Regulated Phosphorylation and Dephosphorylation of GAL4, a Transcriptional Activator

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Regulated phosphorylation and dephosphorylation of GAL4, a transcriptional activator

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In yeast, galactose triggers a rapid GAL4-dependent induction of galactose/melibiose regulon [GAL/MEL] gene transcription, and glucose represses this activation. We discovered that alterations in the physical state of the GAL4 protein correlate with activation and repression of the GAL/MEL genes. Using Western immunoblot assay, we observe two electrophoretic forms of GAL4 protein—GAL4i and GAL4n—in noninduced cells. In the absence of glucose, the addition of galactose to such cells results in the rapid appearance of a third and slower-migrating form, GAL4in, which differs from at least GAL4i by phosphorylation. GAL80-deletion cells that constitutively transcribe galactose-responsive genes due to the lack of the GAL80 protein, an antagonist of the GAL4 protein, exhibit GAL4in without galactose addition. Addition of glucose, which results in rapid repression of galactose gene transcription, triggers a rapid elimination of GAL4in and an increase in GAL4i.

Cycloheximide experiments provide evidence that the galactose- and glucose-triggered GAL4 protein mobility shifts are due to post-translational modification. GAL4in is labeled with [32P]phosphate in vivo; in vivo 35S-labeled GAL4in could be converted by phosphatase treatment in vitro to GAL4i. We present a model proposing that phosphorylation state changes in the GAL4 protein are key to modulating its activity.

[Key Words: GAL4, Saccharomyces cerevisiae, phosphorylation, transcriptional activator, carbon catabolite repression]

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whether by galactose addition or deletion of the GAL80 gene. GAL4m is converted rapidly to a more rapidly migrating form in response to glucose in either the presence or absence of GAL80. We present evidence consonant with the notion that this glucose response is the result of dephosphorylation of GAL4m.

Results

GAL4 protein is altered physically in response to galactose

By immunoblot analysis of GAL4 in extracts of yeast cells, we detect at least three electrophoretic forms of GAL4 protein, which we designate as GAL4i, GAL4n, and GAL4m, with apparent sizes, respectively, of 100, 105, and 108 kD (see below). These forms are detected in both wild-type cells and cells that are amplified for GAL4 protein production.

GAL4i and GAL4n are observed in extracts prepared from SC252 (wild-type) cells grown on the noninducing carbon sources glycerol and lactic acid (gly/lac; Fig. 1B, lanes 1 and 5). Addition of galactose to the culture results in the appearance of GAL4m as well as the disappearance of GAL4n within 30 min (Fig. 1B, lane 6). If galactose remains in the culture for a longer period of time (galactose catabolism being well established; Adams 1972; Broach 1979; Yarger et al. 1984; Torchia and Hopper 1986), we again detect GAL4n (Fig. 1B, lanes 8 and 9, which represent 2 and 4 hr post galactose addition, respectively). Although GAL4m and GAL4n are not clearly resolved in lanes 8 and 9 (Fig. 1B), the increased width of the upper band indicates that both are present.

Deletion of GAL80 allows formation of GAL4m in the absence of galactose

Because deletion of the GAL80 gene (gal80D) results in constitutive activation of GAL/MEL transcription, we compared the electrophoretic profiles of GAL4 protein from strains differing genetically only at the GAL80 locus. Deletion of GAL80 produced a dramatic effect: GAL4m was prominent in extracts from gal80D but not GAL80 cells grown on noninducing gly/lac media (cf. lanes 1 or 5 between Fig. 1B,C). Thus, a form of GAL4, GAL4m, which appears in GAL80 cells only in response to galactose, appears in gal80D cells in the absence of galactose. Clearly then, the GAL80 protein prevents an alteration of the GAL4 protein that normally occurs in response to galactose.

The results illustrated in Figure 1C indicate further that GAL80 is not the only activity involved in modulating the abundance of GAL4m. For example, addition of galactose to the gal80D gly/lac culture resulted in a transient disappearance of GAL4m (Fig. 1C, cf. lanes 6 or 7 with lane 5). GAL4m was reduced to undetectable levels within 30 min after galactose addition, but was visible again within 2 hr (Fig. 1C, cf. lanes 6 or 7 with...
Regulated phosphorylation of GAL4 protein

Both the GAL4i species we detect from yeast and GAL4 protein produced in *E. coli* comigrate (Fig. 3) and exhibit the electrophoretic mobility expected of the full-length GAL4 protein [predicted molecular mass of 99,350 daltons; Laughon and Gesteland 1984]. GAL4ni and GAL4im migrate more slowly than GAL4i (e.g., Figs. 1–3 and 4B).

To determine whether post-translational events might give rise to the multiple electrophoretic forms of GAL4, we employed an inhibitor of protein synthesis. Previous work [Perlman and Hopper 1979] established that inhibition of protein synthesis with cycloheximide does not prevent the normal rapid galactose-triggered appearance of GAL transcripts. Using cycloheximide in a similar protocol here, we observe that both GAL4ni and GAL4im appear to form at the expense of GAL4i upon galactose addition in the absence of protein synthesis [Fig. 3]. In Figure 3, both GAL4i and GAL4ni are observed prior to cycloheximide addition, the GAL4ni band being the more intense of the two [Fig. 3, lanes 1}. By 1 hr in the presence of cycloheximide [Fig. 3, + cycloheximide lane 0], the intensity of the two bands had changed to favor GAL4ni. A similar change was not observed in the control [cf. Fig. 3, lanes 0]. Within 30 min of galactose addition to both control and cycloheximide-containing flasks, a shift in intensity favoring more slowly migrating forms of GAL4 was observed [Fig. 3, lanes 0.5]. In each case, the ‘upward’ intensity shift included an increase in both GAL4ni and GAL4im although GAL4im appears less abundant (and probably for this reason appears less clearly resolved from GAL4ii in the presence of cycloheximide [Fig. 3, + GAL lanes 1]. Addition of glucose to both control and cycloheximide-containing flasks removed GAL4im and increased the intensity of GAL4ni and GAL4i.

**Figure 2.** Immunoblotting analysis of GAL4 protein in GAL80 and gal80D cells grown for several generations in the presence of galactose. Yeast strains SC413 (lane 4D), SC414 (lane 4D), SC252 [lane wt], and SC285 [lane 80D] were grown at least five generations in gly/lac media containing 2% galactose. Extracts were prepared by Method A. Equivalent amounts of Coomassie Blue-staining protein were assayed by immunoblotting. Molecular weights of standards are indicated in kilodaltons. Electrophoretic forms of GAL4 (I, II, III) are indicated in the GAL4 STD lane by arrows.

**Figure 3.** Immunoblotting evidence for post-translational modification of GAL4 protein. ADH1 GAL4 GAL80 yeast strain SC386 was grown in gly/lac media. The culture was sampled (lane −1) and split into two flasks. One flask was supplemented with 100 μg/ml cycloheximide [Perlman and Hopper 1979]. After 1 hr, zero time samples [lane 0] were removed from each flask, galactose was added (+GAL), and incubation continued with samples removed after 0.5 and 1 hr. Immediately after the 1-hr sampling, glucose was added, and final samples [lane +GLC] were removed 30 min later. Whole-cell extracts were prepared [method B], and 40 μg of protein was loaded in each lane. A 0.5-ng amount of GAL4 protein produced in *E. coli* mixed with extract from SC413 [lane gal4D yeast] was included as a standard [lane E. coli GAL4].

*GAL4im is reduced severely in response to glucose catabolism*

Severe glucose repression of GAL/MEL transcription occurs even in the absence of the GAL/MEL-specific negative regulator, GAL80. We reasoned that if GAL4im either expedites or results from the rapid induction phase, its abundance may be reduced by glucose catabolite repression. However, we could not test this notion using glucose-grown strains because we were unable to detect any forms of GAL4 protein in glucose-grown strains unless GAL4 production was amplified [data not shown]. To test for a glucose response, we added glucose to cells in which we could detect GAL4 protein. Glucose addition to wild-type (SC252) cells where GAL4im previously was induced by galactose resulted in a rapid disappearance of GAL4im [Fig. 1B, cf. lane 7 with lanes 10–12; see Fig. 1A for time and carbon source information]. The glucose response was equally dramatic in a gal80D mutant growing in the absence of galactose. GAL4im disappeared within 30 min following glucose addition to gly/lac-grown gal80D cells [Fig. 1C, cf. lanes 2 or 3 with lane 5]. When glucose was added in the presence of galactose, GAL4im did not reappear within 2 hr, kinetics observed for GAL4im reappearance without glucose addition [Fig. 1C, cf. lanes 8 and 12]. Clearly then, the glucose-triggered elimination of GAL4im occurs independently of GAL80, a protein that blocks GAL4im appearance in gly/lac-grown cells.
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Figure 4. Immunoprecipitation and Western blotting analysis of yeast cell extracts labeled in culture with $^{32}$Porthophosphate. Extracts from yeast strains SC413, SC252, and SC340 labeled in culture with $^{32}$Porthophosphate (see Materials and methods) were analyzed by immunoprecipitation (A) and by immunoblotting (B). (A) Autoradiogram of electrophoresed $^{32}$P immunoprecipitates. (Lane 1) Immune serum; (lane P) preimmune serum. Immunoprecipitation reactions were prepared using 77,000 Cerenkov cpm; volumes of primary extracts required were 100, 52, and 49 µl from SC413, SC252, and SC340, respectively. The positions of prestained molecular weight standards are indicated in kilodaltons. The position of $^{32}$P-GAL4 is indicated by the arrow to the right. Exposure was for 24 hr without an intensifying screen. (B) Immunoblotting analysis of $^{32}$P-labeled extract prepared from strain SC340. An 8-µl aliquot of primary extract from $^{32}$P-labeled SC340 was analyzed by immunoblotting (lane 2). A 0.5-ng amount of GAL4 produced in E. coli strain mixed with unlabeled extract from SC413 was included as a standard (lane 1). The positions of electrophoretic forms GAL4III and GAL4I are indicated. GAL4II was not visible under these conditions. A and B are not aligned for molecular mass comparisons.

Phosphorylation accounts for the reduced mobility of GAL4III

Protein phosphorylation provides a reversible means of modulating both conformation and catalytic activity (Sprang et al. 1988), and protein phosphorylation often alters mobility during denaturing SDS–PAGE. For example, phosphorylation of a yeast transcriptional regulator, the heat shock transcription factor (HSF), was shown to reduce its migration during SDS–PAGE, as well as correlate with increased transcription from HSF sensitive promoters (Sorger and Pelham 1988).

We find that GAL4 is phosphorylated in yeast (Fig. 4A). A $^{32}$P-labeled protein band displaying electrophoretic mobility characteristic of GAL4III was detected by immunoprecipitation of extracts from a GAL4-overproducing strain SC340 upon galactose induction in the presence of $^{32}$Porthophosphate. This band is identified as a form of GAL4 protein because it was not detected in isogenic wild-type (SC252) or gal4D (SC413) cells, or with preimmune serum. Under these conditions, levels of GAL4 protein found within wild-type SC252 cells are at least 60-fold lower than in the nearly isogenic GAL4-overproducing strain SC340 (data not shown). Presumably, the lower abundance of GAL4 protein in the wild-type strain coupled with the level of background in the $^{32}$P-labeled immunoprecipitates prevented detection of $^{32}$P-GAL4 in wild-type cells. We were able to detect GAL4 metabolically labeled with $^{35}$Smethionine in galactose-induced SC252 cells upon extended fluorography because of the lower background in those experiments (data not shown).

It should be emphasized that the absence of $^{32}$P-labeled species corresponding to GAL4II and/or GAL4I in the immunoprecipitation experiment shown in Figure 4A does not indicate that these species were not $^{32}$P-labeled in the same experiment. As illustrated in Figure 4B, the relative abundances of GAL4I and GAL4II (not visible) are reduced noticeably in the GAL4 overproducer during galactose induction in the phosphate-limiting media. For this reason, they would not be visible above the background even if they were $^{32}$P-labeled. The lowered relative abundance of GAL4I and GAL4II observed during galactose induction of the GAL4-overproducing strain (SC340) in the phosphate-limiting media used for these experiments is reproducible.

Covalent linkage of the $^{32}$P label to GAL4III, the major GAL4 band detected by immunoprecipitation, was confirmed by two-dimensional paper electrophoresis after limited acid hydrolysis (Fig. 5). Phosphoserine and phosphothreonine, but not phosphotyrosine, were detected.

Figure 5. Phosphoamino acid analysis of $^{32}$P-labeled GAL4 protein. Aliquots of primary extracts from SC413 and SC340 (see Fig. 4) were immunoprecipitated and analyzed by two dimensional paper electrophoresis following excision and partial acid hydrolysis of the major $^{32}$P-GAL4 band. Direction of electrophoresis at the indicated pH is given by the arrow. [Pi] Free label; [pPep] incompletely hydrolyzed peptides; [Orig] origin. Pi and pPep were assigned according to Cooper et al. (1983).
In a parallel control experiment employing the nearly isogenic gal4 deletion strain SC413, extraction and analysis of the corresponding gel slice revealed 50-fold less phosphoamino acids and phosphopeptides [data not shown]. Phosphoamino acid analyses on forms GAL4i and GAL4m could not be done because these forms were not detected in the 32P-labeling experiment [see above].

**Phosphatase treatment increases the electrophoretic mobility of GAL4m**

Phosphatase treatment of 32P- and 35S-labeled immunoprecipitates was carried out to determine whether the labeled band migrating as GAL4m could be dephosphorylated to yield electrophoretic forms GAL4i or GAL4m. Immunoprecipitates of GAL4 from radiolabeled, galactose-induced SC340 were treated with calf intestinal alkaline phosphatase [CIP] in the presence and absence of inorganic phosphate, a phosphatase inhibitor (Fernley 1971). In the absence of inhibitor, CIP treatment increased the mobility of the [35S]GAL4 from the mobility characteristic of GAL4m to that of GAL4i [Fig. 6]. 32P-labeled GAL4m was also sensitive to phosphatase treatment, in that it was no longer visible following phosphatase treatment in the absence of added inhibitor. Whether the 32P-labeled material visible after phosphatase treatment in the absence of inhibitor is physically identical to GAL4i produced in vivo remains unclear. Although phosphatase treatment apparently did remove one or more phosphates altering the electrophoretic mobility of GAL4 protein, it is certainly possible that other phosphorylated residues that do not affect GAL4’s electrophoretic mobility are refractory to CIP treatment. We have not yet succeeded in clearly resolving a labeled species that comigrates with GAL4m by limited phosphatase treatment of GAL4m in vitro. Whether GAL4m is also related to GAL4i by phosphatase-sensitive covalent modification remains to be demonstrated.

**Discussion**

**GAL4m correlates with high-level expression of GAL/MEL genes**

This work reveals a striking correlation between the presence of GAL4m and the expression state of the system. This correlation is realized in terms of GAL80 effects, galactose effects, and the glucose effect.

We find that GAL4m is prominent in gal80D cells grown in gly/lac, but is not detected in GAL80 wild-type cells grown long term on galactose. These cell types and growth conditions have been compared previously for GAL/MEL expression, and it is evident that GAL/MEL expression in gal80D cells grown on gly/lac is twofold higher than that in wild-type GAL80 cells grown long term on galactose (Torchia et al. 1984). An additional example of the correlation in terms of a GAL80 effect is our finding that GAL4m is prominent in gal80D but not GAL80 cells grown long term on galactose media. In comparing the expression levels in gal80D and GAL80 strains grown long term on galactose, Torchia et al. (1984) observed 1.5-fold higher galactokinase [GAL1] and 1.2-fold higher a-galactosidase [MEL1] levels [enzymes and transcripts] in the gal80D cells.

The GAL4m pattern exhibits a remarkable parallel to expression state changes during the early-phase induction response in GAL80 cells. In cells grown on gly/lac the GAL/MEL gene transcripts are at very low levels, but in response to galactose addition the levels increase 100- to 1000-fold [for review, see Johnston 1987] and attain maximal levels within 1–2 hr (Yarger et al. 1984; Torchia and Hopper 1986). We detect only GAL4i and GAL4m in gly/lac-grown, noninduced GAL80 wild-type cells. By 30 min after galactose addition, GAL4i has been replaced by GAL4m. However, within 2–4 hr of galactose addition GAL4m reappears, and some time after 4 hr GAL4m begins to disappear and is no longer detected in GAL80 cells grown long term on galactose. But even this post-induction disappearance of GAL4m appears to correlate with an expression state change. The GAL1 transcript reaches a maximal level by 1 hr after galactose addition and decreases to about 75% of the maximal level within 3 hr after galactose addition (Yarger 1981).

The occurrence of GAL4m associated with gal80D cells grown in either gly/lac or long term on galactose, as well as its appearance in GAL80 cells in response to galactose, is suppressed dramatically in response to glucose. The disappearance of GAL4m is correlated with the transition to the glucose-repressed state. That glucose addi-
tion rapidly and severely reduces synthesis of the GAL/MEL regulon enzymes was well documented some time ago (Adams 1972; Kew 1974). By recent quantitative experiments employing a GAL10-LacZ fusion construct, Ma and Botstein (1986) demonstrated a 50-fold decrease in LacZ expression in response to a shift from galactose to galactose plus glucose. Our experiments using shifts from galactose to galactose plus glucose show that GAL4 remains intact within 30 min after the addition of glucose to either 1-hr galactose-induced wild-type cells or gal80D cells growing in gly/lac.

Finally, the transient disappearance of GAL4 that we observe upon galactose addition to gal80D cells grown in gly/lac may strengthen this correlation further. This transient disappearance most likely reflects transient self carbon catabolite repression, a phenomenon well documented in bacteria (Magasanik 1961), because we do not observe the transient disappearance of GAL4 in gal80D gal1D gal7D gal10D cells, which lack galactose catabolic enzymes.

On the basis of striking consistency in the occurrence of GAL4 relative to the expression state of the system, we propose that the presence of GAL4 is correlated with maximal or nearly maximal GAL4-dependent transcription.

GAL4 is a phosphoprotein and arises at the expense of GAL4.

In response to galactose, GAL4 appears to form post-translationally, either directly or indirectly at the expense of GAL4. From the experiments reported here it is unclear whether or not GAL4 is an intermediate leading from GAL4 to GAL4. The event producing electrophoretic-form GAL4 is most likely phosphorylation because the GAL4 protein can be labeled in vivo with 32P, and immunoprecipitated, 35S-labeled GAL4 is converted by in vitro phosphatase treatment to a faster electrophoretic form migrating with GAL4. On the basis of these results and the results presented above, we propose that the transcriptional activator GAL4 undergoes carbon-responsive and GAL80-responsive phosphorylation and dephosphorylation in vivo.

Glucose triggers alteration of the GAL4 protein

Our results provide direct evidence for the physical change of a transcriptional activator protein brought about by carbon catabolite or glucose repression. We show here that glucose addition rapidly alters GAL4 protein in vivo in a manner consistent with dephosphorylation. The effect does not require GAL80 protein and therefore most likely represents at least one point at which the GAL4 protein is directly affected by glucose repression control.

Interestingly, our results differ from those of Cherry et al. (1989), who present indirect evidence that glucose triggers a phosphorylation inactivation of ADR1, a transcriptional activator of the yeast ADH2 gene. In the case of ADR1, phosphorylation is proposed to occur in response to the presence of glucose, the opposite of what we propose for GAL4. Examination of the predicted amino acid sequences of the ADR1 and GAL4 proteins provides yet another contrast. ADR1 contains a consensus phosphorylation site for cAMP-dependent protein kinase that appears important for proper regulation of ADR1-dependent transcription (Cherry et al. 1989). Our analysis of the published GAL4 sequence does not reveal such a motif. Instead, GAL4 appears to contain multiple sequences that might serve as phosphorylation sites for cAMP-independent casein kinases I and/or II (Hathaway and Traugh 1982; Edelman et al. 1987).

In light of our results, we note that several potential phosphate acceptors occur within a segment of the GAL4 protein that is important for transcriptional activation [activation region II; Ma and Ptashne 1987a], and within the carboxy-terminal 30 amino acids required for repression by GAL80 protein [Johnston et al. 1987; Ma and Ptashne 1987b]. These candidate sites are currently under investigation.

Proposed model: regulated phosphorylation modulates GAL4 activity

Overall, our data provide evidence for post-translational changes in the physical state of the GAL4 protein that occur in response to conditions known to affect GAL4 protein-dependent transcription. The different physical states of GAL4 protein indicated by the multiple electrophoretic forms observed most likely represent GAL4 species of differing activity. We favor a model (Fig. 7) in which a phosphorylation event leading to GAL4 for-
readily phosphorylated. It also remains to be determined which GAL4-specific functions are affected by post-translational modification (DNA binding or activation) and what protein modifies GAL4.

Materials and methods

Yeast strains and growth media

Genetic variants of yeast strain SC252 [Sp21R; Johnston and Hopper 1982] were created by lithium acetate transformation [Ito et al. 1983] using linearized plasmid DNA [Rothstein 1983]. Strain SC285 was constructed by replacement of the wild-type GAL80 locus with a gal80 construct lacking an internal 0.6-kb BglII fragment. Yepl3 was used as a cotransforming vector [Braoch et al. 1979]. LEU4 transformatants were screened for constitutive MELI expression by a chromogenic overlay assay [Post-Beittenmiller et al. 1984], and the deletion was confirmed by Southern analysis [Southern 1975] of EcoRI-cut genomic DNA [T. Torchia and L. Mylin, unpubl.].

Yeast strain SC386 (TDD16-1C), a generous gift of T. Torchia, was obtained by sporulation [Sherman et al. 1986] of diploid 4063-2 (Torchia and Hopper 1986). SC386 is GAL4 GAL80 gal(1,10,7)/ (ADH1-GAL4-Ura3). The integrated ADH1-GAL4 construct was described previously [Johnston et al. 1986; Baker et al. 1987], the radiation-induced deletion allele of the GAL1 GAL10 GAL7 cluster, gal(1,10,7)/, was isolated by D. Hawthorne and has been characterized by St. John and Davis (1981).

Strains SC413 (gal4 deletion in SC252) and SC340 (SC252 containing an integrated construct in which the GALA-coding region is fused to the GAL10 promoter, and overproduces GAL4 protein upon galactose addition) have been described elsewhere [Schultz et al. 1987; Mylin et al. 1989]. GAL4 was disrupted in SC285 with LEU2 as described [Mylin et al. 1989], producing strain SC414.

Yeast strains were maintained on YEPD liquid media (Torchia and Hopper 1986). The 5× succinate/NaOH-buffered synthetic liquid media used for most experiments was essentially as described [Mylin et al. 1989], except that 0.05% dextrose was omitted from noninducing gly/lac media, and leucine was included at 300 mg/liter for synthetic complete media. Unless otherwise indicated, experiments were initiated using gly/lac and c/lac cultures (ODx = 0.15–0.3) shaken at 30°C. Glucose (GLC) or galactose (GAL) were added as concentrated stock solutions to final concentrations of 2% (wt/vol), as indicated in the figure legends. Metabolic labeling with 35S-methionine (Amersham, SI:1015) was performed in the same media lacking methionine after galactose addition.

Cells were labeled with 32Porthophosphate (JCN, 64014) in phosphate-adjusted, citrate-buffered, synthetic complete liquid media [Toh-E et al. 1973] supplemented with 1/5× phosphate-depleted YEP [Bostian et al. 1980], 3% glycerol, and 2% ethanol. Cultures were incubated with the isotope (1 mCi/ml culture) for 2 hr in this media, followed by addition of 2% galactose for another 2 hr to induce GAL4 protein overproduction.

Preparation of yeast extracts and immunoblotting

Yeast extracts used for immunoblot analysis were prepared essentially as described [Schultz et al. 1987], except that 20 µg/ml aprotinin (Sigma) was included in breaking buffers (method A). Alternatively, unfractionated cell homogenates were solubilized by addition of 5× electrophoresis sample buffer (Schultz et al. 1987) directly to the cell homogenates prior to separation from the glass beads, heated, and clarified by centrifugation (method B).

Immunoblotting analysis was performed as described [Mylin et al. 1989], except that electrophoresis proceeded for 3 hr, blots were incubated overnight at 4°C in blocking solution, and incubations with primary and secondary antibodies were increased to 4 and 2 hr, respectively. Extracts prepared from a yeast strain displaying electrophoretic forms GAL4, GAL4p, and GAL4m were included as standards on most blots. GAL4 protein produced in an E. coli strain [Johnston et al. 1986; Mylin et al. 1989] was mixed with extract from SC413 for use on some blots. Protein estimations were performed as described (Schultz et al. 1987). Prestained molecular weight standards (~Highs; Bethesda Research Laboratories) were included on all gels; the molecular masses indicated are the apparent sizes given by the supplier.

Preparation and immunoprecipitation of metabolically labeled yeast extracts

Yeast strains were labeled in culture as described above. Cells were separated from culture media by centrifugation after chilling on ice, resuspended and pelleted twice in ice-cold water, and stored as pellets at –70°C after freezing on dry ice. Cell homogenates were prepared by intermittent vortexing of the cell pellets with 0.5 ml of 0.45-mm glass beads and 0.3 ml of buffer A [50 mM NaPO4, pH 7.2], 5 mM EDTA, 1 mM DTT, 1 mM PMFSF, 0.2 mM Na3VO4, 50 mM NaF, 2 µM pepstatin A, 0.6 µM leupeptin, and 20 µg/ml aprotinin]. Primary extracts were prepared from unfractionated homogenates by addition of 0.3 ml of buffer B [buffer A containing 2% SDS], heated at 100°C for 5 min with intermittent vortexing, and clarified by centrifugation at room temperature at 50,000g for 10 min.

Reaction mixtures for immunoprecipitation were prepared as follows using aliquots of extracts containing equal amounts of total trichloroacetic acid (TCA)-precipitable radioactivity. Each tube received a 0.1-ml total of labeled primary extract and combined buffers A and B, 0.9 ml of buffer C [50 mM NaPO4 (pH 7.2), 130 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1.1 mg/ml of bovine serum albumin, and 10 mM EDTA], and 8 µl of either preimmune or anti-GAL4 serum [Mylin et al. 1989]. Tubes were agitated gently at 4°C for 2 hr. A total of 20 µl of Pansorbin [Calbiochem] was then added, and agitation continued for 2 hr. Immunoprecipitates were collected by centrifugation at 10,000g for 5 min at 4°C, washed twice with resuspension in buffer C containing 0.2% SDS (C-0.2%) and pelleting through C-0.2% containing 1 M sucrose, washed once with resuspension in C-0.2%, rinsed without resuspension with cold water, solubilized by heating in 80 µl of 1× electrophoresis sample buffer for 7 min at 100°C, and clarified by centrifugation at 10,000g for 5 min. Then 10-µl aliquots were electrophoresed as described above. Gels were fixed in 50% methanol, 10% acetic acid for 2 hr, followed by 10% methanol, 7% acetic acid for 2 hr, rinsed with water, dried, and exposed to Kodak XAR5 film. Gels containing 35S-labeled proteins additionally were impregnated with Autorfluor [National Diagnostics] prior to drying.

Phosphoamino acid analysis

Solubilized immunoprecipitates were fractionated on preparative mini slab gels. 32P|GAL4 protein was located by autoradiography, excised (as well as the corresponding region from a gal4D control gel), electroeluted using an Elutrap (Schleicher & Schuell), concentrated by precipitation with TCA, and rinsed with ethanol and diethyl ether. Precipitates were solubilized by
addition of 15 µl of 0.1 N NaOH followed by 0.6 ml of constant boiling HCl (Pierce), heated at 110°C for 2 hr, and dried under vacuum. Next, 100 µl of pH 1.9 electrophoresis buffer containing 5 µg each of unlabeled phosphoserine, phosphothreonine, and phosphoarginine (pSer, pThr, and pTyr, respectively) were added to each hydrolysate. The majority of the 32P radioactivity was solubilized after extended vortexing at room temperature. An additional 10 µg of each unlabeled phosphoamino acid standard was added to the samples, which then were spotted on individual 20 x 20-cm sheets of Whatman 3MM paper. Flatbed electrophoresis was performed in the first dimension at pH 1.9 for 50 min (3750 volts), and in the second at pH 3.5 for 20 min as described [Cooper et al. 1983]. The positions of unlabeled standards were determined with ninhydrin before exposing the papers to X-ray film with intensifying screens for 2 weeks at -70°C.

Alkaline phosphatase treatment of immunoprecipitates

Immunoprecipitates [see above] were solubilized in 1 x electrophoresis sample buffer, clarified by brief centrifugation, and aliquots diluted 10-fold into 20 µl phosphatase reaction mixtures. Phosphatase reaction mixtures contained 50 mM Tris (pH 8.0), 0.1 mM EDTA, 1 mM DTT, 1 mM PMSE, 20 µg/ml Aprotinin, 0.6 µM leupeptin, 2 µM pepstatin A, 1 mM MgCl2, and 4 units of calf intestinal alkaline phosphatase [Boehringer–Mannheim, 713 023] in addition to components supplied by the now 10-fold diluted electrophoresis sample buffer. Sodium phosphate 10 mM was included as indicated. Incubations were preformed at 37°C for 1 hr, followed by the addition of 0.25 volumes of fresh electrophoresis sample buffer, heating, and electrophoresis.

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