Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 10510–10514, December 1991
Genetics

GAL4 is phosphorylated as a consequence of transcriptional activation

(yeast activators/GAL4 mutants/protein kinases)

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ABSTRACT **GAL4 protein isolated from yeast in which it is active is phosphorylated predominantly on two different serine residues. One of these was identified as Ser-837; substitution of this residue for alanine has no detectable effect on transcriptional activation by GAL4. Phosphorylation at Ser-837 requires that both the DNA binding and transcriptional activation functions be intact. We propose that some phosphorylations of GAL4, including that at Ser-837, occur concomitantly with activation of transcription.**

The yeast transcriptional activator GAL4 controls expression of the genes required for galactose catabolism (for review, see ref. 1). Several mechanisms regulate the activity of GAL4 in response to growth media carbon sources. In the absence of galactose, transcriptional activation by DNA-bound GAL4 is inhibited by interaction with the regulatory protein GAL80. Inhibition is relieved upon galactose addition; a metabolite of galactose, produced by the GAL3 gene product, is thought to act as a regulatory ligand for GAL80. In addition, GAL4-regulated genes are repressed by several mechanisms promoting the preferential use of glucose (2).

Previous experiments have identified several functional domains of GAL4 (ref. 3; see Fig. 4*B*). Residues 1–147 contain a zinc-requiring DNA-binding motif and a region mediating dimer formation (4, 5). Either of two acidic regions, termed activating regions I and II (residues 148–238 and 768–881, respectively), can activate transcription when fused independently to the DNA-binding domain (3, 6). Lying between the two activating domains is a central region (CR; residues 239–767) for which a function has not yet been identified; incidental to our major conclusions, we show here that CR inhibits activity of GAL4 when only one of the activating domains of GAL4 is present.

It has been suggested that phosphorylation of GAL4 may be required for transcriptional activation (7). GAL4 phosphorylation is known to correlate with its ability to activate transcription. Thus, GAL4 protein inhibited by GAL80 is unphosphorylated, but it becomes rapidly phosphorylated upon induction with galactose. In gal80$^-$ cells, GAL4 is phosphorylated in the absence of galactose (8). Several GAL4 missense mutants that are impaired for transcriptional activation were found to be unphosphorylated; pseudorevertants of these mutants restored phosphorylation (7).

In this report, we present evidence suggesting that several of the phosphorylations of GAL4 occur as a consequence of, but are not required for, transcriptional activation. We find that these phosphorylations are dependent on both a DNA-binding and a transcriptional activation function, and at least one is unnecessary for activity. We propose that GAL4 is phosphorylated during interaction with the transcriptional initiation complex.

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MATERIALS AND METHODS

Plasmids, GAL4 Mutants, and Yeast Strains. Yeast strain YT6::171 (*MATα, gal4, gal80, ura3, his3, ade2, ade1, lys2, trp1, ara1, leu2, met, URA3::GAL1–LacZ*) was used for most experiments (9). YT6G80::171 is YT6::171 with *GAL80* integrated at *LEU2* using the vector YIplac128 (10). Most of the GAL4 deletion derivatives have been described (3). The GAL4-(1–147 + 239–881) mutant has a deletion from the *Xba* I to the *Cla* I site; GAL4-(1–147 + 239–848) was derived from this construct by insertion of an *Xba* I linker at the *Mlu* I site. Plasmids for expression of GAL4 were as follows: pMA210 (3); pDN3, which expresses GAL4 from the ADH1 promoter on YEplac112 (10); pMH76 (provided by M. Hollis, ICI Biotech), which is an ARS–CEN vector with GAL4 expressed from the ADH1 promoter; and YCpG4, which is YCplac22 (10) with a *Bam*HI/*Hin*dIII insert containing the GAL4 promoter and coding sequence. Oligonucleotide-directed point mutants were created as described by Kunkel (11). Oligonucleotide mutants in which threonine was changed to alanine, in activating region II, were at the following residues: 784, GCT; 786, GCT; 796–797, GCAGCT; 799, GCA; 823, GCG; 841, GCG; 844, GCT.

Metabolic Labeling, Immunoprecipitations, and β-Galactosidase Assays. Labeling with [^{35}S]methionine and immunoprecipitation were as described (12), except that all of the buffers were supplemented with phosphatase and protease inhibitors [5 mM NaF/2 mM $ZnCl_2$/aprotinin (10 μg/ml)/1 mM leupeptin/10 mM phenylmethylsulfonyl fluoride]. For phosphatase treatment, immune complexes were incubated with 0.5 unit of potato acid phosphatase (Boehringer Mannheim) at 37°C for 20 min in 50 mM Pipes buffer (pH 6.5). Cultures (10 ml) for metabolic labeling with [^{32}P]orthophosphate were starved 3 hr in phosphate-depleted medium prepared as described (13), washed three times in 150 mM NaCl/10 mM Tris·HCl, pH 7.0, and resuspended in 100 μl of the same mixture. [^{32}P]Orthophosphate (1–10 mCi; 1 Ci = 37 GBq; ICN) was added, and the cells were labeled at room temperature for 40 min. Acid hydrolysis of labeled proteins and phosphoamino acid analysis were as detailed (14). Transcriptional activation by GAL4 was determined by measuring β-galactosidase activity in yeast extracts, as described by Himmelfarb *et al.* (9).

RESULTS

Transcriptionally Active GAL4 Is Phosphorylated on at Least Two Serine Residues. Fig. 1 shows that wild-type GAL4, isolated from yeast in which it is active, migrates on SDS/polyacrylamide gels as multiple species, designated a, b, and c. Treatment with phosphatase converts forms b and c into form a (lanes WT), suggesting that forms b and c are

Abbreviation: CR, central region.

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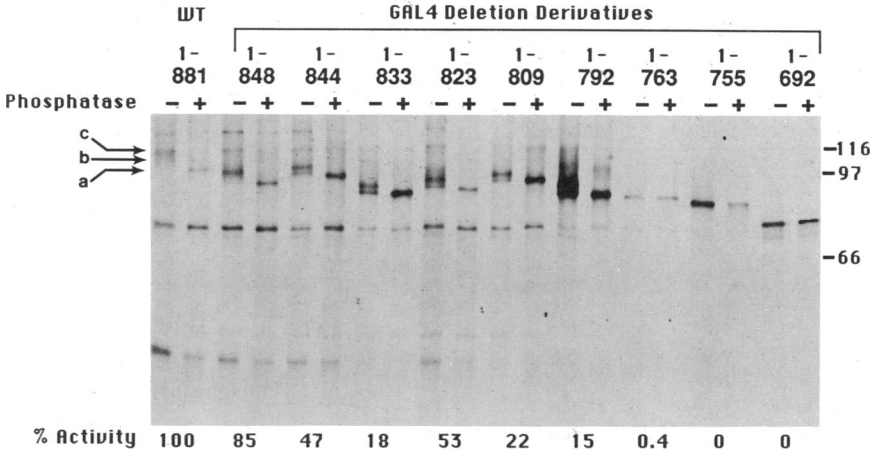

FIG. 1. Phosphorylation of wild-type GAL4 and C-terminal deletion derivatives. [^{35}S]Methionine-labeled wild-type GAL4-(1–881), or GAL4 C-terminal deletion derivatives bearing the indicated residues (3), were immunoprecipitated from gal80$^-$ yeast. One half of each sample was treated with phosphatase (lanes +), and the remainder was left untreated (lanes −). The proteins were resolved on an SDS/7.5% polyacrylamide gel and visualized by autoradiography. Relative transcriptional activation of a *GAL1–LacZ* reporter gene, as a percentage of wild-type GAL4, is shown below the lanes for each derivative. Migration of molecular mass standards is shown on the right (kDa). Three species of wild-type GAL4, designated a, b, and c, are indicated on the left.

produced by phosphorylation of form a. We observe, as have others (7, 8), that the slower migrating phosphorylated species cannot be detected in GAL80$^+$ yeast grown in glycerol (uninduced conditions) but are detectable within 2 hr after galactose addition. In gal80$^-$ yeast, GAL4 forms b and c can be detected even in the absence of galactose (data not shown; see also ref. 8). For reasons we do not yet understand, we find variability in detection of form b in our labeling experiments (compare lanes WT in Fig. 1 to lanes WT in Figs. 2*B* and 3*A*). In contrast, form c is easily detected and its presence correlates with GAL4 activity. Several experiments suggest that forms b and c are produced by separate single phosphorylations. In pulse–chase experiments, form c appears to be produced directly from form a and, furthermore, shortened phosphatase treatment of GAL4 directly converts form c into form a (data not shown). Phosphoamino acid analysis of wild-type GAL4 yielded only phosphoserine (Fig. 2*A*). We conclude that transcriptionally active GAL4 is phosphorylated on at least two serine residues, which cause alterations in electrophoretic mobility.

Phosphorylation of Ser-837 Is Required for Production of Form c. To locate these modifications, we examined a series of C-terminal GAL4 deletion derivatives (3). Fig. 1 shows that deletion of GAL4's activating region II (residues 764–881) eliminates all of the slower migrating forms. Thus, GAL4-(1–763) and the two smaller derivatives shown in Fig. 1 migrate as single species that are unaffected by phosphatase treatment. In contrast, GAL4-(1–792) and all larger C-terminal deletion derivatives, all bearing portions of activating region II, have at least one of the phosphorylated species. We then changed, either individually or in pairs, each of the 14 serine residues to alanine within activating region II of full-length GAL4 (Table 1). Each of these mutant proteins was analyzed by metabolic labeling and immunoprecipitation (Fig. 2*B*). We found that only one of these mutations altered the mobility of GAL4 on SDS/PAGE; the Ser-837 → alanine mutant produced form b but not form c. We conclude that Ser-837 is a major site of GAL4 phosphorylation necessary for production of form c.

Two additional experiments suggest that Ser-837 is the only site of phosphorylation within activating region II that produces an alteration in electrophoretic mobility. First, starting with the Ala-837 mutant, each of the remaining 13 serines in activating region II was mutated to alanine to produce a series of double and triple mutants, each bearing Ala-837. Each of these mutants migrated on SDS gels and responded to phosphatase identically as did GAL4 Ala-837 (data not

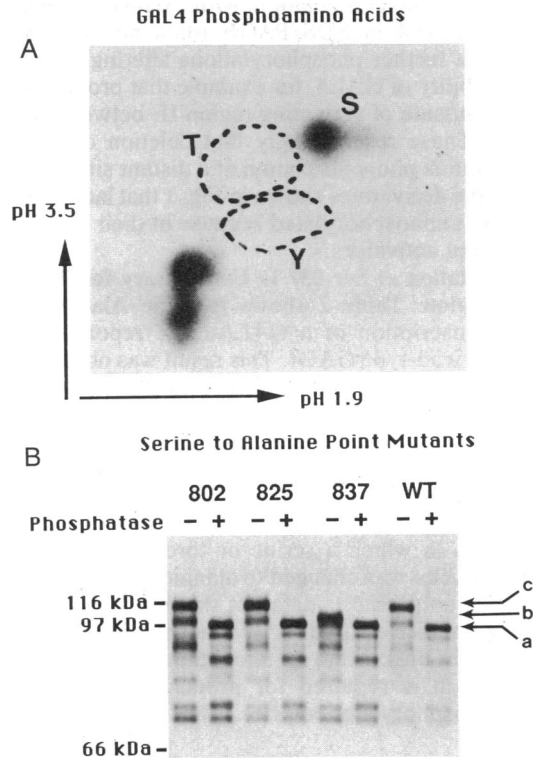

FIG. 2. Ser-837 is a major site of GAL4 phosphorylation. (*A*) Phosphoamino acid analysis of wild-type GAL4. Yeast (gal80$^-$) expressing wild-type GAL4 were metabolically labeled with [^{32}P]orthophosphate, and GAL4 protein was isolated by immunoprecipitation and purification on an SDS/polyacrylamide gel. Acid-hydrolyzed samples were mixed with phosphoamino acid standards and resolved by two-dimensional electrophoresis at pH 1.9 and pH 3.5. Migration of the standards is indicated as follows: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. (*B*) Ser-837 is required for production of GAL4 form c. Representative immunoprecipitations of the serine to alanine point mutants described in Table 1 are shown. Lanes: 802, GAL4 Ser-802 to alanine; 825, Ser-825 to alanine; 837, Ser-837 to alanine; WT, wild-type GAL4. Migration of the three species of GAL4, designated a, b, and c, is indicated on the right.

Table 1. GAL4 activity is unaffected by a major phosphorylation in activating region II: Serine to alanine point substitutions created in activating region II

Serine residue(s)	Mutation	Altered mobility?	Activity affected?
762	GCT	No	No
773	GCT	No	No
779, 780	GCCGCA	No	No
782	GCC	No	No
788, 789	GCTGCC	No	No
801	GCT	No	No
805	GCA	No	No
810, 811	GCCGCT	No	No
825	GCT	No	No
833	GCA	No	No
837	GCA	Yes	No

Eleven oligonucleotides were used to create alanine point substitutions of every serine residue within GAL4 activating region II. The mutants were expressed in yeast and assayed for alteration in electrophoretic mobility; a sample of these is shown in Fig. 2*B*. Transcriptional activation of a *GAL1–LacZ* reporter gene was measured for each mutant.

shown). Second, although we have only detected phosphoserine on GAL4, we mutated each of the threonines between residues 762 and 848 and found that none of these changes, either alone or in combination with Ala-837, altered the mobility of GAL4 in SDS/PAGE (data not shown). We conclude that further phosphorylations altering the electrophoretic mobility of GAL4, for example that producing form b, must lie outside of activating region II, between residues 1 and 762. These results imply that deletion of activating region II inhibits phosphorylation at a distant site. We argue below that the derivatives shown in Fig. 1 that lack activating region II are unphosphorylated because of their inactivity as transcriptional activators.

Phosphorylation at Ser-837 Is Unnecessary for Transcriptional Activation. Table 2 shows that the Ala-837 mutant activates transcription of a *GAL1–LacZ* reporter gene as efficiently as wild-type GAL4. This result was observed both in a gal80$^-$ strain grown on glycerol or galactose and in a GAL80$^+$ strain induced with galactose. Consistent with these results, GAL80$^+$ yeast strains expressing GAL4 Ala-837 from its natural promoter, on plasmid YCpG4, grow as well as do those expressing wild-type GAL4, with galactose as the sole carbon source (data not shown). Furthermore, none of the mutations in which a serine or threonine in activating region II of GAL4 was changed to alanine affected transcriptional activation (Table 1; data not shown).

DNA Binding Is Required for Production of GAL4 Form c. Experiments in Figs. 3 and 4*A* show that a functional DNA-binding domain is required for production of the slower migrating phosphorylated species. Thus, a deletion derivative of GAL4 lacking residues 1–74 [GAL4-(75–881); Fig. 4*A*], which cannot bind to DNA, lacks both phosphorylated species. Moreover, GAL4 derivatives bearing point mutations in the zinc-requiring DNA-binding motif (15) lack phosphorylated species c and yield only traces of form b (Fig. 3*A*). The DNA-binding defect of one of these mutants, Pro-26 to leucine, is partially corrected by high concentrations of zinc (16). Fig. 3*B* shows that when cells containing this mutant are grown in high levels of zinc, the phosphorylated form c of GAL4 can be detected. These DNA-binding-defective mutants appear to be more unstable, relative to wild-type GAL4, as immunoprecipitates of these mutants usually contain more degraded GAL4 protein (Fig. 3; data not shown).

Table 2. GAL4 activity is unaffected by a major phosphorylation in activating region II: Phosphorylation at Ser-837 is not required for GAL4 activity

	gal80$^-$		GAL80$^+$	
	Gly	Gal	Gly	Gal
GAL4	918	1123	16	289
GAL4 A-837	886	1020	14	273

Units of β-galactosidase activity induced by wild-type GAL4 and the Ala-837 mutant (A-837). Yeast strains YT6::171 (gal80$^-$) and YT6G80::171 (GAL80$^+$), bearing plasmid pMH76 expressing the GAL4 derivatives, were grown in glycerol (Gly) or were induced 2 hr with galactose (Gal) before assaying for β-galactosidase activity. Data are an average of three samples; the standard error varied between 10% and 15%.

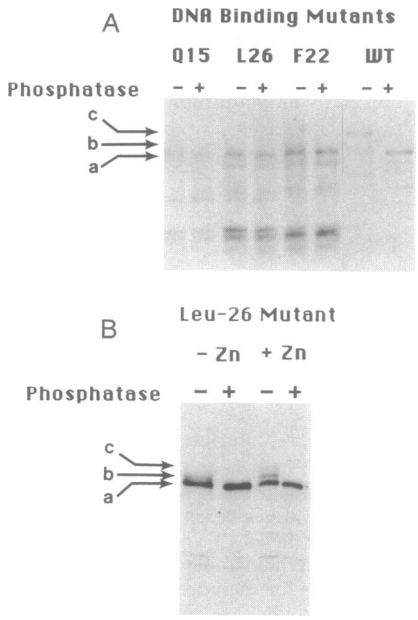

FIG. 3. DNA binding is required for production of the major GAL4 phosphorylated species. (*A*) Mutations in the zinc-requiring DNA-binding motif inhibit GAL4 phosphorylation. Shown are immunoprecipitates of full-length GAL4 derivatives with the following point mutations: Q15, Arg-15 to Gln; L26, Pro-26 to Leu; F22, Ser-22 to Phe; WT, wild-type GAL4. Samples were either treated with phosphatase (lanes +) or were left untreated (lanes −) before electrophoresis. Migration of species a, b, and c of wild-type GAL4 is indicated on the left. (*B*) High zinc concentrations partially restore normal phosphorylation of the Leu-26 mutant. Yeast expressing the Leu-26 mutant were grown and labeled without zinc (− Zn) or in the presence of 25 mM zinc sulfate (+ Zn). Immunoprecipitates were treated with phosphatase (lanes +) or were left untreated (lanes −) before resolving on 7.5% polyacrylamide gels.

Transcriptional Activation Function of GAL4 Is Required for Production of the Slower Migrating Phosphorylated Forms. Fig. 4*A* shows that GAL4 derivatives capable of efficient DNA binding require transcriptional activation function for production of the phosphorylated species. Consider first the Ser-837 phosphorylation required for production of form c. GAL4 deletion derivatives only become phosphorylated at Ser-837 if they can activate transcription. For example, GAL4-(1–147) fused to activating region II (1–147 + 763–881) or to both regions I and II (1–238 + 763–881) efficiently activate transcription and also produce phosphorylated species. For both of these derivatives, and for GAL4-(1–238 + 768–848), changing Ser-837 to alanine eliminates the phosphorylated species (data not shown). In contrast, we find that inactive deletion derivatives, despite bearing Ser-837, are unphosphorylated. Thus, GAL4-(1–147 + 768–848) and a derivative lacking activating region I, GAL4-(1–147 + 239–

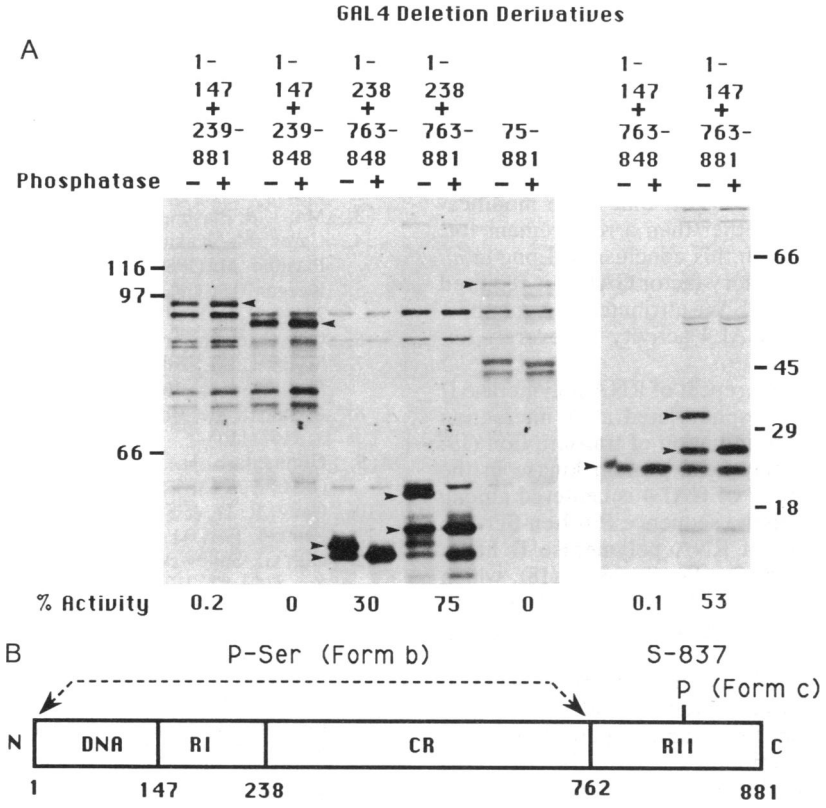

FIG. 4. Transcriptional activation is required for production of the phosphorylated GAL4 species. (*A*) Shown are immunoprecipitates of GAL4 deletion derivatives bearing the indicated residues, resolved on an SDS/7.5% polyacrylamide gel (*Left*), or an SDS/10% polyacrylamide gel (*Right*). Bands representing GAL4 derivatives, and their phosphorylated species, are indicated with arrowheads. For each phosphorylated derivative shown in this figure, changing Ser-837 to alanine eliminates the slower migrating phosphorylated form, although in these instances we observe minor phosphorylated species (data not shown). Relative transcriptional activation of a *GAL1–LacZ* reporter gene by each derivative is shown below the lanes. Migration of molecular mass standards (kDa) is indicated. (*B*) Functional domains identified by deletion analysis (3) are shown: DNA, DNA-binding domain; RI, activating region I; RII, activating region II. A major phosphorylation at Ser-837 is required for the presence of GAL4 form c; a second phosphorylation producing form b lies outside of activating region II between residues 1 and 762.

881), are both very weak activators and, although bearing Ser-837, are unphosphorylated (Fig. 4).

The fact that both GAL4-(1–147 + 768–881) and GAL4-(1–238 + 768–848) are phosphorylated demonstrates that neither residues 148–767 nor residues 849–881 are required for recognition by the protein kinase that phosphorylates GAL4 at Ser-837. Nevertheless, the derivative GAL4-(1–147 + 768–848), which is transcriptionally inactive, is unphosphorylated, suggesting that GAL4's transcriptional activation function is required for phosphorylation at Ser-837. We suggest that this site becomes phosphorylated when the activating regions of GAL4 are engaged in transcriptional activation.

Although we do not know the precise location of the phosphorylation responsible for production of form b, we note that deletion of activating region II (residues 763–881) from full-length GAL4 severely impairs activity and inhibits this phosphorylation [see GAL4-(1–763); Fig. 1]. As mentioned in the Introduction, the inactivity of deletion derivatives lacking either activating region I or activating region II reveals a previously unrecognized property of the CR of GAL4 lying between activating regions I and II (residues 239–762; Fig. 4*B*). In the presence of CR, both activating regions are required for function; in the absence of CR, as reported (3), either activating region is active when fused to GAL4-(1–147).

DISCUSSION

Our experiments, taken together, suggest that GAL4 is phosphorylated as a consequence of transcriptional activation. The following observations support this conclusion. (*i*) Wild-type GAL4 isolated from cells in which it is working as a transcriptional activator is phosphorylated on at least two serine residues to produce several slower migrating species; these phosphorylated species are absent in cells in which GAL4 is inactive. Thus, GAL4 forms b and c cannot be detected in GAL80$^+$ cells grown in the absence of galactose, but they appear after induction. In gal80$^-$ cells, the phosphorylated species are present in the absence of galactose. (*ii*) GAL4 has one major site of phosphorylation within activating region II, at Ser-837; a second major phosphorylation must lie outside of activating region II. Changing Ser-837 to alanine has no effect on transcriptional activation by GAL4. (*iii*) Inactivation of GAL4's DNA-binding domain by mutation eliminates the phosphorylated species, even in gal80$^-$ cells. Partial restoration of DNA binding of one such mutant by excess zinc also restores the major phosphorylation. (*iv*) Careful examination of the proteins produced by a series of deletion derivatives indicates that the activation function of GAL4 is necessary for production of the phosphorylated species. For example, deletion of activating region I from an otherwise intact molecule eliminates phosphorylation at Ser-837. Deletion of activating region II also inactivates GAL4 and eliminates the phosphorylation located outside of activating region II.

Our results are supported by the observations of Mylin and coworkers (7, 8), who demonstrated a correlation between GAL4 activity and the presence of two phosphorylated species, designated II and III. They found that several missense mutations that disrupt GAL4 activity prevent for-

mation of forms II and III. Revertants of these mutants reestablished production of the phosphorylated species. Also, a series of mutants impaired for DNA binding were shown to lack form III. We believe the species isolated in our immunoprecipitations, designated forms b and c, are identical to their forms II and III, respectively. We show here that the phosphorylation producing form c is irrelevant for transcriptional activation, and we suggest that these modifications are a consequence of, rather than a requirement for, GAL4 activity. Consistent with this conclusion, Long *et al.* (17) have shown that the accessory factor GAL11 is required for production of GAL4 form III. We attribute this hypophosphorylation to the fact that GAL4 activity is severely impaired in cells lacking GAL11 (9).

The C-terminal heptapeptide repeat of RNA polymerase II (18) is known to become phosphorylated after interaction with the promoter and before initiation of transcription (19, 20), suggesting that there may be a protein kinase in the transcription complex. Ser-837 of GAL4 is centered among several proline residues, with the sequence Pro-Leu-Ser-Pro (21). The C-terminal repeat of RNA polymerase II has a similar sequence (Pro-Thr-Ser-Pro-Ser-Tyr-Ser) (18), which is known to be phosphorylated (20, 22). GAL4 may be phosphorylated by the same or by a similar kinase during activation of transcription.

Our results raise the possibility that phosphorylation of other transcriptional activator proteins could be a consequence of activator function. For example, phosphorylation of the yeast heat shock transcription factor (23, 24), the yeast STE12 protein (25), and the glucocorticoid receptor (26, 27), is known to correlate with transcriptional activation. Similarly, when simian virus 40, which requires the factor SP1 for transcription, infects HeLa cells, phosphorylation of SP1 is induced (28). In the latter case, a protein kinase has been identified that phosphorylates SP1 in a DNA-binding-dependent manner (28). It is conceivable that GAL4 could be phosphorylated by a similar yeast kinase, but the kinase would have to be specifically inhibited by deletions that impair GAL4 activity. Consequently, we prefer the hypothesis that GAL4 phosphorylation occurs by an activation-dependent kinase.

We thank Howard Himmelfarb for providing yeast strains, Jun Ma for GAL4 deletion mutants, and Mark Johnston for DNA-binding mutants. We are also grateful to Michael Green and Brendan Bell for helpful comments on the manuscript. This work was supported by grants from the National Cancer Institute and Medical Research Council of Canada to I.S., and the American Cancer Society to M.P. I.S. is a Research Scientist of the National Cancer Institute of Canada.

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