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Feedback Inhibition of the Yeast Ribosomal Protein Gene *CRY2* Is Mediated by the Nucleotide Sequence and Secondary Structure of *CRY2* Pre-mRNA

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The *Saccharomyces cerevisiae CRY1* and *CRY2* genes, which encode ribosomal protein rp59, are expressed at a 10:1 ratio in wild-type cells. Deletion or inactivation of *CRY1* leads to 5- to 10-fold-increased levels of *CRY2* mRNA. Ribosomal protein 59, expressed from either *CRY1* or *CRY2*, represses expression of *CRY2* but not *CRY1*. *cis*-Acting elements involved in repression of *CRY2* were identified by assaying the expression of *CRY2-lacZ* gene fusions and promoter fusions in *CRY1 CRY2* and *cry1-Δ CRY2* strains. Sequences necessary and sufficient for regulation lie within the transcribed region of *CRY2*, including the 5′ exon and the first 62 nucleotides of the intron. Analysis of *CRY2* point mutations corroborates these results and indicates that both the secondary structure and sequence of the regulatory region of *CRY2* pre-mRNA are necessary for repression. The regulatory sequence of *CRY2* is phylogenetically conserved; a very similar sequence is present in the 5′ end of the *RP59* gene of the yeast *Kluyveromyces lactis*. Wild-type cells contain very low levels of both *CRY2* pre-mRNA and *CRY2* mRNA. Increased levels of *CRY2* pre-mRNA are present in *mtr* mutants, defective in mRNA transport, and in *upf1* mutants, defective in degradation of cytoplasmic RNA, suggesting that in wild-type repressed cells, unspliced *CRY2* pre-mRNA is degraded in the cytoplasm. Taken together, these results suggest that feedback regulation of *CRY2* occurs posttranscriptionally. A model for coupling ribosome assembly and regulation of ribosomal protein gene expression is proposed.

The expression of ribosomal genes is coordinately regulated so that equimolar amounts of rRNAs and ribosomal proteins accumulate for assembly into ribosomes. The rate of synthesis of ribosomal molecules is also tightly coordinated with the physiological state of cells (reviewed in references 72 and 73). Coordinate synthesis of yeast ribosomal proteins is controlled primarily at the level of transcription of their genes through one of two common upstream activating sequences, UAS_{RPG} or UAS_{T} (reviewed in references 72 and 73). Posttranscriptional controls provide mechanisms for fine-tuning the expression of individual ribosomal protein (rp) genes (1, 7, 14, 41, 50, 53, 64, 69).

Balanced accumulation of ribosomal proteins in *Escherichia coli* results from feedback regulation of their expression (reviewed in references 47 and 48). Certain *E. coli* ribosomal proteins, when synthesized in excess of the rRNAs to which they bind in the assembling ribosome, repress expression of their own operons. Four eukaryotic rp genes have also been found to be autogenously regulated. Yeast ribosomal protein L32 binds to a structure comprising the 5′ exon and the first few nucleotides of the intron of *RPL32* pre-mRNA and blocks its splicing (15, 67). L32 also regulates the translation of its own mRNA through a similar secondary structure formed in the 5′ end of the mature mRNA (8). Expression of the yeast rp gene *RPL2* is autogenously controlled at the level of mRNA accumulation (53). The *Xenopus laevis* gene that encodes the homolog of yeast rpL2 is also feedback regulated but at the level of pre-mRNA splicing (5). Transcription of the mammalian *RPS14* gene that encodes the homolog of yeast rp59 is autogenously regulated (62).

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To understand balanced expression of yeast rp genes, one must take into account the fact that about half of the yeast ribosomal proteins are encoded by two genes and half are encoded by single-copy genes (reviewed in references 72 and 73). In the cases examined, both copies of the duplicated genes are expressed, at ratios ranging from 1:1 to 10:1 (1, 13, 49, 54, 57, 63, 68). mRNAs and proteins expressed from single-copy rp genes as well as from duplicated rp genes accumulate in equimolar amounts in the cell (20, 29). How this balanced expression occurs is unclear.

To investigate the mechanism for balanced expression of yeast rp genes, we are studying the *CRY1* and *CRY2* genes, encoding rp59. This protein is an essential component of 40S ribosomal subunits and is necessary for their assembly. *CRY1* mRNA is present at 8- to 10-fold-higher levels than *CRY2* mRNA in wild-type cells (49). However, yeast cells in which *CRY1* is deleted grow at nearly wild-type rates and contain about 80% of wild-type amounts of *CRY* mRNA and 40S ribosomal subunits (49). These results suggest that expression of rp59 from *CRY2* might be increased upon deletion of *CRY1.*

In this study, we investigated the mechanism of regulation of *CRY1* and *CRY2*. We found that the level of *CRY2* mRNA is increased about 5- to 10-fold when *CRY1* is deleted or inactivated. Expression of *CRY2* but not *CRY1* is repressed by rp59 expressed from either *CRY* gene. *cis*-Acting regulatory sequences, including the 5′ exon and the first 62 nucleotides of the intron of *CRY2*, are necessary and sufficient for repression of *CRY2*. Analysis of point mutations in the regulatory region demonstrated that repression is mediated by the secondary structure as well as the sequence of *CRY2* pre-mRNA. *CRY2* pre-mRNA accumulates in *mtr* and *upf1* mutants blocked in nuclear export or cytoplasmic turnover of RNA, indicating that in wild-type cells, *CRY2* pre-mRNA is normally exported to the cytoplasm and degraded. We discuss models for regulation of

TABLE 1. Yeast strains used in this study

Strain	Relevant genotype	Source or reference
JTY81	*MAT*α *CRY1 cry2-Δ1*::*LEU2 his3-Δ200 trp1-Δ1 leu2-Δ2 ura3-167*	49
JTY82	*MAT***a** *cry1-Δ2*::*TRP1 CRY2 his3-Δ200 trp1-Δ1 leu2-Δ2 ura3-167*	49
JTY83	*MAT*α *CRY1 CRY2 his3-Δ200 trp1-Δ1 leu2-Δ2 ura3-167*	49
JWY3245	*MAT***a** *cry1-Δ2*::*TRP1 CRY2 his3-Δ200 trp1-Δ1 leu2-Δ2 ura3-167*::*CRY2-lacZ*	This study
JWY3246	*MAT*α *CRY1 CRY2 his3-Δ200 trp1-Δ1 leu2-Δ2 ura3-167*::*CRY2-lacZ*	This study
JWY3249	*MAT***a** *cry1-Δ2*::*TRP1 CRY2 his3-Δ200 trp1-Δ1 leu2-Δ2 ura3-167*::*CRY1-lacZ*	This study
JWY3250	*MAT*α *CRY1 CRY2 his3-Δ200 trp1-Δ1 leu2-Δ2 ura3-167*::*CRY1-lacZ*	This study
T12	*MAT***a** *ura3-52 leu2-Δ1 mtr1-2*	A. Tartakoff
T8	*MAT***a** *ura3-52 his3-Δ200 mtr2-1*	A. Tartakoff
T34	*MAT***a** *ura3-52 mtr3-1*	A. Tartakoff
T140	*MAT***a** *ura3-52 leu2 mtr12-1*	A. Tartakoff
yRP582	*MAT***a** *ura3-52 leu2 rpb1-1*	R. Parker
yRP689	*MAT***a** *ura3-52 leu2 rpb1-1 xrn1*::*URA3*	R. Parker
B-9037	*MAT*α *cyc1-512 trp2-1 ura3-52*	F. Sherman
B-9046	*MAT*α *cyc1-512 trp2-1 ura3-52 upf1*::*URA3*	F. Sherman
JWY3303	*MAT***a** *ura3-52 mtr3-1* pZL37	This study
JWY3304	*MAT***a** *ura3-52 mtr3-1 cry1-Δ1*::*URA3* pZL37	This study

CRY2 and for coupling the rate of ribosome assembly to regulation of expression of the duplicated *CRY* genes.

MATERIALS AND METHODS

Strains and nucleic acids. The yeast strains used in this work are described in Table 1. The *CRY1-lacZ* (*Hin*dIII-*Bgl*II), *CRY2-lacZ* (*Eco*RI-*Bgl*II), and *CRY2-lacZ* (*Eco*RI-*Nru*I) "intronless" fusions are integrated at the *ura3-167* locus of yeast strains JTY82 and JTY83. All other *CRY2-lacZ* fusion constructs were on *CEN6*-containing plasmids and were transformed into JTY82 and JTY83 without integration.

Established procedures were used for genetic manipulation of yeast strains (59). DNA was transformed into yeast cells treated with lithium acetate (26). In order to integrate *CRY1-lacZ* or *CRY2-lacZ* fusion constructs into the yeast genome, plasmids containing the fusions were linearized within *URA3* by digestion with *Stu*I and transformed into JTY82 and JTY83. Genomic Southern blot analysis confirmed the expected pattern of integration at *ura3-167*. *E. coli* NM522 (Stratagene, La Jolla, Calif.) was used to propagate plasmids, and *E. coli* CJ236 (Bethesda Research Laboratories, Gaithersburg, Md.) was used for site-directed mutagenesis. DNA manipulation and Southern analyses were performed as described before (11).

Oligonucleotides. The oligonucleotides used for this study were as follows: ZL1, 5′-GGGAATTCCTTCGTCATCTTGAAACATGG-3′; ZL4, 5′-GGCTT GACAAATTCG-3′; ZL6, 5′-GTTATCGCGAGCTTGAACAAGGTCGTTAG CCATTCTTAATTG-3′; ZL11, 5′-CCCGATCACAGATCTCCAACTG-3′; ZL 12, 5′-GGGTGAGCGCAGATCTCTATAACACAATAG-3′; ZL13, 5′-GCG TAAATTCTAGACAACACCAAAAACTTGGG-3′; ZL14, 5′-GGGAATTCG AAGACTGTTTTCTACATATC-3′; ZL15, 5′-GCAATTCTAGAGACAGAA ACAGG-3′; ZL18, 5′-GCATACAGAAGGAATAGGATCCGCTTTTATACT CG-3′; ZL22, 5′-CTCTTGCGATGAAGCATGCATACAGAAGG-3′; ZL23, 5′-CCCCTTTCACATACCGTTAGC-3′; ZL24, 5′-CCCCTTTCAGGTACCGT TAGC-3′; ZL28, 5′-CGTTAGCCATTCTTAAAAGCTTGTTATTGGGAGTT G-3′; SWF5, 5′-GGAGTTGTTGATTTCAGATCTTTTTCTCTCTTGCG-3′; Z L29, 5′-CCCCTTTCACGTAGCGTTAGCCATTC-3′; and ZL30, 5′-GAATAA CTAAATGCTTTTAAACAGG-3′.

Construction of plasmids. All fusions of *lacZ* to *CRY1* or *CRY2* were constructed by using the plasmid vectors of Myers et al. (46). The nucleotides of *CRY2* are numbered with respect to the major transcription start site, designated +1, which is 33 nucleotides 5′ of the initiator ATG. Plasmids pZL54 and pZL50 containing *CRY1-lacZ* or *CRY2-lacZ* gene fusions at codon 107 or 108, respectively, were described by Paulovich et al. (49).

Plasmid pZL33 is the *Eco*RI-*Nru*I *CRY2-lacZ* gene fusion cloned in the yeast integrating plasmid YIp354 (46). The intronless *CRY2-lacZ* fusion plasmid pZL35 was derived from pZL33 by precise deletion of the *CRY2* intron, achieved by PCR with oligonucleotides ZL6 and ZL1. The resulting fragment was digested with *Eco*RI and *Nru*I and cloned into the *Eco*RI and *Sma*I sites of plasmid YIp354 to produce pZL35. pZL33 was converted to pZL37 containing a *CEN6* sequence by in vivo recombination (40). pZL37 was used as the backbone for making subsequent fusions and as the parent reporter construct to assay regulation of *CRY2* in subsequent experiments. pZL101 was derived from pZL37 but contains a *Bam*HI site at nucleotide −44 upstream of *CRY2*, created by site-directed mutagenesis (30) with oligonucleotide ZL18.

DNA containing the *Saccharomyces cerevisiae RP28* gene (43) was generated by PCR amplification of the genomic sequences with oligonucleotides ZL14 and ZL15. The PCR-amplified DNA was digested with *Eco*RI and *Xba*I and cloned into plasmid YEp357 (46) to construct plasmid pZL106, containing an *RP28-lacZ* gene fusion. In this construct, *lacZ* was fused in frame with *RP28* at codon 65. A 0.3-kb *Eco*RI-*Bam*HI fragment from this plasmid was used to replace the 0.5-kb *Eco*RI-*Bam*HI fragment of pZL101 to construct plasmid pZL103, containing *RP28-CRY2-lacZ*, in which the promoter of *RP28* is upstream of the *Eco*RI-*Nru*I *CRY2-lacZ* gene fusion.

To construct the *CRY2-RP28* hybrid intron, a *Bgl*II site was created at nucleotide +105 within the *CRY2* intron by site-directed mutagenesis with oligonucleotide ZL11. The *Bgl*II-*Hin*dIII fragment from the resulting plasmid was replaced by the *Nru*I-*Hin*dIII fragment of *RP28-lacZ* in pZL106 to construct plasmid pZL107. The resulting *CRY2-RP28-lacZ* fusion has the *CRY2* promoter sequence and 5′ exon plus the first 62 nucleotides of the *CRY2* intron fused to nucleotide 163 within the *RP28* intron. This construct also contains the *RP28* 3′ exon fused in frame at codon 65 to *lacZ*.

The *RP28-CRY2-RP28-lacZ* fusions were constructed by using the *CRY2-RP28-lacZ* fusion. A *Bgl*II or a *Hin*dIII site was created upstream of nucleotide +1 or nucleotide +28 of *CRY2* by site-directed mutagenesis with oligonucleotide SWF5 or ZL28, respectively. The *Eco*RI-*Bam*HI fragment or the *Eco*RI-*Hin*dIII fragment of *RP28* from plasmid pZL106 was used to replace the *Eco*RI-*Bgl*II fragment or the *Eco*RI-*Hin*dIII fragment of the *CRY2-RP28-lacZ* fusion to construct the *RP28-CRY2-RP28-lacZ* (*Bgl*II) and *RP28-CRY2-RP28-lacZ* (*Hin*dIII) fusions, respectively. In the *Bgl*II fusion, all of the 5′ nontranscribed sequences of *CRY2* were replaced by those of *RP28*. In the *Hin*dIII fusion, sequences upstream of nucleotide +28 of *CRY2* were replaced by those of *RP28*. Therefore, the *Hin*dIII fusion utilizes the transcription start site of *RP28*. Both fusions contain the *CRY2-RP28* hybrid intron that has the first 62 nucleotides of the *CRY2* intron fused to the 3′ 284 nucleotides of the *RP28* intron.

A 2.2-kb *Hin*dIII fragment containing the *CRY1* gene (31) was cloned into pRS313 (60) to construct pRS313CRY1. A 3.0-kb *Eco*RI-*Bam*HI fragment containing *CRY2* was cloned into pRS313 to construct pRS313CRY2. The stop codon TAG was inserted in frame with the open reading frame of *CRY1* after codon 18 or in frame with the open reading frame of *CRY2* after codon 19 in plasmids pRS313CRY1 and pRS313CRY2, respectively, by site-directed mutagenesis with oligonucleotide ZL13.

S1 nuclease protection assay. S1 nuclease protection assays were performed as described previously (49). A 1.3-kb *Eco*RI-*Bgl*II *CRY2* fragment extending from nucleotide −551 5′ of *CRY2* to nucleotide +765 at codon 108 of *CRY2* was ^{32}P labeled at the *Bgl*II site and used to detect both *CRY2* mRNA and pre-mRNA. This probe also detects *CRY1* mRNA, since the nucleotide sequences of the 3′ exons of *CRY2* and *CRY1* are very homologous. However, the sequences differ slightly at the 5′ ends of the respective 3′ exons (49). Therefore, several shorter *CRY2* DNA fragments are protected against S1 nuclease digestion when hybridized to *CRY1* mRNA than when hybridized to *CRY2* mRNA (data not shown). To circumvent this problem, a 2.0-kb *Eco*RI-*Cla*I fragment from plasmid pZL37 containing the *CRY2-lacZ Eco*RI-*Nru*I fusion was ^{32}P labeled at the *Cla*I site in *lacZ* and used to detect *CRY2-lacZ* pre-mRNA and mRNA. This probe unambiguously distinguishes *CRY2-lacZ* pre-mRNA and *CRY2-lacZ* mRNA from *CRY1* mRNA. There are two protected fragments corresponding to *CRY2-lacZ* mRNA; the last nucleotide of the *CRY2* intron is the same as the last nucleotide of the 5′ exon, resulting in two protected fragments that differ by one extra nucleotide. An 0.8-kb *Xba*I-*Bgl*II fragment from ribosomal protein gene *RPL1* (11), ^{32}P labeled at the *Bgl*II site, was used to detect *RPL1* mRNA. The identity of all of the protected bands was verified by their presence or absence in *cry1-Δ* or *cry2-Δ* strains or by their size, confirmed by comparison to ^{32}P-end-labeled *Bst*EII-digested λ DNA as molecular size markers. Relative amounts of RNAs

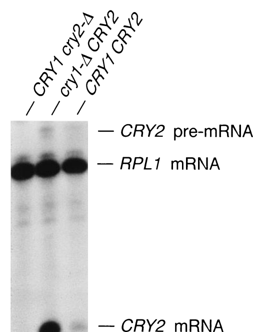

FIG. 1. Level of *CRY2* mRNA is increased when *CRY1* is deleted. RNA was extracted from *CRY1 cry2*-Δ (JTY81), *cry1*-Δ *CRY2* (JTY82), and *CRY1 CRY2* (JTY83) strains, hybridized to the ^{32}P-labeled *Eco*RI-*Bgl*II *CRY2* fragment and *Xba*I-*Bgl*II *RPL1* fragment, digested with S1 nuclease, subjected to electrophoresis on a denaturing polyacrylamide gel, and exposed to X-ray film. The positions of protected DNAs corresponding to *CRY2* pre-mRNA, *CRY2* mRNA, and *RPL1* mRNA are indicated. *RPL1* mRNA was used as a loading control. For clarity, shorter protected fragments resulting from hybridization to *CRY1* RNA are not included.

were quantified with an Ambis radioanalytic imaging system (Ambis Inc., San Diego, Calif.).

β-Galactosidase assays. In order to assay β-galactosidase expressed in yeast colonies grown on solid medium, patches of cells grown on YEPD or synthetic medium were replica plated to medium containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 200 μg/ml) and incubated for 2 days at 30°C. For quantitative β-galactosidase assays, cells grown to log phase in YEPD or synthetic medium were harvested, pelleted by centrifugation, and frozen at −80°C. Cells were lysed by vortexing with glass beads, and β-galactosidase was measured as described by Deshmukh et al. (11). Each assay was done at least twice with two independent transformants. β-Galactosidase activity is expressed as units of β-galactosidase per milligram of protein.

Screen for *CRY2* regulatory mutants. Random mutations that derepress *CRY2-lacZ* were generated by PCR coupled with in vivo gap repair (45). Oligonucleotides ZL4 and ZL12 were used to amplify a fragment extending from nucleotide −287 5′ of *CRY2* to nucleotide +302 within the *CRY2* intron. PCR amplification was done under several different conditions predicted to cause misincorporation of nucleotides (37, 76). An *Sph*I site was generated upstream of nucleotide −22 of *CRY2-lacZ* in pZL37 by site-directed mutagenesis with oligonucleotide ZL22. The resulting plasmid, pZL144, was digested with *Sph*I and *Bgl*II (at nucleotide +105 of *CRY2*) to generate a gap in the plasmid. The PCR fragment was cotransformed with the gapped plasmid into JTY83. Ura^{+} transformants were screened on plates containing X-Gal for mutants in which *CRY2-lacZ* was derepressed. Plasmids were extracted from blue colonies as well as some white colonies and retransformed into JTY83. The mutations were sequenced by PCR (3, 21). Expression of most of the mutant *CRY2-lacZ* RNAs was also assayed by S1 nuclease protection.

RNA secondary-structure analysis. The RNA secondary structure predicted to form within the 5′ end of *CRY2* pre-mRNA was identified by using the University of Wisconsin Fold program (12), accessed through the Pittsburgh Supercomputing Center.

RESULTS

The level of *CRY2* mRNA is increased when *CRY1* is deleted. To determine whether expression of *CRY2* is increased upon deletion of *CRY1*, we measured the amount of *CRY2* mRNA in yeast strains JTY83 (*CRY1 CRY2*), JTY82 (*cry1*-Δ *CRY2*), and JTY81 (*CRY1 cry2*-Δ) by an S1 nuclease protection assay. *CRY2* mRNA is present at approximately 10-fold-higher levels in *cry1*-Δ *CRY2* strains than in *CRY1 CRY2* strains (Fig. 1). mRNA expressed from *RPL1*, a ribosomal protein gene whose expression is not affected by deletion of *CRY1* (37a, 44), was used as a loading control. Low levels of *CRY2* pre-mRNA were detected in both *CRY1 CRY2* and *cry1*-Δ *CRY2* strains (Fig. 1). This result was corroborated by examining the levels of *CRY2* mRNA upon termination of transcription of *GAL-CRY1* in a *cry1*-Δ *CRY2 GAL-CRY1* strain (44). The level of *CRY2* mRNA was increased after shifting this strain from galactose-containing medium to glucose-containing medium, in which transcription of *CRY1* is repressed (data not shown). The relative expression of *CRY1* or *CRY2* was not changed when a *CRY1 CRY2* strain was shifted from galactose-containing medium to glucose-containing medium (data not shown). Taken together, these results indicate that expression of *CRY2* is repressed in *CRY1 CRY2* cells but derepressed upon deletion or inactivation of *CRY1*.

Expression of *CRY2* but not *CRY1* is repressed in *CRY1 CRY2* strains. The amount of *CRY1* mRNA is not detectably higher in *CRY1 cry2*-Δ strains than in *CRY1 CRY2* strains (data not shown). Since *CRY2* contributes only a minor proportion of rp59 in wild-type strains, the level of rp59 might not be sufficiently altered in *CRY1 cry2*-Δ strains to noticeably affect expression of *CRY1*. To determine whether expression of *CRY1* is sensitive to the levels of rp59 expressed from *CRY1* and *CRY2*, we assayed expression of a *CRY1-lacZ* gene fusion in *cry1*-Δ *CRY2* and *CRY1 CRY2* strains. *CRY1-lacZ* and *CRY2-lacZ* gene fusions were constructed in which *lacZ* was fused in frame at codon 107 of *CRY1* and at codon 108 of *CRY2* (49). One copy of each of these gene fusions was integrated at the *ura3-167* locus of JTY82 and JTY83.

The *CRY2-lacZ* gene fusion responds to deletion of *CRY1* in a manner identical to that of the wild-type *CRY2* gene. The level of *CRY2-lacZ* mRNA is 5.1-fold higher in the *cry1*-Δ *CRY2* strain (JWY3245) than in the *CRY1 CRY2* strain (JWY3246) (Fig. 2, lanes 1 and 2). A similar result was obtained when β-galactosidase activity expressed from this fusion was assayed (see Fig. 4, construct A). Thus, a fusion of *CRY2* to *lacZ* can be used as an accurate reporter for regulation of *CRY2* expression. In contrast, nearly identical levels of *CRY1-lacZ* mRNA and β-galactosidase activity were detected in both *cry1*-Δ *CRY2* (JWY3249) and *CRY1 CRY2* (JWY3250) strains containing the *CRY1-lacZ* fusion (Fig. 2, lanes 3 and 4). Therefore, expression of *CRY1-lacZ* is not regulated like that of *CRY2-lacZ* or *CRY2*. We conclude that *CRY2* but not *CRY1* is repressed in *CRY1 CRY2* cells.

rp59 expressed from either *CRY1* or *CRY2* can repress *CRY2*. To demonstrate that functional rp59 protein is necessary for repression of *CRY2*, we tested whether a nonsense mutation in *CRY1* causes derepression of *CRY2*. JWY3245, a *cry1*-Δ *CRY2* strain containing the *CRY2-lacZ* reporter, was transformed with a plasmid containing wild-type *CRY1* (pRS313CRY1) or with a plasmid containing *CRY1* bearing a nonsense mutation after codon 18 (pRS313cry1stop). *CRY2-lacZ* is derepressed in both JWY3245 and JWY3245 transformed with the vector pRS313 but repressed when plasmid-borne wild-type *CRY1*

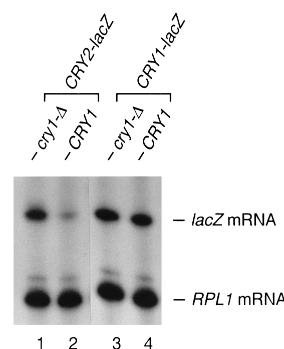

FIG. 2. Expression of *CRY2-lacZ* but not *CRY1-lacZ* is repressed by rp59. RNA was extracted from JTY82 (*cry1*-Δ *CRY2*) and JTY83 (*CRY1 CRY2*) strains carrying *CRY2-lacZ* or *CRY1-lacZ* integrated at *ura3-167* and subjected to the S1 nuclease protection assay with ^{32}P-end-labeled *RPL1* and *CRY2-lacZ* DNA probes.

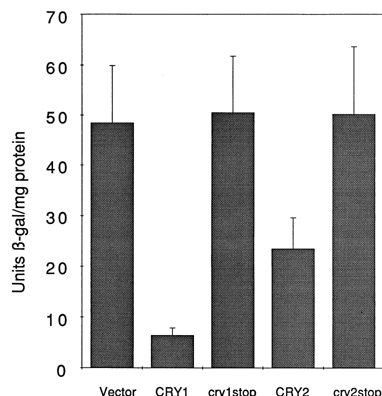

FIG. 3. rp59 protein expressed from either *CRY1* or *CRY2* can repress *CRY2*. Yeast strain JWY3245 (*cry1*-Δ *CRY2 CRY2-lacZ*) was transformed with the plasmid vector pRS313, pRS313 bearing either wild-type *CRY1* (pRS313CRY1) or wild-type *CRY2* (pRS313CRY2), or a plasmid containing a nonsense allele of *CRY1* (pRS313cry1stop) or *CRY2* (pRS313cry2stop). The values represent the average β-galactosidase activities measured in four independent experiments with two different transformants (n = 8).

is present (Fig. 3). However, in cells transformed with pRS313cry1stop, *CRY2-lacZ* is derepressed. The derepression of *CRY2-lacZ* does not result from increased turnover of *CRY1* mRNA containing the early nonsense codon, since *CRY1* mRNA expressed from pRS313cry1stop is present at levels identical to those expressed from pRS313CRY1 (data not shown). Thus, derepression of *CRY2-lacZ* is a result of diminished amounts of functional rp59.

The amino acid sequence of rp59 encoded by *CRY2* is 95% identical to that of rp59 encoded by *CRY1* (32, 49). In addition to the conservative substitutions at codon 72 (K→R) and codon 123 (S→C), the rp59 polypeptides encoded by *CRY1* and *CRY2* differ by several amino acids at their amino termini. To test whether rp59 expressed from *CRY2* could repress *CRY2*, we transformed a plasmid bearing *CRY2* (pRS313 CRY2) into JWY3245. Expression of *CRY2-lacZ* is repressed in these transformants, indicating that rp59 expressed from *CRY2* can also repress *CRY2-lacZ*. Introduction of an in-frame stop codon after codon 19 of *CRY2* abolishes repression of *CRY2-lacZ* (Fig. 3).

The amount of β-galactosidase expressed in pRS313CRY2 transformants is about threefold higher than in pRS313CRY1 transformants (Fig. 3). We infer from this result that *CRY2* on pRS313CRY2 is repressed by rp59 and expressed at lower levels than *CRY1* on pRS313CRY1, resulting in decreased repression of *CRY2-lacZ*. Alternatively, rp59 protein encoded by *CRY2* may function less well as a repressor of *CRY2* than rp59 encoded by *CRY1.*

Identification of *cis*-regulatory sequences necessary and sufficient for repression of *CRY2*. To identify *cis*-acting sequences at the *CRY2* locus that are necessary and sufficient for its repression, we constructed a series of *CRY2-lacZ* promoter fusions and gene fusions and assayed their expression in *CRY1 CRY2* and *cry1*-Δ *CRY2* strains (Fig. 4). β-Galactosidase expression was measured for constructs A to E. In addition, levels of *CRY2-lacZ* mRNA and pre-mRNA were assayed by S1 nuclease protection for each construct as well as the wild-type *CRY2* gene. As described above, the *Eco*RI-*Bgl*II *CRY2-lacZ* fusion at codon 108 of *CRY2* (Fig. 4, construct A) behaves similarly to wild-type *CRY2* (Fig. 2). The *Eco*RI-*Nru*I *CRY2-lacZ* gene fusion that lacks all but the first 16 nucleotides of the 3′ exon of *CRY2* is also regulated like *CRY2* (Fig. 4, construct B). Precise deletion of the *CRY2* intron from the *Eco*RI-*Nru*I *CRY2-lacZ* gene fusion results in constitutive derepression of this construct in *CRY1 CRY2* strains (Fig. 4, construct C).

Because the 5′ nontranscribed sequences (NTS) of *CRY1* and *CRY2* are not identical except for conserved promoter elements (49), the specific repression of *CRY2* could be mediated by sequences in the 5′ NTS of *CRY2.* To test whether the 5′ NTS of *CRY2* is necessary for regulation of *CRY2*, sequences upstream of nucleotide −44 of *CRY2* or *CRY2-lacZ* were replaced with the 5′ NTS from two other yeast genes, *RP28* and *GAL1*, whose expression is not affected by deletion of *CRY1* (data not shown). Expression of both promoter fusion constructs is regulated like that of the wild-type *CRY2* gene (Fig. 4, construct D, and data not shown). Thus, the 3′ NTS, most of the 3′ exon, and the 5′ NTS upstream of nucleotide −44 of *CRY2* are not required for repression of *CRY2*, whereas the intron is necessary.

To identify more precisely the regulatory sequences within the *CRY2* intron, a *CRY2-RP28* hybrid gene was constructed (Fig. 4, construct E). This chimeric gene contains the 5′ NTS, the 5′ exon, and the first 62 nucleotides of the *CRY2* intron (at nucleotide +105 of *CRY2*) followed by the 3′ 284 nucleotides of the *RP28* intron plus the first 81 nucleotides of the *RP28* 3′ exon, fused in frame to *lacZ*. Expression of this *CRY2-RP28-lacZ* tripartite hybrid gene is derepressed in a *cry1*-Δ strain and repressed in a *CRY1* strain, indicating that the 3′ 346 nucleotides of the *CRY2* intron are not necessary for its repression.

To further delineate essential regulatory sequences and to identify sequences sufficient for regulation, two *RP28-CRY2-RP28-lacZ* hybrid genes were constructed in which the only *CRY2* sequence present is the transcribed sequence of *CRY2* between either nucleotides +1 and +105 (Fig. 4, construct F) or nucleotides +28 and +105 (Fig. 4, construct G). Both constructs are repressed in *CRY1 CRY2* cells but derepressed in *cry1*-Δ *CRY2* cells, like the wild-type *CRY2* gene (Fig. 4). Taken together, these results demonstrate that sequences between nucleotides +28 and +105 of *CRY2* are sufficient for repression of *CRY2.*

Results with each of the fusion constructs were similar for both β-galactosidase and S1 nuclease assays, although derepression ratios were approximately twofold higher in β-galactosidase assays. Expression of constructs A to C, integrated in single copy into the genome, was lower than that of construct D, present on a *CEN* plasmid at 1 to 5 copies per cell. Low levels of β-galactosidase and *CRY2-lacZ* mRNA were expressed from constructs E to G even in *cry1*-Δ cells, and large amounts of *CRY2-lacZ* pre-mRNA were present in both *CRY1* and *cry1*-Δ cells. These results with constructs E to G are consistent with previous observations that chimeric introns are poorly spliced (18, and references therein). The lower derepression ratios for constructs E to G may also reflect the presence of additional regulatory elements in the intron of *CRY2*. The high ratio for construct E may result from errors in measuring very low levels of expression in *CRY1* cells.

Nucleotide sequence of the regulatory region of *CRY2* is phylogenetically conserved. To assess the importance of the regulatory sequences of *CRY2*, we compared the sequence of *CRY2* with that of genes encoding rp59 or its homologs in other organisms. As shown in Fig. 5, the sequence from nucleotides +20 to +103 of *S. cerevisiae CRY2* is 79% identical to that of *RP59* of the yeast *Kluyveromyces lactis* (33). The extent of sequence identity decreases 3′ of nucleotide +103; there is no obvious conservation of sequences between the 3′ 240 nucleotides of the *CRY2* intron and the 3′ 590 nucleotides of the *RP59* intron except for the branch point sequences and the 3′ splice sites.

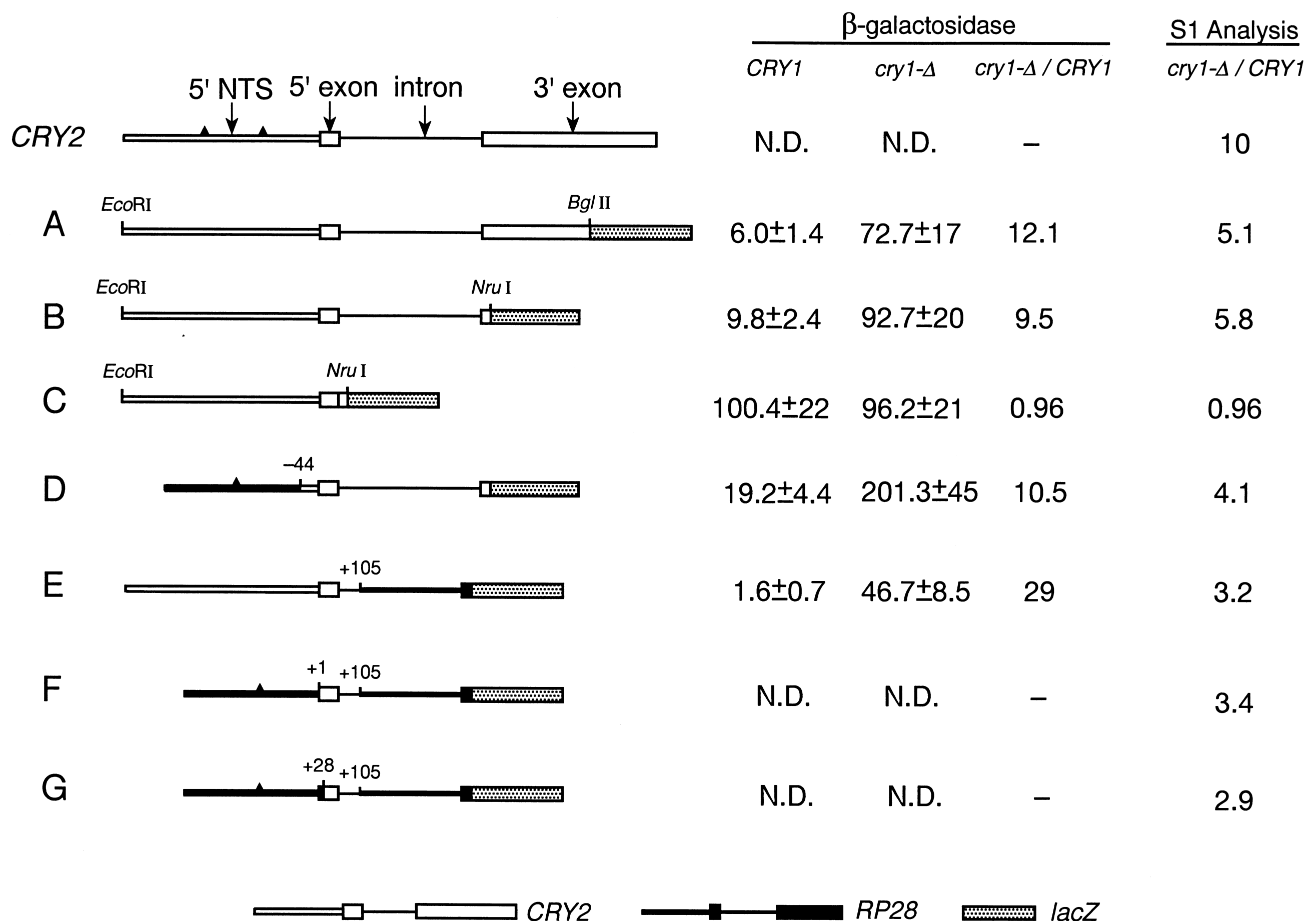

FIG. 4. Nucleotide sequences necessary and sufficient for repression of *CRY2* are confined to the transcribed region of *CRY2*, including the 5′ exon and first 62 nucleotides of the intron of *CRY2*. The top line is a diagram of *CRY2*. The UAS_{RPG} sequences present in the 5′ NTS of *CRY2* and *RP28* are represented by small triangles. The structure and expression of each construct in *CRY1 CRY2* and *cry1-Δ CRY2* strains are shown. The 5′ NTS (thin box), exons (thick box), and introns (line) of *CRY2* and *RP28* and *lacZ* (dotted box) are shown as indicated at the bottom of the figure. The restriction sites used for cloning (constructs A, B, and C) and the junctions between *CRY2* and *RP28* in constructs D, E, F, and G are also indicated. The ratios of the level of *CRY2* mRNA or *CRY2-lacZ* mRNA in *cry1-Δ CRY2* versus *CRY1 CRY2* cells are shown in the last column. Constructs: A, *Eco*RI-*Bgl*II *CRY2-lacZ* fusion; B, *Eco*RI-*Nru*I *CRY2-lacZ* fusion; C, *Eco*RI-*Nru*I intronless *CRY2-lacZ* fusion; D, *RP28-CRY2-lacZ* fusion driven by the *RP28* promoter; E, *CRY2-RP28-lacZ* fusion containing a hybrid *CRY2-RP28* intron; F, *RP28-CRY2-RP28-lacZ* fusion containing *CRY2* sequence from nucleotides +1 to +105; G, *RP28-CRY2-RP28-lacZ* fusion containing *CRY2* sequence from nucleotides +28 to +105. β-Galactosidase activity is shown in units per milligram of protein. N.D., not determined.

The similarity between *S. cerevisiae CRY2* and *K. lactis RP59*, especially in the intron sequences, may be significant; noncoding sequences of other *S. cerevisiae* genes and their *K. lactis* homologs usually are not conserved. For example, the introns of the *ACT1* genes in the two species bear no resemblance (9). An exception is *RPL32*. Two short stretches of sequences in the 5′ exon (including untranslated nucleotides) and the 5′ end of the introns are conserved and are the target for feedback regulation of *RPL32* (8, 15, 67). There is no similarity in the nucleotide sequences of the introns of *S. cerevisiae CRY1* and *CRY2* except for the conserved 5′ splice site, branch point sequence, and 3′ splice site. This is consistent with the observation that *CRY2* but not *CRY1* is repressed by rp59.

Both the nucleotide sequence and the secondary structure of *CRY2* pre-mRNA are important for regulation. The results described above show that the regulatory sequence of *CRY2* lies entirely within the transcribed portion of the gene. Repression of *CRY2* might occur at the level of transcription, via the regulatory sequences identified within the 5′ transcribed portion of the gene, or posttranscriptionally, mediated by the nucleotide sequence and structure of *CRY2* pre-mRNA. The first 105 nucleotides of *CRY2* pre-mRNA, comprising the regulatory region, are predicted to form a secondary structure with a ΔG of -20.3 kcal/mol (-84.9 kJ/mol) (Fig. 6).

To further delineate the regulatory sequences of *CRY2* and to begin to test whether the sequence or structure of *CRY2* pre-mRNA is important for regulation, we screened for random mutations that derepress *CRY2*. Mutations were generated by PCR coupled with in vivo recombination (45) and targeted to the interval between nucleotides -257 and $+302$ of the *CRY2-lacZ Eco*RI-*Nru*I fusion construct. Derepressed mutants were identified by screening for increased expression of β-galactosidase from *CRY2-lacZ* in a *CRY1 CRY2* wild-type strain. Subsequently, the levels of *CRY2-lacZ* pre-mRNA and mRNA in these mutants in both *CRY1* and *cry1-Δ* strains were assayed by S1 nuclease protection. By this approach, 24 different point mutations that derepress *CRY2-lacZ* in a *CRY1 CRY2* strain were recovered (Fig. 6). We also sequenced a number of *CRY2-lacZ* alleles that were not derepressed and identified 12 different silent mutations that have little or no effect on regulation of *CRY2* expression (Fig. 6). All of the mutations that derepress *CRY2-lacZ* lie between nucleotides +38 and +89 of

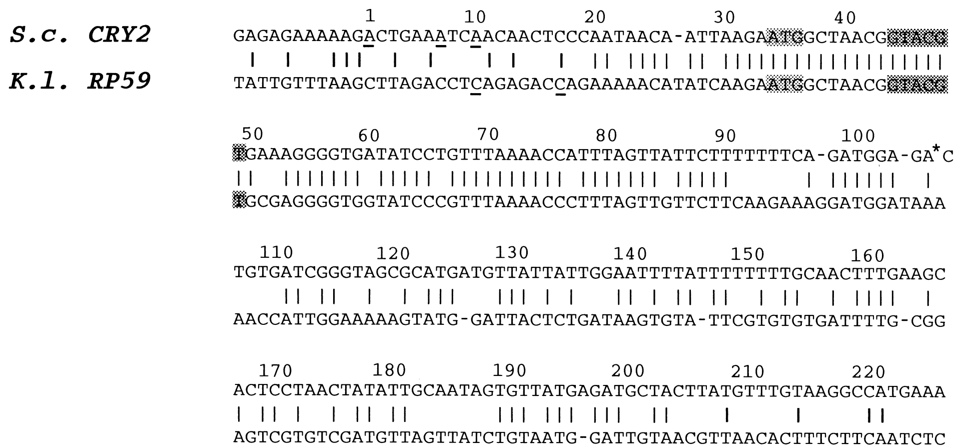

FIG. 5. Nucleotide sequence of the regulatory region of the *CRY2* gene from *S. cerevisiae* is quite similar to sequences in the 5′ end of the *RP59* gene from *K. lactis*. The transcription start sites of each gene are underlined. The translation initiation codon ATG and the 5′ splice site GTACGT of each gene are shaded. Nucleotide 105, which is the 62nd nucleotide of the intron of *CRY2*, is indicated by an asterisk. Identical nucleotides are indicated by vertical lines.

CRY2. In contrast, the silent mutations were found both inside and outside of this region. Seven of the silent mutations occur at positions near the 5′ or 3′ end of the regulatory region, suggesting that the regulatory region of *CRY2* might be shorter than that defined by the fusion constructs.

Eleven of the mutations that cause derepression of *CRY2-lacZ* disrupt predicted base pairs in the *CRY2* pre-mRNA. Four other mutations create G · U base pairs that might significantly weaken the stems. These results suggest that the secondary structure of *CRY2* pre-mRNA is important for regulation. Eight mutations are in nucleotides predicted to be unpaired, suggesting that the nucleotide sequence of *CRY2* RNA is important as well. One mutation, G54A, replaces a G · U base pair with an A · U base pair, potentially increasing the stability of the predicted secondary structure. All of these mutations cause derepression of *CRY2-lacZ*, although the level of derepression varies (Fig. 7). All of the mutations recovered that derepress *CRY2* are in nucleotides conserved between *S. cerevisiae* and *K. lactis*. Some of the silent mutations are also in conserved nucleotides. Some mutations were recovered multiple times from independent PCRs. However, mutations were not recovered in many of the nucleotides in this interval. Thus, the mutagenesis is far from saturated. Because the screen for derepression of *CRY2-lacZ* relies on β-galactosidase expression, mutations that interfere with splicing of *CRY2-lacZ* pre-mRNA or translation of *CRY2-lacZ* mRNA would not have been recovered by this approach.

To examine whether the predicted secondary structure rather than just the sequence of *CRY2* pre-mRNA per se is important for regulation, we constructed two pairs of compensatory double mutations (C38U and G82A and G43U and C76A [circled in Fig. 6]) that restore the predicted base-pairings. While each of the single mutations results in increased levels of *CRY2-lacZ* mRNA in *CRY1 CRY2* cells, expression of *CRY2-lacZ* is decreased to levels closer to those in the wild type in each double mutant (Fig. 8). Therefore, restoration of the predicted base-pairings also restores repression of *CRY2*. Taken together, these results with mutations in the regulatory region of *CRY2* demonstrate that the nucleotide sequence and

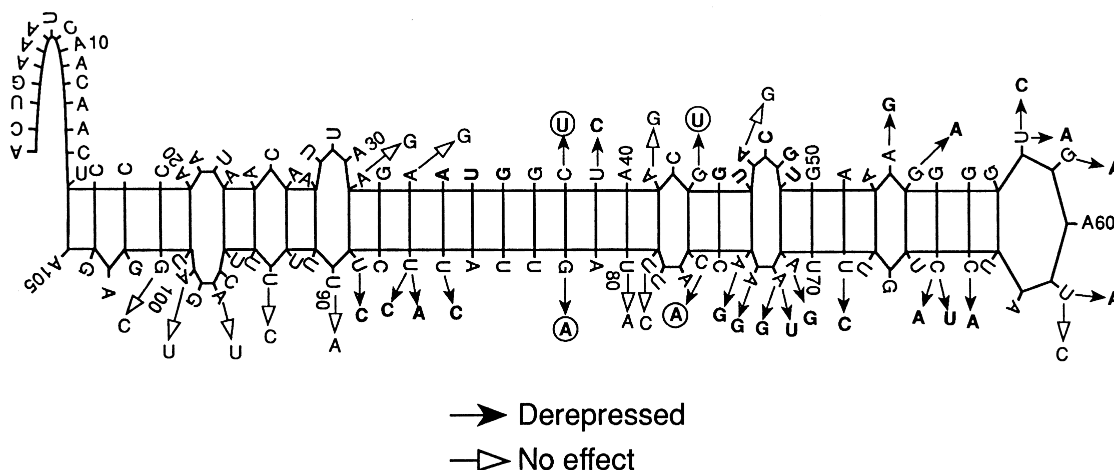

FIG. 6. Predicted secondary structure of the 5′ end of *CRY2* pre-mRNA and point mutations obtained by PCR or site-directed mutagenesis. Nucleotides are numbered according to their position in the *CRY2* transcript. The translation initiation codon AUG and the 5′ splice site GUACGU are in boldface. Point mutations are indicated above the sequence. Changes marked by solid arrows cause derepression of *CRY2-lacZ*, while those depicted by open arrows have no effect. The two compensatory double mutations at nucleotides +38 and +82 and nucleotides +43 and +76 are circled.

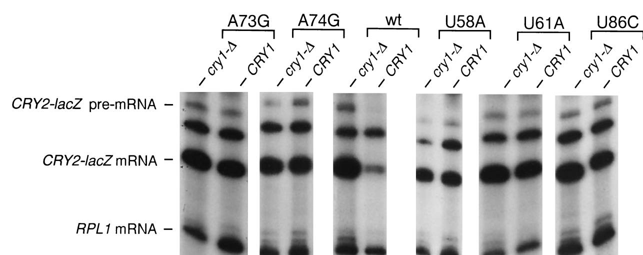

FIG. 7. Point mutations lead to derepression of *CRY2*. RNA was extracted from JTY82 (*cry1*-Δ *CRY2*) and JTY83 (*CRY1 CRY2*) transformants carrying wild-type *CRY2-lacZ* or *CRY2-lacZ* containing point mutations, as indicated, and subjected to the S1 nuclease protection assay. Mutations are named by listing the wild-type nucleotide, the nucleotide position within the *CRY2* transcript, and the mutant nucleotide. The samples were run on separate gels. The unlabeled band contains the DNA probe.

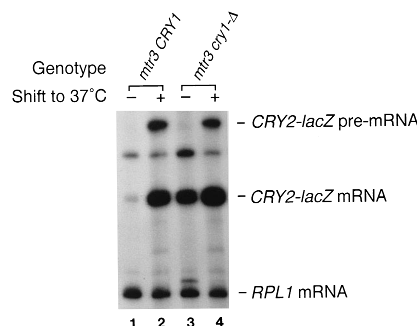

FIG. 9. *CRY2-lacZ* pre-mRNA and mRNA accumulate in an *mtr3* mutant defective in nuclear export of polyadenylated RNA. RNA was extracted from *mtr3 CRY1* (JWY3303) and *mtr3 cry1*-Δ (JWY3304) strains containing plasmid-borne *CRY2-lacZ* grown at 23°C (lanes 1 and 3) and after a shift to 37°C for 2 h (lanes 2 and 4) and subjected to the S1 nuclease protection assay.

the predicted secondary structure of *CRY2* pre-mRNA are important for repression.

***CRY2* is regulated posttranscriptionally.** At what specific step(s) does repression of *CRY2* occur? It is not obvious that splicing of *CRY2* pre-mRNA is inhibited, since very little *CRY2* pre-mRNA can be detected in *CRY1 CRY2* cells in which *CRY2* is repressed (Fig. 1). *CRY2* pre-mRNA may be transcribed but specifically targeted for degradation before it has an opportunity to be spliced. Alternatively, assembly of *CRY2* pre-mRNA into splicing complexes could be specifically blocked, so that unspliced pre-mRNA is then degraded by default. In either case, turnover of the *CRY2* pre-mRNA might occur in the nucleus or upon export to the cytoplasm, as observed for inefficiently spliced pre-mRNAs that contain early nonsense codons (24). To test these hypotheses, we assayed the effects of blocking polyadenylated mRNA export from the nucleus in *mtr* mutants (27) and of inactivating mRNA turnover in *upf1* and *xrn1* mutants (25, 34).

Plasmid pZL37 containing the *CRY2-lacZ EcoRI-NruI* fusion was transformed into an *mtr3* temperature-sensitive mutant strain, and *CRY2-lacZ* pre-mRNA and mRNA levels were assayed by S1 nuclease protection analysis. Low levels of *CRY2-lacZ* pre-mRNA and mRNA were detected in *mtr3* cells grown at 23°C (Fig. 9, lane 1), as observed in wild-type *MTR3* cells (Fig. 8, lane 2). However, both *CRY2-lacZ* pre-mRNA and mRNA accumulate to high levels after *mtr3* cells are shifted to 37°C for 2 h (Fig. 9, lane 2). Similar results were obtained with three other *mtr* mutants tested, *mtr1*, *mtr2*, and *mtr12* (data not shown) (27, 28). The accumulation of *CRY2* pre-mRNA is not due to a heat shock effect, since no accumulation of *CRY2* pre-mRNA was detected after an *MTR3* strain was shifted to 37°C (data not shown).

We took advantage of the fact that the block of nuclear RNA export in *mtr* mutants precludes cytoplasmic turnover of transcripts to assess whether transcription or splicing of *CRY2* pre-mRNA is derepressed in *cry1*-Δ versus *CRY1* cells. In general, transcription and splicing are not perturbed in *mtr* mutants. For example, the amount of spliced *CRY1* mRNA is identical in *mtr* strains grown at 23 or 37°C (27). The total amount of *CRY2-lacZ* transcripts (pre-mRNA plus mRNA) present in an *mtr3 CRY1* strain is approximately equal to that which accumulates in an *mtr3 cry1*-Δ strain when both are grown at 37°C (Fig. 9, compare lanes 2 and 4). Furthermore, the ratio of unspliced *CRY2-lacZ* pre-mRNA to spliced *CRY2-lacZ* mRNA (P/M ratio) is 2.5-fold lower in *mtr3 cry1*-Δ cells than in *mtr3 CRY1* cells when both are shifted to 37°C for 2 h (Fig. 9, lanes 2 and 4).

To examine whether *CRY2* pre-mRNA is normally degraded in *CRY1* cells, we measured amounts of *CRY2* pre-mRNA in *upf1* and *xrn1* mutants. The *UPF1* gene product is involved in the decay of cytoplasmic mRNAs containing early nonsense codons (24, 34). *CRY2* pre-mRNA contains a number of in-frame stop codons within its intron, which is located near the 5′ end of the transcript (49). The yeast *XRN1* gene encodes a 5′→3′ exoribonuclease (22, 25, 45a). Higher levels of *CRY2* pre-mRNA are present in *upf1*-Δ and *xrn1*-Δ cells than in otherwise isogenic *UPF1* and *XRN1* cells, respectively (Fig. 10). Taken together, these results with *mtr*, *upf1*-Δ, and *xrn1*-Δ mutants suggest that *CRY2* pre-mRNA is transcribed in repressed cells but spliced inefficiently. In derepressed *cry1*-Δ cells, *CRY2* pre-mRNA is spliced more efficiently to generate higher levels of *CRY2* mRNA.

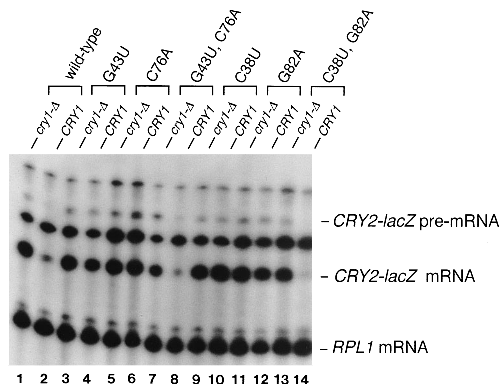

FIG. 8. Compensatory double mutations that restore base-pairing of *CRY2* pre-mRNA also restore repression of *CRY2*. RNA was extracted from JTY82 (*cry1*-Δ *CRY2*) and JTY83 (*CRY1 CRY2*) transformants carrying wild-type *CRY2-lacZ* or *CRY2-lacZ* containing different point mutations and compensatory double mutations, as indicated, and subjected to the S1 nuclease protection assay.

DISCUSSION

Balanced expression of yeast ribosomal proteins cannot be explained by a simple model of coordinate transcription of each of the rp genes via common and equally functional enhancer and promoter elements and *trans*-acting transcription factors. The dosage of rp genes is not equal; half are encoded by two functional genes, and half are encoded by one gene. The number and sequence of enhancer elements are not identical; half of the rp genes contain one copy of either the UAS_{RPG} or

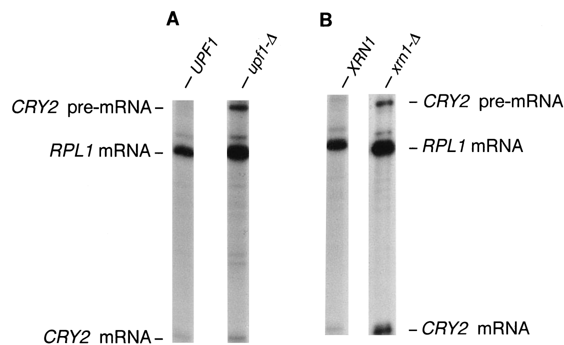

FIG. 10. *CRY2* pre-mRNA accumulates in yeast strains that are defective for cytoplasmic RNA turnover. RNA was extracted from *UPF1* (B-9037) and *upf1*-Δ (B9046) strains (A) and *XRN1* (yRP582) and *xrn1*-Δ (yRP689) strains (B) and subjected to the S1 nuclease protection assay with ^{32}P-end-labeled *RPL1* and *CRY2* DNA probes.

UAS_T enhancer, and half contain two UAS_{RPG} elements. The relative functionality of the UASs has not been quantified. This complexity is exemplified by the duplicated *CRY1* and *CRY2* genes encoding rp59. In wild-type cells, rp59 accumulates to levels equal to those of other rps (20, 44). *CRY1* is expressed at 8- to 10-fold-higher levels than *CRY2* despite the fact that *CRY1* contains one functional UAS_{RPG} and *CRY2* contains two UAS_{RPG}s. The functionality of these two UASs 5′ of *CRY2* has not been examined. The specific repression of *CRY2* but not *CRY1* described in this paper could account for the higher levels of expression of *CRY1* than of *CRY2* in wild-type cells.

From these results, we propose the following model in which expression of the two *CRY* genes could be balanced with that of other ribosomal protein genes, including single-copy genes, and coupled to the rate of assembly of ribosomes. rp59 encoded by both *CRY* genes is synthesized in the cytoplasm and imported to the nucleus, where it assembles into the 40S ribosomal subunit in the nucleolus. We assume that in wild-type *CRY1 CRY2* cells, there is a small pool of unassembled rp59 protein, expressed from both *CRY1* and *CRY2*, present in the nucleoplasm prior to its assembly into ribosomes. This unassembled rp59 may directly or indirectly repress *CRY2* expression via sequences present in the 5′ end of the *CRY2* pre-mRNA. When *CRY1* is deleted or inactivated, the pool of unassembled rp59 may shrink. Thus, *CRY2* is derepressed to levels sufficient to support nearly wild-type rates of assembly of 40S ribosomal subunits. This model explains the observation that *cry1*-Δ *CRY2* strains contain 80% of wild-type levels of *CRY* mRNA and 40S ribosomal subunits (49).

Our experiments demonstrate that regulation of *CRY2* is mediated by the secondary structure as well as the nucleotide sequence of *CRY2* RNA and therefore is unlikely to operate via *CRY2* DNA. Some mutations in the regulatory sequence that cause derepression are in predicted unpaired nucleotides, suggesting a requirement for specific sequences or RNA structures more complex than those in the predicted model. rp59 or other regulatory molecules may bind to and stabilize the secondary structure of *CRY2* pre-mRNA, leading to repression (Fig. 11). *CRY2* is derepressed in the absence of free rp59. Thus, the extent of repression or derepression could be a function of the amount of unassembled rp59 within the nucleus, determined by the rate of its assembly into ribosomes. The predicted stem-loop and bulge structures of the regulatory region of *CRY2* pre-mRNA are reminiscent of other known regulatory sequences recognized by RNA-binding proteins. Additional experiments are under way to determine the structure of *CRY2* RNA, to examine its importance, and to ascertain whether rp59 binds directly to the RNA.

The low, repressed levels of *CRY2* pre-mRNA and mRNA in *CRY1 CRY2* cells might result from specific effects on either transcription, splicing, localization, or turnover of *CRY2* pre-mRNA. The observation that the total amounts of *CRY2* transcripts are not increased in *mtr3 cry1*-Δ versus *mtr3 CRY1* mutant strains suggests that transcription of *CRY2* pre-mRNA is not attenuated via structure or sequences in the 5′ end of the transcript.

There are a growing number of examples showing that RNA secondary structure participates in the regulation of splicing by specifying splice sites, by interfering directly with splice site selection, or by functioning as a negative *cis*-acting element (reviewed in reference 4). Long-range base pair interactions within introns have a positive effect on splicing by shortening the effective distance between the branch point and the splice sites (10, 18). On the other hand, stable hairpins that sequester splicing signals strongly inhibit splicing (19, 38). The 5′ splice site of *CRY2* pre-mRNA is located within a stem-loop region in the predicted secondary structure, which might make it inaccessible to or unrecognizable by the splicing machinery.

A block in splicing of yeast pre-mRNA usually is evident from accumulation of unspliced pre-mRNA or of splicing intermediates (reviewed in reference 71). However, significant amounts of unspliced *CRY2* pre-mRNA might not accumulate if splicing of *CRY2* pre-mRNA were blocked prior to stable association of the pre-mRNA with components of the splicing apparatus. Under these conditions, the pre-mRNA might be degraded either in the nucleus or upon export to the cytoplasm, as previously observed for inefficiently spliced yeast pre-mRNAs (24). When nuclear export is blocked in *mtr* mutants, both *CRY2* pre-mRNA and mRNA accumulate. Under these conditions, deletion of *CRY1* results in more efficient splicing of *CRY2* pre-mRNA, reflected by a decreased P/M ratio (52). This result suggests that *CRY2* pre-mRNA splicing is affected by rp59.

To begin to determine whether repression of *CRY2* occurs upstream or downstream of particular steps in spliceosome assembly and splicing, we tested whether repression is epistatic with respect to different *prp* mutants blocked at different steps in the splicing pathway. No epistasis was observed for *prp39*, *prp3*, *prp4*, *prp8*, *prp11*, *prp17*, *prp18*, *prp20*, *prp22*, and *prp24* (data not shown) (23, 66). Thus, repression may occur upstream of *PRP39*, which is necessary for the formation of the

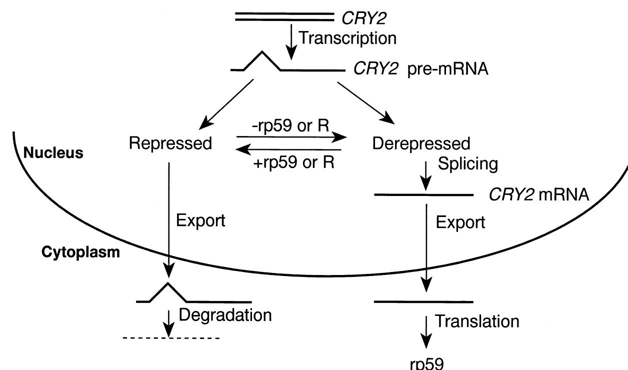

FIG. 11. Model for regulation of *CRY2*. *CRY2* pre-mRNA is targeted for nuclear export and cytoplasmic degradation when it is repressed, whereas it can associate with the splicing machinery and is spliced under derepressed conditions. rp59 or other regulatory molecules (R) may bind to and stabilize the secondary structure of *CRY2* pre-mRNA, leading to repression.

earliest splicing complex, the commitment complex (39). *cis*-Acting mutations in the 5′ splice site or in the branch point sequence that block splicing but do not alter the predicted secondary structure of *CRY2* pre-mRNA do not affect repression of *CRY2* (37b). These observations support the model that repression of *CRY2* might occur upstream of *PRP39*, prior to the formation of the commitment complex. Several results suggest that *CRY2* pre-mRNA is spliced inefficiently even under derepressed conditions. Unspliced *CRY2* pre-mRNA is present not only in *CRY1 CRY2* cells but also in *cry1*-Δ *CRY2* strains (Fig. 1) and in *mtr3* strains shifted to 37°C (Fig. 9), in which *CRY2* pre-mRNA may be retained in the nucleus.

An alternative model for repression of *CRY2* is that the sequence and structure of *CRY2* transcripts specifically target them for nuclear export before they are committed to the splicing pathway. Most pre-mRNAs are completely spliced before being exported to the cytoplasm, suggesting that introns may function as nuclear retention signals until splicing is completed (35). RNA secondary structure has been implicated in the export of several different mRNAs. For example, changes in the stem-loop structure at the 3′ end of histone mRNA affect its nucleocytoplasmic transport (70). The human immunodeficiency virus type 1 Rev protein promotes the nuclear export of unspliced or partially spliced RNAs via its interaction with a highly structured viral RNA sequence, the Rev response element (42). Studies carried out by Fischer et al. provide evidence that Rev directly activates the export of pre-mRNA molecules harboring the Rev response element and does not interfere with pre-mRNA splicing (17). Our observation that *CRY2* pre-mRNA accumulates to high levels in *mtr* mutants when export of mRNA is blocked suggests that in wild-type repressed cells, unspliced *CRY2* pre-mRNA is exported to the cytoplasm and degraded. We cannot rule out the possibility that import of rp59 into the nucleus is blocked in these *mtr* mutants at the nonpermissive temperature, resulting in derepression of *CRY2*. However, the *mtr* mutants used in this study are not defective in the import of Nop1p or histone H2B into the nucleus (27). Although the experiments with the *mtr* mutants suggest that *CRY2* pre-mRNA is degraded in the cytoplasm, we cannot distinguish whether splicing is specifically blocked or export is specifically stimulated by rp59.

The results of He et al. (24) suggest that inefficiently spliced pre-mRNAs that are exported to the cytoplasm are substrates for the nonsense codon-mediated RNA decay pathway. The 5′→3′ exoribonuclease Xrn1p degrades transcripts containing nonsense codons in response to *cis*-acting downstream elements in the RNA and *trans*-acting Upf proteins (22). The increased levels of *CRY2* pre-mRNA in *upf1*-Δ and *xrn1*-Δ cells suggest that some portion of unspliced *CRY2* pre-mRNA may be degraded by this pathway after it is exported from the nucleus. We cannot rule out whether some *CRY2* pre-mRNA is specifically degraded in the nucleus, e.g., before it can be spliced.

The mechanism of feedback regulation of *CRY2* is reminiscent of that for bacterial rp genes, in that the sequence and secondary structure of the *CRY2* transcript are the target for repression. The binding sites for *E. coli* L1, L10, S7, and S8 ribosomal proteins, like that predicted for *CRY2*, are characterized by bulges and loops within short helical segments of the rp transcripts (reviewed in references 47 and 74). However, it has not yet been tested whether rp59 binds directly to *CRY2* pre-mRNA, as is the case for bacterial ribosomal proteins and yeast rpL32 (67, 74). Repression is coupled to ribosome assembly in *E. coli* and occurs primarily at the level of translation. Repression of *CRY2* occurs in the nucleus, where the presence of unassembled ribosomal proteins may be sensed, to possibly couple expression with assembly.

In some respects, the feedback regulation of *CRY2* is similar to that of *RPL32* of *S. cerevisiae*. In both cases, the sequences and the structure of the 5′ ends of the transcripts are responsible for the regulation (15; this study). However, the mechanisms of regulation may differ. L32 protein binds to *RPL32* pre-mRNA and blocks its splicing by preventing the association of the U2 small nuclear ribonucleoprotein (67). Under these conditions, *RPL32* pre-mRNA accumulates. If *CRY2* is indeed repressed at the level of splicing, it might be blocked at an earlier step, e.g., prior to commitment complex formation, since unspliced *CRY2* pre-mRNA does not accumulate in repressed cells. The mammalian homolog of rp59, rpS14, can bind to *RPS14* mRNA and to short antisense RNAs expressed from the *RPS14* gene. It has also been shown that rpS14 inhibits transcription of *RPS14* in vitro (62). It will be of interest to determine to what extent the mechanisms of feedback regulation of the yeast and human genes encoding these rps are similar.

rp59 is a representative of the S14 ribosomal proteins that are highly conserved across species. For example, the amino acid sequences of rp59 protein encoded by the *S. cerevisiae CRY1* and *CRY2* genes and the rpS14 protein encoded by the mammalian *RPS14* gene are 80% identical (31, 55). However, the number, size, and location of introns within genes encoding rp59 and its homologs differ radically (2, 6, 33, 51, 55, 65). The yeast genes contain one intron interrupting codon 3 or 4, whereas the metazoan genes contain multiple introns at different locations (56). Interestingly, the expression of the human *RPS14* gene is feedback regulated at the level of transcription (62). However, none of the metazoan S14 genes contain any significant sequence similarity to yeast *CRY2* outside of codon regions.

Two other pairs of yeast ribosomal protein genes, *SSM1A* and *SSM1B* and *RPL4A* and *RPL4B*, may be subject to feedback regulation similar to that for *CRY1* and *CRY2*. For each of these pairs of genes, deletion of one gene leads to increased expression of the other but not vice versa (48a, 51a). It remains to be seen whether other uncharacterized ribosomal protein gene pairs are subject to the same kind of control as *CRY1* and *CRY2*.

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REFERENCES

1. **Abovich, N., L. Gritz, L. Tung, and M. Rosbash.** 1985. Effect of RP51 gene dosage alterations on ribosome synthesis in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **5:**3429–3435.
2. **Aerne, B. L., C. D. Baader, V. Schmid, and P. Schuchert.** 1994. The sequence of a cDNA encoding ribosomal protein S14 from the hydrozoan *Podocoryne carnea* reveals high evolutionary conservation. Gene **140:**243–246.
3. **Allard, M. W., D. L. Ellsworth, and R. L. Honeycutt.** 1991. The production of single-stranded DNA suitable for sequencing using the polymerase chain

reaction. BioTechniques **10:**24–26.
4. **Balvay, L., D. Libri, and M. Y. Fiszman.** 1993. Pre-mRNA secondary structure and the regulation of splicing. BioEssays **15:**165–169.
5. **Bozzoni, I., P. Fragapane, F. Annesi, P. Pierandrei-Amaldi, F. Amaldi, and E. Beccari.** 1984. Expression of two *Xenopus laevis* ribosomal protein genes in injected frog oocytes: a specific splicing block interferes with the L1 RNA maturation. J. Mol. Biol. **180:**987–1005.
6. **Brown, S. J., D. D. Rhoads, M. J. Stewart, B. Van Slyke, I.-T. Chen, T. K. Johnson, R. E. Denell, and D. J. Roufa.** 1988. Ribosomal protein S14 is encoded by a pair of highly conserved, adjacent genes on the X chromosome of *Drosophila melanogaster*. Mol. Cell. Biol. **8:**4314–4321.
7. **Dabeva, M. D., M. A. Post-Beittenmiller, and J. R. Warner.** 1986. Autogenous regulation of splicing of the transcript of a yeast ribosomal protein gene. Proc. Natl. Acad. Sci. USA **83:**5854–5857.
8. **Dabeva, M. D., and J. R. Warner.** 1993. Ribosomal protein L32 of *Saccharomyces cerevisiae* regulates both splicing and translation of its own transcript. J. Biol. Chem. **268:**19669–19674.
9. **Deshler, J. O., G. P. Larson, and J. J. Rossi.** 1989. *Kluyveromyces lactis* maintains *Saccharomyces cerevisiae* intron-encoded splicing signals. Mol. Cell. Biol. **9:**2208–2213.
10. **Deshler, J. O., and J. J. Rossi.** 1991. Unexpected point mutations activate cryptic 3′ splice site by perturbing a natural secondary structure within a yeast intron. Genes Dev. **5:**1252–1263.
11. **Deshmukh, M., Y.-F. Tsay, A. G. Paulovich, and J. L. Woolford, Jr.** 1993. Yeast ribosomal protein L1 is required for the stability of newly synthesized 5S rRNA and the assembly of 60S ribosomal subunits. Mol. Cell. Biol. **13:**2835–2845.
12. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
13. **Donovan, D. M., M. P. Remington, D. A. Stewart, J. C. Crouse, D. J. Miles, and N. J. Pearson.** 1990. Functional analysis of a duplicated linked pair of ribosomal protein genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:** 6097–6100.
14. **El-Baradi, T. T. A. L., C. A. F. M. Van der Sande, W. H. Mager, H. A. Raue, and R. J. Planta.** 1986. The cellular level of yeast ribosomal protein L25 is controlled principally by rapid degradation of excess protein. Curr. Genet. **10:**733–739.
15. **Eng, F. J., and J. R. Warner.** 1991. Structural basis for the regulation of splicing of a yeast messenger RNA. Cell **65:**797–804.
16. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
17. **Fischer, U., S. Meyer, M. Teufel, C. Heckel, R. Lührmann, and G. Rautmann.** 1994. Evidence that HIV-1 Rev directly promotes the nuclear export of unspliced RNA. EMBO J. **17:**4106–4112.
18. **Goguel, V., and M. Rosbash.** 1993. Splice site choice and splicing efficiency are positively influenced by pre-mRNA intramolecular base pairing in yeast. Cell **72:**893–901.
19. **Goguel, V., Y. Wang, and M. Rosbash.** 1993. Short artificial hairpins sequester splicing signals and inhibit yeast pre-mRNA splicing. Mol. Cell. Biol. **13:**6841–6848.
20. **Gorenstein, C., and J. R. Warner.** 1976. Coordinate regulation of the synthesis of eukaryotic ribosomal proteins. Proc. Natl. Acad. Sci. USA **73:**1547–1551.
21. **Gyllensten, U. B.** 1989. PCR and DNA sequencing. BioTechniques **1:**700–708.
22. **Hagan, K. W., M. J. Ruiz-Echevarria, Y. Quan, and S. W. Peltz.** 1995. Characterization of *cis*-acting sequences and decay intermediates involved in nonsense-mediated mRNA turnover. Mol. Cell. Biol. **15:**809–823.
23. **Hartwell, L. H.** 1967. Macromolecular synthesis in temperature-sensitive mutants in yeast. J. Bacteriol. **93:**1662–1670.
24. **He, F., S. W. Peltz, J. L. Donahue, M. Rosbash, and A. Jacobson.** 1993. Stabilization and ribosome association of unspliced pre-mRNAs in a yeast *upf1*$^-$ mutant. Proc. Natl. Acad. Sci. USA **90:**7034–7038.
25. **Heyer, W., A. W. Johnson, U. Reinhart, and R. D. Kolodner.** 1995. Regulation and intracellular localization of *Saccharomyces cerevisiae* strand exchange protein 1 (Sep1/Xrn1/Kem1), a multifunctional exonuclease. Mol. Cell. Biol. **15:**2728–2736.
26. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
27. **Kadowaki, T., S. Chen, M. Hitomi, E. Jacobs, C. Kumagai, S. Liang, R. Schneiter, D. Singleton, J. Wisniewska, and A. M. Tartakoff.** 1994. Isolation and characterization of *Saccharomyces cerevisiae* mRNA transport-defective (*mtr*) mutants. J. Cell Biol. **126:**649–659.
28. **Kadowaki, T., D. Goldfarb, L. M. Spitz, A. M. Tartakoff, and M. Ohno.** 1993. Regulation of RNA processing and transport by a nuclear guanine nucleotide release protein and members of the Ras superfamily. EMBO J. **7:**2929–2937.
29. **Kim, C. H., and J. R. Warner.** 1983. Messenger RNA for ribosomal proteins in yeast. J. Mol. Biol. **165:**79–89.
30. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
31. **Larkin, J. C., J. R. Thompson, and J. L. Woolford, Jr.** 1987. Structure and expression of the *Saccharomyces cerevisiae CRY1* gene: a highly conserved ribosomal protein gene. Mol. Cell. Biol. **7:**1764–1775.
32. **Larkin, J. C., and J. L. Woolford, Jr.** 1983. Molecular cloning and analysis of the *CRY1* gene: a yeast ribosomal protein gene. Nucleic Acids Res. **11:**403–420.
33. **Larson, G. P., and J. J. Rossi.** 1991. Altered response to growth rate changes in *Kluyveromyces lactis* versus *Saccharomyces cerevisiae* as demonstrated by heterologous expression of ribosomal protein 59 (*CRY1*). Nucleic Acids Res. **19:**4701–4707.
34. **Leeds, P., S. W. Peltz, A. Jacobson, and M. R. Culbertson.** 1991. The product of the yeast *UPF1* gene is required for rapid turnover of mRNAs containing a premature translational termination codon. Genes Dev. **5:**2303–2314.
35. **Legrain, P., and M. Rosbash.** 1989. Some *cis*- and *trans*-acting mutants for splicing target pre-mRNA to the cytoplasm. Cell **57:**573–583.
36. **Lesser, C. F., and C. Guthrie.** 1993. Mutational analysis of pre-mRNA splicing in *Saccharomyces cerevisiae* using a sensitive new reporter gene, *CUP1*. Genetics **133:**851–863.
37. **Leung, D. W., E. Chen, and D. V. Goeddel.** 1989. A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. Technique **1:**11–15.
37a. **Li, Z.** Unpublished data.
37b. **Li, Z., and J. Woolford.** Unpublished data.
38. **Liu, H., G. J. Goodall, R. Kole, and W. Filipowicz.** 1995. Effects of secondary structure on pre-mRNA splicing: hairpins sequestering the 5′ but not the 3′ splice site inhibit intron processing in *Nicotiana plumbaginifolia*. EMBO J. **14:**377–388.
39. **Lockhart, S. R., and B. C. Rymond.** 1994. Commitment of yeast pre-mRNA to the splicing pathway requires a novel U1 small nuclear ribonucleoprotein polypeptide, Prp39p. Mol. Cell. Biol. **14:**3623–3633.
40. **Ma, H., S. Kunes, P. Schatz, and D. Botstein.** 1987. Plasmid construction by homologous recombination in yeast. Gene **58:**201–216.
41. **Maicas, E., F. G. Pluthero, and J. D. Friesen.** 1988. The accumulation of three yeast ribosomal proteins under conditions of excess mRNA is determined primarily by fast protein decay. Mol. Cell. Biol. **8:**169–175.
42. **Malim, M. H., J. Hauber, S.-Y. Le, J. V. Maizel, and B. R. Cullen.** 1989. The HIV-1 Rev trans-activator acts through a structural target sequence to activate nuclear export of unspliced viral mRNA. Nature (London) **338:**254–257.
43. **Molenaar, C. M. T., L. P. Woudt, A. E. M. Jansen, W. H. Mager, and R. J. Planta.** 1984. Structure and organization of two linked ribosomal protein genes in yeast. Nucleic Acids Res. **12:**7345–7358.
44. **Moritz, M., A. G. Paulovich, Y.-F. Tsay, and J. L. Woolford, Jr.** 1990. Depletion of yeast ribosomal proteins L16 or rp59 disrupts ribosome assembly. J. Cell Biol. **111:**2261–2274.
45. **Muhlrad, D., and R. Parker.** 1992. Mutations affecting stability and deadenylation of the yeast *MFA2* transcript. Genes Dev. **6:**2100–2111.
45a. **Muhlrad, D. C., J. Decker, and R. Parker.** 1995. Turnover mechanisms of the stable yeast *PGK1* mRNA. Mol. Cell. Biol. **15:**2145–2156.
46. **Myers, A. M., A. Tzagoloff, D. M. Kinney, and C. J. Lusty.** 1986. Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions. Gene **45:**299–310.
47. **Nomura, M.** 1990. History of ribosome research: a personal account, p. 3–55. *In* W. E. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger, and J. R. Warner (ed.), The ribosome: structure, function, and evolution. American Society for Microbiology, Washington, D.C.
48. **Nomura, M., R. Gourse, and G. Baughman.** 1984. Regulation of the synthesis of ribosomes and ribosomal components. Annu. Rev. Biochem. **53:**75–117.
48a. **Ohtake, Y., and R. Wickner.** Personal communication.
49. **Paulovich, A. G., J. R. Thompson, J. C. Larkin, Z. Li, and J. L. Woolford, Jr.** 1993. Molecular genetics of cryptopleurine resistance in *Saccharomyces cerevisiae*: expression of a ribosomal protein gene family. Genetics **135:**719–730.
50. **Pearson, N. J., H. M. Fried, and J. R. Warner.** 1982. Yeast use translational control to compensate for extra copies of a ribosomal protein gene. Cell **29:**347–355.
51. **Perelman, D., and J. C. Boothroyd.** 1990. Lack of introns in the ribosomal protein gene S14 of trypanosomes. Mol. Cell. Biol. **10:**3284–3288.
51a. **Petitjean, A., and F. Lacroute.** Personal communication.
52. **Pikielny, C. W., and M. Rosbash.** 1985. mRNA splicing efficiency in yeast and the contribution of nonconserved sequences. Cell **41:**119–126.
53. **Presutti, C., S.-A. Caifre, and I. Bozzoni.** 1991. The ribosomal protein L2 in *S. cerevisiae* controls the level of accumulation of its own mRNA. EMBO J. **10:**2215–2221.
54. **Presutti, C., A. Lucioli, and I. Bozzoni.** 1988. Ribosomal protein L2 in *Saccharomyces cerevisiae* is homologous to ribosomal protein L1 in *Xenopus laevis*. J. Biol. Chem. **263:**6188–6192.
55. **Rhoads, D. D., A. Dixit, and D. J. Roufa.** 1986. Primary structure of human ribosomal protein S14 and the gene that encodes it. Mol. Cell. Biol. **6:**2774–2783.
56. **Rhoads, D. D., and D. J. Roufa.** 1991. Molecular evolution of the mammalian

ribosomal protein gene, *RPS14*. Mol. Biol. Evol. **8:**503–514.

57. **Rotenberg, M. O., M. Moritz, and J. L. Woolford, Jr.** 1988. Depletion of *Saccharomyces cerevisiae* ribosomal protein L16 causes a decrease in 60S ribosomal subunits and formation of half-mer polyribosomes. Genes Dev. **2:**160–172.
58. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
59. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
60. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *S. cerevisiae*. Genetics **122:**19–27.
61. **Struhl, K.** 1982. The yeast *his3* promoter contains at least two distinct elements. Proc. Natl. Acad. Sci. USA **79:**7385–7389.
62. **Tasheva, E. S., and D. J. Roufa.** 1995. Regulation of human *RPS14* transcription by intronic antisense RNAs and ribosomal protein S14. Genes Dev. **9:**304–316.
63. **Tornow, J., and G. M. Santangelo.** 1994. *Saccharomyces cerevisiae* ribosomal protein L37 is encoded by duplicated genes that are differentially expressed. Curr. Genet. **25:**480–487.
64. **Tsay, Y.-F., J. R. Thompson, M. O. Rotenberg, J. C. Larkin, and J. L. Woolford, Jr.** 1988. Ribosomal protein synthesis is not regulated at the translational level in *Saccharomyces cerevisiae*: balanced accumulation of ribosomal proteins L16 and rp59 is mediated by turnover of excess protein. Genes Dev. **2:**664–676.
65. **Tyler, B. M., and K. Harrison.** 1990. A *Neurospora crassa* ribosomal protein gene, homologous to yeast *CRY1*, contains sequences potentially coordinating its transcription with rRNA genes. Nucleic Acids Res. **18:**5759–5765.
66. **Vijayraghavan, U., M. Company, and J. Abelson.** 1989. Isolation and characterization of pre-mRNA splicing mutants of *Saccharomyces cerevisiae*. Genes Dev. **3:**1206–1216.
67. **Vilardell, J., and J. R. Warner.** 1994. Regulation of splicing at an intermediate step in the formation of the spliceosome. Genes Dev. **8:**211–220.
68. **Vincent, A., and S. W. Liebman.** 1992. The yeast omnipotent suppressor SUP46 encodes a ribosomal protein which is a functional and structural homolog of the *Escherichia coli* S4 *ram* protein. Genetics **132:**375–386.
69. **Warner, J. R., G. Mitra, W. F. Schwindinger, M. Studeny, and H. M. Fried.** 1985. *Saccharomyces cerevisiae* coordinates the accumulation of yeast ribosomal proteins by modulating mRNA splicing, translational initiation, and protein turnover. Mol. Cell. Biol. **5:**1512–1521.
70. **Williams, A. S., T. C. I. Ingledue, B. K. Kay, and W. F. Marzluff.** 1994. Changes in the stem-loop at the 3′ terminus of histone mRNA affects its nucleocytoplasmic transport and cytoplasmic regulation. Nucleic Acids Res. **22:**4660–4666.
71. **Woolford, J. L., Jr.** 1989. Nuclear pre-mRNA splicing in yeast. Yeast **5:**439–457.
72. **Woolford, J. L., Jr.** 1991. The structure and biogenesis of yeast ribosomes. Adv. Genet. **29:**63–118.
73. **Woolford, J. L., Jr., and J. R. Warner.** 1991. The ribosome and its synthesis, p. 587–626. *In* J. Broach, E. Jones, and J. Pringle (ed.), The molecular biology of the yeast *Saccharomyces*: genome dynamics, protein synthesis, and energetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
74. **Zengel, J. M., and L. Lindahl.** 1994. Diverse mechanisms for regulating ribosomal protein synthesis in *Escherichia coli*. Prog. Nucleic Acid Res. Mol. Biol. **47:**331–370.
75. **Zengel, J. M., and L. Lindahl.** 1990. *Escherichia coli* ribosomal protein L4 stimulates transcription termination at a specific site in the leader of the S10 operon independent of L4-mediated inhibition of translation. J. Mol. Biol. **213:**67–78.
76. **Zhou, Y., X. Zhang, and R. H. Ebright.** 1991. Random mutagenesis of gene-sized DNA molecules by use of PCR with Taq DNA polymerase. Nucleic Acids Res. **19:**6052.