MOLECULAR AND CELLULAR BIOLOGY, Feb. 1989, p. 384–389
0270-7306/89/020384-06$02.00/0

Vol. 9, No. 2

Context Affects Nuclear Protein Localization in *Saccharomyces cerevisiae*

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Received 23 August 1988/Accepted 24 October 1988

Proteins destined for the nucleus contain nuclear localization sequences, short stretches of amino acids responsible for targeting them to the nucleus. We show that the first 29 amino acids of GAL4, a yeast DNA-binding protein, function efficiently as a nuclear localization sequence when fused to normally cytoplasmic invertase, but not when fused to *Escherichia coli* β-galactosidase. Moreover, the nuclear localization sequence from simian virus 40 T antigen functions better when fused to invertase than when fused to β-galactosidase. A single amino acid change in the T-antigen nuclear localization sequence inhibits the nuclear localization of simian virus 40-invertase and simian virus 40–β-galactosidase in *Saccharomyces cerevisiae*. From these results, we conclude that the relative ability of a nuclear localization sequence to act depends on the protein to which it is linked.

Nuclear localization sequences are defined as short stretches of amino acids that are sufficient to target proteins to the nucleus (reviewed in references 5 and 28). Their function has been demonstrated by mutational analysis and gene fusion experiments. For example, a mutation at amino acid 128 of simian virus 40 (SV40) large T antigen causes the mutant T antigen to accumulate in the cytoplasm (12, 15). Mutations to either side of amino acid 128 have a lesser effect on T-antigen nuclear localization. Gene fusions encoding portions of T antigen fused to a normally non-nuclear protein, such as pyruvate kinase, yield nuclear imported proteins (13). By these analyses, a short stretch of amino acids was defined as both necessary and sufficient for T-antigen nuclear import. Similar experiments have revealed nuclear localization sequences at the N terminus of GAL4, a well-characterized DNA-binding protein of *Saccharomyces cerevisiae* (29), and in a number of other nuclear proteins (2, 4, 9, 20, 24, 32). These nuclear localization sequences share little primary sequence homology, although they do have a high content of basic amino acids. Furthermore, in at least one case, the ability of the SV40 T-antigen sequence to promote nuclear import depends on where it resides within the imported protein (25).

We do not know how nuclear localization sequences function. By one model, they are recognized by a receptor that may be located at the nuclear surface (21, 23) or in the cytoplasm. Following binding at the nuclear pore, proteins are imported through the pore in an ATP-dependent process (6, 22).

We designed the following experiments to further define the nuclear localization sequence from GAL4. Previously, we showed that the first 74 GAL4 amino acids were sufficient to target a GAL4–β-galactosidase fusion protein to the yeast nucleus (29). Mutations causing single amino acid changes throughout this region of the protein affect nuclear localization to varying degrees (30). We now show that β-galactosidase fusion proteins containing the first 61, 34, or 29 GAL4 amino acids are no longer nuclear localized. On the other hand, the first 29 GAL4 amino acids, when fused to normally cytoplasmic invertase, are sufficient to localize that protein to the nucleus. By this analysis, a nuclear localization sequence has been defined, but its ability to function depends on the protein to which it is fused.

We have also investigated the ability of the SV40 T-antigen nuclear localization sequence to function in *S. cerevisiae*. When fused to the N terminus of either invertase or β-galactosidase, the resulting proteins are localized to the nucleus. However, the nuclear localization of SV40-invertase is more complete than that of SV40–β-galactosidase.

MATERIALS AND METHODS

Construction of gene fusions. (i) *GAL4-lacZ* gene fusions. P_{ADH}-*GAL4* was excised as a *Bam*HI-*Xho*I fragment from plasmid PS118, which contains $P_{(ADH)}$-$GAL4_{(1-74)}$-*lacZ* in YEp213 (29). The fragment was introduced into RB486, a derivative of pBR322 with the *Pvu*II site converted to *Xho*I. The resulting plasmid was digested with *Xho*I, treated with Bal31 exonuclease for varying lengths of time, treated with Klenow, and ligated with *Bam*HI linkers. The resulting deletions were sequenced by the dideoxy method (26). Deletions ending at GAL4 amino acids 61, 34, and 29 were fused to *lac*Z at a *Bam*HI site corresponding to amino acid 7 of LacZ in YEp213.

(ii) *GAL4-SUC2* gene fusions. All *SUC2* fusions were made in pSEY304 (1), which contains a truncated form of *SUC2* that is missing its own signal sequence and has a polylinker inserted at codon 3 of mature invertase. To facilitate construction of some of the gene fusions, PSEY304 was digested with *Hin*dIII, treated with Klenow to generate blunt ends, and ligated with *Xho*I linkers to create pMN2. $GAL4_{(1-74)}$-*SUC2* was generated by excision of the P_{ADH}-*GAL4*-containing fragment from the corresponding *lacZ* fusion and ligation into the *Bam*HI-*Xho*I site of pMN2. $GAL4_{(1-29)}$-*SUC2* was created by ligation of the *Bam*HI fragment carrying $P_{(ADH)}$-$GAL4_{(1-29)}$ into the *Bam*HI site of pSEY304.

(iii) SV40 gene fusions. An oligonucleotide with the sequence

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5'AGCTTATGCCCAAGAAGAAGCGGAAGGTCCC 3'
      ATACGGGTTCTTCTTCGCCTTCCAGGGAGCT
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was synthesized and introduced into PS118 that had been previously treated with *Hin*dIII-*Xho*I to remove GAL4 sequences. For the SV40 T-antigen mutant, an oligonucleotide with the sequence

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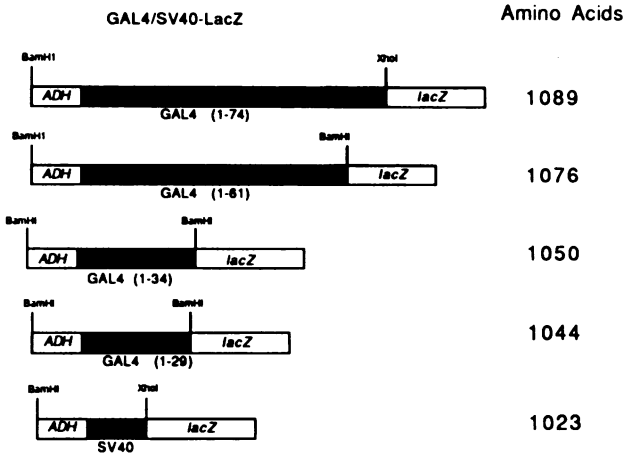

FIG. 1. *GAL4* and SV40-*lacZ* gene fusions under control of the *ADH1* promoter. $GAL4_{(1\text{-}74)}$-*lacZ* contained on plasmid PS118 has been described previously (29). New truncated *GAL4* genes bearing 5′ sequences were created by cutting PS118 at the unique *Xho*I site, treating with *Bal*31 exonuclease and DNA polymerase large fragment (Klenow), and ligating with *Bam*HI linkers. The truncated *GAL4* genes with the *ADH1* promoter were ligated to the *Bam*HI site corresponding to amino acid 7 encoded in the *lacZ* gene. SV40-*lacZ* was made with an oligonucleotide encoding the amino acid sequence shown in Fig. 6. Each oligonucleotide also contained *Hin*dIII and *Xho*I sites for introduction into PS118 in place of *GAL4* sequences.

5′ AGCTTATGCCCAAGACGAAGCGGAAGGTCCC 3′
ATACGGGTTCTGCTTCGCCTTCCAGGGAGCT

was synthesized and introduced into *lacZ* in the same manner. SV40-*SUC2* fusions were generated by excising the $P_{(ADH)}$-SV40 BamHI-*Xho*I-containing fragment from the corresponding *lacZ* fusion and ligating it into pMN2.

Immunoprecipitations. Cells were labeled with 0.1 mCi of $Na_2{}^{35}SO_4$ (31) for 30 min, and cell lysates were prepared as described previously (30). LacZ fusion proteins were detected by precipitation with 50 μl of mouse monoclonal anti-β-galactosidase followed by 50 μl of a 10% (wt/vol) suspension of formalin-fixed, sodium dodecyl sulfate-washed *Staphylococcus aureus*. Following elution from the *Staphylococcus aureus* in sample buffer (14), the samples were analyzed by sodium dodecyl sulfate-gel electrophoresis on a 6% polyacrylamide gel. Invertase fusion proteins were detected in the same way, except that 5 μl of rabbit anti-invertase was used and electrophoresis was on a 10% polyacrylamide gel.

Immunofluorescence. Cells were prepared for immunofluorescence and β-galactosidase fusion proteins were detected as described previously (30). To visualize invertase fusion proteins, rabbit anti-invertase antibody (from R. Schekman) was used at 1:10,000 dilution, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) (Miles Laboratories) at a 1:200 dilution.

Strains. *S. cerevisiae* strains used were DB745 (*MATα adel-100 leu2-3 leu2-112 ura3-52*) and SEY2101 (*MATa suc2-Δ9 ura3-52 leu2-3 leu2-112 gal2 ade2-101*). *Escherichia coli* strains MM294 (*end hsdR thi pro*) and JM107 [Δ(*lac-pro*) *thi rpsl supE endA* F′ *proAB lacI*^{q} *ZΔM15*] were used for construction and maintenance of plasmids.

Other methods. β-Galactosidase was assayed as described previously (8). Media for growing yeasts and *E. coli* are described in references 27 and 18, respectively. Yeast transformation was performed by the lithium acetate method of Ito et al. (10). Oligonucleotides were synthesized by a Applied Biosystems model 380A DNA synthesizer. DNA manipulations were performed as described in reference 17.

RESULTS

Fusions between GAL4 and β-galactosidase. We wished to define further the information within the GAL4 protein necessary for its nuclear localization. The first 74 amino acids of GAL4 have been shown previously to be sufficient to localize *E. coli* β-galactosidase to the yeast nucleus (29). Intact β-galactosidase is, on the other hand, not localized to any compartment within the yeast cell, consistent with it being a cytoplasmic protein (19, 30).

We constructed gene fusions between portions of *GAL4* encoding the first 61, 34, and 29 amino acids and *lacZ* (Fig.

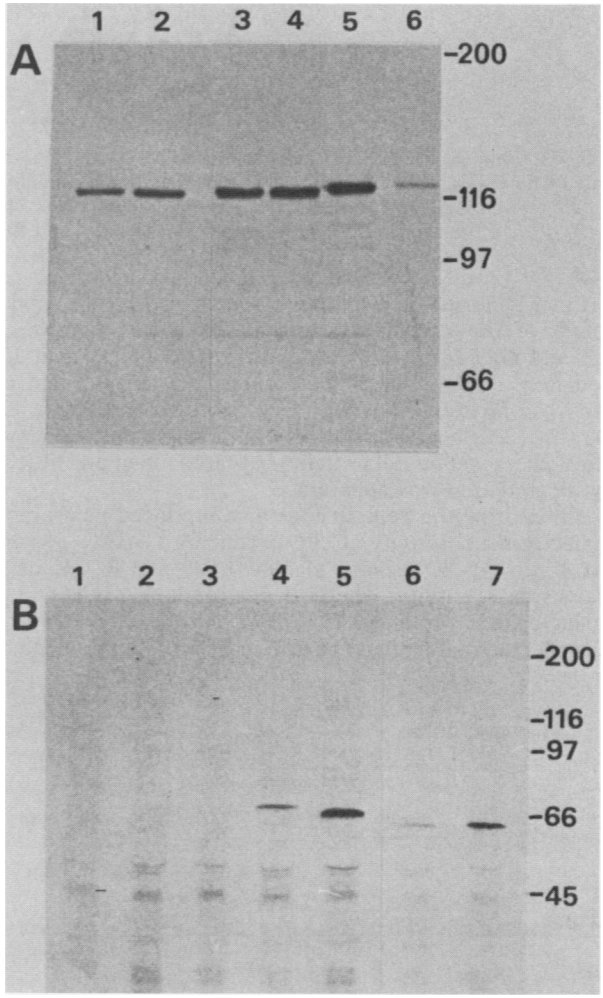

FIG. 2. Immunoprecipitation of proteins from cells producing LacZ and SUC2 chimeric proteins with anti-β-galactosidase (A) or anti-invertase (B). (A) Analysis was performed with lysates of yeast cells bearing the following gene fusions: lane 1, SV40-*lacZ*; lane 2, mutant SV40-*lacZ*; lane 3, $GAL4_{(1\text{-}29)}$-*lacZ*; lane 4, $GAL4_{(1\text{-}34)}$-*lacZ*; lane 5, $GAL4_{(1\text{-}61)}$-*lacZ*; lane 6, $GAL4_{(1\text{-}74)}$-*lacZ*. (B) Analysis with anti-invertase was performed on cells bearing the following gene fusions: lane 4, $GAL4_{(1\text{-}74)}$-*SUC2*; lane 5, $GAL4_{(1\text{-}29)}$-*SUC2*; lane 6, SV40-*SUC2*; lane 7, mutant SV40-*SUC2*. Lanes 1 and 2, *suc2* cells alone; lane 3, cells bearing pSEY304.

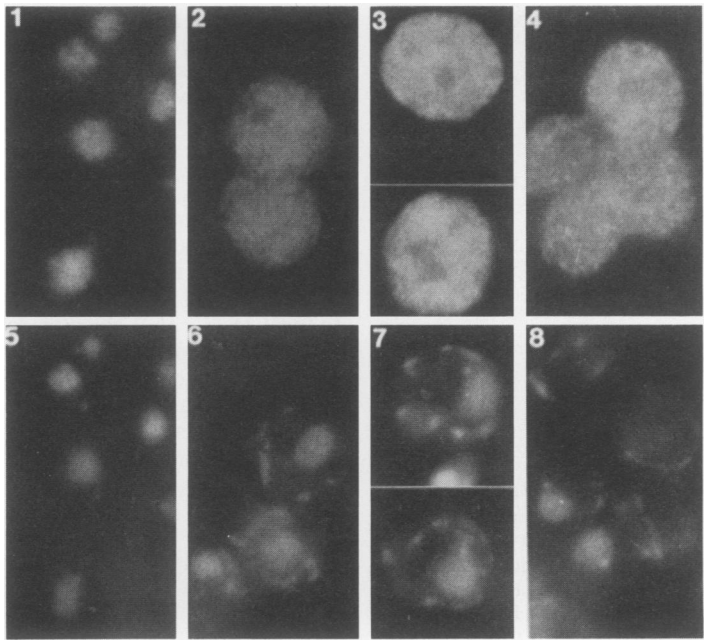

FIG. 3. Immunofluorescence of cells producing GAL4-LacZ fusion proteins. Cells were prepared for indirect immunofluorescence and treated with either mouse monoclonal anti-β-galactosidase antibody followed by FITC-conjugated anti-mouse IgG (panels 1 to 4) to identify the chimeric proteins or 4',6-diamidino-2-phenylindole (panels 5 to 8) to identify cell nuclei. Panels 1 and 5, *GAL4*$_{(1-74)}$-*lacZ*; panels 2 and 6, *GAL4*$_{(1-61)}$-*lacZ*; panels 3 and 7, *GAL4*$_{(1-34)}$-*lacZ*; panels 4 and 8, *GAL4*$_{(1-29)}$-*lacZ*.

1). Each gene fusion was expressed in yeast cells, and a protein of the predicted size was produced. *GAL4*$_{(1-74)}$-*lacZ* and *GAL4*$_{(1-61)}$-*lacZ* yielded proteins of 124 and 122 kilodaltons (kDa), respectively (Fig. 2A, lanes 5 and 6). *GAL4*$_{(1-34)}$-*lacZ* and *GAL4*$_{(1-29)}$-*lacZ* yielded proteins of 119 kDa (Fig. 2A, lanes 3 and 4). By immunoprecipitation with anti-β-galactosidase, all proteins appeared intact and little, if any, degradation was apparent.

Cells bearing the gene fusions also produced measurable β-galactosidase activity. Cells producing GAL4$_{(1-61)}$– and GAL4$_{(1-29)}$–β-galactosidase yielded 1,049 and 647 U of β-galactosidase activity, whereas cells producing GAL4$_{(1-74)}$–β-galactosidase produced 35 U. The difference in activity is also reflected in the relative amounts of protein produced by the gene fusion-bearing cells (Fig. 2A, cf. lane 6 and lanes 3 to 5).

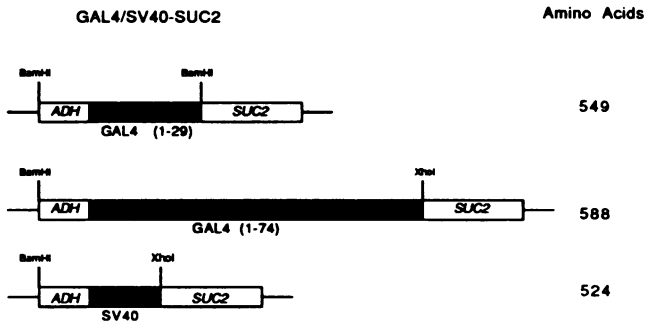

FIG. 4. *GAL4* and *SV40-SUC2* gene fusions under control of the *ADH1* promoter. Portions of *GAL4* encoding GAL4 amino acids 1 to 74 and 1 to 29 were introduced into pSEY304 (1) so that in-frame fusions between *GAL4* and *SUC2* were created. Fusions containing SV40 used the same oligonucleotides as *lacZ* fusions described in the legend to Fig. 1. All fusions were expressed from the *ADH1* promoter and were contained in pSEY304, which also bears *URA3* and a 2μm origin of replication.

None of the GAL4–β-galactosidase fusion proteins except GAL4$_{(1-74)}$–β-galactosidase (Fig. 3, panel 1) was localized to the nucleus as judged by immunofluorescence with anti-β-galactosidase antibody. GAL4$_{(1-61)}$–, GAL4$_{(1-34)}$–, and GAL4$_{(1-29)}$–β-galactosidases all appeared distributed throughout the cells (Fig. 3, panels 2 to 4). By this analysis, we cannot say that the protein is totally excluded from the nucleus. This staining pattern is the same as that observed for intact β-galactosidase (30), which can enter the yeast nucleus to some extent but does not accumulate there (19).

The protein distribution is not a consequence of overproduction. Each protein fusion, when expressed from a single integrated gene, still showed the same nonnuclear distribution. Moreover, when the *ADH1* promoter on *GAL4*$_{(1-29)}$-*lacZ* was substituted with the weaker *GAL4* promoter so that the amount of protein produced was comparable to that from *ADH-GAL4*$_{(1-74)}$-*lacZ*, the distribution remained the same, although the immunofluorescence was much fainter (data not shown). Moreover, the half-lives of GAL4$_{(1-74)}$–β-galactosidase and GAL4$_{(1-61)}$–β-galactosidase are both about 5 to 6 h (30; P. Silver, unpublished results).

Fusions between *GAL4* and *SUC2*. Fusion proteins containing the first 74 or the first 29 GAL4 amino acids fused to invertase were localized to the yeast nucleus. Invertase, the product of the *SUC2* gene (3), is made as either a constitutive cytoplasmic form or an inducible secreted form (3). The cytoplasmic form lacks the N-terminal signal sequence necessary for secretion. Gene fusions were constructed that contained portions of GAL4 encoding the first 74 or the first 29 amino acids fused to *SUC2* at codon 3 of mature invertase (Fig. 4). The gene fusions were introduced into *S. cerevisiae* via a 2μm-containing vector and expressed from the *ADH1* promoter. Yeast cells bearing these gene fusions produced

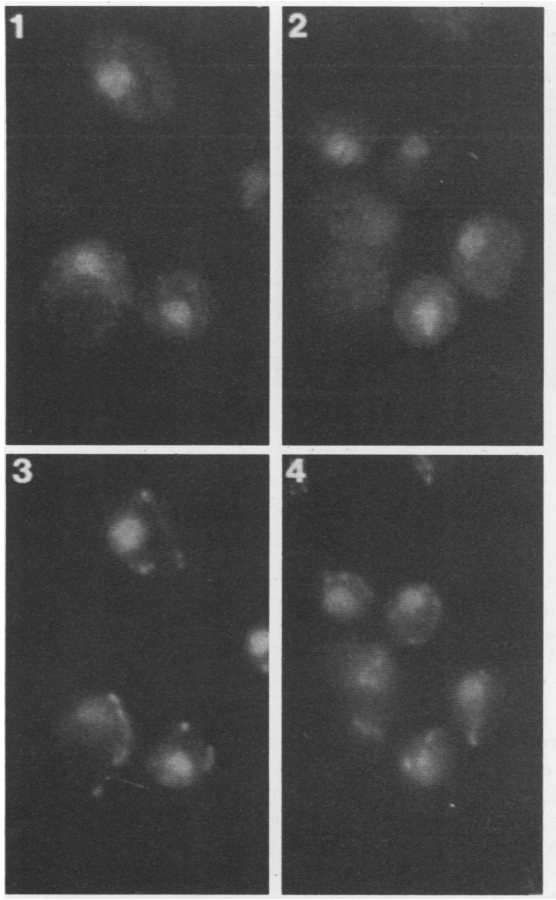

FIG. 5. Immunofluorescence of cells producing GAL4-invertase fusion proteins. Cells were prepared for indirect immunofluorescence and treated with either rabbit anti-invertase, followed by FITC-conjugated anti-rabbit IgG (panels 1 and 2), to identify the fusion proteins or DAPI (panels 3 and 4) to identify cell nuclei. Panels 1 and 3, $GAL4_{(1-74)}$-*SUC2*; panels 2 and 4, $GAL4_{(1-29)}$-*SUC2*.

proteins of the approximate predicted size, as determined by immunoprecipitation of radiolabeled cell extracts. $GAL4_{(1-74)}$-*SUC2* produced a 65-kDa protein (Fig. 2B, lane 4) and $GAL4_{(1-29)}$-*SUC2* produced a 61-kDa protein (Fig. 2B, lane 5). When assayed by immunofluorescence with anti-invertase antibody, both $GAL4_{(1-74)}$-invertase (Fig. 5, panel 1) and $GAL4_{(1-29)}$-invertase (Fig. 5, panel 2) were mainly associated with the nucleus. The staining pattern was similar to that seen for $GAL4_{(1-74)}$–β-galactosidase (Fig. 3, panel 1).

A nuclear localization sequence from SV40 T antigen functions in yeast cells. An oligonucleotide encoding the SV40-T-antigen nuclear localization sequence (Fig. 6) was synthesized and fused to either *lacZ* or *SUC2* to yield in-frame fusion proteins (Fig. 1 and 4, respectively). When introduced into yeast cells, proteins of the predicted molecular size were produced: 117 kDa in the case of SV40–β-galactosidase (Fig. 2A, lane 1) and 60 kDa in the case of SV40-invertase (Fig. 2B, lane 6). The SV40–β-galactosidase (Fig. 7, panel 1) and the SV40-invertase (Fig. 8, panel 1) were localized to the nucleus as judged by immunofluorescence. The nuclear localization of SV40-invertase was more complete (less cytoplasmic staining) than that of SV40–β-galactosidase. In fact, the SV40-invertase showed the strongest nuclear localization of any fusion protein we have observed in yeast cells.

A single amino acid change in the SV40 nuclear localization sequence abolished its function in *S. cerevisiae*. An oligonucleotide with the lysine at position 4 (equivalent to lysine 128 of mature T antigen; Fig. 6) converted to threonine was synthesized and fused in-frame to *lacZ* and *SUC2*. When introduced into yeast cells, fusion proteins of the predicted molecular size were produced (Fig. 2A, lane 2, and Fig. 2B, lane 7). Both mutant SV40–β-galactosidase (Fig. 7, panel 4) and SV40-invertase (Fig. 8, Panel 4) were no longer nuclear localized, as judged by immunofluorescence.

DISCUSSION

We show that the first 29 amino acids of the yeast DNA-binding protein GAL4 are sufficient to localize normally cytoplasmic invertase to the yeast nucleus. However, the same 29 amino acids, when fused to the N terminus of β-galactosidase, do not localize that fusion protein to the nucleus. The nuclear localization sequence from SV40 T antigen functions in *S. cerevisiae*, and a mutation that inactivates its function in mammalian cells has the same effect in yeast cells.

Tests of nuclear localization sequence function in yeast cells often use β-galactosidase fusion proteins (9, 19, 20, 30). β-Galactosidase is a large hydrophilic protein of the *E. coli* cytoplasm, which normally forms a tetramer. For this reason, β-galactosidase does not readily cross membranes and should contain no information for localization to any intracellular compartment in a eucaryotic cell. However, we wanted to test the ability of nuclear localization sequences to target a true yeast cytoplasmic protein to the nucleus. The cytoplasmic form of invertase is missing the N-terminal amino acid signal sequence. Fusions of heterologous proteins to the N terminus of invertase maintain invertase activity and have been used extensively to study protein sorting to the yeast vacuole (1, 11). We now show that, in two different constructions, invertase bearing a nuclear localization sequence appears to be more completely localized to the nucleus than is β-galactosidase.

The apparent context dependence of the first 29 GAL4 amino acids (Fig. 6) and the SV40 nuclear localization sequence in promoting nuclear protein import could be explained in one or more of the following ways.

Protein size. The monomeric molecular weight of β-galactosidase is about twice that of invertase. The first 74 GA14 amino acids may contain several shorter nuclear localization sequences. A single targeting sequence contained in the first 29 GAL4 amino acids and the SV40 sequence may not be sufficient to completely transport a protein as large as β-galactosidase. Proteins with multiple nuclear localization sequences have been shown to be more efficiently imported into the nucleus (7, 16).

GAL4	Amino Acids 1-29	M K L L S S I E Q A C D L C R L K K L K C S K E K P K C A
SV 40 T Antigen		M P K K K R K V

FIG. 6. Sequence of GAL4 amino acids 1 to 29 and the SV40 T-antigen nuclear localization sequence.

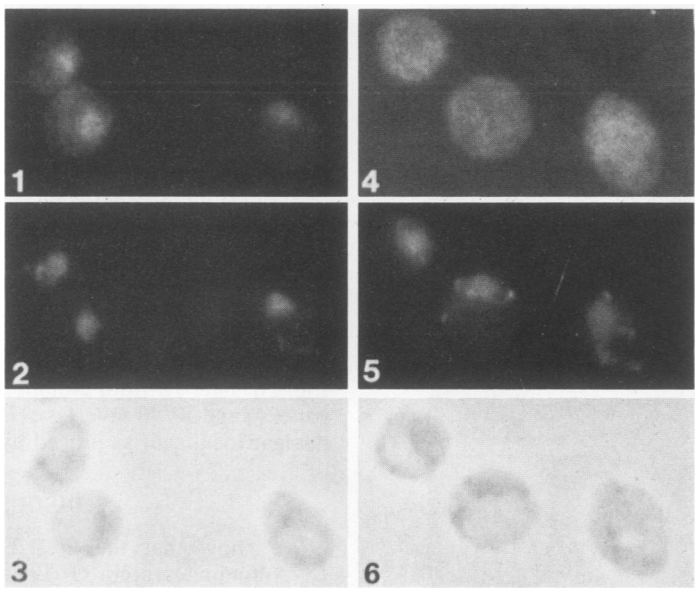

FIG. 7. Immunofluorescence of cells bearing SV40-*lacZ* gene fusions. Cells were prepared for indirect immunofluorescence and treated with either mouse monoclonal anti-β-galactosidase, followed by FITC-conjugated anti-mouse IgG (panels 1 and 4), or DAPI (panels 2 and 5). Panels 3 and 6 are the corresponding cells photographed by phase contrast. Panels 1 to 3, Wild-type SV40-*lacZ*; panels 4 to 6, mutant SV40-*lacZ*.

Oligomerization. Active β-galactosidase is a tetramer. We do not know whether it is imported into the nucleus as a tetramer or a monomer. Deletion of amino acids 62 to 74 may increase the ability of the protein to tetramerize in the cytoplasm. The resulting 450-kDa tetramer may then be too large for efficient localization with a partially active localization sequence.

Protein structure. Invertase may be contributing structural or sequence information that is missing from the first 29 GAL4 amino acids and cannot be provided by β-galactosidase. The position of the peptides within the protein seems unlikely to explain the difference in their ability to act since they are all placed at the N terminus of the fusion proteins.

The SV40 T-antigen nuclear localization sequence is one

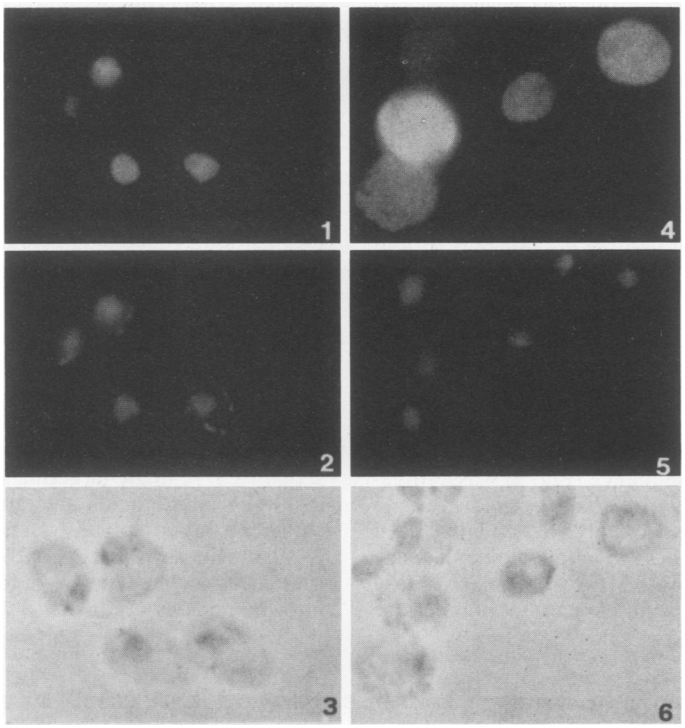

FIG. 8. Immunofluorescence of cells bearing SV40-*SUC2* gene fusions. Cells were prepared for indirect immunofluorescence and treated with either rabbit polyclonal anti-invertase, followed by FITC-conjugated anti-rabbit IgG (panels 1 and 4), or DAPI (panels 2 and 5). Panels 3 and 6 are the corresponding cells photographed by phase contrast. Panels 1 to 3, Wild-type SV40-*SUC2*; panels 4 to 6, mutant SV40-*SUC2*.

of the best characterized of such sequences. This sequence can also function to target heterologous proteins to the yeast nucleus (Fig. 7 and 8). The efficiency with which the SV40 peptide functions also appears to depend on the size or sequence of the protein to which it is fused. A single amino acid change, which allowed the original identification of the sequence as a nuclear localization sequence in mammals (12, 15), also eliminates its nuclear localization activity in yeast cells. Taken together, these results confirm the commonality between the mechanism of nuclear protein transport in fungi and mammals.

ACKNOWLEDGMENTS

We thank S. Emr for pSEY304 and SEY2101 and R. Schekman for anti-invertase antibodies. We thank S. Chapman and X. Yang for technical assistance, M. Bernstein and H. Blumberg for advice, and M. Flocco for DNA synthesis.

This work was supported by Public Health Service grant GM36373-01 from the National Institutes of Health and in part by a Presidential Young Investigator Award from the National Science Foundation. M.N. is supported by Public Health Service Institutional NRSA grant GM07312.

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