SSL1, a suppressor of a *HIS4* 5'-UTR stem-loop mutation, is essential for translation initiation and affects UV resistance in yeast

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The *SSL1* locus was identified as a *trans*-acting suppressor that restores *HIS4* expression despite a stem-loop structure in the 5'-UTR. *SSL1* encodes an essential protein of 52 kD with features characteristic of a protein with multiple zinc fingers. The mechanism of *SSL1* suppression is not related to altering *his4* transcription or removing the stem-loop sequence from the 5'-UTR; rather, 3- to 5-fold increases in *His4* translational expression are observed indicating a post-transcriptional mechanism for *SSL1* suppression. *SSL1* suppressor mutants that are conditional for growth have altered polysome profiles at the restrictive temperature, and their cell-free extracts are thermolabile in their ability to translate exogenously added mRNA. In addition, the mechanism of suppression appears to be specific for stem-loop structures placed near the 5' end of the message as opposed to a stem-loop located at a downstream position in the 5'-UTR. These observations suggest a role for this protein in promoting translation initiation presumably at the level of ribosomal binding to mRNA. Surprisingly, *SSL1* suppressor mutations that are shown to confer an *in vivo* and *in vitro* defect in translation initiation also rendered yeast hypersensitive to UV irradiation. This latter phenotype was observed previously with a mutation in the *SSL2* suppressor gene, which encodes the yeast homolog of the human gene *ERCC-3*, for which a defective form causes xeroderma pigmentosum. In light of the related effects of mutations in the *SSL1* and *SSL2* genes, the encoded proteins may functionally interact both to promote DNA repair and perform an essential function during translation initiation.

[Key Words: Translation initiation; DNA repair; Stem-loop; *Saccharomyces cerevisiae*; *SSL1* gene]

Received August 27, 1992; revised version accepted October 26, 1992.

A ribosomal scanning model has been proposed to account for molecular events between the preinitiation complex and mRNA that lead to initiation of protein synthesis in higher eukaryotes (Kozak 1991a). The basic features of this model are that the 40S ribosomal subunit, in association with initiation factors and tRNA^{Met}, binds near or at the 5' end of a mRNA and migrates in a 3' direction in search of the first AUG initiator codon. Although this model is based on sound experimental evidence, it remains unclear which initiation factors might facilitate the direct binding of the ribosome to mRNA or confer the ability of the preinitiation complex to scan the mRNA. One of the best candidates for promoting ribosomal binding/scanning of mRNA is the eukaryotic initiation factor eIF-4F (Hershey 1991). This complex is composed of three subunits: eIF-4E, the 7-methyl-G, cap-binding protein; eIF-4A, the prototype DEAD box RNA helicase; and p220, a subunit of unknown function. On the basis of the biochemical characterization of this factor and the associated subunits, a model has been put forth to explain at least one of the initial events in translation initiation (Sonnenberg 1988; Thach 1992). In this model, eIF-4E binds the cap structure of mRNA, which

brings eIF-4A, the helicase subunit of eIF-4F, to the mRNA. eIF-4A, in conjunction with another translation initiation factor, eIF-4B, is then proposed to unwind mRNA, removing secondary structure to provide a single-stranded region on the mRNA for the ribosome to bind. Although eIF-4E and eIF-4A are no doubt essential for translation initiation, a fundamental question in translation initiation is whether these factors function *in vivo* in the mechanistic aspects suggested by this model. Also, what other known, or as yet unidentified, factors might be involved in promoting ribosomal binding/scanning of mRNA, let alone the mechanistic details.

Recently, we have reported an alternative approach to attempt to identify the components of the translation initiation complex that might participate in ribosomal binding/scanning of mRNA (Gulyas and Donahue 1992). The strategy of this approach is to block ribosomal binding/scanning of mRNA by placing a stem-loop structure mutation in the 5' untranslated region (5'-UTR) of the *HIS4* gene of yeast and select genetically for suppressor mutations that overcome the block in translation initiation. Suppressor mutations that are unlinked to *HIS4*

would therefore be prime candidates for identifying genes that encode factors now possessing altered specificities for the initiation process and overcome the inhibitory effect of the secondary structure in the *HIS4* leader. This selection scheme resulted in the identification of four suppressor genes, *SSL1–SSL4*, which restore a *His4*⁺ phenotype when mutated. Characterization of the *SSL2* gene has shown that it encodes an essential gene product with motifs characteristic of RNA helicase/nucleic acid-binding proteins (Gulyas and Donahue 1992). *SSL2* does not restore expression at *HIS4* by removing the stem-loop sequence from either the DNA or the *HIS4* message. Instead, the characterization of *SSL2* suppression is consistent with a post-transcriptional mechanism of suppression and suggests that a helicase protein other than eIF-4A may be important during the early events of translation initiation. Interestingly, the *SSL2* gene is the yeast homolog of the human gene *ERCC-3* (Gulyas and Donahue 1992). A defective form of the *ERCC-3* gene has been identified in a patient with xeroderma pigmentosum and clinical symptoms of Cockayne's syndrome (Weeda et al. 1990). An *SSL2* allele made to resemble this defective form of *ERCC-3* confers a UV-sensitive defect to yeast cells when present as the sole genomic copy of *SSL2*. In contrast, an *SSL2* suppressor mutation did not confer UV sensitivity to yeast. This suggested that *SSL2* and *ERCC-3* might have dual functions: a nonessential function related to DNA repair, as suggested by the repair defects observed in mutated forms of these genes, and a second essential function that is related to translation initiation. Alternatively, *SSL2* and, by extrapolation, *ERCC-3*, might have a single function in gene expression, which leads to an alteration of the expression of DNA repair genes.

Here, we report the characterization of the *SSL1* suppressor gene. *SSL1* encodes an essential protein with multiple Cys-X₂-Cys sequences located in its carboxyl end, suggestive of a protein that functions in nucleic acid binding. *SSL1* suppressor mutants restore *His4* expression by a post-transcriptional mechanism that appears to have specificity for the position and sequence of the stem-loop in the 5'-UTR. Polysome analysis and cell-free in vitro translation assays of conditional *SSL1* strains indicate that *Ssl1* is essential for yeast translation. Taken together, these data implicate *Ssl1* as a novel translation factor that is required for an early event during the translation initiation process in yeast. Interestingly, *SSL1* suppressor strains also confer a UV sensitive phenotype to yeast. In light of other observations that we have made, these data appear to indicate that *SSL1* and *SSL2* gene products functionally interact to promote DNA repair and/or translation initiation.

Results

Characterization of the wild-type *SSL1* gene

The *SSL1* suppressor genes were identified as spontaneous *His*⁺ revertants of *His4*[−] yeast strains that contained the *his4-316* allele (S.P. Miller and T.F. Donahue, unpubl.). The *his4-316* allele contains a 36-bp insertion

sequence with perfect dyad symmetry in the DNA region that corresponds to the *HIS4* 5'-UTR. Upon transcription of *his4-316*, a stem-loop structure is predicted to reside 10 nucleotides downstream from the *HIS4* transcriptional start position and 50 nucleotides upstream from the *HIS4* AUG start codon. Six revertants were characterized genetically to contain *SSL1* suppressor mutations: JJ636 (*SSL1-1*), JJ637 (*SSL1-2*), JJ638 (*SSL1-3*), JJ576 (*SSL1-4*), JJ578 (*SSL1-5*), and JJ582 (*SSL1-6*). The former three strains were also temperature-sensitive (*Ts*[−]) for growth on enriched medium.

The wild-type *SSL1* gene was identified as part of a 7.4-kb DNA insert from a YCp50 wild-type genomic clone bank, on the basis of its ability to complement the recessive *Ts*[−] phenotype associated with the *SSL1* suppressor strains JJ636, JJ637, and JJ638. A 4.2-kb *EcoRI–BamHI* DNA fragment was subcloned into the integrating vector YIp5 and used to transform an *SSL1* wild-type strain to *Ura*⁺. *Ura*⁺ transformants were then crossed to a *Ts*[−] *Ura*[−] *SSL1* suppressor strain and subjected to tetrad analysis. All *Ura*⁺ ascospores were *Ts*⁺, whereas all *Ura*[−] ascospores were *Ts*[−], indicating that the DNA region is derived from the *SSL1* locus.

The ability to complement the *Ts*[−] phenotype associated with the *SSL1* suppressor strains was localized to a 2.5-kb *XhoI–PvuII* DNA fragment. Also, the presence of this fragment on a YCp50 vector in these suppressor strains reduces the ability of the strains to grow on SD-histidine medium. Both observations are similar to those made about the dominant/recessive nature of these phenotypes in diploid cells (S.P. Miller and T.F. Donahue, unpubl.) and indicated that the 2.5-kb *XhoI–PvuII* DNA fragment contains the intact *SSL1* wild-type gene. The DNA sequence of this fragment is shown in Figure 1. An open reading frame (ORF) of 461 amino acids that begins with an ATG is contained in this sequence and is predicted to encode a protein with a calculated *M_r* value of 52,289. Southern analysis showed that this region is unique in the haploid yeast genome (data not shown). Northern analysis of total and poly(A)⁺ RNA isolated from wild-type and *SSL1* suppressor strains identified a unique 1.5-kb transcript derived from this region (data not shown). Primer extension analysis (data not shown) identified three major transcriptional start sites at −45, −37, and −29 relative to the ATG that defines the 461-amino-acid ORF.

One copy of this 461-amino-acid ORF was disrupted in a wild-type diploid strain using the *URA3*⁺ gene. The resulting diploid transformants were subjected to tetrad analysis. Among 44 tetrads dissected, 31 showed a segregation pattern of two viable to two inviable spores, whereas 13 tetrads had one viable spore. All viable spores were *Ura*[−], indicating that disruption of this ORF results in cell inviability. The lethal effect of this gene disruption can be rescued by first transforming this diploid strain with a *LEU2*⁺, *CEN4* plasmid that either contained the 4.2-kb *EcoRI–BamHI* DNA fragment or the 461-amino-acid ORF under the control of a galactose-inducible promoter. Antibodies directed against the carboxyl terminus-coding region of this ORF, as part of a

XhoI
CTCGAGGTACTTACGGAACAAC

ATCAGCGTTAGCTGCCTTCTCTCCACGTCCATGGATCGTTGTGACATGTTTTTCATTGTATTGATTTGCTGACACTCTACTTCTCAGCAACAACA
AAGTAAACTTTAATCTATCATACTTTGAATCTTTATATAGCTGTATATATGCACCCGCTGAGATGCTCATCGAGATTGCTCATCCTCTGAAAAGGC
TGTCTGTACACCACCATGTTTCCGCCGACCAATGACAGTTCCTTAAGGGAACTCAAAGAGACTATATTAACCTAAGGAGGATCAACAGATATGCAAA
AAAAACAATAGTTATGACGCACTCGAACCAACCAACACCCCTATAATTAATGTTGATATGATAGCGACTCGAAATTTGCCAATAAACCTGTCT
ATTACGCTCATCTACTTGAGAGTAATATATCGAAGTTCGAGATACTCCGCACAGGCTCTGGGTGAGGTTTCTTTGCTATTGTATAGTTTTCAAGA
TACGTAAAAGTCTTCATCTTATCTTATATAGATGACAGGTGATTACTAGTGGGGCAAGTCGGCAGCTAATTGCCTGTGTGCGGAAGGATTGTGCAGTAGA

1
ATGGCTCTGTAGTTATTTTTCAGAACTCTGAAGAGGATGAGGATAGAGTTGCCATTACTAGAAGAACTAAGAGACAAGTTCATTTTGTGGCGAGGAGATGAT
MetAlaProValValIleSerGluSerGluGluAspGluAspArgValAlaIleThrArgArgThrLysArgGlnValHisPheAspGlyGluGlyAspAsp

35
CGGTAGACCAACAGCAACAGCAGCAGCTTAAGCCACAGGATAGGATAAATCATGTGACAGCTAAGAAGAAGAAAGGCTCTCGAATAGAACCTGCAA
ArgValAspGlnGlnGlnGlnHisSerSerHisArgAspArgAspLysHisValGlnArgLysLysLysArgLeuSerAsnArgAsnLeuGln

69
GGTTCAATGGTGGGTACGCTTGGGAGGATGAGATCAAGAGAAGTTGGGATTGGTGAAAGTGGACGATGAAGGGGATATGGCATCTCTTGTGGCTAGCATA
GlySerAsnGlyGlyTyrAlaTrpGluSerTrpAspLeuValLysValAspAspGluGlyAspMetAlaSerLeuValAlaSerIle

103
GTGAGGCCAGAAAGAACCTACTGCTAAGAAAAACATAACGCCATACCAAGAGGTTATTCAGAAAGTTTAAATATTAACACTAGATTGTAGTGAAGCTATG
ValGluAlaArgLysLysArgThrAlaLysLysAsnIleThrProTyrGlnArgGlyIleIleArgSerLeuIleLeuThrLeuAspCysSerGluAlaMet

137
TTGGAAAAGGATTGAGACCAACAGGATGCCATGATTATCCAGTACGCCATAGATTTTGTCCATGAATTTTTCGACCAAAATCCGATATCTCAATGGGT
LeuGluLysAspLeuArgProAsnArgHisAlaMetIleIleGlnTyrAlaIleAspPheValHisGluPhePheAspGlnAsnProIleSerGlnMetGly

171
ATTATCATATGAGAAACGGTTTGGCAACATGGTTCAGCCAACTTAGCGGAAACCCGAGGATCACATTGATCGGTTGAATCCATCAGGAAACAAGAACCG
IleIleIleMetArgAsnGlyLeuAlaGlnLeuValSerGlnValSerGlyAsnProGlnAspHisIleAspAlaLeuLysSerIleArgLysGlnGluPro

205
AAGGAAACCTTCATTGCAAAATGCAATGGAAATGGCAAGAGCCCTTTATTCGCTGTCTGCGCATGTGCAAGGGAAGTACTGATTGTATTGGTAGT
LysGlyAsnProSerLeuGlnAsnAlaLeuGluMetAlaArgGlyLeuLeuLeuProValProAlaHisCysThrArgGluValLeuIleValPheGlySer

239
CTTCCCAACTGATCCTGGAGATATTCACCAACTATCGATTCTGTTAGTTCTGAGAAAAATAGAGTGAAGTTTGGGGTTATCAGCTCAAGTGGCCATA
LeuSerThrThrAspProGlyAspIleHisGlnThrIleAspSerLeuValSerGluLysIleArgValLysValLeuGlyLeuSerAlaGlnValAlaIle

273
TGTAAGAATATGCAAGGCAACGAATATGGTGATGAATCTTCTATAAAATTTTACTCGATGAACTCACTTAAAGGAGCTATTAAACGAGGCTGTACC
CysLysGluLeuCysLysAlaThrAsnTyrGlyAspGluSerPheTyrLysIleLeuLeuAspGluThrHisLeuLysGluLeuPheAsnGluAlaValThr

307
CCTTTACCCGTTAACAAAATTAATAGGGTTTACACTCGTAAAGATGGGGTTCCGACAAGGATATTCGAAGATACTCCAATTTTGTTCGTGCCATTCC
ProLeuProValAsnLysIleAsnLysGlyPheThrLeuValLysMetGlyPheProThrArgIlePheGluAspThrProThrPheCysSerCysHisSer

341
AAGTTAGTTTACGGCGGATACTTCTGTCCCAATTTGTCATAGTAAAGTTTGTTCATTGCCCCACAGTCTGTCATGTTGTGACTTGATGCTAATCCTTTCTACC
LysLeuValTyrGlyGlyTyrPheCysProAsnCysHisSerLysValCysSerLeuProThrValCysProCysCysAspLeuMetLeuIleLeuSerThr

375
CACCTGGCCAGATCATATCACCATTCTGATGCGGTAAAAACATTTCGAGAAGTACCTACACAGAAAAATTCGATCAGAAGACTGCTTTAGTTGCCAGTCA
HisLeuAlaArgSerTyrHisHisLeuMetProLeuLysThrPheAlaGluValProThrThrGluLysPheArgSerGluAspCysPheSerCysGlnSer

409
AGATTTCCTACTGAAAAACCAATAAATGGTAACTACTGCAAGTTCTCGCTATCGTTGTGAAGATTGTAACAAGAATTTTGTGTGACTGCGATGTG
ArgPheProIleLeuLysAsnHisLysAsnGlyLysLeuLeuThrSerSerArgTyrArgCysGluAspCysLysGlnGluPheCysValAspCysAspVal

443
TTCTCCATGAATCCCTCATAACTGCCAGGCTGTGAATCCAAAGCCGTAATACTTAACGAAAACTCTGTCAGGCTATACACGATCACTCTCCATCAT
PheIleHisGluIleLeuHisAsnCysProGlyCysGluSerLysProValIleThrTer

ATAACAAATCCAAGTAGTAGAGAAGAAATAGTTTTTGTGGACATATACTACATAATTATTACAAAAATAACCTCATTTAATGTCCTATTGTTTATTA
CGAGAAATCTTAATAGCACTTACGGCATTAAAGATATTTATTACTATTTTCAATTATCAGGCACTTATCAACTAAAAAATAAATTTCTTGT
TTTCAATAATATTAATAATTTTGAATAAATATCGTTACATTCTATCATAATGTCTCTACACGGTACAACCAACTCACAATTTCTATTGAGTGGG
CGAGAGGTTTGAATTTTTTCTTACTGGGCTGTGTAGGGGTCATAGATGTGCTGTTGAATGAGTGAGGGTTGGAAGGTGCAATAGGTTTCTGTGGA
GATTTAACAAAACACTGTGCGTATCCGGCTTTCTCCCTGCTTCCAGCTG

PvuII

Figure 1. DNA sequence of the wild-type *SSL1* gene. The DNA sequence of the 2.5-kb *XhoI*–*PvuII* DNA fragment was confirmed for both strands. The 461-amino-acid sequence corresponding to the *SSL1* gene product is numbered from the ATG (+1). The arrowheads designate the transcriptional start sites that mapped at –45, –37, –29 relative to this first ATG that defines the *SSL1* DNA-coding region, as determined by primer extension analysis. The asterisks (left to right) indicate the positions of the nucleotide change found in the *SSL1-6* suppressor allele [GAA (Glu) to AAA (Lys); amino acid position +159], the *SSL1-4* mutant allele [TCA (Ser) to TTA (Leu); amino acid position +209], and the *SSL1-1* allele [GGG (Gly) to AGG (Arg)] and *SSL1-3* mutant allele [CGG (Gly) to CGG (Arg)] the latter two alleles containing different mutations that altered the same amino acid at position +265 in the *SSL1* DNA-coding region. Underlined are the eight Cys- X_n -Cys motifs located in the carboxyl end of the *SSL1* DNA-coding region. The nucleotide sequence presented in this paper has been assigned GenBank accession number Z17385.

TrpE fusion protein, detect a protein in yeast extracts with an apparent M_r of 50 kD, which is consistent with the calculated M_r of the 461-amino-acid ORF (S.P. Miller and T.F. Donahue, unpubl.). These studies indicate that the intact 461-amino-acid DNA-coding region is translated in yeast and encodes a protein that is essential for cell viability.

Characterization of the *SSL1* suppressor genes

Each mutation of *SSL1* that conferred both the Ts^- and the His^+ suppressor phenotypes was mapped by the gap–

duplex repair method. A 4.2-kb *EcoRI*–*BamHI* DNA fragment as part of YCp50 was restricted at the *Clal* site within the 461-amino-acid ORF and used to transform the Ts^- , *SSL1* suppressor strains JJ636, JJ637, and JJ638 to Ura^+ . Transformation of these strains with intact plasmid yields primarily Ts^+ transformants with a weak suppressor phenotype. In contrast, transformation of these strains with plasmid restricted at the *Clal* site resulted in a high incidence of Ts^- transformants with a stronger His^+ phenotype. This indicated that the *Clal* site was near the site of the mutation that conferred the recessive Ts^- and the weak codominant His^+ suppres-

sor phenotype. Plasmids were isolated from these Ts⁻ transformants. In addition, the identical DNA region was isolated from the *SSL1* suppressor strains JJ576 and JJ582 by the integration–excision method. The DNA sequence of the entire 461-amino-acid DNA-coding region contained on each of the corresponding plasmids was then determined. Each DNA sequence identified a single-base change that altered an amino acid in this ORF (Fig. 1). The Ts⁻ *SSL1* suppressor strains JJ636 (*SSL1-1*) and JJ637 (*SSL1-2*) each contained the same mutation that altered a glycine (GGG) residue at amino acid position +265 to arginine (AGG), whereas JJ638 (*SSL1-3*) had a different base change but contained the same glycine (GGG) to arginine (CGG) change at +265. JJ576 (*SSL1-4*) contained a serine (TCA) to leucine (TTA) change at amino acid +209, whereas JJ582 (*SSL1-6*) contained a glutamic acid (GAA) to lysine (AAA) change at amino acid position +159. On the basis of these and the above studies, we conclude that this 461-amino-acid DNA-coding region encodes the *SSL1* suppressor gene product.

Searches of DNA and protein data banks did not identify any previously characterized gene or gene product to be related to the *SSL1* gene or encoded protein. However, searches did pick out amino acid similarities with a number of zinc finger proteins, because the *SSL1* gene product contains a number of sequence motifs common to zinc finger proteins. There are six Cys-X₂-Cys, one Cys-X₃-Cys, and one Cys-X₂-His sequence motifs that are localized to the carboxy-terminal region of the protein (Fig. 1).

SSL1 suppression does not alter his4-316 transcription or remove the stem-loop sequence from mRNA

In light of the fact that *SSL1* does not correspond in sequence to any previously characterized gene or protein, especially one involved in translation initiation, we characterized the expression of the *his4-316* gene in an *SSL1-1* strain to gain insight into the mechanism of suppression and the *in vivo* function of the *SSL1* gene product. To address whether alterations in the transcriptional process at *his4-316* or removal of the stem-loop sequence from the mRNA were potential mechanisms of *SSL1* suppression, we determined the levels, structure, and start position of the *his4-316* transcript. As shown in Figure 2A, Northern analysis indicates that the steady-state level of the *his4-316* transcript is similar in isogenic *SSL1*⁺ and *SSL1-1* strains. Densitometric scanning of the autoradiograph indicated that the relative ratio of the *his4-316* transcript steady-state level to the actin transcript steady-state level was 0.52 in the *SSL1* wild-type strain and 0.46 in the *SSL1-1* suppressor strain. Comparable ratios of *his4-316* mRNA to actin mRNA were also observed in independent experiments. This suggests that an increase in the level of the *his4-316* transcripts is not the mechanism by which *SSL1-1* restores a His⁺ phenotype to this strain.

Primer extension analysis of the *his4-316* transcripts (Fig. 2B) identified a termination position near the base of the insertion mutation. This does not reflect the start

point of the *HIS4* transcript, which has been well-documented to initiate 60 nucleotides upstream of the AUG in the wild-type *HIS4* allele (Donahue et al. 1982; Nagawa and Fink 1985). Instead, this position, as has been described previously (Cigan et al. 1988; Gulyas and Donahue 1992), reflects the termination position of the primer extension reaction that maps to position -54, 4 nucleotides upstream from the site of the insertion of the stable stem-loop structure present in the *his4-316* message. No primer extension product exists either 5' or 3' to this termination position, indicating that the *his4-316* message derived from the *SSL1*⁺ and *SSL1-1* strains are equivalent, with all molecules possessing a stem-loop structure at the site of the insertion.

S1 nuclease protection analysis was performed to assess the start position of the *his4-316* transcript in the *SSL1*⁺ and the *SSL1-1* suppressor strains. For these experiments a ³²P-labeled, single-stranded DNA fragment corresponding to the complementary antisense strand of *his4-316* mRNA, including the stem-loop insertion sequence, was used for hybridization. As shown in Figure 2C, two different sized DNA fragments are protected, both of which are identified using RNA isolated from either the *SSL1*⁺ or the *SSL1-1* suppressor strain. The smaller fragment maps an S1 nuclease-sensitive site at 77 nucleotides upstream from the AUG that defines the start of translation at *HIS4* (position -77; see Fig. 2C). Presumably this site does not correspond to the 5' end of the *his4-316* message, because a termination position site at position -77 was not identified by primer extension analysis, but only the site of the insertion at position -54 (Fig. 2B). We believe that the S1 nuclease-sensitive site that maps to position -77 corresponds in position to a site within the stem-loop structure (Fig. 2C). We interpret this result to be attributed to a population of mRNA : DNA duplex that maintains intramolecular base-pair interactions at the site of the insertion such that a stem-loop structure exists in the DNA. The stem-loop structure within this DNA therefore provides a single-stranded region that can be cleaved by S1 nuclease and maps near position -77 (Fig. 2C).

The larger protected fragment maps an S1 nuclease-sensitive site at 96 nucleotides from the *HIS4* AUG start codon (Fig. 2C). This corresponds precisely to the total length of the wild-type *HIS4* leader, 60 nucleotides, and the length of the stem-loop structure, 36-nucleotides. We believe that this is the result of a second population of mRNA : DNA duplexes in the hybridization reaction, which is completely base-paired, including the inserted region of dyad symmetry. S1 cleavage of this duplex therefore identifies the 5' end of the *HIS4* message that is predicted to correspond to position -96 (Fig. 2C) if the stem-loop structure has no effect on the normal transcription initiation process at *HIS4*. More importantly, the observation that the same two DNA fragments are protected in an *SSL1-1* suppressor strain as that found in an *SSL1*⁺ strain and the apparent ratio of each protected product is also the same (Fig. 2C, cf. the intensity of the -77 vs. the -96 nucleotide protected fragment) illustrates that no change in the *his4-316* transcription pat-

Figure 2. Analysis of *his4-316* mRNA from an *SSL1*⁺ and an *SSL1-1* suppressor strain. (A) Northern analysis. Total RNA was prepared from the *SSL1* wild-type strain JJ567 and the *SSL1-1* suppressor strain JJ636. RNA (50 μ g) was separated on a 1.0% agarose gel, transferred to Nytran, and hybridized with ³²P-labeled probes from the *HIS4* DNA-coding region and the actin gene DNA-coding region. (B) Primer extension analysis. Total RNA (50 μ g) isolated from strains JJ567 (*SSL1*⁺) and JJ636 (*SSL1-1*) was used as a template. The primer and procedure is described in Material and methods and in Cigan et al. (1988). Lanes G, A, T, and C represent a DNA sequence ladder generated with the same primer and a plasmid that contains an *EcoRI* restriction site between positions -51 and -50 in the *HIS4* leader. This plasmid was used as the starting plasmid to construct *his4-316*, and the DNA sequence is only used as a marker to map the position of the terminated primer extension reaction. Below the autoradiograph is a schematic interpretation of the primer extension reaction in which the antisense ³²P-

labeled oligonucleotide (short thick line), is annealed to the *HIS4* DNA-coding sequence and the reverse transcriptase extends the oligonucleotide up to the site of the insertion (I). The products of the reaction detect the presence of the stable stem-loop structure in the *his4-316* message, which prevents the identification of the 5' end of the transcript. (C) S1 nuclease protection analysis. Total RNA was isolated from the wild-type *SSL1*⁺ strain 1427-15C and the *SSL1-1* suppressor strain 1427-27C. RNA (100 μ g each) was hybridized with uniformly, ³²P-labeled single-stranded DNA that contains the antisense strand of the upstream region of the *his4-316* allele, as described in Materials and methods. The resulting hybrids were treated with S1 nuclease, and the S1-resistant fragments were analyzed on an 8% polyacrylamide sequencing gel. Two major S1 nuclease-protected products are identified. The size of each product was determined from a DNA sequencing ladder that was coelectrophoresed. For clarity, the size of each fragment is interpreted, via the schematic diagram, relative to the position of the AUG codon in the *HIS4* DNA-coding region. One product maps an S1 nuclease site at position -77 within the stem-loop structure. The other product maps an S1 nuclease site at position -96, which is equal in length to the wild-type leader region (60 nucleotides) and the insertion sequence (36 nucleotides). For Northern analysis and primer extension and S1 nuclease studies, substitution of total RNA isolated from a *his4* deletion strain showed that all signals are specific to the *his4-316* transcript.

tern occurs as a result of *SSL1* suppression. Hence, our analysis indicates that the levels, structure, and start position of the *his4-316* transcript are similar in *SSL1* wild-type, and suppressor strains and suggests that the mechanism of *SSL1* suppression is related to a post-transcriptional process.

SSL1 suppression restores *HIS4* expression by a post-transcriptional mechanism

Further evidence that suppression occurs post-transcriptionally was determined by β -galactosidase assays of extracts prepared from *SSL1* wild-type and suppressor strains that contained a *his4-316-lacZ* fusion construct. As shown in Table 1, extracts derived from *SSL1* suppressor strains have higher levels of His4- β -galactosidase activity than extracts derived from an isogenic *SSL1* wild-type strain that contain the same *his4-316-lacZ* construct. The *SSL1-1* and *SSL1-3* alleles lead to a higher level of expression, being fivefold above the activity

measured from the *SSL1* wild-type control strain, whereas *SSL1-4* and *SSL1-6* result in a threefold increase in expression. In contrast, *SSL1* suppression events do not lead to increased levels of β -galactosidase activity when a wild-type *HIS4-lacZ* fusion construct is contained in these strains (Table 1). However, two *SSL1* suppressor alleles, *SSL1-1* and *SSL1-3*, appear to lower *HIS4-lacZ* expression to ~50% of the levels detected in an isogenic wild-type strain. We do not understand the basis for this effect. Nevertheless, these assays and our characterization of the *his4-316* transcript, as described above, suggest that *SSL1* overcomes the inhibitory effects of stem-loop by a post-transcriptional mechanism.

A Ts⁻, SSL1 suppressor strain has an in vivo polysome defect

As mentioned previously, three of the *SSL1* suppressor strains that we have identified, JJ636 (*SSL1-1*), JJ637 (*SSL1-2*), and JJ638 (*SSL1-3*), are also *Ts⁻* for growth on

Table 1. Efficiency of suppression

Strain	Genetic background	Plasmid	β -Galactosidase specific activity	Fold increase over wild type
JJ567	<i>SSL1</i> ⁺	<i>his4-316-lacZ</i>	8	1
JJ636	<i>SSL1-1</i>	<i>his4-316-lacZ</i>	43	5
JJ638	<i>SSL1-3</i>	<i>his4-316-lacZ</i>	39	5
JJ576	<i>SSL1-4</i>	<i>his4-316-lacZ</i>	28	3
JJ582	<i>SSL1-6</i>	<i>his4-316-lacZ</i>	24	3
JJ567	<i>SSL1</i> ⁺	<i>HIS4-lacZ</i>	627	1
JJ636	<i>SSL1-1</i>	<i>HIS4-lacZ</i>	307	0.5
JJ638	<i>SSL1-3</i>	<i>HIS4-lacZ</i>	298	0.5
JJ576	<i>SSL1-4</i>	<i>HIS4-lacZ</i>	627	1
JJ582	<i>SSL1-6</i>	<i>HIS4-lacZ</i>	583	1

enriched medium. On the basis of the genetic selection scheme that we employed, one simple interpretation of the *Ts*[−] growth phenotype is that at the restrictive temperature (37°C), the Ssl1 protein is thermolabile and cannot perform its essential function during the translation initiation process, which leads to the inhibition of protein synthesis and the inhibition of cell growth.

To test this possibility, duplicate cultures of a *Ts*[−], *SSL1-1* suppressor strain were grown at the permissive temperature (23°C), and then one culture was shifted to 37°C for an approximate generation time (2 hr). Extracts were prepared from both cultures, fractionated by sucrose gradients, and polysome profiles were compared. Figure 3A shows the virtually identical polysome profiles from two extracts prepared from an isogenic *SSL1* wild-type strain: One from a culture grown only at 23°C, and the other from a culture that had been shifted to 37°C for 2 hr. In contrast, the two polysome profiles derived from an isogenic *Ts*[−], *SSL1-1* suppressor strain, JJ636, under the identical growth regimens, are different from each other (Fig. 3B). Although normal polysome profiles are observed when this strain is grown at the permissive temperature, an aberrant profile is observed after this strain is shifted to the restrictive growth temperature. The aberrant profile portrays a reduction in the amount of polysomes and an increased amount of the 80S ribosomal peak, which represents a nonspecific association of accumulated, free 40S and 60S ribosomal subunits (Yoon and Donahue 1992). This is consistent with the notion that the thermolabile *SSL1* gene product cannot perform its essential function in translation initiation. As a result of this block in translation initiation, ribosomes finish translating mRNA but cannot recycle back into the translation process. This leads to a reduction in the number of polysomes and the accumulation of ribosomal subunits, as depicted in the altered profiles at the elevated temperature (Fig. 3). Similar alterations in polysome profiles have also been observed at the restrictive temperature for *Ts*[−] mutants that encode other thermolabile gene products involved in translation initiation (Cigan et al. 1991; Yoon and Donahue 1992).

SSL1 cell-free extracts exhibit an inherent thermolabile defect in mRNA translation

The polysome defect described above is consistent with the notion that the *SSL1* gene encodes an essential component for translation initiation, which is thermolabile as a result of the suppressor mutation. Alternatively, similar results would be expected if the *SSL1* suppressor gene encoded a thermolabile transcription factor that regulated the expression of translation initiation components. Perhaps, as a result of the suppressor mutation, Ssl1 causes the overexpression of an essential translation initiation factor at the permissive temperature, which leads to suppression at *HIS4*. It would therefore stand to reason that inactivation of the thermolabile Ssl1 protein at the restrictive temperatures would then result in lower levels of the translational component and thus indirectly reduce the translational activity.

To differentiate between these mechanistic interpretations, the in vitro thermolability of cell-free mRNA translation was analyzed with extracts prepared from isogenic wild-type and *Ts*[−], *SSL1* suppressor strains grown at the permissive temperature. The logic behind this experiment is that we anticipate that the thermolabile nature of the Ssl1 protein would be inherent in extracts prepared from cells grown at the permissive

Figure 3. Polysome analysis. Duplicate cultures of either wild-type or *Ts*[−] *SSL1-1* strains were grown to an OD₆₀₀ of 1.0 in SD complete medium at 23°C. One of the duplicate cultures was then shifted to 37°C, and both the 23°C and 37°C cultures were incubated for 2 hr more. Cells from each culture were harvested, cell extracts were prepared, and 50 OD₂₆₀ units from each extract was fractionated on a 17–57% sucrose gradient. Gradients were collected from the top to the bottom using an ISCO 640 density gradient fractionator and monitored by the ISCO UA-5 absorbance-fluorescence detector. Gradients are plotted as OD₂₆₀ units versus fraction numbers (1.2 ml) from the top to the bottom of the gradient. (A) Polysome profiles prepared from the *SSL1*⁺ parent strain JJ567 grown at 23°C (left) and 37°C (right). (B) Polysome profile of the isogenic *Ts*[−] *SSL1-1* suppressor strain JJ636 grown at 23°C (left) and 37°C (right).

temperature. Therefore, only if *SSL1* encodes a thermolabile translation factor would the incubation of this extract at an elevated temperature lead to a decrease in its ability to translate exogenously added mRNA. We would not expect that incubation of the extract at elevated temperature would lead to decreased protein synthesis if *SSL1* encoded a thermolabile transcription factor. For these experiments, cell-free extracts, depleted of endogenous mRNA with micrococcal nuclease, were preincubated for increasing lengths of time at an elevated temperature and then assayed for the ability to translate exogenously added mRNA. However, this experiment was complicated by the fact that wild-type yeast extracts are inactive for cell-free translation at 37°C (Mandel and Trachsel 1989). To circumvent this problem the extracts were preincubated under less severe temperature conditions (32°C) and then assayed at 23°C.

As shown in Figure 4, extracts prepared from isogenic wild-type and *Ts⁻ SSL1* suppressor strains differ dramatically from each other in their sensitivity to in vitro heat inactivation. Whereas [³⁵S]methionine incorporation in a wild-type extract (solid circles) was only moderately affected by preincubation at 32°C, extracts from the *Ts⁻ SSL1* suppressor strains *SSL1-1* (open squares) and *SSL1-3* (open triangles) were strongly inhibited by an identical preincubation treatment. For example, wild-type extracts retained ~80% of their activity after 6 min of pre-

incubation at 32°C. In contrast, an identical treatment of an *SSL1-1* or *SSL1-3* suppressor extract inhibited mRNA translation to 16% or 8% of the untreated control extracts, respectively. Moreover, the *Ts⁻ SSL1* suppressor extracts were completely inactive after only 9 min at 32°C, whereas wild-type extracts retained ~55% of their original activity. We believe that this difference in thermal sensitivity is not the result of different levels of translational factors in these extracts, as both extracts in the absence of heat treatment are nearly identical in activity (see legend to Fig. 4) and show no difference in sensitivity to m⁷GTP inhibition (S.P. Miller and T.F. Donahue, unpubl.). Instead, these results indicate that the thermolability of protein synthesis is an inherent property of the *Ts⁻ SSL1* suppressor extracts and agrees with the notion that *SSL1* encodes a thermolabile translation factor. It is also worth noting that substitution of poly(U) for total RNA in this experiment did not result in the inhibition of poly(U) translation (S.P. Miller and T.F. Donahue, unpubl.). The *Ts⁻ SSL1* suppressor extracts retained >95% of their activity even after 12 min of preincubation at 32°C and were indistinguishable from an isogenic wild-type extract. This seems to illustrate further that the inherent thermolability of protein synthesis in *Ts⁻ SSL1* extracts is not the result of a defect in the elongation process but, rather, translation initiation.

Figure 4. In vitro heat inactivation profiles of cell-free translation extracts from wild-type and *SSL1* suppressor strains. Nuclease-treated extracts (0.45 µg) from permissive cultures of wild-type (●), *SSL1-1* (□), and *SSL1-3* (△) strains were preincubated in parallel reactions at 32°C for increasing amounts of time as indicated. The extracts were immediately transferred to an incubator and incubated further at 23°C with all other components required for mRNA translation. Aliquots (9 µl) were removed after 15 min of incubation and analyzed for hot acid-insoluble radioactivity. The results are expressed as the percent of activity as compared with the unheated control extracts. The 100% values were 10,105 cpm for wild-type, 9,091 cpm for *SSL1-1* and 9,341 cpm for *SSL1-3*.

Changes in the sequence or position of the stem-loop in the HIS4 5' UTR can alter suppression

An important observation obtained from the β-galactosidase assays is that *SSL1* suppressor mutants increase the level of *His4* expression when a stem-loop is present in the mRNA but not when the stem-loop is not present (Table 1). This implies that the mechanism of suppression is dependent on having the stem-loop in the mRNA. One interpretation of this result is that the suppressor mutation confers upon Ssl1 affinity for the stem-loop structure that facilitates translation of this message. Alternatively, Ssl1 does not have newly acquired affinity for the stem-loop but has the general ability to overcome the inhibitory effect of any stem-loop placed in the *HIS4* leader, perhaps facilitating a general "unwinding" activity that promotes improved translation of the message.

To gain further insight into the mechanism of *SSL1* suppression, we tested whether the *His⁻* phenotype caused by the inhibitory effect of stem-loop structures that are either different in sequence or length or located at a different position in the *HIS4* leader region was capable of being suppressed by an *SSL1* suppressor strain. The *his4-316* allele used for the selection of the *SSL* suppressor mutation contains a 36-bp insertion sequence that is composed of two repeating units of the sequence 5'-CCCGGATCCGGG-3' flanked by *EcoRI* restriction sites (Gulyas and Donahue 1992). We therefore increased this insertion to three repeating units to increase the dyad symmetry length (48 nucleotides) and decrease the predicted ΔG° value (-44.3) of the stem-loop between positions -51 and -50 and constructed a strain to con-

tain this mutation at the *HIS4* locus. As shown in Table 2, crosses of this strain (*his4-317*) to an *SSL1* suppressor strain that contains a *his4* deletion allele give rise to meiotic ratios of $2^+ : 2^-$, $1^+ : 3^-$, and $0^+ : 4^-$ on SD-histidine plates. These meiotic ratios are indicative of the ability of *SSL1* to act in *trans* and restore His4 expression despite this alternative stem-loop structure mutation in the leader.

We also constructed yeast strains containing insertion mutations between positions -51 and -50 that deviate significantly in sequence from the repeating sequence unit found in the *his4-316* allele but still contain dyad symmetry. One sequence, 5'-GGAATTGTCCTCCTGCCTCCTCCTTAAGGAGGCAGGAGGACAATTCC-3', was 47 bp in length ($\Delta G^\circ = -38.1$). This strain (*his4-318*), when crossed with an *SSL1* suppressor strain that contains a *his4* deletion, also gave rise to the meiotic ratios $2^+ : 2^-$, $1^+ : 3^-$, and $0^+ : 4^-$ (Table 2), indicative of the ability of *SSL1* to restore expression to this *his4* stem-loop mutation strain. However, a third construct, 5'-GGAATTCGCGCGCGCGATATCGCGCGCGCGGAATTC-3', failed to be suppressed by *SSL1* (Table 2; *his4-319*), as indicated by only His⁻ meiotic products being identified in crosses to a *SSL1* suppressor strain that contains a *his4* deletion. Although this latter sequence resembles the insertion sequence in the *his4-316* allele in part, the stem is predicted to be richer in contiguous G:C/C:G base pairs, the loop is predicted to be different in sequence from the *his4-316* loop, and the ΔG° value is lower (-32.5) than that of *his4-316* (-27.3), although not lower than the ΔG° value calculated for the *his4-317* (-44.3) and *his4-318* (-38.1) insertions, both of which are suppressible by *SSL1-1*. Taken together, these results seem to indicate that there may be flexibility in the mechanism of *SSL1* suppression possibly tolerating some changes in the sequence or structure of the stem-loop placed between positions -51 and -50 .

To determine whether the position of the stem-loop in the leader was important for the ability of *SSL1* to suppress at *HIS4*, we constructed a strain to contain a stem-loop mutation inserted between positions -27 and -20 in the *HIS4* leader such that the stem-loop structure would be located 33 nucleotides downstream from the 5' end of the message and 20 nucleotides upstream from the AUG start codon. The sequence in-

serted at this position was the 36-bp insertion sequence that was used to construct the *his4-316* allele. When this strain (*his4-320*) is crossed to an *SSL1* suppressor strain that contains a *his4* deletion allele, only His⁻ meiotic products are obtained (Table 2), indicative of the inability of *SSL1* to restore His4 expression to this stem-loop mutant. In addition, not only is this insertion sequence derived from the *his4-316* allele "nonsuppressible" when located at this downstream site, but reducing this sequence to only one of the repeating core sequences still does not allow *SSL1* to restore His4 expression (data not shown). These results suggest that the mechanism of *SSL1* suppression may be specific for the position of the stem-loop being nearer the 5' end of the message.

Two *SSL1* suppressor strains exhibit hypersensitivity to UV light

As mentioned previously, studies in our laboratory also identified the *SSL2* gene as a *trans*-acting suppressor of the stem-loop structure mutation at *HIS4* (Gulyas and Donahue 1992). Characterization of *SSL2* showed that it is the yeast homolog of the human gene *ERCC-3* for which a mutated form was identified in a patient with the DNA repair disease, xeroderma pigmentosum, and clinical symptoms of Cockayne's disease (Weeda et al. 1990). However, *SSL2* suppression does not result in removal of the stem-loop sequence from the *his4-316* mRNA nor does it confer a defect in DNA repair as measured by increased sensitivity to UV. Only when an *SSL2* allele (*SSL2-XP*) was constructed to resemble the defective form of the *ERCC-3* gene found in a xeroderma pigmentosum patient did yeast become hypersensitive to UV light with 10^4 times more yeast cells killed at UV dosages of 15 J/m² or greater as compared with a wild-type strain (Gulyas and Donahue 1992). In crosses between *SSL2-1* and *SSL1-1* suppressor strains, we noticed that haploid yeast strains containing both suppressor mutations resulted in the loss of the His⁺ suppressor phenotype that is evident in strains containing either suppressor gene (Gulyas and Donahue 1992). This constitutes genetic evidence that the two gene products either interact or function in a related pathway. In light of this functional interaction between the *SSL1* and *SSL2*

Table 2. Tetrad analysis of His⁻, alternative stem-loop strains crossed to *SSL1-3*, *his4* deletion strains

Stem-loop mutation				Segregation of His ⁺ phenotype in tetrads					
allele	position in leader	length (nucleotides)	ΔG°	(3 spores)			(4 spores)		
				$2^+ : 1^-$	$1^+ : 2^-$	$0^+ : 3^-$	$2^+ : 2^-$	$1^+ : 3^-$	$0^+ : 4^-$
<i>his4-317</i>	$-51/-50$	48	-44.3	1	4	1	5	9	3
<i>his4-318</i>	$-51/-50$	47	-38.1	2	7	9	12	11	6
<i>his4-319</i>	$-51/-50$	38	-32.5	0	0	4	0	0	15
<i>his4-320</i>	$-27/-20$	36	-27.3	0	0	14	0	0	7

The strains containing the alternative stem-loop mutation alleles were EP496 (*his4-317*), EP1734 (*his4-318*), EP1751 (*his4-319*), and CD21 (*his4-320*) and were analyzed in crosses with either of the *his4-401* deletion strains 603-7B or EP1568, both of which contain the *SSL1-3* suppressor allele derived from JJ638.

suppressor genes, we tested whether *SSL1* suppressor strains might render yeast cells hypersensitive to UV light.

As shown in Figure 5, two Ts^- *SSL1* suppressor strains, JJ636 (*SSL1-1*) and JJ638 (*SSL1-3*), are hypersensitive to UV irradiation when compared with the UV sensitivity profile of the isogenic *SSL1* wild-type strain JJ567. Although neither suppressor strain is as sensitive to low doses of UV as was the *SSL2-XP* strain (Gulyas and Donahue 1992), these strains are as hypersensitive to UV at the highest doses (Fig. 5; 40–60 J/m²) as the *SSL2-XP* strain. In contrast, neither of the isogenic Ts^+ suppressor strains JJ576 (*SSL1-4*) nor JJ582 (*SSL1-6*) is hypersensitive to UV; both are as resistant to UV as the *SSL1*⁺ strain (data not shown). It is important to note that although JJ636 and JJ638 have different single-base changes in the *SSL1* DNA-coding region, each mutation alters amino acid position +265 from a glycine to an arginine codon (Fig. 2). Hence, the UV-sensitive phenotype observed in both strains is specific for the same amino acid substitution in the *SSL1* DNA-coding region.

One difference between JJ636 and JJ638, and the other

Figure 5. UV-sensitivity profile of *SSL1* wild-type and suppressor strains. Cells were grown to stationary phase in liquid YEPD, diluted, and plated onto YEPD plates; sets of plates were then exposed immediately to UV light for various times. UV-irradiated cells were incubated overnight in the dark at room temperature and then transferred to a 30°C incubator. Colonies were counted and compared with the number of colonies obtained on plates that were not irradiated. The number of colonies was multiplied by the dilution factor and divided by the starting cell population. The log of this fraction (ordinate) was then plotted against the UV dose (abscissa). UV irradiation doses were calculated using the model UVX digital radiometer (UVP, Inc.). Symbols for yeast strains: (▲) JJ567 (*SSL1*⁺); (□) JJ636 (*SSL1-1*); (○) JJ638 (*SSL1-3*); (△) 301-4D (*sui1-1*); (◇) 304-1D (*sui2-1*). The UV-sensitivity profiles for strains JJ576 (*SSL1-4*) and JJ582 (*SSL1-6*) were also assayed and were no different than profiles for strain JJ567, 301-4D, or 304-1D.

Table 3. Phenotypes of strains assayed for UV sensitivity

Strain	Relevant allele	Doubling time (min)	<i>his4-316</i> suppression ^a	UV hyper-sensitivity
JJ567	<i>SSL1</i>	90	–	–
JJ636	<i>SSL1-1</i>	128	+	+
JJ638	<i>SSL1-3</i>	132	+	+
JJ576	<i>SSL1-4</i>	102	+	–
JJ582	<i>SSL1-6</i>	102	+	–
301-4D	<i>sui1-1</i>	146	ND	–
304-1D	<i>sui2-1</i>	161	ND	–

^a[ND] Not determined.

strains, JJ567, JJ576, and JJ582, is that the former two strains grow significantly poorer than the latter strains (Table 3). This led us to speculate that perhaps a mutation in any translation initiation factor that results in an adverse effect on gene expression, as exhibited by poor growth properties, may indirectly confer hypersensitivity to UV to yeast. Perhaps, this may be a result of lowering the level of expression of a protein that is critical/rate-limiting to the DNA repair process. To test this possibility, we assayed a *sui1* and a *sui2* mutant for UV-hypersensitive phenotypes, each of which contains a mutation in a gene that is important for translation initiation in yeast and confers poor growth properties on the cell. The *sui1* and *sui2* mutants were identified in our laboratory as suppressors of initiator codon mutations at the *HIS4* locus in yeast (Castilho-Valavicius et al. 1990). The *sui1* suppressor gene encodes a putative translation initiation factor that is essential for the start site selection process in yeast (Yoon and Donahue 1992). The *sui2* suppressor gene encodes the α -subunit of eIF-2 that is essential for ternary complex formation (GTP-dependent binding of tRNA^{Met}) and has been implicated genetically to mediate ribosomal recognition of the AUG start codon (Donahue et al. 1988; Cigan et al. 1989; Dever et al. 1992).

As shown in Figure 5, neither the *sui1* nor the *sui2* suppressor mutant is hypersensitive to UV, with each displaying the same profile observed for the wild-type control strain. More importantly, both the *sui1* and *sui2* strains grow more poorly than the *SSL1-1* and *SSL1-3* strains, which showed increased sensitivity to UV (Table 3). Thus, neither a general defect in translation initiation nor poor growth properties as a result of this defect appears to confer a DNA repair defect in yeast as measured by hypersensitivity to UV light. Instead, the UV-hypersensitive phenotype appears to be a direct consequence of specific types of mutations not only in the *SSL1* gene but in the *SSL2* gene as well.

Discussion

By using a genetic selection scheme at the *HIS4* locus in yeast, we have implicated a novel gene product encoded by the *SSL1* gene to be involved in the early steps of translation initiation. The basic strategy of the selection

scheme is that mutations in components of the translation initiation complex that function in ribosomal binding/scanning of mRNA would bypass the inhibitory effect of a stem-loop structure in the leader region by either promoting unwinding of the secondary structure or allowing the ribosome to bind 3' to the site of the mutation. The characterization of the *SSL1* gene and its mechanism of suppression is in agreement with our selection strategy. The *SSL1* gene encodes a gene product that has features suggestive of a protein that might interact with nucleic acids (Fig. 1). It is rich in stretches of basic residues in the amino-terminal end of the protein and contains a number of Cys- X_2 -Cys motifs that are found in proteins known or suspected to interact with RNA or DNA (Berg 1986; Klug and Rhodes 1987). In addition, *SSL1* suppressor strains do not appear to alter *HIS4* transcription or remove the stem-loop structure either from the *HIS4* DNA or mRNA but, rather, suppress the inhibitory effects of the stem-loop mutation by a post-transcriptional mechanism. Most importantly, Ts^- *SSL1* suppressor strains have thermolabile defects in their polysome profiles and the yeast cell-free in vitro translation system. This constitutes convincing in vivo and in vitro evidence that this protein performs an essential function during translation initiation.

The fact that an *SSL1* suppressor strain is capable of suppressing the inhibitory effect of stem-loops when positioned 10 nucleotides downstream from the 5' end of the message but not when located 33 nucleotides downstream from the 5' end of the message could suggest that this protein functions at a step that is coincident with ribosomal binding to mRNA. This suggestion seems reasonable if one assumes that 33 nucleotides of leader sequence 5' to the stem-loop is sufficient to support ribosomal binding to the *his4* message, but the Ssl1 suppressor protein does not function in the scanning process and therefore cannot overcome the scanning defect imposed by the downstream stem-loop mutation. In addition, we have found that when the stem-loop derived from *his4-316* is positioned 44 nucleotides downstream of the 5' end of the message and 36 nucleotides upstream of the AUG, in an artificial *HIS4* leader construct, it also cannot be suppressed by *SSL1-1* (data not shown). This might also suggest that the ribosome is not simply bypassing the stem-loop by binding 3' to the stem-loop, as 36 nucleotides is sufficient in length to accommodate a ribosome-binding event (Kozak 1991b). Instead, these results suggest that the mechanism of suppression is dependent on the position of the stem-loop near the 5' end of the message, perhaps to overcome a mutational block in ribosomal binding at this position.

If Ssl1 functions in promoting ribosomal binding to mRNA, then we suspect that it functions with the *SSL2* gene product in achieving this goal. A number of observations support this suspicion. First, both suppressor genes were isolated by the same selection scheme. Second, the *SSL2* suppression spectrum is identical to that observed for the *SSL1* suppressor (Tables 1 and 2); *SSL2* suppresses the same stem-loop structures positioned

near the 5' end of the *HIS4* message but does not suppress stem-loop structures located farther downstream (K.D. Gulyas and T.F. Donahue, unpubl.). Third, and most important, a haploid strain containing both *SSL1-1* and *SSL2-1* suppressor mutations results in loss of the His^+ suppressor phenotype, suggestive of two gene products that either interact or function in a related pathway. In addition, this interaction is an allele-specific effect, as it is only observed in strains that contain one of the alleles *SSL1-1*, *SSL1-2*, or *SSL1-3*, in combination with the *SSL2-1* allele (T.F. Donahue, unpubl.); the former three alleles contain the same amino acid substitution in the *SSL1* DNA-coding region. In light of these relationships between *SSL1* and *SSL2*, it is tempting to speculate that Ssl1 might function as an mRNA-binding protein and the RNA helicase-like suppressor gene product Ssl2 functions in unwinding the secondary structure from mRNA. This is reminiscent of the model whereby eIF-4E, the cap binding protein, functions in bringing eIF-4A, the helicase, to mRNA to remove the secondary structure and provide a single-stranded region for the ribosome to bind (Sonnenberg 1988; Thach 1992). Alternatively, Ssl1 could function in a fashion analogous to eIF-4B. eIF-4A alone is incapable of unwinding RNA:RNA duplex in vitro in the absence of eIF-4B (Rozen et al. 1990). eIF-4B is thought to provide RNA-binding specificity for eIF-4A to associate with RNA (Milburn et al. 1990; Rozen et al. 1990). Hence, Ssl1 could provide mRNA or rRNA-binding activity that allows Ssl2 to associate with mRNA or be in proximity to mRNA to promote unwinding activity.

One surprising outcome of our analysis is the observation that specific alleles of *SSL1* confer yeast hypersensitive to UV light. This phenotype establishes another functional relationship between the *SSL1* and *SSL2* gene products, both of which apparently play an important role in the DNA repair process. This represents an enigma in our analysis as we have not yet resolved the functional connection as to how mutations in both genes can result in either suppression of stem-loop structures in the *HIS4* leader or a DNA repair defect in yeast. However, we can envision three possible mechanistic models that would relate these diverse phenotypes. Unfortunately, none of the mechanisms can be supported unequivocally by the available data. One possible explanation for these results is that both genes function only in DNA repair. We offer this model, as it has been speculated that the RNA:DNA duplex displacement activity observed with the *RAD3* gene product might be related to removing mRNA that is being transcribed from damaged single-stranded template DNA to facilitate DNA repair (Bailly et al. 1991). Suppressor mutations in either gene would therefore result in an Ssl1-Ssl2 complex acquiring the totally new function of unwinding mRNA to remove the secondary structure that leads to suppression at *HIS4*. Although we cannot rule out this possibility for Ssl2, as it has not been directly implicated in translation, it seems rather remote for Ssl1, as we have compelling evidence that this gene product is directly involved in protein synthesis; Ts^- , *SSL1* suppressor strains have in

vivo and in vitro defects in translation initiation (Figs. 3 and 4; S.P. Miller and T.F. Donahue, unpubl.).

A second possibility is that both Ssl1 and Ssl2 have dual functions: a function in translation initiation that is essential for cell viability, and a second function in DNA repair that is not essential for viability. As a complex in the nucleus, Ssl1 and Ssl2 might function in the DNA repair process; and as a complex in the cytoplasm, both function in the translation initiation process. This is a very interesting possibility, and we have no data to refute this potential mechanism. The observation that a specific deletion mutation in the *SSL2* gene (*SSL2-XP*), made to resemble the mutation identified in the *ERCC-3* gene of a xeroderma pigmentosum patient, was necessary to render a DNA repair defect in yeast could suggest that a specific region of the protein is dedicated solely to interacting with DNA or participating in the DNA repair process.

A third possible mechanism as to why mutations in *SSL1* and *SSL2* suppress stem-loop structure mutations and affect DNA repair is that both gene products only function in translation initiation and control the expression of DNA repair genes. Of all the possible mechanisms put forth, an alteration in gene expression that impacts on DNA repair would appear to be the most conventional explanation for the "connection" between suppression and UV hypersensitivity. The only requirement that needs to be imposed is that a DNA repair gene or a gene that controls the expression of a DNA repair gene must be subject to translational control. The observation that a single mutation in *SSL1* affects both DNA repair and translation initiation may mean that both phenotypes have a common functional denominator. On the other hand, the observation that *SSL2-XP* has such a drastic consequence on DNA repair but does not significantly impair the growth of yeast (Gulyas and Donahue 1992), as might be expected of a mutation that would alter translation initiation, clouds a clear understanding of how DNA repair might be linked to translation initiation. However, one possible explanation for this observation is that Ssl1 might play a more general role in translation initiation, with one of its roles being the control of expression of a DNA repair gene. Ssl2 also might play an important part in translation, but with its action limited to a subset of mRNAs, including a DNA repair enzyme. This might explain why a complete deletion of this gene is lethal to yeast but a specific mutation might affect only DNA repair—a limited region of this protein perhaps dedicated to promote an interaction with a region of mRNA that encodes a DNA repair enzyme. This raises the intriguing possibility that the expression of some mRNAs may be controlled by specific secondary structures and that Ssl1 and Ssl2 function in removing such structures. In essence, not all mRNAs are created equal at the translational level. Hence, the translation of standard mRNAs may be more dependent on eIF-4F, for example, whereas less standard mRNAs may be more dependent on Ssl1 and Ssl2. Therefore, by putting a secondary structure in the *HIS4* message and selecting for expression, we have demanded that factors that nor-

mally remove specific secondary structures in some mRNAs acquire a new specificity for the stem-loop at *HIS4*. If this proves to be the case, it would seem that the genetics of yeast has provided a powerful approach to the discovery of a translational regulatory mechanism that is not apparent by straightforward biochemical approaches. In any case, our genetic studies appear to have been effective in identifying a novel translation factor that is essential for the early steps of translation initiation.

Materials and methods

Yeast strains and genetic methods

The yeast strains used in this analysis are related to TD28 (*MAT α ura3-52 ino1-13*), a derivative of yeast strain S288C (*MAT α*). Standard genetic techniques and media used for our analysis have been described (Sherman et al. 1979). The *SSL1* suppressor strains JJ576 (*SSL1-4*), JJ578 (*SSL1-5*), JJ582 (*SSL1-6*), JJ636 (*SSL1-1*), JJ637 (*SSL1-2*), and JJ638 (*SSL1-3*) were identified in the same fashion as that reported for the *SSL2* suppressor strain (Gulyas and Donahue 1992; S.P. Miller and T.F. Donahue, unpubl.).

For quantitating the effects of *SSL1* suppression events on *His4* expression, in-frame *HIS4-lacZ* and *his4-316-lacZ* fusion genes as part of YCp50 were each used to transform the isogenic strains JJ567 (*SSL1⁺*), JJ576 (*SSL1-4*), JJ582 (*SSL1-6*), JJ636 (*SSL1-1*), and JJ638 (*SSL1-3*) to Ura⁺. The construction of these plasmids has been described previously (Cigan et al. 1988) and contains all 5' noncoding sequences that are necessary for the transcription and regulation of *HIS4* expression. Strains were grown in SD medium lacking uracil, and extracts were prepared and assayed for β -galactosidase activities as described previously (Donahue and Cigan 1988).

Construction of the yeast strain containing the *his4-317* secondary structure allele (previously referred to as -51/-50B3) has been described (Cigan et al. 1988). Yeast strains containing the alternative secondary structure alleles *his4-318* and *his4-319* were constructed in the identical fashion as strains containing the *his4-316* and *his4-317* alleles (Cigan et al. 1988), with the exception that oligonucleotides of different sequences were inserted at the unique *EcoRI* site located between positions -51 and -50 in the *HIS4* leader region. Each of the self-complementary oligonucleotides 5'-AATTGTCCTCCTGCCTCCTCCT-TAAGGAGGCAGGAGGAC-3' and 5'-AATTCGCGCGCGCGGATATCGCGCGCGCGG-3' was used to construct the *his4-318* and *his4-319* alleles, respectively. A yeast strain containing the *his4-320* allele was constructed by first introducing a unique *EcoRI* site in the *HIS4* leader by site-directed mutagenesis. The oligonucleotide 5'-TAATACAAGGAATTCCAAAATTT-3' was used for this experiment and results in changing the sequence of positions -27 to -20 in the *HIS4* leader from TAGTTTAC to GGAATTCC. This allowed us to introduce the 36-bp insertion sequence contained in the *his4-316* allele, via the unique *EcoRI* site, to a position more 3' in the *HIS4* leader. The methods employed for this construction and the subsequent construction of yeast strains containing the *his4-320* allele are the same as described previously for other site-directed and insertion mutagenesis studies of the *HIS4* leader region (Cigan et al. 1988). The presence and number of inserts in the *HIS4* region were confirmed either by Maxam and Gilbert DNA sequencing (1980) or by the double-stranded chain-termination method (Sanger et al. 1977). ΔG° values of the stem-loop structures were calculated using the Zuker and Jaeger MUFOLD computer program (Jaeger et al. 1989). The ability of *SSL1* to sup-

press the alternative stem-loop structure mutants, EP496 (*Mata his4-317 ura3-52 ino1-13*), EP1734 (*Mata his4-318 ura3-52 ino1-13*), EP1751 (*Mata his4-319 ura3-52 ino1-13*), and CD21 (*Mata his4-320 ura3-52 ino1-13*) was assessed by tetrad analysis from crosses with either of the *his4* deletions strains 603-7B (*Mata SSL1-3 his4-401 leu2-3, -112 ino1-13*) or EP1568 (*Mata SSL1-3 his4-401 ura3-52 leu2-3, -112*); the latter two strains contain the *SSL1-3* suppressor allele that was derived from mutant JJ638.

UV sensitivity of yeast strains JJ567 (*SSL1*⁺), JJ576 (*SSL1-4*), JJ582 (*SSL1-6*), JJ636 (*SSL1-1*), JJ638 (*SSL1-3*), 301-4D (*Mata sui1-1 his4-303 ura3-52 leu2-3, -112*), and 304-1D (*Mata sui2-1 his4-303 ura3-52*) was measured as described previously (Gulyas and Donahue 1992). The genetic, molecular, and biochemical characterizations of the *sui1-1* and *sui2-1* strains used in these experiments have been reported previously (Cigan et al. 1989; Castilho-Valavicius et al. 1990; Yoon and Donahue 1992). The data presented in Figure 5 represent the average of two independent experiments, with each data point being the average of four plates per UV dosage.

Isolation and characterization of wild-type and mutant *SSL1* alleles

The wild-type *SSL1* gene was cloned from a YCp50 wild-type genomic clone bank (Rose et al. 1987). Four positive clones were identified on the basis of complementation of the recessive Ts⁻ phenotype associated with yeast strains 555-8C (*SSL1-1*), 556-4B (*Mata SSL1-2 his4-316 ura3-52 ino1-13*), or 557-4B (*SSL1-3*), which are ascospore derivatives of *SSL1* suppressor strains, JJ636, JJ637, and JJ638, respectively. Restriction analysis of these clones indicated that the inserts were related and derived from the same chromosomal location. The smallest clone, which contained a *Sau3A* DNA insert of ~7.4 kb was used for subsequent characterizations of the wild-type *SSL1* gene.

To establish that the cloned DNA fragments were derived from the *SSL1* chromosomal region, a 4.2-kb *EcoRI*–*Bam*HI DNA fragment was subcloned into the *EcoRI* and *Bam*HI sites of the integrating plasmid YIp5 to generate plasmid p1030. Plasmid p1030 was then restricted at the unique *Clal* site (Fig. 1), which resides in the *SSL1* DNA-coding region, and this linearized plasmid was used to transform the wild-type *SSL1* strain JJ567 to Ura⁺. Ura⁺ transformants were then crossed to the Ura⁻, Ts⁻ *SSL1* suppressor strain 556-4D (*Mata SSL1-2 his4-316 ura3-52 ino1-13*), an ascospore derivative of JJ637, and diploids were analyzed by tetrad analysis. Subcloning experiments for localization of the wild-type *SSL1* gene were performed with the yeast single-copy *URA3* plasmid YCp50. The *SSL1* gene was localized to a *Xho*HI–*Bam*HI fragment of ~3.0 kb. Subsequent removal of the small 0.3-kb *Pvu*II fragment contained in the distal end of this region was shown not to affect its ability to complement the Ts⁻ phenotype associated with *SSL1* suppressor strains. This allowed us to localize the *SSL1* wild-type gene to reside on the 2.5-kb *Xho*I–*Pvu*II DNA fragment (Fig. 1). The complete DNA sequence of this fragment was determined for both strands.

A YCp50 plasmid, p1023, containing the wild-type *SSL1* gene as part of a 4.2-kb *EcoRI*–*Bam*HI fragment, was used to isolate the Ts⁻ *SSL1* suppressor alleles by the gap–duplex repair method (Orr-Weaver et al. 1983). p1023 was restricted at the *Clal* site located within the coding region of the *SSL1* gene (Fig. 1). The Ts⁻ *SSL1* suppressor strains JJ636, JJ637, and JJ638 were transformed with the linearized plasmid, and Ura⁺ Ts⁻ transformants with a His⁺ phenotype, indicative of the presence of an *SSL1* suppressor mutation on the plasmid, were used to isolate *SSL1* mutant alleles. The identical DNA region was iso-

lated from the Ts⁺ *SSL1* suppressor strains JJ576 and JJ582 by the integration and excision method (Winston et al. 1983). For these experiments plasmid p967 was used, which contains an *EcoRI*–*Clal* DNA fragment of ~2.6 kb in YIp5. The presence of a single mutation in each of the *SSL1* suppressor alleles was confirmed by DNA sequence analysis of the entire *SSL1* DNA-coding region.

Analysis of *SSL1* expression

Plasmid p1022 was constructed to perform gene disruption experiments (Rothstein 1983) of the *SSL1*⁺ DNA-coding region. This plasmid contains the 4.2-kb *EcoRI*–*Bam*HI fragment ligated into the *EcoRI* and *Bam*HI site of pBR322. This plasmid was restricted at the unique *Clal* site in the *SSL1* DNA-coding region, and the site was blunt ended using reverse transcriptase and dNTPs. The *Hind*III ends of a 1.1-kb DNA fragment containing the *URA3*⁺ gene were blunt ended by the same procedure, and this fragment was ligated into the blunt ended *Clal* site of plasmid p1022 to generate plasmid p1029. Plasmid p1029 was then restricted with *EcoRI* and *Bam*HI. This fragment contains the *SSL1* wild-type gene disrupted by the *URA3*⁺ gene and was used to transform the diploid strain EKP81 (*Mata/Mata his4-316/his4-316 ura3-52/ura3-52 leu2-3, -112/leu2-3, -112*) to Ura⁺. Two transformant EKP99 and EKP105 strains were confirmed by Southern analysis to contain an intact copy of the *SSL1* gene and an *SSL1::URA3*⁺ disruption allele. EKP99 and EKP105 strains were sporulated and analyzed by tetrad analysis. For Southern blotting, a ³²P-labeled probe complementary to the *SSL1* DNA-coding region was generated using the universal primer and a M13mp10 phage that contained the 1.1-kb *Clal*–*Pvu*II fragment.

To determine whether translation of the wild-type *SSL1* DNA-coding region could rescue the disrupted *SSL1::URA3*⁺ allele, plasmid p1375 was constructed by first ligating an *EcoRI*–*Bam*HI fragment of ~820 bp containing the *GAL1*–*GAL10* promoter and regulatory region (Johnston and Davis 1984) into the corresponding restriction sites of the *LEU2*⁺, *CEN4* vector pSB32 (Rose and Broach 1991) to generate plasmid p1345. A *Bam*HI restriction site was introduced 9 nucleotides upstream from the ATG codon that defines the start of translation of the *SSL1* DNA-coding region by site-directed mutagenesis. This allowed us to subclone the entire *SSL1* DNA-coding region and distal sequences as part of a 2.5-kb *Bam*HI DNA fragment into the *Bam*HI site of plasmid p1345 such that the transcription of the *SSL1* DNA-coding region was under the control of the galactose-inducible *GAL1* promoter region. This plasmid, p1375, was then used to transform the *SSL1::URA3*⁺ diploid strains EKP99 and EKP105 to Leu⁺. Transformants were sporulated and dissected on either YEPD or YEP galactose (YEP-Gal) plates. Antibodies directed against the *SSL1* gene product were generated from a TrpE–Ssl1 fusion protein expressed in *Escherichia coli*. The *SSL1*⁺ DNA-coding region from amino acid +251 to +461 contained on a 2.0-kb *Clal*–*Bam*I DNA fragment was fused in-frame to the carboxyl end of the *trpE* DNA-coding region in the pATH3 expression vector (Dieckmann and Tzagoloff 1985). The methods used for inducing the expression of the *trpE*–*SSL1* plasmid (p986) in *E. coli*, extracting the antigen, and immunizations have been reported (Donahue et al. 1988).

RNA analysis

Northern blot, primer extension, and S1 nuclease protection analysis were performed with total RNA. The procedure for RNA isolation has been described previously (Cigan et al. 1988).

For Northern analysis of the *SSL1* transcript, RNA was isolated from the *SSL1* wild-type strain 1427-15C [*Mata his4-316 ura3-52*] and the *SSL1* suppressor strain 1427-27C [*Mata SSL1-1 his4-316 ura3-52*]. A ³²P-labeled probe was generated from the 2.0-kb *KpnI*–*Clai* DNA fragment containing the upstream wild-type *SSL1* DNA-coding region. Northern analysis of the *his4-316* transcript was performed with RNA isolated from the wild-type *SSL1* strain JJ567 and the isogenic *SSL1-1* suppressor strain JJ636. The *HIS4* probe was derived from a 2.7-kb *EcoRI* fragment that encodes the downstream *HIS4* DNA-coding region (Donahue et al. 1982). As an internal control for the *his4-316* Northern analyses, filters were washed of counts and incubated with a probe from a 3.0-kb *EcoRI*–*BamHI* fragment containing the entire actin gene DNA-coding region. All probes were made by the random primer method, and 50 µg of RNA was probed from each strain. Conditions for Northern blots have been reported (Donahue et al. 1983).

For primer extension analysis of the *SSL1*⁺ transcript, 10 µg of poly(A)⁺ RNA from strain EP831 was hybridized with an oligonucleotide, 5'-CCCTCGCCATCAAAATG-3', which is complementary to positions +79 to +95 in the *SSL1* DNA-coding region. This strain contains the *SSL1* gene on a high-copy plasmid, as described above, and was used for this experiment, as we could not detect an extension product with RNA isolated from a single-copy *SSL1* strain, presumably owing to the low abundance of the transcript. For primer extension analysis of the *his4-316* transcript, 50 µg of total RNA, isolated from strains JJ567 (*SSL1*⁺) and JJ636 (*SSL1-1*) was incubated with the oligonucleotide 5'-CATCAATTAACGGTAGAATCGG-3', which is complementary to position +10 to +31 in the *HIS4* DNA-coding region. The conditions for primer extension have been described previously (Cigan et al. 1988), except that 6 units of AMV reverse transcriptase was used for each analysis and primer extension reactions were incubated at 42°C for 90 min.

S1 nuclease protection experiments of the *his4-316* transcript were performed as described by Burke (1984), with minor modifications. A single-stranded DNA template was constructed by subcloning a 796-bp *PvuII*–*XhoI* fragment from the upstream region of the *his4-316* allele into the *SmaI* and *SalI* sites of phage vector M13mp18. This template (mp18::*his4-316*) contains ~100 nucleotides of the *HIS4* DNA-coding region, a 96 nucleotides 5'-UTR inclusive of the inserted region of dyad symmetry (*his4-316* mutation), and ~600 nucleotides of the *HIS4* promoter and regulatory region (Donahue et al. 1982; Cigan et al. 1988). Primer annealing was performed by heating the template (750 ng) and M13 universal sequencing primer (50 ng) in a 50 µl reaction mixture at 90°C for 3 min and then cooling slowly to room temperature. At the end of incubation, unlabeled dATP, dGTP, and dTTP at a final concentration of 100 µM were added. To prepare the probe, 10 pmoles of [α-³²P]dCTP (3000 Ci/mmol) and 1.0 unit of DNA polymerase I (Klenow fragment) were added to the primer annealed template and incubated for 20 min at room temperature. The synthesis was chased by the addition of 1 µl of 10 mM dCTP and incubated further for 20 min at room temperature. The reaction was stopped by heat inactivation at 65°C for 15 min. Probe labeled with ³²P was digested with *PvuII*, and the single-stranded probe was isolated by electrophoresis of the restriction digest on a 4% polyacrylamide strand separation gel containing 8 M urea. Single-stranded probe was hybridized with total RNA (100 µg) in a buffer containing 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl, and 80% deionized formamide at 80°C for 10 min and then incubated for 16 hr at 42°C. The hybridization mixture was incubated for 30 min at 37°C with 100 units of S1 nuclease in 150 µl of S1 buffer (250 mM NaCl, 30 mM sodium acetate at pH 4.6, 4.5 mM zinc sulfate, 20 µg of sonicated calf thymus DNA). S1 nuclease di-

gestions were terminated by the addition of 0.5 M ammonium acetate, 10 mM EDTA, and 5 µg of tRNA and then ethanol precipitated. S1-protected fragments were analyzed on 8% polyacrylamide sequencing gels with a sequencing ladder generated from mp18::*his4* and the universal primer.

Polysome analysis

Polysome profiles of the Ts⁻ *SSL1-1* strain JJ636 were analyzed from cultures incubated under permissive (23°C) and restrictive (37°C) temperature conditions and compared with those of the isogenic *SSL1*⁺ parent strain JJ567. The growth conditions and methods used to prepare extracts and polysomes from wild-type and *SSL1* suppressor strains are identical to those described previously (Yoon and Donahue 1992), with the exception of two modifications: Cells were grown in SD complete medium, as the Ts⁻ effect was more pronounced in this medium than in YEPD medium; and 17–57% sucrose gradient conditions were used to enhance the resolution of the polysomes.

Cell-free translation

The *SSL1* suppressor strains JJ636 (*SSL1-1*) and JJ638 (*SSL1-3*) were analyzed in a cell-free translation system in comparison to the isogenic parent strain JJ567 (*SSL1*⁺). Cells were grown at 28°C to an OD₆₀₀ of ~1.0 in 1 liter of YEPD, harvested, and washed; cell-free extracts were then prepared in parallel from each culture, as described by Hofbauer et al. (1982). Protein concentrations were determined spectrophotometrically (Groves et al. 1968), with bovine serum albumin as a standard.

The two-step incubation procedure (Gasior et al. 1979) was employed to assay cell-free translation of mRNA. In the first step, endogenous mRNA was digested by treating the extracts (10 mg/ml of protein) with 20–40 µg/ml of micrococcal nuclease (Sigma) in the presence of 1 mM CaCl₂. After 10–12 min at 23°C the reactions were placed on ice and then stopped by the addition of EGTA (pH 7.5) to a final concentration of 2 mM. In the second step, aliquots (45 µl) from the mRNA-depleted extracts were incubated with all of the components required for protein synthesis, including total yeast RNA and a radioactive amino acid. The final reaction mixtures (75 µl) contained 0.45 mg of nuclease-treated extract, 60 mM HEPES–KOH (pH 7.5), 160 mM KO–Ac, 3 mM Mg(O–Ac)₂, 3 mM dithiothreitol, 0.5 mM ATP, 0.1 mM GTP, 2 mM glucose-6-phosphate, 20 mM creatine phosphate, 5 µg of creatine phosphokinase, 50 mM each of 19 unlabeled amino acids (omitting methionine), 20 µg of total yeast RNA, and 0.3 µM L-[³⁵S]methionine (600–1000 Ci/mole). Yeast total RNA was prepared as described previously (Cigan et al. 1988). The incubations were carried out at 23°C. Aliquots (9 µl) were removed at various times, and the amount of [³⁵S]methionine incorporation into hot (90°C) 5% trichloroacetic acid-insoluble protein was determined as described previously (Husain and Leibowitz 1986), except that the discs were washed only once with acid following the boiling step.

Cell-free translation of the synthetic template poly(U) was carried out under reaction conditions that measure only polypeptide chain elongation (Gasior et al. 1979). Reaction mixtures (75 µl) contained 0.45 mg of nuclease-treated extract, 20 µg of poly(U), 5 µg of yeast tRNA^{Phe}, 12.5 µM L-[U-¹⁴C]phenylalanine plus buffered salts, DTT, ATP, GTP, glucose-6-phosphate, creatine phosphate, and creatine phosphokinase, as described above except that the Mg²⁺ concentration was increased to 14 mM. The reactions were incubated at 23°C, and at various times aliquots were removed and analyzed for poly-[¹⁴C]phenylalanine synthesis by determining the amount of radioactivity that was incorporated into hot acid-insoluble protein as described above.

The in vitro heat inactivation experiments were performed as described previously (Mandel and Trachsel 1989), with minor modifications. Aliquots (45 μ l) from the mRNA-depleted extracts (10 mg/ml of protein) were preincubated in parallel at 32°C for various lengths of time as indicated in Figure 4. Immediately following this preincubation, all of the components required for cell-free translation were added and the reactions (75 μ l) were then incubated at 23°C. Aliquots (9 μ l) were removed from each translation reaction after 5, 10, 15, 20, 30, and 40 min of incubation and analyzed as described above for hot acid-insoluble radioactivity. For Figure 4, only the 15-min time point is presented.

Acknowledgments

We thank members of our laboratory for helpful discussions, Colleen Dixon for technical assistance, Jim Brown for doing the Zuker program analysis, and Peter Cherbas and Mimi Zolan for helpful suggestions on the manuscript. This work was supported by U.S. Public Health Service grant GM32263 from the National Institutes of Health awarded to T.F.D.

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SSL1, a suppressor of a HIS4 5'-UTR stem-loop mutation, is essential for translation initiation and affects UV resistance in yeast.

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Genes Dev. 1992, **6**:

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