UC Irvine
UC Irvine Previously Published Works

Title
Polyamine Regulation of Ornithine Decarboxylase Synthesis in Neurospora crassa

Permalink
https://escholarship.org/uc/item/1nv2r039

Journal
Molecular and Cellular Biology, 20(8)

ISSN
0270-7306

Authors
Hoyt, Martin A
Broun, Mariya
Davis, Rowland H

Publication Date
2000-04-01

DOI
10.1128/mcb.20.8.2760-2773.2000

Copyright Information
This work is made available under the terms of a Creative Commons Attribution License, availalbe at https://creativecommons.org/licenses/by/4.0/

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Molecular and Cellular Biology, Apr. 2000, p. 2760–2773 Vol. 20, No. 8
0270-7306/00/$04.00+0
Copyright © 2000, American Society for Microbiology. All Rights Reserved.

Polyamine Regulation of Ornithine Decarboxylase Synthesis in *Neurospora crassa*

MARTIN A. HOYT,† MARIYA BROUN,‡ and ROWLAND H. DAVIS*

Department of Molecular Biology and Biochemistry, University of California Irvine, Irvine, California 92697-3900

Received 23 November 1999/Returned for modification 20 December 1999/Accepted 21 January 2000

Ornithine decarboxylase (ODC) of the fungus *Neurospora crassa*, encoded by the *spe-1* gene, catalyzes an initial and rate-limiting step in polyamine biosynthesis and is highly regulated by polyamines. In *N. crassa*, polyamines repress the synthesis and increase the degradation of ODC protein. Changes in the rate of ODC synthesis correlate with similar changes in the abundance of *spe-1* mRNA. We identify two sequence elements, one in each of the 5′ and 3′ regions of the *spe-1* gene of *N. crassa*, required for this polyamine-mediated regulation. A 5′ polyamine-responsive region (5′ PRR) comprises DNA sequences both in the upstream untranscribed region and in the long 5′ untranslated region (5′-UTR) of the gene. The 5′ PRR is sufficient to confer polyamine regulation to a downstream, heterologous coding region. Use of the β-tubulin promoter to drive the expression of various portions of the *spe-1* transcribed region revealed a 3′ polyamine-responsive region (3′ PRR) downstream of the coding region. Neither changes in cellular polyamine status nor deletion of sequences in the 5′-UTR alters the half-life of *spe-1* mRNA. Sequences in the *spe-1* 5′-UTR also impede the translation of a heterologous coding region, and polyamine starvation partially relieves this impediment. The results show that *N. crassa* uses a unique combination of polyamine-mediated transcriptional and translational control mechanisms to regulate ODC synthesis.

Ornithine decarboxylase (ODC) catalyzes an initial, rate-limiting reaction in the biosynthesis of the polyamines, the conversion of the amino acid ornithine to the divalent polyamine putrescine (1,4-diaminobutane). Putrescine undergoes two subsequent aminopropyl transfer reactions, in which it is converted first to the trivalent spermidine and then to the tetravalent spermine. Polyamines are essential for the growth of normal, and particularly neoplastic, cell types (25, 38), but excess spermidine and spermine are toxic (8, 28).

ODC is a one of the most highly regulated enzymes of eukaryotic cells, its activity varying over a 100-fold range. ODC activity responds to extracellular signals such as mitogens and growth factors and to changes in the intracellular concentrations of the polyamines themselves. Polyamine regulation of ODC activity is unusual in that the end products do not act as allosteric effectors of this initial enzyme but control only the synthesis and degradation of the ODC protein (reviewed in references 6 and 41).

In the filamentous fungus *Neurospora crassa*, ODC is encoded by the *spe-1* gene (3, 9). The regulation of ODC in this fungus resembles that in other eukaryotic organisms. Polyamines reduce the rate of synthesis and increase the rate of degradation of ODC protein (1). However, unlike all other eukaryotes in which polyamine-mediated regulation has been studied, changes in the rate of synthesis ODC in *N. crassa* are correlated with similar changes (ca. 10-fold) in the abundance of *spe-1* mRNA (43). Previously, we identified two regions of the *spe-1* gene that affect its expression (27). An upstream activation region (UAR) was required for normal expression. Its elimination reduced *spe-1* mRNA abundance approximately fivefold, with some loss of regulation by polyamines. The second region was a 473-bp *Afl*III-*Nru*I segment of the region encoding the long 5-untranslated region (5′-UTR) of the mRNA. Deletion of this segment resulted in high levels of *spe-1* expression and greatly diminished polyamine regulation.

Here we report a general analysis of sequences required for polyamine regulation of *spe-1* mRNA and ODC activity in *N. crassa*. We show that regulation of *spe-1* mRNA involves interactions between sequence elements in the DNA encoding the 5′-UTR of the mRNA and regions upstream of or overlapping the start of transcription, as well as an independently acting 3′ activator element. In addition, we identify an element in the *spe-1* 5′-UTR that modestly regulates the translation of the ODC coding region in response to polyamines.

MATERIALS AND METHODS

***N. crassa* strains and growth conditions.** The *N. crassa* arginaseless strain IC3 (*aga*) was used as the wild-type control in this study. Plasmids were transformed into strains IC2747-22a (*his-3 aga*), IC54 (*spe-1*::*hph his-3 aga*), and IC2794-5 (*spe-1 inl his-3 aga*) as indicated below. The specific alleles of the genes carried by these strains were *aga(UM906), his-3(Y155M261), inl(89601),* and *spe-1(JP209)*. The isolation of the strain carrying a disrupted *spe-1* gene (*spe-1*::*hph*) is described below.

All strains carry the *aga* mutation, which eliminates arginase activity and renders the strains unable to catabolize arginine as a source of ornithine (5). The addition of 1 mM arginine to these strains leads to feedback inhibition of ornithine synthesis from glutamate and, because arginine catabolism is blocked, results in ornithine and polyamine starvation. Under these circumstances, ODC activity is highly derepressed and polyamine synthesis is confined to the formation of small amounts of the polyamine analogs cadaverine (1,4-diaminopentane) and aminopropylcadaverine (24). These analogs, formed by the inefficient decarboxylation of lysine by derepressed ODC activity (24) and subsequent aminopropylation, allow indefinite, slow growth of arginine-treated *aga* cultures (4).

N. crassa strains were grown and maintained by standard methods (2). Cultures were provided with 1 mM spermidine trihydrochloride to support the growth of *spe-1* mutant strains and in some cases to test the effect of polyamine supplementation. In other cases, 5 mM ornithine was added to restore cellular polyamines to arginine-grown cultures. Mutants deficient in inositol synthase (*inl*) were supplemented with 100 μg inositol per ml of culture. Cultures of *his-3* mutants were supplemented with 100 μg of histidine per ml.

Plasmid constructions and *N. crassa* transformation. (i) Nested deletions of the *spe-1* UAR. Plasmid pPHL2 (27) consists of a *Bgl*II-*Hin*dIII cassette contain-

* Corresponding author. Mailing address: Department of Molecular Biology and Biochemistry, University of California Irvine, Irvine, CA 92697-3900. Phone: (949) 824-5872. Fax: (949) 824-8551. E-mail: rhdavis@uci.edu.

† Present address: Department of Microbiology and Immunology, University of California San Francisco, San Francisco, CA 94143-0414.

‡ Present address: Department of Developmental and Cell Biology, University of California Irvine, Irvine, CA 92697-2300.

Downloaded from http://mcb.asm.org/ on August 31, 2017 by UNIV OF CALIFORNIA IRVINE

FIG. 1. Restriction map of the *spe-1* gene and flanking sequences found in plasmids pPHL1 and pPH1. The boxed area represents the transcribed region, beginning with the right-pointing arrow. The coding sequence, interrupted by one intron, is shown in black. Abbreviations: MCS, multiple-cloning site; B, *Bgl*II; V, *Eco*V; C, *Cla*I; R, *Eco*RI; P, *Pst*I; S, *Sac*I; A, *Afl*III; N, *Nru*I; K, *Kpn*I; Sa, *Sal*I; H, *Hin*dIII.

ing the entire *spe-1* gene (Fig. 1) in a pDE1-based transformation vector. This plasmid was used as starting material for 5′-to-3′ deletions from the *spe-1* upstream *Pst*I site using an exonuclease III/mung bean nuclease deletion kit (Stratagene) as specified by the manufacturer. The *Bgl*II site in the multiple-cloning site of the pDE1 vector was used to linearize the plasmid and then filled in with α-thiophosphate nucleoside triphosphates. Deletions were initiated from an adjacent *Eco*RI site (also in the pDE1 multiple-cloning site) immediately upstream of the *spe-1 Pst*I site. The blunt, deleted plasmid ends resulting from the exonuclease III/mung bean nuclease reactions were ligated with *Bgl*II linkers, digested with *Bgl*II, recircularlized, and used to transform *Escherichia coli* DH1 cells. A set of plasmids (see Fig. 5) carrying nested deletions of the *spe-1* 5′ region were identified by restriction analysis, and their 5′ ends were determined by manual sequencing with T7 primers using Sequenase version 2.0 (U.S. Biochemical Corp.) as specified by the manufacturer. These plasmids were used to transform strain IC2794-5.

(ii) *spe-1* 5′-UTR deletions. Deletions within the long (622-bp) *spe-1* 5′-UTR were constructed using convenient restriction sites: *Afl*III (at position +101 from the transcription start site), *Bsu*36I (+266), *Stu*I (+403), and *Nru*I (+574). The starting material for the deletions was plasmid pSS1 (27), containing the 1.3-kb *spe-1 Sac*I-*Sal*I region in a pSP72 vector (Promega), in which the *Afl*III site of the vector had been obliterated. The pSS1 plasmid was digested with two of the appropriate restriction endonucleases, and where necessary (*Afl*III and *Bsu*36I digests), overhanging ends were filled in using Klenow DNA polymerase. The doubly digested plasmid was recircularized, and the *Sac*I-*Sal*I fragment was cloned into *spe-1* sequences in pPH1 (27). The *spe-1* genes containing 5′-UTR deletions were cloned into pDE1 transformation vectors, creating plasmids pMH11 (*Afl*III-*Bsu*36I deleted), pMH12 (*Bsu*36I-*Stu*I deleted), pMH13 (*Stu*I-*Nru*I deleted), and pMH15 (*Afl*III-*Stu*I deleted). Construction of plasmid pDPH1 (*Afl*III-*Nru*I deleted) was described previously (27). These plasmids were used to transform strain IC54.

(iii) *spe-1*::*qa-2* constructs. For construction of *spe-1*::*qa-2* chimeric genes, a 0.7-kb *spe-1 Sac*I-*Nru*I fragment (containing most of the *spe-1* 5′-UTR) was joined to a 0.7-kb *Asp*718-*Bam*HI *qa-2* fragment from plasmid pMSK338 (32). The *qa-2 Asp*718 site lies 13 bp upstream of the initiation codon of the coding region of *N. crassa* catabolic dehydroquinase. The *Nru*I end of the *spe-1* fragment was blunt-end ligated to the *Asp*718 end of the *qa-2* fragment that had been filled by treatment with Klenow polymerase, and the *spe-1*::*qa-2* fragment was inserted into a *Sac*I-*Bam*HI-cut pSP72 vector to create plasmid pMH34. Similarly, a 262-bp *Sac*I-*Afl*III *spe-1* fragment (lacking most of the *spe-1* 5′-UTR) was blunt-end ligated to the *Asp*718-*Bam*HI *qa-2* fragment, after the *Afl*III and *Asp*718 ends had been filled in, and inserted into a *Sac*I-*Bam*HI-cut pSP72 vector to create plasmid pMH35. The upstream region of the *spe-1* gene was reconstituted in the *spe-1*::*qa-2* vectors by insertion of a 0.8-kb *Bgl*II-*Sac*I *spe-1* cassette (containing the UAR) from pPH1 into the *Bgl*II-*Sac*I-cut pMH34 and pMH35 by ligation, generating plasmids pMH36 and pMH37, respectively. The entire *spe-1* 5′-UTR was joined to the *qa-2* coding region at the translation initiation codon using gene splicing by overlap extension (15). The *spe-1* sequences were amplified as a 211-bp fragment from plasmid pSS1 using the sense *spe-1* primer MH1 (5′-CGTACCGACACCGACCCCCC-3′) and the antisense splice primer SQ2 (5′-ATGTGACGGGGGGACGCCATATCCCAAGATTTGACTG-3′). A 0.4-kb *qa-2* fragment was amplified from plasmid pMSK338 using the sense splice primer SQ1 (5′-CAGTCAAATCTTGGGATATGGCGTCCCCCCGTCACAT-3′) and the antisense *qa-2* primer Q1 (5′-CACATGAACCTCCACAAACG-3′). The PCR products from these reactions were then combined and used as templates for splice overlap extension PCR with primers MH1 and Q1. The product was attached to upstream *spe-1* sequences in plasmid pMH79. Transformation vectors were generated by ligation of *Bgl*II-*Hin*dIII cassettes containing the entire *spe-1*::*qa-2* genes from pMH36, pMH37, and pMH79 into *Bgl*II-*Hin*dIII-cut pDE1 vectors, generating plasmids pMH40, pMH41, and pMH82, respectively. These plasmids were used to transform strain IC2747-22a.

(iv) *tub*::*spe-1* constructs. The basic constructs used as starting material for construction of chimeric *tub*::*spe-1* genes (see Fig. 7 and 8 and Results) were generated by introducing a *Bam*HI linker 10 bp upstream of the *spe-1* initiation codon and 25 bp downstream of the 5′ end of the *tub* transcribed region (21), and an *Eco*RI linker immediately following the *spe-1* and *tub* termination codons. The *spe-1* coding region was amplified from plasmid pPH1 with primers MH8 (5′-TTATCACCCAGGATCCTCTTGGGATATGGTTATGCCGAC-3′) and MH9 (5′-AAGCTGCACGGAATTCTTACAATCCCAAGAGCGCCATAGC-3′), which introduced 5′ *Bam*HI and 3′ *Eco*RI sites (underlined), respectively. The *tub* 5′- and 3′-UTRs and vector sequences of plasmid pβT6, which carries the β-tubulin gene (20), were amplified with primers MH11 (5′-ACGCATCTTGGGATCCTGGTGATGACGAACACGGGTCTAT-3′) and MH10 (5′-CCTTGAGGGCGAATTCTAAATCATTCCACTCAACATTCAG-3′), which introduced 5′ *Bam*HI and 3′ *Eco*RI sites, respectively. The PCR products were digested with *Bam*HI and *Eco*RI, and the *spe-1* coding region was inserted between the *tub* untranslated sequences by ligation to create plasmid pMH25. Several subcloning steps followed to place a *Bgl*II site upstream of the *Sal*I site at the 5′ end of the *tub* promoter sequences to create plasmid pMH30. This plasmid consists of a *Bgl*II-*Hin*dIII cassette containing the *spe-1* coding region flanked by *tub* 5′- and 3′-UTRs in a pSP72-derived vector. The *Bgl*II-*Hin*dIII cassette from plasmid pMH30 was subcloned into the *Bgl*II-*Hin*dIII-cut pDE1 vector, generating the transformation vector pMH33. A *tub*::*spe-1* chimeric gene containing the *spe-1* coding and 3′ regions attached to the *tub* promoter and 5′-UTR was created by ligating a *spe-1 Not*I-*Hin*dIII fragment from pPH1 into the similarly cut pMH33 to create plasmid pMH42.

To create chimeric genes with the *tub* promoter attached to different lengths of the *spe-1* gene, *Bam*HI sites were introduced as linkers into the *spe-1* 5′-UTR at the *Afl*III or *Nru*I site. Plasmid pSS1 was digested with *Afl*III, and pSP3 (containing a 3.2-kb *spe-1 Sac*I-*Hin*dIII cassette) was digested with *Nru*I. The linearized plasmids were treated with Klenow polymerase, *Bam*HI linkers (Stratagene) were ligated onto the filled in ends, and the plasmids were digested with *Bam*HI and recircularized. The resulting plasmids were digested with *Bam*HI and *Dsa*I, and the resulting fragments, containing portions of the *spe-1* 5′-UTR and coding region, were used to replace the *Bam*HI-*Dsa*I *spe-1* sequences in pMH42. This resulted in plasmids containing the *tub* promoter fused to the *spe-1* 5′-UTR at the former positions of *Afl*III (in pMH45) and *Nru*I (in pMH47) sites, now replaced by *Bam*HI linkers. A *Bam*HI site (underlined) was introduced at the 5′ end of the *spe-1* transcribed region by PCR amplification with primer MH37 (5′-CAAGTCCAACCTACCTCTTGGATCCTTTCTCACCCTTCT-3′) and a downstream antisense *spe-1* primer GB9 (5′-TGCGGAGGAAAAGCTCGGCG-3′). The PCR product was digested with *Bam*HI and *Dsa*I, and the 0.7-kb *spe-1* fragment was used to replace *Bam*HI-*Dsa*I *spe-1* sequences in pMH42. The resulting plasmid, pMH86, contained the *tub* promoter fused to the major transcription start site (+1) of the *spe-1* gene. In plasmid pMH107, the *spe-1 Not*I-*Hin*dIII 3′ region of pMH86 was replaced with that of pMH30, described above, in which the *tub* 3′ region follows the *spe-1* coding region. These plasmids were used to transform strain IC54.

PCR method. PCRs used in cloning procedures were carried out using the Expand high-fidelity PCR system (Boehringer Mannheim). Each reaction mixture contained 100 pmol of each primer, 0.1 μg of template DNA, 200 nM each deoxyribonucleoside triphosphate, 2.5 U of Expand enzyme mix, and 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 mM KCl) in a 100-μl volume. The reactions were performed in a PTC-100 thermal controller (MJ Research) for 15 cycles (denaturing for 2 min at 94°C [4 min for the first cycle only], annealing for 2 min at 55°C, and extension for 2 min at 68°C). Products of the appropriate size were purified by agarose gel electrophoresis.

Transformation. Plasmid DNA (5 μg) was used for transformation of *N. crassa* spheroplasts as previously described (20). The pDE1-derived vectors, containing various *spe-1* constructs, were used to target the entire transforming plasmid DNA to the *his-3* locus. *N. crassa* strains carry the Y155M261 allele of *his-3*, which has a point mutation in the 3′ region of the coding region; the transforming plasmid lacks a 5′ region of the gene. A His^+ phenotype results only when the truncated *his-3* gene of the plasmid recombines homologously with the mutant allele in the recipient (see Fig. 3 in reference 27). Transformants were selected and screened as previously described (27) for those carrying single copies of the *spe-1* plasmids integrated at the *his-3* locus. This allowed *spe-1* expression of different transformants to be determined with the constructs in a common chromosomal context. *N. crassa* transformants were made using recipients carrying either the wild-type $spe\text{-}1^+$ allele or the disrupted *spe-1* gene described below.

Construction of a strain with a *spe-1* gene disruption (IC54, *spe-1*::*hph*). Plasmid pGS1 carries the 5.0-kb *Hin*dIII fragment that includes the entire *spe-1* gene (43). We replaced the 1.3-kb *Sac*I-*Sal*I segment, containing the *spe-1* promoter and transcription and translation starts with the bacterial hygromycin phosphotransferase gene (*hph*) in reverse orientation (Fig. 2A). The source of the *hph* gene was plasmid pCSN43 (36), in which the *hph* gene is transcribed from the *Aspergillus nidulans trpC* promoter. The 5.0-kb *spe-1*::*hph* insert of the resulting plasmid (pMB1) was used for homologous replacement of the endogenous *spe-1* gene of strain IC2747-22a (*his-3 aga*) by transformation and selection of hygromycin-resistant (Hyg^r) colonies followed by screening for those that were Spe^-. One transformant identified by Southern analysis that met the criteria for homologous replacement by the disrupted *spe-1* fragment was backcrossed to a *his-3 aga* strain of the opposite mating type, and a Spe^- Hyg^r isolate was selected and designated strain IC54. This strain behaves as expected with respect to diagnostic probes of its DNA (Fig. 2B), and it lacks detectable *spe-1* mRNA under repressing and derepressing conditions (results not shown).

Inhibition of RNA synthesis by thiolutin. Thiolutin, an inhibitor of all three RNA polymerases, has been used previously for measurements of mRNA half-life in *Saccharomyces cerevisiae* (14). Thiolutin was kindly provided to us by Pfizer, Inc., and a 1.5-mg/ml stock solution was prepared in dimethyl sulfoxide. The effects of thiolutin on the transcription of total cellular RNA were measured

Downloaded from http://mcb.asm.org/ on August 31, 2017 by UNIV OF CALIFORNIA IRVINE

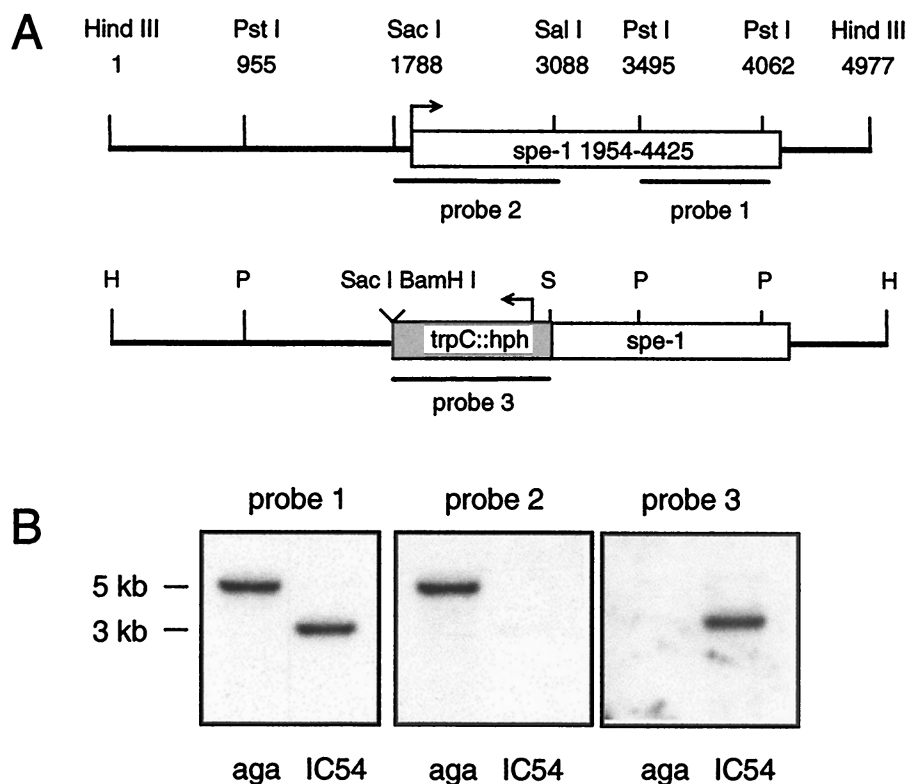

FIG. 2. Construction of a *spe-1*::*hph* deletion-insertion mutant. (A) Restriction maps of the wild-type *N. crassa spe-1* genomic region (top) and the *spe-1*::*hph* deletion-insertion allele (bottom). Boxes indicate the *spe-I* (open) and *trpC*::*hph* (shaded) transcribed regions. Arrows indicate the direction of transcription. Restriction fragments used to probe Southern and Northern blots are indicated. Distances from the 5′ *Hin*dIII site are given in base pairs. Abbreviations: B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I. (B) Southern blot of *N. crassa* genomic DNA from strains IC3 (*aga*) and IC54 (*spe-1*::*hph his-3 aga*) digested with *Bam*HI and *Hin*dIII and probed with the probes indicated in panel A.

by the addition of 4×10^{-2} μCi of [^{3}H]uridine and 50 nmol of cold uridine per ml of an *aga* culture (strain IC3, inoculated with 10^6 conidia per ml) during exponential growth in Vogel's minimal medium. The cells were labeled for 10 min before the addition of 0, 1.5, or 3 μg of thiolutin per ml of culture. Culture samples (5 ml) were added to an equal volume of ice-cold 10% trichloroacetic acid (TCA), and the cells were then collected and washed with cold 5% TCA on 5-μm-pore-size membrane filters (Micron Separations Inc.). The filters were boiled for 10 min in 5% TCA, and the acid-soluble radioactivity was determined using a scintillation counter. The effects of thiolutin on translation in strain IC3 were measured in similar cultures growing in the presence of 1.25×10^{-2} μCi of [^{35}S]methionine and 50 nmol of cold methionine per ml of culture. Cells were labeled for 10 min before thiolutin was added, as in the transcription experiments. At given times after thiolutin addition, 5-ml culture samples were collected on 5-μm membrane filters. The filters were washed with 5% cold TCA and counted.

***N. crassa* RNA analyses.** *N. crassa* total RNA was prepared as previously described (43), with minor modifications. Wet mycelial pads were collected by filtration, frozen at −80°C, lyophilized overnight, and powdered by vortexing in a 7- by 100-mm polypropylene tube with a spatula. Solubilization of RNA in later steps was routinely followed by a 2-min centrifugation step (10,000 × *g*) to remove insoluble material. RNA was resuspended and stored in deionized formamide.

Total RNA was denatured in 1× MOPS buffer–37% formaldehyde (2:15 [vol/vol]; 1× MOPS buffer is 200 mM morpholinepropanesulfonic acid [MOPS], 50 mM sodium acetate, and 10 mM EDTA [pH 7.0]) by heating to 65°C for 3 min and separated by electrophoresis on 6% formaldehyde–1% agarose gels made in 1× MOPS buffer. RNA was transferred to Nytran nylon membranes (Schleicher & Schuell) by capillary transfer using 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate) (31). The membranes were UV cross-linked as specified by the manufacturer and prehybridized in 50% deionized formamide–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–5× Denhardt's reagent–0.1% sodium dodecyl sulfate (SDS)–0.1 mg of sheared salmon testis DNA per ml (31). Hybridization was carried out in the same buffer overnight at 42°C using random-primed ^{32}P-labeled DNA probes. A 0.5-kb *Eco*RI-*Kpn*I fragment from pCS1 (to probe for *spe-1* mRNA [43]), a 1.2-kb *Sst*I fragment from pβT6 (for β-tubulin mRNA [20]), or a 0.7-kb *Asp*718-*Bam*HI fragment from plasmid pMSK338 (for *qa-2* mRNA [32]) were used as probes. Hybridized membranes were washed three times at room temperature for 5 min with 2× SSPE–0.1% SDS and three times at 65°C for 30 min with 0.1 × SSPE–0.1% SDS. Signals from hybridized membranes were visualized by autoradiography, and scanned autoradiographs were quantified by densitometry with Adobe Photoshop software. A standard series of autoradiographic signals was also scanned and quantified to determine the linear range of values obtainable by this method. Multiple autoradiographic exposures were quantified for each experiment, and only values within the linear range were used for mRNA determinations.

Primer extension analysis was carried out using the avian myeloblastosis virus reverse transcriptase primer extension system (Promega). Between 10 and 25 μg of total RNA in deionized formamide was precipitated with 4 volumes of ethanol and resuspended in a total volume of 11 μl of 1× primer extension (PE) buffer (50 mM Tris-HCl [pH 7.5], 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM spermidine, 1 mM each dATP, dCTP, dGTP, and dTTP) with 100 fmol of the end-^{32}P-labeled *spe-1* primer (5′-CCGTGTTAGATACGGTTGCC-3′). The primer and RNA were heated to 83°C for 5 min and annealed at 55°C for 20 min. The annealed primers were extended by the addition of 9 μl of the extension mix (final concentration, 1× PE buffer, 2.8 mM sodium pyrophosphate, and 1 U of avian myeloblastosis virus reverse transcriptase) and given a 30-min incubation at 42°C. Extension products were resolved on a denaturing 6% polyacrylamide gel and visualized by autoradiography.

Polysome analyses. Methods for polysome analysis were adapted from those previously described (44). Standard exponential cultures were treated with cycloheximide (final concentration, 50 μg/ml) 5 min prior to harvesting. Mycelia were homogenized in buffer (10 mM KCl, 10 mM $MgCl_2$, 30 mM triethanolamine, and 1 mM EGTA, treated with 0.01% diethylpyrocarbonate, and sterilized by autoclaving) in a 30-ml Bead Beater chamber (Biospec) with acid-washed 0.5-mm-diameter glass beads. Nuclei and mitochondria were removed from the homogenate by centrifugation at 12,000 × *g* for 10 min at 4°C. A volume of the supernatant containing 4 absorbance at 260 nm (A_{260}) units of the supernatant was layered on a 11.6-ml linear gradient (10 to 40% sucrose in 50 mM Tris-HCl [pH 7.6]–25 mM KCl–3 mM $MgCl_2$ treated with 0.01% diethylpyrocarbonate). Polysomal RNA was sedimented through the gradients by centrifugation in a Beckman SW41 Ti rotor at 35,000 rpm for 90 min at 4°C. The A_{254} profile was determined by pumping the gradient through a Gilson 111B UV detector. Approximately 0.5-ml fractions were collected from the bottom of the gradient into

Downloaded from http://mcb.asm.org/ on August 31, 2017 by UNIV OF CALIFORNIA IRVINE

microcentrifuge tubes containing 0.5 ml of isopropanol and precipitated in isopropanol overnight at −20°C.

Polysomal RNA was extracted from the precipitated fractions by the sequential addition of 100 μl of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 M β-mercaptoethanol), 10 μl 2 M sodium acetate, 100 μl of water-saturated phenol, and 20 μl of chloroform-isoamyl alcohol (48:1). Samples were mixed, and the phases were separated by centrifugation (3,000 × *g* for 10 min at 4°C). The aqueous phase was precipitated with 220 μl of isopropanol at −20°C. Precipitated RNA was resuspended in 10 μl of deionized formamide and stored at −80°C until analyzed by Northern blotting.

Enzyme assays. ODC activity was measured in duplicate 5-ml permeabilized cell samples by previously described methods (4, 43).

Catabolic 5-dehydroquinase activity was assayed in extracts of lyophilized mycelial powders as described by Hautala et al. (13). The lyophilized powders, in extraction buffer (0.1 M K^+PO_4 [pH 7.5], 0.4 mM dithiothreitol, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride) at a ratio of 1 g of powder per 20 ml of buffer, were resuspended on a vortex mixer and mixed further on an orbital shaker for 30 min at room temperature. The extract was cleared by centrifugation, and protamine sulfate was added to a final concentration of 0.14% and mixed for 15 min on an orbital shaker. The precipitated nucleic acids were removed by centrifugation, and the extract was then heated to 71°C for 10 min to inactivate biosynthetic dehydroquinase. The precipitated proteins were removed by centrifugation, and the supernatant was dialyzed against extraction buffer overnight at 4°C. Enzyme activity was measured in reaction mixtures containing 100 mM Tris-HCl (pH 7.4), 0.2 mM 5-dehydroquinic acid (kindly provided by M. E. Case), 0.2 mM EDTA, and 1/12 volume of dialyzed cell extract. Activity was measured as the rate of appearance of the product, 5-dehydroshikimic acid, by monitoring the change in A_{240}. Protein was determined by the method of Lowry et al. (18), using 100 μl of the cell extract.

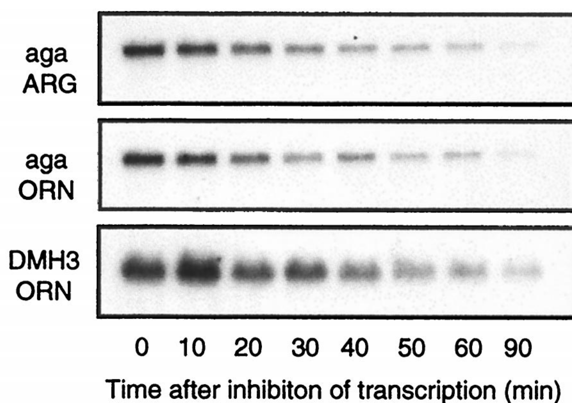

FIG. 3. Effects of polyamines on the stability of *spe-1* mRNA. Cultures of strain IC3 (*aga*) or transformant DMH3 (Δ5′-UTR) were starved for polyamines by growth on 1 mM arginine. Transcription was inhibited by the addition of thiolutin (final concentration, 1.5 μg/ml) at time zero. Incubation was continued in arginine-containing medium (ARG) or in cultures supplemented with ornithine (5 mM) to restore polyamine synthesis (ORN). Total RNA was extracted, and 10 μg from each time point was subjected to Northern blotting, using a 0.5-kb *Nru*I-*Sal*I fragment of a *spe-1* cDNA as a probe. rRNA was visualized on the gels prior to transfer with ethidium bromide to confirm equivalent sample loading.

RESULTS

As noted in the introduction, previous studies revealed that deletion of two regions of the *spe-1* gene, the UAR and a segment encoding the 5′-UTR of the mRNA, affect its expression. The results suggested that the UAR includes an enhancer-like element and that sequences in the 5′-UTR encode a negatively acting, polyamine-responsive regulatory element. Two questions might be asked regarding the mechanism of polyamine regulation of *spe-1* mRNA. (i) Because the polyamine-responsive element appears to reside within the transcribed region of the gene, is regulation exerted through changes in the stability of the mRNA? (ii) Are the sequences in the 5′-UTR able to impart polyamine regulation to other genes? We show here that although sequences in the 5′-UTR negatively affect expression, they are insufficient to impart polyamine regulation to reporter constructs in the absence of upstream *spe-1* sequences. Furthermore, we demonstrate that in the absence of any upstream untranscribed and 5′-UTR *spe-1* sequences (hereafter collectively referred to as the 5′ polyamine-responsive region [5′ PRR]), a 3′ polyamine-responsive region (3′ PRR) remains. The 3′ PRR was identified using a heterologous promoter driving the expression of *spe-1* coding and 3′ regions. The effects of the 3′ PRR are obscured by high levels of expression in *spe-1* genes lacking regulatory elements in the 5′-UTR.

Effects of polyamines and 5′-UTR sequences on the stability of *spe-1* mRNA. Because we had previously identified elements affecting the expression of *spe-1* mRNA within the *spe-1* transcribed region, we asked whether these elements, or polyamines themselves, altered the stability of *spe-1* mRNA. To determine the half-life of cytoplasmic *spe-1* mRNA, we inhibited transcription with the drug thiolutin at 1.5 μg/ml in exponential-phase cultures. This concentration inhibited total-RNA synthesis by 75% and inhibited translation by 20%. Higher concentrations of thiolutin were more effective in inhibiting transcription. However, they also inhibited translation to an extent that might seriously compromise the degradation of short-lived mRNAs (30). The residual transcription at the lower concentration of thiolutin should not affect our conclusions, since our interest is in differences in the rate of mRNA decay, rather than the true half-life.

The inhibition of transcription in a polyamine-starved (arginine-grown) culture of the *aga* strain (IC3) resulted in the disappearance of *spe-1* mRNA with a half-life of approximately 15 min (Fig. 3, top panel). To determine the effects of polyamines, ornithine was added to an arginine-grown culture following the inhibition of transcription in these experiments. Normally, supplementation of the growth medium from inoculation with spermidine is sufficient to repress ODC activity (27). However, cellular polyamine pools are restored only slowly by the direct addition of spermidine to the growth medium of starved cultures, owing to poor uptake (4, 7). The addition of ornithine leads to its rapid conversion to excess putrescine and spermidine by the derepressed ODC in these cells (23). Restoration of polyamine synthesis by this method had no effect on the turnover of *spe-1* mRNA compared to that in the polyamine-starved culture (Fig. 3, middle panel). Similar experiments were carried out with cultures of a transformant (DMH3) in which the *Afl*III-*Nru*I region of the *spe-1* 5′-UTR had been deleted and in which *spe-1* expression was highly derepressed even in the presence of excess ornithine (27). The stability of the mutant mRNA was similar to that of the normal transcript in both the presence and absence of polyamines despite the deletion of the 5′-UTR sequences (Fig. 3, bottom panel). We conclude that polyamine regulation does not involve changes in the stability of cytoplasmic *spe-1* mRNA, since neither excess polyamine levels nor removal of the negatively acting 5′-UTR sequence altered the rate of *spe-1* mRNA turnover.

Determination of *spe-1* mRNA transcription start sites. Previous S1 endonuclease protection experiments (43) indicated that *spe-1* mRNA transcription was initiated at three adjacent sites, 535, 534, and 533 nucleotides upstream of the first ATG in the transcribed region. We carried out primer extension analysis to confirm this result, using an antisense *spe-1* primer with a 5′ end located 87 nucleotides downstream from the previously determined *spe-1* transcription start site. Primer extension failed to detect the 5′ end determined by S1 nuclease protection in both the control IC3 strain (Fig. 4A, lanes 2 and

Downloaded from http://mcb.asm.org/ on August 31, 2017 by UNIV OF CALIFORNIA IRVINE

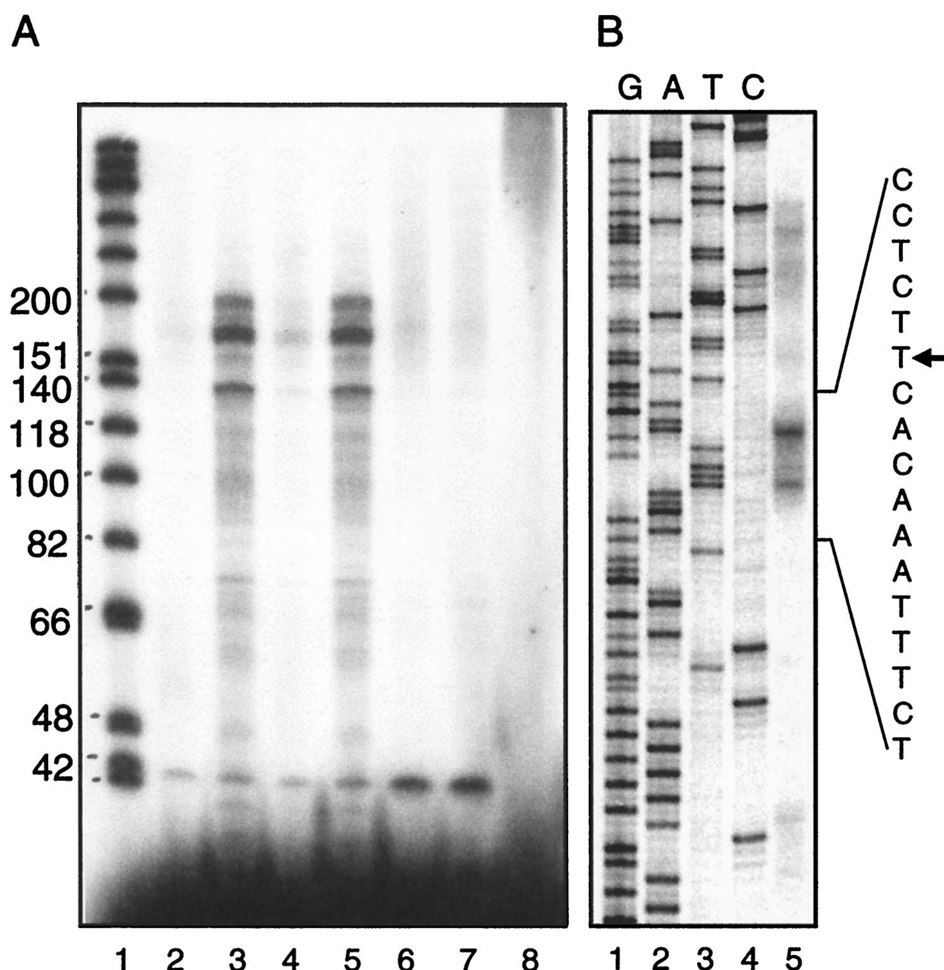

FIG. 4. Determination of the 5′ ends of *spe-1* transcripts by primer extension analysis. (A) Total RNA was isolated from cultures of strains IC3 (*aga*), IC54 (*spe-1*::*hph his-3 aga*), and transformant DMH1, supplemented as indicated below with 1 mM spermidine (SPD), 1 mM arginine (ARG), or 100 μg of histidine per ml (HIS). Total RNA (20 μg) was analyzed by primer extension analysis using the MH12 primer. Lanes: 1, φX174/*Hin*cII molecular weight marker; 2, IC3/SPD; 3, IC3/ARG; 4, DMH1/SPD; 5, DMH1/ARG; 6, IC54/SPD+HIS; 7, IC54/HIS; 8, no RNA. (B) The 5′ ends of the extension products from total RNA of the IC3 strain, grown in the presence of arginine, were determined by comparing primer extension products (lane 5) to dideoxynucleotide chain termination sequencing reactions (lanes 1 to 4) with a *spe-1* DNA template using the same primer. The sequence of the sense DNA strand is given to the right, with the major extension product indicated by the arrow.

3) and a transformant (DMH1) carrying a normal copy of the *spe-1* gene at the *his-3* locus (lanes 4 and 5). Instead of the expected ca. 87-nucleotide transcript extension product, transcripts with 5′ ends ranging in size from about 140 to 200 nucleotides were found. The major product was 173 nucleotides. We calculated that this product belonged to a transcript whose 5′ end was 622 nucleotides upstream from the first ATG in the *spe-1* transcribed region (Fig. 4B). We arbitrarily designate the 5′ end of this transcript the actual start site (+1) of the *spe-1* transcript. Other, less abundant transcripts that initiated 5′ and 3′ to the major transcript were also seen. The conclusion that the primer extension analysis detected bona fide *spe-1* transcripts was based on the following observations. (i) In both the IC3 and DMH1 strains, the abundance of the extension products was derepressed by polyamine starvation (Fig. 4A, compare lane 2 with lane 3 and compare lane 4 with lane 5). (ii) No extension products were detectable in the IC54 strain, in which the corresponding *spe-1* sequences were been deleted (lanes 6 and 7). (iii) Finally, we detected sequences corresponding to the new 5′ end of the *spe-1* transcripts independently by reverse transcription-PCR analysis (results not shown).

Effects of upstream sequences on *spe-1* expression and polyamine regulation. A *Hin*dIII genomic fragment (GenBank accession no. L16920) includes a *Pst*I-*Hin*dIII region sufficient for normal expression of the *spe-1* gene (27). Deletion of sequences between the upstream *Pst*I site, 1,000 bp 5′ of the transcription start site, and a *Sac*I site 167 bp 5′ of +1 (a region defined as the UAR) leads to a three- to fivefold reduction in mRNA abundance without eliminating its regulation by polyamines (27). To better define upstream sequences affecting expression, a series of transformants carrying 5′-to-3′ nested deletions extending from the upstream *Pst*I site to a site downstream of the *spe-1* transcription start site was constructed (Fig. 5A). The *spe-1* constructs carrying these deletions were targeted by transformation to the *his-3* locus of strain IC2794-5, which lacked detectable ODC activity, in earlier studies. No *spe-1* mRNA or ODC activity was detectable in the untransformed recipient strain (a chromosomal aberration mutant), even in polyamine-starved cultures. The P2 transformant in this series, carrying the full-length UAR, had somewhat (but reproducibly) lower ODC activity and *spe-1* mRNA than did comparable transformants carrying the same plasmid in the IC54 ODC-null strain. However, the effect of deleting the

Downloaded from http://mcb.asm.org/ on August 31, 2017 by UNIV OF CALIFORNIA IRVINE

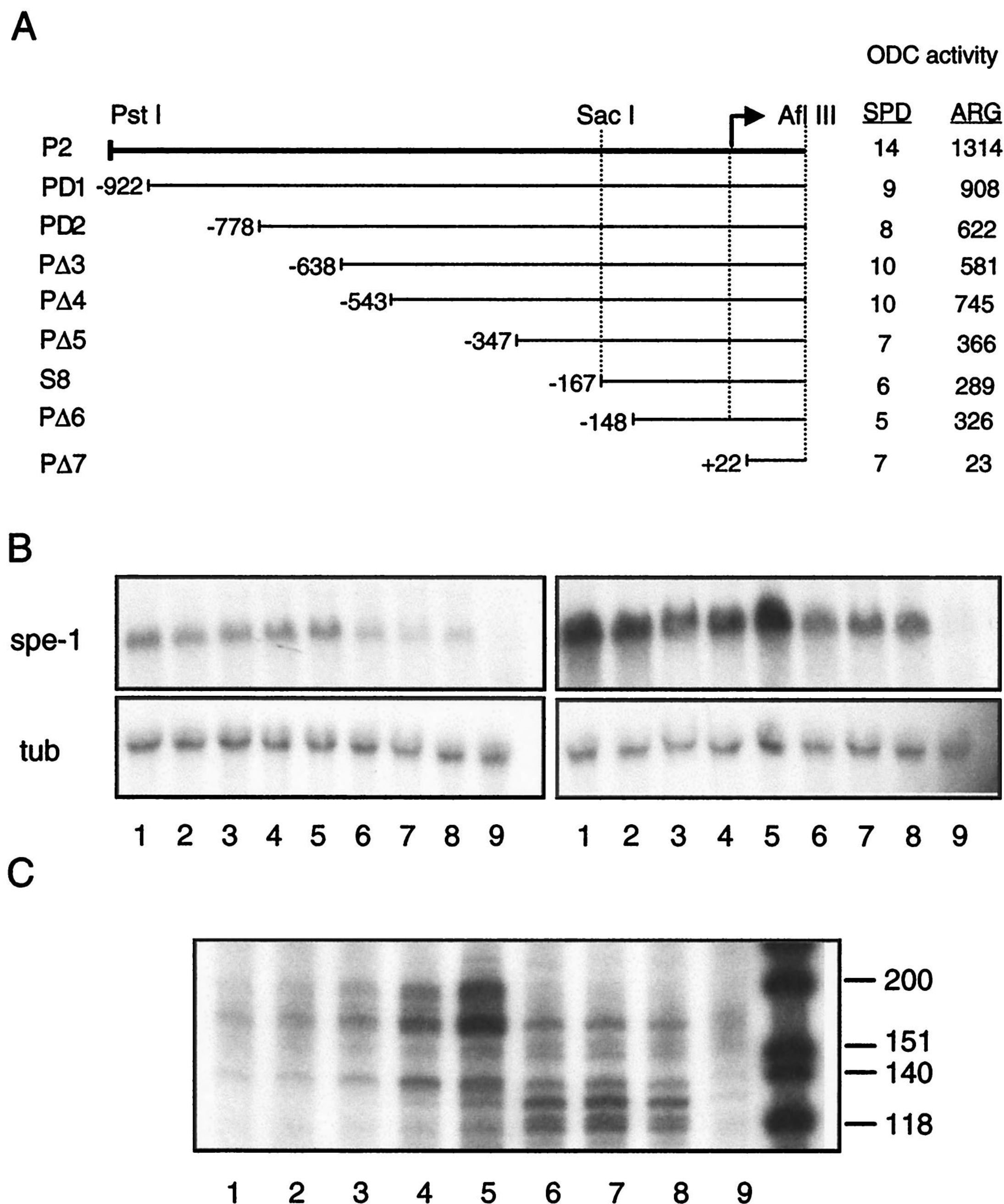

FIG. 5. Effects of 5′-to-3′ deletions of the *spe-1* upstream region on ODC activity and derepression of *spe-1* mRNA. (A) Schematic representation of the wild-type (P2) and deleted *spe-1* genes integrated at the *his-3* locus of strain IC2794-5. Distances from the major transcription start site, indicated by the arrow, are given in base pairs. The relative positions of the *Pst*I (−1000), *Sac*I (−167), and *Afl*III (+97) sites are also shown. ODC activity (in units per milligram of protein) of the transformants grown with 1 mM spermidine (SPD) or 1 mM arginine (ARG) are given to the right. (B) Northern blots of total RNA (10 μg) from repressed (left) and derepressed (right) cultures of these transformants were probed with *spe-1* cDNA (*spe-1*) or a fragment of the β-tubulin gene (*tub*), the latter as a loading control. Lanes: 1, P2; 2, PΔ1; 3, PΔ2; 4, PΔ3; 5, PΔ4; 6, PΔ5; 7, S8; 8, PΔ6; 9, PΔ7. (C) Approximately 25 μg of total RNA from the derepressed transformants was analyzed by primer extension reactions with the MH12 primer to determine the 5′ ends of their *spe-1* transcripts. The molecular size marker on the right is given in nucleotides. Lanes are labeled as in panel B.

entire *Pst*I-*Sac*I sequence of the *spe-1* gene on ODC activity and mRNA abundance (approximately fivefold decrease in polyamine-starved cultures) in the two types of transformant was similar.

In the deletion series, both ODC activity and *spe-1* mRNA abundance diminished to similar extents in transformants as more of the upstream region of the *spe-1* gene was removed (Fig. 5A and B), confirming previous evidence (27). The deletion of sequences upstream of −778 (in transformant PΔ2 [Fig. 5B, lane 3]) led to a ca. 50% decrease in *spe-1* expression in the polyamine-starved cultures; further deletion to −347 (transformant PΔ5 [lane 6]) reduced expression to 25% of the control. Similar decreases in the ODC activity and *spe-1* mRNA of these transformants occurred in spermidine-supplemented cul-

Downloaded from http://mcb.asm.org/ on August 31, 2017 by UNIV OF CALIFORNIA IRVINE

tures. The deletion that overlapped the *spe-1* transcription start site (transformant PΔ7 [lane 9]) almost completely eliminated expression under both growth conditions. In all transformants carrying upstream deletions, there was some derepression of *spe-1* mRNA abundance in cells starved for polyamines. However, the deletion of the UAR clearly reduced the amplitude of the response to polyamines (compare the effect of polyamines on transformants P2 [lane 1] and S8 [lane 7]).

Primer extension was used to determine the 5′ ends of *spe-1* transcripts in transformants with these upstream deletions. Removal of upstream sequences had no effect on transcription start site selection until sequences between −543 and −347 were eliminated (transformant PΔ5 [Fig. 5C, lane 6]). The deletion of this region greatly reduced initiation from the normal start site and led to the appearance of new transcripts with 5′ ends 40 to 60 nucleotides downstream (lanes 6 to 8). This change in the site of transcript initiation occurred although the original start site remained intact. The deletion that overlapped +1 (in transformant PΔ7 [lane 9]) eliminated all transcripts detectable with the primer.

The *spe-1* UAR and 5′-UTR impart polyamine regulation to a heterologous coding and 3′ region. We next asked whether the UAR and 5′-UTR of the *spe-1* gene would confer polyamine regulation to a heterologous downstream reporter gene. We attached various portions of the *spe-1* 5′ region to the coding and 3′ region of the *N. crassa qa-2* gene (Fig. 6A) and determined the effects of polyamines on expression of the chimeric gene. The *qa-2* gene encodes catabolic dehydroquinase, an enzyme involved in quinic acid utilization, and *qa-2* mRNA is undetectable in wild-type strains in the absence of quinic acid supplementation (22). In quinate-induced cultures, manipulation of cellular polyamine levels had no effect on *qa-2* expression (data not shown).

In transformant SMH13 (Fig. 6A), *spe-1* sequences upstream of the *Afl*III site (at +101) in the 5′-UTR were fused to the *qa-2* coding and 3′ region. Polyamines had little or no effect on expression of the *spe-1*::*qa-2* mRNA detected by a *qa-2* probe, in this transformant (Fig. 6B). If the chimeric gene also included the *spe-1 Afl*III-*Nru*I region (in transformant SMH12), expression of the resulting *spe-1*::*qa-2* mRNA was reduced in spermidine-supplemented cultures but was derepressed threefold upon polyamine starvation. The effects of polyamines on *spe-1*::*qa-2* mRNA in this transformant were modest but repeatable and were always greater than in the transformant lacking the *Afl*III-*Nru*I sequences. The further inclusion of sequences between the *Nru*I site and the *spe-1* translation initiation codon (at +623) in transformant SMH41 resulted in slight increases of *spe-1*::*qa-2* mRNA abundance in both polyamine-supplemented and starved cultures compared to the SMH12 transformant and in a slight increase in the regulatory amplitude.

Transformants carrying the *spe-1*::*qa-2* chimeric gene integrated at the *his-3* locus also contained a functional *spe-1* gene at its normal chromosomal location. This allowed us to compare the expression of the *spe-1* and *spe-1*::*qa-2* genes in transformant SMH41 by Northern analysis with probes of the 5′ *spe-1* transcribed sequences. In a polyamine-starved culture, expression of the *spe-1*::*qa-2* mRNA is much lower than that of the endogenous *spe-1* gene (Fig. 6C, 0 min). We measured the turnover of *spe-1*::*qa-2* mRNA using thiolutin and compared it to that of *spe-1* mRNA in the same transformant (Fig. 6C). Both mRNAs disappeared with similar half-lives, approximately 15 min, indicating that changes in stability cannot account for the differences in expression.

These results indicate that *spe-1* sequences upstream of the *Afl*III site in the 5′-UTR, including sequences in the UAR, are incapable of imparting significant polyamine regulation in the absence of downstream *spe-1* sequences. The inclusion of sequences in the *Afl*III-*Nru*I region in addition to these upstream sequences results in polyamine regulation of the chimeric gene. However, polyamines did not regulate the *spe-1*::*qa-2* genes to same extent as they regulated the normal *spe-1* gene, and its expression was lower. This raised the possibility that *spe-1* sequences downstream of the 5′-UTR were required for normal expression (but not stability) and/or regulation of *spe-1* mRNA. We next asked whether the *Afl*III-*Nru*I region of the 5′-UTR, necessary for polyamine regulation, was sufficient in itself to impart regulation to a heterologous upstream promoter and what role, if any, downstream sequences play.

Downstream sequences confer promoter-independent polyamine regulation of *spe-1* mRNA. To test whether the polyamine regulation imparted by the *spe-1* 5′-UTR in the *spe-1*::*qa-2* transformants was independent of upstream untranscribed sequences, we placed all transcribed *spe-1* sequences and its 3′ flank downstream of a heterologous promoter, that of the *N. crassa* β-tubulin (*tub*) gene (Fig. 7A). (This promoter can force constitutive expression of the normally inducible *N. crassa cys-3* gene [21].) The abundance of the full-length *spe-1* mRNA in transformant DMH43, carrying such a construct, responded to changes in the cellular polyamine status (Fig. 7B). However, the ca. 4-fold regulation of *spe-1* mRNA in this transformant was somewhat attenuated compared to the 10-fold regulation in the DMH1 transformant, carrying a normal *spe-1* gene (27, 43). The result shows that at least some of the regulatory amplitude is imparted by *spe-1* DNA sequences within the transcribed region, independently of the *spe-1* promoter.

We next asked whether sequences in the 5′-UTR required for repression of the normal *spe-1* gene were responsible for the regulated expression of the *tub*::*spe-1* chimeric gene. Various 5′ deletions were made in the *spe-1* 5′-UTR, and the remainder of the gene was attached to the *tub* promoter (Fig. 7A). In transformant DMH26, all *spe-1* 5′UTR sequences downstream of the *Afl*III site (at +101) were attached to the *tub* promoter. The expression and polyamine regulation of *tub*::*spe-1* mRNA in DMH26 (Fig. 7B) were similar to those in DMH43, demonstrating that sequences between +1 and *Afl*III are dispensable for regulation of the chimeric gene.

Transformant DMH27 carries the portion of the *spe-1* gene downstream of the *Nru*I site at +574 attached to the *tub* promoter, eliminating most of the *spe-1* 5′-UTR. This transformant has greatly increased mRNA accumulation and diminished regulation compared to transformants in which most of the *spe-1* 5′-UTR is present (DMH43 or DMH26 [Fig. 7B]). This behavior of DMH27, lacking sequences upstream of *Nru*I, resembles the effect of deleting the *Afl*III-*Nru*I segment in the intact *spe-1* gene (27) (see below). Thus, the *Afl*III-*Nru*I segment of the 5′-UTR negatively affects expression from the *tub* promoter, as it does in the *spe-1*::*qa-2* constructs and the wild-type *spe-1* gene.

We then tested a chimeric gene, in transformant DMH11, in which the *tub* promoter is joined to *spe-1* sequences 9 bp upstream of the translation initiation codon (at +613). The absence of most of the sequences from the *Nru*I site to the start codon in this transformant had two surprising effects. First, it caused a lower abundance of *tub*::*spe-1* mRNA compared to that in transformant DMH27 (*tub* promoter fused at the *Nru*I site of *spe-1*), suggesting that this 40 bp region contains a positively acting sequence element. Deletion of this sequence in an otherwise intact *spe-1* gene reduced expression by 50% but had no effect on polyamine regulation (results not shown).

Downloaded from http://mcb.asm.org/ on August 31, 2017 by UNIV OF CALIFORNIA IRVINE

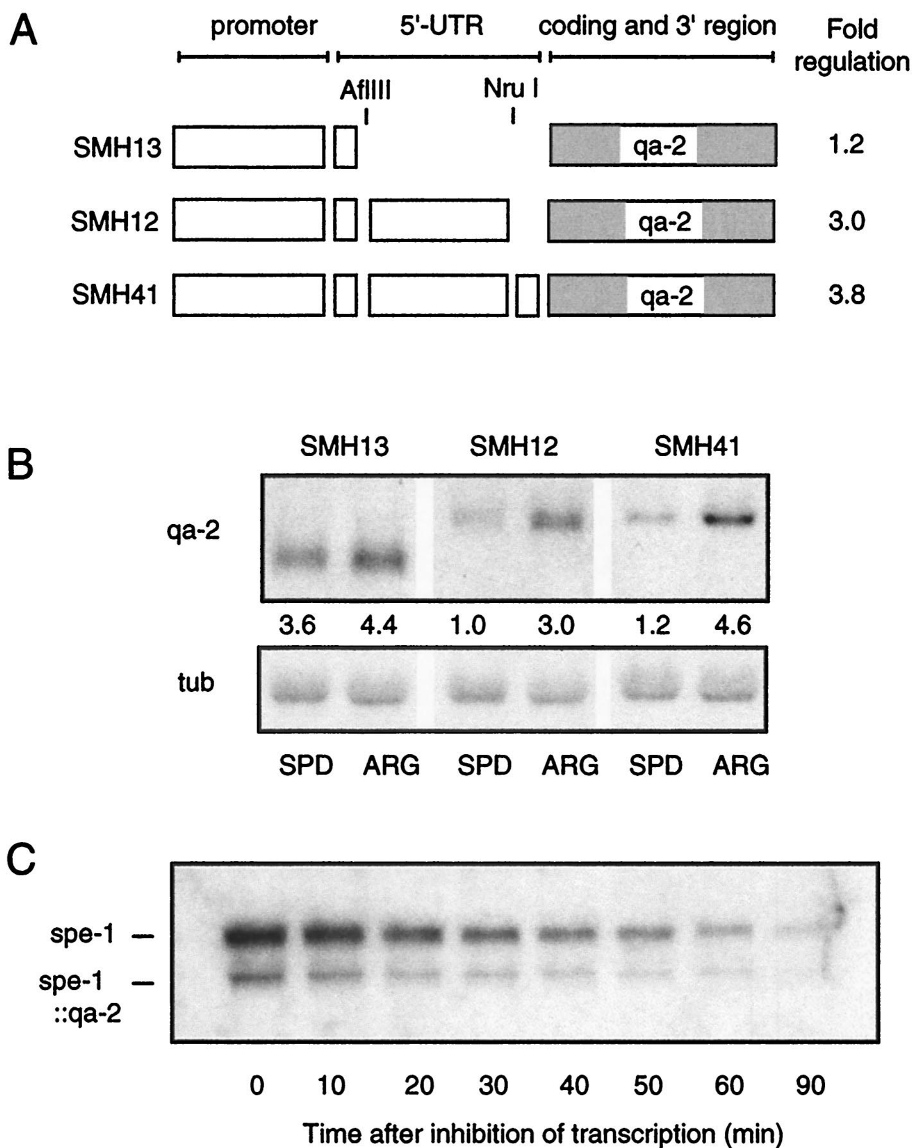

FIG. 6. Regulation of *spe-1*::*qa-2* chimeric genes by polyamines. (A) Schematic diagram of the *spe-1*::*qa-2* genes used to transform strain IC2747-22a. Functional regions of each gene are listed across the top. Open boxes represent *spe-1* sequences, while shaded boxes represent *qa-2* sequences. The relative positions of the *Afl*III (+101) and *Nru*I (+574) sites within the *spe-1* 5′-UTR are indicated. The fold regulation of *spe-1*::*qa-2* mRNA in each transformant, derived from the Northern blot analysis in panel B, shown on the right, is the ratio of *qa-2* mRNA abundance in derepressed over repressed cultures after normalization with *tub* mRNA. (B) Northern blot analysis of 10 μg of total RNA from repressed (SPD) or derepressed (ARG) cultures of the transformants. Northern blots were probed with *qa-2*- or *tub*-specific probes. The amount of *spe-1*::*qa-2* mRNA relative to that in SPD-grown SMH12 is given below the *qa-2* panels. The order of transformants on the Northern blots has been rearranged for ease of presentation, preserving the relative positions of the autoradiographic signals. (C) The turnover of *spe-1* and *spe-1*::*qa-2* mRNA in the SMH41 transformant was determined after inhibition of transcription as described in the legend of Fig. 3. The *spe-1 Afl*III-*Nru*I sequence was used as a probe for Northern blots.

We designated this region the downstream activation region (DAR). The second surprising effect of extending the deletion of the 5′-UTR by the additional 40 nucleotides was the restoration of polyamine regulation to the level observed in DMH43 (*tub* promoter joined to *spe-1* at +1) and DMH26 (joined at *Afl*III). Thus, the *Afl*III-*Nru*I segment, part of a major regulatory element in transformants carrying the *spe-1* gene and *qa-2* chimeras, appears wholly dispensable in those carrying the *tub*::*spe-1* chimeras. Therefore, DMH27 and DMH11, both lacking the *Afl*III-*Nru*I segment, may differ not in whether they have a remaining regulatory element but, rather, in their ability to express it. The high, constitutive expression of the *tub*::*spe-1* chimera in DMH27 may limit further derepression upon polyamine starvation, while DMH11, lacking the DAR, may express the gene at a low enough level to display its full regulatory range.

If our assumption that the high level of expression limits the regulation of genes lacking the *spe-1 Afl*III-*Nru*I segment is correct, lowering expression by removal of sequences other than the DAR should have similar effects. To that end, we compared polyamine regulation of transformants carrying *spe-1* genes lacking either the 5′-UTR *Afl*III-*Nru*I region (DMH3) or both the 5′-UTR and positively acting sequences in the UAR (DMH4) (Fig. 8A). Reducing expression by re-

Downloaded from http://mcb.asm.org/ on August 31, 2017 by UNIV OF CALIFORNIA IRVINE

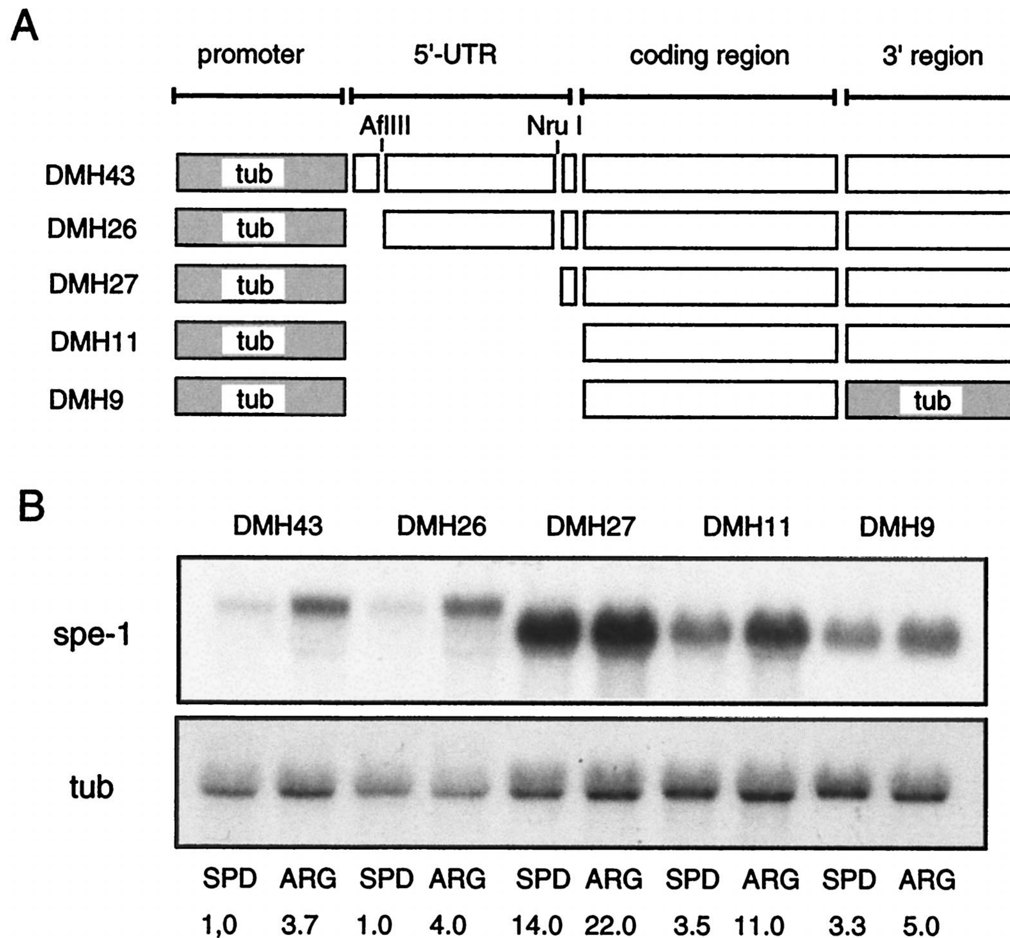

FIG. 7. Expression of various *spe-1* and chimeric transcripts driven by the β-tubulin (*tub*) promoter of *N. crassa*. (A) Schematic diagram of *tub*::*spe-1* genes introduced into strain IC54, with functional regions of each gene listed across the top. The *spe-1* sequences are represented by open boxes; and *tub* sequences are represented by shaded boxes. The positions of the *Afl*III and *Nru*I sites in the *spe-1* 5′-UTR are shown. (B) Northern blots of 10 μg of total RNA from repressed (SPD) or derepressed (ARG) cultures, probed with *spe-1* cDNA or the coding region of *tub* DNA. The relative abundance of *tub*::*spe-1* mRNA in each transformant, normalized to *tub* mRNA and relative to that in DMH43/SPD, is given below the panel.

moving the UAR might once again allow the full regulatory amplitude of constructs lacking the *Afl*III-*Nru*I segment to become apparent. Indeed, the reduced expression of *spe-1* mRNA lacking both the positively acting UAR and the negatively acting *Afl*III-*Nru*I segment in transformant DMH4 resulted in greater polyamine regulation of this mRNA compared to that in transformant DMH3, in which the UAR was still present (Fig. 8B). This result supports the finding with the *tub*::*spe-1* transformants that the high level of expression in the absence of the *Afl*III-*Nru*I sequences limits the amplitude of regulation.

We make the important additional inference that the regulation of *spe-1* mRNA expression in DMH4 and *tub*::*spe-1* mRNA expression in DMH11, DMH43, and DMH26 (the last two including the *Afl*III-*Nru*I segment) must be governed by sequences within their coding or 3′ regions. In this connection, we can also infer that both the UAR and the 5′-UTR are essential for the action of the 5′ PRR: the removal of the UAR from an otherwise normal *spe-1* gene causes a substantial reduction in the regulatory amplitude (compare lanes 1 and 7 in Fig. 5) to a level comparable to that imparted by the 3′ region in DMH4, DMH11, DMH43, and DMH26.

The *spe-1* 3′ region contains a positively acting, polyamine-responsive element. The question of the location of the remaining regulatory element was addressed by replacing sequences downstream of the *spe-1* coding region in transformant DMH11 (lacking 5′-UTR sequences) with the corresponding region of the *tub* gene. This yielded a *tub*::*spe-1* chimeric gene (in transformant DMH9) in which the only *spe-1* region that remained was the ODC coding sequence. The *tub*::*spe-1* mRNA of DMH9 was unresponsive to polyamine starvation, although its expression in spermidine-supplemented cultures was equivalent to that in DMH11 (Fig. 7B). These results imply that the *spe-1* 3′ region contains sequences required for polyamine-mediated derepression. In-frame deletions within the ODC coding region had no effect on polyamine regulation or the expression of the resulting *spe-1* mRNAs (results not shown). We refer to the *spe-1* sequences downstream of the ODC termination codon as the 3′ PRR. This segment contains not only the region encoding the 3′ UTR of *spe-1* mRNA but also the untranscribed region extending to the *Hin*dIII site some 552 nucleotides downstream.

The amplitude of regulation of *tub*::*spe-1* mRNA expressed from the *tub* promoter in the DMH43, DMH26, and DMH11

Downloaded from http://mcb.asm.org/ on August 31, 2017 by UNIV OF CALIFORNIA IRVINE

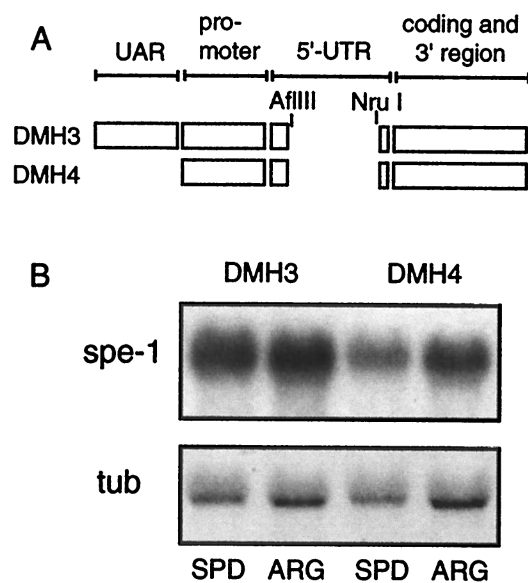

FIG. 8. Role of the UAR on the regulation of *spe-1* genes lacking the *Afl*III-*Nru*I segment of the 5′-UTR. (A) Schematic of *spe-1* genes lacking 5′-UTR sequences in which the *spe-1* UAR is either present (DMH3) or absent (DMH4). The positions of the *Afl*III and *Nru*I sites of the 5′-UTR are indicated. (B) Northern blot analysis of repressed (SPD) and derepressed (ARG) cultures of the transformants. Northern blots were hybridized with probes derived from *spe-1* cDNA or the coding region of the *tub* gene.

transformants is about fourfold in all cases (Fig. 7B). Because the chimeric *tub*::*spe-1* gene in each of these transformants includes the *spe-1* 3′ PRR, we sought to confirm the inference above that the sequences in the 3′ PRR are solely responsible for the regulation seen in these transformants. The *spe-1* 3′ sequence in the DMH43 *tub*::*spe-1* gene was replaced with the 3′ end of the *tub* gene, leaving the *spe-1* 5′-UTR sequences and coding region flanked by the *tub* promoter and the *tub* 3′ region (transformant DMH52) (Fig. 9A). The expression of *tub*::*spe-1* mRNA in this transformant showed little response to changes in cellular polyamine levels and resembled the behavior of *tub*::*spe-1* mRNA in transformant DMH9 (containing only the ODC coding region), even though the *Afl*III-*Nru*I segment was present in the *tub*::*spe-1* chimeric gene (Fig. 9B). We conclude that the 3′ PRR is wholly responsible for the polyamine regulation observed in transformant DMH43, in which the β-tubulin promoter is attached to the entire *spe-1* transcribed region and its downstream flank. In addition, these results indicate again that the *Afl*III-*Nru*I segment cannot confer polyamine regulation in the absence of upstream, untranscribed *spe-1* sequences. We also infer that the presence of the 3′ PRR in the *spe-1* transformant lacking both the UAR and 5′-UTR (DMH4) accounts for its polyamine responsiveness.

We also tested the regulatory effect of the 3′ PRR by replacing the 3′ region of a normal *spe-1* gene with that of the *tub* gene in transformant DMH10 (Fig. 9A). The replacement of *spe-1* 3′ sequences with those of *tub* had little effect on *spe-1*::*tub* mRNA (Fig. 9C) or ODC activity (results not shown) in spermidine-supplemented cultures compared to those in a transformant carrying the normal *spe-1* gene (DMH1). However, neither the *spe-1*::*tub* mRNA nor ODC activity in the DMH10 transformant was derepressed in polyamine-starved cultures to the same extent as in the DMH1 transformant. This result indicates that the 3′ PRR is required for part, but not all, of the observed normal depression of *spe-1* mRNA in response to polyamine starvation. This supports the conclusions already reached by examination of polyamine regulation in the *spe-1*::*qa-2* transformants and suggests that the 5′ and 3′ regulatory elements act independently.

Regulation of ODC translation by polyamines and the *spe-1* 5′-UTR. Change in the rate of ODC translation has been proposed as a regulatory feature in mammalian cells (25). Because sequences in the 5′-UTR might affect the translation of *spe-1* mRNA as well as its abundance, we tested the effects of three segmental deletions within the 5′-UTR on ODC translation, and in the process, on mRNA expression (Fig. 10). The abundance of *spe-1* mRNA increased upon deletion of the proximal *Afl*III-*Bsu*36I region (+101 to +266) or the middle *Bsu*36I-*Stu*I region (+266 to +403), but deletion of both was required to duplicate the effect of removing most of the 5′-UTR (*Afl*III-*Nru*I). Deletion of the *Stu*I-*Nru*I region had no effect on mRNA abundance. Therefore, either a negatively acting sequence overlaps the *Bsu*36I site or there are at least two such elements, one in each of the first two segments.

We assessed effects on translation by comparing the ratio of ODC activity (and thus of protein) to *spe-1* mRNA abundance in repressed cultures of transformants carrying the deletions above. The comparison revealed that the increases of ODC activity were three- to fourfold greater than the increases of *spe-1* mRNA abundance in transformants lacking the *Bsu*36I-*Stu*I region (shown qualitatively in Fig. 10 [compare DMH1, DMH5, and DMH6]). This result suggests that removal of the *Bsu*36I-*Stu*I region eliminates an impediment to translation of the *spe-1* coding region and that this effect is distinct from or overlaps effects on mRNA abundance. The sequence of the *spe-1* 5′-UTR in this (or any other) region contains no upstream open reading frame or obvious secondary structure that might impede scanning ribosomes.

Polyamines increase the turnover of ODC protein (1), making ODC activity a poor reporter of translational regulation of ODC by polyamines. We therefore used transformants carrying *spe-1*::*qa-2* genes to determine the effects of the *spe-1* 5′-UTR and polyamines upon translation of the dehydroquinase coding region. Dehydroquinase activity and *spe-1*::*qa-2* mRNA abundance were compared in transformants carrying *spe-1*::*qa-2* genes with (SMH12) and without (SMH13) the *spe-1* 5′-UTR *Afl*III-*Nru*I region (Table 1). In repressed cultures, deletion of the *spe-1* 5′-UTR led to 3.4-fold-greater *spe-1*::*qa-2* mRNA abundance and 10-fold-higher enzyme activity. We interpret these results to indicate that the translational efficiency of the mRNA rises by about threefold when the *Afl*III-*Nru*I region is removed. We conclude that the presence of the *spe-1* 5′-UTR impedes the translation of the coding region in the *spe-1*::*qa-2* gene, as it does in the *spe-1* gene itself.

In derepressed cultures, deletion of the 5′UTR sequence led to an approximately 1.8-fold increase in *spe-1*::*qa-2* mRNA and a 2.8-fold increase in enzyme activity, nominally about a 1.5-fold increase in translational efficiency. Indeed, in the transformant with deletion of the 5′UTR, polyamine starvation had little effect on either parameter. We conclude that polyamine starvation partially relieves the translational impediment imposed by 5′-UTR. We have previously inferred that polyamine starvation inhibits translation generally (43), and it is of interest that the relief of the translational block in the *spe-1* mRNA mitigates that effect for this particular mRNA.

A reduction in the efficiency of ODC translation could be due to a reduction in either the frequency of ribosomes initiating at the ODC start codon or the elongation rate of translating ribosomes. Reduced initiation should correlate with fewer ribosomes associated with *spe-1* mRNA, while reduced elongation rates should correlate with greater ribosomal loading of *spe-1* mRNA. Polysomal profiles of various cultures,

Downloaded from http://mcb.asm.org/ on August 31, 2017 by UNIV OF CALIFORNIA IRVINE

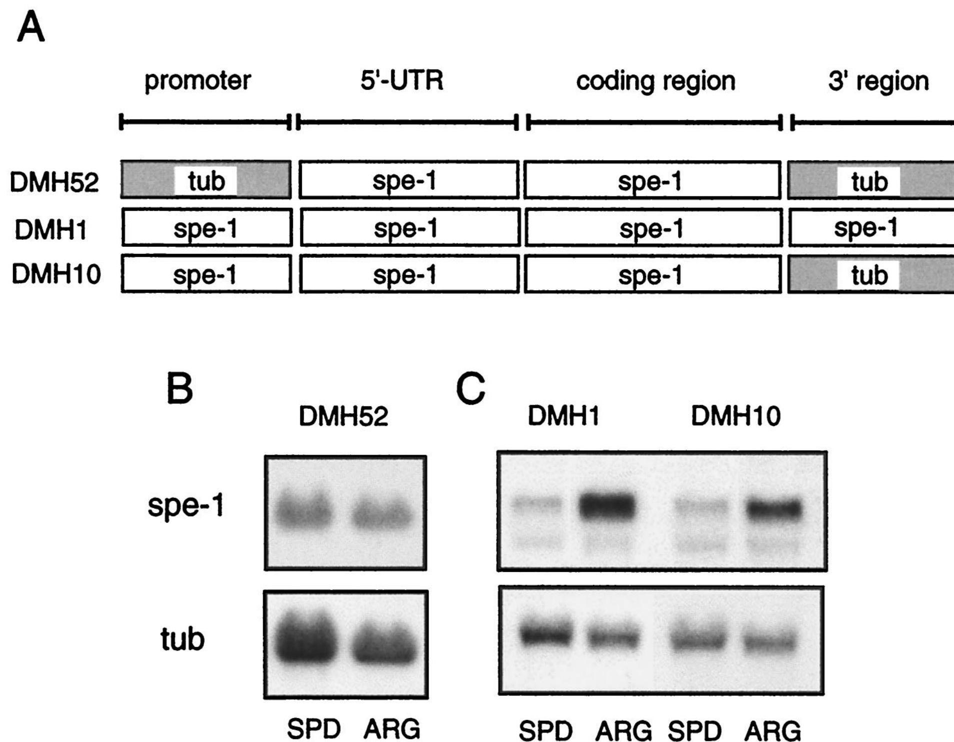

FIG. 9. Effects of replacing the *spe-1* 3′ region with that of the *tub* gene on *spe-1* and *tub*::*spe-1* gene expression. (A) Schematic of *tub*::*spe-1* constructs in transformants DMH1 (wild-type *spe-1* gene), DMH52, and DMH10. (B) Northern blot analysis of repressed (SPD) and derepressed (ARG) cultures of transformant DMH52. (C) Northern blot analysis of similar cultures of transformants DMHI and DHMI0. Blots were probed with *spe-1* cDNA or the coding region of the *tub* gene.

after probing with *spe-1* probes, revealed that *spe-1* mRNA could be found in both monosomal and polysomal fractions (Fig. 11). In repressed cultures, all the *spe-1* mRNA was associated with ribosomes, indicating that repression was not due to a failure to recruit ribosomes. The removal of the 5′UTR (transformant DMH3) led to a modest increase in the average polysome size. Polyamine starvation led to greater ribosomal loading of both wild-type *spe-1* mRNAs and those in which the 5′-UTR had been removed. The results of polysome analysis are consistent with greater ribosomal loading or their impeded elongation or both under conditions of polyamine starvation.

DISCUSSION

We have shown that expression of *spe-1* mRNA depends upon both negatively and positively acting elements of the gene and that the full range of polyamine regulation requires the independent action of both upstream (5′ PRR) and downstream (3′ PRR) PRRs. The 5′ PRR comprises a negatively acting element in the region encoding the 5′-UTR and a collaborating, enhancer-like element in the 5′ UAR of the gene. The positively acting *spe-1* 3′ PRR lies downstream of the coding region, but its exact location is not known. Its effects are obscured in strains lacking the 5′-UTR, owing to the high level of expression caused by such deletions. In addition, modest translational regulation is imparted by an element in the 5′-UTR of *spe-1* mRNA that impedes translation of the downstream coding region. The 5′ and 3′ PRRs, each exerting 3- to 4-fold regulation, can account for the full 10- to 12-fold regulatory amplitude of the mRNA of the intact *spe-1* gene.

Polyamine regulation by the 5′ PRR in *N. crassa* cells is a nuclear phenomenon, most probably transcription, that reduces the abundance of *spe-1* mRNA. This conclusion is based on the findings that (i) the stability of *spe-1* mRNA is not altered by manipulations of cellular polyamine pools or by the removal of the 5′-UTR and (ii) regulation is governed in part by a sequence upstream of the start of transcription. At present, we cannot distinguish between regulatory mechanisms acting at the initiation of the *spe-1* transcript and those that might act on its continued elongation or termination, nor can we exclude potential effects of the 5′-UTR on nuclear export of the *spe-1* mRNA. Because the 5′ PRR and 3′ PRR act independently, it is possible that more than one of these steps in *spe-1* expression are targets of regulation.

The sequences governing *spe-1* expression are distributed over an unusual length of DNA, both upstream and downstream of the coding region. In particular, it is rare that sequences more than 300 bp upstream of the normal transcription start site determine the position of the latter. It is also unusual for sequences almost 600 bp 3′ of the transcription start (the DAR, just 5′ to the coding region) or 3′ to the coding region to influence mRNA abundance without having any effect on its stability. Recently, Pollard et al. (27a) demonstrated that polyamines act to repress the transcription of a subset of genes in the yeast *Saccharomyces cerevisiae*, potentially by stabilizing condensed chromatin states. In addition, they showed the activity of the GCN5 histone acetyltransferase antagonizes these transcriptional effects of polyamines. We suggest that the sequences affecting *spe-1* mRNA abundance may be part of a larger chromatin domain that may be remodeled or modified as cells respond to polyamines.

The 5′ PRR is particularly complex. It includes sequences in both the upstream, untranscribed region and the 5′-UTR. The −1000 to −778 region (within the UAR) contains several poly(dA-dT) sequences characteristic of upstream transcrip-

Downloaded from http://mcb.asm.org/ on August 31, 2017 by UNIV OF CALIFORNIA IRVINE

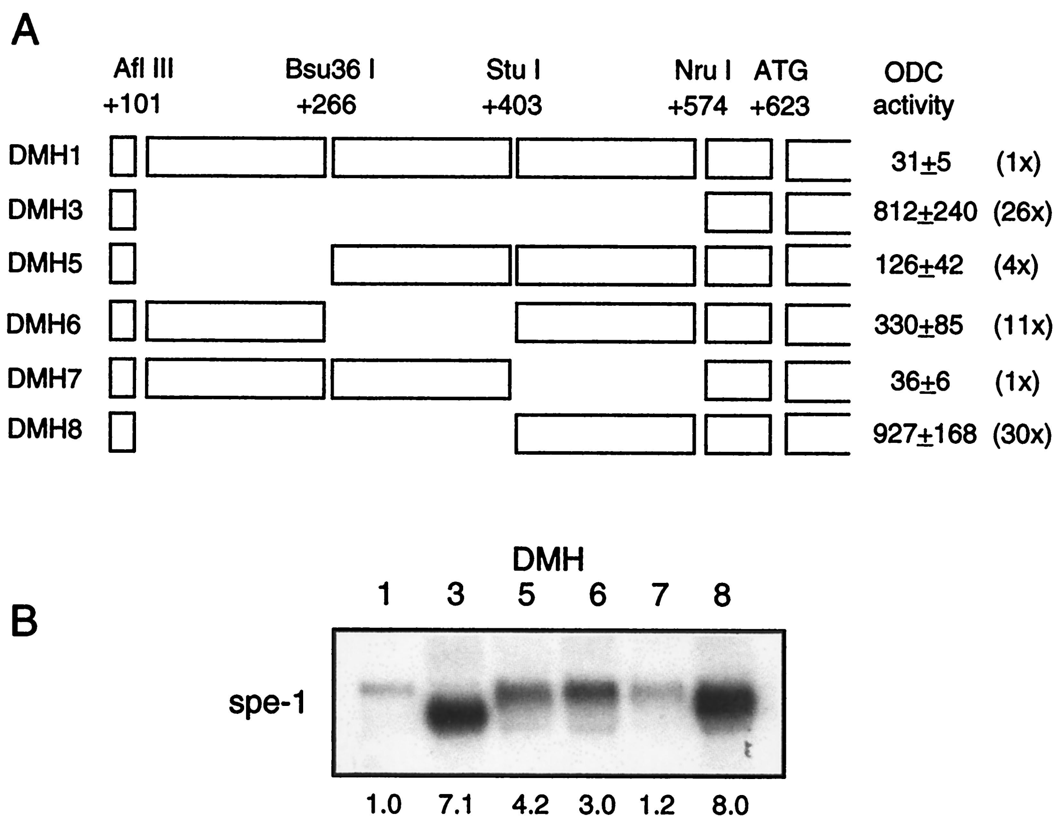

FIG. 10. Effects of deletions within the 5′-UTR on *spe-1* mRNA abundance in repressed cultures. (A) Schematic of *spe-1* 5′-UTR deletions. The positions of restriction sites used to make the deletions and the ODC start codon are indicated, along with their distances from the start of transcription. The ODC activity (mean milliunits per milligram of protein ± standard deviation) and activity relative to the normal repressed level (1×) in spermidine-grown cultures of each transformant is given on the right. (B) Northern blot analysis of the transformants grown under repressing conditions. The numbers given across the top correspond to the transformant numbers in panel A. Northern blots were probed with fragments of *spe-1* cDNA, and loading was normalized by hybridization with a *tub* gene probe. The relative abundance of *spe-1* mRNA in each transformant, normalized to *tub* mRNA (data not shown) and relative to that in DMH1, is given below the panel.

tional activators in other *N. crassa* and *S. cerevisiae* genes (11, 37). Curiously, three copies of an 11-bp conserved sequence element found in the UAR [CCCTCC(A/T)CCAC] do not appear to play much of a role in regulation, as judged from the deletion analysis. Clearly, the role of the UAR will have to be examined in the absence of the 3′ PRR. Because deletion of sequences upstream of and overlapping the transcription start site (−148 to +22, in transformant PΔ7) impairs expression and affects initiation site selection, we cannot exclude the possibility that polyamine-responsive regulatory sequences also reside in this region. Furthermore, although sequences in the 5′-UTR are required for regulation (as demonstrated in the *spe-1*::*qa-2* transformants) and have negative effects on expression (in all contexts in which they were tested), more extensive analysis of this region is required to determine whether these effects are attributable to a single sequence element.

In yeast (10) and mammalian (34) cells, ODC mRNA abundance is unresponsive to fluctuations in the levels of cellular polyamines. In fact, the response of ODC mRNA abundance to polyamine status in *N. crassa* is unique among eukaryotes. However, regulation of ODC in *N. crassa* and mammalian cells has some common features. In both, polyamines reduce ODC activity by accelerating the degradation of ODC protein (1, 33). Second, ODC mRNA from most organisms has an extremely long 5′-UTR. In mammalian cells, the ca. 200-nucleotide 5′-UTR is GC rich and may form stable secondary structures (42). The removal of these sequences increases the rate of translation of the ODC coding region (16, 19, 29). However,

TABLE 1. Effects of *spe-1* 5′-UTR sequences and polyamine starvation on translation of the *qa-2* coding region

Transformant	*spe-1*::*qa-2* mRNA[a] in:		DHQ activity[b] in:		DHQ/mRNA[c]		
	SPD	ARG	SPD	ARG	SPD	ARG	ARG/SPD[d]
SMH12	1.0	1.8	101	347	101	192	1.9
SMH13	3.4	3.3	1,017	993	299	301	1.0

[a] Relative abundance of *spe-1*::*qa-2* mRNA in spermidine- (SPD) or arginine (ARG)-supplemented cultures, as determined by Northern blot analysis and normalied to *tub* mRNA.
[b] Dehydroquinase (DHQ) specific activity in nanomoles of dehydroshikimate per milligram of protein at 37°C.
[c] Translational efficiency of *spe-1*::*qa-2* mRNA, as measured by DHQ specific activities divided by the relative abundances of *spe-1*::*qa-2* mRNA.
[d] The effect of polyamine starvation is measured as the translation of *spe-1*::*qa-2* mRNA in ARG cultures divided by that in SPD cultures.

Downloaded from http://mcb.asm.org/ on August 31, 2017 by UNIV OF CALIFORNIA IRVINE

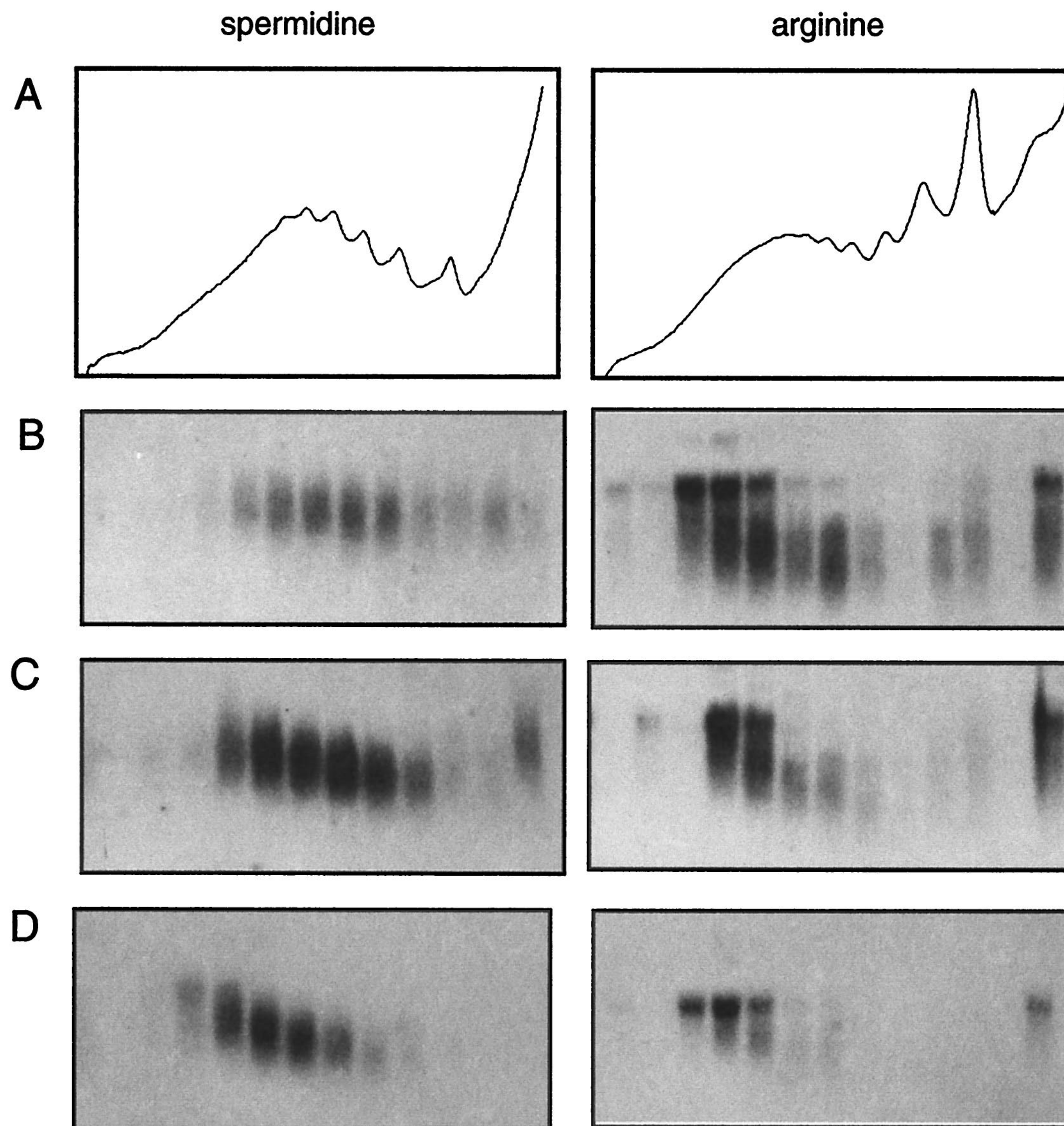

FIG. 11. Analysis of polysomal mRNA. Data from spermidine- and arginine-grown cultures are shown on the left and right, respectively, and the tops of the gradients are to the right. (A) A_{254} profile of 10 to 40% sucrose gradients. (B) Northern blot analysis of *spe-1* mRNA from the *aga* strain (IC3). (C) Northern blot analysis of *spe-1* mRNA from transformant DMH3, lacking the 5′-UTR sequences between the *Afl*III and *Nru*I sites. (D) Northern blot analysis of *tub* mRNA of strain IC3 shown in panel B (*tub* mRNA from DMH3 was similar). Polysomal fractions are aligned below their approximate positions in the gradient.

polyamines do not appear to act through these sequences to regulate ODC translation (39–41), but some debate on this issue remains (26, 35). Our results show that the long 5′-UTR of the *N. crassa spe-1* mRNA impedes translation of the ODC coding region, although it lacks the obvious secondary structures found in mammalian ODC mRNA (43). The 5′-UTR of mammalian ODC mRNA also contains a short upstream open reading frame, absent in the *N. crassa spe-1* 5′-UTR, but this open reading frame has little effect. Our results indicate that polyamine starvation partially relieves the impediment to translation imposed by the *spe-1* 5′-UTR of *N. crassa*. While the *spe-1*::*qa-2* chimeric genes used in our studies of translation lacked *spe-1* 3′-UTR sequences, further investigations are required to determine the role, if any, of *spe-1* 3′-UTR sequences in ODC translation in *N. crassa*, as they appear to have in the mammalian system (12, 17).

The regulation of *spe-1* mRNA expression by polyamines distinguishes *N. crassa* polyamine regulation from that in all other eukaryotic organisms studied to date. Why does *N. crassa* go to such extraordinary lengths to regulate ODC activity, employing mechanisms acting at almost every level of gene expression? The constitutive derepression of ODC in transformants lacking the 5′-UTR does not adversely affect their growth in laboratory culture, and other mechanisms appear to prevent spermidine accumulation to toxic levels (27). The information so far indicates that most organisms do not require precise control of cellular polyamine levels (6). To accomplish this, an allosteric enzyme regulatory mechanism would be required, and no organism known displays a polyamine-responsive ODC enzyme. The mechanisms governing ODC activity in *N. crassa* appear simply adequate to maintain the small amount of polyamine synthesis needed to sustain growth while preventing wasteful use of nitrogen over the long term (6). While the inefficient and redundant modes of regulation ap-

Downloaded from http://mcb.asm.org/ on August 31, 2017 by UNIV OF CALIFORNIA IRVINE

pear to be individually rather insignificant, together they may contribute substantially in nature to the fitness of the organism.

ACKNOWLEDGMENTS

This work was supported by Public Health research grant 35120 from the National Institute of General Medical Sciences and bridge funding from the University of California, Irvine, Office of Research and Graduate Studies. M.A.H. was a trainee supported by Public Health Services NIH predoctoral training grant GM07311.

We thank John W. Pitkin, Alan Kanehl, and Janet Ristow for the construction and Northern analysis of *Neurospora* strains carrying deletions of the UAR, and we thank Laura Williams-Abbott for assistance with polysome analysis. We also thank Mary E. Case for a gift of dehydroquinate, Matthew Petroski for helpful discussions, and Pfizer Laboratories for a gift of Thiolutin used in these studies. We are especially grateful to Philip Coffino for his critical reading of the manuscript.

REFERENCES

1. **Barnett, G. R., M. Seyfzadeh, and R. H. Davis.** 1988. Putrescine and spermidine control degradation and synthesis of ornithine decarboxylase in *Neurospora crassa*. J. Biol. Chem. **263:**10005–10008.
2. **Davis, R. H., and F. J. deSerres.** 1970. Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. **17A:**79–143.
3. **Davis, R. H., L. V. Hynes, and P. Eversole-Cire.** 1987. Nonsense mutations of the ornithine decarboxylase structural gene of *Neurospora crassa*. Mol. Cell. Biol. **7:**1122–1128.
4. **Davis, R. H., G. N. Krasner, J. J. DiGangi, and J. L. Ristow.** 1985. Distinct roles of putrescine and spermidine in the regulation of ornithine decarboxylase in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA **82:**4105–4109.
5. **Davis, R. H., M. B. Lawless, and L. A. Port.** 1970. Arginaseless *Neurospora*: genetics, physiology, and polyamine synthesis. J. Bacteriol. **102:**299–305.
6. **Davis, R. H., D. R. Morris, and P. Coffino.** 1992. Sequestered end products and enzyme regulation: the case of ornithine decarboxylase. Microbiol. Rev. **56:**280–290.
7. **Davis, R. H., and J. L. Ristow.** 1988. Polyamine transport in *Neurospora crassa*. Arch. Biochem. Biophys. **267:**479–489.
8. **Davis, R. H., and J. L. Ristow.** 1991. Polyamine toxicity in *Neurospora crassa*: protective role of the vacuole. Arch. Biochem. Biophys. **285:**306–311.
9. **Eversole, P., J. J. DiGangi, T. Menees, and R. H. Davis.** 1985. Structural gene for ornithine decarboxylase in *Neurospora crassa*. Mol. Cell. Biol. **5:**1301–1306.
10. **Fonzi, W. A.** 1989. Regulation of *Saccharomyces cerevisiae* ornithine decarboxylase expression in response to polyamine. J. Biol. Chem. **264:**18110–18118.
11. **Frederick, G. D., and J. A. Kinsey.** 1990. Nucleotide sequence and nuclear protein binding of the two regulatory sequences upstream of the am (GDH) gene in *Neurospora*. Mol. Gen. Genet. **221:**148–154.
12. **Grens, A., and I. E. Scheffler.** 1990. The 5′- and 3′-untranslated regions of ornithine decarboxylase mRNA affect the translational efficiency. J. Biol. Chem. **265:**11810–11816.
13. **Hautala, J. A., J. W. Jacobson, M. E. Case, and N. H. Giles.** 1975. Purification and characterization of catabolic dehydroquinase, an enzyme in the inducible quinic acid catabolic pathway of *Neurospora crassa*. J. Biol. Chem. **250:**6008–6014.
14. **Herrick, D., R. Parker, and A. Jacobson.** 1990. Identification and comparison of stable and unstable mRNAs in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**2269–2284.
15. **Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease.** 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene **77:**61–68.
16. **Ito, K., K. Kashiwagi, S. Watanabe, T. Kameji, S. Hayashi, and K. Igarashi.** 1990. Influence of the 5′-untranslated region of ornithine decarboxylase mRNA and spermidine on ornithine decarboxylase synthesis. J. Biol. Chem. **265:**13036–13041.
17. **Lorenzini, E. C., and I. E. Scheffler.** 1997. Co-operation of the 5′ and 3′ untranslated regions of ornithine decarboxylase mRNA and inhibitory role of its 3′ untranslated region in regulating the translational efficiency of hybrid RNA species via cellular factor. Biochem. J. **326:**361–367.
18. **Lowry, O. H., N. J. Rosebrough, A. C. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
19. **Manzella, J. M., and P. J. Blackshear.** 1990. Regulation of rat ornithine decarboxylase mRNA translation by its 5′-untranslated region. J. Biol. Chem. **265:**11817–11822.
20. **Orbach, M. J., E. B. Porro, and C. Yanofsky.** 1986. Cloning and characterization of the gene for beta-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. Mol. Cell. Biol. **6:**2452–2461.
21. **Paietta, J. V.** 1992. Production of the CYS3 regulator, a bZIP DNA-binding protein, is sufficient to induce sulfur gene expression in *Neurospora crassa*. Mol. Cell. Biol. **12:**1568–1577.
22. **Patel, V. B., M. Schweizer, C. C. Dykstra, S. R. Kushner, and N. H. Giles.** 1981. Genetic organization and transcriptional regulation in the *qa* gene cluster of *Neurospora crassa*. Proc. Natl. Acad. Sci. USA **78:**5783–5787.
23. **Paulus, T. J., and R. H. Davis.** 1981. Regulation of polyamine synthesis in relation to putrescine and spermidine pools in *Neurospora crassa*. J. Bacteriol. **145:**14–20.
24. **Paulus, T. J., P. Kiyono, and R. H. Davis.** 1982. Polyamine-deficient *Neurospora crassa* mutants and synthesis of cadaverine. J. Bacteriol. **152:**291–297.
25. **Pegg, A. E.** 1988. Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy. Cancer Res. **48:**759–774.
26. **Persson, L., E. L. Wallstrom, S. Nasizadeh, C. Dartsch, A. Jeppsson, A. Wendt, and J. Holmgren.** 1998. Regulation of mammalian ornithine decarboxylase. Biochem. Soc. Trans. **26:**575–579.
27. **Pitkin, J., M. Perriere, A. Kanehl, J. L. Ristow, and R. H. Davis.** 1994. Polyamine metabolism and growth of *Neurospora* strains lacking *cis*-acting control sites in the ornithine decarboxylase gene. Arch. Biochem. Biophys. **315:**153–160.

27a. **Pollard, K. J., M. L. Samuels, K. A. Crowley, J. C. Hanson, and C. L. Peterson.** 1999. Functional interaction between GCN5 and polyamines: a new role for core histone acetylation. EMBO J. **18:**5622–5633.

28. **Poulin, R., J. K. Coward, J. R. Lakanen, and A. E. Pegg.** 1993. Enhancement of the spermidine uptake system and lethal effects of spermidine overaccumulation in ornithine decarboxylase-overproducing L1210 cells under hyposmotic stress. J. Biol. Chem. **268:**4690–4698.
29. **Pyronnet, S., S. Vagner, M. Bouisson, A. C. Prats, N. Vaysse, and L. Pradayrol.** 1996. Relief of ornithine decarboxylase messenger RNA translational repression induced by alternative splicing of its 5′ untranslated region. Cancer Res. **56:**1742–1745.
30. **Ross, J.** 1995. mRNA stability in mammalian cells. Microbiol. Rev. **59:**423–450.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
32. **Schweizer, M., M. E. Case, C. C. Dykstra, N. H. Giles, and S. R. Kushner.** 1981. Identification and characterization of recombinant plasmids carrying the complete *qa* gene cluster from *Neurospora crassa* including the qa-1$^+$ regulatory gene. Proc. Natl. Acad. Sci. USA **78:**5086–5090.
33. **Seely, J. E., and A. E. Pegg.** 1983. Effect of 1,3-diaminopropane on ornithine decarboxylase enzyme protein in thioacetamide-treated rat liver. Biochem. J. **216:**701–707.
34. **Sertich, G. J., and A. E. Pegg.** 1987. Polyamine administration reduces ornithine decarboxylase activity without affecting its mRNA content. Biochem. Biophys. Res. Commun. **143:**424–430.
35. **Shimogori, T., K. Kashiwagi, and K. Igarashi.** 1996. Spermidine regulation of protein synthesis at the level of initiation complex formation of Met-tRNAi, mRNA and ribosomes. Biochem. Biophys. Res. Commun. **223:**544–548.
36. **Staben, C., B. Jensen, M. Singer, J. Pollock, M. Schechtman, J. Kinsey, and E. Selker.** 1989. Use of a bacterial hygromycin B resistance gene as a dominant selectable marker in *Neurospora*. Fungal Genet. Newsl. **36:**79–81.
37. **Struhl, K.** 1985. Naturally occurring poly(dA-dT) sequences are upstream promoter elements for constitutive transcription in yeast. Proc. Natl. Acad. Sci. USA **82:**8419–8423.
38. **Tabor, C. W., and H. Tabor.** 1984. Polyamines. Annu. Rev. Biochem. **53:**749–790.
39. **van Daalen Wetters, T., M. MacRae, M. Brabant, A. Sittler, and P. Coffino.** 1989. Polyamine-mediated regulation of mouse ornithine decarboxylase is posttranslational. Mol. Cell. Biol. **9:**5484–5490.
40. **van Steeg, H., C. T. Van Oostrom, H. M. Hodemaekers, L. Peters, and A. A. Thomas.** 1991. The translation in vitro of rat ornithine decarboxylase mRNA is blocked by its 5′ untranslated region in a polyamine-independent way. Biochem. J. **274:**521–526.
41. **Wallström, E. L., and L. Persson.** 1999. No role of the 5′ untranslated region of ornithine decarboxylase mRNA in the feedback control of the enzyme. Mol. Cell. Biochem. **197:**71–78.
42. **Wen, L., J. K. Huang, and P. J. Blackshear.** 1989. Rat ornithine decarboxylase gene. Nucleotide sequence, potential regulatory elements, and comparison to the mouse gene. J. Biol. Chem. **264:**9016–9021.
43. **Williams, L. J., G. R. Barnett, J. L. Ristow, J. Pitkin, M. Perriere, and R. H. Davis.** 1992. Ornithine decarboxylase gene of *Neurospora crassa*: isolation, sequence, and polyamine-mediated regulation of its mRNA. Mol. Cell. Biol. **12:**347–359.
44. **Wong, L. J., and G. A. Marzluf.** 1980. Sequence complexity and abundance classes of nuclear and polysomal polyadenylated RNA in *Neurospora crassa*. Biochim. Biophys. Acta **607:**122–135.

Downloaded from http://mcb.asm.org/ on August 31, 2017 by UNIV OF CALIFORNIA IRVINE