Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 5951–5955, October 1984
Biochemistry

Amino terminus of the yeast *GAL4* gene product is sufficient for nuclear localization

(β-galactosidase fusion proteins/immunofluorescence/positive regulatory protein)

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Contributed by Mark Ptashne, June 14, 1984

ABSTRACT We have studied the intracellular compartmentalization in yeast of *Escherichia coli* β-galactosidase bearing heterologous amino acid sequences at its amino terminus. Chimeras containing as few as 74 NH_2-terminal amino acids of GAL4, a yeast positive regulatory protein, at the amino terminus accumulate in the cell nucleus. This and other results are consistent with the proposal that the *GAL4* gene product mediates positive control by binding to DNA and that the information for nuclear localization resides in its amino terminus. The amino acid sequence of the GAL4 amino terminus does not agree with the previously proposed consensus sequences responsible for nuclear localization. The β-galactosidase activity in cells bearing the non-nuclear chimeric proteins is 10-fold greater than in cells bearing chimeric proteins that specifically concentrate in the nucleus.

The *GAL4* gene product is required for transcriptional induction of the genes (*GAL1*, *-7*, and *-10*) necessary for galactose metabolism in the yeast *Saccharomyces cerevisiae* (1–3). The *GAL4* gene has been cloned (refs. 4 and 5; unpublished results) and shown to encode a 99-kDa protein (6). It has been proposed that the *GAL4* gene product mediates positive control by binding to specific sites on the DNA upstream of the starting site of transcription (7–9), thereby stimulating RNA polymerase II-dependent transcription.

We know very little about how certain proteins accumulate in the nucleus. Nuclear proteins injected into the *Xenopus* oocyte cytoplasm move to the nucleus, indicating that nuclear association is independent of protein synthesis and processing (reviewed in ref. 10). The carboxyl terminus of the *Xenopus* protein, nucleoplasmin, was found to be necessary for passage of the complete protein into the nucleus, suggesting that specific amino acids may act as nuclear determinants (11).

To determine whether *GAL4* encodes protein sequences capable of directing the gene product to the nucleus, its presumed site of action, we have taken the following approach. Gene fusions with various portions of the *GAL4* gene fused to the 5′ end of the *Escherichia coli lacZ* gene were constructed and introduced into yeast cells. The intracellular location of the resultant chimeric proteins was determined by indirect immunofluorescence using antibody to β-galactosidase. We show that as few as 74 GAL4 NH_2-terminal amino acids are sufficient to localize a GAL4–β-galactosidase chimeric protein to the cell nucleus. Unexpectedly, the amount of chimeric GAL4–β-galactosidase in the cell correlates with its intracellular location. Nuclear-associated chimeras are present at $\approx$10% the levels of non-nuclear-associated chimeric proteins.

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MATERIALS AND METHODS

Strains. Plasmids were constructed and maintained in *E. coli* MM294 (*end hsdR thi pro*). The *S. cerevisiae* strain used was DB745 (*ade 1-100 leu 2-3 leu 2-112 ura 3-52*, constructed in the laboratory of D. Botstein).

Plasmids. The original plasmid containing the intact wild-type *GAL4* gene from *S. cerevisiae* was recovered from a library of yeast DNA in YEp13 (provided by K. Nasmyth) by complementation of a *gal4* strain (unpublished results). The DNA sequence of the first 400 nucleotides was determined by the method of Maxam and Gilbert (unpublished results; ref. 6). Plasmid pAAH5, generously provided by G. Ammerer, contains the alcohol dehydrogenase (*ADH1*) promoter and terminator from *S. cerevisiae*, as well as *LEU2* and a 2-μm origin of replication. The region of the *E. coli lacZ* gene encoding the COOH-terminal sequence of β-galactosidase was from pMC1403 (12). The *URA3–lacZ* fusion (13) was the gift of M. Rose. All DNA constructions were done as described (14).

Materials. *E. coli* β-galactosidase, aprotinin, Percoll, 4′,6-diamidino-2-phenylindole (DAPI), and poly(L-lysine) were purchased from Sigma. Zymolyase 60,000 and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antiserum were purchased from Miles. Glusulase was purchased from Endo Laboratories (New York). Bovine gamma globulin was purchased from Calbiochem. Bovine serum albumin fraction V was purchased from United States Biochemical (Cleveland, OH). Affinity-purified goat anti-rabbit IgG, horseradish peroxidase conjugate, and horseradish peroxidase substrate were purchased from Bio-Rad.

Media. All plasmid-bearing yeast were grown in minimal selective medium: 7 mg of yeast nitrogen base without amino acids and 0.7 mg of amino acid mixture without leucine (15) per liter of 2% glucose.

β-Galactosidase Assays. Cells were grown to a density of 2×10^7 cells per ml, concentrated 10-fold, and assayed as described (16). Aliquots of purified nuclei were assayed in the same manner.

Immunofluorescence. Cells were prepared for indirect immunofluorescence according to the protocol of Adams and Pringle (17). After the cells were fixed with formaldehyde, digested with Glusulase and Zymolyase, and applied to the slide, 0.5 μg of affinity-purified rabbit anti-β-galactosidase in phosphate-buffered saline (P_i/NaCl; 150 mM NaCl/2 mM NaH_2PO_4/10 mM Na_2HPO_4) containing bovine serum albumin at 1 mg/ml was applied. The slide was incubated at room temperature for 2 hr, washed three times with P_i/NaCl, and incubated at room temperature for 2 hr with affinity-purified FITC-coupled goat anti-rabbit IgG. The second antibody was removed and DAPI, a DNA-specific stain, at 1 μg/ml in

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; P_i/NaCl, phosphate-buffered saline.

H_2O, was applied for 5 min. The slides were viewed using a 100× objective on a Zeiss microscope equipped for fluorescein epi-illumination fluorescence microscopy.

Immunoblots. Cells (2 ml at 2×10^7 cells per ml) were harvested by centrifugation, resuspended in 0.2 ml of Laemmli sample buffer (18) containing glass beads, mixed on a Vortex for 2 min, and heated at 100°C for 5 min. Samples (50 μl) of this cell homogenate were loaded onto each lane of a standard 6% polyacrylamide Laemmli-type gel (18). The proteins were subjected to sodium dodecyl sulfate/PAGE and electrophoretically transferred to nitrocellulose (19). The nitrocellulose blot was incubated at 37°C for 1 hr in P_i/NaCl containing 3% bovine serum albumin, then for 3 hr at room temperature with affinity-purified rabbit anti-β-galactosidase in P_i/NaCl containing 3% bovine serum albumin and 0.1% bovine gamma globulin. The blot was washed for 2 hr in P_i/NaCl, incubated at room temperature for 2 hr with affinity-purified goat anti-rabbit IgG conjugated with horseradish peroxidase, washed at 37°C for 1 hr in P_i/NaCl containing 3% bovine serum albumin and 0.1% bovine gamma globulin, and developed with horseradish peroxidase substrate.

Nuclei. Nuclei were prepared by the method of Baum and Thorner (personal communication) with the following modifications: Cells (300 ml at 5×10^7 cells per ml) were harvested by centrifugation (5000 × *g*, 10 min), resuspended in 8 ml of 1 M sorbitol/20 mM potassium phosphate buffer, pH 6.5/0.5 mM $CaCl_2$/0.5% 2-mercaptoethanol/0.5 mM phenylmethylsulfonyl fluoride containing Zymolyase 60,000 at 0.25 mg/ml, and incubated at room temperature until spheroplasts had formed. The spheroplasts were collected by centrifugation (3500 × *g*, 3 min), resuspended in 2 ml of lysis buffer (0.2% Nonidet P-40/10 mM Tris·HCl, pH 7.4/10 mM NaCl/5 mM $MgCl_2$/0.5 mM $CaCl_2$/0.5 mM phenylmethylsulfonyl fluoride containing aprotinin at 1 μg/ml), and homogenized in a Teflon-glass homogenizer until lysis, monitored microscopically, was complete. Crude nuclei were collected by centrifugation (3000 × *g*, 5 min), resuspended in 30 ml of 50% (wt/vol) Percoll/40 mM Pipes, pH 6.8/10 mM $MgCl_2$/0.5 mM phenylmethylsulfonyl fluoride/0.05% Triton X-100, and centrifuged at 21,000 × *g* for 35 min in a Ti30 rotor. The nuclear band was harvested and the Percoll was removed by 10-fold dilution with 0.01 M potassium phosphate buffer (pH 6.5) and centrifugation at 5000 × *g* for 10 min to sediment the nuclei.

Table 1. β-Galactosidase activity of *GAL4–lacZ* fusions

Fusion	β-Galactosidase, unit(s)
$GAL4_{(1-147)}$–*lacZ*	0.3
$GAL4_{(1-752)}$–*lacZ*	0.1
$P_{ADH1}GAL4_{(1-752)}$–*lacZ*	5
$P_{ADH1}GAL4_{(1-147)}$–*lacZ*	11
$P_{ADH1}GAL4_{(1-74)}$–*lacZ*	6
$P_{ADH1}GAL4_{(79-752)}$–*lacZ*	77
$P_{ADH1}GAL4_{(79-147)}$–*lacZ*	123

Plasmids bearing the indicated gene fusions were introduced into DB745. Cells were grown in "leucine dropout medium" (15), pH 7, containing 2% glucose. β-Galactosidase activity was assayed as previously described (16).

RESULTS

We wished to determine whether there were amino acid sequences encoded by the *GAL4* gene that could confer specific nuclear localization on a normally non-nuclear protein. Toward this end, we constructed gene fusions between *GAL4* and the *E. coli lacZ* gene. Chimeras expressed in yeast, bearing NH_2-terminal substitutions in β-galactosidase, retain enzymatic activity (16). We used indirect im-

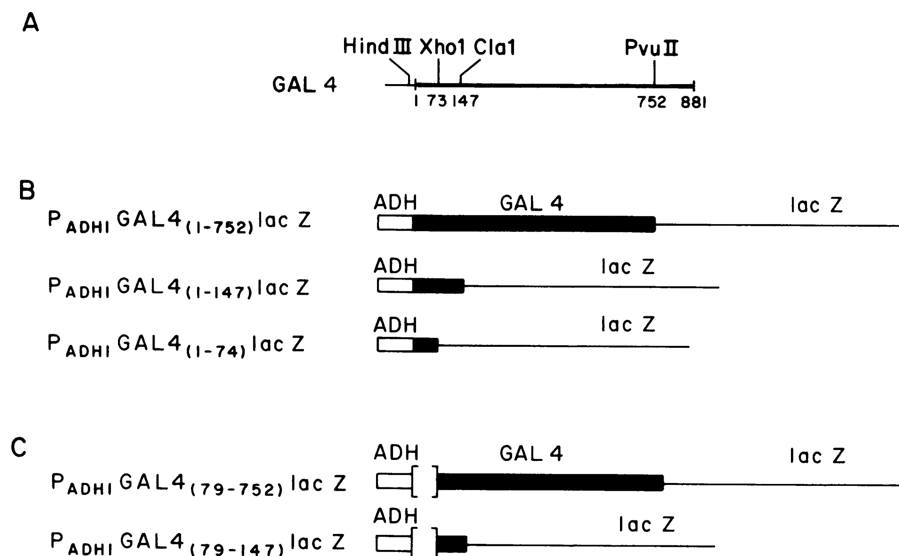

FIG. 1. (*A*) Restriction map of the *GAL4* gene. The *GAL4* gene was cloned on a 5.2-kilobase insert in the yeast vector YEp13. The predicted first methionine codon is at nucleotide 443 of the insert (labeled 1). The *GAL4* gene has been shown to encode an 881-amino acid protein product (20). The number below each restriction site represents the amino acid codon at which it occurs. The *Hin*dIII site was introduced into the *GAL4* promoter ≈15 base pairs upstream from the first methionine codon. (*B*) *GAL4–lacZ* gene fusions under the control of the *ADH1* promoter. The *ADH1* promoter P_{ADH1}, from pAAH5, was substituted for the wild-type *GAL4* promoter at the *Hin*dIII site. Truncated *GAL4* genes bearing 5′ sequences were created by cutting at one of the sites: *Pvu* II, *Cla* I, or *Xho* I (see *A*) and filling in the ends with DNA polymerase large fragment (Klenow) where necessary. The truncated *GAL4* genes were ligated to a similarly treated *Bam*HI site corresponding to amino acid 7 encoded in the *lacZ* gene. The numbers in parentheses indicate GAL4 amino acids. (*C*) Deletions in the $P_{ADH1}GAL4$–*lacZ* gene fusions. The *ADH1* promoter was placed adjacent to the second in-frame methionine codon of *GAL4* in the $P_{ADH1}GAL4_{(1-752)}$–*lacZ* and the $P_{ADH1}GAL4_{(1-147)}$–*lacZ* fusions by eliminating the *Hin*dIII–*Xho* I fragment; the two gene fusions were cut with restriction enzymes *Hin*dIII and *Xho* I, treated with Klenow fragment to fill in the recessed ends, and blunt-end ligated. The deletions are indicated by the brackets. *GAL4* DNA is represented by the thick line, *lacZ* DNA (not drawn to scale) is represented by the thin line. The open box represents DNA sequences required for *ADH1* promoter activity; no *ADH1* amino acid-coding sequences are present.

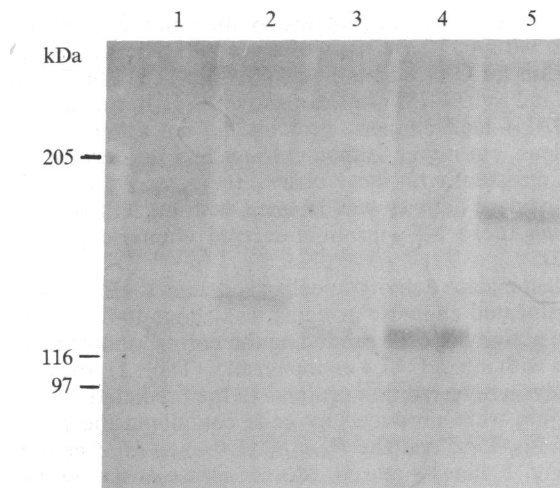

FIG. 2. Immunoblots of proteins from cells producing GAL4–lacZ chimeric proteins. Analysis was performed with lysates of yeast cells bearing the following gene fusions: lane 1, P_{ADH1}-$GAL4_{(1-752)}$–*lacZ*; lane 2, $P_{ADH1}GAL4_{(1-147)}$–*lacZ*; lane 3, P_{ADH1}-$GAL4_{(1-74)}$–*lacZ*; lane 4, $P_{ADH1}GAL4_{(79-147)}$–*lacZ*; lane 5, P_{ADH1}-$GAL4_{(79-752)}$–*lacZ*.

munofluorescence with anti-β-galactosidase antibody to detect the intracellular location of the chimeric proteins. We assumed that intact β-galactosidase would not accumulate in the yeast nucleus (see below), and that the GAL4–β-galactosidase chimeras that did concentrate in the nucleus did so because they contained nuclear "address sequences" of GAL4 protein.

We fused DNA that encodes the large COOH-terminal region of β-galactosidase to three sites in the DNA that encodes the GAL4 amino terminus, maintaining an open reading frame in each case. These three sites (Fig. 1*A*) were at positions encoding amino acids 752, 147, and 74 of the total of 881 amino acids predicted by the *GAL4* DNA sequence (6). When placed in yeast on 2-μm vectors, the $GAL4_{(1-147)}$–*lacZ* and $GAL4_{(1-752)}$–*lacZ* gene fusions yielded β-galactosidase activity, albeit at very low levels (Table 1). No GAL4–β-galactosidase chimeric protein was detectable by immunoblot analysis of cell lysates from strains harboring these gene fusions. This low level of β-galactosidase activity properly reflects the low level of production of *GAL4* mRNA when cells are grown in glucose (4). Growth in galactose, a condition that relieves glucose repression, increased enzyme levels 2- to 7-fold (data not shown), a level not high enough for our analysis.

To increase the intracellular levels of the chimeric proteins, we replaced the wild-type *GAL4* promoter with the strong yeast alcohol dehydrogenase (*ADH1*) promoter in the three *GAL4–lacZ* fusions (Fig. 1*B*). Yeast carrying the gene fusions with the *ADH1* promoter on 2-μm vectors yielded β-galactosidase activity 40- to 60-fold higher than cells bearing the wild-type *GAL4–lacZ* gene fusions (Table 1). Immunoblot analysis (Fig. 2, lanes 1, 2, and 3) showed that proteins of the predicted molecular weights were produced by cells harboring the $P_{ADH1}GAL4$–*lacZ* hybrid genes. Cells bearing the $P_{ADH1}GAL4_{(1-752)}$–*lacZ*, $P_{ADH1}GAL4_{(1-147)}$–*lacZ*, and $P_{ADH1}GAL4_{(1-74)}$–*lacZ* gene fusions produced proteins of 195, 130, and 123 kDa, respectively. The gel used in this analysis could not be used to detect differences of <2 kDa.

We determined the intracellular location of the chimeric proteins by indirect immunofluorescence of whole yeast cells (Fig. 3). The immunoreactive material was found pre-

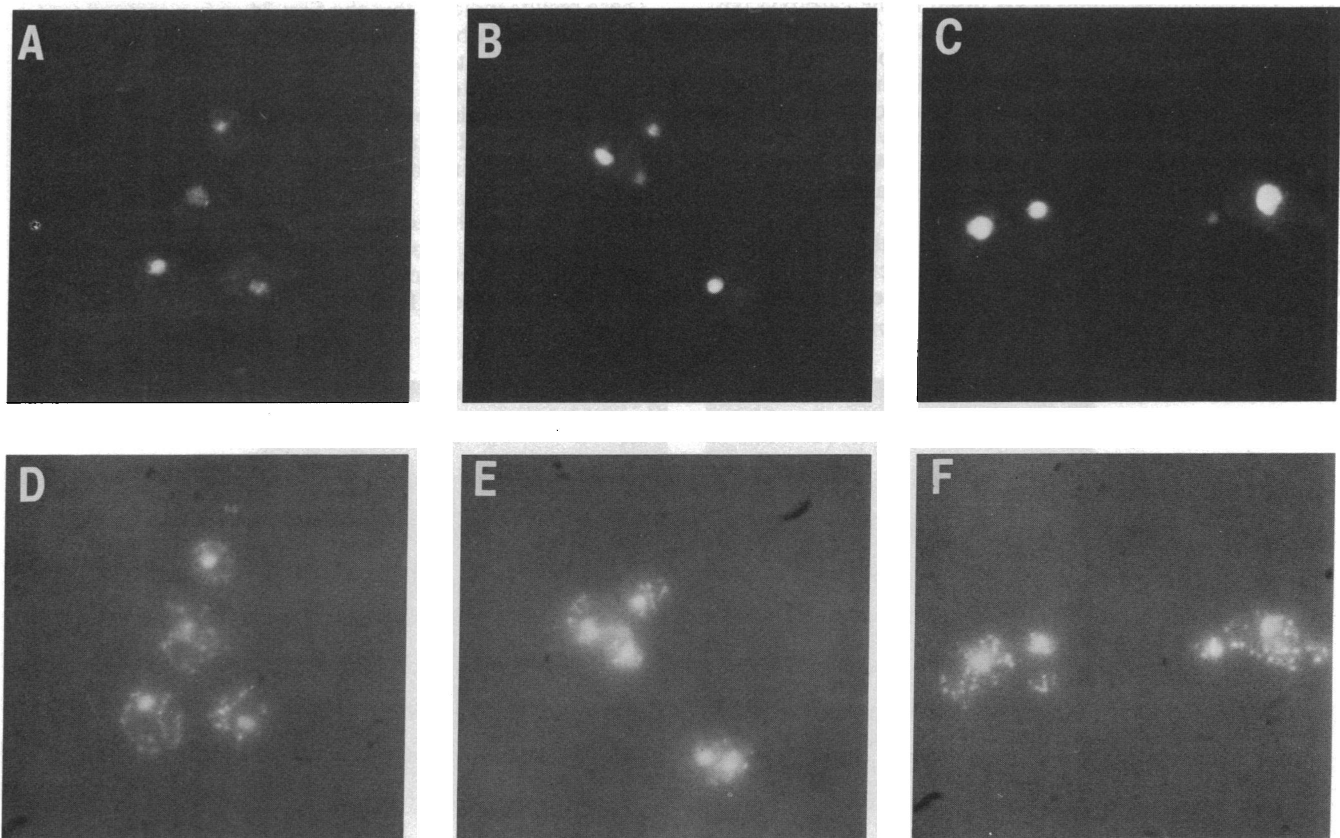

FIG. 3. Immunofluorescence of cells producing nuclear-associated GAL4–lacZ chimeric proteins. Cells were prepared for indirect immunofluorescence and treated with either rabbit anti-β-galactosidase antibody, followed by FITC-conjugated anti-rabbit IgG (*A–C*) to identify the chimeric proteins, or DAPI (*D–F*) to identify the cell nuclei. *A* and *D*, $GAL4_{(1-74)}$–*lacZ*; *B* and *E*, $GAL4_{(1-147)}$–*lacZ*; *C* and *F*, $GAL4_{(1-752)}$–*lacZ*.

Table 2. β-Galactosidase activity associated with nuclei from cells producing GAL4-lacZ fusion proteins

Fusion	% total β-galactosidase activity in nuclear fraction
$P_{ADHI}GAL4_{(1-752)}$-lacZ	20
$P_{ADHI}GAL4_{(1-147)}$-lacZ	25
$P_{ADHI}GAL4_{(1-74)}$-lacZ	52
$P_{ADHI}GAL4_{(79-752)}$-lacZ	3
$P_{ADHI}GAL4_{(79-147)}$-lacZ	1
URA3–lacZ	4

The specific activity of β-galactosidase per unit protein was determined in the nuclear fraction as well as in a total cell extract.

dominantly in the cell nucleus of cells bearing the P_{ADHI}-$GAL4_{(1-74)}$-*lacZ* (*A*), the $P_{ADHI}GAL4_{(1-147)}$-*lacZ* (*B*), and the $P_{ADHI}GAL4_{(1-752)}$-*lacZ* (*C*) gene fusions. These results indicate that as few as 74 NH_2-terminal GAL4 amino acids, when fused to the carboxyl terminus of β-galactosidase, direct the chimeric protein to the cell nucleus.

The amounts of β-galactosidase activity associated with nuclei isolated from cells bearing the *GAL4–lacZ* gene fusions were consistent with the immunofluorescence results. We observed nuclear-associated β-galactosidase activity ranging from 20% (for the $P_{ADHI}GAL4_{(1-752)}$-*lacZ* fusion) to 52% (for the $P_{ADHI}GAL4_{(1-74)}$-*lacZ* fusion) of the total activity in the cells (Table 2). The variability in the activity of the chimeric proteins isolated with nuclei is consistent with reports (21) that other nuclear-associated proteins show 30%–50% cofractionation with yeast nuclei.

To show that nuclear localization of GAL4–lacZ fusion proteins is dependent upon specific GAL4 sequences, we analyzed a strain bearing a *URA3–lacZ* gene fusion. The chimeric protein produced from this gene fusion contains 18 URA3 NH_2-terminal amino acids fused to the carboxyl terminus of β-galactosidase. The intracellular location of the *URA3* gene product is not known but is thought to be nonnuclear. The anti-β-galactosidase-specific fluorescence was uniformly distributed throughout these cells (data not shown) rather than specifically associated with the nucleus. Nuclei isolated from cells producing the URA3–lacZ chimeric protein contained very little β-galactosidase activity (Table 2).

The results presented thus far suggest that the nuclear determinant for the yeast *GAL4* gene product lies in the first 74 amino acids. To test this idea further, we eliminated the first 78 amino acids from the $GAL4_{(1-147)}$-lacZ and the $GAL4_{(1-752)}$-lacZ hybrid proteins by moving the *ADH* promoter to within 13 base pairs of a second in-frame methionine codon (at GAL4 amino acid 79, Fig. 1*C*). The 79–147 (Fig. 4) and the 79–752 (data not shown) NH_2-terminal truncated GAL4–lacZ chimeric proteins did not accumulate in the nucleus, but rather formed extranuclear fluorescent material. Consistent with these observations, very little of the β-galactosidase activity was isolated with nuclei from cells producing these NH_2-terminal-deleted chimeric proteins (Table 2).

β-Galactosidase activity in cells producing these NH_2-terminal-truncated chimeric proteins was about 10-fold higher than the activity in cells producing the corresponding fusion proteins with intact GAL4 amino termini (Table 1). Immunoblot analysis revealed that proteins of the predicted molecular weights were produced by cells containing the P_{ADHI}-$GAL4_{(79-752)}$-*lacZ* and the $P_{ADHI}GAL4_{(79-147)}$-*lacZ* gene fusions (Fig. 2, lanes 4 and 5). Moreover, we observed more immunoreactive protein in lysates of cells producing the chimeric proteins bearing truncated amino termini. These results indicate that the increased β-galactosidase activity is a consequence of increased amounts of the chimeric proteins.

DISCUSSION

We have demonstrated that the yeast *GAL4* gene encodes amino acid sequences that, when fused to the normally nonnuclear protein, *E. coli* β-galactosidase, are capable of directing the chimeric protein to the yeast cell nucleus. By indirect immunofluorescence and cell fractionation, we showed that β-galactosidase accumulates in the cell nucleus in cells producing chimeric proteins with 752, 147, and 74 GAL4 NH_2-terminal amino acids at their amino terminus. These results indicate that the first 74 amino acids of *GAL4*-encoded protein bear a determinant for specific nuclear association. Consistent with this proposal is the observation that chimeric proteins with GAL4 amino acids 79–147 or 79–752 fused to β-galactosidase are not nuclear-associated (see below). The GAL4–lacZ fusion proteins that lack the 78 NH_2-terminal amino acids of GAL4 form distinct non-nuclear aggregates (Fig. 4). This is in contrast to our observations of cells bearing the non-nuclear URA3–lacZ chimeric protein. In that case, fluorescent β-galactosidase was distributed uniformly throughout the cells (data not shown). We do not understand the different behavior of the two non-nuclear proteins.

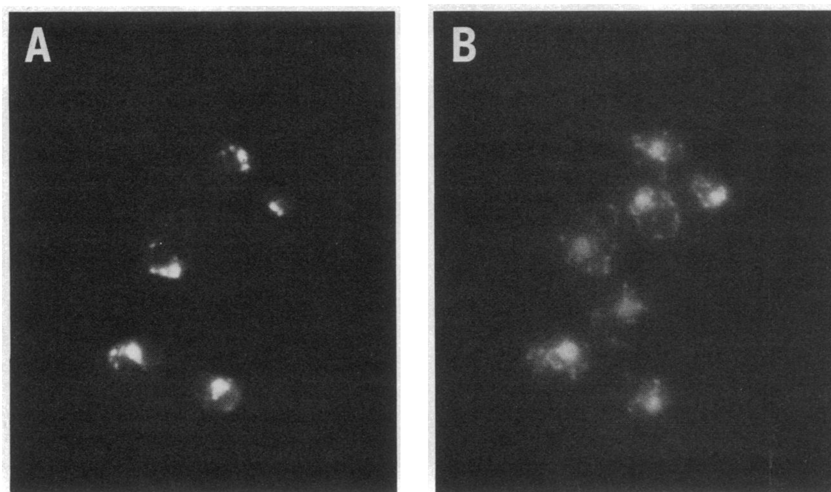

FIG. 4. Indirect immunofluorescence of cells producing $GAL4_{(79-147)}$-lacZ. *A*, anti-β-galactosidase antibody followed by FITC-conjugated anti-rabbit IgG; *B*, DAPI-stained.

Replacement of the wild-type *GAL4* promoter with the yeast *ADH1* promoter resulted in a 40- to 60-fold increase in the β-galactosidase activity in cells bearing the *GAL4–lacZ* gene fusions. Even when produced at this higher level, the GAL4–lacZ chimeric proteins bearing intact GAL4 NH_2 termini accumulated in the nucleus, suggesting that the nucleus can accommodate much more than wild-type levels of the GAL4 protein. An unexpected finding was that the chimeric proteins that lack the 78 NH_2-terminal amino acids of GAL4, which do not accumulate in the nucleus, were produced at even higher levels. It is possible that the mRNAs are transcribed or translated at a faster rate and that the exclusion from the nucleus is related coincidentally to the higher level of expression. Alternatively, it is possible that nuclear transport somehow limits the amount of protein that is made. For example, there may be a limited number of nuclear-envelope-associated receptors that recognize a nuclear determinant in the 74 GAL4 NH_2-terminal amino acids. Once these sites are saturated, newly synthesized protein that can no longer bind to or translocate into the nucleus would instead be degraded. It is also possible that the synthesis of GAL4 protein is autoregulated and that its first 74 amino acids are responsible for this regulation. The hypothetical site for this regulation would have to be encoded in the first 200 nucleotides of the *GAL4* coding sequence since we have replaced the *GAL4* promoter with the *ADH1* promoter in our gene fusions.

Similar experiments with the yeast α2 gene product have led Hall *et al.* (22) to suggest that some nuclear proteins contain a specific sequence, Lys-Ile-Pro-Ile-Lys, that is responsible for targeting them to the nucleus. This particular sequence does not occur in the region of GAL4 protein that we have determined to be sufficient for nuclear transport.

We especially thank Mark Rose for invaluable assistance with the immunofluorescence, John Douhan III for providing anti-β-galactosidase antiserum, and Susan Varnum for technical assistance. We also thank Roger Brent, Doug Melton, Jackie Sharon, Robin Wharton, and Mark Zoller for helpful discussions and for their comments on the manuscript. This work was supported by National Institutes of Health Grant GM 32308 to M.P. and by postdoctoral fellowships to P.S. from the American Cancer Society (PF-2201) and the National Institutes of Health (GM 08871) and by a Paul Mazur Fellowship from Harvard University to L.K.

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