase activity is expressed per microgram of protein of yeast lysate. The numbers shown are the average and standard deviations obtained from three independent colonies that were assayed in parallel.

presence and in the absence of CHX. Expression of the full-length Gal4 protein was able to completely inhibit growth on plates containing CHX (Fig. 2). Therefore, expression of the full-length Gal4 protein was able to confer complete sensitivity to CHX.

Next we wanted to determine if CHX sensitivity could be conferred only as the result of the coexpression of Gal4BD:c-Rel with Gal4AD:p40. We first needed to determine if the expression of either Gal4BD:c-Rel or Gal4AD:p40 alone would confer sensitivity to CHX. Therefore, Gal4BD was expressed with Gal4AD:p40 in the yeast strain CL9. Expression of Gal4BD with Gal4AD:p40 in the presence of CHX did not confer sensitivity to CHX as compared with coexpression of Gal4BD with Gal4AD (Fig. 2). Next, Gal4BD:c-Rel was expressed with Gal4AD in CL9. Expression of Gal4BD:c-Rel with Gal4AD in the presence of CHX resulted in partial inhibition of cell growth as determined by a significant reduction in colony size as compared with the size of colonies observed when Gal4BD is expressed with Gal4AD. In contrast, expression of Gal4BD:c-Rel with Gal4AD:p40 in CL9 conferred complete sensitivity to CHX. Therefore, complete growth inhibition is dependent upon the expression of both the Gal4BD:c-Rel and the Gal4AD:p40 fusion proteins.

We next determined whether the manifestation of the CHX-sensitive phenotype in the reverse two-hybrid system correlated with β -galactosidase activity. Consistent with previous data obtained in the strain GGY:171 (19), expression of Gal4BD with Gal4AD:p40 did not result in elevated β -galactosidase activity compared with expression of Gal4BD with Gal4AD (Fig. 2). Expression of Gal4BD:c-Rel with Gal4AD resulted in a 7000-fold increase in β -galactosidase activity, reflecting the potent transactivation domain within the C-terminus of c-Rel (31–34). Coexpression of Gal4BD:c-Rel with Gal4AD:p40 resulted in a further increase in β -galactosidase activity, to ~11 000-fold greater than that obtained from coexpression of Gal4BD with Gal4AD. Expression of the full-length Gal4 protein alone resulted in a 15 000-fold increase in β -galactosidase activity as compared with coexpression of Gal4BD with Gal4AD. Thus, the ability of the full-length Gal4 protein or various combinations of Gal4 fusion proteins to increase β -galactosidase activity correlated with the extent to which the proteins conferred

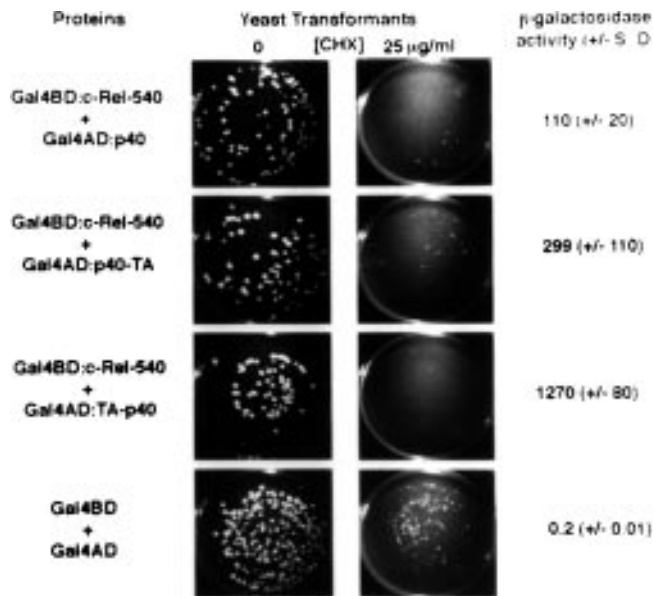


Figure 5. Localization of the C-terminal transactivation domain of c-Rel to the Gal1 promoter in *trans* can confer growth inhibition. The indicated combinations of proteins were expressed in the *S.cerevisiae* CHX-resistant *cyh2* yeast strain CL9. Equivalent aliquots of the transformed yeast were plated in the absence and the presence of 25 μ g/ml CHX. Cell lysates obtained from CL9 transformants grown on plates lacking CHX were assayed for levels of β -galactosidase activity by a chemiluminescent assay. β -galactosidase activity is expressed per microgram of protein of yeast lysate. The numbers shown are the average and standard deviations obtained from three independent colonies that were assayed in parallel. The results here and in Figure 2 were obtained from the same experiment.

sensitivity to CHX. In particular, although expression of full-length Gal4BD:c-Rel with Gal4AD was able to confer partial CHX sensitivity, complete inhibition of cell growth was dependent upon the expression of Gal4BD:c-Rel with Gal4AD:p40. That complete inhibition of cell growth was dependent upon the coexpression of Gal4BD:c-Rel with Gal4AD:p40 suggested that association of c-Rel and p40 was required to confer sensitivity to CHX.

The reverse two-hybrid system can select against p40 proteins that contain an intact ankyrin repeat domain

Three domains within p40 have been defined: (i) an N-terminal domain that is responsible for cytokine-dependent phosphorylation and degradation of p40 (3–10); (ii) a central ankyrin repeat domain that is required for association with and cytoplasmic retention of c-Rel (1,2,19); and (iii) a C-terminal PEST domain that is specifically required for inhibition of DNA-binding (1,2). To determine which domains of Gal4AD:p40 were necessary to confer complete sensitivity to CHX when coexpressed with Gal4BD:c-Rel, the CL9 yeast strain was cotransformed with a plasmid that expressed full-length Gal4BD:c-Rel and with plasmids that expressed various Gal4AD:p40 mutants. Expression of Gal4BD:c-Rel with either a Gal4AD:p40 fusion protein containing a 70 amino acid (aa) N-terminal truncation (Gal4AD:p40- Δ N70, Fig. 6) or with a Gal4AD:p40 fusion protein containing an 18 aa C-terminal truncation (Gal4AD:p40-300, Fig. 6) resulted in complete sensitivity to CHX (data not shown).

Expression of Gal4BD:c-Rel with a Gal4AD:p40 fusion protein containing an internal deletion that removed a portion of its ankyrin repeat domain (Gal4AD:p40- Δ ank, Fig. 6) did not confer complete

sensitivity to CHX. Rather, only a slight reduction in colony size as compared with coexpression of Gal4BD and Gal4AD was observed (Fig. 4). Therefore, the ankyrin repeat domain of p40 is required to confer complete sensitivity to CHX when Gal4AD:p40 and Gal4BD:c-Rel are coexpressed.

Next, we determined if reduced sensitivity to CHX correlated with reduced levels of β -galactosidase activity. Expression of Gal4AD:p40- Δ N70 or Gal4AD:p40-300 with Gal4BD:c-Rel in CL9 resulted in levels of β -galactosidase activity that were equivalent to coexpression of Gal4AD:p40 with Gal4BD:c-Rel (data not shown). However, expression of Gal4AD:p40- Δ ank with Gal4BD:c-Rel resulted in a level of β -galactosidase activity that was ~2% of the level of β -galactosidase activity detected when Gal4BD:c-Rel was expressed with wild-type Gal4AD:p40 (Fig. 4). Thus, the level of β -galactosidase activity and CHX sensitivity correlated with the Gal4AD:p40 mutants that were tested. The inability of Gal4AD:p40- Δ ank to confer sensitivity to CHX when coexpressed with Gal4BD:c-Rel suggests that the reverse two-hybrid system is able to detect mutant proteins that are unable to associate with their partner protein.

The C-terminal transactivation domain of c-Rel is necessary for complete growth inhibition in the reverse two-hybrid system

To determine which domains of Gal4BD:c-Rel were necessary to confer complete sensitivity to CHX when coexpressed with Gal4AD:p40, truncated Gal4BD:c-Rel fusion proteins were expressed with Gal4AD:p40 in strain CL9. Gal4AD:p40 was expressed with Gal4BD:c-Rel-540 and with c-Rel truncated at residue 355 (Gal4BD:c-Rel-355; Fig. 6) in the CL9 yeast strain. Figure 3 shows that expression of Gal4BD:c-Rel-540 with Gal4AD:p40 was not inhibitory towards growth of CL9 on plates containing CHX and that further truncation of the Gal4BD:c-Rel protein to residue 355 slightly increased the size of the viable colonies. Thus, the C-terminal transactivation domain of c-Rel is required for complete growth inhibition in the reverse two-hybrid system.

Truncated Gal4BD:c-Rel proteins expressed with Gal4AD:p40 were assayed for β -galactosidase activity as shown in Figure 3. Truncation of Gal4BD:c-Rel to amino acid 540 resulted in a decrease in β -galactosidase activity to ~3.9% of the β -galactosidase activity detected when full-length Gal4BD:c-Rel was expressed with Gal4AD:p40. Truncation of Gal4BD:c-Rel to amino acid 355 resulted in a reduction of β -galactosidase activity to ~0.35%. Therefore, the level of β -galactosidase activity correlated with the extent of sensitivity to CHX when truncated Gal4BD:c-Rel proteins were expressed with Gal4AD:p40.

Localization of the C-terminal transactivation domain of c-Rel to the Gal1 promoter increases CHX sensitivity in the reverse two-hybrid system

Although expression of full-length Gal4BD:c-Rel with Gal4AD:p40 resulted in complete growth inhibition in the presence of CHX, expression of full-length Gal4BD:c-Rel with Gal4AD resulted in only partial inhibition of growth in the presence of CHX. As expression of Gal4BD:c-Rel-540 with Gal4AD did not confer significant sensitivity to CHX (presumably due to removal of the potent C-terminal transactivation domain of c-Rel), we desired to manipulate the reverse two-hybrid system such that Gal4BD:c-Rel-540 could be used as the 'bait'

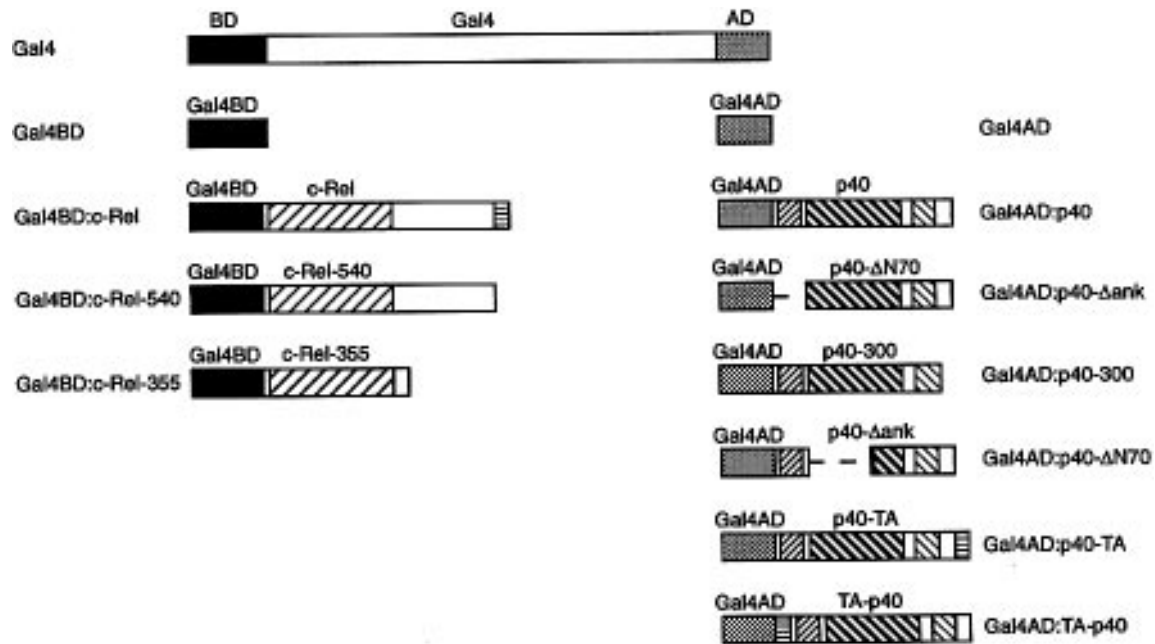


Figure 6. The Gal4, wild-type and mutant c-Rel, and wild-type and mutant p40 proteins. The full-length Gal4 protein is diagramed at the top. The Gal4 DNA-binding domain, Gal4BD () and the Gal4 activation domain, Gal4AD (), are shown directly below full-length Gal4. The full-length and truncated c-Rel proteins were expressed as fusion proteins with Gal4BD and are diagramed directly beneath Gal4BD. The c-Rel protein contains an N-terminal Rel Homology Domain () and a potent C-terminal transactivation domain (). The wild-type and mutant p40 proteins were expressed as fusion proteins with Gal4AD and are diagramed directly beneath Gal4AD. The p40 protein contains an N-terminal regulatory domain (), a central ankyrin repeat domain (), and a C-terminal PEST domain ().

protein. However, expression of Gal4BD:c-Rel-540 with Gal4AD:p40 was not sufficient to confer complete CHX sensitivity. To enhance the manifestation of the CHX-sensitive phenotype when Gal4BD:c-Rel-540 was expressed with Gal4AD:p40, the C-terminal transactivation domain of c-Rel was fused to Gal4AD:p40. The C-terminal transactivation domain of full-length c-Rel was fused to Gal4AD:p40 either at the C-terminus of p40 (Gal4AD:p40-TA; Fig. 6) or at the N terminus of p40 (Gal4AD:TA-p40; Fig. 6) in the expectation that the addition of the c-Rel transactivation domain to the Gal4AD:p40 fusion proteins would increase transcription of *CYH2* upon association with Gal4BD:c-Rel-540.

Coexpression of Gal4BD with either the Gal4AD:p40-TA or the Gal4AD:TA-p40 fusion protein had no effect on either CHX sensitivity or on β -galactosidase activity (data not shown). Coexpression of Gal4BD:c-Rel-540 with Gal4AD:p40-TA did not markedly increase growth inhibition in the presence of CHX as compared with coexpression of Gal4BD:c-Rel-540 with Gal4AD:p40 (Fig. 5). Expression of Gal4BD:c-Rel-540 with Gal4AD:p40-TA resulted in levels of β -galactosidase activity that were ~3-fold greater than the levels of β -galactosidase activity that resulted from the expression of Gal4BD:c-Rel-540 with Gal4AD:p40. However, β -galactosidase activity obtained from coexpression of Gal4BD:c-Rel-540 with Gal4AD:p40-TA was still approximately one-tenth that of β -galactosidase activity obtained from coexpression of Gal4BD:c-Rel with Gal4AD:p40. Therefore, fusion of the C-terminal transactivation domain of Gal4BD:c-Rel to Gal4AD:p40 at the C-terminus of p40 was not sufficient to enhance the manifestation of the CHX-sensitive phenotype and Gal1 promoter-dependent gene expression.

Coexpression of Gal4BD:c-Rel-540 with Gal4AD:TA-p40 resulted in nearly complete sensitivity to CHX as compared with

coexpression of Gal4BD:c-Rel-540 with Gal4AD:p40 (Fig. 5). Furthermore, coexpression of Gal4BD:c-Rel-540 with Gal4AD:TA-p40 resulted in an ~12-fold increase in β -galactosidase activity as compared with coexpression of Gal4BD:c-Rel-540 with Gal4AD:p40 (Fig. 5). The level of β -galactosidase activity obtained from coexpression of Gal4BD:c-Rel-540 with Gal4AD:TA-p40 was approximately half that obtained from coexpression of full-length Gal4BD:c-Rel with Gal4AD:p40 (Fig. 5). Therefore, the C-terminal transactivation domain of Gal4BD:c-Rel fused to the N-terminus of p40 enhances the manifestation of the CHX-sensitive phenotype in the reverse two-hybrid system, presumably due to increased transcription of *CYH2* from the Gal1 promoter.

DISCUSSION

We have developed the reverse two-hybrid system as a genetic scheme to select against protein/protein interactions. The reverse two-hybrid system takes advantage of the ability of the *S.cerevisiae* wild-type *CYH2* gene product to confer sensitivity to cycloheximide in a dominant manner over the mutant *cyh2* gene product which confers resistance to cycloheximide (30). The Gal1 promoter was utilized to drive expression of the *CYH2* gene, such that transcriptional activation of the Gal1 promoter by the full-length Gal4 protein resulted in restoration of complete cycloheximide sensitivity. Complete CHX sensitivity in a CHX-resistant *cyh2* yeast strain was also conferred when the wild-type Gal4BD:c-Rel fusion protein was coexpressed with the wild-type Gal4AD:p40 fusion protein. Expression of a mutant p40 protein in the reverse two-hybrid system that was unable to associate with c-Rel did not confer sensitivity to CHX. Therefore, the reverse

two-hybrid system could provide an effective selection system against specific protein/protein interactions in yeast.

Sensitivity to cycloheximide in the reverse two-hybrid system is presumably due to the formation of a protein/protein complex at the Gal1 promoter. Since the yeast strain that we constructed also contains the *lacZ* gene driven by the Gal1 promoter, we were able to determine whether CHX sensitivity correlated with levels of β -galactosidase activity. The degree of CHX sensitivity correlated with the levels of β -galactosidase activity for each combination of proteins expressed. Although we did not directly measure *CYH2* mRNA expression directly, it is firmly established that association of Gal4 fusion proteins results in the transcriptional transactivation of a gene driven by the Gal1 promoter (23). Thus, it is likely that the cycloheximide sensitivity in the reverse two-hybrid system is a direct consequence of the association of Gal4 fusion proteins at the Gal1 promoter with consequent transcriptional activation of the *CYH2* gene.

Several aspects of our results suggest that a threshold level of transcriptional activation of the *CYH2* gene may be required for complete sensitivity to cycloheximide. First, even though the c-Rel protein contains a potent C-terminal transcriptional activation domain (31–34), expression of full-length Gal4BD:c-Rel with Gal4AD was only able to confer partial sensitivity to CHX. Instead, complete sensitivity to CHX required the coexpression of Gal4AD:p40 fusion proteins that are able to strongly associate with the Gal4BD:c-Rel fusion protein. Most strikingly, while coexpression of Gal4BD:c-Rel-540 with Gal4AD:p40 did not confer complete sensitivity to cycloheximide, coexpression of Gal4BD:c-Rel-540 with Gal4AD:TA-p40 gave nearly complete sensitivity to CHX. Thus, localization of both the C-terminal transactivation domain of c-Rel and the Gal4 activation domain to the Gal1 promoter was necessary for protein/protein interactions to confer complete CHX sensitivity. Taken together, these results suggest that a threshold level of *CYH2* expression is necessary to confer complete sensitivity to cycloheximide. The threshold level of *CYH2* expression necessary to confer complete sensitivity to cycloheximide may be affected by a number of factors, including the strength of a particular protein/protein interaction, growth temperature, and the concentration of cycloheximide in the plates on which the transformants are grown.

As the two-hybrid system has been used to study a wide variety of *in vivo* protein/protein interactions, the reverse two-hybrid system could likewise be suitable to study a wide variety of protein/protein interactions. In the reverse two hybrid system, we demonstrated that the lack of association between two fusion proteins results in the complete lack of cell growth in the presence of CHX. Therefore, the reverse two-hybrid system may be suitable for the rapid screening of randomly generated mutant proteins that are no longer able to associate with their partner protein. Experiments are currently underway to identify mutant c-Rel proteins which can no longer associate with p40. Additionally, the reverse two-hybrid system could potentially be used to identify other proteins which can disrupt a particular protein/protein complex. For example, the reverse two-hybrid system may be particularly suitable for screening expression libraries to identify regulators of protein/protein interactions. Finally, the reverse two hybrid system could be used to screen for drugs that abolish a specific protein/protein interaction. Therefore, the reverse two-hybrid system will likely become a potent tool in the study of protein/protein interactions.

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