MOLECULAR AND CELLULAR BIOLOGY, Sept. 1996, p. 4879–4887 Vol. 16, No. 9
0270-7306/96/$04.00+0

Phosphorylation of Gal4p at a Single C-Terminal Residue Is Necessary for Galactose-Inducible Transcription

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Received 3 April 1996/Returned for modification 23 May 1996/Accepted 28 June 1996

Gal4p regulates expression of genes necessary for galactose catabolism in *Saccharomyces cerevisiae*. We have previously shown that phosphorylation of Gal4p requires both its DNA binding and transcriptional-activation functions and have suggested that phosphorylation occurs as a consequence of interaction with general transcription factors. In this study, we show that phosphorylation occurs rapidly on a limited fraction of overexpressed Gal4p present in a sodium dodecyl sulfate-extractable subcellular fraction while a significant fraction remains stably unphosphorylated. Taken together with our previous observations, we conclude that Gal4p is phosphorylated only if it becomes localized to the nucleus and is capable of both DNA binding and transcriptional activation. We demonstrate that Gal4p is multiply phosphorylated at both the C and N termini, and we identify the precise locations of three sites of phosphorylation at serines 691, 696, and 699. Of these sites, only serine 699 must be phosphorylated for galactose-inducible transcription to occur. Mutation of S-699 to alanine significantly impairs *GAL* induction by galactose in *GAL80*$^+$ cells but does not affect transcriptional activation by Gal4p in *gal80*$^-$ cells. In *gal80*$^-$ cells, Gal4p phosphorylation, including that of serine 699, is stimulated by the presence of both galactose and glucose, indicating that phosphorylation at this site is not specifically activated by galactose. Serine 699 phosphorylation requires Gal4p's DNA binding function and is influenced by the function of the RNA polymerase II holoenzyme component Gal11p. These results suggest that a phosphorylation on Gal4p, likely resulting from interaction with the holoenzyme, modulates the induction process by regulating interaction between Gal4p and Gal80p.

Gal4p activates transcription of genes necessary for catabolism of galactose in the budding yeast *Saccharomyces cerevisiae*. Gal4p activity and expression of the *GAL* genes are stringently regulated by the availability of galactose and glucose (11, 19). Galactose induces the *GAL* genes, while glucose, the preferred source of fermentable carbon, causes strong repression of *GAL* transcription even when galactose is present (12). Gal4p activity is regulated by the function of Gal80p, a protein which binds to the C-terminal 30 amino acids of Gal4p and presumably prevents interaction with the general initiation factors under noninducing conditions (17, 22). Several observations have suggested that Gal4p and Gal80p do not dissociate in response to galactose but rather undergo a conformational change that allows interaction of Gal4p's activation domains with general initiation complex components (17, 28). Rapid induction by galactose requires the product of *GAL3*, a protein with unknown function which is structurally similar to the galactokinase encoded by *GAL1*. Overexpression of either *GAL3* or *GAL1* causes induction of the *GAL* genes in the absence of galactose, suggesting that Gal3p may cause an alteration in the Gal4p-Gal80p complex in response to galactose (5). Consistent with this hypothesis, Gal3p and Gal80p have been shown to interact in vitro and in vivo (38). These results are supported by earlier experiments with a temperature-sensitive *GAL3* allele, which demonstrated that Gal3p itself, rather than a metabolite, is required to maintain the induction process (27). In the absence of Gal3p, the *GAL* genes are induced by galactose over a period of days rather than hours, an effect which has been called long-term adaptation (4).

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Gal4p has been shown to be phosphorylated in a manner which correlates with its ability to activate transcription (24, 25, 32). Gal4p in cells expressing Gal80p is unphosphorylated in noninducing conditions but becomes phosphorylated upon induction by galactose, as judged by the appearance of more slowly migrating species in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (24). We have previously identified a site of phosphorylation in Gal4p's C-terminal activating region 2 (AR2), at serine 837, which when phosphorylated causes a large decrease in the mobility of [^{35}S]methionine-labeled Gal4p in SDS-PAGE (32). Rather than proving that this phosphorylation is required for activation, our results instead indicated that Gal4p phosphorylation likely occurs as a consequence of transcriptional activation (32). Supporting this conclusion is the observation that elimination of the DNA binding function by mutation impairs phosphorylation at Gal4p serine 837. Also, we generally find that Gal4p deletion derivatives are unphosphorylated unless they are capable of activating transcription, even if they bear regions containing phosphorylation sites (32, 36). Thus, for example, deletion of AR1 (residues 148 to 238; see Fig. 4A and 5A) eliminates both the transcriptional-activation function of Gal4p and its phosphorylations, including the one at the distantly located serine 837. Similarly, deletion of AR2 (residues 768 to 881) prevents both transcriptional activation and the appearance of the remaining phosphorylations which we know to reside outside AR2 (references 32 and 36 and this study). Thus, we have previously suggested that Gal4p may be phosphorylated as a consequence of its interaction with general transcription factor components (32). Consistent with this hypothesis is a series of observations which demonstrate a correlation between transcriptional-activation function and phosphorylation (20, 25, 28). Gal4p purified from yeast cells grown under conditions of galactose induction bears at least one phosphorylation,

whereas Gal4p purified from yeast cells grown under noninducing conditions is unphosphorylated (28). Overexpression of *GAL3* and *GAL1* causes the occurrence of Gal4p phosphorylation in the absence of galactose (5). Also, the RNA polymerase holoenzyme component Gal11p has been shown to be necessary for both efficient Gal4p phosphorylation and transcriptional activation (10, 20, 37). All of these results are intriguing because they imply a role for phosphorylation in regulation of Gal4p's interaction with the holoenzyme.

In this study we examine phosphorylation of a series of deletion derivatives and determine that Gal4p may be phosphorylated on at least seven different serine residues. We identify the precise location of a cluster of three phosphorylations within a C-terminal region and find that one of them, at serine 699, is necessary for galactose-inducible transcription. In our analysis, serine 699 phosphorylation causes a minor physical shift in Gal4p's mobility in SDS-PAGE which can be observed only with immunoprecipitation of C-terminal fusion derivatives. In contrast to its requirement for galactose induction in *GAL80*$^+$ cells, phosphorylation at serine 699 is not required for transcriptional activation in cells lacking Gal80p. We suggest that phosphorylation at this site may regulate interaction between Gal4p and Gal80p to modulate the induction process. Our results are significant not only because we demonstrate the importance of a critical phosphorylated residue for Gal4p function but also because we have found at least three other phosphorylations which have no effect on Gal4p function by our analysis, one of which is the major site of phosphorylation in vivo.

MATERIALS AND METHODS

Yeast strains. Most experiments were performed with yeast strains YT6::171 (*MAT*α *gal4 gal80 ura3 his3 ade2 lys2 trp1 ara1 leu2 met URA3*::*GAL1-lacZ*), YT6G80::171 (*MAT*α *gal4 gal80 ura3 his3 ade2 lys2 trp1 ara1 leu2 met URA3*::*GAL1-lacZ LEU2*::*GAL80*) (10, 32), and YM707::171 (*MAT***a** *ura3 his3 ade2 lys2 trp1 tyr1 met gal4 URA3*::*GAL1-lacZ*) (12). The *gal11*$^-$ and *GAL11P* strains, which were derived from YT6::171, contained a disruption of *GAL11* with *TRP1* and expressed the dominant *GAL11P* allele, respectively (10).

Plasmids and oligonucleotide mutagenesis. For expression of *GAL4* derivatives bearing deletion and point mutations, we used plasmid vectors with *TRP1* markers as follows: pDN3, which expresses *GAL4* from the *ADH1* promoter on a 2μm vector (32); pMH76, which produces Gal4p from the *ADH1* promoter on an ARS-CEN plasmid (31, 36); and YCpG4, which produces Gal4p from its natural promoter on an ARS-CEN plasmid vector. YCpG4 was constructed by insertion of a *Bam*HI-*Hin*dIII *GAL4* genomic fragment into YCplac22 (8). Plasmids pCD21X, pCD56X, and pMA246, which have been described previously, are 2μm vectors with a *HIS3* selectable marker and express Gal4p deletion derivatives from the *ADH1* promoter (23). Vectors expressing the Gal4p DNA-binding domain (DBD) and deletion derivatives Δ322 and Δ768 were produced from pMH76 (36). Oligonucleotide-directed mutations were constructed in plasmid pGSH, which contains a *Sal*I-*Hin*dIII C-terminal-encoding *GAL4* fragment in pGEM3Z(f)+. Oligonucleotides for generation of serine-to-alanine mutations were as follows: A-691, CAACAATATCGCTGTTAAATATG; A-696, TAAATATGTTGCTCCTGGCTCAG; and A-699, TTCTCCTGGCGCAGTAGGGCCTT. The substitution of alanine for serine at position 837 has been described previously (32). Double substitutions of A-691, A-696, and A-699 with A-837 were created in plasmid pGSHA837 (32). The triple and quadruple Ser-to-Ala derivatives were created in pGSHA691A837 and pGSHA691A696A837, respectively. *GAL4* Δ683 derivatives were generated by digestion of wild-type (WT) or mutant pGSH plasmids with *Acc*III-*Sac*I, treatment with T4 DNA polymerase, and religation. *Eco*RI-*Hin*dIII C-terminal-encoding fragments from these intermediates were then inserted into pY2 (31) to create in-frame fusions with the Gal4p DBD. Mutant DNAs encoding the serine-to-alanine mutations were subcloned into plasmid YCpG4 and pDN3 with *Sal*I-*Mlu*I. *GAL4* derivatives Δ701, Δ723, and Δ739 were generated by digesting *GAL4* Δ683 with *Eco*RI, followed by treatment with nuclease *Bal* 31. Fragment ends were made blunt by treatment with the Klenow fragment of DNA polymerase I and then ligated to *Eco*RI linkers. *Bal* 31-digested products were then cloned as *Eco*RI-*Hin*dIII fragments into pY2 and sequenced. Derivatives containing in-frame fusions with the Gal4p DBD were used (see Fig. 4A).

Plasmids expressing the DNA-binding-defective Y14 Gal4p Δ683 and Y14 Gal4p Δ683 bearing substitutions of alanine for serine at positions 691, 696, and 837 were constructed by subcloning *Xho*I-*Mlu*I fragments from their respective pY2 expression constructs into pDN3Y14 (32). The *HIS3* 2μm plasmids expressing Gal4p Δ683 and Gal4p Δ683 bearing serine-to-alanine mutations at positions 691, 696, and 837 from the *ADH1* promoter were constructed by subcloning *Xho*I-*Mlu*I fragments into pMA210 as described above (23).

Immunoprecipitation and phosphatase treatment of Gal4p protein in vitro. Cells expressing *GAL4* derivatives were labeled with [^{35}S]methionine and lysed, and the lysate was immunoprecipitated with rabbit anti-Gal4p DBD polyclonal antibodies and treated with phosphatase in vitro as described previously (32). Unless otherwise specified, cells were grown to an A_{600} of 1.0 in minimal selective medium containing glycerol, ethanol, and lactic acid; glucose was added to a final concentration of 2% 30 min prior to harvesting the cells for labeling. Cells were labeled for 20 min in methionine-free medium in the presence of 2% glucose (unless otherwise specified). For pulse-chase labeling of WT Gal4p, cells were grown to mid-log phase and glucose was added as described above. The cells were harvested and washed three times in methionine-free medium containing 2% glucose, prewarmed to 30°C, prior to addition of [^{35}S]methionine at a concentration of 1 mCi/ml. The cells were labeled for 5 min at 30°C, immediately pelleted by microcentrifugation, and then resuspended in an equivalent volume of medium containing 25 mM unlabeled methionine. A sample was taken immediately for lysis and immunoprecipitation (zero-time chase). The remaining labeled cells were incubated in the presence of unlabeled methionine at 30°C, and samples were taken at 10, 20, and 30 min for lysis and immunoprecipitation.

β-Galactosidase assays. β-Galactosidase activity was assayed in cell extracts prepared by lysis with glass beads as described previously (10, 32). Cells were grown to an A_{600} of 1.0 in selective medium containing glycerol, ethanol, and lactic acid. Galactose was added to a final concentration of 2%, and cells were lysed 8 h later for determination of β-galactosidase activity (36).

RESULTS

WT Gal4p is phosphorylated on multiple sites. Several previous reports have described phosphorylated forms of Gal4p whose appearance correlates with the transcriptional-activation function. These phosphorylated species have been designated forms II and III when observed by Western blotting (immunoblotting) (24) or b and c when determined by immunoprecipitation (32). Our previous analysis using immunoprecipitation of [^{35}S]methionine-labeled protein demonstrated that Gal4p is phosphorylated on at least two sites, one of which was identified as a major site of phosphorylation (serine 837) (32). Serine 837 phosphorylation is necessary for the appearance of form c, which we believe to be the same as the Gal4p form III detected by Western blotting. However, in this paper we present data which demonstrate that Gal4p is phosphorylated on many more sites than are implied by the designation of specific species on SDS-PAGE. Immunoprecipitates of WT Gal4p from cells labeled with [^{35}S]methionine for 20 min contain two major Gal4p species of approximately 100 and 120 kDa (Fig. 1, lane 1); each of these species migrates as a diffuse band. Excess phosphatase treatment of the immunoprecipitates in vitro converts the major 120-kDa species into the 100-kDa form (lane 2). When the phosphatase reaction is limited to a shorter time with lower concentrations of enzyme, it becomes apparent that there are many more phosphorylated species of WT Gal4p than can be resolved into distinct bands by SDS-PAGE (lanes 4 to 7). Phosphatase concentrations of 0.5, 1, and 3 U/ml produced an obvious smear of labeled species which migrate between the non-phosphatase-treated 120- and 100-kDa species. The variety of species that can be generated by limiting phosphatase treatment suggests that WT Gal4p is likely phosphorylated on many more sites than the two which we previously predicted (32).

GAL4 is normally expressed at very low levels (9), and we have found that overexpression of Gal4p produces phenotypes which make it difficult to efficiently label with [^{32}P]orthophosphate in vivo. These phenotypes include delayed stationary-phase recovery, enhanced thermotolerance, and greater resilience to lysis (30), all of which appear to be aggravated under the conditions of phosphate starvation that are required for phosphate labeling. Therefore, we have been unable to reliably obtain sufficient incorporated phosphate label in immunoprecipitated Gal4p to perform tryptic phosphopeptide analysis

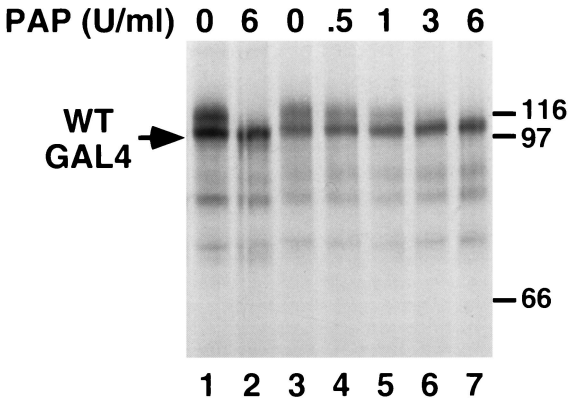

FIG. 1. Limited phosphatase treatment of WT Gal4p demonstrates multiple phosphorylated forms. WT *GAL4* was expressed from the *ADH1* promoter on plasmid vector pDN3 in yeast strain YT6::171 (*gal80*$^-$) and pulse-labeled with [^{35}S]methionine for 20 min. Gal4p was recovered by immunoprecipitation and either resolved by SDS-PAGE directly (lanes 1 and 3) or treated in vitro with potato acid phosphatase (PAP) at a concentration of 6 U/ml (lanes 2 and 7), 0.5 U/ml (lane 4), 1 U/ml (lane 5), or 3 U/ml (lane 6) for 30 min (lane 2) or 15 min (lanes 4 to 7). Migration of molecular mass standards is indicated (in kilodaltons).

and have had to rely on identification of phosphorylation sites by virtue of their effect on the mobility of Gal4p derivatives in SDS-PAGE (32).

A limited fraction of overexpressed Gal4p is phosphorylated. We have previously suggested that Gal4p phosphorylation occurs as a consequence of its interaction with targets in the general initiation complex and that it might be phosphorylated by general transcription factor components (32). This argument is supported by experiments which demonstrate that mutations of the DNA-binding motif eliminate phosphorylation in a distant part of the protein (32). If this model is correct, we would also expect that only a limited fraction of overexpressed Gal4p could become available for phosphorylation, since there are likely to be a limited number of binding sites for Gal4p on the genome. To test this notion, we performed pulse-chase labeling of Gal4p produced by expression from the *ADH1* promoter on a 2μm plasmid vector in *gal80*$^-$ cells. Figure 2 shows that phosphorylated species of Gal4p are detectable following a 5-min pulse-labeling with [^{35}S]methionine, as determined by the presence of more slowly migrating, phosphatase-sensitive species (Fig. 2, 0-min chase). After a 10-min chase in the presence of excess unlabeled methionine, we found that approximately 30% of the total labeled Gal4p

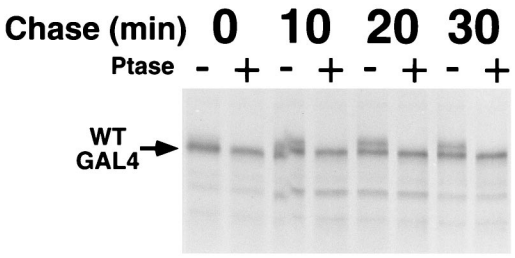

FIG. 2. A limited fraction of overexpressed Gal4p is phosphorylated. WT *GAL4* was expressed in yeast strain YT6::171 (*gal80*$^-$) on plasmid vector pDN3 and labeled for 5 min with [^{35}S]methionine in selective medium containing 2% glucose. The cells were then washed and incubated in selective medium containing excess unlabeled methionine for the indicated number of minutes prior to immunoprecipitation. The samples were left untreated (Ptase −) or treated with phosphatase in vitro (Ptase +) prior to analysis by SDS-PAGE.

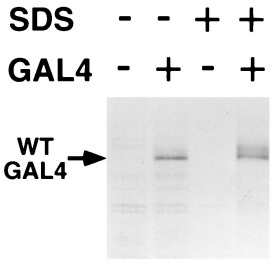

FIG. 3. Gal4p is unphosphorylated in a cytoplasmic (nuclei-removed) cellular fraction. Yeast strain YT6::171 (*gal80*$^-$) bearing plasmid pMH76 (GAL4 +) or YCplac22 (GAL4 −) was labeled with [^{35}S]methionine and lysed in radioimmunoprecipitation assay buffer containing 1% NP-40 without (−) or with (+) 0.1% SDS. Particulate material was removed from the samples by centrifugation, and Gal4p was recovered by immunoprecipitation and analyzed by SDS-PAGE. Samples lysed in the absence of 0.1% SDS represent the nuclei-removed fraction, while samples from cell extracts prepared with 0.1% SDS represent total cell extracts.

becomes converted into the more slowly migrating, phosphatase-sensitive species (Fig. 2, 10-min chase). We observed that this phosphorylated Gal4p-to-unphosphorylated Gal4p ratio did not change significantly beyond 10 min and persisted for at least 30 min (compare 10-min and 30-min lanes). If interaction between Gal4p and its kinase was freely diffusible, we would expect to observe a gradual accumulation of phosphorylation on most of the Gal4p produced during the pulse-labeling. Rather, because we observe that a significant proportion of overexpressed Gal4p remains stably unphosphorylated, we conclude that accessibility of Gal4p to its kinase is physically limited.

A second prediction of our model, that Gal4p is phosphorylated as a consequence of its transcriptional-activation function, is that overexpressed Gal4p which is not translocated to the nucleus should also be unphosphorylated. Although we have been unable to effectively isolate nuclear fractions of *gal80*$^-$ cells overexpressing *GAL4*, we performed a simple test of this prediction by comparing immunoprecipitates of Gal4p from soluble clarified cell extracts prepared with the nonionic detergent Nonidet P-40 (NP-40) in the presence or absence of SDS. NP-40 has been used in lysates for preparation of intact yeast nuclei (18), whereas SDS is required at a concentration of 0.1% in radioimmunoprecipitation assay buffer for disruption of nuclear membranes (14, 29). We found that Gal4p that immunoprecipitated from the supernatant of NP-40-solubilized extracts prepared in the absence of SDS (nuclei removed) was largely unphosphorylated, whereas, in contrast, a significant fraction of Gal4p that immunoprecipitated from extracts prepared with 0.1% SDS (total cellular) appeared to be phosphorylated, as judged by more slowly migrating species on SDS-PAGE (Fig. 3). The fact that the phosphorylated forms of Gal4p are not observed in extracts from which nuclei were removed is consistent with the notion that Gal4p must undergo nuclear translocation to become phosphorylated.

Localization of multiple phosphorylations on the Gal4 protein. Upon examining protein produced by a series of *GAL4* deletion derivatives, we have localized additional phosphorylated residues to a C-terminal region between residues 683 and 701 and within residues 1 to 238 of the N terminus. In previous experiments, we demonstrated that only Gal4p deletion derivatives which can activate transcription become phosphorylated (32, 36). This phenomenon is illustrated in Fig. 4 and 5. The large central region of Gal4p contains a segment which strongly inhibits transcriptional activation when either AR1 or AR2 is deleted (Fig. 4A and 5A, Inhibitory) (36). Note that

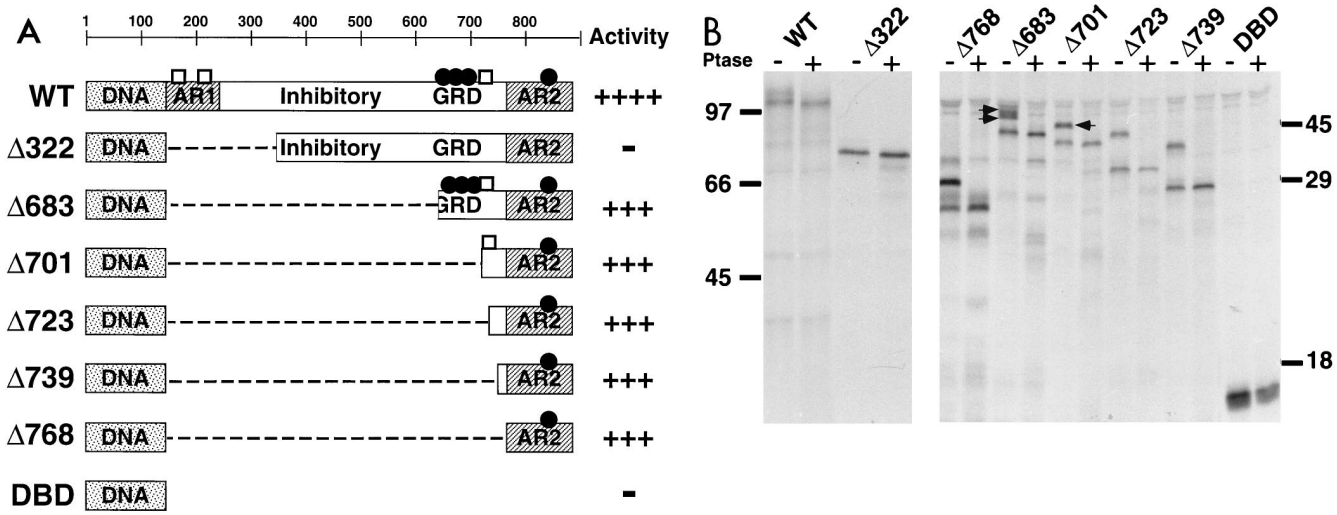

FIG. 4. Localization of phosphorylation sites within the C terminus of Gal4p. (A) Schematic representation of WT Gal4p (WT) and derivatives bearing C-terminal Gal4p fragments fused to the DBD. Relative amounts of *GAL1-lacZ* transcription activated by each derivative, as determined by β-galactosidase activity, are indicated (++++, 100% activity; −, no activity). Gal4p functional regions indicated are as follows: DNA, DBD; Inhibitory, inhibitory region (36); GRD, glucose response domain (36). ●, phosphorylation; □, predicted phosphorylation. (B) Immunoprecipitates of [^{35}S]methionine-labeled Gal4p derivatives depicted in panel A. Samples were left untreated (Ptase −) or were treated with phosphatase (Ptase +) in vitro prior to being resolved by SDS–7.5% PAGE (WT and Δ322) or SDS–10% PAGE (Δ768, Δ683, Δ701, Δ723, Δ739, and DBD). Phosphatase-sensitive species of Δ683 and Δ701 are indicated with arrows. Positions of molecular mass standards (in kilodaltons) are indicated.

derivative Δ322, which contains the DBD fused to the C-terminal residues 322 to 881, is lacking AR1 but contains most of the inhibitory segment, is incapable of activating transcription, and was also found to be unphosphorylated (Δ322, Fig. 4). Similarly, the C-terminally truncated derivative CD21X contains a significant fraction of the inhibitory segment but is deleted of AR2; this mutant is inactive as a transcriptional activator and was also found to be unphosphorylated (CD21X, Fig. 5). In both cases, elimination of the entire inhibitory segment restored both the transcriptional-activation function and phosphorylation. Specifically, note that derivative Δ683, which contains the DBD fused to C-terminal residues 683 to 881, activated transcription efficiently and also appeared to be multiply phosphorylated (Δ683, Fig. 4). Likewise, derivative MA246, which consists of the N-terminal 238 residues, activates transcription by virtue of the presence of AR1 (23) and, surprisingly, was also found to be phosphorylated (MA246, Fig. 5).

The presence of multiple phosphatase-sensitive species on the Gal4p Δ683 derivative (Fig. 4B) indicated that there must be at least one phosphorylated amino acid, in addition to serine 837 (32), within the region from residue 683 to 881. To localize the additional phosphorylation(s), we constructed and examined a nested set of truncated C-terminal fragments fused to the DBD (Fig. 4A). In this analysis, we found that deletion of residues 683 to 701 eliminated a phosphorylated species,

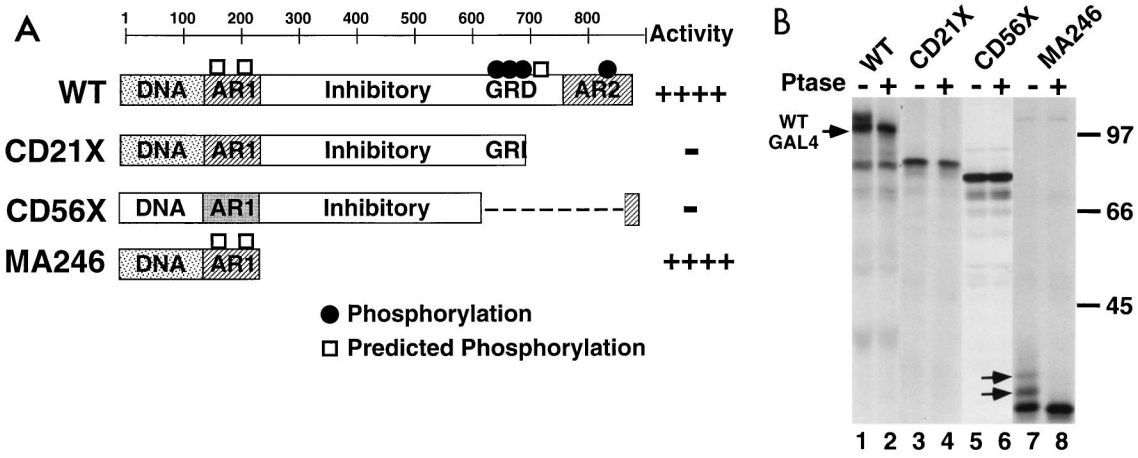

FIG. 5. Localization of phosphorylation sites within the N terminus of Gal4p. (A) Schematic representation of WT Gal4p (WT) and derivatives bearing C-terminal deletions. Relative amounts of *GAL1-lacZ* transcription activated by each derivative, as determined by β-galactosidase activity, are indicated (++++, 100% activity; −, no activity). Gal4p functional regions indicated are as follows: DNA, DBD; Inhibitory, inhibitory region (36); GRD, glucose response domain (36). (B) Immunoprecipitates of [^{35}S]methionine-labeled Gal4p derivatives depicted in panel A. Samples were left untreated (Ptase −) or were treated with phosphatase (Ptase +) in vitro prior to being resolved by SDS–10% PAGE. Phosphatase-sensitive species of derivative MA246 are indicated with arrows. Positions of molecular mass standards (in kilodaltons) are indicated.

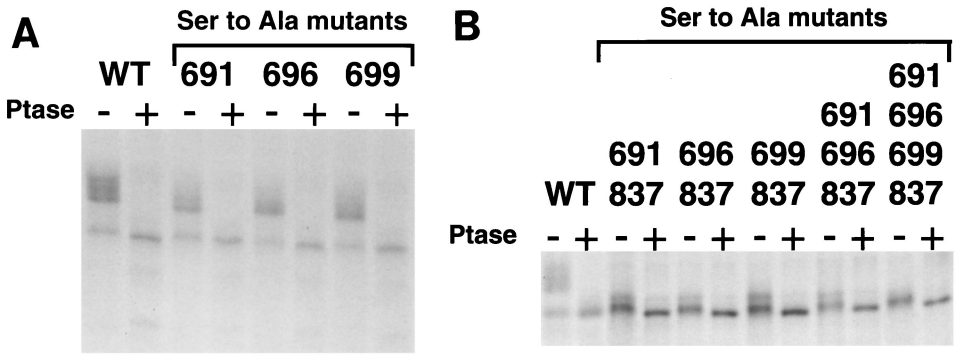

FIG. 6. Identification of a cluster of C-terminal phosphorylated serines. (A) Immunoprecipitates of [^{35}S]methionine-labeled Gal4p Δ683 WT or Δ683 derivatives bearing a serine-to-alanine mutation at position 691, 696, or 699. (B) Immunoprecipitates of [^{35}S]methionine-labeled Gal4p Δ683 WT or Δ683 derivatives bearing multiple serine-to-alanine mutations at the indicated residues (top). Samples were left untreated (Ptase −) or were treated with phosphatase in vitro (Ptase +) prior to analysis by SDS–10% PAGE.

suggesting that at least one phosphorylated residue must reside within this region (compare derivatives Δ683 and Δ701, Fig. 4). Derivatives bearing C-terminal fragments spanning from position 701 (Δ701), 723 (Δ723), 739 (Δ739), or 768 (Δ768) to position 881 were found to have only one major distinctive phosphorylated species (Fig. 4B). The large difference in mobilities of the phosphorylated species produced by these derivatives is characteristic of phosphorylation at serine 837 (32, 36).

Although we have not performed an extensive analysis of the N-terminal phosphorylations, the results shown in Fig. 4 and 5 suggest that Gal4p may have two phosphorylations within AR1 as well. We observed two phosphorylated species in immunoprecipitates of derivative MA246 (Fig. 5B, MA246) but no detectable phosphorylated species in immunoprecipitates of the DBD expressed independently (DBD, Fig. 4B). The simplest interpretation of these data is that AR1 must contain two sites of phosphorylation. However, since the DBD expressed on its own does not activate transcription in vivo, we cannot exclude the possibility that there are phosphorylated residues between amino acids 1 to 147 which are prevented from becoming phosphorylated when AR1 is deleted. We do not suspect this to be the case since we observe only a single phosphorylated species on derivative Δ768, which activates transcription efficiently and which we know to be phosphorylated on serine 837 (32, 36). Therefore, we propose that in addition to the C-terminal phosphorylations, Gal4p may have two additional phosphorylated residues within AR1 (see Fig. 4A and 5A).

Gal4p is phosphorylated on a cluster of serines at residues 691, 696, and 699. We were previously able to identify the phosphorylation at serine 837 because of the large change in mobility that it causes in SDS-PAGE (32). In contrast, the remaining phosphorylations in the C terminus of Gal4p cause only small shifts in mobility (compare derivatives Δ683 and Δ701). Because the additional phosphorylated species are more easily resolved on the Δ683 derivative than on WT Gal4p (see reference 29), we used mutagenesis of this derivative to identify the locations of phosphorylated residues between 683 and 701. Individual alanine substitutions for each hydroxyl amino acid within this region were made at serines 691, 696, and 699 and expressed as Δ683 derivatives (Fig. 6A). Note that our previous analysis of phosphoamino acids generated from WT Gal4p indicated that most, if not all, phosphorylated residues were serines (32). Mutation of each of the serines at positions 691, 696, and 699 to alanines caused an alteration in the phosphatase-sensitive species of Gal4p Δ683 derivative in SDS-PAGE (Fig. 6A), suggesting that each of these serines may become phosphorylated. We also created Δ683 derivatives with multiple serine-to-alanine mutations (Fig. 6B). The contribution of serine 837 phosphorylation to the large shift in mobility of phosphorylated species is illustrated by the fact that changing this residue to alanine individually in the Δ683 derivative (data not shown) or by double substitutions with A-691, A-696, or A-699 significantly increases the mobility of phosphatase-sensitive species (Fig. 6B, compare WT Δ683 to Δ683-A691A837, Δ683-A696A837, and Δ683-A699A837). We find that the distinct phosphatase-sensitive species in the Δ683-A837 derivatives cannot be eliminated until the serines at positions 691, 696, and 699 are all changed to alanine (Fig. 6B and data not shown). Most notably, a distinct phosphorylated species is observed on a derivative bearing a triple substitution of alanines at positions 691, 696, and 837; however, in contrast, a corresponding quadruple mutant bearing substitutions at positions 691, 696, 699, and 837 has lost this species. Taken together, the experiments shown in Fig. 4 and 6 suggest that in addition to the phosphorylation at serine 837, which causes a major shift in mobility, Gal4p likely has a cluster of phosphorylations which occur at positions 691, 696, and 699, any one of which appears to cause a smaller shift in mobility.

It should also be noted that the derivative bearing serine-to-alanine mutations at all four of the suspected sites of phosphorylation still migrates as a diffuse band on SDS-PAGE which is sharpened by treatment with phosphatase in vitro (Fig. 6B; compare Ptase − and Ptase + for 691 696 699 837). This indicates that the Δ683 derivative must contain at least one phosphorylation in addition to the likely sites at positions 691, 696, 699, and 837. On the basis of our current data, we predict that this phosphorylation occurs between residues 701 and 768 (see Fig. 4A and 5A).

Phosphorylation at Gal4p serine 699 is required for galactose induction. To examine whether phosphorylation at serine 691, 696, or 699 is required for Gal4p activity, we created individual alanine substitutions at each of these residues in a single-copy plasmid vector which expresses full-length Gal4p from its own promoter (YCpG4). Induction by galactose and transcriptional activation were determined for each by measuring β-galactosidase activity expressed from a *GAL1-lacZ* reporter gene in *GAL80*$^+$ and *gal80*$^-$ cells. We found that of these mutations, only *GAL4* S699A (i.e., the mutation resulting in the substitution of alanine for serine at position 699 of Gal4p) caused a defect in galactose induction in *GAL80*$^+$ cells

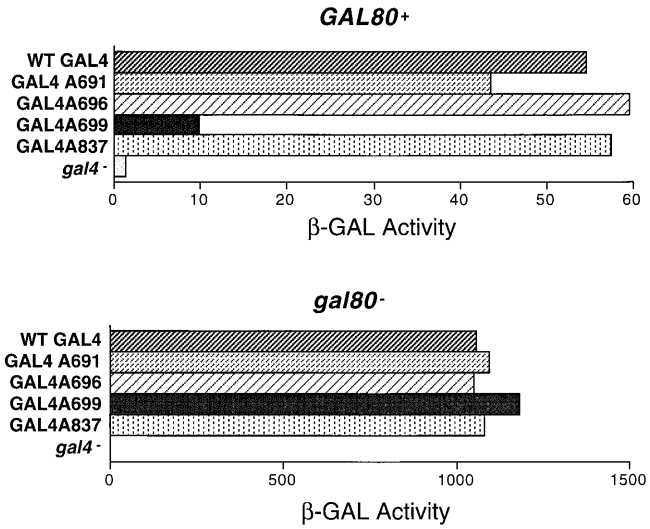

FIG. 7. Phosphorylation at serine 699 is necessary for galactose-inducible transcription. Yeast strains YT6G80::171 (*GAL80*$^+$, top panel) and YT6::171 (*gal80*$^-$, bottom panel) were transformed with control vector YCplac22 (*gal4*$^-$), a plasmid expressing full-length WT *GAL4* from its own promoter on plasmid vector YCpG4 (WT), or YCpG4 bearing the indicated serine-to-alanine mutations at position 691 (GAL4 A691), 696 (GAL4 A696), 699 (GAL4 A699), or 837 (GAL4 A837). Cells were grown in selective medium and induced with galactose 8 h prior to harvesting and determination of β-galactosidase (β-GAL) activity. Results are averages of data from three samples; the standard error for all results was less than 15%.

(Fig. 7, top panel). After 8 h in 2% galactose, cells expressing Gal4p S699A had only approximately 20% of the β-galactosidase activity of the WT. None of the other Gal4p derivatives bearing serine-to-alanine mutations had significant defects in *GAL1-lacZ* expression in *GAL80*$^+$ cells, including Gal4p S837A, in which a major phosphorylation is eliminated (32). These results suggest that among the four likely sites of C-terminal Gal4p phosphorylation, only serine 699 phosphorylation is necessary for efficient induction in response to galactose. *S. cerevisiae* YT6G80::171 was generated by reintegration of a WT *GAL80* allele into the *gal4*$^-$ *gal80*$^-$ strain YT6::171 (32) and induces relatively slowly with galactose compared with other S228C strains. To confirm that the galactose induction defect caused by the substitution of alanine for serine at position 699 was not due to a peculiarity of YT6G80::171, we also examined induction in *S. cerevisiae* YM707::171 (12). We found the effect of the S699A mutation to be essentially the same in both strains, although *GAL1-lacZ* induction by WT Gal4p in YM707::171 is higher than in YT6G80::171 (data not shown). We believe that inactivity of Gal4p bearing the S699A substitution cannot be due to instability because its synthesis could be detected by immunoprecipitation as readily as WT Gal4p or the other proteins with Ser-to-Ala mutations (data not shown).

In contrast to the induction defect displayed by Gal4p S699A, we find that this protein activates transcription as efficiently as WT Gal4p in cells lacking the negative regulator Gal80p (*gal80*$^-$; Fig. 7, bottom panel). All of the Gal4p derivatives bearing serine-to-alanine mutations activated *GAL1-lacZ* transcription to approximately the same level. From the differential effect of S699A in *GAL80*$^+$ and *gal80*$^-$ cells, we conclude that phosphorylation of Gal4p on serine 699 is necessary for efficient induction by galactose but is not required for Gal4p to activate transcription. These results suggest that phosphorylation of Gal4p at serine 699 may be involved in regulating the conformation of the Gal4p-Gal80p complex.

Serine 699 phosphorylation requires Gal4p's DNA binding function and is influenced by the product of *GAL11*. Results shown in Fig. 4 and 5 and our previous observations (32, 36) suggest that all of the Gal4p phosphorylations that we detect as phosphatase-sensitive species on SDS-PAGE occur only on derivatives which are capable of activating transcription. To examine whether phosphorylation at serine 699 has the same requirement for DNA binding as serine 837 phosphorylation (32), we examined phosphorylation of Gal4p Δ683 derivatives bearing mutations of cysteine 14 (C-14) to tyrosine (Y-14) within the N-terminal zinc cluster DNA-binding motif. This mutation impairs DNA binding in vitro and activation of transcription by Gal4p in vivo (12). We found that phosphorylation of both the WT Gal4p Δ683 derivative (W, Fig. 8A) and a derivative bearing a triple substitution of alanines for serines 691, 696, and 837 (T, Fig. 8A) was significantly impaired by the Y-14 mutation relative to their counterparts bearing WT DBDs (compare Y14 with C14 DBD). Since the triple substitution of alanines for serines leaves only the serine 699 phosphorylation site intact, this result demonstrates that efficient serine 699 phosphorylation requires Gal4p's DNA binding function. Note that although phosphorylation is impaired on the Y-14 derivatives, some phosphorylation is observed, particularly on Y-14 of Gal4p Δ683 WT. Residual phosphorylation may reflect weak DNA binding by the Y-14 mutants or interaction with general initiation factors in the absence of DNA binding due to its high level of expression.

It has been previously demonstrated that the RNA polymerase II holoenzyme component Gal11p is required for formation of the most slowly migrating Gal4p phosphorylated species. To determine whether phosphorylation at serine 699 requires *GAL11*, we examined phosphorylation of Gal4p Δ683 derivatives (Fig. 8B) in *gal80*$^-$ yeast strains in which *GAL11* was disrupted (*gal11*$^-$) or that expressed the dominant

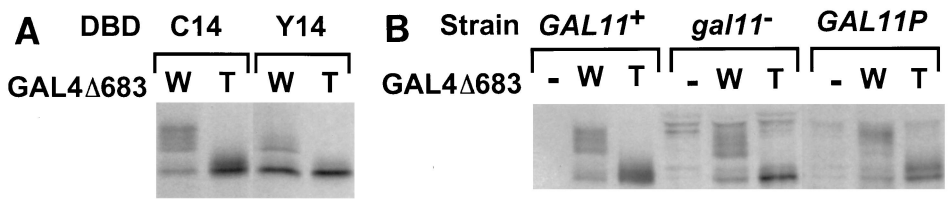

FIG. 8. (A) Gal4p phosphorylation at serine 699 is inhibited by a DBD mutation. Immunoprecipitates of Gal4p were prepared from YT6::171 expressing WT Gal4p Δ683 (W) or Gal4p Δ683 bearing the triple substitution of alanines for serines at positions 691, 696, and 837 (T) in yeast vector pDN3 with the WT DBD (C14) or a DNA-binding-defective DBD (Y14). (B) Gal4p phosphorylation at serine 699 is influenced by Gal11p. Immunoprecipitates were prepared from cells expressing no Gal4p (−), WT Gal4p Δ683 (W), or Gal4p Δ683 bearing the triple substitution of alanines for serines at positions 691, 696, and 837 (T) from the *ADH1* promoter on a *HIS3* 2μm vector in yeast strain YT6::171 (*GAL11*$^+$), in YT6::171 in which *GAL11* was disrupted (*gal11*$^-$), or in cells expressing the dominant *GAL11P* allele (*GAL11P*). Samples were analyzed by SDS–10% PAGE.

GAL11P allele (*GAL11P*). We found that in a *gal80*$^-$ background the absence of Gal11p caused only slight impairment of phosphorylation of the WT Gal4p Δ683 derivative, although there was a noticeable decrease in the abundance of the most slowly migrating phosphorylated form (compare Gal4p Δ683 W in *GAL11*$^+$ and *gal11*$^-$ lanes). However, we found that phosphorylation of the Gal4p Δ683 derivative bearing serine-to-alanine mutations at positions 691, 696, and 837 was impaired significantly in *gal11*$^-$ cells relative to the *GAL11*$^+$ strain (compare Gal4p Δ683 T in *GAL11*$^+$ and *gal11*$^-$ lanes). This result demonstrates that efficient phosphorylation at serine 699 requires the product of *GAL11*, since the major phosphorylation on this derivative requires serine 699 (Fig. 6B). Consistent with this observation, we found that the Gal4p Δ683 derivatives appeared to be hyperphosphorylated in yeast cells expressing the dominant *GAL11P* allele (Fig. 8B, *GAL11P*). In this strain, most of the WT Gal4p Δ683 derivative appears as the more slowly migrating species (Fig. 8B, *GAL11P*, W). Furthermore, the Gal4p Δ683 derivative bearing the triple serine-to-alanine mutations at positions 691, 696, and 837 produces a more distinctive band of more slowly migrating phosphorylated species than is observed in cells expressing WT *GAL11*. The mutant protein produced by *GAL11P* is thought to exert its dominant effect by causing artificial recruitment of the RNA polymerase holoenzyme to Gal4p (2). The observation that *GAL11P* causes hyperphosphorylation of Gal4p Δ683 derivatives while disruption of *GAL11* inhibits serine 699 phosphorylation supports the hypothesis that phosphorylation at this site results from interaction between Gal4p and holoenzyme components.

Gal4p phosphorylation is stimulated by galactose and glucose in *gal80*$^-$ cells. Despite its requirement for galactose induction in *GAL80*$^+$ cells, we believe that serine 699 phosphorylation cannot be a mechanism to effect a galactose-specific change in Gal4p-Gal80p because we detect this modification in response to glucose as well as galactose in *gal80*$^-$ cells. Glucose represses transcription of the *GAL* genes through several different mechanisms. However, the inhibitory effect of glucose on *GAL* transcription can be effectively eliminated by deletion of *GAL80* and overexpression of Gal4p (13, 36). We found that WT Gal4p overexpressed in *gal80*$^-$ cells growing in nonfermentable sources of carbon was unphosphorylated, as judged by its mobility on SDS-PAGE (Fig. 9A, Gly). Addition of either galactose or glucose caused the rapid appearance of apparently identical phosphorylated species on SDS-PAGE (Fig. 9A, Gal and Glu). Although we cannot say whether all of the Gal4p phosphorylations are identical in galactose- and glucose-stimulated cells, we know that serine 699 phosphorylation can occur in the presence of glucose because the immunoprecipitates shown in Fig. 6 were performed with cells labeled in medium containing 2% glucose and the results are identical to those of experiments performed with galactose (data not shown). We conclude from these results that phosphorylation of overexpressed Gal4p can be stimulated by either galactose or glucose in *gal80*$^-$ cells. These results suggest that the kinase which phosphorylates Gal4p, including serine 699, may be inactive in cells growing in nonfermentable sources of carbon but can be activated by either glucose or galactose. Alternatively, the presence of galactose or glucose may inhibit a specific phosphatase to allow the appearance of Gal4p phosphorylation.

In contrast to what was observed in *gal80*$^-$ cells, we find that the presence of Gal80p inhibits Gal4p phosphorylation in response to glucose but not galactose, even when Gal4p is overexpressed (Fig. 9B). As with *gal80*$^-$ cells, we found that Gal4p is unphosphorylated in *GAL80*$^+$ cells growing in glycerol (Fig. 9B, Gly). Addition of galactose caused the appearance of a significantly higher proportion of phosphorylated species than did glucose in *GAL80*$^+$ cells producing Gal4p from the *ADH1* promoter (Fig. 9B, compare Glu and Gal). Contrast this result with that seen for Gal4p produced in *gal80*$^-$ cells, which was efficiently phosphorylated in response to glucose as well as galactose (Fig. 9A) (compare Glu GAL80 − and + in Fig. 9B). These results demonstrate that Gal80p prevents phosphorylation of Gal4p in glucose even though the kinase may be active.

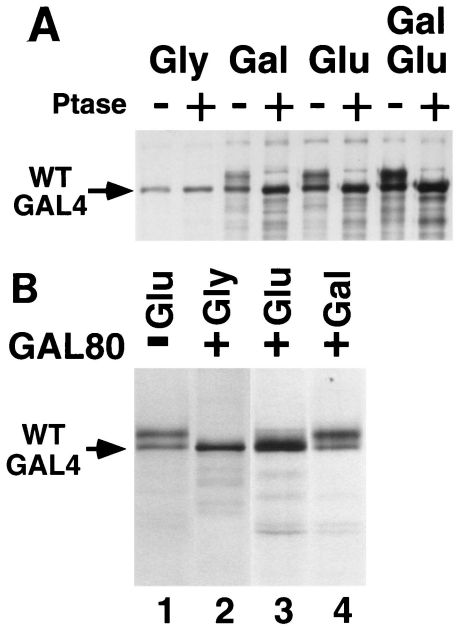

FIG. 9. (A) Gal4p phosphorylation is stimulated by glucose or galactose in *gal80*$^-$ cells. YT6::171 cells expressing full-length WT *GAL4* from plasmid pMH76 were labeled for 20 min with [^{35}S]methionine in medium containing glycerol, ethanol, and lactic acid (Gly) or medium containing 2% galactose (Gal) and/or 2% glucose (Glu). Gal4p was recovered by immunoprecipitation and either left untreated (Ptase −) or treated with phosphatase in vitro (Ptase +) prior to analysis by SDS–7.5% PAGE. (B) Gal80p inhibits Gal4p phosphorylation in glucose. Strains YT6::171 (GAL80 −; lane 1) and YT6G80::171 (GAL80 +; lanes 2 to 4) expressing full-length WT Gal4p (WT) from plasmid pMH76 were labeled for 20 min with [^{35}S]methionine in medium containing glycerol, ethanol, and lactic acid (Gly, lane 2) or in medium containing 2% galactose (Gal, lane 4) or 2% glucose (Glu, lanes 1 and 3). Gal4p was recovered by immunoprecipitation and analyzed by SDS–7.5% PAGE.

DISCUSSION

Our results demonstrate that Gal4p may be phosphorylated on at least seven different serine residues under conditions in which it can activate transcription. We previously identified a site of phosphorylation at serine 837 which causes a large alteration in the mobility of Gal4p in SDS-PAGE (32). In the present study, we localized two phosphorylation sites within the N-terminal 238 amino acids of Gal4p, identified a cluster of serine phosphorylation sites at residues 691, 696, and 699, and predicted that there is an additional phosphorylation site within residues 701 to 768. The analysis described in Fig. 1 indicated that Gal4p is likely to have many more phosphorylated species than are implied by designation of specific bands on SDS-PAGE (24, 32); this prediction is confirmed by identification of four specific phosphorylation sites and localization of three additional sites. Of the four likely sites of phosphorylation we have identified, only serine 699 is required for galactose-inducible transcription under the conditions of our as-

say. We demonstrate that phosphorylation at serine 699 is necessary for galactose-inducible transcription in *GAL80*$^+$ cells but is unnecessary for transcriptional activation by Gal4p in *gal80*$^-$ cells.

We have previously suggested that Gal4p phosphorylation occurs as a consequence of its interaction with the general initiation complex (32). The evidence in support of this hypothesis is as follows. First, in WT yeast cells, Gal4p becomes phosphorylated only under conditions in which it can activate transcription (24). Second, Gal4p requires both its DNA binding and transcriptional-activation functions for efficient appearance of all of the phosphorylations we detect (25, 32, 36). Third, only a limited fraction of overexpressed Gal4p which is present in an SDS-soluble fraction of the cell, likely reflecting nuclear localization, becomes available for phosphorylation. Fourth, the RNA polymerase holoenzyme component Gal11p, which is required for transcriptional activation by Gal4p (2, 10, 26, 37), is necessary for efficient Gal4p phosphorylation (20). We found that serine 699 phosphorylation is particularly dependent upon *GAL11* in *gal80*$^-$ cells. Furthermore, the dominant *GAL11P* allele (10) causes hyperphosphorylation of Gal4p. The mutant protein encoded by *GAL11P* has been shown to cause artificial recruitment of the holoenzyme to Gal4p (2). These observations are consistent with the hypothesis that Gal4p becomes phosphorylated only when it is localized in the nucleus, bound to DNA, and capable of activating transcription. We therefore reiterate our previous hypothesis that Gal4p phosphorylation, including that at serine 699, occurs as a consequence of interaction with the general transcriptional initiation-RNA polymerase II holoenzyme complex (32).

If the above hypothesis is correct, there are several strong candidates for the Gal4p kinase in vivo. At least two cyclin-dependent protein kinases have been identified as components of the RNA polymerase holoenzyme. TFIIH has been shown to contain a protein kinase which phosphorylates the C-terminal domain of RNA polymerase II (21). This enzyme was identified as the cyclin-dependent kinase (CDK) Kin28p in yeast cells and as MO15 in mammalian cells (6, 7, 34). Also, the SRB-mediator holoenzyme subcomplex includes a CDK-cyclin pair encoded by *SRB10* and *SRB11*, respectively (34), which were also identified in genetic screens for suppressors of glucose repression as *SSN3-SSN8* (15) and *GIG2-GIG3* (1). The latter results imply that the Srb10-Srb11 cyclin-CDK pair may be involved in negative regulation of the holoenzyme complex (3). A further possibility is that the Gal4p kinase associates transiently with the holoenzyme complex as part of its regulation. A cyclin-CDK pair which may fall into this category is the pair of proteins encoded by *CTK1* and *CTK2*, which were identified biochemically by their ability to phosphorylate the C-terminal domain of RNA polymerase II (16, 35). Experiments investigating the possible relationship between these protein kinases and Gal4p phosphorylation are currently in progress.

Whatever the nature of the kinase, our results predict that its activity towards Gal4p may be regulated by the availability of fermentable sources of carbon and not specifically by galactose. Both glucose and galactose were found to stimulate Gal4p phosphorylation in *gal80*$^-$ cells, but only galactose stimulates Gal4p phosphorylation in *GAL80*$^+$ cells. These results demonstrate that the kinase which phosphorylates Gal4p may be activated by either glucose or galactose and that Gal80p prevents Gal4p phosphorylation in response to glucose. An alternative possibility is that the Gal4p kinase is constitutively active but the occurrence of Gal4p phosphorylations in medium with galactose or glucose is caused by specific inhibition of a phosphatase. In either case, we suggest that the occurrence of Gal4p phosphorylations does not result from a specific galactose-regulated protein kinase but rather is controlled by the availability of a fermentable source of carbon. Consistent with this prediction, we find that the S699A mutation severely compromises galactose induction in *gal3*$^-$ cells (long-term adaptation) (30), suggesting that phosphorylation at serine 699 occurs independently of the Gal3p- and galactose-specific induction mechanism.

Gal11p has previously been shown to be required for appearance of the most slowly migrating Gal4p phosphorylated species (20). Our data are consistent with this observation and suggest that phosphorylation at serine 699 is particularly dependent upon Gal11p. In *gal80*$^-$ cells, phosphorylation of the WT Gal4p Δ683 derivative is only moderately affected by the absence of Gal11p; there is a noticeable effect only on the most slowly migrating Gal4p Δ683 species in *gal11*$^-$ cells. However, phosphorylation at serine 699, as indicated by examination of the Gal4p Δ683 derivative bearing substitutions of alanine for serine at positions 691, 696, and 837, is significantly impaired by deletion of *GAL11. GAL11* was identified in genetic screens by its requirement for efficient utilization of galactose (37) and as a dominant suppressor of a weak *GAL4* allele (10). Gal11p has been demonstrated to be a component of the RNA polymerase II holoenzyme (2). When produced by expression in *Escherichia coli*, the dominant *GAL11P*-encoded protein, unlike WT Gal11p, interacts with the dimerization region of Gal4p in vitro and thus has been proposed to cause artificial recruitment of the holoenzyme in vivo (2). In this study we found that *GAL11P* causes hyperphosphorylation of Gal4p. It is likely that this hyperphosphorylation results from the enhanced interaction between Gal4p and the holoenzyme by virtue of direct contact with the mutant Gal11Pp.

By what mechanism could the normal function of Gal11p influence Gal4p phosphorylation? One possibility is that Gal4p may normally interact with Gal11p in vivo but this interaction may require a modification on either protein which would not be present when they are produced by expression in *E. coli*. In this scenario, it is possible that the *GAL11P* mutation may suppress the requirement for an in vivo modification, thus generating a dominant phenotype. However, as attractive as this model may be, the fact that Gal11p deleted of 10 amino acids spanning the site affected by the *GAL11P* mutation (Asn 342 to Ile) functions as well as WT Gal11p in vivo tends to suggest that these two proteins do not normally interact. A second possibility is that Gal11p may be involved in regulating the activity of the Gal4p kinase or its interaction with the holoenzyme. Direct interaction between Gal4p and Gal11p would not be necessary for the latter mechanisms. In either case, its requirement for Gal4p phosphorylation may ultimately be informative in elucidation of the function of Gal11p.

Since phosphorylation at serine 699 is unnecessary for transcriptional activation by Gal4p in *gal80*$^-$ cells but is required for induction in cells expressing Gal80p, we suggest that this phosphorylation is involved in regulating interaction between Gal4p and Gal80p. Since Gal4p phosphorylation appears to occur as a consequence of its interaction with general initiation factors, we propose a model in which galactose causes an initial transient alteration in the Gal4p-Gal80p complex, allowing Gal4p to contact its targets in the general initiation complex. We suggest that during initial interaction with holoenzyme-general initiation components, Gal4p acquires its phosphorylations, including that at serine 699. Phosphorylation at serine 699 may stabilize an active conformation of Gal4p-Gal80p and allow unimpeded transcriptional activation by Gal4p's activation domains. This model is supported by the observation that eliminating the phosphorylation site at serine 699 by mutation

does not prevent induction altogether but rather simply makes the induction process slower (Fig. 7 and data not shown).

Given the requirement of serine 699 phosphorylation for Gal4p function, one might think it curious that it is relatively difficult to detect. In fact, a full-length Gal4p derivative bearing a substitution of alanine for serine at position 699, but which is otherwise WT, is indistinguishable from WT Gal4p when recovered from *gal80*$^-$ cells and examined by SDS-PAGE (data not shown). Also, even with the Gal4p Δ683 derivatives, phosphorylation at serine 699 does not typically appear to be as abundant as serine 837 phosphorylation. Only in *GAL11P* cells can we observe a significant phosphorylated species attributable to serine 699 phosphorylation. Therefore, this individual phosphorylation is obscured by the presence of Gal4p's multiple phosphorylations and only becomes obvious in [^{35}S]methionine-labeled deletion derivatives bearing multiple serine-to-alanine mutations (Fig. 6). We argue that a critical regulatory phosphorylation may be more difficult to detect because its presence is likely to be more strictly controlled than phosphorylations which are nonfunctional or which have redundant functions. For example, it may be expected that the presence of a phosphate at serine 699 may be subject to control by a specific phosphatase which catalyzes its removal under conditions in which Gal4p needs to be switched off. Further experiments examining regulation of phosphorylation at serine 699 and identification of the relevant protein kinases will be required to clarify the role of phosphorylation in the regulation of Gal4p function.

ACKNOWLEDGMENTS

We thank John Rohde, J. Martin Hirst, Brendan Bell, Neena Kuriakose, and K. Amy Olson for comments on the manuscript and Y. Brickman for technical assistance. We are also grateful to the late Howard Himmelfarb for providing the *gal11* and *GAL11P* yeast strains.

This research was supported by a grant from the Medical Research Council of Canada. I.S. is a Research Scientist of the National Cancer Institute of Canada.

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