Mutations that alter both localization and production of a yeast nuclear protein

Pamela A. Silver, Annette Chiang, and Ingrid Sadler

Department of Biology, Princeton University, Princeton, New Jersey 08544 USA

The first 74 amino acids of the yeast GAL4 gene product are sufficient to localize a GAL4-β-galactosidase chimeric protein to the yeast nucleus. Chimeric proteins missing the first 74 GAL4 amino acids, but containing almost all of the rest of GAL4, are not localized to the nucleus and are expressed at higher levels than their nuclear counterparts. On this basis, point mutations within GAL4, which reduce nuclear localization and increase production of a normally nuclear GAL4-β-galactosidase fusion protein, were isolated and sequenced. The effect of these mutations on the localization and expression of the intact GAL4 protein was examined. The degree to which the mutant proteins are excluded from the nucleus varies, but all mutations cause overproduction of the protein. Point mutations altering two of the six cysteine residues of the GAL4 putative 'zinc finger' abolish gene activation by intact GAL4; however, mutations in nearby residues have no effect on GAL4-dependent gene activation.

[Key Words: GAL4; nuclear targeting; zinc fingers; protein localization]

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Nuclear proteins contain stretches of amino acids responsible for their localization to the nucleus (for review, see Silver and Hall 1988). At least two consensus nuclear targeting sequences have been proposed (Hall et al. 1984; Kalderon et al. 1984). Affinity for DNA alone appears to be neither necessary nor sufficient for nuclear protein targeting (Paucha et al. 1985; Silver et al. 1986), arguing that proteins do not enter the nucleus by diffusion through the pore and remain due to interactions with DNA. Instead, recent studies indicate that nuclear protein uptake is a saturable process and can be competed by an excess of a specific nuclear targeting peptide (Goldfarb et al. 1986; Lanford et al. 1986). These results suggest that nuclear protein uptake is a receptor-mediated process.

By using gene fusions, we have previously shown that the first 74 amino acids of the GAL4-gene product are sufficient to localize a GAL4-β-galactosidase chimeric protein to the nucleus of the yeast Saccharomyces cerevisiae [Silver et al. 1984]. The GAL4-gene product is an 881-amino-acid DNA-binding protein (Laughon and Gesteland 1982; Giniger et al. 1985) that activates the genes involved in galactose metabolism [Douglas and Hawthorne 1972, St. John and Davis 1981]. The information for specific DNA binding is also contained in the first 74 GAL4 amino acids [Keegan et al. 1986]. However, substitution of the first 74 GAL4 amino acids with a prokaryotic DNA-binding protein does not result in nuclear localization [Silver et al. 1986]. Moreover, a chimeric protein containing GAL4 amino acids 79-752 fused to β-galactosidase is excluded from the nucleus, demonstrating that the first 74 GAL4 amino acids are also necessary for nuclear localization. We also observed that nonnuclear GAL4-β-galactosidase fusion proteins were produced at levels 10-fold higher when compared to nuclear GAL4-β-galactosidase proteins [Silver et al. 1984].

We now report the isolation and characterization of a new class of GAL4-linked point mutations that affect both the production and the nuclear localization of a chimeric GAL4-β-galactosidase and intact GAL4 protein.

Results

Identification of single amino acid changes in GAL4

We have isolated mutations within the GAL4 segment of a GAL4–lacZ hybrid gene that lead to the overexpression of this gene fusion. A plasmid carrying the gene fusion encoding the first 74 GAL4 amino acids fused to almost all of Escherichia coli β-galactosidase [Fig. 1] was mutagenized and introduced into yeast, and transformant colonies were screened for their levels of β-galactosidase expression on X-gal indicator plates [for details of the mutagenesis, see Materials and methods]. A number of mutants were identified that expressed elevated levels of β-galactosidase [Table 1]. All those linked to the plasmid involved amino acid changes within the GAL4 segment of the plasmid and were identified by DNA sequencing.

Mutations affect nuclear localization of GAL4-β-galactosidase

Fusion of the first 74 GAL4 amino acids to β-galactosi-
lacZ
ADH1
GAL4

Figure 1. Diagram of plasmid PS118 containing the GAL4-lacZ gene fusion under the control of the ADH1 promoter (P\textsubscript{ADH}) (open arrow). DNA encoding the first 74 amino acids of GAL4 is contained between the HindIII and XhoI sites. The truncated GAL4 gene was ligated to lacZ (open line) at a site corresponding to amino acid 7 of β-galactosidase (Silver et al. 1984). The ADH–GAL4-lacZ gene fusion is contained between the BamHI and SalI sites of the yeast 2-μm vector YEp213 (Sherman et al. 1983).

dase results in complete nuclear localization of β-galactosidase as determined by immunofluorescence [Fig. 2A–C; Silver et al. 1984]. On the other hand, intact β-galactosidase is not localized to any intracellular compartment when subjected to a similar analysis [Fig. 2D–E; Moreland et al. 1987].

Mutations causing increased expression of GAL4-β-galactosidase also affect the degree of nuclear localization of the protein to varying degrees. Cells expressing mutant forms of GAL4-β-galactosidase were inspected by immunofluorescence with anti-β-galactosidase antibodies. The patterns of immunofluorescence differed for each mutant protein. However, two general types of staining patterns with respect to the nucleus were observed. Conversion of serine 6 to phenylalanine resulted in the first and most extreme pattern of staining (Fig. 2F–G); there was almost a complete failure of the immunofluorescence signal throughout the cell and somewhat concentrated at the cell periphery. The remaining eight mutant proteins gave staining patterns of the second general type; the immunofluorescence appeared throughout the cell and somewhat concentrated at the cell periphery. The two most extreme effects were seen by conversion of cysteine 38 to serine, which resulted in only a 1.6-fold increase in activity, and conversion of lysine 20 to glutamate, which resulted in a 15-fold increase in activity, as compared to the normal protein. Mutations at GAL4 amino acid glutamate 8, cysteine 21, and tyrosine 40 caused approximately a threefold increase in β-galactosidase activity. Mutations at amino acids serine 6, arginine 51, and histidine 53 resulted in about a 6-fold increase in activity, and the mutation at amino acid lysine 43 resulted in a 13-fold increase in the amount of β-galactosidase activity.

The amounts of wild-type and mutant GAL4-β-galactosidase proteins synthesized during a 30-min period varied in proportion to their β-galactosidase activities. Cells producing normal or mutant forms of GAL4-β-galactosidase were radioactively labeled for 30 min and lysed immediately, and the resulting cell lysate was immunoprecipitated with anti-β-galactosidase. A radioactively labeled band of protein of the expected size [124 p-Galactosidase units] was de-

Table 1. Single amino acid changes in GAL4 that alter Gal-β-galactosidase nuclear localization and expression

| GAL4 amino acid change | β-Galactosidase units | Complementation |
|------------------------|-----------------------|-----------------|
|                        | multicopy chromosomal | of gal4         |
| None                   | 35                    | 6               | +               |
| Ser-6 to Phe           | 207                   | 11              | +               |
| Glu-8 to Lys           | 108                   | 4               | +               |
| Lys-20 to Glu          | 515                   | 13              | +               |
| Cys-21 to Arg          | 93                    | 9               | +               |
| Cys-38 to Ser          | 59                    | 1               | +               |
| Tyr-40 to His          | 130                   | N.D.            | +               |
| Lys-43 to Glu          | 461                   | N.D.            | +               |
| Agr-51 to Gly          | 221                   | 4               | +               |
| His-53 to Pro          | 197                   | N.D.            | +               |

β-Galactosidase activity was determined for DB745 transformed with P\textsubscript{ADH}–GAL4-lacZ, normal or mutant, on a multicopy plasmid or integrated into the chromosome (see Materials and methods), s.e. 15–23%. Complementation of gal4 was determined in ML171 by the ability to induce GAL1–lacZ on Xgal indicator plates containing galactose. (N.D.) Not determined.
Nuclear protein localization

Figure 2. Immunofluorescence of cells producing normal and mutant forms of GAL4-β-galactosidase. Cells were prepared for immunofluorescence and treated with a mouse anti-β-galactosidase monoclonal antibody, followed by FITC-conjugated anti-mouse IgG, to localize the LacZ fusion proteins (Panels A,D,F,H,J,L,N,P,R,T,V) and DAPI to stain the cell DNA (Panels B,E,G,I,K,M,O,Q,S,U,W). [Panels A–C] Cells producing normal GAL4_{1,-741}β-galactosidase. C shows the same cells viewed by phase contrast. [Panels D–E] Cells producing intact β-galactosidase. The remaining panels show immunofluorescence and DAPI stains of cells producing GAL4_{1,-741}β-galactosidase with the following amino acid changes: [F,G] Ser 6 to Phe; [H,I] Glu 8 to Lys; [J,K] Lys 20 to Asp; [L,M] Cys 21 to Arg; [N,O] Cys 38 to Ser; [P,Q] Tyr 40 to His; [R,S] Lys 43 to Asp; [T,U] His 53 to Pro; [V,W] Arg 51 to Gly. Cells were prepared for immunofluorescence as described in Materials and methods. Exposure times for all immunofluorescence pictures were equal.
kD) was observed for cells producing normal GAL4-β-galactosidase (Fig. 3A, lane 2). Cells producing no LacZ fusion proteins showed no labeled protein in the corresponding region of the autoradiographed gel (Fig. 3A, lane 1). Cells producing mutant forms of GAL4-β-galactosidase all showed radioactively labeled proteins of the correct size (Fig. 3A, lanes 3–11). Moreover, there were no labeled proteins of lower molecular size, except for those nonspecifically and variably bound to the antibody–Staphylococcus aureus complex. Over the 30-min labeling period, cells producing proteins mutated at GAL4 amino acids lysine 20 (Fig. 3A, lane 5) and lysine 43 (Fig. 3A, lane 9) produced the highest amount of radioactively labeled protein, as compared to the wild-type protein (Fig. 3A, lane 2). Cells producing proteins with mutations at serine 6 (Fig. 3A, lane 3), tyrosine 40 (Fig. 3A, lane 4), histidine 53 (Fig. 3A, lane 11) (for identification of the new amino acids, see Table 1). (Lane 12) Immunoprecipitation from cells producing intact β-galactosidase. Lower molecular weight bands are proteins that bind nonspecifically to the antibody–S. aureus complex and are precipitated to variable extents.

Figure 3. Mutations in GAL4 affect the production of GAL4-β-galactosidase. (A) Relative synthesis rates of GAL4-β-galactosidase. Cells were pulse labeled with SO4\(^{2-}\) for 30 min and assayed immediately for GAL4-β-galactosidase by immunoprecipitation. Analyses were performed from cells transformed with YEp213, which does not encode lacZ (lane 1); cells transformed with PS118 (Fig. 1), which encodes normal GAL4\(_{174}\)-β-galactosidase (lane 2); or cells bearing gene fusions encoding amino acid changes at GAL4 amino acids Ser6 (lane 3), Glu 8 (lane 4), Lys 20 (lane 5), Cys 21 (lane 6), Cys 38 (lane 7), Tyr 40 (lane 8), Lys 43 (lane 9), and Arg 51 (lane 10), His 53 (lane 11) (for identification of the new amino acids, see Table 1). (Lane 12) Immunoprecipitation from cells producing intact β-galactosidase. Lower molecular weight bands are proteins that bind nonspecifically to the antibody–S. aureus complex and are precipitated to variable extents. (B) Degradation rates of GAL4-β-galactosidase. The rate of degradation of normal and mutated GAL4\(_{174}\)-β-galactosidase was assayed by pulse labeling transformed cells for 30 min with \(^{35}\)SO\(^{4}\) and chasing with unlabeled SO\(^{4}\)\(^{2-}\), precisely as described in Materials and methods. Analyses were performed from cells transformed with PS118 (Panel 1) or cells bearing gene fusions encoding amino acid changes at GAL4 amino acids Ser 6 (Panel 2), Lys 20 (Panel 3), and Arg 51 (Panel 4). Aliquots from each culture were removed and analyzed by immunoprecipitation with anti-β-galactosidase antibody at 0, 4, and 15 hr after the addition of the unlabeled SO\(^{4}\)\(^{2-}\), as indicated at the bottom of the panels. Cells transformed with a plasmid producing intact β-galactosidase were analyzed at 0 and 15 hr after the addition of unlabeled SO\(^{4}\)\(^{2-}\) (Panel 5). The positions of protein standards (kD) are shown at the side of each gel.
3A, lane 8), arginine 51 (Fig. 3A, lane 10), and histidine 53 (Fig. 3A, lane 11) showed slightly higher levels of labeled protein, as compared to cells producing proteins with mutations at glutamate 8 (Fig. 3A, lane 4), cysteine 21 (Fig. 3A, lane 6), and cysteine 38 (Fig. 3A, lane 7), and all produced more protein than cells producing the unmutated protein (Fig. 3A, lane 2).

Overproduction is not due to increased protein stability or plasmid copy number

The relative rate of degradation of several of the mutant proteins was not significantly different from the normal GAL4-β-galactosidase, and all the proteins appeared to be very stable. The rate of turnover of the normal and mutant proteins was measured by radioactively labeling cells that expressed the fusion proteins for 30 min and then chasing with unlabeled sulfate for up to 15 hr. In preliminary experiments, it was determined that the half-life of the fusion proteins was >2 hr. Lysates were prepared from cells immediately and at 4 and 15 hr after the addition of the chase. The amount of radioactively labeled GAL4-β-galactosidase present was determined by immunoprecipitation with anti-β-galactosidase antibody. At 4 hr after the addition of the chase, approximately one half of the unmutated GAL4-β-galactosidase was still present, and by 15 hr the protein was almost completely degraded (Fig. 3B, panel 1). A similar pattern of degradation was observed for proteins mutated at serine 6 (Fig. 3B, panel 2), lysine 20 (Fig. 3B, panel 3), and arginine 51 (Fig. 3B, panel 4); approximately one half of the labeled protein remained after 4 hr of chase. Experiments with the other mutant proteins yielded similar results (data not shown). On the other hand, intact β-galactosidase was even more stable than any of the GAL4-β-galactosidase hybrids; almost all of the labeled protein remained even after 15 hr of chase (Fig. 3B, panel 5).

The differences between mutant and parental gene fusions in β-galactosidase levels lessened significantly when GAL4-β-galactosidase was produced from a single copy of the gene integrated into the chromosome at URA3. Cells containing a single copy of GAL4-lacZ produced 6 units of β-galactosidase activity (Table 1). Cells containing a single copy of gene fusions encoding changes at amino acids lysine 20, serine 6, and cysteine 21 caused a relative increase of 2-, 2-, and 1.5-fold, respectively, and the other mutants showed no detectable increase. In contrast, on multicopy plasmids, these same mutations caused increases of 15-, 6-, and 3-fold, respectively. Cells producing the protein with a mutation at cysteine 38 showed a significant decrease in the level of β-galactosidase activity. If the difference in activity observed when the fusion proteins were expressed from multicopy plasmids was due to decreased protease sensitivity of the mutant proteins, one would not expect this difference to be affected by the gene copy number. This is consistent with the finding that the half-lives of the parental and mutant fusion proteins are approximately the same (see above).

The overproduction of mutant GAL4-β-galactosidase cannot be accounted for by an increased plasmid copy number. The amount of wild-type plasmid (PS118) relative to the chromosomal URA3 DNA did not fluctuate by more than a factor of two when DNA was prepared from different transformants (Fig. 4, lanes 2–4). DNA prepared from cells bearing mutants that gave the highest level of overproduction (Fig. 4, lanes 4–9) showed less than a twofold difference in the amount of plasmid relative to the wild-type plasmid. However, the patterns of localization of the fusion proteins remained identical to that of proteins produced from multicopy plasmids (data not shown), except that the intensity of the immunofluorescence staining was reduced.

Effect of mutations on GAL4 expression and activity

All mutations were placed into intact GAL4. Lysates from yeast cells overproducing intact GAL4 from the ADH1 promoter were examined by immunoblot analysis with anti-GAL4 antibody. As visualized in this way, GAL4 protein was modified to an apparently higher molecular weight form. Two bands specifically reacted with anti-GAL4 (Fig. 5, lane 5). The faster migrating band corresponded to a protein with an apparent molecular weight of 99,000 and comigrated with intact GAL4 extracted from a strain of E. coli that overproduces GAL4 (Fig. 5, lanes 2 and 15). An apparent molecular weight of 99,000 is in good agreement with the calculated molecular weight of 99,350 for GAL4 (Laughon and Gesteland 1982). The slower migrating band (Fig. 5, lane 5) corre-
Figure 5. Mutations affect the level of intact GAL4 protein. Cell extracts from DB745 containing plasmids encoding normal and mutated GAL4 and E. coli JM107, without or with pLK76-7, were subjected to SDS–PAGE (7%) gel. Proteins were transferred to nitrocellulose and probed with rabbit anti-GAL4 antibody and goat anti-rabbit IgG conjugated with horseradish peroxidase. [Lane 1] JM107, [lanes 2 and 15] JM107/pLK76-7, [lane 3] DB745/YEp213, [lane 4] DB745 transformed with a plasmid expressing GAL4 from its own promoter, [lane 5] DB745 transformed with a plasmid causing the overproduction of GAL4 from the ADH promoter. The remaining lanes contain lysates prepared from DB745 transformed with plasmids that cause the overproduction from the ADH promoter of GAL4 with amino acid changes at Cys 38 (lane 6), Arg 51 (lane 7), Ser 6 (lane 8), Glu 8 (lane 9), Lys 20 (lane 10), Lys 43 (lane 11), His 53 (lane 12), Cys 21 (lane 13), and Tyr 40 (lane 14). The positions of protein standards (kD) are shown at the side of the gel. Proteins corresponding to molecular weights of 80,000 and 61,000 reacted nonspecifically with the anti-GAL4 antiserum by two criteria; both proteins were still observed when a strain deleted for GAL4 was similarly examined, and they were not observed when affinity-purified anti-GAL4 antibody was used to probe a similar blot.

Some mutations affect localization of intact GAL4 protein

Intact GAL4 protein is localized exclusively to the cell nucleus. Cells producing intact GAL4 protein from the ADH1 promoter on a multicopy plasmid were examined by immunofluorescence, using an anti-GAL4 antibody [Fig. 6A–C]. About 25% of the cells showed faint nuclear-associated immunofluorescence. Cells not overproducing GAL4 did not stain with the antibody [Fig. 6P], even though the strain was GAL4 and, thus, produced a small amount of GAL4 protein.

Mutations in the first 74 GAL4 amino acids affected normally nuclear localization of intact GAL4 to varying
Figure 6. Localization by immunofluorescence of normal and mutant GAL4 protein. DB745 cells transformed with plasmids encoding intact normal GAL4 [panels A–C], mutated forms of GAL4 [panels D–O], or no GAL4 [panel P] were prepared for immunofluorescence, as described in Materials and methods, and treated with an affinity-purified rabbit anti-GAL4 antibody followed by FITC-conjugated anti-rabbit IgG [panels A, D, I, L, M, P] and DAPI [panels B, E, H, K, M, O]. Cells producing mutations at Lys 20 [panels D–I], Lys 43 [panels G–I], Cys 38 [panels J–K], His 53 [panels L–M], and Arg 51 [panels N–O]. Cells containing normal GAL4 and GAL4 with mutations at Lys 20 and Lys 43 are viewed by phase contrast in panels C, F, and I, respectively.
degrees. GAL4 mutated at lysine 20 appeared still mainly nuclear localized (Fig. 6D–F), although the intensity of immunofluorescence was increased, as compared to the staining seen with normal GAL4 (cf. Fig. 6D and A). GAL4 mutated at lysine 43 showed mainly nuclear-associated immunofluorescence with some increase in cytoplasmic staining, as compared to cells producing normal GAL4. Cells producing intact GAL4 mutated at cysteine 38 (Fig. 6J–K), histidine 53 (Fig. 6L–M), and arginine 51 (Fig. 6N, O) showed an even greater increase in the amount of cytoplasmic immunofluorescence. GAL4 mutated at cysteine 21 also showed a faint increase in nonnuclear staining, but the signal was too faint to be adequately reproduced photographically. GAL4 mutated at serine 6, glutamate 8, and tyrosine 40 could not be seen at all by immunofluorescence.

Discussion

We have identified nine point mutations within the first 74 codons of GAL4 that result in single amino acid changes. Changes between GAL4 amino acids 6 and 53 affect both the degree of nuclear localization and the level of production of a GAL4-β-galactosidase fusion protein.

Within intact GAL4, these mutations have similar effects. Mutant GAL4 proteins are overproduced, as are mutant GAL4-β-galactosidase proteins. Some mutant GAL4 proteins are also mislocalized, although to a lesser extent than the corresponding GAL4-β-galactosidase protein. The presence of GAL4 amino acids 75–881 may assist amino acids 1–74 in assuming a conformation required for proper localization.

The nine amino acid changes that cause nonnuclear GAL4-β-galactosidase to accumulate are distributed throughout GAL4 amino acids 1–74. Although all the changes are nonconservative, there is no consistent pattern of replacement with respect to charge or hydrophobicity. This suggests that the entire 1–74 region is somehow important for nuclear localization. In contrast, other nuclear targeting sequences are very short; e.g., the SV40 sequence PKKKRKV (Kalderon et al. 1984) is sufficient for nuclear localization of β-galactosidase in yeast [I. Sadler and P.A. Silver, unpubl.]

This paradox might be resolved in several ways. The first 74 GAL4 amino acids could contain several shorter nuclear localization sequences, as has been proposed for MATa2 [Hall 1986; Silver and Hall 1988] and polyoma large T antigen [Richardson et al. 1986]. Mutations in one might reduce nuclear localization only partially. Alternatively, they could be altering the folding of the first 74 GAL4 amino acids and, hence, obscure a shorter nuclear targeting sequence. Protein context has been shown to affect the function of nuclear localization signals [Roberts et al. 1987]. Another possibility is that misfolding of the protein could result in aberrant binding to other cellular components, as is the case of mutant forms of influenza hemagglutinin that fail to be transported out of the endoplasmic reticulum [Gething et al. 1986]. Finally, the entire amino-terminal 74 amino acids may be recognized for nuclear localization, and a perturbation anywhere in its sequence could disrupt its ability to promote nuclear transport.

Overproduction of GAL4-β-galactosidase protein is unlikely to directly cause improper localization. Mutant proteins expressed from a single gene copy are also mislocalized. Also, one mutation, lysine 20 to glutamate, causes nonnuclear GAL4-β-galactosidase to accumulate. However, intact GAL4 bearing this mutation is almost completely nuclear localized but still overproduced. More likely, lack of complete localization is due to failure of the mutant proteins to interact properly with the localization apparatus.

The lysine 20 to glutamate mutation has a variable effect on protein localization, depending on the protein context. Similarly, the mutation cysteine 38 to serine has only a small effect on nuclear localization and production of GAL4-β-galactosidase. On the other hand, intact GAL4 bearing this mutation is overproduced and found in both the cytoplasm and the nucleus. These apparent inconsistencies further reveal the importance of overall protein context for the function of nuclear localization sequences (Roberts et al. 1987).

The mutations were isolated because they cause an increase in β-galactosidase activity from a GAL4-lacZ fusion carried on a multicopy plasmid, as assayed on X-gal indicator plates. In principle, such an increase could result from an increase in plasmid copy number, transcription, mRNA stability, translation, protein stability, or enzyme activity. However, the plasmid copy number [Fig. 4] and protein stability [Fig. 3B] in the mutants is the same as that of the parental construction, and the mutants show an increase in the level of fusion protein synthesized [Fig. 3A], rather than an alteration in specific activity. Therefore, the difference in levels of β-galactosidase activity between the mutants and their parent appears to result from transcription, mRNA stability, or translation, i.e., in some aspect of the production of the protein. All fusions are expressed from the same promoter, making differences in transcription levels unlikely. [Preliminary experiments indicate only slight differences in steady-state RNA levels, which are not, by themselves, sufficient to account for differences in protein levels [P. Silver, unpubl.]. A limited cellular factor may be necessary for translation of the normal GAL4 protein, hence, limiting the level to which it can be overproduced. The mutations identified here might alter the interaction of the GAL4 amino-terminus with this factor and allow more efficient translation.

In other cases, localization of proteins to the nucleus is correlated with a decrease in production of the localized protein relative to similar, nonlocalized controls. Hall et al. [1984] showed that yeast strains with nuclear-localized MATa2-β-galactosidase fusion proteins exhibited less β-galactosidase activity than strains with nonlocalized fusions. Similarly, Moreland et al. [1985] examined fusions between β-galactosidase and yeast ribosomal protein L3, which is localized to the nucleus prior to assembly into ribosomes. Fusions that contain a complete nuclear targeting sequence were synthesized.
at lower levels than corresponding proteins lacking the targeting sequence. We suggest that the underlying control mechanism in these cases, as well as for GAL4, may be the same.

By overproducing GAL4 from the strong ADH1 promoter, we have identified two forms of GAL4 protein. Two of the GAL4-linked mutations, at arginine 51 and histidine 53, appear to block the production of the higher molecular weight form of GAL4. We do not know how this new form of GAL4 is modified. One possibility is that the active form of GAL4 is modified by phosphorylation by the protein kinase encoded by SNF1 (Celanza and Carlson 1986). Genes regulated by GAL4 are subject to glucose repression (for review, see Johnston 1987a). snf1 cells are also gal+1, suggesting that SNF1 may exert its effect via GAL4.

The mutations we have identified in the GAL4 amino-terminus that alter nuclear localization have varying effects on the ability of GAL4 to act as a positive regulator. The GAL4 DNA-binding domain is also contained in the first 74 amino acids and contains six cysteines that may be involved in chelating a metal such as zinc [Miller et al. 1985; Johnston 1987]. We have now identified two mutations, one at cysteine 21 and the other at cysteine 38, that eliminate the ability of GAL4 to complement a gal4 strain. Because the GAL4 DNA-binding and nuclear localization determinants overlap, it would not be surprising if some mutations disrupt both functions.

Materials and methods

Strains and plasmids

S. cerevisiae strains used were DB745 (ade1-100 leu2-3 leu2-112 ura3-52), and ML171 (leu2-3 leu2-112 ade1-100 gal4 ura3:: GAL1-lacZ, provided by M. Lamphier). E. coli strains MM294 [end hsdRI thi pro] and JM107 [Δlac pro] thi strA supE were provided by N. Kleckner) was used for plasmid mutagenesis. E. coli DH5α [lac pro] thi strA supE M13, which was subjected to dideoxy sequencing (Sanger et al. 1987). Two different mutant plasmids contained the same mutation at the codon corresponding to tyrosine 40.

GAL4-linked mutations were reconstructed into the intact GAL4 by placing the GAL4 HindIII–XhoI fragment into a plasmid that contained the intact GAL4 gene expressed from the ADH1 promoter [Silver et al. 1984]. Chromosomally integrated forms of GAL4–lacZ were prepared by inserting the BamHI–Sall fragment (Fig. 1) into the integrating plasmid YEp5 (Botstein and Davis 1982). The resulting plasmid (PS106) was digested with ApaI to target integration to the URA3 locus.

Immunofluorescence

Cells were grown in minimal selective media with 2% glucose at 30°C and prepared for indirect immunofluorescence as described previously [Silver et al. 1984], with the following modifications. Cells were fixed by adding formaldehyde to a final concentration of 3.5% [vol/vol] directly to the growing culture by overproducing GAL4 from the strong ADH1 promoter, we have identified two forms of GAL4 protein. Two of the GAL4-linked mutations, at arginine 51 and histidine 53, appear to block the production of the higher molecular weight form of GAL4. We do not know how this new form of GAL4 is modified. One possibility is that the active form of GAL4 is modified by phosphorylation by the protein kinase encoded by SNF1 (Celanza and Carlson 1986). Genes regulated by GAL4 are subject to glucose repression (for review, see Johnston 1987a). snf1 cells are also gal+1, suggesting that SNF1 may exert its effect via GAL4.

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GAL4-linked mutations were reconstructed into the intact GAL4 by placing the GAL4 HindIII–XhoI fragment into a plasmid that contained the intact GAL4 gene expressed from the ADH1 promoter [Silver et al. 1984]. Chromosomally integrated forms of GAL4–lacZ were prepared by inserting the BamHI–Sall fragment (Fig. 1) into the integrating plasmid YEp5 (Botstein and Davis 1982). The resulting plasmid (PS106) was digested with ApaI to target integration to the URA3 locus.

Immunofluorescence

Cells were grown in minimal selective media with 2% glucose at 30°C and prepared for indirect immunofluorescence as described previously [Silver et al. 1984], with the following modifications. Cells were fixed by adding formaldehyde to a final concentration of 3.5% [vol/vol] directly to the growing culture and incubated with gentle shaking for 90 min at 30°C. After washing and digestion with zymolyase 60,000 [Kirin], the cells were placed on a polylysine coated multiwell slide [Flow Laboratories] and immersed in cold methanol and acetone. Antibodies were diluted in 1 mg/ml bovine serum albumin [BSA] [Sigma] in phosphate-buffered saline [PBS] [0.04 M K2HPO4, 0.01 M KH2PO4, 0.15 M NaCl]. To visualize GAL4–β-galactosidase antibody [from T. Mason] at a dilution of 1 : 500 was used, followed by FITC-conjugated goat anti-mouse IgG [Boehringer Mannheim] at 1 : 500 dilution. To visualize intact GAL4 [Fig. 4], affinity-purified rabbit anti-GAL4 antibody [from C. Debouck] was used at 1 : 100 dilution, followed by FITC-conjugated goat anti-rabbit IgG [Miles] at 1 : 200. Anti-GAL4 antibody was affinity purified by passing it over a Sepharose column to which a peptide containing GAL4 amino acids 1–147 [from M. Hollis and M. Ptashne] had been coupled. Cells were viewed at 1000× magnification with either a Zeiss Photo III microscope or Zeiss Axioskop, both equipped for fluorescence. Exposure times for immunofluorescence were 30 sec in all cases.
Pulse-labeling experiments

Cells were grown at 30°C to a cell density of $1 \times 10^7$ cells/ml in 5 ml of media [Wickerham 1946], supplemented with 0.1 mM ammonium sulfate, collected by centrifugation (2500g for 5 min), and resuspended in 1 ml prewarmed sulfate-free media with 0.1 mCi Na$_2$HPO$_4$ (574 mCi/m mole, DuPont NEN). Following incubation for 30 min at 30°C, ammonium sulfate (5 mM final concentration) and cycloheximide (1 mM final concentration) were added. At the indicated time points, cells (0.3 ml) were immediately chilled, collected by centrifugation (5000g, 2 min), resuspended in 0.5 ml cold buffer A [150 mM NaCl/50 mM Tris-Cl (pH 7.5)/5 mM EDTA/1% (vol/vol) Triton X-100/1 $\mu$g/ml leupeptin and pepstatin] and glass beads to one quarter the final volume, and lysed by three 30-sec agitations on a VWR vortex mixer. The lysate was centrifuged (14,000g, 3 min), and the resulting supernatant was analyzed as described below.

Immunoprecipitation

Immunoprecipitation of GAL4-β-galactosidase was achieved by the addition of 50 μl of the mouse monoclonal anti-β-galactosidase to 0.3 ml of cell lysate for 1 hr at 4°C, followed by 50 μl of a 10% (wt/vol) suspension of formalin-fixed, SDS-washed S. aureus (from M. Resh) for an additional 1 hr at 4°C. The S. aureus were then sedimented (14,000g, 3 min, 4°C), washed twice with buffer A (0.5 ml), and washed once with buffer A containing 0.1% SDS. The final cell pellet was resuspended in 50 μl sample buffer [Laemmli 1970], boiled for 5 min and sedimented for 5 min at 14,000g, and the supernatant was analyzed by SDS-gel electrophoresis [Laemmli 1970] and fluorography [Chamberlin 1979].

Plasmid levels

Total DNA was isolated from plasmid bearing cells (2 x 10$^8$ cells/ml) grown in selective media [Sherman et al. 1983], separated by electrophoresis in a 0.6% agarose gel and transferred to nitrocellulose with the method of Southern (1975). The plasmid YIp5 was used as the nick-translated probe.

Immunoblotting

Immunoblotting was used to quantify the amount of intact GAL4 protein. Cells (5 ml of 1 x 10$^7$ cells/ml) were collected by centrifugation (2600g, 5 min), resuspended in 1 ml of 0.25 M NaOH/1% β-mercaptoethanol, and incubated for 10 min on ice. Then, 0.16 ml of trichloroacetic acid (50%) was added, and the cells were incubated for 10 min on ice and collected by centrifugation (14000g, 10 min). The pellet was washed with cold acetone (1 ml), allowed to air-dry, resuspended in 0.2 ml sample buffer, boiled for 5 min, and electrophoresed on a 7% SDS-polyacrylamide gel [Laemmli 1970]. Equal amounts of protein were loaded for each lysate, as determined by Coomassie staining of an identical gel run in parallel. Following electrophoresis, the proteins were transferred to nitrocellulose with a Polyblot Electroblotter (ABN). The nitrocellulose blot was blocked and incubated with antibodies, as described previously [Silver et al. 1984]. Rabbit serum containing anti-GAL4 antibody (from S. Chapman) and goat anti-rabbit IgG conjugated with horseradish peroxidase (BioRad) were each used at 1:200 dilution.

E. coli extracts were prepared from JM107 transformed with pLK76-7. Cells were grown in LB containing ampicillin (20 μg/ml). At OD$_{600}$ of 1, IPTG was added (final concentration 5 mM). Cells were then incubated for 2 hr at 37°C, collected by centrifugation (14,000g, 5 min), and lysed by resuspension in sample buffer and boiling for 5 min.

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References

Botstein, D. and R.W. Davis. 1982. Principles and practice of recombinant DNA research with yeast. In The Molecular biology of the yeast Saccharomyces cerevisiae. Metabolism and gene expression (ed. J.N. Strathem, E.W. Jones, and J.R. Broach), pp. 607–636. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Celenza, J.L. and M. Carlson. 1986. A yeast gene that is essential for release from glucose repression encodes a protein kinase. Science 233: 1123–1127.

Chamberlin, J.P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. Anal. Biochem. 98: 132–135.

Douglas, H. and D. Hawthorne. 1972. Uninducible mutants in the gal 1 locus of Saccharomyces cerevisiae. J. Bacteriol. 109: 1139–1143.

Gething, M.-J., K. McCammon, and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: The role of folding in intracellular transport. Cell 46: 939–950.

Ginger, E., S.M. Vamum, and M. Ptashne. 1985. Specific DNA binding of GAL4, a positive regulatory protein of yeast. Cell 40: 767–774.

Goldfarb, D.S., J. Gariepy, G. Schoolnik, and R.D. Kornberg. 1986. Synthetic peptides as nuclear location signals. Nature 322: 641–644.

Guarente, L. and M. Ptashne. 1981. Fusion of Escherichia coli lacZ to the cytochrome c gene of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. 78: 2199–2203.

Hall, M.N. 1986. Nuclear protein localization signals in yeast. In Yeast cell biology. [ed. J. Hicks], pp. 421–425. Alan R. Liss, New York.

Hall, M.N., L. Hereford, and I. Herskowitz. 1984. Targeting of E. coli beta-galactosidase to the nucleus in yeast. Cell 36: 1057–1065.

Ito, H., Y. Fukado, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells with alkali cations. J. Bacteriol. 153: 163–168.

Johnston, M. 1987a. A model fungal gene regulatory mechanism: The GAL genes of Saccharomyces cerevisiae. Microbiol. Rev. 51: 458–476.

———. 1987b. Genetic evidence that zinc is an essential co-factor in the DNA binding domain of GAL4 protein. Nature 328: 353–355.

Kalderon, D., B.L. Roberts, W.D. Richardson, and A.E. Smith. 1984. A short amino acid sequence able to specify nuclear location. Cell 39: 499–509.

Keegan, L.P., G. Gill, and M. Ptashne. 1986. Separation of DNA
binding from the transcription-activating function of a eukaryotic regulatory protein. Science 231: 699–704.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227: 680–685.

Lanford, R.E., P. Kanda, and R.C. Kennedy. 1986. Induction of nuclear transport with a synthetic peptide homologous to the SV40 T antigen transport signal. Cell 46: 575–582.

Laughon, A. and R.F. Gesteland. 1982. Primary structure of the Saccharomyces cerevisiae GAL4 gene. Mol. Cell. Biol. 4: 260–267.

Miller, J. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Miller, J., A.D. McLachlan, and A. Klug. 1985. Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. EMBO J. 4: 1609–1614.

Moreland, R.B., H.G. Nam, L.M. Hereford, and H.M. Fried. 1985. Identification of a nuclear localization signal of a yeast ribosomal protein. Proc. Natl. Acad. Sci. 82: 6561–6565.

Moore, R.B., G.L. Langevin, R.H. Singer, R.L. Garcea, and L.M. Hereford. 1987. Amino acid sequences that determine the nuclear localization of yeast histone H2B. Mol. Cell. Biol. 7: 4048–4057.

Paucha, E., D. Kalderon, W.D. Richardson, R.W. Harvey, and A.E. Smith. 1985. The abnormal location of cytoplasmic SV40 large T is not caused by failure to bind to DNA or p53. EMBO J. 4: 3235–3240.

Richardson, W.D., B.L. Roberts, and A.E. Smith. 1986. Nuclear location signals in polyoma virus large-T. Cell 44: 77–85.

Roberts, B.L., W.D. Richardson, and A.E. Smith. 1987. The effect of protein context on nuclear location signal function. Cell 50: 465–475.

St. John, T. and R. Davis. 1981. The organization and transcription of the galactose gene cluster of Saccharomyces. J. Mol. Biol. 152: 285–315.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. 74: 5463–5467.

Sherman, F., G. Fink, and C. Lawrence. 1983. Methods in yeast genetics, revised ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Silver, P.A. and M.N. Hall. 1988. Transport of proteins into the nucleus. In Protein transfer and organelle biogenesis, [ed. R.C. Das and P.W. Robbins]. pp. 747–769. Academic Press, New York.

Silver, P., R. Brent, and M. Ptashne. 1986. DNA binding is not sufficient for nuclear localization of regulatory proteins in Saccharomyces cerevisiae. Mol. Cell. Biol. 6: 4763–4766.

Silver, P., L. Keegan, and M. Ptashne. 1984. Amino terminus of the yeast GAL4 gene product is sufficient for nuclear localization. Proc. Natl. Acad. Sci. 81: 5951–5955.

Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503–517.

Wickerham, L.J. 1946. A critical evaluation of the nitrogen assimilation tests commonly used in the classification of yeasts. J. Bacteriol. 52: 293–301.
Mutations that alter both localization and production of a yeast nuclear protein.

P A Silver, A Chiang and I Sadler

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