Mutations that increase the activity of a transcriptional activator in yeast and mammalian cells

(GAL4 protein/acidic activator)

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ABSTRACT Activating region I of GAL4 protein is a stretch of amino acids, positioned adjacent to the DNA-binding region, that activates transcription in yeast and, as we show here, in mammalian cells. Here we describe mutations located throughout a 65-amino acid region that increase the activation function of region I. Most of these mutations replace positively charged amino acids in the region with neutral ones, although we also describe substitutions at one position that do not alter the charge of the region. Mutations of region I that alter the activation function in yeast have similar effects on activation when assayed in mammalian cells. When individual mutations that raise the acidity of the activating region are recombined, the activities of the mutant proteins increase with increasing negative charge in both yeast and mammalian cells. These results extend and modify the correlation between acidity and activation and suggest that the requirements for a strong activating region are conserved in yeast and mammals.

GAL4, an 881-amino acid protein, binds to sites in the galactose upstream activation sequence (UAS_G) and stimulates transcription of the divergently transcribed *GAL1* and *GAL10* genes (1–5). Either of two activating regions (region I, amino acids 148–196; region II, amino acids 768–881) stimulates transcription when attached to the DNA-binding domain of GAL4 (residues 1–147); the DNA-binding domain alone fails to activate (6, 7). GAL4 and various GAL4 derivatives also activate transcription in *Drosophila*, plant, and mammalian cells (8–11). In each of these systems, one or more of the activating regions identified in yeast are required for the observed stimulation.

The activating regions of GAL4, like those described for other eukaryotic activators including GCN4 and Herpes virus VP16 (12, 13), are rich in acidic residues, although they share no obvious sequence homology. In fact, a number of different acidic peptides encoded by fragments of *Escherichia coli* DNA function as activating regions when attached to the DNA-binding region of GAL4 protein (14). Acidity, however, is not the sole characteristic of an activating region. Giniger and Ptashne (15) showed that a synthetic peptide designed to form an amphipathic α -helix with negative charges along one surface will activate transcription when fused to the DNA-binding domain of GAL4; a peptide with the same amino acid composition but ordered so that an amphipathic α -helix cannot form, fails to activate. Thus, in general, when comparing activating regions that differ significantly in sequence, and presumably structure, the net negative charge does not strictly correlate with the efficiency of activation (14, 15).

All previously described mutations in activating region I of GAL4 protein that improved the activation function increased acidity of the region, while some, but not all,

mutations that impaired the activation function decreased acidity of the region (16). Here we describe additional mutations that increase the activation function of region I; with the exception of one position at which substitutions do not alter the charge, these mutations increase acidity of the region. Analysis of recombinant proteins bearing multiple amino acid substitutions shows that individual mutations spread over a 65-amino acid region combine nearly additively to improve activation. We show that region I activates transcription in mammalian cells and that proteins bearing single and multiple mutations that are improved for activity in yeast are also more active in mammalian cells.

MATERIALS AND METHODS

Yeast Strains and Media. The yeast strains used each contain a *GAL1-lacZ* fusion integrated at the *URA3* locus of strain GGY1, which is deleted for *GAL4*. Strain GGY1:171 bears UAS_G upstream of the *GAL1-lacZ* fusion, and strain GGY1:SV15 bears a 17-mer upstream of the *lacZ* fusion (16). Yeast cells were made competent for transformation by treatment with lithium acetate (17). Each of the GAL4 derivatives, expressed from the alcohol dehydrogenase promoter, was carried on a single-copy plasmid; pGG22 expressing GAL4(1–881) and pGG23 expressing GAL4(1–238) are as described (16). β -galactosidase assays were performed as described (16). Yeast were grown in selective medium containing 2% galactose, 3% glycerol, and 2% lactic acid (pH 6). The β -galactosidase activities reported are the mean of at least three assays; the standard deviation was always <20%. Activities of the mutant proteins were normalized to the activity of unmutagenized GAL4(1–238) assayed in parallel.

Immunoprecipitations. Five-milliliter cultures of yeast grown in selective media were harvested, washed twice in medium lacking methionine, and labeled for 60 min with 350 μ Ci (1 Ci = 37 GBq) of [³⁵S]methionine in methionine-free minimal medium supplemented with 2% galactose, 3% glycerol, and 2% lactic acid. Labeled cells were washed twice in cold lysis buffer (50 mM Tris, pH 8.0/5 mM MgCl₂/150 mM NaCl) and lysed by mixing with glass beads in 400 μ l of the same buffer. An equal volume of 2 $\times$ RIPA buffer (1 $\times$ RIPA is 10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) was added; the samples were mixed briefly and clarified by centrifugation at 10,000 $\times$ g for 30 min. Supernatants were immunoprecipitated with rabbit anti-GAL4(1–147), as described (18).

Abbreviations: UAS_G, galactose upstream activating sequence; CAT, chloramphenicol acetyltransferase; CHO, Chinese hamster ovary.

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Chloramphenicol Acetyltransferase (CAT) Assays. Five micrograms each of the reporter (pG5BCAT, ref. 19) and effector plasmids and 1 μ g of a β -galactosidase internal control plasmid (pCH110, ref. 20) were transfected into Chinese hamster ovary (CHO) cells by the DEAE-dextran technique. Forty-eight hours posttransfection cells were collected and assayed for CAT activity as described (21). The acetylated forms of chloramphenicol were separated by thin-layer chromatography, and after autoradiography, the spots were excised and quantitated by liquid scintillation counting. The effector plasmids express GAL4 and GAL4 derivatives from the simian virus 40 promoter. GAL4-derivative coding sequences were transferred from their respective yeast vectors into the pECE plasmid (21, 22).

Mutagenesis. All but six of the mutations described here were generated by random mutagenesis and screened in yeast as described in ref. 16. Substitutions of Lys-212 with glutamic acid and glutamine and substitution of Ile-228 with serine, tyrosine, aspartic acid, and glutamic acid were generated by site-directed mutagenesis (23). Mutagenic primers (purchased from Operon) were hybridized to uracil-substituted single-stranded DNA and extended *in vitro* with Klenow fragment. Incorporation of the correct mutation was verified by dideoxy chain-termination sequencing. All the region I mutations are contained within a *Cla* I-*Xba* I fragment (residues 148–236). Mutations were recombined using restriction fragments generated by cutting with either *Cla* I or *Xba* I and the restriction enzymes *Ava* II (cuts at position 189) or *Hpa* I (cuts at position 221).

RESULTS

To characterize the essential features of activating region I of GAL4 we screened in yeast for mutants of GAL4(1–238) improved for activation. As described earlier (16), we ligated a mutagenized DNA fragment encoding region I (residues 148–236) to an unmutagenized fragment encoding the DNA-binding region, GAL4(1–147). The randomly mutagenized pools were introduced into a yeast strain bearing an integrated *GAL1-lacZ* fusion. Cells bearing derivatives of GAL4(1–238) that were improved for activation formed darker blue colonies than the wild type on 5-bromo-4-chloro-3-indolyl β -D-galactosidase (X-gal) indicator plates. Table 1 shows the β -galactosidase activity stimulated by each

Table 1. Improved activators with single amino acid changes

Protein	UAS _G	17-mer
GAL4(1–881)	3320	710
GAL4(1–238)	710	60
GAL4(1–238)		
Arg-166→Thr	970	120
Arg-166→Met	1090	200
Arg-166→Trp	1085	230
His-170→Leu	960	99
His-170→Tyr	1283	258
Lys-188→Asn	1535	160
Arg-207→Pro	1284	236
Lys-212→Gln	929	169
Lys-212→Glu	1440	308
Arg-231→Ser	1030	160

Improved activators bearing single amino acid substitutions that increase the negative charge of activating region I. Single-copy plasmids encoding the indicated GAL4 derivatives were introduced into Δ gal4 yeast bearing an integrated *GAL1-lacZ* fusion gene with either UAS_G (bearing four GAL4-binding sites) or the near-consensus 17-mer GAL4-binding site upstream. β -Galactosidase activities were determined as described. Each mutation, with the exception of substitutions at position 212, was isolated from the randomly mutagenized pool as described in ref. 16.

FIG. 1. Mutations (in one-letter code) that increase activity of GAL4(1–238) do not increase abundance. Single-copy plasmids expressing each of the indicated GAL4(1–238) derivatives were introduced into a Δ gal4 yeast strain. The GAL4(1–238) derivatives were metabolically labeled with [³⁵S]methionine and immunoprecipitated with anti-GAL4(1–147) antiserum. The sample volume loaded on a polyacrylamide gel was normalized relative to background bands in the immunoprecipitation. We believe the aberrant mobility of the protein bearing three amino acid substitutions is a direct consequence of those substitutions (substitutions that alter mobility without affecting posttranslational modification have been described; ref. 24); the mobility of this derivative is unaffected by treatment with phosphatase (data not shown).

of the mutant proteins in yeast from a *GAL1-lacZ* fusion bearing upstream either a single near-consensus GAL4-binding site (the 17-mer) or the UAS_G (which contains four GAL4-binding sites). Several of these mutants have been described and are included for comparison (16).

All mutations described in Table 1 increase both the activity and the negative charge of activating region I. Each single amino acid substitution in Table 1 (except substitutions at position 212; see below) increases the net negative charge of GAL4(1–238) from -7 to -8 by replacing a positively charged amino acid, arginine or lysine, with a neutral one; this results in an average 3-fold increase in activity when the mutant is assayed on a single site.

Our random mutagenesis did not yield any substitutions of lysine-212, and we therefore used site-directed mutagenesis to introduce either glutamine or glutamic acid at this position. As shown in Table 1, substitution of Lys-212 with glutamine, which increases net negative charge by -1 , increased the activity 3-fold as assayed on a single site. Introducing a negatively charged residue at this position further increased activation; substitution of Lys-212 with glutamic acid, which increases the charge by -2 , results in an activator 5-fold more active than wild-type GAL4(1–238). We do not believe that these mutations simply increase the abundance or stability of GAL4(1–238) because none of the mutant proteins examined was significantly overexpressed as determined by immunoprecipitation analysis (see Fig. 1).

Table 2. Improved activators with no increase in negative charge

Protein	UAS _G	17-mer
GAL4(1–881)	3320	710
GAL4(1–238)	710	60
GAL4(1–238)		
Ile-228→Thr	1637	136
Ile-228→Ser	1460	140
Ile-228→Tyr	1086	204
Ile-228→Asp	1621	293
Ile-228→Glu	1880	297

Improved activators bearing substitutions that do not affect the net charge of the activating region. Single-copy plasmids encoding the indicated GAL4 derivatives were introduced into Δ gal4 yeast bearing an integrated *GAL1-lacZ* fusion gene with either UAS_G or the near-consensus 17-mer GAL4-binding site upstream. β -Galactosidase activities were determined as described.

Table 3. Improved activators with multiple amino acid changes

Protein	UAS _G	17-mer
GAL4(1-881)	3320	710
GAL4(1-238)	710	60
GAL4(1-238)		
Arg-166→Trp and Lys-188→Gln	1948	380
His-170→Leu and Arg-207→Pro	1628	276
Arg-166→Trp, Lys-188→Gln, and Arg-207→Pro	2484	477
Arg-166→Trp, Lys-188→Gln, Arg-207→Pro, and Arg-231→Ser	2566	563
Arg-166→Trp, Lys-188→Gln, Arg-207→Pro, Lys-212→Gln, and Arg-231→Ser	2020	572
Arg-166→Trp, Lys-188→Gln, Arg-207→Pro, Lys-212→Glu, and Arg-231→Ser	2350	556

Improved activators bearing multiple amino acid substitutions that increase the negative charge of activating region I. Single-copy plasmids encoding the indicated GAL4 derivatives were introduced into *Δgal4* yeast bearing an integrated *GAL1-lacZ* fusion gene with either UAS_G or the near-consensus 17-mer GAL4-binding site upstream. β -Galactosidase activities were determined as described.

We have also isolated substitutions at one position in activating region I that increases the activity without altering the net charge. A mutant protein bearing an Ile→Thr substitution at position 228 was isolated from the randomly mutagenized pool; we introduced other substitutions at position 228 by site-directed mutagenesis. As shown in Table 2, mutant proteins in which Ile-228 has been replaced by threonine, serine, or tyrosine activated transcription 2- to 3-fold better than wild-type GAL4(1-238) as assayed on a single site. Substitution of Ile-228 by the acidic amino acids glutamic acid or aspartic acid increased activation 5-fold; the activity of these mutant proteins is similar to that of proteins in which the net charge is increased by -2. Immunoprecipitation analysis of mutant proteins bearing substitutions at position 228, shown in Fig. 1, does not reveal any significant

difference in abundance or electrophoretic mobility compared with unmutagenized GAL4(1-238).

When independently isolated mutations were recombined into a single protein, the activity in yeast increased with increased negative charge (see Table 3; summarized in Fig. 3). For example, although substituting Arg-166 with tryptophan improved activation $\approx$ 4-fold and replacing Lys-188 with asparagine increased activation $\approx$ 3-fold, a mutant protein bearing substitutions at both positions 166 and 188 activated transcription 6-fold better than wild type, as assayed on a single site. Replacing Arg-207 with proline in this double mutant raised the activity further, to 8-fold better than wild type. And GAL4(1-238) bearing four amino acid substitutions that increase the negative charge from -7 to -11 was 9-fold more active than wild-type GAL4(1-238) and 80% as active as full-length GAL4(1-881) (25). Further increasing the negative charge of region I by substituting glutamine or glutamic acid for Lys-212 in the quadruple mutant protein did not further increase the activity.

The data in Tables 1-3 show that each mutant protein is increased for activity as measured both on the UAS_G and the 17-mer, although there is no simple correlation between the activities measured in these two assays. GAL4 binds cooperatively to the four sites in UAS_G (26); presumably, interactions between GAL4 molecules (direct or indirect) account for the variation in activity between the 17-mer and the UAS_G (27).

Fig. 2 shows that mutations of activating region I that alter the activation function in yeast have similar effects on activation in mammalian cells. Plasmids expressing each of the indicated GAL4 derivatives were cotransfected into CHO cells with a reporter plasmid bearing five GAL4-binding sites upstream of a minimal adenovirus E1b promoter expressing the CAT-encoding gene. The CAT activity stimulated by each of the GAL4 derivatives is shown in Fig. 2. A mutation that replaces Asp-199 with tyrosine reduced activation $\approx$ 40-fold in yeast (16) and, as shown in Fig. 2, severely impaired activation in CHO cells. Substituting the positively charged residue Lys-212 with glutamine improved activation $\approx$ 2-fold in mammalian cells.

FIG. 2. Increased negative charge of activating region I increases activation in CHO cells. Each indicated GAL4 derivative was cotransfected into CHO cells with a reporter bearing five near-consensus GAL4-binding sites upstream of the adenovirus E1B minimal promoter expressing CAT. CAT activities stimulated by each GAL4 derivative are shown. The CAT assays were normalized for variations in transfection efficiency by comparison with an internal control plasmid expressing β -galactosidase (data not shown). The double mutant in GAL4(1-238) is Arg-166→Trp, Lys-188→Gln. The triple mutant is Arg-166→Trp, Lys-188→Gln, Arg-207→Pro. The quadruple mutant in GAL4(1-238) is Arg-166→Trp, Lys-188→Gln, Arg-207→Pro, and Arg-231→Ser. The quintuple mutant is Arg-166→Trp, Lys-188→Gln, Arg-207→Pro, Lys-212→Gln, and Arg-231→Ser. Activity of each of these mutants in yeast is shown in Tables 1 and 3, and the activities in both yeast and mammalian cells are summarized in Fig. 3. Immunoprecipitation analysis suggests that the failure of the Asp-199→Tyr mutant protein to activate transcription is not due to decreased protein levels (data not shown).

Moreover, as shown in Fig. 2 and summarized in Fig. 3, when individual mutations that increase the negative charge of region I are recombined into a single protein, the activity increased with increased negative charge in mammalian cells as well as in yeast. The relative activities of the region I mutants in both yeast and mammalian cells versus the net negative charge of the activating region are plotted in Fig. 3. In both systems there is an overall increase in activity with increased negative charge. For example, a mutant in which four positive charges in region I have been replaced with uncharged residues activated transcription better than the triple mutant in both yeast and mammalian cells. There are, however, differences in the behavior of particular activators in the two systems. For example, as mentioned above, substituting glutamine or glutamic acid for Lys-212 in the quadruple mutant failed to further increase activity in yeast; the additional mutation did, however, increase the activity in mammalian cells. In CHO cells, the quintuple mutant of GAL4(1-238) that bears a net negative charge of -12 activated about 8-fold better than wild-type GAL4(1-238) and 95% as well as intact GAL4.

DISCUSSION

Activating region I of GAL4 is rich in charged amino acids, containing 7 positively and 14 negatively charged residues over a stretch of 90 amino acids. Taken with our previous work (16), we have now isolated from a randomly mutagenized pool nine mutants bearing single amino acid substitutions that increase the activity of this region in GAL4(1-238). At all but one of the six positions altered in our mutants, the mutation increases the negative charge of the region by replacing a positively charged residue, arginine or lysine, with a neutral one. Because replacing Arg-166 with any of three different neutral residues improved activation, it appears that the increase in activity results from loss of the positively charged residue. At one position where substitution of a basic residue (Lys-212) by a neutral residue increased activity, introduction of an acidic residue by site-

FIG. 3. Activity of GAL4 region I increases with increasing negative charge. The activity of various GAL4(1-238) derivatives in both yeast and mammalian cells versus the net negative charge of the activating region is plotted. The β -galactosidase activity stimulated from a *GAL1-lacZ* fusion with the 17-mer upstream, and the percent acetylation in the CAT assays are each expressed as percent of wild-type GAL4(1-881) activity. The β -galactosidase data are shown in Tables 1 and 3. CAT activities were determined in an experiment parallel to that shown in Fig. 2, but assay time was reduced. The net negative charge of region I is taken as (Asp + Glu) - (Arg + Lys). Because their pK_a is strongly influenced by the local environment, histidine residues were not considered positively charged in determining the net charge of activating region I. There are three histidines in region I, at least one of which, His-170, may be positively charged because removal of this particular residue improves activation (see Table 1).

directed mutagenesis increased activity still further. As noted earlier (16), none of the mutants isolated from the randomly mutagenized pool substituted a negatively charged residue for a neutral one, a finding that is not obviously attributable to bias in the mutagenesis procedure (28). Perhaps only those negatively charged residues that are appropriately positioned in the activating region contribute to function, and a more general requirement for a strong activating region is the absence of positively charged residues.

The bacteriophage λ repressor uses an acidic amphipathic α -helix to stimulate transcription (29-31). A recent study of the activation α -helix of λ repressor revealed that at one position an acidic residue (Glu-34) is essential for activation (32). At the three remaining solvent-exposed positions of the activating helix, negatively charged residues favor activation, although certain noncharged residues work equally well. Positively charged amino acids interfere with efficient activation by λ repressor, just as they do for region I of GAL4.

We also isolated from the randomly mutagenized pool a substitution at position 228 that improves activation without altering the net charge of the activating region, Ile $\rightarrow$ Thr. Using site-directed mutagenesis, we replaced the isoleucine at position 228 with serine and tyrosine, both of which increase activation, and glutamic acid and aspartic acid, which increased activation still further. These mutations could allow the region to adopt a conformation more favorable for activation. Alternatively, the substituted residues at position 228 could directly participate in the activation reaction, perhaps by forming hydrogen bonds with the target. The observation that replacing Ile-228 with a negatively charged residue improves activation more than substitution with a neutral residue may suggest that amino acid 228 is positioned to interact with the target. In contrast to an earlier suggestion (27), we do not believe that the proteins bearing substitutions at position 228 are differentially modified.

Perhaps our most striking result is that when single amino acid substitutions that increase the activity of activating region I are combined, they contribute nearly additively to improve activation. Comparison of these region I derivatives, which differ only by single-amino acid changes, shows a strong correlation between the net negative charge of the activating region and the efficiency of activation. Thus, up to five amino acid substitutions, located throughout a 65-amino acid region, each of which increases the negative charge of the activating region can be combined to generate progressively better activators. This result suggests that the activating region includes a large and, perhaps, flexible surface that can interact with the target. GCN4 proteins bearing deletions in the activating region show a stepwise loss of activity that is also consistent with a large activating region, many parts of which can contribute to function (33).

Our mutants of GAL4(1-238), isolated in yeast, also alter the activity of the protein in mammalian cells. In particular, the activity of proteins bearing multiple mutations increases with increased negative charge in mammalian cells, as in yeast. The activating regions of GAL4 have been proposed to activate transcription by interacting with a protein component of the transcription machinery (27, 34, 35). Both RNA polymerase and the TATA-binding factor, which are highly conserved between yeast and mammalian cells, have been proposed as targets (27, 35-39). Our results suggest that the surface of the target that interacts with, at least, certain activators must be very similar in yeast and mammalian cells.

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