© 1996 Oxford University Press
Nucleic Acids Research, 1996, Vol. 24, No. 5 943–950

Nutritional and growth control of ribosomal protein mRNA and rRNA in *Neurospora crassa*

Thomas P. Cujec+ and Brett M. Tyler*

Department of Plant Pathology, University of California, Davis, CA 95616, USA

Received September 25, 1995; Revised and Accepted January 23, 1996

ABSTRACT

The effects of changing growth rates on the levels of *40S* pre-rRNA and two r-protein mRNAs were examined to gain insight into the coordinate transcriptional regulation of ribosomal genes in the ascomycete fungus *Neurospora crassa*. Growth rates were varied either by altering carbon nutritional conditions, or by subjecting the isolates to inositol-limiting conditions. During carbon up- or down-shifts, r-protein mRNA levels were stoichiometrically coordinated. Changes in *40S* pre-rRNA levels paralleled those of the r-protein mRNAs but in a non-stoichiometric manner. Comparison of *crp-2* mRNA levels with those of a *crp-2::qa-2* fusion gene indicated no major effect from changes in *crp-2* mRNA stability. *Crp-2* promoter mutagenesis experiments revealed that two elements of the *crp-2* promoter, –95 to –83 bp (Dde box) and –74 to –66 bp (CG repeat) important for transcription under constant growth conditions, are also critical for transcriptional regulation by a carbon source. Ribosomal protein mRNA and rRNA levels were unaffected by changes in growth rates when the cultures were grown under inositol-limiting conditions, suggesting that, under these conditions, transcription of the ribosomal genes in *N.crassa* was regulated independently of growth rate.

INTRODUCTION

Ribosomes are essential for viability and their synthesis requires a major metabolic effort on the part of the cell (1,2). Consequently, it is presumably advantageous for the cell to closely regulate the synthesis of their components. Regulation of ribosome synthesis typically occurs at two levels: (i) control of the number of ribosomes in response to varying cellular demands, and (ii) regulation of the synthesis of the ribosomal components to ensure that each component is present in sufficient and equimolar amounts.

In *Escherichia coli*, changing growth rates due to changing environmental or nutritional conditions results in a rapid adjustment in the number of ribosomes and is paralleled by changes in rRNA and r-protein levels (3–5). The regulation of ribosomal gene expression appears to occur primarily at the translational level through an autogenous feedback mechanism (6).

In contrast, in *Saccharomyces cerevisiae*, transcriptional control plays a major role in the regulation of ribosomal gene expression during changes in carbon (7–9) or nitrogen conditions (10), or during amino acid starvation (11). During carbon shifts, changes in r-protein mRNA levels in yeast are stoichiometrically coordinated with changing 35S rRNA levels (7,12). Expression of most yeast r-protein genes is coordinated at the transcriptional level by virtue of a conserved upstream activator sequence (UAS_{rpg}) found in the promoters of most genes (7,8,13,14). A repressor–activator protein (RAP1), (15) also called TUF (16) or GRF (17), binds the UASrpg sequence and plays a role in coordinating transcription of r-protein genes (13,18). A 45 bp sequence at the 3′-end of the rRNA enhancer region is known to be both necessary and sufficient for transcriptional activation during a carbon upshift from ethanol to glucose (9). The regulatory factor(s) which binds to this region have not yet been identified, and consequently its role, if any, in coordinating transcription by RNA polymerases I and II is unknown. Although regulation of r-protein gene expression in yeast occurs primarily at the transcriptional level, post-transcriptional control mechanisms such as mRNA splicing (19) and protein degradation (20,21) are also operative in some cases.

In higher eukaryotes, the regulation of ribosomal gene expression is not as well understood as in yeast. In mouse, the promoter elements required for transcription of three r-protein genes (*rpL30*, *rpL32* and *rpL16*) have been identified, but nothing is known about their possible role in coordinating the transcription (22–24). In other organisms such as *Xenopus* (25) or *Drosophila* (26), translational control plays a major role in regulating r-protein expression during embryogenesis or differentiation.

In *N.crassa*, rRNA and r-protein levels change rapidly in response to changing carbon nutrition sources (27,28). In contrast, tRNA, DNA and total cellular protein levels do not change until rRNA and r-protein levels have stabilized at levels characteristic of the new media (1). More recent work has demonstrated that the mRNA levels of four r-proteins decrease rapidly and coordinately to 2–3% of preshift levels following a carbon downshift from sucrose to quinic acid (29). Together, these results suggest that the expression of r-protein genes in *N.crassa* may be regulated at the transcriptional level. They also emphasize the particular sensitivity of ribosomal gene expression to growth conditions.

In *N.crassa*, expression of unlinked structural genes involved in complex metabolic processes are often coordinated at the transcriptional level through the presence of conserved promoter elements (30–34). The promoters of six cytoplasmic ribosomal

* To whom correspondence should be addressed

+Present address: Departments of Medicine, Microbiology and Immunology, Howard Hughes Medical Institute, University of California, San Francisco, CA 94143-0724, USA

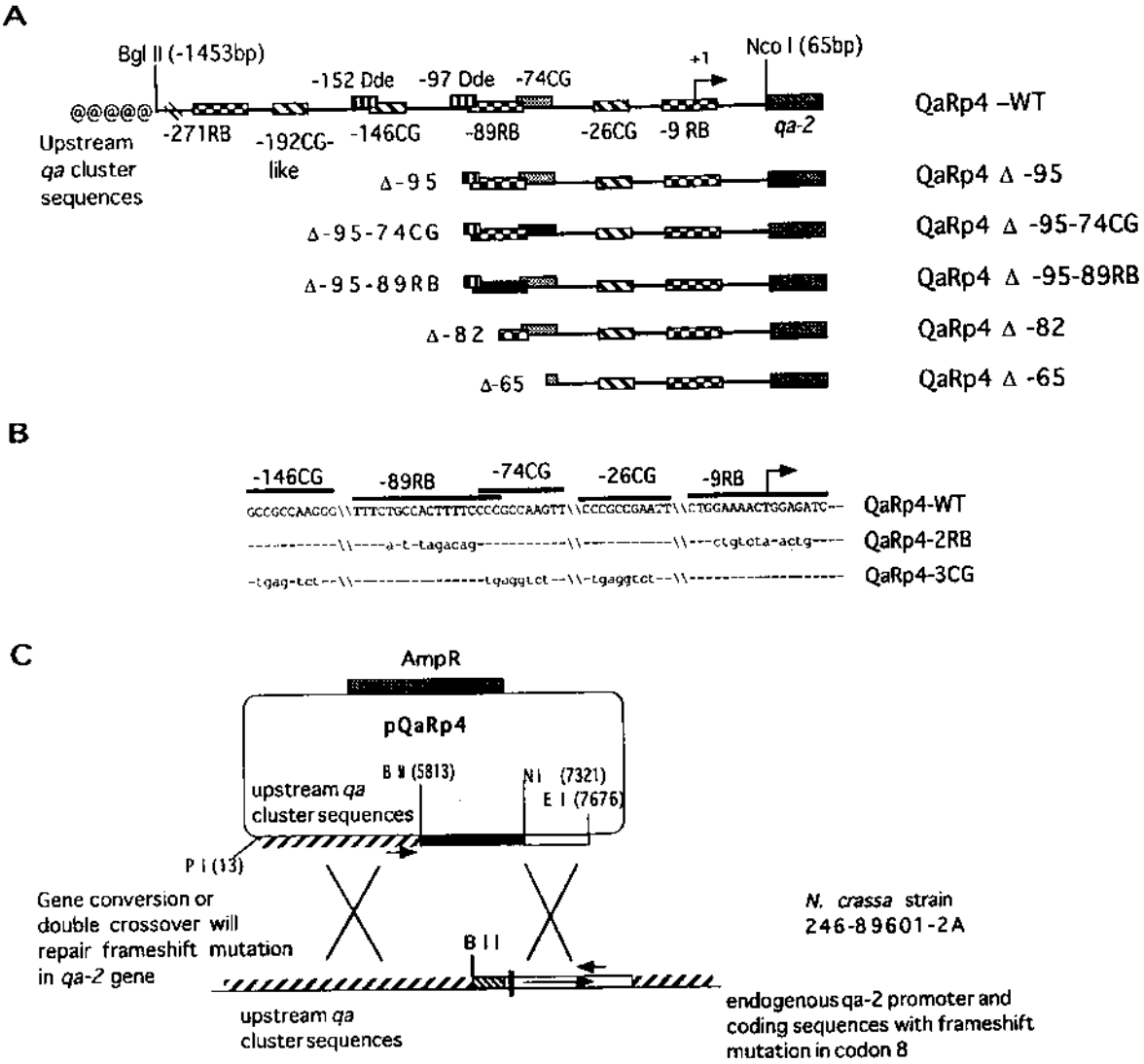

Figure 1. (**A**) Schematic diagram of *crp-2* promoter sequences. *Crp-2* promoter (1.45 kbp) and untranslated leader sequences (65 bp), fused to downstream *qa-2* coding sequences (355 bp) and flanked by upstream *qa* cluster (@@@) are shown. Conserved Ribo box, Dde box and CG repeat sequences are boxed. Deleted promoters (Δ-95, Δ-82, Δ-65) are indicated. Deletions are labeled according to the position of the last wild-type residue. Plasmid designations are indicated to the right. (**B**) Wild-type sequences of conserved regions are shown on the top line. Slashes represent sequences not shown. Lower case letters correspond to substitution mutations. Dashes represent wild-type sequences. (**C**) Strategy for targeting *crp-2* promoter sequences to the *qa-2* locus in *N.crassa*. Pertinent restriction sites and their relative positions are shown. *Crp-2* promoter sequences (*crp-2*), truncated *qa-2* coding sequences (*qa-2 3′*Δ) and upstream *qa* cluster sequences are indicated. The small left and right pointing arrows designate PCR primers BT 8.21 and BT 9.21. Restriction sites are as follows: BII, *Bgl*II; EI, *Eco*RI; NI, *Nco*I and PI, *Pst*I.

protein (*crp*) genes and a translation elongation factor (*tef-1*) (35) in *N.crassa* have been sequenced to date (29,36–39; I. de la Serna and B. Tyler, unpublished). Several conserved elements are present in some or all of the promoters of these genes, as well as in the transcriptional regulatory regions of the 5S and *40S* rRNA genes. These include the CG repeat (29), the Taq box (29), the Ribo box element (29,40) and a Dde box sequence (Fig. 6). The Ribo box is required for transcription of the 5S rRNA genes *in vitro* (40) and *in vivo* (41). The Ribo box and Dde box are required for transcription from the *40S* promoter *in vitro* (42), but have not been tested *in vivo*. Conserved elements within the ribosomal gene promoters have the potential to play roles in coordinating transcription by the three classes of RNA polymerases.

Mutational analysis of the *crp-2* promoter has identified six sequences important for optimal transcription *in vivo* during exponential growth under constant nutritional conditions (Cujec and Tyler, submitted to *Mol. Gen. Genet.*). Optimal transcriptional efficiency is dependent upon unidentified elements between –245 and –189 bp and between –48 and +9 bp, as well as on sequences from –153 to –147 bp (includes most of the –152 Dde box) and on sequences from –95 to –83 bp (includes all of the –97 Dde box sequences) (Fig. 1A). In addition, the –74 CG repeat is also critical for optimal transcription and is redundant to an upstream element (probably another CG repeat) between –189 and –154 bp.

In order to gain a better understanding of the mechanisms involved in the transcriptional regulation of r-genes in *N.crassa*, the effects of changing carbon sources and different growth rates on r-protein mRNA and rRNA levels were determined. *Crp-2* promoter mutants were used to identify sequence elements involved in regulation.

MATERIALS AND METHODS

DNA manipulation

DNA restrictions and ligations were done as described (43). DNA probes (100 ng) were 5′-end-labeled using 50 µCi (Amersham) [γ-^{32}P]ATP and T_4 polynucleotide kinase. DNA probes were 3′-end-labeled by incubation with the Klenow fragment (4 U) in the absence of dNTPs for 10 min before adding [α-^{32}P]dCTP (50 µCi, Amersham) and the remaining dNTPs (0.2 mM).

Mutant strains

Construction of the *crp-2* promoter mutations (Fig. 1A and B) used in this study and their targeting to the *qa-2* locus were as described (41; Cujec and Tyler, submitted). Transformant 246(pQaRp4-WT) contains 1.45 kbp of *crp-2* promoter and leader sequences fused to the ATG codon of 355 bp of 5′ *qa-2* (catabolic dehydroquinase) coding sequences. Transformants, 246(pQaRp4Δ-95), 246(pQaRp4Δ-82) and 246(pQaRp4Δ-65) contain 5′ deletion mutations in the *crp-2* promoter sequences of *crp-2::qa-2*. *Crp-2::qa-2* mRNA levels in 246(pQaRp4Δ-95), 246(pQaRp4Δ-82) and 246(pQaRp4Δ-65) were 19%, 4% and 3% of wild-type levels [in 246(pQaRp4-WT)] respectively, during steady-state growth in sucrose media (Cujec and Tyler, submitted to *Mol. Gen. Genet.*). Transformants 246(pQaRp4-2RB) and 246(pQaRp4-3CG) contain substitution mutations in either the conserved Ribo box or CG repeat sequences of the *crp-2::qa-2* fusion. The double Ribo box mutation reduced transcription to 69% of wild-type levels, while the triple CG repeat increased transcription slightly (136%) during growth in sucrose (41). Transformants 246(pQaRp4Δ-95-89RB) and 246(pQaRp4Δ-95-74CG) contain substitution mutations in either the –89 Ribo box or the –74 CG repeat element in the context of a 5′ deletion to –95 bp. These mutations decreased transcription to 17% [246(pQaRp4Δ-95-89RB)], or to 2% [246(pQaRp4Δ-95-74CG)] of wild-type levels (41) during exponential growth in sucrose media.

Growth conditions

As appropriate, 4×10^7 conidia or 5–10 g wet mycelia were inoculated into 2 l flasks containing 400 ml minimal Vogel's liquid media (44) and shaken (300 r.p.m.) at 25°C in the dark. For the carbon shift experiments, the media were supplemented with inositol (50 µg/ml) and either sucrose (1.5%) or glycerol (1.0% w/v) as carbon source. In the inositol depletion experiments, minimal Vogel's media contained sucrose (1.5%) and either 0.7, 5.0 or 50 µg/ml of filter-sterilized inositol.

In the carbon downshift experiments, conidia were inoculated into sucrose media and grown to exponential phase (16 h). The mycelia were harvested by filtration through miracloth (Calbiochem), rinsed with several volumes of distilled water, and divided into three aliquots. One aliquot (2–3 g) was returned to sucrose media for 2 h, another aliquot was transferred to glycerol media for 2 h, while the remaining aliquot was grown in glycerol for 8 h. Harvested mycelia was frozen in liquid N_2 immediately and stored at –80°C. In the carbon upshift experiments, conidia were initially inoculated into sucrose media to permit spore germination and mycelial outgrowth. After 16 h, the mycelia were harvested, washed with 2–3 vol water, and transferred to glycerol. After 16 h in glycerol, the mycelia were harvested again and divided into three aliquots. One aliquot was returned to glycerol for 2 h, another was transferred to sucrose for 2 h, and the remaining mycelia were grown in sucrose for 8 h.

In the inositol deprivation experiments, conidia from the appropriate transformants were grown in minimal media supplemented with inositol (5 µg/ml) for 20 h to permit mycelial growth. The mycelia (40 g wet weight/flask) were then harvested and 10 g wet mycelia were inoculated into culture media (400 ml) containing either 0.7 or 50 µg/ml inositol. In all cases, the inositol was filter sterilized before addition to the autoclaved media. At 6 h intervals for the next 12 h, half the media (including mycelia) was removed and 200 ml fresh media containing the appropriate inositol concentration was added to each flask. After the final media replenishment (t = 12 h), 20 ml of media was removed and the wet and dry weights of the collected mycelia determined. In order to determine the growth rates at different inositol concentrations, additional 20 ml aliquots were obtained at t = 15, 16.5, 18, 19.5 and 21 h. At 16.5 and 21 h, 150 ml media was removed from the flasks and the mycelia stored for subsequent RNA extraction.

RNA manipulation

Total RNA was extracted from frozen mycelia and poly(A$^+$) RNA purified using a oligo-dT-cellulose column essentially as described by Patel *et al.* (45). The S1 nuclease hybridization assay was used to quantify the mRNAs of interest (46). The DNA probes used were a 567 bp *Bam*HI–*Sty*I (3′-end-labeled) fragment for the β-tubulin gene (47), a 580 bp *Bst*BI–*Eco*NI (5′-end-labeled) fragment for *crp-1* (36), a 213 bp *Bst*YI–*Eco*RI (5′-end-labeled) fragment for *crp-2* (40) and a 112 bp *Xho*I–*Rsa*I (5′-end-labeled) fragment for the *40S* pre-rRNA (42). The *qa-2* (30) probe was obtained by cloning 336 bp of *qa-2* sequences (*Ava*I–*Sph*I) present on a *Ava*I–*Sal*I fragment into pUC18 (*Ava*I–*Sal*I) and then digesting with *Ava*I and *Nde*I (680 bp, 3′-end-labeled). The *40S* probe spans the transcription initiation site and hybridizes to sequences in the external transcribed spacer, upstream of an efficient RNA processing site. These sequences are rapidly degraded during processing of the *40S* pre-RNA transcript (42). Consequently, the amount of *40S* pre-rRNA hybridizing to the DNA probe more accurately reflects rRNA transcription rates than the level of the stable mature rRNAs. All probes were gel purified prior to hybridization. The β-tubulin, *qa-2*, *crp-1* and *crp-2* probes were hybridized simultaneously to 5 µg poly(A$^+$) RNA for 16 h at 58°C, while the *40S* pre-rRNA, β-tubulin, *crp-1* and *crp-2* probes were hybridized to 100 µg of total RNA overnight at 56°C. After digestion with 50 U of S1 nuclease (Boehringer Mannheim), the products were separated on a 5% polyacrylamide–7 M urea gel. Protected DNA fragments were visualized by autoradiography and bands quantified either by the AMBIS radioanalytical system (San Diego, CA) or by phosphoimager analysis (Sunnyvale, CA).

RESULTS

Effect of carbon downshifts on r-protein mRNA and *40S* pre-rRNA levels

The *crp-2* protein in *N.crassa* is homologous to S11 in *E.coli* (48) and rp59 (CRY1) in *S.cerevisiae* (2) and is assumed to be essential for viability. In order to assay different *crp-2* promoter mutations, and to judge effects on mRNA stability, the promoter of the *qa-2* gene (catabolic dehydroquinase) was replaced with wild-type or mutant *crp-2* promoters, using gene targeting (Cujec and Tyler, submitted to *Mol. Gen. Genet.*). A transformant containing

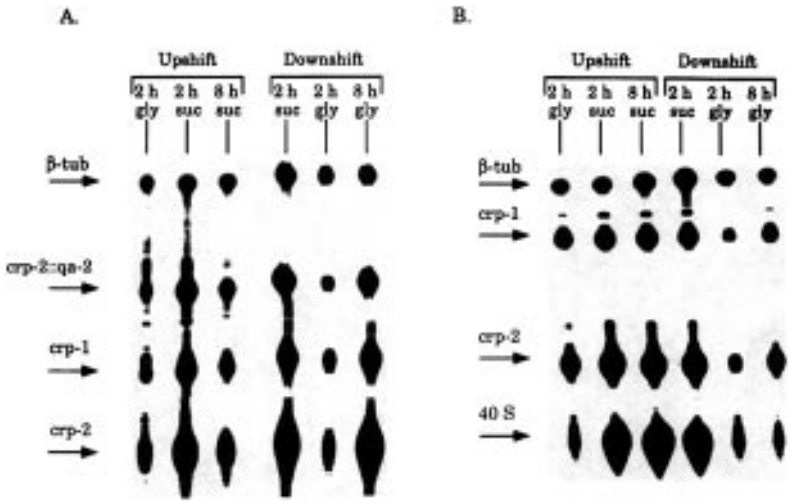

Figure 2. S1 nuclease hybridization assays were used to determine the effect of carbon up- or down-shifts on levels of r-protein (*crp-1, crp-2*) mRNAs (A and B), *crp-2::qa-2* mRNA (A) and 40S rRNA (B) transformant 246(QaRp4-WT). DNA probes (indicated on the left) were hybridized simultaneously to either 5μg poly(A^+) RNA (**A**), or 100 μg total RNA (**B**) extracted at appropriate intervals (h) from mycelia grown in media containing either sucrose (suc), or glycerol (gly) as a carbon source. Details of the experimental procedures are described in Material and Methods.

wild-type *crp-2* promoter sequences integrated at the *qa-2* locus, 246(pQaRp4-WT), was grown first in sucrose containing medium, then transferred to glycerol containing medium (carbon downshifts). The S1 nuclease assay was used to simultaneously quantify *crp-1, crp-2, crp-2::qa-2* and β-tubulin mRNA levels at various times during the shifts. In some experiments, a DNA probe hybridizing to processed regions of the *40S* pre-rRNA was also used in order to determine the effects of changing carbon nutritional sources on *40S* transcription levels. *Crp-2::qa-2, crp-1, crp-2* and *40S* pre-RNA levels were standardized to those of β-tubulin and are expressed as a fraction of transcript levels at 2 h sucrose (Figs 2 and 3).

Following a carbon downshift from sucrose to glycerol, *crp-1, crp-2* and *crp-2::qa-2* mRNA levels in the *N.crassa* transformant 246(pQaRp4-WT) were generally reduced to 10–20% of preshift levels after 2 h in glycerol and returned to 40–70% of preshift levels after 8 h in glycerol (Figs 2 and 3A). Similarly, *40S* pre-rRNA levels decreased to 10% of preshift levels after 2 h in glycerol. However unlike mRNA levels, rRNA levels had only returned to 25% of preshift levels after 8 h in glycerol (Figs 2 and 3A).

In these experiments, *qa-2* mRNA levels from the *crp-2::qa-2* fusion were closely coordinated with those of the endogenous *crp-1* or *crp-2* genes. These results demonstrate that during carbon downshifts, transcription from the *crp-2::qa-2* fusion accurately reflects transcription from the endogenous *crp-2* promoter, and that the *crp-2* promoter and 5′ leader sequences in 246(pQaRp4-WT) contain *cis*-acting elements sufficient for proper transcriptional regulation. They also suggest that there is no significant regulation at the level of mRNA stability.

Effect of carbon upshifts on rp mRNA and *40S* pre-rRNA levels

The mRNA levels of the *crp-1, crp-2* and *crp-2::qa-2* genes in 246(pQaRp4-WT) were closely coordinated during carbon upshifts (Figs 2 and 3B). Two hours after transfer to sucrose to glycerol, mRNA levels were 1.5–2.0-fold higher than those of cultures in glycerol. After 8 h in sucrose, the *crp-1, crp-2* and *crp-2::qa-2* mRNA levels decreased to levels 15–50% higher than those prior to the shift. In contrast, *40S* pre-rRNA levels were 7-fold greater than preshift levels after 2 h in sucrose and remained 5-fold greater after 8 h in sucrose (Fig. 3B). These results demonstrate that r-protein mRNA levels change coordinately during changes in carbon sources. Under these conditions, *40S* pre-rRNA levels are also coordinated with r-protein mRNA levels, however, the coordination is not stoichiometric.

Effect of *crp-2* promoter mutations on carbon shift regulation

In order to identify the carbon-responsive element(s) in the *crp-2* promoter, transformants containing 5′ deletions in the *crp-2* sequences integrated at the *qa-2* locus were subjected to carbon shifts. Initial experiments demonstrated that a promoter deletion to –153 bp, which reduced transcription in sucrose media to 45% of wild-type, did not affect the transcriptional regulation of the *crp-2::qa-2* gene during either a carbon up or downshift (data not shown). A 5′ deletion to –95 bp reduced *crp-2::qa-2* mRNA levels to 20% of wild-type levels during steady growth in sucrose media but did not affect the transcriptional regulation of the *crp-2::qa-2* gene during carbon shifts (Fig. 4A). In the downshift experiments, *crp-2::qa-2* mRNA levels in 246(pQaRp4Δ-95) dropped to 11% of sucrose levels after 2 h in glycerol and then returned to 68% of preshift levels after 8 h in glycerol (Fig. 4A). Following an upshift to sucrose media, *crp-2::qa-2* mRNA levels in 246(pQaRp4Δ-95) increased to 212% of pre-shift levels after 2 h in sucrose before decreasing to 123% of pre-shift levels after 8 h in sucrose (Fig. 4B). The decrease at 8 h may be due to the cultures entering stationary phase. In both the up- and downshifts, changes in *crp-2::qa-2* mRNA levels in 246(pQaRp4Δ-95) were similar to those normally observed in the control transformant 246(pQaRp4-WT).

A deletion mutation to –65 bp in *crp-2::qa-2* almost completely abolished transcription during growth in sucrose (3% of wild-type; Cujec and Tyler, submitted to *Mol. Gen. Genet.*). In 246(pQaRp4Δ-65), *crp-2::qa-2* mRNA levels did not follow the same pattern as those in 246(pQaRp4-WT) during either a carbon

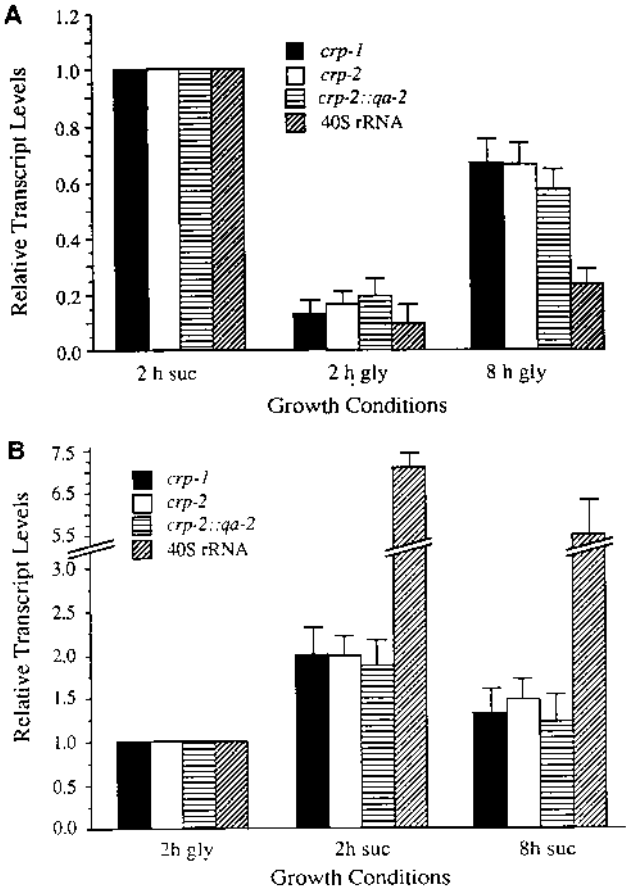

Figure 3. Effect of a carbon downshift (**A**) and a carbon upshift (**B**) on *crp-2::qa-2* mRNA and *40S* pre-rRNA levels relative to *crp-1* and *crp-2* in strain 246(pQaRp4-WT). Growth conditions are indicated on the X-axis. Numbers refer to the time (h) in sucrose (suc) or glycerol (gly) media. All transcript levels (Y-axis) were determined by the S1 nuclease assay, and were standardized to β-tubulin then expressed relative to the levels in cultures grown in sucrose for 2 h (2 h suc) (A) or relative to the levels in cultures grown for 2 h in glycerol (2 h gly) (B). *Crp-2::qa-2* mRNA and *40S* pre-rRNA levels were determined in separate experiments. Data is an average of two to six replicates. Error bars are standard errors.

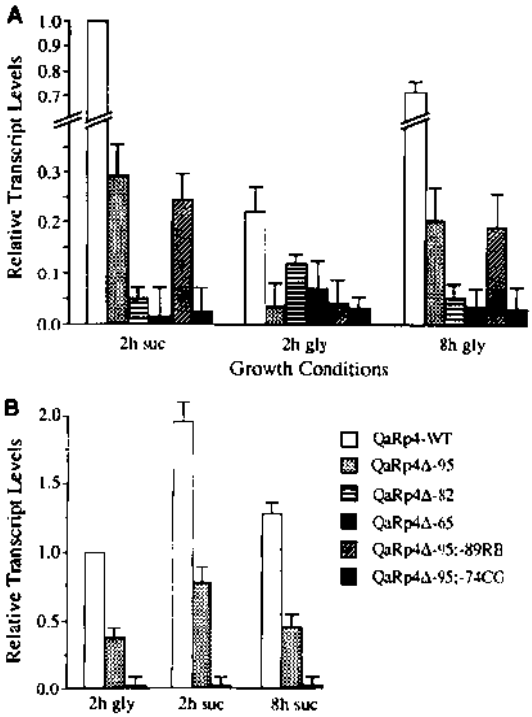

Figure 4. Effect of *crp-2* promoter mutations on *crp-2::qa-2* mRNA levels in *N.crassa* following a carbon downshift (**A**) or a carbon upshift (**B**). Transformant designations correspond to those in Figure 1A. The *crp-2::qa-2* mRNA levels (Y-axis) were determined by the S1 nuclease assay, and were standardized to β-tubulin then expressed relative to the levels in 246(pQaRp4-WT) cultures grown in sucrose for 2 h (2 suc) (A) or relative to the levels in cultures grown for 2 h in glycerol (2 h gly) (B). *Crp-2::qa-2* mRNA levels in 246(pQaRp4Δ-95) and 246(pQaRp4Δ-65) were determined in experiments separate from 246(pQaRp4Δ-82), 246(pQaRp4Δ-95;–89RB) and 246(pQaRp4Δ-95;–74CG). The histogram patterns are the same for both A and B. Data are averages of two to six replicates. Error bars are standard errors.

up- or down-shift. In the downshift experiments, *crp-2::qa-2* mRNA levels in this mutant appeared to increase 4-fold following the shift to glycerol (0.03–0.12) and remained 50% greater than preshift levels (0.06), even after 8 h in glycerol (Fig. 4A). Following an upshift to sucrose, relative *crp-2::qa-2* levels in 246(pQaRp4Δ-65) increased slightly (from 0.03 to 0.04) after transfer to sucrose and then remained unchanged at 0.04, even after 8 h in sucrose (Fig. 4B). These results suggest that a *crp-2* promoter element between –95 and –66 bp is required for regulation of *crp-2* mRNA levels during changes in carbon nutritional conditions.

In order to further delimit which sequences from –95 to –66 bp are important for transcriptional regulation, the effects of additional *crp-2* promoter mutations on *crp-2::qa-2* mRNA levels were determined during a carbon downshift. During steady state growth in sucrose, *crp-2::qa-2* mRNA levels in 246(pQaRp4Δ-95-89RB), 246(pQaRp4Δ-95-74CG) and 246(pQaRp4Δ-82) are typically 17, 2 and 4% of wild-type levels 246(pQaRp4-WT) respectively (Fig. 4A). In the context of a –95 bp deletion, substitution mutations in the –89 Ribo box [246(pQaRp4Δ-95-89RB)] did not affect the regulation of *crp-2::qa-2* mRNA levels (Fig. 4A). Similar to the control transformant 246(QaRp4-WT), *crp-2::qa-2* mRNA levels in 246(pQaRp4Δ-95-89RB) decreased to 26% of sucrose levels following a downshift from sucrose to glycerol before increasing to 78% of pre-shift levels after 8 h in glycerol. Although the low levels of *crp-2::qa-2* mRNA observed in the 246(pQaRp4Δ-95-74CG) mutant during constant growth in sucrose media (2% of wild-type levels) hindered our ability to accurately quantify changes in mRNA levels during the carbon downshift, no obvious changes in mRNA levels were observed. In 246(pQaRp4Δ-82), *crp-2::qa-2* mRNA levels increased 3-fold (0.04–0.12) following a downshift from sucrose to glycerol, before decreasing to levels similar to those observed in the sucrose-based media (0.045). These results are similar to those obtained with 246(pQaRp4Δ-65) and together they suggest that residues from –95 to –83 bp and from –73 to –66 bp are critical for the regulation of *crp-2* mRNA levels during a carbon nutritional shift.

Since the Ribo box and the CG repeat elements are conserved among the promoters of various ribosomal genes in *N.crassa* (29,36,38,39), we postulated that they may play an important role in transcriptional regulation during changing nutritional conditions. Consequently, the effects of Ribo box and CG repeat substitution mutations on *crp-2::qa-2* mRNA levels were determined during

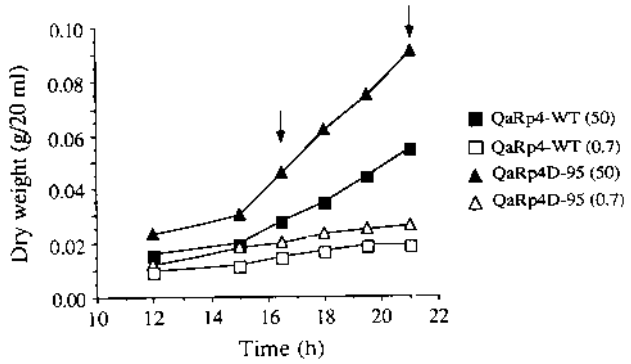

Figure 5. Effect of inositol concentrations on growth of *N.crassa* isolates 246(pQaRp4-WT) and 246(pQaRp4Δ-95). Bracketed numbers beside isolate designations represent inositol concentrations (μg/ml) in the media. Abscissa denotes the time elapsed following transfer of mycelia to media containing 50 or 0.7 μg/ml inositol. Dry weights are indicated on the Y-axis. Vertical arrows denote time of RNA extractions. Details of experimental procedures are given in the text.

carbon down or upshifts. Isolates containing a double Ribo box mutation [246(pQaRp4-2RB)], or a triple CG repeat mutation [246(QaRp4-3CG)] (Fig. 1B) in the *crp-2::qa-2* fusion were subjected to carbon up- or down-shifts. A triple CG repeat substitution mutation in the *crp-2* promoter (QaRp4-3CG) did not affect characteristic changes in *crp-2::qa-2* mRNA levels relative to 246(pQaRp4-WT) during the carbon shifts (data not shown). Similarly, transcriptional regulation of the *crp-2::qa-2* gene in 246(pQaRp4-2RB) paralleled that of 246(pQaRp4-WT).

Effect of inositol concentrations on growth rates and ribosomal transcript levels

In an attempt to determine whether the ribosomal genes respond to carbon source or to growth rate directly, r-protein mRNA and *40S* pre-rRNA levels were compared in cultures growing in sucrose media at different growth rates. Growth rates were varied by growing the transformants, which are inositol auxotrophs, in media containing either sufficient (50 μg/ml) or limiting (0.7 μg ml) inositol concentrations.

Based on preliminary experiments, the growth rates of 246(pQaRp4-WT) and 246(pQaRp4Δ-95) were compared at regular intervals beginning 12 h after transfer to media containing either 50 or 0.7 μg/ml inositol. After 16.5 h in media containing 50 μg/ml inositol, the growth rate of 246(pQaRp4-WT) was three times greater (5 mg/h) than in 0.7 μg/ml inositol (1.6 mg/h) while the growth rate of 246(pQaRp4Δ-95) was six times greater (11 mg/h versus 1.7 mg/h) (Fig. 5). After 21 h, both transformants had ceased to grow under inositol-limiting conditions, while their growth rates under sufficient inositol conditions remained about the same as at 16.5 h.

Next, *crp-1*, *crp-2*, *crp-2::qa-2* mRNA and *40S* pre-rRNA transcript levels were compared in these cultures. Despite the differences in growth rates, *crp-1*, *crp-2* and *crp-2::qa-2* transcript levels in 246(pQaRp4-WT) and 246(pQaRp4Δ-95) were similar regardless of whether the isolate was grown in 50 μg/ml inositol or 0.7 μg/ml (Table 1).

Regardless of the inositol concentration, *crp-2::qa-2* mRNA levels in 246(pQaRp4Δ-95) were ~30% of those in 246(pQaRp4-WT). After 21 h the transformants had essentially stopped

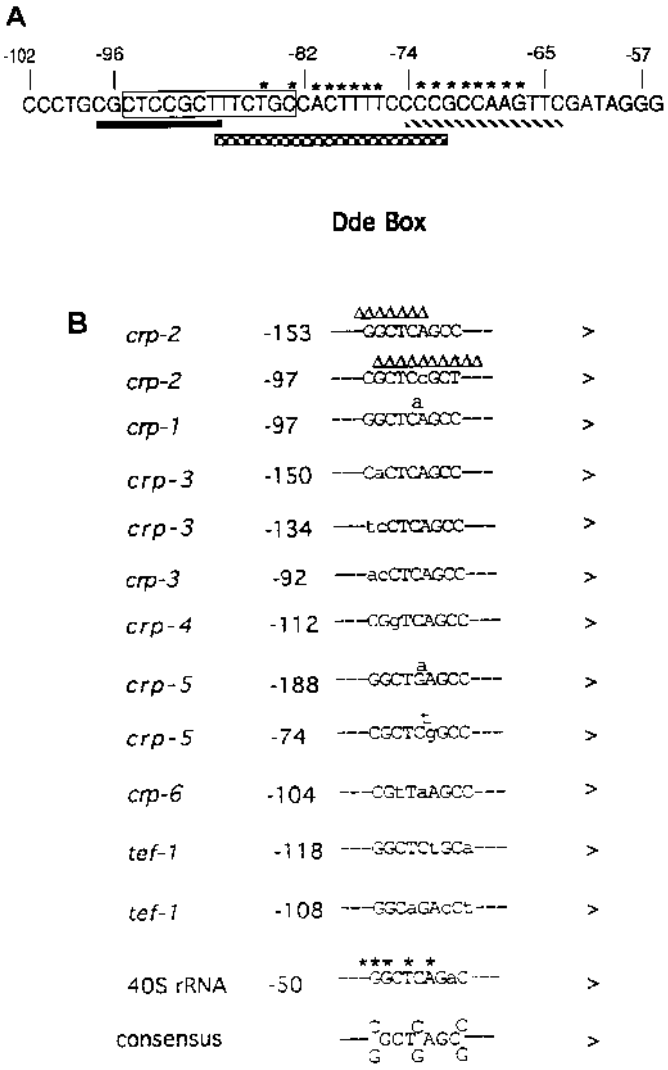

Figure 6. *Crp-2* promoter sequences required for transcriptional regulation during a carbon up- or down-shift (**A**). Outlined numbers represent *crp-2* promoter deletions in *N.crassa* isolates 246(pQaRp4Δ-95) and 246(pQaRp4Δ-65). Outlined letters represent the *crp-2* promoter element required for transcriptional regulation. Boxed and asterisked residues identify those required for transcription during exponential growth in sucrose media and correct transcription initiation respectively. Solid bar represents the –97 Dde box, the checked bar the –89 Ribo box and the stripped bar the –74 CG repeat.

growing in inositol-limiting media. In spite of this, *crp-1*, *crp-2* and *crp-2::qa-2* mRNA levels in these cultures were 66–83% of those in cultures having growth rates of 6 or 11 mg/h (Table 1). At 16.5 and 21 h, *40S* pre-rRNA levels in 246(pQaRp4-WT) were unaffected by inositol concentrations in the media. However, in 246(pQaRp4Δ-95), *40S* pre-rRNA levels were 3–4-fold higher under inositol-limiting concentrations.

DISCUSSION

In *N.crassa*, *40S* pre-rRNA and r-protein mRNA levels change rapidly following changes in carbon nutritional conditions (27–29). This fact, and the presence of conserved elements in the transcription regulatory regions of r-protein and rRNA genes suggest that transcriptional regulation may play an important role in the

Table 1. Effect of inositol concentration on *crp-1*, and *crp-2::aq1–2* mRNA and *40S*rRNA levels in *N.crassa*

Time[1]	16.5 h				21 h			
Isolate[2]	QaRp4-WT		QaRp4Δ-95		QaRp4-WT		QaRp4Δ-95	
Inositol (μg/ml)	50	0.7	50	0.7	50	0.7	50	0.7
RNA Levels[3]								
crp-1	1.23	1.37 (1.11)[4]	1.36	1.38 (1.01)	2.09	1.38 (0.66)	1.71	1.17 (0.68)
crp-2	2.79	3.24 (1.16)	3.07	3.31 (1.08)	4.42	2.94 (0.67)	3.80	2.57 (0.68)
crp-2::qa-2	1.09	1.35 (1.24)	0.29	0.32 (1.10)	1.48	1.23 (0.83)	0.29	0.21 (0.72)
40S rRNA	0.81	0.91 (1.12)	0.75	2.43 (3.24)	0.91	1.02 (1.12)	0.47	1.91 (4.06)

[1]Represents number of hours following transfer of mycelia to media containing the indicate inositol concentration.
[2]Isolate designatins are as in Figure 1A.
[3]Values represent relative RNA levels after standardization to β-tubulin transcript levels.
[4]Values in parentheses represent mRNA or rRNA levels in 0.7 μg/ml inositol relative to those in 5 μg/ml inositol.

expression of ribosomal genes in *N.crassa* (29,40,42). As a first step in gaining a better understanding of how ribosomal genes in *N.crassa* are regulated at the transcriptional level, rRNA and r-protein mRNA levels were compared in cultures having different growth rates. Growth rates were varied either by shifting cultures from one carbon source to another, or by subjecting them to inositol-limiting concentrations. In addition, elements required for transcriptional regulation *in vivo* were identified using mutations in the promoter of the r-protein gene, *crp-2*.

This study demonstrated that r-protein mRNA levels in *N.crassa* are stoichiometrically coordinated during a carbon upshift from glycerol to sucrose, or a downshift from sucrose to glycerol. During the carbon shifts, *40S* pre-rRNA levels changed coordinately with r-protein mRNA levels. However, *40S* pre-rRNA levels appeared more responsive to the quality of the carbon nutrient source than r-proteins genes. The lack of stoichiometry between r-protein mRNA and rRNA levels may reflect the nature of the assays (transient pre rRNA versus mRNA), or the possibility that post-transcriptional mechanisms may also be important in balancing the expression of ribosomal genes.

Transcription of the *crp-2::qa-2* fusion gene in 246(pQaRp4-WT) is entirely dependent upon the *crp-2* promoter during exponential growth in sucrose media (Cujec and Tyler, submitted to *Mol. Gen. Genet.*). In the carbon up- and down-shift experiments described in this study, *crp-2::qa-2* mRNA levels in 246(pQaRp4-WT) were closely coordinated with those of the endogenous *crp-1* and *crp-2* genes. Thus, the 1.5 kbp of *crp-2* promoter sequences fused to the *qa-2* gene in 246(pQaRp4-WT) are sufficient to confer normal transcriptional regulation on the *qa-2* gene during carbon shifts. This observation also indicates that the observed changes in *crp-2* mRNA levels are unlikely to be due to differences in mRNA stability. This is supported by the observation that upstream promoter deletions, which do not affect the mRNA, abolished regulation.

In order to identify the carbon-responsive element(s) in the *crp-2* promoter, *N.crassa* isolates containing mutant *crp-2* promoter sequences were subjected to carbon shifts and the effects of these mutations on *qa-2* mRNA levels determined. Since the *crp-2* promoter is strong, it was possible to measure changes in mRNA levels even when a mutant promoter retained only 3–4% activity. *Crp-2* promoter sequences from –95 to –83 bp and from –73 to –66 bp were identified as being required for transcriptional regulation during carbon shifts. The region from –95 to –83 bp in the *crp-2* promoter matches the Dde box consensus sequence (–97 Dde box) while the region from –73 to –66 bp matches the –74 CG repeat consensus. Sequences homologous to the Dde box consensus element are also present in another functional upstream element in the *crp-2* promoter (–152 Dde box), as well as in the promoters of the six other *N.crassa* r-proteins sequenced to date, a translation elongation factor (*tef-1*) and in the 40S rRNA promoter (Fig. 6) (29,36,37,39,42; I. de la Serna and B. Tyler, unpublished). Deletion of the –152 Dde box in the *crp-2* promoter resulted in a 2-fold decrease in transcription during growth in sucrose (Cujec and Tyler, submitted to *Mol. Gen. Genet.*), while a substitution mutation in the Dde box of the 40S rRNA promoter caused a 7-fold drop in transcription *in vitro* (42). Together, these results suggest that *crp-2* promoter sequences required for optimal transcription under constant growth conditions (Dde box and CG repeat) are also required for transcriptional regulation during changes in nutritional sources. Consequently, the transcriptional regulation of the *crp-2* gene in *N.crassa* appears analogous to that of numerous r-protein genes in *S.cerevisiae*; the UAS_{rpg} element found in the promoters of most yeast r-protein genes is required for transcription under optimal growth condi-

tions as well as following a nutritional downshift (7,8,13,14,18). Efforts are presently underway to identify the protein(s), which might bind the –97 Dde box and the –74 CG repeat and to determine how they interact with the basal transcription machinery to regulate transcription under constant, as well as changing nutritional conditions.

In *S.cerevisiae* and *N.crassa*, changing carbon nutritional conditions affect the regulation of r-genes as well as genes involved in carbon metabolism (7,12,27,28). Different carbon sources also affect the growth rates of both organisms (1,18). Consequently, it is unclear whether changes in ribosomal gene transcript levels following a carbon shift are due to changes in growth rate which in turn alter transcription rates, or whether the signaling pathways which normally detect changes in carbon status interact directly with the transcription machinery responsible for r-gene expression. One way to address this question is to vary the growth rates of *N.crassa* without altering the carbon status of the media. The cofactor, inositol is involved in membrane structure and has no nutritional value (49). Consequently in these experiments, growth rates were varied by adjusting inositol concentrations in the presence of a constant source of carbon (1.5% sucrose).

Inositol starvation caused a 3–6-fold reduction in the growth rates of 246(pQaRp4-WT) and 246(pQaRp4Δ-95). However, despite the differences in growth rates, the r-protein mRNA and *40S* pre-rRNA levels were similar. The unresponsiveness of r-protein mRNA and *40S* pre-rRNA levels to different growth rates suggests that transcription is dependent upon the carbon source in the media and not on the growth rate of the culture. This is further supported by the observation that sharp drops in r-protein and rRNA expression only occur during a downshift from sucrose to glycerol, but not from glycerol to no carbon source (R. Ballica, I. de la Serna and B. Tyler, unpublished). The fact that the difference in *crp-2::qa-2* mRNA levels between transformants 246(pQaRp4-WT) and 246(pQaRp4Δ-95) remained similar regardless of inositol concentrations suggests that the *crp-2* promoter does not have a regulatory element upstream of –95 bp which is specifically required for transcription during slow growth.

ACKNOWLEDGEMENTS

We thank Eva Steinberger for performing some of the oligonucleotide mutagenesis, Felipe Arredondo for oligonucleotides and assistance in screening transformants and Jeff Hall for photography. This work was supported by National Institutes of Health grant R01 GM42178.

REFERENCES

1 Alberghina, L. and Sturani, E. (1981) *Microbiol. Rev.* **45**, 99–122.
2 Warner, J.R. (1989) *Microbiol. Rev.* **53**, 256–271.
3 Dennis, P.P. and Bremer, H. (1974) *J. Mol. Biol.* **89**, 233–239.
4 Maaloe, O. and Kjeldgaard, W.O. (1966) *Control of Macromolecular Synthesis. A study of DNA, RNA and Protien Synthesis in Bacteria.* W.A. Benjamin, New York.
5 Waldron, C. and Lacroute, F. (1975) *J. Bacteriol* **122**, 855–865.
6 Nomura, M., Gouse, R. and Baughmen, G. (1984) *Ann. Rev. Biochem.* **53**, 75–117.
7 Donovan, D.M. and Pearson, N.J. (1986) *Mol. Cell. Biol.* **6**, 2429–2435.
8 Herruer, M.H., Mager, W.H., Woudt, L.P., Nieuwint, R.T.M., Wassenaar, G.M., Groeneveld, P. and Planta, R.J. (1987) *Nucleic Acids Res.* **15**, 10133–10144.
9 Morrow, B.E., Johnson, S.P. and Warner, J.R. (1993) *Mol. Cell. Biol.* **13**, 1283–1289.
10 Kraig, E., Haber, J.E. and Roshash, M. (1982) *Mol. Cell. Biol.* **2**, 1199–1204.
11 Warner, J.R. and Gorenstein, C. (1978) *Nature* **275**, 338–339.
12 Kief, D.R. and Warner, J.R. (1981) *Mol. Cell. Biol.* **1**, 1007–1015.
13 Mager, W.H. and Planta, R.J. (1991) *Mol. Cell. Biochem.* **104**, 181–187.
14 Nieuwint, R.T.M., Mager, W.H., Maurer, K.C.T. and Planta, R.J. (1989) *Curr. Genet.* **15**, 247–251.
15 Shore, D. and Nasmyth, K. (1987) *Cell* **51**, 721–732.
16 Huet, J., Cottrell, P., Cool, M., Vignais, M.-L., Thiele, D., Marck, C., Buhler, J.-M., Sentenac, A. and Fromageot, P. (1985) *EMBO J.* **4**, 3539–3547.
17 Buchman, A.R., Kimmerly, W.J., Rine, J. and Kornberg, R.D. (1988) *Mol. Cell. Biol.* **8**, 210–225.
18 Kraakman, L.S., Griffioen, G., Zerp, S., Groeneveld, P., Thevelein, J.M., Mager, W.H. and Planta, R.J. (1993) *Mol. Gen. Genet.* **239**, 196–204.
19 Dabeva, M.D., Post-Bettenmiller, M.A. and Warner, J.R. (1986) *Proc. Natl Acad. Sci. USA* **83**, 5854–5857.
20 Maicas, E., Pluthero, F.G. and Friesen, J.D. (1988) *Mol. Cell. Biol.* **8**, 169–175.
21 Warner, J.R., Mitra, G., Schwindenger, W.F., Studeny, M. and Fried, H.M. (1985) *Mol. Cell. Biol.* **5**, 1512–1521.
22 Hariharan, N., Kelley, D.E. and Perry, R.P. (1989) *Genes Dev.* **3**, 1789–1800.
23 Hariharan, N. and Perry, R.P. (1989) *Nucleic Acids Res.* **17**, 5323–5337.
24 Hariharan, N. and Perry, R.P. (1990) *Proc. Natl Acad. Sci. USA* **87**, 1526–1530.
25 Mariottini, P. and Amaldi, F. (1990) *Mol. Cell. Biol.* **10**, 816–822.
26 Kay, M.A. and Jacobs-Lorena, M. (1985) *Mol. Cell. Biol.* **5**, 3583–3592.
27 Sturani, E., Costantini, M.G., Zippel, R. and Alberghina, F.A.M. (1976) *Exper. Cell Res.* **99**, 245–252.
28 Sturani, E., Magnani, F. and Alberghina, F.A.M. (1973) *Biochimica et Biophysica Acta.* **319**, 165–173.
29 Shi, Y. and Tyler, B.M. (1991) *Nucleic Acids Res.* **19**, 6511–6517.
30 Geever, R.F., Huiet, L., Baum, J.A., Tyler, B.M., Patel, V.B., Rutledge, B.J., Case, M.E. and Giles, N.H. (1989) *J. Mol. Biol.* **207**, 15–34.
31 Fu, Y.H. and Marzluf, G.A. (1990) *Proc. Natl Acad. Sci. USA* **87**, 5331–5335.
32 Kang, S. and Metzenburg, R.L. (1993) *Genetics* **133**, 193–202.
33 Metzenberg, R.L. (1979) *Microbiol. Rev.* **43**, 361–383.
34 Paietta, J.V. (1992) *Mol. Cell. Biol.* **12**, 1568–1577.
35 Ichi-Ishi, A. and Inoue, H. (1995) *Jpn. J. Genet.* **70**, 273–287.
36 Kreader, C.A. and Heckman, J.E. (1987) *Nucleic Acids Res.* **15**, 9027–9042.
37 Tarawneh, K.A., Anumula, K.R. and Free, S.J. (1994) *Gene* **147**, 137–140.
38 Tyler, B.M. and Harrison, K. (1990a) *Nucleic Acids Res.* **18**, 5759–5765.
39 Wang, Z., Tarawneh, K.A. and Free, S.J. (1993) *Curr. Genet.* **23**, 330–333.
40 Tyler, B.M. (1987) *J. Mol. Biol.* **196**, 801–811.
41 Cujec, T.P. (1994) Effects of promoter mutations and changing nutritional conditions on ribosomal protein mRNA and rRNA levels in *Neurospora crassa*. Ph.D. Thesis. University of California, Davis.
42 Tyler, B.M. (1990) *Nucleic Acids Res.* **18**, 1805–1811.
43 Maniatis, T., Fritsch, E.F. and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
44 Case, M.E., Schweizer, M., Kushner, S.R. and Giles, N.H. (1979) *Proc. Natl Acad. Sci. USA* **76**, 5259–5263.
45 Patel, V.B., Schweizer, M., Dybstra, C.C., Kushner, S.R. and Giles, N.H. (1981) *Proc. Natl Acad. Sci. USA* **78**, 5783–5787.
46 Tyler, B.M., Geever, R.F., Case, M.E. and Giles, N.H. (1984) *Cell* **36**, 493–502.
47 Orbach, M.J., Porro, E.B. and Yanofsky, C. (1986) *Mol. Cell. Biol.* **6**, 2452–2461.
48 Held, W.A., Ballou, B., Mizushima, S. and Nomura, M. (1974) *J. Biol. Chem.* **249**, 3103–3111.
49 Fuller, R.C. and Tatum, E.L. (1956) *Amer. J. Bot.* **43**, 361–365.