Identification of a nuclear localization signal of a yeast ribosomal protein

(Saccharomyces cerevisiae/hybrid proteins/ribosome assembly)

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ABSTRACT To identify a signal involved in transporting a ribosomal protein to the nucleus, we constructed hybrid genes encoding amino-terminal segments of yeast ribosomal protein L3 joined to the amino-terminal end of the entire Escherichia coli beta-galactosidase molecule. The subcellular locations of the corresponding hybrid proteins in yeast were determined by in situ immunofluorescence. The first 21 amino acids of L3 were sufficient to localize beta-galactosidase to the nucleus. This region shows limited homology to portions of other nuclear proteins identified as essential for their transport. Larger fusion proteins were also localized to the nucleus. However, a hybrid protein containing all but the 14 carboxyl-terminal amino acids from L3 initially failed to localize; this defect was corrected by inserting a glycine- and proline-containing bridge between the L3 and beta-galactosidase moieties. The renaturated protein was able to associate with ribosomes, suggesting that, in addition to entering the nucleus, this hybrid polypeptide was assembled into 60S ribosomal subunits that were subsequently exported to the cytoplasm.

Ribosome biosynthesis is a complex yet highly efficient process (1). Anywhere from 50 to 75 different ribosomal proteins and several RNA molecules are each synthesized in equal molar amounts and assembled at a rate that is precisely matched to the requirements of a particular growth condition and that leaves no unassembled components. In eukaryotic organisms, most ribosomal proteins are transported into the nucleus as complexes with nascent ribosomal RNA transcripts (1). Thus, even if each protein were produced at the same rate in the cytoplasm, this factor would be of little benefit to the economy of ribosome biosynthesis unless the ribosomal proteins were also delivered to the nucleus rapidly and coordinately. That ribosomal proteins do indeed meet this criterion is borne out by pulse-labeling experiments; within minutes of their synthesis most ribosomal proteins are found in the nucleus (2).

As the nuclear transport of ribosomal proteins is rapid and coordinated, mechanisms must exist to direct the proteins to their assembly site and it is likely that the proteins themselves contain the information that determines their destination. With this view, we have undertaken to identify nuclear transport signals in ribosomal proteins by using gene-fusion techniques. A series of genes were constructed specifying hybrid proteins containing at their amino terminus various extents of yeast ribosomal protein L3 followed by the Escherichia coli beta-galactosidase polypeptide sequence. The locations of the hybrid proteins in yeast were determined by in situ immunofluorescence staining with anti-beta-galactosidase antibody. Our results show that the first 21 amino acids of L3 are sufficient to direct beta-galactosidase to the nucleus. This 21 amino acid signal shows limited homology to regions of other nuclear proteins that have been identified as essential for their transport.

MATERIALS AND METHODS

Strains. Plasmids used in this study were propagated in E. coli strain MC1061 (araD139 Δara–leu7697 ΔlacX74 GalUA–GalK–hsr– hsm+ strA), provided by M. Casadaban (University of Chicago) and Saccharomyces cerevisiae strain DBY745 (Mata ura3-52 ade1-101 leu2-3 leu2-112) from D. Botstein (Massachusetts Institute of Technology, Cambridge).

Construction of L3–beta-Galactosidase Hybrid Genes. Restriction enzyme fragments of the yeast TIM1 gene, which encodes ribosomal protein L3 (3), were joined at either a Sma I or a BamH I restriction site adjacent to the beta-galactosidase coding sequence in plasmid pTCM-PP (4, 5) to produce a continuous translational reading frame fusing the L3 amino acid sequence to beta-galactosidase. Table I lists the resulting plasmids and the particular fragments of TIM1 (see Fig. 1) which they contain. All TIM1-lacZ junctions were verified by DNA sequencing (not shown).

Plasmid pTCM-PPneo is a modified version of pTCM-PP. In addition to the 373 amino acid codons of L3 joined to the beta-galactosidase sequence in pTCM-PP, pTCM-PPneo contains three tandem Neo I oligonucleotide linkers (CCCATGGG; New England Biolabs) inserted between the L3 and beta-galactosidase DNA segments and was obtained by ligating 2 pmol of the Pvu II (position 465)–Pvu II (+1147) fragment of TIM1 in the presence of 80 pmol of linker prior to inserting the fragment in pJT24. The linker addition resulted in the sequence -Ala-His-Gly-Pro-Met-Gly-Pro-Trp–bridging the L3 and beta-galactosidase portions of the encoded protein (see Results).

NaDODSO4/PAGE Analysis of L3–beta-Galactosidase Hybrid Proteins. Twenty-five milliliters of cells grown to 2 × 10^7 per ml were harvested, washed in NET-NP (150 mM NaCl, 5 mM EDTA, 50 mM Tris Cl, pH 7.4/0.5% Nonidet P-40/0.02% NaN3) (6), resuspended with 0.5 ml of NET-NP containing protease inhibitors [chymostatin, leupeptin, and pepstatin A (each at 1 μg/ml) and 1 mM phenylmethylsulfonyl fluoride], transferred to a 1.5-ml Eppendorf tube, and disrupted by shaking with glass beads. After centrifugation for 1 min at 15,000 × g, the supernatant was saved, the pellet was washed twice with 0.2 ml of NET-NP containing protease inhibitors, and the three supernatants were pooled and incubated for 1 hr at 0°C with 45 μg of affinity-purified anti-beta-galactosidase antibody. After 1 hr, antigen–antibody complexes were retrieved by adding 0.1 ml of a 10% suspension of formalin-fixed Staphylococcus aureus cells (Bethesda Research Laboratories) and continuing incubation at 0°C for 30 min. The cells were pelleted, washed three times in NET with 0.05% Nonidet P-40, suspended in 62.5 mM Tris Cl, pH 6.8/2% NaDODSO4/10% (vol/vol) glycerol/5% (vol/vol) 2-mercapto-

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Abbreviation: DAPI, 4',6-diamidino-2-phenylindole.
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ethanol/0.005% bromphenol blue, and heated at 100°C for 3 min. After brief centrifugation, the supernatant was electrophoresed in a gel composed of 7% acrylamide and 0.35% N,N′-diallyltartardiamide (Sigma) and containing 0.1% NaDodSO4. Gels were stained in 7% acetic acid/50% methanol/0.2% Coomassie blue R-250.

**Indirect Immunofluorescence Localization of L3-β-Galactosidase Proteins in Yeast.** The cellular location of fusion proteins was visualized by treating cells prepared for immunofluorescence (7) with a rabbit anti-β-galactosidase antibody followed by a fluorescein-conjugated goat anti-rabbit IgG antibody. Nuclei were visualized by staining with the nonintercalating DNA dye 4′,6-diamidino-2-phenylindole (DAPI; Aldrich). After fixation and removal of cell walls, cells were applied to polylysine-coated toxoplasmosis slides (Vellco Glass). Affinity-purified rabbit anti-β-galactosidase antibodies and affinity-purified fluorescein-conjugated goat anti-rabbit IgG antibodies were prepared according to Kilmartin et al. (8). Protocols for antibody treatment of slides and DAPI staining were as described (9).

**Sedimentation Analysis of β-Galactosidase.** Yeast polyribosomes were extracted and sedimented in sucrose gradients exactly as described (10). Polyribosomes were converted to 80S monosomes by including in the cell extracts (prepared without heparin and diethylpyrocarbonate) RNase A at 10 μg/ml. Gradient fractions were diluted in Z buffer and assayed for β-galactosidase (11).

**RESULTS**

**Construction of L3-β-Galactosidase Genes.** To identify a region of a ribosomal protein responsible for its nuclear localization, we joined various amino-terminal portions of the gene TCM1, which encodes yeast ribosomal protein L3 (3), to the amino-terminal end encoding the entire coding sequence of the E. coli lacZ gene. Since β-galactosidase (Mr 116,000) is too large to diffuse freely into the nucleus (12), any segment of L3 causing nuclear association of β-galactosidase is thereby implicated as containing a signal involved in transport. Thus, five restriction enzyme fragments of TCM1 were selected whose promoter distal ends (shown in boldface in Fig. 1) encompass from 1% (5 amino acids) to 96% (373 amino acids) of the TCM1 coding sequence. These fragments were inserted into the plasmid cloning vector pJT24 (4, 5), placing the L3 sequence upstream and adjacent to that of β-galactosidase. The particular restriction enzyme fragments of TCM1 inserted into pJT24 were chosen also on the basis of their nucleotide sequence (13), which indicated that the ribosomal protein and β-galactosidase segments would be joined in a continuous translational reading frame without additional modification of the DNA fragments. When introduced into yeast cells, each of the five plasmids led to β-galactosidase production (the parent plasmid pJT24 does not direct β-galactosidase synthesis), under direction of the TCM1 transcriptional signals.

**Properties of Hybrid Proteins.** Before localizing hybrid proteins in the cell, we sought information about the relative amount of each protein produced by the various plasmids. Thus β-galactosidase was assayed by measuring hydrolysis of o-nitrophenyl β-D-galactoside in crude extracts of the yeast transformants (11). The results, given in Table 1, show that levels of β-galactosidase vary over a 50-fold range from one plasmid-bearing strain to the next. This variability was not a consequence of differences in the frequency of transcription of the hybrid genes, as each has a promoter of equivalent strength (5). In addition, some differences were too great to be accounted for by plasmid copy number (data not shown). Rather, the variability was likely due to detrimental effects of some of the hybrid proteins upon cell viability, since, as Table 1 shows, the presence of these plasmids in cells was coincident with reduced growth rates.

To verify that the plasmids directed synthesis of hybrid proteins of the correct sizes, cell extracts were incubated with anti-β-galactosidase antibody. The immunoprecipitates were recovered and analyzed by NaDodSO4/7% PAGE (Fig. 2). As expected, cells carrying either plasmid pTCM-RR or plasmid pTCM-HA yielded a single immunoprecipitable protein with an electrophoretic mobility essentially equal to that of authentic β-galactosidase (the fusion protein encoded by these two plasmids contain only 5 and 21 amino acids, respectively, joined to β-galactosidase, too few to have been detected as a difference in electrophoretic mobility. Cells transformed with plasmid pTCM-PP yield a product whose estimated molecular weight is 157,000, close to the predicted size of 155,000 calculated from addition of 373 amino acids to β-galactosidase. In contrast, plasmids pTCM-SS and pTCM-HB routinely yielded two immunoprecipitable β-galactosidase proteins, one corresponding in size to that of the full-length hybrid and one corresponding to β-galactosidase. Fig. 2 shows this phenomenon for the 99 amino acid fusion (lane 10×HB). Apparently, the ribosomal protein moiety of 36 and 99 amino acids were cleaved from these two hybrid proteins. Cleavage of these proteins was not peculiar to L3-β-galactosidase hybrids, since it occurred with a protein consisting of 110 amino acids of another ribosomal protein.

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**Table 1. Properties of L3-β-galactosidase fusion proteins in yeast**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Restriction fragment of TCM1 joined to lacZ*</th>
<th>Amino acids of L3 joined to β-galactosidase</th>
<th>β-Galactosidase, units</th>
<th>Location of hybrid protein</th>
<th>Cell-doubling time, hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.6</td>
</tr>
<tr>
<td>pTCM-RR</td>
<td>Rsa I(−94)→Rsa I(+)</td>
<td>1–5</td>
<td>700</td>
<td>Cytoplasmic</td>
<td>3.8</td>
</tr>
<tr>
<td>pTCM-HA</td>
<td>Hpa I(−229)→Hpa I(+)</td>
<td>1–21</td>
<td>400</td>
<td>Nuclear</td>
<td>6.4</td>
</tr>
<tr>
<td>pTCM-SS</td>
<td>Sau3A(−138)→Sau3A (+136)</td>
<td>1–36</td>
<td>15</td>
<td>Nuclear</td>
<td>6.4</td>
</tr>
<tr>
<td>pTCM-HB</td>
<td>Hpa I(−229)→Bgl II(+)</td>
<td>1–99</td>
<td>13</td>
<td>Nuclear</td>
<td>5.4</td>
</tr>
<tr>
<td>pTCM-PP</td>
<td>Pvu II(−465)→Pvu II(+)</td>
<td>1–373</td>
<td>100</td>
<td>Cytoplasmic</td>
<td>4.0</td>
</tr>
<tr>
<td>pTCM-PPnoc</td>
<td>Pvu II(−465)→Pvu II(+)</td>
<td>1–373</td>
<td>77</td>
<td>Nuclear (primarily)</td>
<td>5.4</td>
</tr>
</tbody>
</table>

*See Fig. 1.
†See ref. 11.
L29 (14), joined to β-galactosidase (see Fig. 2). Because of the difficulty in inhibiting yeast vacuolar proteasines, the proteolysis of these hybrid proteins may have occurred in vitro during incubation of extracts with antibody; clarification of this phenomenon may require use of strains lacking endogenous proteases (15). Nonetheless, it is clear that the plasmids directed synthesis of proteins of the correct sizes. Finally, Fig. 2 illustrates that levels of β-galactosidase measured from o-nitrophenyl β-D-galactoside assays are reflect- ed in the yield of hybrid protein recovered by immunoprecipitation.

Subcellular Localization. The subcellular locations of the hybrid proteins described above were determined by in situ immunofluorescence with anti-β-galactosidase IgG (7–9). Cells were stained both with antibody, to detect β-galactosidase proteins, and with DAPI, which stains the nucleus by binding to DNA. Fig. 3 shows representative fluorescence micrographs for cells transformed with pTCM-RR and pTCM-HA. The nuclei of cells transformed with pTCM-HA showed strong fluorescence staining with β-galactosidase antibody as did cells carrying pTCM-SS and pTCM-HB (data not shown), whereas cells transformed with pTCM-RR lacked any localized staining and exhibited fluorescence throughout the cell. Thus, the first 21 amino acids of ribosomal protein L3 were capable of localizing β-galacto- sidase to the nucleus. At this level of resolution, we cannot determine whether the proteins are inside the nucleus or sequestered in the nuclear envelope.

Although hybrid proteins containing the first 21, 36, or 99 amino acids of L3 were associated with the nucleus, the fusion protein containing 373 amino acids of L3 failed to localize (Fig. 4B). The failure of this protein to localize was surprising, since it contains all but the carboxyl-terminal 14 amino acids of L3 and should most closely resemble L3 itself. However, this fusion protein was largely insoluble, since most (75–80%) of the β-galactosidase activity could be sedimented by low-speed centrifugation after cell disruption. Thus, we speculated that the L3 portion of the protein was prevented from folding properly by its association with β-galactosidase, leading to insolubility and inability to be transported. We discovered, however, that insertion of 8 additional amino acids between the L3 and β-galactosidase moieties of this hybrid produced a protein that was targeted to the nucleus (see pTCM-PP_Nco, in Fig. 4). The extra 8 amino acids, Ala-His-Pro-Met-Gly-Pro-Trp, are not derived from L3 but are encoded in three tandem Nco I oligonucle- otide linkers inserted at the L3-β-galactosidase junction of pTCM-PP. Fortuitously, the original 373 amino acid fusion contained a proline residue at the L3-β-galactosidase junction which would be expected to have aided in isolating the L3 and β-galactosidase domains if simple secondary structure alone is considered. Secondary-structure predictions (16) suggested the presence of an α-helix ending at residue 361 followed by a β-pleated sheet structure up to residue 372 and a short (4 amino acid) turn at the fusion junction (data not shown). This line of reasoning led us to choose the Nco I oligomer for insertion, as it provided additional proline and glycine residues with which to separate the L3 and β-galactosidase moieties. The 8 amino acid insertion was predicted to extend the turn to 10 amino acids, which could act as a hinge between the two proteins and more effectively separate them from one another. This proposition is reasonable in view of the fact that adjacent secondary structures in proteins have been found to interact (reviewed in ref. 17). Whatever was the defect in the original hybrid protein that prevented its nuclear localization, it is indeed dramatic that an 8 amino acid insertion into a protein of nearly 1400 amino acids would alter its nuclear localization and biochemical properties (see below).

Ribosomal Association of Hybrid Protein. Although the peptide bridge between L3 and β-galactosidase permitted the 373 amino acid fusion to localize to the nucleus, considerable fluorescence was usually seen in the cytosol. This incomplete localization may simply indicate that the peptide bridge was not an ideal solution to the protein's transport defect. Alternatively, since this fusion contains all but the carboxyl-
terminal 14 amino acids of L3, perhaps acquisition of transport enabled this protein to assemble into a 60S ribosomal subunit and be exported back to the cytoplasm. To determine whether the fusion protein was associated with ribosomes, polyribosomes were sedimented in a sucrose gradient and fractions were assayed for β-galactosidase activity. As a control, ribosomes were also sedimented from cells containing the 5 amino acid fusion. While all of the β-galactosidase activity of the 5 amino acid fusion was found at the top of the gradient, β-galactosidase activity from the large fusion resided not only at the top of the gradient but in a region containing 60S subunits and 80S monosomes and in a region containing polyribosomes (Fig. 5). Thus, at least by sedimentation, the hybrid protein behaved as if it were associated with ribosomes. A similar analysis of cells containing the original 373 amino acid fusion (without the linker) did not show β-galactosidase in either the monosome or polysome region (data not shown). A further demonstration of the association of the hybrid protein (containing the peptide bridge) with ribosomes was found by treating extracts with a small amount of RNase, which converted polyribosomes into 80S monosomes. After such treatment, the polysome peak of β-galactosidase was absent but considerable β-galactosidase activity was still found in the 80S region. The β-galactosidase activity associated with ribosomes was unlikely due to enzyme molecules in the process of being translated. Absence of as few as 10 of the carboxyl-terminal amino acids completely inactivates the enzyme (18), and it is unlikely that there were enough chains completed beyond this point, but still unreleased, to account for the fact that nearly half the β-galactosidase activity was found in the ribosomal portions of the gradient. Thus, although we do not know that the hybrid protein is properly assembled into the ribosomes, it appears that it is nonetheless associated with ribosomes and this association probably explains the cytoplasmic fluorescence of antibody-stained cells. We note also that a β-galactosidase fusion to ribosomal protein 51A has also been found to associate with ribosomes (L. Gritz, N. Abovich, J. L. Teem, and M. Rosbash, personal communication).

DISCUSSION

In this study we have asked whether a ribosomal protein contains the information to direct its transport to the nucleus. Our results show that the amino-terminal 21 amino acids of yeast ribosomal protein L3 are sufficient to localize E. coli β-galactosidase to the nucleus. These results confirm our expectation that ribosomal proteins should contain signals that direct the proteins efficiently to their site of assembly, as one factor ensuring their economical synthesis. So far, studies of nuclear localization similar to the one reported here have examined primarily DNA binding proteins (9, 19, 20). Our results show that another class of proteins, often overlooked as far as nuclear transport is concerned, is also amenable to questions about mechanisms of protein trafficking.

As far as the mechanism of localization is concerned, we cannot say how the 21 amino acid domain of L3 functions. It is generally assumed that proteins enter the nucleus by way of the nuclear pore, although the evidence is limited. In a recent elegant study, Feldherr et al. (21) injected nucleoplasm-coated gold particles into Xenopus oocytes and showed that the particles not only accumulated in the nucleus but could be seen passing through a nuclear pore complex (21). As the first 21 amino acids of L3 have a net charge of +5, it seems more likely that, if this region is a signal for specific uptake by the nucleus, it would interact with the pore rather than the hydrophobic barrier presented by the nuclear envelope. However, a large variety of materials also pass out of the nucleus by way of the pore, including ribosomal subunits. In Tetrahymena, the exit rate has been calculated to be about 45 large and small subunits transported per pore per minute (22). Whether incoming proteins compete for the same pores is not known. Possibly, ribosomal proteins are somehow recognized elsewhere in the nuclear envelope by way of a signal such as described here for L3. In any case, in whatever way the amino terminus of L3 functions, it probably acts differently from the familiar signal sequence in
proteins transported through the endoplasmic reticulum (23, 24). First, unlike secreted proteins which are synthesized as precursors from which the signal sequence is cleaved, ribosomal proteins do not undergo proteolytic processing; translation of ribosomal protein mRNAs in vitro yields products indistinguishable in size from proteins extracted from mature ribosomes (25, 26). Also, amino-terminal amino acid analyses of many yeast ribosomal proteins, including L3, correspond to that predicted from DNA sequence (36). Secondly, nuclear localization of ribosomal proteins probably need not involve a mechanism of cotranslational transport whereby an emerging domain of a nascent ribosomal polypeptide directs the transfer of the ribosomal protein to the nuclear envelope prior to completion of translation, as is the case for secreted proteins. If this were so, then the fusion protein which contains 96% of L3 (derived from pTCM-PP) should have been localized to the nucleus, irrespective of the behavior of the distal part of the protein. We interpret the finding that nuclear localization of this protein occurred only after insertion of a peptide bridge between the L3 and β-galactosidase moieties to indicate that the original fusion protein was folded in some manner as to conceal the nuclear localization signal, before that signal could be accessed by the nuclear envelope. Of course, it is possible that the original fusion is targeted during translation but the finished product is blocked in transport and released to the cytoplasm. We note, however, that when ribosomal protein S6, isolated from 40S subunits of Xenopus laevis, is injected into the cytoplasm of Xenopus oocytes, the protein first enters the nucleus and is later found assembled into subunits (27). Similarly histones and nucleoplasmin also enter the nucleus as mature proteins (28–30). Thus, although cotranslational transport is not ruled out, we favor the idea that ribosomal proteins are transported in a finished form. Lack of a cotranslational transport mechanism is also consistent with the observation that ribosomal proteins are not found to be synthesized in any particular location in eukaryotic cells (31, 32).

The amino-terminal domain of L3 which directs it to the nucleus is of further interest from the standpoint of its sequence and predicted secondary structure. The requirements for nuclear localization have been examined in detail with simian virus 40 (SV40) large tumor (T) antigen. A naturally occurring mutant defective for transport of T antigen has been described (33). A single substitution of asparagine for lysine in position 128 is responsible for the defect. Further, the minimal sequence required for targeting SV40 large T antigen to the nucleus has been defined elegantly by Kalderon and coworkers (20, 34). The region from residue 126 to residue 132 has been shown to be sufficient for nuclear localization. In addition a substitution of threonine for lysine-128 destroys nuclear localization, whereas substitutions in positions 129–131 yield partially localized proteins. The primary sequence of SV40 large T antigen from residue 126 to residue 132 (+Pro-Lys-Lys128, Lys-Arg-Lys-Val-124) is not strictly homologous to any part of the amino-terminal 21 amino acids of L3. However, the overall motif of a proline residue followed by several basic amino acids (+Pro-Arg-Lys20-Arg- in L3) is noted with strict homology at Lys-20/Lys-128. A similar motif has been observed for yeast histone H2B (unpublished observation). Further, a common secondary structure can be predicted for the regions of yeast H2B and SV40 large T antigen that have been determined by mutation to be responsible for their localization (data not shown). Site-specific-mutation analysis of L3 may provide a means to test whether a common nuclear transport signal exists between L3 and SV40 T antigen. If the sequences are homologous in function, we predict that substitutions at Lys-20 of L3 (analogous to Lys-128 of T antigen) should eliminate its nuclear localization. Finally, since the sequences for many other yeast ribosomal proteins are known (35), it will be interesting to compare the amino-terminal region of L3 with those of these other proteins for potential nuclear localization signals.

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