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Cycloheximide resistance in yeast: the gene and its protein

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ABSTRACT

Mutations in the yeast gene CYH2 can lead to resistance to cycloheximide, an inhibitor of eukaryotic protein synthesis. The gene product of CYH2 is ribosomal protein L29, a component of the 60S ribosomal subunit. We have cloned the wild-type and resistance alleles of CYH2 and determined their nucleotide sequence. Transcription of CYH2 appears to initiate and terminate at multiple sites, as judged by S1 nuclease analysis. The gene is transcribed into an RNA molecule of about 1082 nucleotides, containing an intervening sequence of 510 nucleotides. The splice junction of the intron resides within a codon near the 5' end of the gene. In confirmation of peptide analysis by Stocklein et al. (1) we find that resistance to cycloheximide is due to a transversion mutation resulting in the replacement of a glutamine by glutamic acid in position 37 of L29.

INTRODUCTION

In the yeast Saccharomyces cerevisiae resistance to cycloheximide, an inhibitor of polypeptide elongation, can arise by mutation in the gene CYH2 (2). Mutations at the CYH2 locus are manifest simultaneously in ribosomes which become resistant to the inhibitor and in alterations in the amino acid sequence of a large subunit ribosomal protein, L29 (1). We have cloned a resistant allele of CYH2 and have shown that it is indeed a unique gene coding for ribosomal protein L29 (3). The easily identifiable phenotype displayed by CYH2 makes it an attractive tool for studies of the regulation of ribosome synthesis in a eukaryotic organism.

Our interest in ribosomes stems from the fact that, in all eukaryotic cells, the 70 to 75 ribosomal proteins are each synthesized at equal rates, yielding an equimolar amount of each protein, regardless of growth conditions (reviewed in 4,5). The use of cloned ribosomal protein genes has allowed us to determine that, in yeast, this coordinated synthesis is brought about by regulation of the transcription of ribosomal protein messenger RNAs and by regulation of their translation (6,7). Although the molecular

details of these control processes are not yet known, they operate in a cell whose ribosomal protein genes are dispersed throughout the genome and in which some ribosomal proteins are encoded by more than a single gene (5). Furthermore, several lines of evidence suggest that most, but not all, ribosomal protein genes of yeast contain an intervening sequence (8,9,10). By the same evidence CYH2 was classified as an intron containing gene (3). In striking contrast, of nearly five dozen other yeast chromosomal genes examined, only the gene for actin is found to have an intron (11,12). Whether the prevalence of introns in yeast ribosomal protein genes is related to the regulation of their expression is not known at this time.

In view of the fact that CYH2 is a member of a large, tightly regulated family of genes and is an intron-containing yeast gene with an identifiable phenotype, we have determined its DNA sequence and the coordinates of its transcription products as a prelude to using the gene for studies of the regulation of ribosome synthesis. We find that CYH2 is transcribed into an RNA molecule of approximately 1082 nucleotides from which an intron of 510 nucleotides is removed. The 5' splice junction, which appears within a codon, matches the canonical 5' splice junction sequence described by Mount (13). The resulting message codes for a protein of 148 amino acids. Resistance to cycloheximide is brought about by transversion of a C to a G, resulting in the change of glutamine to glutamic acid in position 37 of the amino acid sequence.

METHODS

Strains, Plasmids and Phages: S. cerevisiae strain ts368 was used as a source of RNA. This strain carries a temperature sensitive allele of rna2, a gene which is required for processing of ribosomal protein gene transcripts (8). The plasmid YIp5-S2, carrying the wild type or resistance allele of the CYH2 gene, was described previously (3). The plasmid YIp5cyh2, which carries a 5.4 kb Bam/Hind fragment containing the functional CYH2 gene was subcloned from the above plasmid as described (3).

The phages M13 mp8 and mp9 were used to transform the E. coli strain K12 JM103 (14).

DNA Sequence Determination: The 5.4 kb fragment was isolated and mapped for restriction sites by standard methods (15). Fragments were subcloned in M13 mp8 and mp9 either as defined, isolated fragments or by shotgun techniques (14), and the sequence determined by the dideoxy chain termination method (16).

S1-Nuclease Mapping: The method of Berk and Sharp (18) was used to map the

5' and the 3' termini of the gene. Yeast RNA was isolated and poly $(A)^+$ RNA was purified as described (17). 10-20 µg of poly$(A)^+$ RNA was mixed with a double stranded DNA fragment labeled in vitro at one 5' end with T4 polynucleotide kinase or at a 3' end with DNA polymerase. The nucleic acids were precipitated with ethanol, dissolved in 80% or 65% v/v formamide in 40 mM PIPES pH 6.5, 400 mM NaCl and 1 mM EDTA, and denatured for 15 min at 85° C. Hybridization was performed for 4 hours at 44° C. The hybridization mixture was diluted with 30 volumes of 280 mM NaCl, 30 mM NaOAc (pH 4.5), 4.5 mM $Zn(OAc)_2$, 20 µg/ml denatured calf thymus DNA and 33 or 66 units of S1 nuclease (Sigma Co.). Nuclease digestion was performed for different times (10 to 60 min) at different temperatures (0° C to 37° C). The RNA protected fragments were precipitated with isopropanol, washed several times with 80% ethanol, dried under vacuum, dissolved in formamide/dye solution, heated for 3 min at 90° C, quickly chilled and analyzed on 8% or 6% polyacrylamide/urea sequencing gels.

RESULTS AND DISCUSSION

Evidence for an Intron in CYH2 and Determination of Direction of Transcription

While we originally isolated CYH2 as part of a 12 kb DNA fragment, preliminary analysis narrowed the location of the coding sequence for protein L29 to a central 2 kb EcoR1 fragment (3). However, this fragment was found to be unable to confer resistance to cycloheximide when reintroduced into cells, suggesting it was missing a portion of the gene. For sequence analysis, therefore, we subcloned a 5.4 kb BamHI/HindIII segment carrying a functional resistance allele of CYH2 (see Fig. 1).

Previous experiments suggested that CYH2 contains an intron (3). In wild type cells, RNA corresponding to this gene is about 550 nucleotides in length. In a strain unable to splice precursor mRNA due to a temperature sensitive mutation in the gene rna2 (8), a larger transcript of about 1050 nucleotides accumulates.

To confirm the existence of an intron in CYH2 and to establish its location, the 1.2 kb HincII/EcoR1 fragment was 5' labeled at the EcoR1 site, denatured (Fig. 1), hybridized to RNA isolated from rna2 cells growing at the permissive or the restrictive temperature, and treated with S1 nuclease (18). It is evident from Figure 2 that using RNA isolated from cells growing under permissive conditions, the major protected DNA segment is approximately 315 bp, the product of hybridization to the mature mRNA, while a minor product of approximately 880 bp is also seen. RNA from cells maintained at the non-permissive temperature gave the opposite result, i.e. the 880 nucleotide

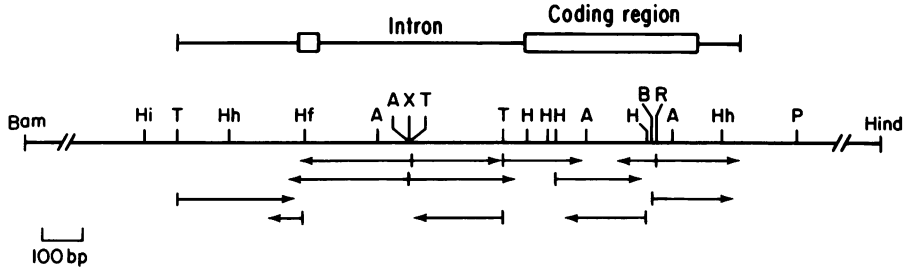


Figure 1. Position of restriction endonuclease sites and strategy for DNA sequencing. Arrows indicate direction and extent of sequence determination from the indicated sites. [A, AluI; B, BglII; H, HpaII; Hf, HinfI; Hh, HhaI; Hi, HincII; P, PstI; R, EcoR1; T, TaqI; X, XhoI].

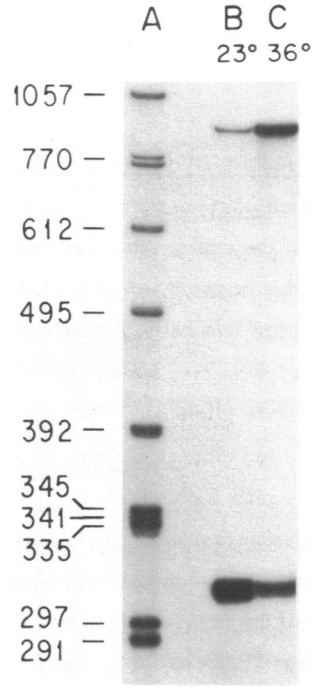


Figure 2. Determination of the intron with S1 nuclease mapping. The 5' labeled Eco/Hinc fragment (Fig. 1) was hybridized to poly $(A)^+$ RNA prepared from cells of strain ts368 growing at the permissive (Lane B) or the restrictive (Lane C) temperature. Lanes B and C show the protected fragments after treatment with S1 nuclease. The length markers (Lane A) are end-labeled HincII fragments of ØX174 RF DNA.

product was substantially more abundant. We conclude that the 3' terminus of the intron is approximately 315 bp upstream from the EcoR1 site, and that the 5' end of the mRNA is some 880 nucleotides upstream from the EcoR1 site.

Sequence of CYH2

Figure 1 shows a map of the restriction sites used to subclone DNA fragments in the filamentous phage M13 and the strategy used to determine the sequence of CYH2 by the dideoxy chain termination method (16).

While the complete amino acid sequence of L29 has never been determined, two fragments of the protein have been analyzed. Itoh et al. (19) determined the N-terminal sequence to be: PRO-SER-ARG-PHE-TYR. Stocklein et al. (1), in their analysis of cycloheximide sensitive and resistant forms of L29, determined the sequence of a tryptic peptide containing the altered amino acid:

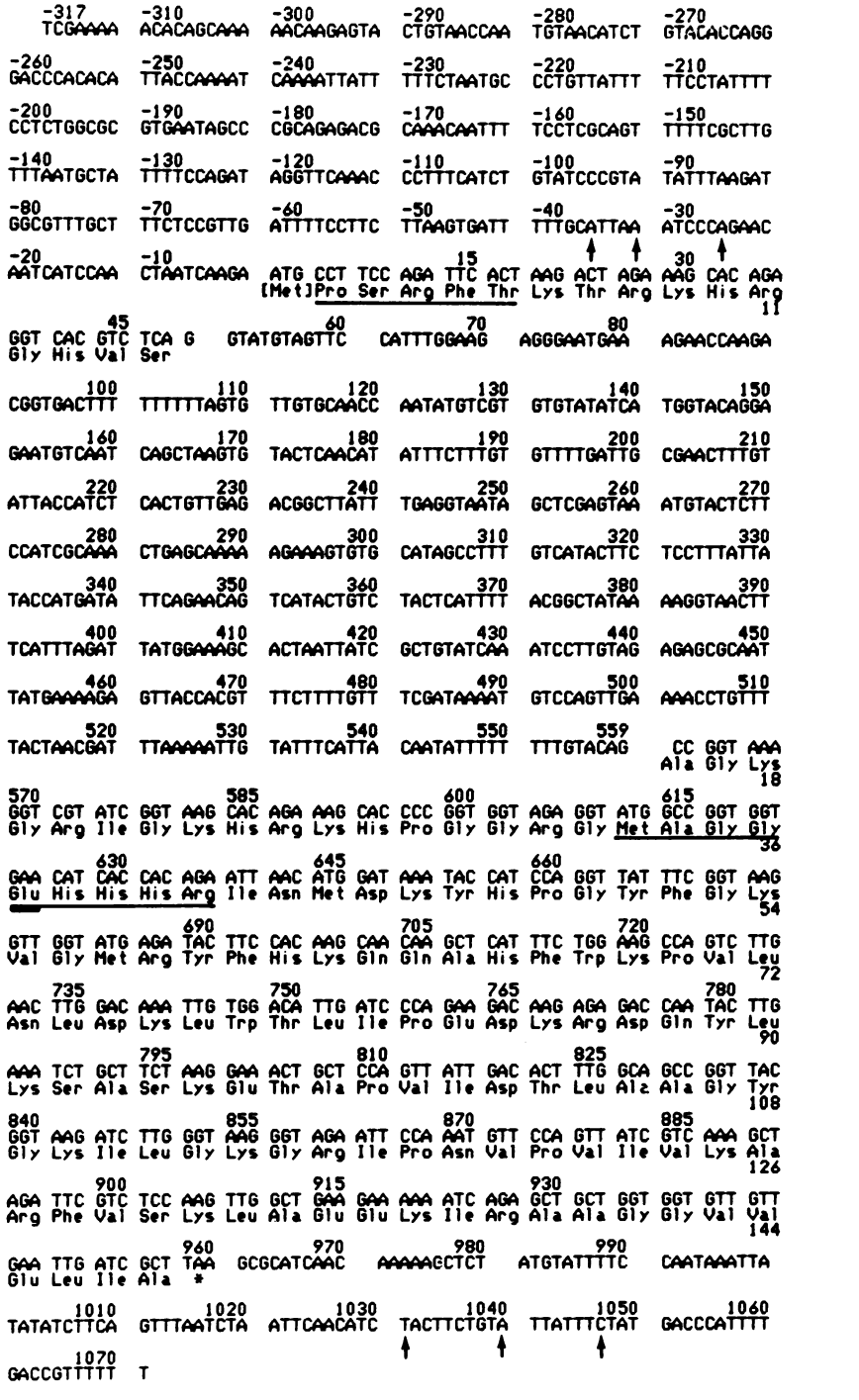
wild type GLY-MET-ALA-GLY-GLY-GLN-HIS-HIS-HIS-ARG

resistant .....-GLU-.....

The DNA sequence containing the CYH2 gene is presented in Figure 3. There is a nucleotide sequence (3 to 18), following an ATG, which corresponds to the predicted N-terminal amino acid sequence. Furthermore, a portion of the DNA sequence (nucleotides 613 to 636) codes for the peptide analyzed by Stocklein et al. (1). This sequence is followed by an open reading frame which terminates at positions 958-960 with a TAA. The EcoR1 site used to map the intron is found at position 860 to 865. About 315 bp upstream from this EcoR1 site, at positions 552-559, there is a 3' acceptor sequence (13). Using the ATG (position 1 to 3) as the presumptive translational start, there is an in-phase stop codon at positions 55 to 57. However, the sequence upstream, at positions 47-55, conforms to the canonical 5' splice junction (13), suggesting that the intron runs from position 50 to 559, consistent with the size predicted from the analysis of RNA (3).

In summary, as indicated in Figure 3, we conclude that the translation of the CYH2 gene initiates at an ATG codon in position 1-3 and terminates at the ochre stop codon, TAA, in position 958-960. Forty-nine nucleotides downstream from the ATG, the structural gene is interrupted by a 510 bp intervening sequence. Ribosomal protein L29 is, therefore, a polypeptide of 148 amino acids with a molecular weight of 16,500 daltons. This size is consistent with electrophoretic estimates of its molecular weight (20,21).

We have also sequenced the wild type allele of CYH2. It is identical except for



a single nucleotide exchange in position 622, where the wild type has a C (Fig. 3) and the resistance allele a G (Fig. 4). The mutation, therefore, leads to the replacement of glutamine by glutamic acid at position 37 of the protein, in accordance with the findings of Stocklein et al. (1). Another mutant form of L29, in which the glutamine is replaced by a lysine (1), is presumably due to the mutation of the C at position 622 to an A.

The Intron of the CYH2 Gene

Because introns are rare in yeast genes, a comparison of those available is warranted. The splice junctions show substantial homology to the consensus sequences compiled by Mount (13):

	Donor (5')														Acceptor (3')						
consensus (13)	C/A	A	G	G	T	A/G	A	G	T	.....	$(T/C)_{11}$			N	C/T	A	G	G
actin (11,12)	C	T	G	G	T	A	T	G	T	.....			G	T	T	T	T	A	G	A
rp S10 (22)	A	A	G	G	T	A	T	G	T	.....			A	T	A	A	C	A	G	T
rp L29	C	A	G	G	T	A	T	G	T	.....	T_8		G	T	A	C		A	G	C

Indeed, for six nucleotides downstream from the 5' donor junction the yeast sequences are identical. Nevertheless deletion mapping of the intron of the actin gene (23) shows that both the G (unpublished) and the T (23) at the beginning of the intron are absolutely required for a correct splice. At the acceptor site such mapping points to an essential sequence which is within the intron and includes the octanucleotide 5' -TACTAACA- 3' near its 3' end (D. Gallwitz, personal communication). The intron of CYH2 contains a similar sequence, at position 511 to 517 (Fig. 3), as does the gene for ribosomal protein S10 (22).

The location of the intron close to the translation initiation site of CYH2 deserves some comment. The intervening sequences in the yeast genes for actin and for ribosomal protein S10 occur after the first and second codon respectively. Electron microscopic evidence suggests that introns are located near the 5' ends of several other ribosomal protein genes as well (24). Because of the close proximity of the 5' splice junction to the 5' end of the mRNAs, it is difficult to argue that the two exons of these genes

Figure 3. DNA sequence of the cyh2 gene and amino acid sequence of ribosomal protein L29. Underlined regions show the N-terminal amino acid sequence and the tryptic peptide determined by amino acid sequencing (see text). The amino acid residues have been numbered from the proline residue, since the initiating methionine is removed in vivo (19). The arrows in the 5' and 3' flanking regions mark the transcriptional initiation and termination sites (see text).

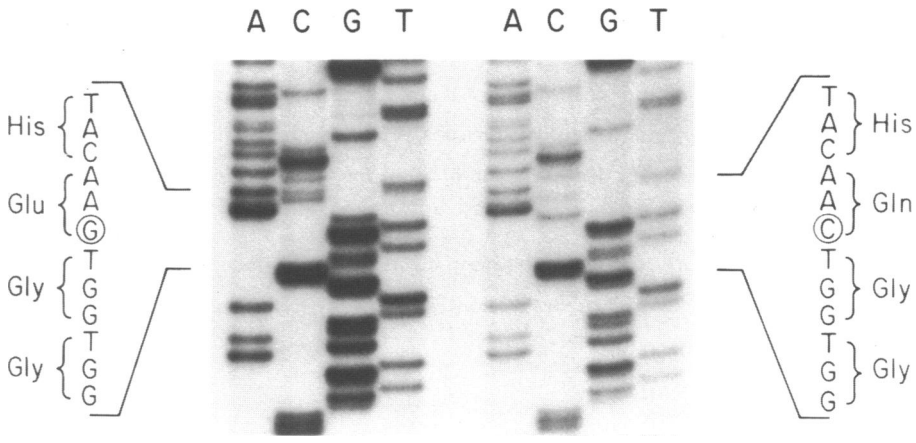


Figure 4. Sequence gels showing the mutation in the CYH2 structural gene which causes the resistance to cycloheximide. Left - the resistance allele; right - the wild type.

represent functional domains of the protein (25). Nevertheless, the introns are all within the coding sequence. Perhaps the location reflects a structural requirement of the RNA processing enzyme(s). Alternatively, the 5' exons may be part of 5' regulatory regions.

Codon Usage and Distribution of Amino Acids in CYH2

The several yeast genes sequenced so far have a strong bias of codon usage (26). The L29 gene shows a similar bias. Only 34 of the 61 possible codons are used and 15 of those extensively. For example, 11 of the 12 arginine codons are AGA; all 18 glycine codons are GGT.

The distribution of basic, acidic, and hydrophobic residues in ribosomal protein L29 is represented in Figure 5. The basic nature of the N terminal region is striking;

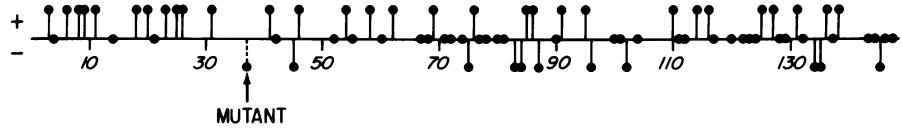


Figure 5. Schematic representation of the amino acid sequence of ribosomal protein L29. The basic residues, arginine and lysine, are indicated above the horizontal line, the acidic residues, aspartate and glutamate, below the line. The hydrophobic residues, tryptophan, phenylalanine, valine, leucine, and isoleucine, are indicated by dots on the line. The mutant glutamate residue is at position 37.

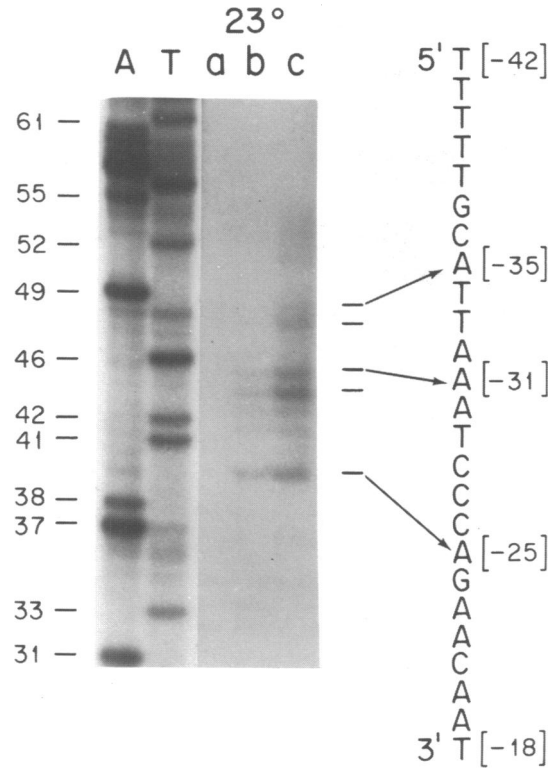


Figure 6. S1 nuclease mapping of the 5' end of the L29 mRNA. The 5' labeled Hinf/Taq fragment (Fig. 1) was hybridized to poly (A)+ RNA from yeast strain ts368 grown at the permissive temperature of 23° C. Lanes a,b,c show the protected length of fragments after hybridization and subsequent treatment with nuclease S1 under different conditions.
a hybridized in 80% formamide, 60 units nuclease for 30 min at 37° C
b hybridized in 65% formamide, 30 units nuclease for 30 min at 37° C
c hybridized in 65% formamide, 30 units nuclease for 30 min at 20° C
The A and T ladder of a known sequence was used to measure the length of the protected fragments.

11 of the first 31 amino acids are lysine or arginine. The first acidic amino acid appears at position 45. With the exception of an aspartic acid at position 102 and a glutamic acid at position 140, all acidic amino acids are adjacent to other charged amino acids. It is noteworthy that the mutation causing resistance to cycloheximide alters two of these generalizations, introducing a lone acidic residue at position 37. In addition the altered amino acid is adjacent to a run of three histidines, an unusual feature suggesting that

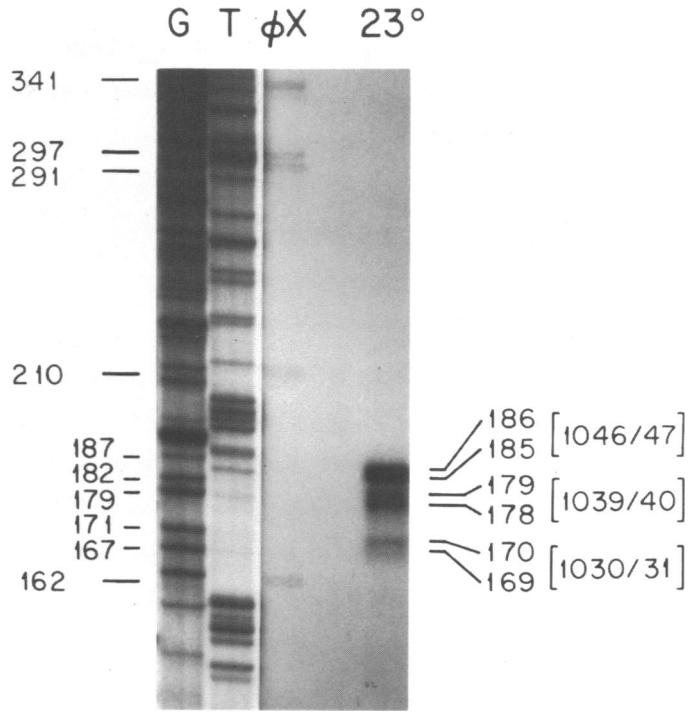


Figure 7. S1 nuclease mapping of the 3' end of the L29 mRNA. The 3' end labeled Eco/Pst fragment (Fig. 1) was hybridized to poly $(A)^+$ RNA from yeast strain ts368 grown at the permissive temperature of 23° C and treated with S1 nuclease. The G and T ladder of a known sequence and radiolabeled HincII fragments from ØX174 RF DNA were used to measure the length of the protected fragments.

this is an important region of the protein. The hydrophobic residues are largely clustered, e.g. at positions 67-81 (9 of 15), at positions 120-129 (6 of 10), and at positions 138-142 (4 of 5). They are rare in the basic N terminal third of the protein.

While mutations in CYH2 reduce the affinity of cycloheximide for the ribosome (27), there is no proof that cycloheximide actually binds to L29. The mutation could alter the conformation of an adjacent site. Indeed, we have found that cells carrying cyh2 together with a mutation which alters protein L3, causing resistance to trichodermin, another inhibitor of elongation, are far less resistant to cycloheximide than cells carrying cyh2 alone. Thus L3 and L29 are probably neighbors in or around the peptidyl transferase site; an alteration in one can have an effect on the other.

Initiation of Transcription

The experiment in Figure 2 shows that the 5' end of the CYH2 mRNA is approximately 880 nucleotides upstream from the EcoR1 site (position +866), i.e. about 25 nucleotides upstream from the initiator codon.

For more precise mapping of transcription initiation we used a 5' labeled HinfI/TaqI fragment of 330 bp. The labeled HinfI site is at position +11, in the structural gene (Fig. 3). For hybridization poly(A)$^+$ messenger RNA was used from strain ts368 grown at the permissive temperature. As can be seen in Figure 6, lane c, a series of protected bands are detected, with major bands of 48/49, 44/45 and 39 bp in size. Since the protected region is AT rich (70%) we varied the hybridization and digestion conditions (Fig. 6, lane a,b,c). The results suggest that the CYH2 gene has three possible transcriptional start sites, the A at -25, the A at -31, and the A at -35 (Fig. 6 and 3). [The S1 nuclease often leaves a double band, due to nibbling at the end (28); therefore we have used the larger of the pairs.] It is noteworthy that all three putative initiators are A, since no caps of the form m^7GpppPyr have been found in yeast (29).

In CYH2, 57 bp upstream from the first transcriptional start point, there is a sequence TATATTTA which is similar to the TATA box present in most eukaryotic genes (30) and may act as a selector for the correct initiation of transcription. Furthermore, at positions -37 to -30 occurs the sequence GCATTAA which is similar to the 'cap' sequence located in or adjacent to the 5' termini of sea urchin histone mRNA and Dyctiostelium mRNA (28,31).

Termination of Transcription

The 3' terminus of the CYH2 message was also mapped using the S1 technique. A 320 bp EcoR1/PstI fragment, whose EcoR1 site is at position 864 within the structural gene, was labeled with α-^{32}P ATP in positions 866 and 867. Following annealing with mRNA and S1 digestion there are 3 protected fragments, all doublets, with sizes of 169/170, 178/179 and 185/186 nucleotides (Fig. 7). This result suggests that CYH2 has three 3' termini and places the transcriptional termination 71 bp, 80 bp and 87 bp downstream from the TAA stop codon (see Fig. 3). The largest protected fragment is the most prominent one, suggesting that position +1047 is the preferred termination site.

Thirty-nine bp upstream from the first mapped transcriptional termination site is an AATAAA, the sequence considered to represent the poly(A) additional signal (32). Zaret and Sherman (33) propose a consensus sequence TAG....TATGTA....TTT to play a role in

transcription termination in yeast genes. In the CYH2 gene this sequence occurs 20 nucleotides downstream from the TAA stop codon and 48 bp before the first poly(A) site (see Fig. 3).

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