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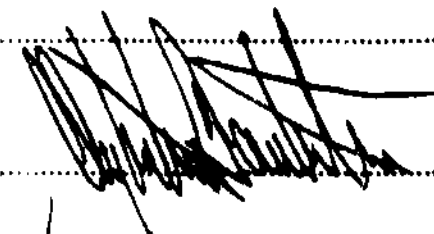
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CLONING AND CHARACTERIZATION
OF
THE KLUYVEROMYCES LACTIS SEC14

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ABSTRACT

The Saccharomyces cerevisiae SEC14 gene has been shown to encode a cytosolic factor required for the export of yeast secretory proteins from the Golgi complex. Sec14p immunoprecipitations have revealed a cross-reactive species in cytosolic extracts of Kluyveromyces lactis. Here I report the cloning and characterization of the K. lactis SEC14 (SEC14^{KL}). This gene was identified by its ability to confer a Ts⁺ phenotype to S. cerevisiae sec14^{ts} strains. Subsequently, it was shown that this gene encoded the sec14p cross-reactive species observed in K. lactis strains. Studies in S. cerevisiae strains carrying SEC14^{KL} in single copy in either a sec14^{ts} or sec14 null background showed that the K. lactis gene product can functionally substitute for SEC14p by the criteria of restoration of wild-type rates of growth, invertase secretion and vacuolar protein localization to such mutant yeast. I also demonstrated that SEC14p is made in at least 5-fold excess in S. cerevisiae strains with respect to the levels of SEC14p that are necessary for wild-type secretory function in this organism. Molecular analysis of SEC14^{KL} revealed that it encodes a polypeptide showing 77% primary sequence identity to S. cerevisiae SEC14p. These findings demonstrate the high level of

functional and structural conservation of SEC14 between
S. cerevisiae and K. lactis.

INTRODUCTION

The Golgi complex plays a central role in the secretory pathway. It is at this organelle that sorting for protein traffic transiting the later stages of this pathway takes place (1). The Golgi complex is characterized by a discontinuous arrangement of membrane-bound cisternae which adopt a stack-like structure. In addition, a biochemical compartmentalization is superimposed on this structural organization. The maintenance of this structural and biochemical compartmentalization is required for proper Golgi function. Thus, not only does the Golgi complex define a control point for the regulation of secretory protein traffic, but its own identity is dictated by the rules governing the correct distribution of proteins between membrane-bound compartments.

Rothman and colleagues were the first to experimentally address the problem of how secretory proteins transverse the Golgi stack. To do this, they reconstituted the process in a cell-free system (2, 3). These studies revealed that intracellular Golgi transport is mediated by bulk carrier vesicles and that ATP and cytosolic factors are required. There is also evidence for the involvement of fatty-acylation reactions and GTP-binding proteins in this process (4, 5).

Presently, the best biochemical understanding of intracisternal transport centers around the events occurring after a transport vesicle has already docked to an acceptor cisternae. An N-ethylmaleimide sensitive factor (NSF) has been purified and shown to be involved at an early stage of the processing of these vesicles before they fuse to the membrane (6). The mechanisms for the formation of such vesicles and their specific targeting to the appropriate acceptor membrane remain unknown. It seems probable that some of the factors which have a role in modulating these events will be cytosolic. Furthermore, it appears that cytosolic factors playing roles in driving Golgi transport functions have been functionally conserved. Dunphy et al. (7) have shown that yeast cytosol will substitute for mammalian cytosol in the reconstituted Golgi transport system.

The fact that mammalian and yeast cytosols are functionally interchangeable supports the use of yeast as a model system for studying Golgi function. In addition, the power of the yeast genetics system offers a complementary approach to the biochemistry which has been developed in the mammalian system. We have been studying the SEC14 gene from the budding yeast Saccharomyces cerevisiae. The SEC14 gene encodes a gene believed to be involved in yeast Golgi function. The identification of this role for the SEC14p was

based on the phenotypic, biochemical, and cytological properties exhibited by sec14^{ts} mutants upon shift to non-permissive conditions (8). We have further demonstrated that the SEC14p most likely plays a direct role in yeast Golgi function. Additionally, SEC14 is essential for yeast viability and SEC14p fractionates primarily to the yeast cytosol (9). As might be predicted from the functional conservation of cytosolic factors observed by Dunphy et al. (7), we found that two divergent yeasts, Kluyveromyces lactis and Schizosaccharomyces pombe, both exhibit polypeptides that are antigenically related to the S. cerevisiae SEC14p (9).

In this thesis, I present a detailed characterization of a K. lactis gene, SEC14^{KL}, which encodes a polypeptide having the properties of both the SEC14p antigenic relatedness and the ability to confer a Ts⁺ phenotype to sec14^{ts} strains. My data indicate that SEC14p^{KL} completely substitutes for SEC14p in S. cerevisiae sec14^{ts} mutants and in mutants carrying haploid-lethal sec14 disruption alleles. I also find that S. cerevisiae exhibits intracellular concentrations of SEC14p that are at least 5-fold in excess of what are required for wild-type growth and secretion. Finally, molecular analysis of SEC14^{KL} indicated a 77% amino acid identity between SEC14p and SEC14p^{KL}. These data demonstrate the high level of structural and functional

conservation between the SEC14p of S. cerevisiae and K. lactis.

EXPERIMENTAL PROCEDURES

Genetic Techniques

Yeast complex (YPD) and Wickerman's minimal defined yeast media have been described (10). Yeast transformations (11), gene disruption methodology (12), and standard meiotic segregation analyses (10) have also been described.

E. coli strains MC1601 (13), DH5 (14) and KK2186 (9) were used for plasmid isolation and maintenance. Standard E. coli media were employed (15). Procedures for alkaline lysis purification of plasmid DNA and transformation of E. coli to ampicillin resistance with plasmid DNA have been reported (16, 15).

Yeast haploid strains employed in this study included CTY1-1A (MATa, ura3-52, Abis3-200, lys2-801am, sec14-1^{ts}) and its SEC14 derivative CTY182. Other isogenic derivatives included CTY235 (CTY1-1A, URA3-52::pCTY28), CTY236 (CTY1-1A, ura3-52::pKL6), CTY237 (CTY235, sec14-129::HIS3) and CTY238 (CTY1-1A, sec14-129::HIS3). pCTY28 was obtained by inserting a 2.0 kb MluI-NruI S. cerevisiae genomic DNA fragment that carried SEC14 into the EcoRI- HindIII sites of YIp5, (VB, 1989). pKL6 was constructed by inserting a 2.2 kb EcoRI-PvuII fragment of the K. lactis genomic insert, that carried SEC14^{KL} (see figure 1) and was placed next to a

polylinker SphI site, into the EcoRI-SphI sites of YIp5. These plasmids were each recombined into the ura3-52 locus of CTY1-1A by linearization at the unique StuI site of the YIp5-borne URA3 gene, followed by transformation of CTY1-1A to Ura⁺. These manipulations yielded strains CTY235 and CTY236, respectively. The CTY237 and CTY238 yeast strains were generated by recombining sec14-129::HIS3 into CTY235 and CTY236, respectively. The sec14-129::HIS3 allele was previously designated SEC14::HIS3, and details of its construction and introduction into yeast have been described (9).

Reagents

All reagents for invertase assays, concavalin A-Sepharose, protein A-Sepharose, and all protease inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes and nucleic acid modifying enzymes were from Bethesda Research Laboratories (Gaithersburg, MD) and Pharmacia Fine Chemicals (Piscataway, NJ). Reagents for SDS-PAGE, agarose gel electrophoresis, and electrophoresis of DNA sequencing reactions were from Bio-Rad Laboratories (Indianapolis, IN). Trans-Label (>1,000 Ci/mmol) was from ICN Radiochemicals (Irvine, CA). α -³²P-dATP (800 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL). Sequenase kit

for DNA sequencing was from United States Biochemical Corp. (Cleveland, OH).

Cloning and Characterization of SEC14^{KL}

Clones containing the SEC14^{KL} were identified by their ability to complement the S. cerevisiae sec14^{ts} defect. The scheme employed to isolate such clones was exactly the same as the one used to obtain the clones of the S. cerevisiae SEC14 (9), with the exception that the transforming DNA was derived from the Salmeron and Johnston K. lactis genomic library propagated in the 2-micron circle vector YEp24 (17). Physical mapping involved deletion and subcloning analysis coupled with phenotypic complementation. Subcloning experiments involved the introduction of defined restriction fragments from the complementing K. lactis DNA into the YEp24 derivative plasmid pSEY18. Deletions within the insert were generated by standard means. Phenotypic complementation was determined by transformation of defined plasmid constructs into CTY1-1A by selection for Ura⁺ at 30°C. Such transformants were subsequently tested for their ability to form single colonies at the restrictive temperature (37°C).

Immunoprecipitations of SEC14p and SEC14p^{KL}

In experiments where SEC14p cross-reactive materials were visualized, the appropriate yeast cells were grown to an early logarithmic growth stage (OD₆₀₀=0.5) in glucose (2%) minimal medium. The culture (0.5 OD₆₀₀ cells) was radiolabeled with 150 uCi of Trans-Label (>1000 Ci/mmol; ICN Radiochemicals, Irvine, CA) for 30 min at 30°C. Incorporation of radiolabel was terminated by the addition of trichloroacetic acid to a 5% final concentration, and cells were subjected to disruption with glass beads. The preparation of clarified extracts, recovery of immune complexes, and evaluation of immunoprecipitates by SDS-PAGE and autoradiography have been described (9).

Invertase Secretion

The appropriate yeast strains were grown to mid-logarithmic phase in YPD medium at 30°C and shaking. Cells were washed with 2 volumes of distilled water, resuspended in glucose (0.1%) YP medium, and incubated for 15 min at 30°C with shaking. The cultures were subsequently shifted to 37°C for 60 min, and total and extracellular invertase activities

were determined (9). Invertase was assayed by the method of Goldstein and Lampen (18). Invertase units were expressed as nanomoles of glucose produced per min at 30°C.

Immunoprecipitations of CPY and PrA

Yeast cells were grown to an early logarithmic growth stage (OD600=0.5) in glucose (2%) minimal medium at 30°C with shaking, then shifted to 37°C for 30 min. The cultures were radiolabeled (0.5 OD600 units of cells) with 150 uCi Trans-Label for 10 min, and chase was initiated by addition of unlabeled methionine and cysteine to final concentrations of 1%. After 20 min, trichloroacetic acid was added to each culture (5% final concentration) and the precipitates were solubilized and subjected to immunoprecipitation, SDS-PAGE, and autoradiography as previously described (9).

DNA Sequencing

Templates for DNA sequence analysis were generated by subcloning the appropriate restriction fragments into the pTZ phagemid vectors of Mead et al. (19). Phage M13K07 was

employed as a helper for template synthesis. Nucleotide sequence analysis employed the chain-termination method of Sanger (20).

RESULTS

Isolation of SEC14^{KL}

We have demonstrated a conservation of *S. cerevisiae* SEC14p structure in the divergent yeasts *Kluyveromyces lactis* and *Schizosaccharomyces pombe* (9). To assess the extent to which this structural similarity reflected a functional similarity, I sought to obtain genomic clones of *K. lactis* genes that could complement the *S. cerevisiae* sec14^{ts} defect. If structural homology is representative of functional homology, the functional SEC14^{KL} should direct the expression in *S. cerevisiae* of the *K. lactis* polypeptide that exhibits immunological cross-reactivity with the *S. cerevisiae* SEC14p. Efforts to isolate clones of the functional SEC14^{KL} were facilitated by the recessive character of sec14-1^{ts} (9) and the ability of *K. lactis* genes to be expressed in *S. cerevisiae* (17, 20, 21). As a result, I was able to define desired clones by direct selection.

In order to obtain potential SEC14^{KL} genes, DNA isolated from a *K. lactis* genomic DNA library maintained in the yeast shuttle plasmid YEp24 was transformed into the *S. cerevisiae* strain CTY1-1A (ura3-52, sec14-1^{ts}). The desired transformants were selected on the basis of their Ura⁺ and Ts⁺ phenotypes. Seven transformants with a Ts⁺ phenotype were obtained from approximately 11,000 Ura⁺ transformants.

To examine whether or not a structural homology coincided with the functional complementation, SEC14p immunoprecipitations (see Experimental Procedures) were performed on the seven Ts⁺ transformants. Figure 1 shows the results of this experiment. Lane 6 shows the profile of CTY1-1A/pKL12. As has been previously shown (9), it contained the S. cerevisiae SEC14p (middle band) and also contained the higher apparent molecular weight species which corresponds to the K. lactis SEC14p. The lower band in this figure seems to be a contaminant frequently observed in these immunoprecipitations. It does not represent a SEC14p cross-reactive species as its precipitation is not competed by unlabeled SEC14p antigen to which the anti-SEC14p was raised (not shown). Strains containing pKL1, pKL2, pKL3, pKL11, and pKL13 (Lanes 1, 2, 3, 5, and 7; Figure 1) also contain the K. lactis SEC14p. However CTY1-1A/pKL4 (Lane 4) clearly does not contain the K. lactis SEC14p. The data indicated that 6 out of 7 of the Ura⁺, Ts⁺ transformants harbored both the Ts⁺ complementing function and the K. lactis polypeptide which is cross-reactive with SEC14p. This result provided evidence that the functional SEC14^{KL} encodes the SEC14p^{KL} detected in K. lactis strains.

One of these strains, CTY1-1A/pKL12 was also tested for plasmid linkage. This demonstrated that the Ts⁺ complementing activity was indeed associated with the plasmid

DNA, thereby ruling out the possibility that CTY1-1A/pKL12 carried a chromosomal suppressor of sec14^{ts}. This plasmid was then used to further characterize SEC14^{KL}. This plasmid contains an insert some 6.3 kb in length. A physical map of this plasmid is shown in Figure 2. Deletion analysis localized the complementing gene to the left of the 3.7 kb SstI fragment defining the right half of the pKL12 insert. Additional subcloning experiments indicated that a 2.2 kb EcoRI- PvuII restriction fragment was necessary and sufficient for the sec14-1^{ts} complementing activity (pKL5, Figure 1). Furthermore, the BamHI site, the two HindIII sites, and two of the three XbaI sites that reside within this fragment were found to lie within the complementing gene. For simplicity, I shall henceforth refer to this insert as SEC14^{KL}.

SEC14^{pKL} Effectively Substitutes for Sec14p in Secretion of Invertase

In order to determine the extent to which SEC14p and SEC14^{pKL} were functionally related, I measured the efficiency of secretory pathway function in *S. cerevisiae* sec14-1^{ts} and sec14 null mutants that bore the SEC14^{KL} in single copy. The

appropriate yeast strains for these experiments were constructed by integrating derivative YIp5 SEC14 or SEC14^{KL} plasmids into the ura3-52 locus of CTY1-1A (see Experimental Procedures). These manipulations generated strains CTY235 and CTY236, respectively. The nature of the expected integration events was confirmed by Southern analysis (not shown).

I observed that CTY235 and CTY236 grew at essentially wild-type rates at 37°C, a normally restrictive temperature for sec14-1^{ts} mutants. This result yielded two conclusions. First, the CTY 235 data indicated that SEC14 was functional when transposed to the ura3-52 locus. In addition, the behavior of CTY236 demonstrated that a single copy of SEC14^{KL} was sufficient to compensate for the sec14-1^{ts} defect. The relative efficiency of this complementation with respect to secretion was estimated by measuring the efficiency of invertase secretion to the cell surface in CTY235 and CTY236 at 37°C. As can be seen from the data in Table 1, the wild-type CTY182 exhibited all of its invertase activity at the cell surface. This was apparent because no latent invertase was detected in these cells. The isogenic sec14-1^{ts} strain (CTY1-1A) exhibited a markedly different invertase profile. Only some 20% of the total invertase was extracellular, and 80% was detected in a latent (i.e., intracellular) form. This distribution defined the uncompensated sec14-1^{ts}

secretory block. Clearly, CTY235 was able to secrete invertase in a wild-type fashion as the extracellular invertase measured for this strain accounted for the total invertase activity (Table 1). This was the expected result since CTY235 harbored the homologous *S. cerevisiae* SEC14 in conjunction with the defective *sec14-1^{ts}* allele. The result that CTY236 also exhibited essentially wild-type secretion of invertase (>95% of the total invertase was extracellular; Table 1) indicated a complete reversal of the *sec14-1^{ts}* secretory block by single copy expression of SEC14^{KL}.

As a further criterion for SEC14^{KL} functional homology to SEC14, invertase assays were performed on strains harboring a *sec14* null mutation which carried a single copy of either SEC14 or SEC14^{KL} at the *ura3-52* locus. The appropriate strains for these experiments were generated by recombining a disrupted copy of SEC14 into CTY235 and CTY236 creating CTY237 and CTY238, respectively (see Experimental Procedures). The nature of these recombination events was confirmed by Southern analysis (data not shown). CTY237 and CTY238 were observed to grow at essentially wild-type rates. This result indicated that SEC14, when transposed to the *ura3-52* locus, could compensate for a disruption of SEC14, normally a lethal event (9). Furthermore, the behavior of CTY238 demonstrated that a single copy of SEC14^{KL} can also compensate for the lethal effect of disrupting SEC14. The

data in Table 1 show the invertase profile for CTY237 and CTY238. CTY237 showed wild-type levels of invertase secretion as would be expected for a strain carrying a wild type copy of SEC14. That CTY238 also exhibited essentially wild-type secretion of invertase (all invertase was extracellular) indicated that SEC14^{KL} in single copy confers a complete reversal of the deleterious effects on invertase secretion of a SEC14 disruption in S. cerevisiae.

SEC14^{pKL} Effectively Substitutes for SEC14^p in Vacuolar Protein Localization

As another test of SEC14 function, vacuolar protein localization was measured in strains containing a single copy of SEC14^{KL} in S. cerevisiae sec14^{ts} and sec14^o mutants. sec14^{ts} mutants show a kinetic defect in vacuolar protein localization (Bankaitis, unpublished results). Carboxypeptidase Y (CPY) and Proteinase A (PrA) are two vacuolar proteins which can be used to monitor vacuolar protein localization. Both of these proteins have precursor forms which are cleaved upon arrival in the vacuole to produce the mature, active forms of these proteins. In both cases the precursor and mature form of the protein can be distinguished by their mobility on SDS-PAGE. The precursor

forms of CPY and PrA migrate at a slower rate (P2CPY, P2PrA; Figure 3) than their mature forms (mCPY, mPrA; Figure 3). In the experiment shown in Figure 3, vacuolar protein localization was measured for the strains shown by a pulse-chase experiment in which cells were grown at the permissive temperature (30°C), shifted to the non-permissive temperature (37°C) for 30 min, labeled for 10 min with [35S] methionine and [35S] cysteine, and chased for 20 min with excess unlabeled methionine and cysteine. CPY and PrA were immunoprecipitated from clarified extracts from these cells, and the immunoprecipitates were evaluated by SDS-PAGE and autoradiography (see Experimental Procedures). This pulse-chase methodology represents conditions for which the wild-type strain will have just chased all radiolabeled pre-vacuolar forms of CPY and PrA to the mature vacuolar species (not shown). Consequently, it represents a very sensitive measure of kinetic or permanent defects in vacuolar protein targeting. Lane 1 of Figure 3 shows the CPY and PrA profile for a wild-type *S. cerevisiae* strain (CTY182). There was very little of the precursor forms CPY and PrA. Most of the CPY and PrA was in the mature form as evidenced by the intense mCPY and mPrA bands. In contrast, the *sec14^{ts}* strain, (CTY1-1A; Lane 2, Figure 3) showed an accumulation of both p2CPY and p2PrA demonstrating the defect in vacuolar protein localization in *sec14^{ts}* strains. However, the

vacuolar block was not complete in these cells as evidenced by the presence of mCPY and mPrA bands. Both CTY235 and CTY237 (Lanes 3 and 5 in Figure 3, respectively) exhibited essentially wild-type profiles of CPY and PrA as would be expected for strains carrying a wild-type copy of SEC14. CTY236 (Lane 4, Figure 3) exhibited a wild-type CPY and PrA profiles as well. These data demonstrated the ability of SEC14^{KL} to compensate for SEC14 in vacuolar protein localization of sec14^{ts} strains. In addition, the S. cerevisiae strain containing a SEC14 disruption and a single copy of SEC14^{KL} also showed a wild-type distribution of CPY and PrA (CTY238; Lane 6, Figure 3). Taken together, these data provided further evidence that SEC14^{KL} was capable of replacing S. cerevisiae SEC14 in vacuolar protein traffic.

SEC14_p is Produced at Reduced Levels when the Gene is Transposed to the Ura3-52 Locus

In order to confirm the presence of SEC14_p or SEC14_p^{KL} in strains CTY237 and CTY238 immunoprecipitations were performed with anti-SEC14_p antibody. These two strains and control strains were grown at 30°C and labelled for 30 min. The results of the immunoprecipitations are seen in Figure 4. Lane 1 shows the result with K. lactis gene on a high copy plasmid transformed in sec14-1^{ts}. The top band has been

shown to correspond to the K. lactis SEC14p while the lower band is the S. cerevisiae protein (9). These serve as markers for the other strains. CTY235 (sec14-1^{ts}/SEC14::ura3-52; Lane 2) showed the expected S. cerevisiae SEC14p band. CTY236 (sec14-1^{ts}/SEC14^{KL}::ura3-52; Lane 3) also showed the band corresponding to S. cerevisiae SEC14p but, in addition this strain exhibited a faint band corresponding to the K. lactis SEC14p. Surprisingly, in CTY237 (Lane 4), the strain which carries its sole copy of SEC14 at the ura3-52 locus also showed a very weak signal for SEC14p. This result indicated that expression of the gene at this locus is dramatically reduced relative to its normal location. This strain also showed a band of low molecular weight corresponding to the amino-terminal portion of SEC14p corresponding to the gene product of the disrupted copy of SEC14. Consistent with this notion, CTY238 (ura3-52::SEC14^{KL}/sec14-129::HIS3) also showed a very faint band corresponding to the SEC14p^{KL}. All of the samples were normalized for total incorporated counts before being loaded onto the gel. This demonstrated that the relatively low amounts of protein seen from this locus was indeed caused by low expression and not other general effects. We estimated this difference to be at least a five-fold decrease in the amount of SEC14p in these strains. However, in all of the

tests described above, these strains behaved in a wild-type fashion.

SEC14^{KL} Nucleotide Sequence

The localization of SEC14^{KL} to a 2.2kb EcoRI-PvuII restriction fragment, coupled with the identification of convenient restriction sites within the SEC14^{KL} gene, facilitated the determination of the SEC14^{KL} nucleotide sequence. The strategy for determining this sequence is diagrammed in Figure 5. In addition, Northern blot analysis was performed to determine the direction of transcription for this gene. Single stranded RNA probes were made by subcloning the BamHI-SstI fragment adjacent to a T7 promoter (19) in both orientations yielding single-stranded RNA probes specific for each strand. Only the probe transcribed in the BamHI to SstI direction hybridized to a Northern blot of RNA from K. lactis (data not shown). These data indicated that the gene is transcribed in direction of SstI to BamHI.

The nucleotide sequence of the gene was determined in both directions. This sequence revealed a large open reading frame extending from downstream of the EcoRV site to just downstream of the first NcoI site (Figure 1). The polarity of this reading frame is consistent with the direction of transcription predicted by the Northern blot analysis. The

open reading frame encodes a polypeptide of 301 amino acid residues ($M_r=34,615$) terminated by two termination codons, UAA and UAG (Figure 6). The predicted molecular weight is, in general, consistent with the mobility of SEC14p^{KL} on SDS-PAGE. However, even though the SEC14p^{KL} is slightly smaller than the S. cerevisiae SEC14p, SEC14p^{KL} migrated at a higher apparent molecular weight on SDS-PAGE. This is most likely due to the fact that SEC14p^{KL} is a more acidic protein.

Probably the most striking feature of this polypeptide is its similarity to the S. cerevisiae SEC14p. Both proteins are of a hydrophilic nature (each has 27% charged amino acids; 9) and exhibit net negative charges at neutral pH ($pI=4.9$ for SEC14p^{KL}, $pI=5.3$ for SEC14p; 9). The amino acid identity between the two polypeptides is 77% (Figure 7). One prominent difference between the two sequences is the lack of an intron in SEC14^{KL}. SEC14 contains a 156 nucleotide intron near the amino terminus of the gene, immediately following the third SEC14 codon (9). These data imply that the S. cerevisiae SEC14 intron probably does not play a crucial role in SEC14 regulation.

DISCUSSION

In this study I have demonstrated that the SEC14^{KL} gene product harbors functional identity, antigenic similarity and a high degree of sequence identity to the S. cerevisiae SEC14p. The ability of the K. lactis SEC14p to functionally substitute for SEC14 was measured by three criteria using strains containing a single copy of SEC14^{KL} in either a sec14-1^{ts} or sec14 null background. The first of these criteria was growth. Strains containing a single copy of SEC14^{KL} in both the sec14-1^{ts} and sec14^o backgrounds were able to grow at wild-type rates at the sec14-1^{ts} non-permissive temperature (37°C). These strains also exhibited wild-type levels of invertase secretion, suppressing the block in invertase secretion seen in S. cerevisiae sec14^{ts} strains. Finally, these strains also showed restored efficiencies of vacuolar protein localization, another function which is aberrant in sec14^{ts} strains. In addition to this functional identity, SEC14p immunoprecipitations of strains carrying the SEC14^{KL} gene yielded a signal corresponding to the same species seen in K. lactis immunoprecipitations with S. cerevisiae SEC14p antisera. The structural similarity implied by this cross-reactivity between the S. cerevisiae and K. lactis SEC14p was supported

by the presence of 77% amino acid identity in the sequence of the two polypeptides.

Although K. lactis resembles S. cerevisiae in that it is a budding yeast with similarities in morphology and life cycle, K. lactis does possess metabolic and genetic variations. For example, in K. lactis the galactose regulon is not repressed by glucose as is the case in S. cerevisiae (20). Additionally, although the K. lactis LAC9 gene is functionally analogous to the S. cerevisiae GAL4 gene, its protein sequence shows low identity to GAL4. Regions of homology between the two proteins are limited to regions which have been implicated as specific functional domains (17). In view of these differences between the two organisms, the conservation of SEC14p between S. cerevisiae and K. lactis supports the notion that SEC14p plays a general role late in Golgi function in the secretory pathway. The divergent yeast Schizosaccharomyces pombe also displayed a SEC14p cross-reactive species in SEC14p immunoprecipitations (9). If this immunoreactive species also proves to be due to a structurally and functionally conserved SEC14 analog in S. pombe, this would provide compelling evidence for a more universal role for the SEC14p in late secretory function.

The only major difference that we detected between the K. lactis and S. cerevisiae SEC14 genes was the lack of an intron in the K. lactis gene. This is perhaps surprising

because the presence of an intron has recently been shown to be conserved between the K. lactis and S. cerevisiae ACT gene (23). The intron splicing signals are also conserved in the K. lactis and S. cerevisiae ACT intron (23). In light of this conservation of introns between the two yeast species, the fact that the SEC14 intron is not conserved in K. lactis suggests that, in the case of SEC14, the intron does not have an important function.

An unexpected finding of this study was the result that S. cerevisiae seems to be synthesizing an excess of SEC14p. In the SEC14p immunoprecipitations on strains containing their sole copy of SEC14 at the ura3-52 locus, SEC14p was found to be present in concentrations at least 5 fold less than when SEC14 is located at its normal locus. However, these strains function at completely wild-type levels for all parameters measured in this study. At this time we have no precise explanation for why the cell is making an excess of SEC14p. One possibility is that SEC14p has another, perhaps non-essential, function. So far we have only one allele of sec14^{ts}. Mutations in other regions of the gene might reveal additional functions for this protein. Alternatively, the cell may have a pool of SEC14p, but only a small fraction of this pool is functionally active at a given time. Fractionation studies on SEC14p have indicated that 65% of SEC14p resides in the cytosol and 35% is associated with

membrane fractions (9). One might envision a situation in which the main pool of SEC14p is cytoplasmic; but when it gets appropriately modified, SEC14p associates with the membrane of the Golgi complex or vesicles budding from the Golgi to perform its cellular function. Experiments are currently underway to investigate whether or not the same ratios are observed in fractionation experiments performed on the strains producing reduced amounts of SEC14p. An altered ratio of SEC14p fractionation between soluble and pelletable fractions would support this sort of model. This work has provided further evidence for the role of SEC14p in the secretory pathway. In addition, the work with the K. lactis SEC14 has raised new questions about the function and regulation of SEC14p in the cell.

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TABLE LEGENDS

Table 1. Invertase assay data. Yeast cells were grown at 30°C in YPD and shifted to 0.1% glucose YP and 37°C for 2 hours. Total and extracellular invertase were determined and expressed in units of activity (see Experimental Procedures). The units of invertase in (i.e., intracellular invertase) was determined by subtracting the units of activity out from the total units of invertase activity.

FIGURE LEGENDS

Figure 1. Strains containing *K. lactis* *sec14^{ts}* complementing clones produce SEC14p^{KL}. CTY1-1A strains containing pKL1, pKL2, pKL3, pKL4, pKL11, KL12, or pKL13 were grown in glucose (2%) yeast minimal medium and allowed to incorporate radiolabel for 30 min at 30°C. Clarified extracts were recovered and immunoprecipitations performed with SEC14p antisera: lane 1, CTY1-1A/pKL1; lane 2, CTY1-1A/pKL2; lane 3, CTY1-1A/pKL3; lane 4, CTY1-1A/pKL4; lane 5, CTY1-1A/pKL11; lane 6, CTY1-1A/pKL12; lane 7, CTY1-1A/pKL13. The positions of the SEC14p and SEC14p^{KL} bands are indicated at the left.

Figure 2. Physical map of the *K. lactis* SEC14 gene. The approx. 6.3-kb genomic insert of the sec14^{ts} complementing plasmid pKL12 (see text) is given. The SEC14^{KL} coding region is defined by the arrow, which also indicates the direction of transcription (see text). The inserts in the lower part of the figure indicated that the strategy for the localization of SEC14^{KL} to a 2.2-kb insert. pKL2 contains a deletion of the region between the two SstI sites. pKL4 contains a deletion of the region between the two BamHI sites. Finally, pKL5 shows the minimum complementing region of PvuII-EcoRI (see text). Restriction site abbreviations are as follows: BamHI (B), EcoRI (E), EcoRV (R), ClaI (C), HindIII (H), NcoI (N), PstI (P), PvuII (Pv), SalI (S), SmaI (Sm), SstI, (Ss), XbaI (X).

Figure 3. SEC14^{p^{KL}} blocks the sec14^{ts} defect in vacuolar protein localization. *S. cerevisiae* strains were grown in glucose (2%) yeast minimal medium at 30°C. After being shifted to 37°C for 30 min, they were allowed to incorporate radiolabel for 10 min. This was followed by a 20 min chase with unlabeled methionine and cysteine (1% final concentration). Immunoprecipitations were performed on clarified extracts with CPY and PrA antisera: lane 1, CTY182; lane 2, CTY1-1A; lane 3, CTY235; lane 4, CTY236; lane 5, CTY237; lane 6, CTY238. The precursor and mature forms of

CPY and PrA are indicated to the left and the SEC14 genotype for each strain is indicated above.

Figure 4. SEC14p immunoprecipitations. S. cerevisiae strains containing a single copy of either SEC14 or SEC14^{KL} at the ura3-52 locus were grown in glucose (2%) minimal yeast medium and allowed to incorporate radiolabeled for 30 min at 30°C. SEC14p immunoprecipitations were performed of clarified extracts. Lane 1 shows CTY1-1A/pKL12. This strain serves as a marker for SEC14p and SEC14p^{KL}, indicated at the left. Lanes 2 (CTY235) and 4 (CTY237) have a single copy of SEC14 at the ura3-52 locus and are sec14^{ts} and sec14^o, respectively, at the normal locus. Lanes 3 (CTY236) and 5 (CTY238) contain a single copy of SEC14^{KL} at the ura3-52 locus and are sec14^{ts} and sec14^o, at the normal locus.

Figure 5. Sequencing strategy for SEC14^{KL}. The K. lactis minimum complementing fragment is shown. The arrows indicate individual sequencing runs. The the arrows with filled-in circles represent sequencing from subclones using the universal primer and the arrows with open boxes represent sequencing from subclones using oligonucleotides synthesized based on the sequence information as primers.

Figure 6. Nucleotide sequence of SEC14^{KL}. The DNA sequence of the SEC14^{KL} coding strand and the predicted primary sequence of its product are shown. Hallmark restriction sites that are referred to in the text are shown.

Figure 7. Amino acid identity between S. cerevisiae and K. lactis SEC14p. The S. cerevisiae and K. lactis SEC14p primary sequences are shown using single letter amino acid abbreviations. Stars indicate identity between the two species at a particular position in the protein sequence. Dashes indicate the absence of an amino acid at a position in the K. lactis SEC14p.

INVERTASE ASSAY DATA

Strain	Genotype	Units of Activity		% Out
		In	Out	
CTY182	<u>SEC14</u>	N.D.	306.2	100.0
CTY1-1A	<u>sec14</u> ^{ts}	388.0	95.7	20.0
CTY235	<u>sec14</u> ^{ts} / <u>URA3::SEC14</u>	1.1	248.2	99.6
CTY236	<u>sec14</u> ^{ts} / <u>URA3::SEC14</u> ^{KL}	7.6	255.1	97.0
CTY237	<u>URA3::SEC14</u> / <u>SEC14</u> _{Hpa} :: <u>HIS3</u>	0.7	258.0	99.7
CTY238	<u>URA3::SEC14</u> ^{KL} / <u>SEC14</u> _{Hpa} :: <u>HIS3</u>	N.D.	222.9	100.0

35

TABLE 1

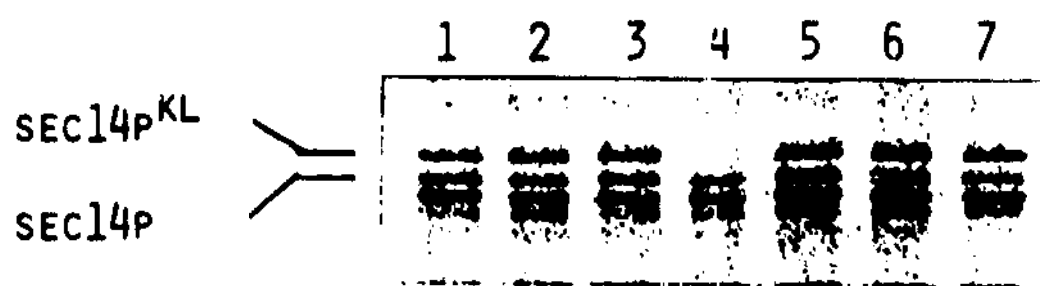
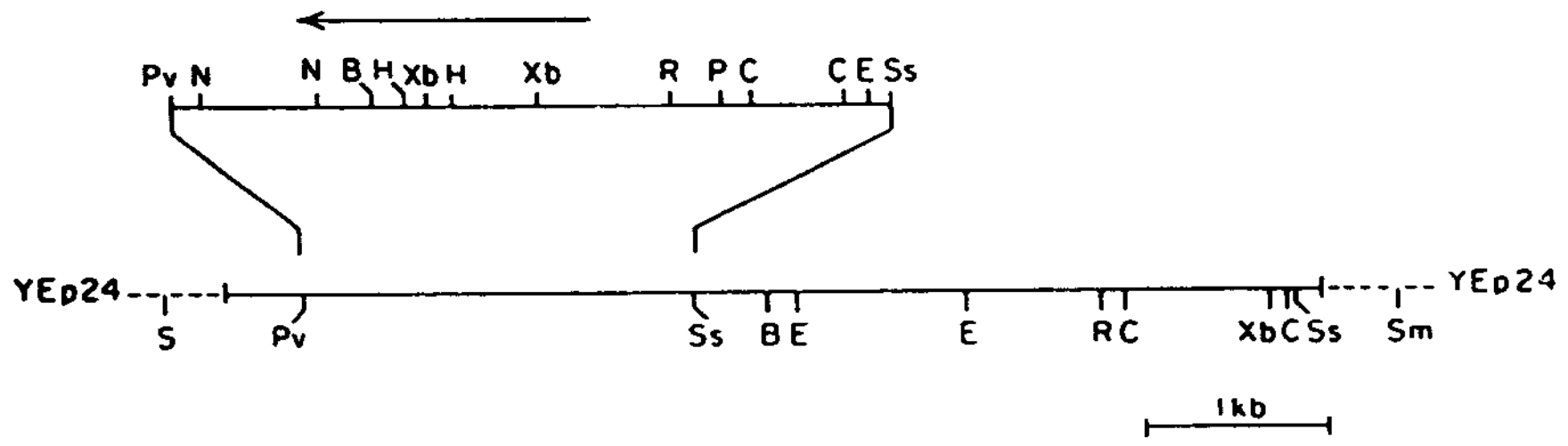


FIGURE 1



Plosmid	Complementation
pKL2	+
pKL4	-
pKL5	+

FIGURE 2

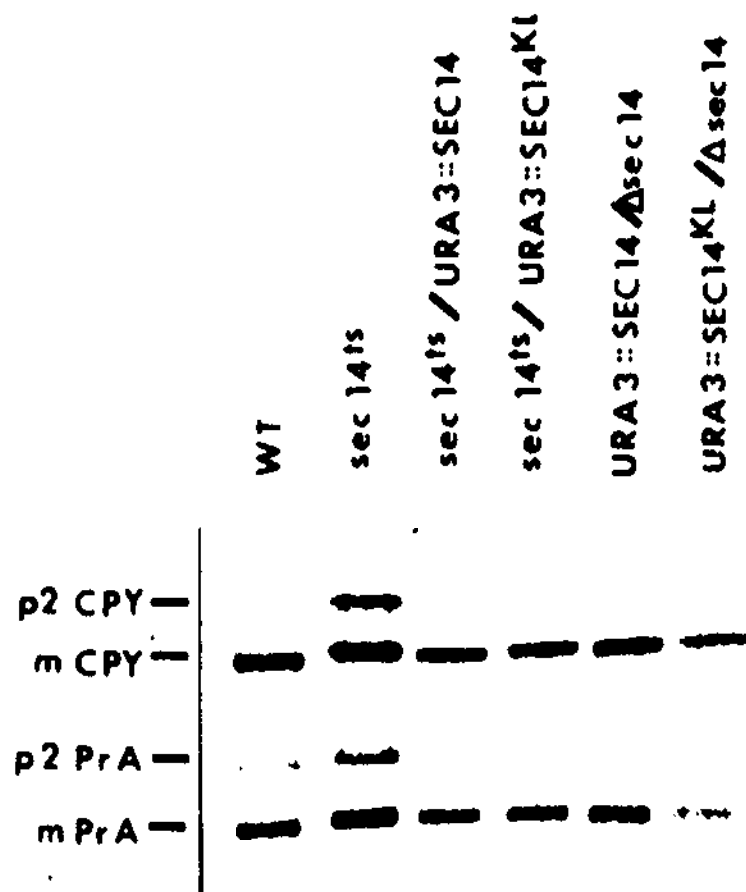


FIGURE 3

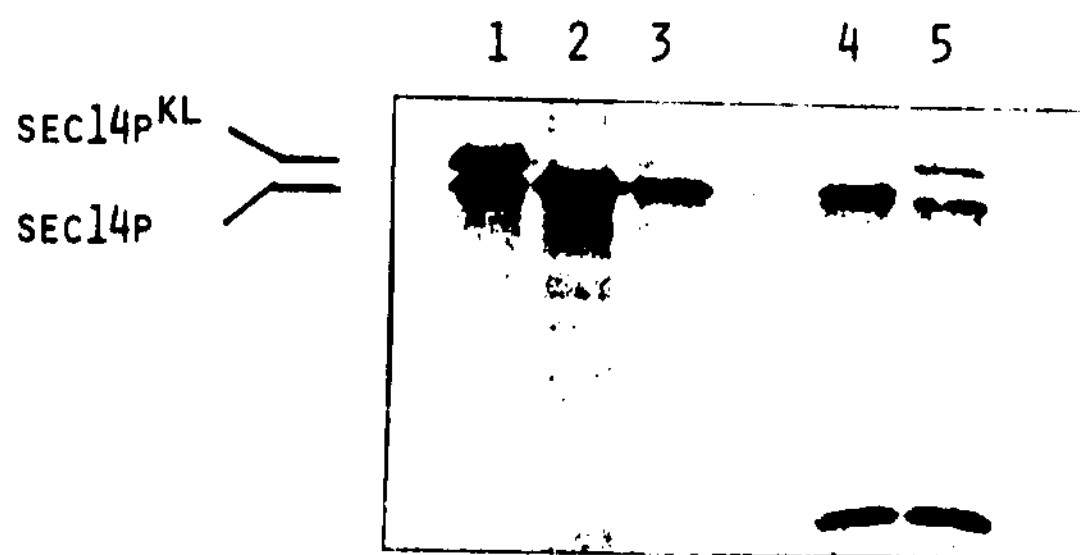


FIGURE 4

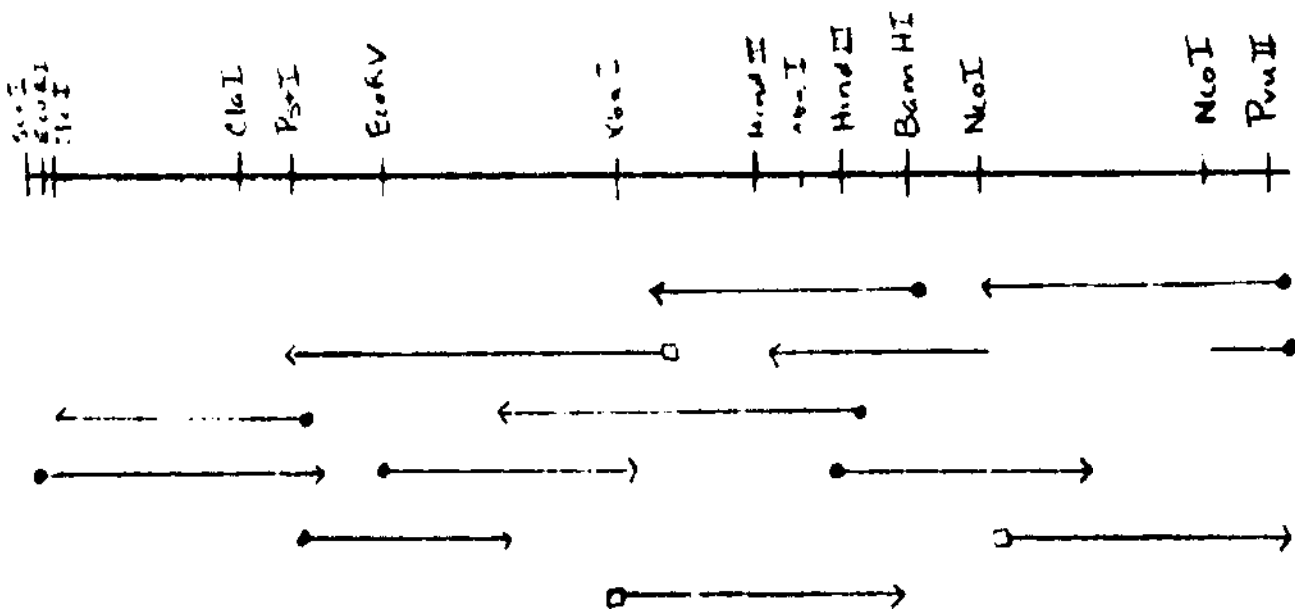


FIGURE 5

-180 -185 -190 -195 -200 -205 -210
 GTATACTAT AGTTTTTCCA TCTATACAGG TAATATTTTT GAGAAGCAAA CTTCAAAATTC CCATTTTCAA CAGTTGGATT
 -215 -220 -225 -230 -235 -240 -245 -250
 ATTATTAOCA CACTTOTTTA AGAAGGTGTC TCGTTCCTTG ATTGATCTAA ATTCATACCA TTGCATTTAG CTAACCCAAA
 -255 -260 -265 -270 -275 -280 -285 -290
 AGTTGACCCC ACGCCCGAAG TTAGATAGAG CCTOTCATC ATG GTA AGT GAA CAA GAA ATT TTA GAA TCA TAC
 MET Val Ser Glu Gln Glu Ile Leu Glu Ser Tyr
 45 60 75 90
 CCC CAG GTT TGT CCT AGT GGA TCA TTG TCT GGA ACA CCA GGC AAT CTT GAT TCA GAA CAA GAA GCC
 Pro Gln Val Cys Pro Ser Gly Ser Leu Ser Gly Thr Pro Gly Asn Leu Asp Ser Glu Gln Glu Ala
 105 120 135 150 165
 AAG TTG AAG GAA TTT AGA GAA TTG TTA GAA TCA TTG GGA TAT AAG GAA AGA CTT GAT GAC TCG ACC
 Lys Leu Lys Glu Phe Arg Glu Leu Leu Glu Ser Leu Gly Thr Lys Glu Arg Leu Asp Asp Ser Thr
 180 195 Met-I 210 225
 TTG TTG COT TTC CTA AAG OCT CGT AAG TTC GAT CTA GAG OCT TCC AAG ATC ATG TAC GAG AAC TGT
 Leu Leu Arg Phe Leu Arg Ala Arg Lys Phe Asp Leu Glu Ala Ser Lys Ile Met Tyr Glu Asn Cys
 240 255 270 285
 GAG AAA TGG AGA AAA GAG TTT GGT GTC ACC ATC TTC GAA GAT TTC CAT TAC GAA GAG AAT ACC
 Glu Lys Trp Arg Lys Glu Phe Gly Val Asp Thr Ile Phe Glu Asp Phe His Tyr Glu Glu Asn Thr
 300 315 330 345 360
 CTT GTC OCT AAA TAC CCA CAA TAC TAT CAC AAG ACC GAC AAC GAT GGT CGT CCC GTT TAC ATT
 Leu Val Ala Lys Tyr Tyr Pro Gln Tyr Tyr His Lys Thr Asp Asn Asp Gly Arg Pro Val Tyr Ile
 375 390 405 420
 GAA GAG CTT GGT TCT GTT AAT TTG ACT CAG ATG TAC AAG ATC ACC ACC CAA GAA CGT ATG CTG AAG
 Glu Glu Leu Gly Ser Val Asn Leu Thr Gln Met Tyr Lys Ile Thr Thr Gln Glu Arg Met Leu Lys
 435 450 Met-III 465 480 495
 AAC TTG GTC TGG GAA TAC GAA OCT TTT GTT AGA TAC AGA TTG CCT OCA TGT TCA AGA AAG OCT GGA
 Asn Leu Val Trp Glu Tyr Glu Ala Phe Val Arg Tyr Arg Leu Pro Ala Cys Ser Arg Lys Ala Gly
 510 525 Met-I 540 555
 TAC CTT GTT GAA ACT TCA TGT ACC ATT CTA GAC TTG AAA GGT ATT TCC ATT TCC AGT OCA GCA CAA
 Tyr Leu Val Glu Thr Ser Cys Thr Leu Ile Asp Leu Lys Gly Ile Ser Ile Ser Ser Ala Ala Gln
 570 585 Met-III 600 615
 GTA TTG AGT TAT GTC AAG GAA OCT TCC AAT ATC GGT CAA AAC TAC TAC CCT GAA CGT ATG GGT AAA
 Val Leu Ser Tyr Val Arg Glu Ala Ser Asn Ile Gly Gln Asn Tyr Tyr Pro Glu Arg Met Gly Lys
 630 645 660 675 690
 TTC TAC TTG ATC AAC OCA CCA TTT GGG TTC TCC ACT OCC TTC AGA TTA TTC AAA CCA TTC TTG GAT
 Phe Tyr Leu Ile Asn Ala Pro Phe Gly Phe Ser Thr Ala Phe Arg Leu Thr Lys Pro Phe Leu Asp
 705 720 735 750
 CCA GTT ACC GTG TCA AAG ATT TTT ATC TTG GGA TCG TCC TAC CAA AAG GAT TTG CTA AAG CAA ATC
 Pro Val Thr Val Ser Lys Ile Phe Ile Leu Gly Ser Ser Tyr Gln Lys Asp Leu Leu Lys Gln Ile
 765 780 795 810 825
 OCT GCC GAA AAC TTG CCA AAA AAA TTT GGT GGT CAA TCC GAG GTC TCA GAA OCA GAA GGC GGT CTA
 Pro Ala Glu Asn Leu Pro Lys Lys Phe Gly Gly Gln Ser Glu Val Ser Glu Ala Glu Gly Gly Leu
 840 855 Met-I 870 885
 TAT CTA TCC GAT ATT GGT CCA TGG AGA GAG GAG GAA TAC ATT GGA CCT GAA GGT GAA GCT CCA AAG
 Tyr Leu Ser Asp Ile Gly Pro Trp Arg Glu Glu Glu Tyr Ile Gly Pro Glu Gly Glu Ala Pro Lys
 900 920 930 940 950 960
 OCT TTT CAA TTG TAA TAG TACCTCCACC GGCATTGCT AGTCTTTTT TCTCCTCTTT TTTCGGCTGA
 Ala Phe Gln Leu *** **
 970 980 990 1000 1010 1020 1030 1040
 GCACITGAG GAAATCCAC TTCAATTTTA CCAGATAGAT ATTAAGTTC AATTGACCGA AAAACCCAAC ATTGTAGGCT
 1050 1060 1070 1080 1090 1100 1110 1120
 ACAGATTGT ACACAAACAT CTTAATTGAT GATTGTAGGT GAATGTAAATG TCCACCACCC ATCAAATTA TAAATCTGTT
 1130 1140 1150 1160 1170 1180 1190 1200
 CATAACGAG ATTCCTAGTA TTTTTTGGT CATCATATAT GTAACCTTAA ATAGCTCCAA ACAAGCAATA CACTATAAAA
 1210 Met-I
 TTAACITTA CCATTTTTA GGCATGCTCA

FIGURE 6

