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Characterization of nuclear localizing sequences derived from yeast ribosomal protein L29

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Two particular seven-amino-acid segments from yeast ribosomal protein L29 caused a non-nuclear reporter protein to associate almost exclusively with the yeast nucleus. The two L29-derived nuclear localizing sequences were identical in five of the seven residues, many of which were basic amino acids. Generally, localization of the reporter protein was most impaired by replacement of the basic residues. A particular Arg residue was unique; substitution by any amino acid including Lys diminished nuclear localization of the reporter protein. In L29 the corresponding Arg 25→Lys substitution within the nuclear localizing sequence distal to the N-terminus was without effect, as evidenced by normal rates of ribosome assembly and cell growth. However, the analogous Arg 8→Lys substitution within the localizing sequence proximal to the N-terminus led to greatly reduced rates of ribosome assembly and cell growth. Finally, when both localizing sequences contained the Arg→Lys substitution a still greater decrease in ribosome assembly and cell growth was observed. These results were as expected if the two short peptide sequences functioned in nuclear localization and/or assembly of yeast ribosomal protein L29.

Key words: ribosomal protein/yeast/nucleus/nuclear localization/mutations

Introduction

Formation of the eukaryotic ribosome initiates when about 70 different ribosomal proteins enter the nucleus, wherein the proteins associate stoichiometrically with four RNA molecules to construct 40S and 60S ribonucleoprotein subunits. Newly assembled ribosomal subunits then exit to the cytoplasm to function in translation. In all cells numerous gene regulatory phenomena tune the initial synthesis of ribosomal components to the demands of cell growth, so the amount of each component synthesized is sufficient for just the required number of ribosomes (Warner, 1989). However, regulation of synthesis of the individual gene products is only one aspect of efficient ribosomal biosynthesis; mechanisms should also exist to promote efficient assembly of ribosomal components. We have therefore undertaken a study to determine how ribosomal proteins translocate from the cytoplasm to the nucleus, an early step in ribosome assembly.

Proteins enter the nucleus through nuclear pores (Feldherr *et al.*, 1984), complex structures that form aqueous channels in the nuclear envelope (Scheer *et al.*, 1988). Since the limit to passage by free diffusion through the pore is ~90 Å (Paine *et al.*, 1975), yet proteins enter the nucleus extremely rapidly (Bonner, 1975; DeRobertis *et al.*, 1978; Dingwall *et al.*, 1982; Feldherr *et al.*, 1983), polypeptides larger than this size limit have been presumed to be assisted in some way through the pore.

Recent studies involving site-specific mutagenesis and/or deletion analyses have established that a number of nuclear proteins possess one or more discrete domains or short peptide sequences that are required for the proteins to enter the nucleus (Dingwall *et al.*, 1982; Kalderon *et al.*, 1984a,b; Davey *et al.*, 1985; Richardson *et al.*, 1986; Lee *et al.*, 1987; Lyons *et al.*, 1987; Picard and Yamamoto, 1987; Dang and Lee, 1988; Kleinschmidt and Seiter, 1988; Loewinger and McKeon, 1988; Maher *et al.*, 1989). Additional protein sequences that function as nuclear localization signals (NLS) have been identified using gene fusions (Hall *et al.*, 1984; Moreland *et al.*, 1985, 1987; Wychowski *et al.*, 1986; Dingwall *et al.*, 1988; Siomi *et al.*, 1988; Nelson and Silver, 1989). Overall, there is limited homology among NLS, although they generally contain predominantly basic amino acids. Considerable latitude exists in what constitutes a functional sequence and, in fact, even non-protein amino acids will suffice; nonetheless, some residues critical for function have been identified in individual NLS (Kalderon *et al.*, 1984a; Colledge *et al.*, 1986; Lanford *et al.*, 1988; Chelsky *et al.*, 1989). Nuclear localization sequences appear to act as ligands for as yet to be defined nuclear pore receptors; following binding to the pore complex, an energy dependent process translocates the proteins through the pore (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988). Cytosolic proteins have been identified that specifically recognize many of these same NLS (Adam *et al.*, 1989; Yamasaki *et al.*, 1989), suggesting that nuclear proteins might be chaperoned to the nuclear pore.

As a first step to determining their mode of nuclear import, we are identifying segments of ribosomal proteins that participate in bringing about their nuclear accumulation. Here we describe two seven-amino-acid, positively charged peptide sequences derived from yeast ribosomal protein L29. The two sequences were identical in five of the seven residues and either was able to bring about essentially complete nuclear association of a reporter protein *in vivo*. Amino acid substitutions in the two NLS were created and many substitutions, including a particular conservative Arg→Lys replacement, abolished localization of the reporter protein. Within L29 itself, the same Arg→Lys change disrupted ribosome assembly; substitution in the localizing sequence proximal to the amino terminus produced a more severe defect than substitution in the more N-terminal distal

sequence. We suggest that the two short peptide sequences are responsible for nuclear localization and/or assembly of yeast ribosomal protein L29.

Results

Initial delineation of nuclear localization sequences derived from ribosomal protein L29

We described previously a series of gene fusions in which portions of yeast ribosomal protein L3 were joined to the amino terminus of *Escherichia coli* β-*galactosidase* (Moreland *et al.*, 1985). The subcellular locations of the fusion proteins were determined in yeast by staining cells with anti-β-galactosidase antibody. In this way a putative nuclear localization sequence was pinpointed to the amino terminal 21 residues of L3. A similar approach was applied to a second ribosomal protein, L29. L29 contains 148 amino acids encoded by two exons of 15 and 133 residues. Originally, three fusion proteins were constructed (H.Fried, unpublished; Nam, 1985). The first placed amino acid residues 1−110 of L29 in frame with β-galactosidase; the resulting protein was localized to the nucleus (data not shown). The second fusion placed amino acid residues 1−16 of L29 in frame with β-galactosidase; this protein was also strongly localized to the nucleus. These results suggested that a possible nuclear localization sequence resided in the first exon of L29. To verify that this was the case we exchanged the first exon of L29 with the first exon from the yeast actin gene, the latter encoding only two amino acids. Contrary to expectation, a β-galactosidase fusion protein containing the first two residues of actin followed by residues 17−110 of L29 was also nuclear localized. Apparently both exons of the L29 gene encoded information sufficient to localize β-galactosidase to the yeast nucleus.

To identify more precisely potential nuclear localization segments, we compared the amino acid sequence contained in the first exon of L29 to that of its larger second exon. The search revealed two seven residue sequences, KTRKHRG (exon 1, residues 6−13) and KHRKHPG (exon 2, residues 23−29) that were identical in five positions. These peptide sequences resembled nuclear localization sequences described for other proteins in that the L29 peptides contained an abundance of basic residues. To determine if these peptide sequences were themselves capable of promoting nuclear localization, we synthesized double stranded DNA oligonucleotides encoding the two peptides, hereafter designated NLS-1 and NLS-2, and inserted the oligomers at the amino terminus of the β-galactosidase gene (the actual fusion sequences were NH_2-MTGS KTRKHRGSGA...*lac*Z and NH_2-MTGSKHRKHPGSGA ...*lac*Z, where underlined residues are derived from L29).

Figure 1 shows the results of indirect immunofluorescence microscopy to determine the subcellular locations of the NLS-β-galactosidase fusion proteins. Both NLS-1 and NLS-2 oligomers, inserted in the correct orientation adjacent to β-galactosidase, produced proteins that were associated with the nucleus (see A−B and C−D in Figure 1). Insertion of the oligomers in the opposite orientation (thereby creating a completely different amino sequence) generated proteins with no apparent localization (not shown). We also inserted the NLS at the junction of a pre-existing fusion protein that contained the first five amino acids of ribosomal protein L3 joined to β-galactosidase (the actual fusion sequence was NH_2-MSHRKYGSKHRKHPGSAL...*lac*Z); the tripartite fusion protein also was nuclear localized (see E−F in Figure 1). These results suggested that the NLS-1 and NLS-2 sequences may be responsible for the nuclear location of L29.

Analysis of mutant NLS

To date there is limited consensus on what sequence or structure of amino acids is required for nuclear localizations since little homology is apparent amongst the reported sequences. Thus far the most extensively characterized NLS is that of the SV40 large T antigen. The T antigen NLS consists of seven amino acids, five of which are basic. Several studies have identified residues important for the function of the SV40 NLS; a particular lysine (Lys 128) near the center of the sequence appears to be the most critical residue, though substitutions by arginine and even some non-protein positively charged amino acids retain partial function (Kalderon *et al.*, 1984a,b; Lanford and Butel, 1984; Colledge *et al.*, 1986; Lanford *et al.*, 1988). We wished to determine whether the NLS sequences derived from L29 were similar in amino acid requirements to the well characterized SV40 NLS and to identify critical amino acids which could then be replaced in L29 to test the function of the peptide sequences in the native ribosomal protein. In the discussion that follows a functional NLS was one that caused nuclear association equal to the wild-type sequence and a non-functional NLS was one that produced a protein displaying essentially uniform staining throughout the cell.

To create an NLS with amino acid substitutions, we re-synthesized the NLS-2-oligomers using the method of Hutchison *et al.* (1985) in which random incorrect nucleotides were incorporated. The pool of NLS-2 double stranded oligomers containing misincorporated nucleotides was inserted adjacent to β-galactosidase exactly as had been done for the wild-type oligonucleotides. Random clones were sequenced and selected mutants analyzed by immunofluorescence localization. Figure 2 lists the single and double substitution mutants that were examined and indicates their localization phenotypes. A comprehensive discussion of the results is not practical here but several salient features are described below.

A number of amino acid substitutions yielded proteins with diminished but not absent nuclear localization, as seen by a more pronounced cytoplasmic background (e.g. mutation [M] 169 in Figure 3B). This intermediate ability to cause nuclear association was noted in previous studies with altered SV40 large T antigen NLS (Kalderon *et al.*, 1984a). We were particularly interested in the effect of substitutions at either of the lysine residues, since nuclear localization mediated by the T antigen NLS was especially sensitive to changes at Lys 128; similarly, nuclear localization mediated by an NLS from yeast histone H2B also depended on a particular lysine residue (Moreland *et al.*, 1987). However, unlike the single critical lysine in the SV40 and histone NLS, *both* Lys 23 and Lys 26 in the L29 NLS were sensitive to substitution by a non-positively charged residue. Replacement of either lysine with uncharged asparagine or methionine abolished nuclear localization while (like the SV40 NLS) arginine was an acceptable substitute (see Figures 2 and 3C).

Replacement of either of the histidines in NLS-2 was generally without effect. Total loss of function was seen only

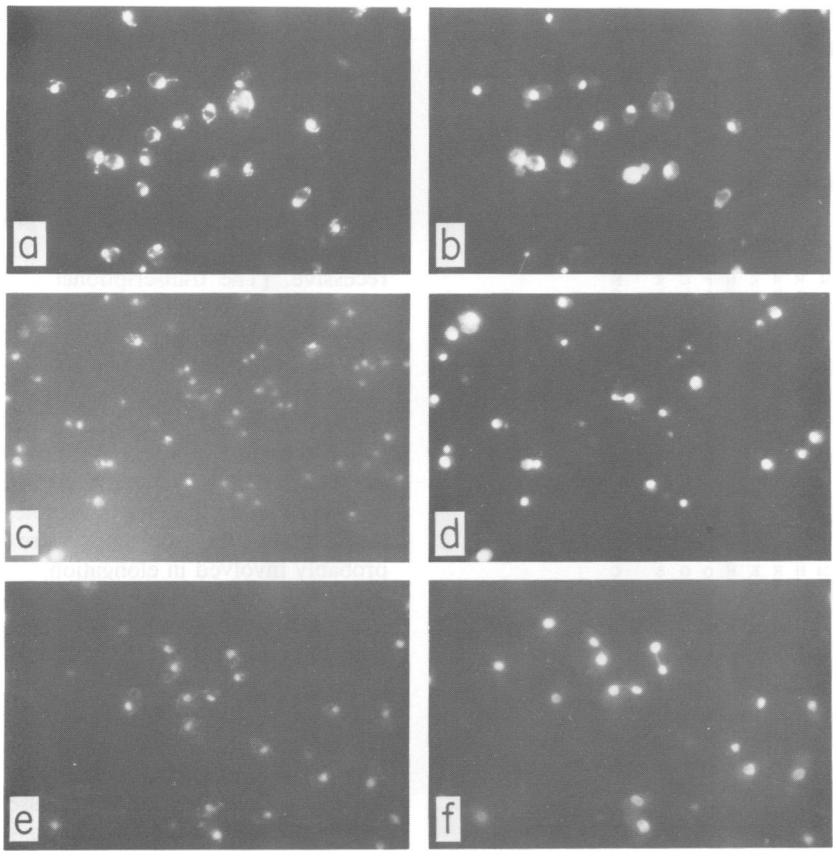

Fig. 1. Indirect immunofluorescence microscopy of yeast cells transformed with plasmids encoding chimeric β-galactosidase polypeptides containing wild-type nuclear localization signals. Samples were prepared for immunofluorescence microscopy as described in Materials and methods. (**A**) and (**B**): cells producing NLS-1−β-galactosidase fusion; (**C** and (**D**): NLS-2−β-galactosidase fusion; (**E**) and (**F**): ribosomal protein L3−NLS-2−β-galactosidase fusion. (A), (C) and (E) show DAPI fluorescence (identifying nuclei), while (B), (D) and (F) show FITC-conjugated antibody fluorescence (indicating β-galactosidase).

when His was replaced with Pro (M 193) or with a negatively charged and sterically dissimilar aspartic acid (M 234). Replacement with a positively charged and sterically dissimilar arginine allowed function. A decrease in function was seen for substitutions of His 24 with leucine (M 250) or of His 27 with tyrosine (M 197). Unexpectedly however, a similar substitution of tyrosine for His 24 (M 215) did not affect nuclear localization, indicating that in this context the two histidine positions were not functionally equivalent.

The amino acid in the NLS-2 sequence which was unique was Arg 25. Thus, while replacement of either of the lysines with arginine did not disrupt nuclear localization, the reverse substitution of lysine for Arg 25 rendered the NLS non-functional (see M 156, Figure 3D); occasionally a cell was seen with a very slight nuclear association. Oddly, substitution of isoleucine for this same Arg 25 did allow for some accumulation, albeit less than normal (M 216, not shown).

A pool of double mutants was also examined and of interest were mutants 160 and 169. M 160 contained both an Arg 25→Lys mutation (which eliminated nuclear localization in M 156; see above) as well as a Gly 29→Arg replacement. Unexpectedly, nuclear localization was maintained in this double mutant (Figure 3F). M 169 had a Lys 26→Gln change (which would have been expected to cause loss of function based on other single mutations in this same lysine) as well as the same Gly 29→Arg substitution that maintained nuclear localization in M 160. Cells expressing M 169 displayed nuclear association but with increased cytoplasmic background (Figure 3B).

Mutagenesis of putative nuclear localization sequences within L29

The preceding results demonstrated that the NLS derived from L29 can bring about nuclear localization of β-galactosidase, with some residues being of considerable importance in this function. To determine if the NLS were functional domains in the ribosomal protein itself, we introduced one of the non-localizing mutations into L29, either at NLS-1 (Arg 8→Lys), NLS-2 (Arg 25→Lys) or both NLS together. The Arg→Lys substitution was selected because this substitution dramatically reduced nuclear association of the reporter protein while being chemically the least dissimilar replacement to do so.

L29 is essential for ribosome assembly and therefore essential for cell growth (Fried *et al.*, 1985). A mutation in the NLS that interfered either with migration of the protein into the nucleus or with any other step in assembly would be expected to impair growth. Thus, we examined the growth rate of cells synthesizing (from single copy plasmids) the mutant L29 polypeptides. Plasmids carrying L29 genes were introduced into yeast strain GCYH-X23. This strain contained a chromosomal L29 gene under transcriptional control of the *GAL10* activation sequence (Fried *et al.*, 1985); as such, transformants grown in galactoside expressed

both the chromosomal (wild-type) L29 protein and the mutant proteins introduced on plasmids, while the same cells grown on glucose expressed only the plasmid encoded proteins.

Substitution of Lys for Arg 25 in NLS-2 had no apparent effect on cell growth. However, the corresponding substitution of Lys for Arg 8 in NLS-1 led to a 50% increase in doubling time, from 0.3 to 0.14 doublings per hour. Furthermore, although the mutation in NLS-2 alone was without effect, when the NLS-1 and NLS-2 mutations were combined cell growth was further reduced to 0.08 doublings per hour. We also measured the growth rate of cells expressing simultaneously the wild-type and mutant L29 proteins. Co-expression of both proteins had no effect on normal growth rate, indicating that the mutations were recessive. [The transcriptional activity of the galactose-requiring chromosome L29 gene was equivalent to that of the plasmid containing L29 genes that were expressed from their normal promoter (Fried *et al.*, 1985).] The significance of a recessive phenotype regarding ribosome assembly is discussed later. In conclusion the reduced growth rates coincident with the Arg→Lys substitutions in L29 suggested that the mutations had interfered with the efficient assembly of ribosomes or that the L29 proteins were competent for assembly but the ribosomes were dysfunctional. [L29 is probably involved in elongation, since resistance to a high cycloheximide concentration, which blocks elongation (Cooper and Bossinger, 1976), is obtained by mutations in L29 (Käufer *et al.*, 1983; Stöcklein and Peipersberg, 1980)].

	Non-mutant sequences									Location
NLS-1	G	S	*K*	*T*	*R*	*K*	*H*	*R*	*G*	S	N
NLS-2	G	S	*K*	*H*	*R*	*K*	*H*	*P*	*G*	S	N
Mut.	**Single mutations in NLS-2**									
226	G	S	N	H	R	K	H	P	G	S	C
161	G	S	R	H	R	K	H	P	G	S	N
193	G	S	K	P	R	K	H	P	G	S	C
214	G	S	K	R	R	K	H	P	G	S	N
215	G	S	K	Y	R	K	H	P	G	S	N
250	G	S	K	L	R	K	H	P	G	S	N>C
156	G	S	K	H	K	K	H	P	G	S	C
195	G	S	K	H	W	K	H	P	G	S	C
266	G	S	K	H	G	K	H	P	G	S	C
269	G	S	K	H	S	K	H	P	G	S	C
221	G	S	K	H	T	K	H	P	G	S	C
216	G	S	K	H	I	K	H	P	G	S	N>C
143	G	S	K	H	R	M	H	P	G	S	C
207	G	S	K	H	R	N	H	P	G	S	C
152	G	S	K	H	R	R	H	P	G	S	N
197	G	S	K	H	R	K	Y	P	G	S	N>C
234	G	S	K	H	R	K	D	P	G	S	C
	Double mutations in NLS-2									
142	G	S	N	L	R	K	H	P	G	S	C
146	G	S	K	E	R	K	Q	P	G	S	C
149	G	S	M	H	R	K	H	Q	G	S	C
151	G	S	K	Q	R	K	Q	P	G	S	C
160	G	S	K	H	K	K	H	P	R	S	N
168	G	S	Q	H	R	K	Y	P	G	S	C
169	G	S	K	H	R	Q	H	P	R	S	N>C
220	G	S	K	L	R	M	H	P	G	S	C
223	G	S	R	H	L	K	H	P	G	S	N

Fig. 2. Effect of various single and double amino acid replacements on localization of NLS-2−β-galactosidase fusion protein. Residues of NLS-1 and NLS-2 shown in italics are ribosomal protein L29 sequence. Localization determined by indirect immunofluorescence. N = nuclear localization; C = no localization; N>C = partial nuclear localization with cytoplasmic fluorescence.

Analysis of ribosomal subunit accumulation

Having established that the NLS mutations impaired cell growth we next wished to determine whether the defect was due to a deficiency in ribosome assembly. Sucrose gradient sedimentation was employed to assess the cellular content

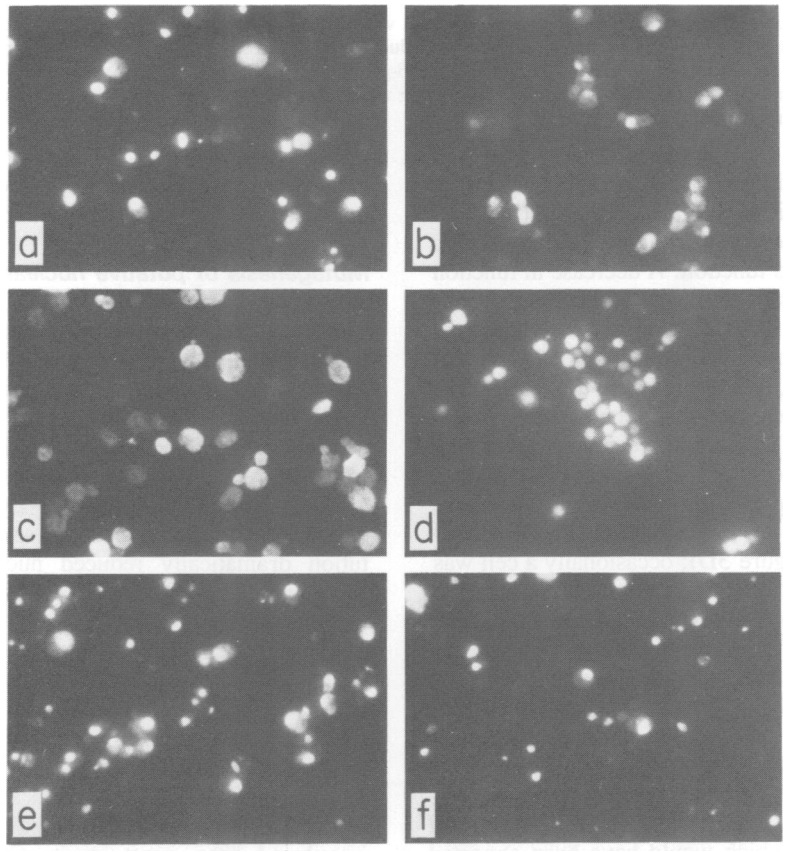

Fig. 3. Indirect immunofluorescence microscopy of yeast cells transformed with plasmids encoding mutant NLS-2 sequences fused to β-galactosidase. Samples were prepared for immunofluorescence microscopy as described in Materials and methods. Only the FITC conjugated anti-β-galactosidase fluorescence is shown. (**A**) Mutation 161 (Lys 23→Arg); (**B**) mutation 169 (Lys 26→Gln plus Gly 29→Arg); (**C**) mutation 143 (Lys 26→Met); (**D**) mutation 156 (Arg 25→Lys); (**E**) mutation 152 (Lys 26→Arg); (**F**) mutation 160 (Arg 25→Lys plus Gly 29→Arg).

of ribosomes. Cells carrying the single copy plasmids described in the preceding section were transferred to glucose for a time sufficient to eliminate synthesis of wild-type L29, after which they were labeled with [^{3}H]uracil (see Materials and methods). Polysomes and ribosomes were isolated from the labelled cells and separated on 7−47% sucrose gradients as described (Warner *et al.*, 1985). Note that the L29 gene contained in the plasmids was the cycloheximide resistance allele (Fried and Warner, 1982). Because the plasmid encoded genes may have conferred resistance, we did not treat cells with cycloheximide prior to extraction as is customarily done to prevent run-off of polyribosomes. Thus, the majority of material seen in the gradients was 80S monosomes and 40S and 60S subunits; only these regions of the gradients are shown in Figure 4.

The relative amount of 40S subunits did not appear to vary appreciably in all samples. However, in comparing the shoulder on the 80S peak, i.e. 60S subunits, plus the 80S peak itself (roughtly fractions 25 to 34) it could be seen that the NLS-1 mutation caused a significant decrease in the amount of 60S + 80S material relative to wild-type. Since the 40S subunit level was unchanged, the mutation probably prevented normal assembly of 60S subunits, of which L29 is a constituent. Note, however, that some 80S ribosomes were still formed in cells producing the NLS-1 mutant protein. Cells producing the NLS-1−NLS-2 double mutant showed a similar deficiency in the ribosome pattern. Finally, the NLS-2 mutation produced little, if any, defect since its ribosome profile was essentially identical to wild-type. Thus, the abundance of ribosomal subunits in cells producing the three mutant L29 proteins correlated with the growth rate of these cells, normal for the NLS-2 mutation and greatly reduced for both the NLS-1 mutant and the double NLS-1−NLS-2 mutant proteins. In summary, the major defect imposed by the latter mutations was an inability to accumulate 60S subunits.

Ribosomal RNA processing

From sucrose gradient analysis the amount of large ribosomal subunits was lower in cells producing NLS-1 or NLS-1−NLS-2 mutant proteins than cells producing wild-type L29. This analysis, nevertheless, did not address the mechanism for reduced subunit accumulation. The decrease in large subunits could have been due either to a reduction in assembly itself or to degradation or instability of large subunits assembled with the altered proteins. To distinguish between these possibilities the initial assembly of ribosomal subunits was characterized by the pattern of maturation of rRNA; proper processing and stability of rRNA is dependent upon ongoing ribosome assembly (Udem and Warner, 1972).

Synthesis of rRNA was monitored by pulse-labeling cells for 5 min with L-[methyl-^{3}H]methionine while processing was observed following a 15 min chase with unlabeled methionine (Kief and Warner, 1981). RNA was extracted, denatured with formaldehyde and separated by size in agarose gels. Cells producing the NLS-2 mutant protein, which were normal in growth rate and ribosome accumulation, displayed the same pattern of precursor rRNA as cells that produced wild-type L29 (Figure 5). However, cells producing the NLS-1 mutant protein, which were found to have a defect in ribosome assembly, displayed a buildup of

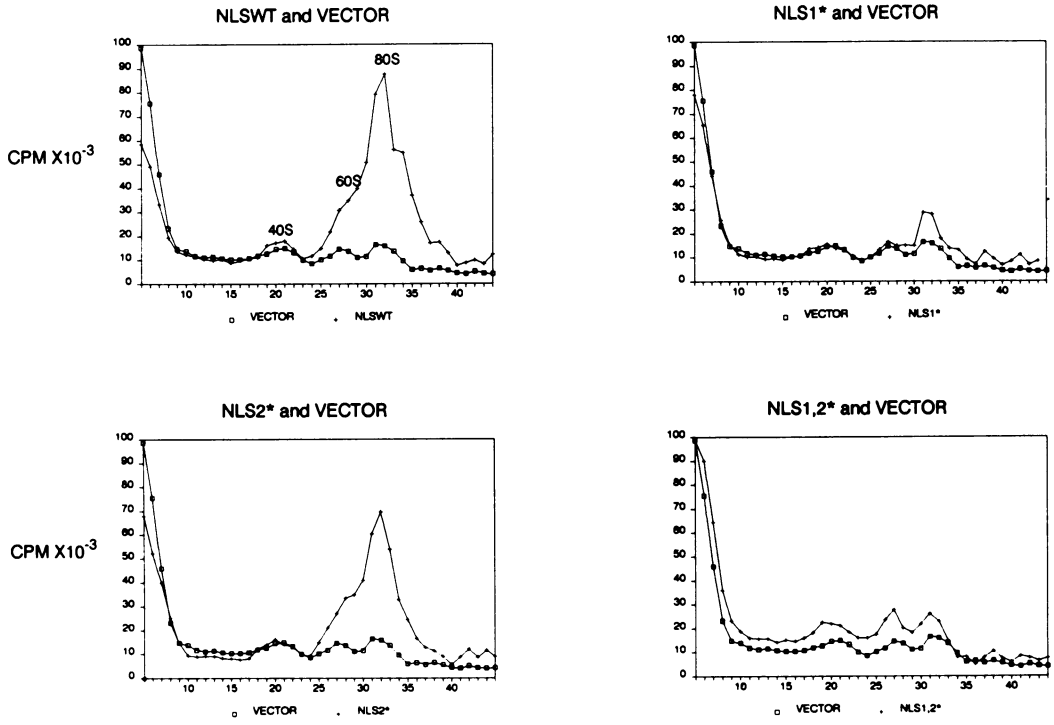

Fig. 4. Polysome profile of yeast strain GCYH-X23 transformed with wild type or mutant L29 genes. Following 2 h incubation in glucose to inactivate the chromosomal (wild-type) L29 gene and permit total elimination of its mRNA, cells were labeled with [^{3}H]uracil for 2.5 h to label ribosomes, synthesis of which was dependent upon the plasmid encoded L29 proteins. Equal amounts of A_{260} material were sedimented individually in sucrose gradients. The left end of each graph was the top of the gradient so the heavier polysome region was to the right. The 40S, 60S and 80S peaks are indicated in the upper left graph. The results for cells that carried the plasmid vector lacking the L29 gene (thus synthesizing no L29 during the labeling period) are reproduced on each graph for reference. NLS1* = L29 with Arg 8→Lys mutation; NLS2* = Arg 25→Lys mutation; NLS1,2* = L29 with both Arg 8 and Arg 25 substituted by lysine.

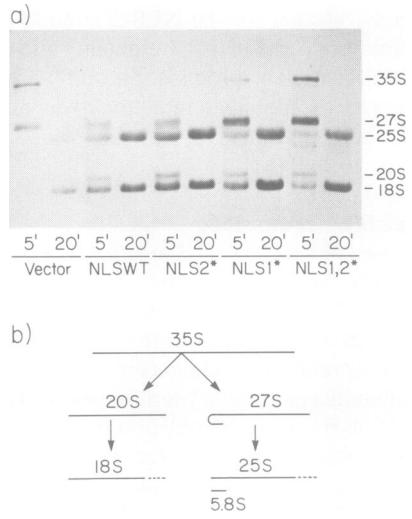

Fig. 5. Processing of RNA in yeast cells producing wild-type or mutant proteins. (**a**) Fluorogram of precursor and mature ribosomal RNA species (labeled on the right) in yeast strain GCYH-X23 transformed with single copy plasmids encoding no L29 protein (Vector), wild-type (NLSWT), or the three different mutant L29 proteins. 5′ and 20′ refer to a 5 min pulse labeling with L-[methyl-^{3}H]methionine followed by a 15 min chase with unlabeled methionine. (**b**) Processing of 35S precursor RNA in yeast cells. Diagram shows sequence of processing events leading to mature rRNA species (Udem and Warner, 1972).

the initial 35S pre-rRNA as well as the 27S large ribosomal subunit pre-rRNA. Similarly, cells producing the NLS-1−NLS-2 double mutant, which had the slowest growth rate, showed an even greater buildup of pre-rRNA, although accumulation was not as extreme as the processing deficit in cells which produced no L29 at all (Vector, Figure 5). In conclusion this analysis demonstrated that the Arg→Lys substitutions in the putative nuclear localization sequences of ribosomal protein L29 led to a reduced assembly of large ribosomal subunits. We suggest, therefore, that these regions of the polypeptide are responsible for nuclear localization and/or assembly of yeast ribosomal protein L29.

Discussion

The purpose of this investigation was to identify a segment or region of a eukaryotic ribosomal protein that may function to localize such a protein to the nucleus. Assembly of ribosomes in eukaryotic cells takes place in the nucleus and thus many ribosomal proteins must be dispatched efficiently to this compartment after their cytoplasmic synthesis. We described two seven-amino-acid sequences derived from yeast ribosomal protein L29 which acted as nuclear localization sequences. Either of the two peptides caused *E.coli* β-galactosidase to associate almost exclusively with the yeast cell nucleus. Single amino acid substitutions were found that reduced or eliminated localization. When incorporated into L29, a particular non-localizing mutation in one of the NLS (Arg 8→Lys in NLS-1) led to impairment of ribosome assembly; the same mutation coupled with the corresponding replacement in the other NLS (Arg 26→Lys in NLS-2) produced a still greater reduction in the rate of ribosome assembly. Diminished ribosome assembly is a phenotype expected of a mutation that imparts to L29 a reduced ability to enter the nucleus.

Delineation of nuclear localization sequences has received considerable attention (see Introduction). The sequences lack extensive homology but are overall generally basic in amino acid composition. This lack of homology may be due to differences in mechanisms of function for different NLS. Alternatively, lack of identity amongst sequences that specify nuclear location may be due to the ability of a receptor(s) to interact with a wide variety of chemically similar protein segments, akin to the recognition process between signal sequences and the signal recognition particle (Verner and Schatz, 1988).

Protein segments that bring about a nuclear location could act as ligands for either a nuclear pore receptor or a cytoplasmic chaperone, either or both of which would facilitate transport of the protein into the nucleus. Alternatively, nuclear localization segments could be those regions of a protein that bind to a non-diffusible nuclear component following passive diffusion of the protein through nuclear pores. In the case of large proteins such as the SV40 T antigen and *Xenopus* nucleoplasmin, it is clearly established that particular peptide sequences are responsible for bringing about facilitated uptake into the nucleus. Conversely, of the many NLS examined to date the NLS from the influenza virus nucleoprotein has been reported to cause nuclear accumulation by a mechanism apparently involving binding to an interior nuclear component (Davey *et al.*, 1985). Note, however, that the influenza nucleoprotein NLS did not show sequence similarity to the majority of reported NLS including that from SV40 large T antigen nor did it contain a concentration of basic residues.

We do not know the mechanism whereby the NLS derived from yeast ribosomal protein L29 promoted nuclear association of the reporter protein β-galactosidase. While β-galactosidase is quite large (the functional form is a tetramer of 116 kd monomers) and therefore should require mediated uptake into the nucleus, the protein has been reported to associate with or enter nuclei on its own (although it did not accumulate in the nucleus; Kalderon *et al.*, 1984b; Moreland *et al.*, 1987). Thus, the L29 NLS could have caused nuclear localization by promoting receptor mediated uptake or the sequences may have brought about nuclear accumulation by binding to an internal component such as rRNA. Indeed L29 is known to bind to 5.8S rRNA (Lee *et al.*, 1983) but specific regions of the protein that engage in binding have not been identified. Nonetheless, for the following reasons we argue that a passive diffusion/binding mechanism was unlikely to have accounted for nuclear localization of L29 NLS−β-galactosidase proteins.

First, rRNA that fails to assemble into ribosomes is rapidly degraded. It is difficult to envision a supply of stable extra-ribosomal 5.8S rRNA sufficient to soak up all of the fusion protein so as to bring about its virtual total nuclear association. Of course, rRNA may not be the only nuclear component with an affinity for a ribosomal protein or a short peptide derived from it. Interestingly, in the course of microscopy some cells were seen to have anti-β-galactosidase antibody staining located in a thin connecting bridge of material between the nuclei of mitotically dividing cells (see E−F in Figure 3). This staining pattern would not be expected if the NLS bound only to rRNA in the nucleolus (the site of rRNA synthesis and ribosome assembly within the nucleus), since cytological evidence has shown that the nucleolus is localized away from the thin connecting neck (Byers, 1982).

Secondly, maintenance of nuclear localization by several of the double substitution mutations is difficult to reconcile with rRNA binding. Were the NLS responsible for binding to a site in 5.8S rRNA (or another interior component), it might be expected that a particular spatial arrangement of amino acid side groups would be required for specific interaction with particular RNA nucleotides. Recall that an Arg→Lys substitution in the centre of NLS-2 (Arg 25) all but abolished nuclear localization. However, an NLS containing this same substitution along with a Gly 29→Arg replacement (M 160) was very efficiently localized. If nuclear localization had been the result of binding to rRNA, it would be necessary to explain how a replacement four residues distal to the original mutation restored correct interaction. Perhaps since the first mutation at residue 25 was a chemically conservative Lys→Arg substitution, further addition of an Arg 29 may have created an environment more conducive for the Lys 25 to engage in the specific 5.8S rRNA contact that ordinarily involves arginine.

Conversely, NLS identified in a number of other proteins show little if any residue to residue identity but most such sequences contain an abundance of basic residues. Since the canonical receptor ligand NLS derived from the SV40 T antigen is known to function in *Xenopus* (Dworetzky *et al.*, 1988; Goldfarb *et al.*, 1986) and in yeast (Nelson and Silver, 1989), yet a sequence identical or highly homologous to the SV40 NLS has not yet been reported in nuclear proteins from either of these organisms, it may be that the receptor(s) with which NLS interact recognize numerous different NLS (different at least from the standpoint of primary amino acid sequence). If the L29 NLS were indeed ligands for that receptor, the addition of another basic residue at position 29 in the NLS-2−β-galactosidase fusion protein may have restored favorable interaction with a receptor that is largely dependent on positive charge.

In the same way that it was not possible to conclude definitively the basis for nuclear localization of the L29 NLS−β-galactosidase proteins, we could not state with certainty that the defects in ribosome assembly caused by mutations in L29 itself were due to inefficient nuclear localization. While the mutations clearly disrupted ribosome assembly, it may be that the mutations simply created proteins less able to assemble once inside the nucleus. While we view the correspondence between loss of nuclear localization of fusion proteins and loss of ribosome assembly as strong evidence that the NLS are in fact responsible for L29's nuclear location, further experiments will be needed to confirm that the mutant proteins are defective in nuclear entry. Since a ribosomal protein that fails to assemble is degraded within minutes (e.g. Maicas *et al.*, 1988) *in vitro* assays will probably be required to assess the ability of the mutant proteins to enter the nucleus.

Nonetheless, our results demonstrate that the mutations in the L29 NLS disrupted initial assembly of the 60S subunit (of which L29 is a component), a consequence expected for diminished nuclear import, rather than the mutations having produced a defect in a post-assembly step or a defect in the finished ribosome itself (i.e., impaired translation). Mutations that permitted normal assembly but resulted in ribosomes less able to carry out translation should be dominant or semi-dominant. This expectation is based on the fact that the 60S subunit is chiefly involved in peptidyl transferase and translocation of the ribosome along the mRNA. Any subunit unable to carry out these tasks would impede progress of a normal subunit positioned upstream; thus, a slow growth phenotype would be evident in cells co-expressing mutant and wild-type L29. No such phenotype was observed, even with the double NLS mutant that, by itself, produced the most severe defect in growth. Secondly, the results for processing and accumulation of rRNA demonstrated that the NLS mutations affected initial assembly of ribosomes. Both the rate of processing and the extent of accumulation of rRNA were diminished in cells producing the NLS-1 or NLS-1−NLS-2 mutant proteins; the double mutant had a more severe reduction in rRNA processing consistent with its slower growth rate compared to the NLS-1 producing cells. If the mutations had created unstable 60S subunits that disassembled later, no decrease in processing of rRNA should have been observed, as processing is coincident with and dependent upon orderly initial assembly. Lastly, it could be argued that the mutations did not affect assembly of L29 *per se* but that binding of the mutant L29 proteins prevented subsequent binding of other ribosomal proteins. This possibility also seems unlikely as such a mutation should also display a dominant or semi-dominant phenotype (Smith, 1977).

In summary, we have identified two segments of yeast ribosomal protein L29 that function as nuclear localization sequences when joined to a reporter protein. Further, mutations within these regions disrupted both reporter protein localization and ribosome assembly. Future experiments will determine the function of these segments in L29.

Materials and methods

Plasmids

Wild-type and mutant L29 NLS peptide sequences were joined to *E.coli* β-galactosidase by insertion of NLS encoding oligonucleotides into plasmid pLGSD5 (Guarente *et al.*, 1982). Plasmid pLGSD5 contained the yeast *CYC1* gene fused at its second codon via a *Bam*HI site to β-galactosidase (*lacZ*); the *CYC1* promoter in pLGSD5 was under control of the yeast *GAL10* gene; transcription of *CYC1−lacZ* was thus dependent upon growth on galactose. L29 NLS oligonucleotides containing *Bam*HI ends were inserted between the *CYC1* and *lacZ* sequences producing fusion genes encoding the first two amino acids of *CYC1* followed by L29 peptide sequences joined to the amino terminus of β-galactosidase. To facilitate sequencing of mutant L29 NLS-*lacZ* fusions (see below), a single stranded phage replication origin (Messing and Vierra, 1988) was installed at the unique *Tth111I* site downstream of *lacZ* in pLGSD5 to produce pZf1, which could readily be isolated as single-stranded DNA and subjected to dideoxy sequencing.

Mutagenesis of L29 NLS β-galactosidase fusion proteins

An essentially random pool of mutant nuclear localization sequences, based on the sequence of NLS-2, was produced by the nucleotide 'doping' method of Hutchison *et al.* (1985) in which incorrect nucleotides are randomly incorporated during oligonucleotide synthesis. Doped oligomers encoding both strands of NLS-2 were synthesized, annealed and inserted at the *Bam*HI site of pZf1 (see above). The ligated mixture was used to transform *E.coli* NM522 [*F'*, *pro*$^+$, *lacI*^{q}*ZdM15*, *hsd*D5, Δ(*lac−pro*)]. Single stranded versions of plasmids containing the NLS sequences were generated by infecting random transformants with helper phage M13K07 and isolating single stranded DNA by standard methods. Mutant NLS sequences were identified by hybridizing the single stranded plasmids to a primer complementary to *lacZ* and sequencing through the region containing the NLS insert. Using a doping level of 2.5%, the non-silent mutation rate was 45%. Single stranded plasmids containing mutations of interest were introduced directly into yeast strain DB745 (*MATα ura3-52 leu2-3, 112ade1-100*) as described by Harinder *et al.* (1982).

Mutagenesis of L29

Mutations in the two nuclear localization sequences of L29, designated NLS-1 and NLS-2, were created by site directed mutagenesis. The mutant L29 genes were constructed in single copy yeast vectors to mitigate the possibility

that over-expression of mutant proteins might obscure their defective phenotypes or (in the case of a dominant mutation) impair viability when introduced in a wild-type strain.

Plasmid YEpCYH (Fried and Warner, 1982) consisted of pBR322 carrying a 5.4 kb *Bam*HI−*Hin*dIII fragment containing *cyh2*, a 1.1 kb *Hin*dIII fragment encoding the yeast *URA3* gene, and a 2.2 kb fragment encompassing the yeast 2 μ multicopy replication origin. The f1 phage replication origin was inserted into YEpCYH at the *Bam*HI site to give pCWT-f1. Mutations in the L29 NLS were created by simultaneously annealing two 5′ phosphorylated mutagenic oligonucleotides to the single stranded form of pCWT-f1 [NLS-1 oligomer; 5′-GACCTCTGTGCTTCTTAGTC; NLS-2 oligomer 5′-CACCGGGGTGCTTCTTGTGC; underlined nucleotides were mutagenic (see Käufer *et al.*, 1983 for L29 sequence)]. Both oligonucleotides were designed to convert an Arg residue to Lys (see Results). The pCWT-f1−NLS oligomer hybrid mixture was used to transform yeast without prior extension of the oligomers (Walder and Walder, 1986). Plasmid DNA from random transformants was isolated (Zakian and Scott, 1982), amplified in *E.coli* and subjected to dideoxy sequencing to identify the mutations. The L29 gene from one plasmid that contained an Arg→Lys substitution in both NLS was sequenced in its entirety to verify that no other mutations had occurred. That being the case, this double NLS mutant gene was cleaved at a *Xho*I site between NLS-1 and NLS-2, and the appropriate fragments were transferred to pCWT-f1 to produce L29 with single NLS mutations. The resulting plasmids were resequenced to confirm the presence of the mutations. Single copy versions of pCWT-f1 carrying wild-type L29 or L29 containing one or the other or both NLS mutations were produced by cleaving the plasmids at the *Bam*HI site 3′ of the L29 gene and at a *Sma*I site upstream of the *URA3* gene (which was actually a the 3′ end of the *URA3* coding sequence) and inserting the *URA3*-L29 fragments between the *Bam*HI and *Pvu*II sites in YEp(CEN3)30 (Fitzgerald-Hayes *et al.*, 1982), the latter carrying the yeast chromosome III centromere.

Immunofluorescence localization of NLS-β-galactosidase proteins

Transformants were grown in 100 ml of minimal media with 2% glucose and subcultured for ~2 h to an A_{660} of 0.4−0.8 in 100 ml of minimal media containing 2% galactose to induce expression of the chimeric proteins under the control of the *GAL10* activating sequence (see above). The level of expression of reporter proteins influenced the relative extent of nuclear localization so it was important to induce all transformants under exactly the same conditions. Indirect immunofluorescence microscopy was carried out essentially as described by Adams and Pringle (1984). Aliquots of 5 ml of 0.5 M potassium phosphate, pH7.4, and 10 ml of 66.7% formaldehyde were added to 100 ml cells and the cells were left to sit for 2 h. Cells were then washed twice with 20 ml 1.2 M sorbitol and converted to protoplasts by suspending for 30−60 min in a mixture of 3 ml 0.5 mg/ml Zymolyase 20T (Seikagaku Kogyo Co., Inc.) plus 10 μl undiluted glusulase (DuPont). Protoplasts were pelleted gently and washed in 10 ml 1.2 M sorbitol. Protoplasts were resuspended in 4 ml 1.2 M sorbitol per 0.2 A_{660} unit and 20 μl of the cell suspension was allowed to settle for 20 min in the wells of polylysine coated toxoplasmosis slides (Bellco Glass). Liquid was removed by aspiration at the edges of the wells and the settled cells were fixed for 10 min at room temperature with 1.2 M sorbitol containing 3.3% formaldehyde. Liquid was again removed and the cells were fixed and permeabilized for 6 min in 100% methanol at −20°C followed by 30 s in 100% acetone at −20°C. Incubation with the primary antibody rabbit anti-β-galactosidase for 2 h to overnight was followed by aspiration and three washings with a drop of PBS−BSA (0.9% NaCl, 66.7 mM potassium phosphate, pH 7.4, 1 mg/ml acetylated BSA, 0.02% sodium azide). The secondary antibody, fluorescein-conjugated goat anti-rabbit IgG, was then added and allowed to remain at room temperature for 2 h. Aspiration and three washings with PBS−BSA and two washings with PBS (0.9% NaCl, 66.7 mM potassium phosphate, pH 7.4, 0.02% sodium azide) was followed by adding 1 drop of 1 μg/ml of the DNA specific dye 4′,6′-diamidino-2-phenylindole (DAPI; Aldrich) to visualize nuclei. The DAPI solution was removed and the stained cells washed once with a drop of PBS. A 15 μl aliquot of PPD-glycerol (9 ml autoclaved glycerol plus 1 ml filter sterilized 10 mg/ml paraphenylenediamine in pH 9.0 sodium bicarbonate) was placed in each well of the side to float the coverslip and slow the rate of photobleaching. Cells were viewed in a Nikon Microphot-FX microscope equipped for DAPI and fluorescein epifluorescence microscopy.

Ribosomal subunit analysis

Single copy plasmids were introduced into GCYH-X23 (*Matα leu2-3, 112 his-3-Δ1 trp1-289 ade⁻ UAS$_G$..cyh2*). Inserted into the chromosomal L29 gene of GCYH-X23 was the activation sequence form *GAL10* (Guarente *et al.*, 1982); consequently, the (wild-type) L29 gene in GCYH-X23 was expressed only when cells were grown in the presence of galactose; introduction of mutant L29 genes into GCYH-X23 and subsequent growth on glucose enabled exclusive measurement of the function of the mutant proteins. A 10 ml sample of cells grown to early log phase in minimal media containing 2% galactose (to maintain expression of the wild-type L29 gene was transferred to 10 ml prewarmed minimal media containing 2% glucose (to terminate expression of the wild-type L29 gene). After 2 h, 150 μCi [^{3}H]uracil (ICN; 45 Ci/mM) was added and the cells were grown for 2.5 h, after which unlabeled uracil was added to 100 mg/ml for 30 min. Cells were harvested, disrupted with glass beads and subunits and polyribosomes separated in 7−47% sucrose gradients (Warner *et al.*, 1985).

Maturation and accumulation of rRNA

GCYH-X23 cells transformed with the single copy plasmids carrying NLS mutations were pre-grown in minimal media with 2% galactose and then subcultured into prewarmed minimal media with 2% glucose as described above. A 10 ml sample of cells at 5 × 10^6 to 1 × 10^7 cells/ml was pulse-labeled for 5 min with 0.5 mCi L-[methyl-^{3}H]methionine (Kief and Warner, 1981); half of the cell suspension was harvested and stored at −80°C and the remainder was incubated (chased) with unlabeled methionine at 100 μg/ml for 15 min. RNA was isolated (Warner and Gorenstein, 1977), denatured with formaldehyde (Maniatis *et al.*, 1982), and separated by size in 1.5% agarose gels containing 2.2 M formaldehyde and 10 mM $NaPO_4$. Gels were treated for fluorography with En3Hance (NEN/Dupont) and exposed to Kodak X-Omat film for 10 or 48 h at −80°C to detect RNA species (Udem and Warner, 1972).

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