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Ornithine Decarboxylase Gene of *Neurospora crassa*: Isolation, Sequence, and Polyamine-Mediated Regulation of Its mRNA

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Ornithine decarboxylase (ODC), which initiates the biosynthesis of the polyamines putrescine, spermidine, and spermine, is encoded by the *spe-1* gene of the fungus *Neurospora crassa*. This gene and its cDNA have been cloned and sequenced. The gene has a single 70-nucleotide intron in the coding sequence. The cDNA, comprising the entire coding region, recognizes a single 2.4-kb mRNA in Northern (RNA) blots. The mRNA transcript, defined by S1 mapping, has an extremely long, 535-base leader without strong secondary-structure features or an upstream reading frame. The translational start of the protein is ambiguous: a Met-Val-Met sequence precedes the Pro known to be the N terminus of the ODC polypeptide. The polypeptide encoded by the *N. crassa spe-1* gene (484 amino acids) has 46% amino acid identity with that of *Saccharomyces cerevisiae* (466 amino acids) and 42% with that of mouse (461 amino acids). Alignment of the longer *N. crassa* sequence with *S. cerevisiae* and mouse sequences creates gaps in different sites in the *S. cerevisiae* and mouse sequences, suggesting that *N. crassa* ODC is closer to an ancestral form of the enzyme than that of either yeast or mouse ODC. *N. crassa* ODC, which turns over rapidly in vivo in the presence of polyamines, has two PEST sequences, found in most ODCs and other proteins with rapid turnover. In striking contrast to other eucaryotic organisms, the variation in the rate of ODC synthesis in response to polyamines in *N. crassa* is largely correlated with proportional changes in the abundance of ODC mRNA. Spermidine is the main effector of repression, while putrescine has a weaker effect. However, putrescine accumulation appears to increase the amount of active ODC that is made from a given amount of ODC mRNA, possibly by improving its translatability. Conversely, prolonged starvation for both putrescine and spermidine leads to the differentially impaired translation of ODC mRNA.

Ornithine decarboxylase (ODC) catalyzes the decarboxylation of ornithine to form putrescine, the first step in polyamine biosynthesis leading to spermidine and spermine formation (Fig. 1). In most organisms, ODC is a dimer of 52,000- to 55,000-Da subunits, the activity of which is highly regulated in response to growth stimuli and to the polyamines themselves (31, 50, 62, 63). The amplitude, the speed, and the unusual mechanisms of regulation of the enzyme suggest to many researchers that ODC and the polyamines have a special relationship to growth. Accordingly, many molecular studies of this enzyme in organisms ranging from bacteria to humans have appeared in the last few years (3, 5, 22, 28, 37).

The most conspicuous features of ODC regulation in mammals in response to polyamines are the control of ODC synthesis without a change in ODC mRNA levels and the polyamine-induced increase in the turnover rate of the enzyme (31). In the fungus *Neurospora crassa*, the regulatory responses of ODC at the level of enzyme protein are the same as in many mammalian systems (3, 16). In this study, we describe the sequence of the cloned ODC gene of *N. crassa*, the nature of its unusual transcript, and a comparison of the deduced primary sequence of ODC with those of other organisms. We present evidence that the polyamine-mediated control of ODC synthesis in *N. crassa* differs in its molecular basis from that in all other organisms studied so far.

(cDNA cloning and studies of regulation were drawn from a thesis submitted by Laura J. Williams in partial fulfillment of the requirement for a Ph.D. from the University of California, Irvine, 1991.)

MATERIALS AND METHODS

Strains and growth. *N. crassa* strains used in this work are (with genotypes) IC3 (*aga*; allele UM906), IC1894-53 (*spe-1 aga*; alleles LV10 and UM906, respectively); IC1475-7 (*spe-1 inl*; alleles PE7 and 89601), IC2568-15 (*spe-2 aga*; alleles JP100 and UM906), and IC1923-48 (*spe-1 qa-2 arom-9 inl*). The *aga* (14), *spe-1* (12, 17), and *spe-2* (52) mutations impose complete deficiencies in the enzymes arginase, ODC, and *S*-adenosylmethionine decarboxylase, respectively (Fig. 1), and the *inl* mutation imposes an inositol requirement. Strain IC1923-48 was isolated from a cross of strains IC1475-7 and DDP9305. Strain DDP9305, obtained from David D. Perkins, contains the mutations *qa-2* and *arom-9*, which together impose auxotrophy for aromatic amino acids, and *inl*. For clarity in regulatory studies, strains will be referred to by their full or partial genotype rather than strain number. In addition to the strains above, the kit of strains designed to map restriction fragment length polymorphisms, prepared by Metzenberg et al. (44), was obtained from the Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City. Strains containing all mutations listed are available from the same source.

Growth and maintenance of *N. crassa* were done as described previously (11). Spermidine trihydrochloride was provided at 1 mM for growth of *spe-1* and *spe-2* mutant

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* Corresponding author.

GLUTAMATE

Feedback —| Inhibition

ARGININE ◄—— ORNITHINE ODC —► PUTRESCINE SPD SYN —► SPERMIDINE SPM SYN —► SPERMINE
                        $spe\text{-}1$ $spe\text{-}3$

aga

ARGINASE

S-ADENOSYLMETHIONINE SAM-DC —► DECARBOXYLATED
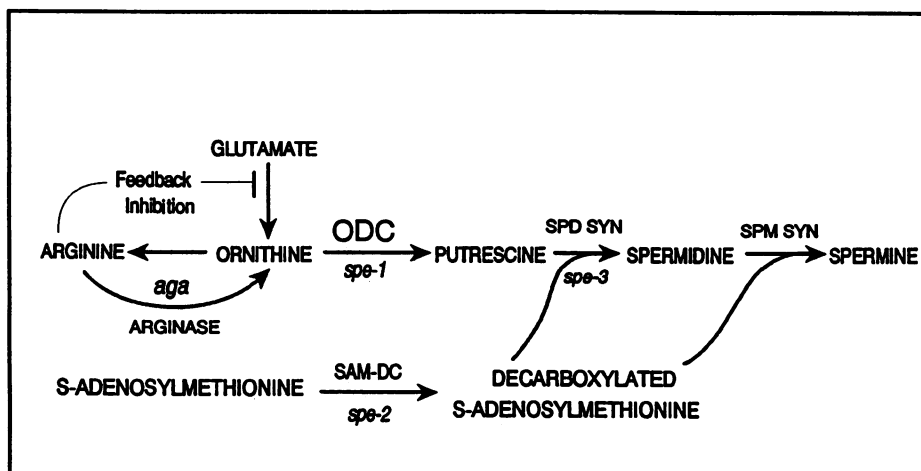 $spe\text{-}2$ S-ADENOSYLMETHIONINE

FIG. 1. The polyamine pathway of *N. crassa*, showing intermediates, enzymes, and structural genes known to control polyamine biosynthetic enzymes. The *aga* mutation grows entirely normally unless arginine is added. Arginine feedback inhibits ornithine biosynthesis at an early step, and the arginase deficiency blocks ornithine formation from arginine. The strain, unable to synthesize ornithine after arginine addition, soon becomes polyamine deficient. Cyclohexylamine (CHA) partially inhibits spermidine synthase (SPD SYN; see text). Abbreviations: SPM SYN, spermine synthase; SAM-DC, *S*-adenosylmethionine decarboxylase.

strains. Strains carrying the mutations *qa-2* and *arom-9* were supplemented with 80 µg of each aromatic amino acid per ml and 2 µg of *p*-aminobenzoic acid per ml. Inositol-requiring strains were grown with 50 µg of inositol per ml. Arginase-less strains, used to manipulate polyamine metabolism (13), were starved for ornithine and polyamines by the addition of 1 mM arginine hydrochloride (14, 49).

The *Escherichia coli* strains used were HB101, BB4^{+}, DH1, JM101 (41), and XL1-Blue (Stratagene), used for much of the subcloning of the original cDNA of *N. crassa*. They were grown in liquid or on solid Luria-Bertani medium (41) with antibiotic additions as needed for plasmid selection. Ampicillin, chloramphenicol, and tetracycline were added to final concentrations of 100 to 200, 10, and 12.5 µg/ml, respectively.

Genetic techniques. Media and techniques for crossing *N. crassa* have been described previously (11). Clones were mapped in the *N. crassa* genome with restriction fragment length polymorphisms between Oak Ridge and Mauriceville strains as described by Metzenberg et al. (44).

Plasmids, libraries, and screening. The cosmid genomic library was that of Vollmer and Yanofsky (67), made in the pSV50 vector, and represented 3,000 clones with 40-kb *N. crassa* genomic inserts maintained in HB101. The benomyl resistance gene, *BmI*[R] (45) was the selectable marker. The plasmid genomic library was that of Akins and Lambowitz (1), made in the pRAL1 vector, and it represented ca. 18,000 *N. crassa* genomic inserts of about 9 kb. The selectable marker was the *N. crassa* *qa-2*[+] gene. Both libraries were screened by sib selection for complementation of *spe-1* auxotrophic recipients. Colony blots of both libraries were also screened with labeled DNA probes as described by Maniatis et al. (41).

A mixed *N. crassa* library, derived from genomic DNA digested with either *Bam*HI or *Bgl*II endonucleases, was made by ligation with *Bam*HI-digested lambda EMBL3 arms (Stratagene). The ligation reaction was packaged in vitro with the Gigapack Gold kit according to the Stratagene protocol and the titers were determined in the bacterial host strain, BB4^{+}. There were 5×10^5 phages present in the

library before amplification. For screening with DNA probes, the library was plated (41) onto a lawn of BB4^{+}.

Two *N. crassa* cDNA libraries in the lambda ZAP vector (46) were made and kindly given to us by M. Sachs in strain XL1-Blue. One contained cDNAs of 6-h mycelial cultures and had at least 324,000 inserts. The other library contained cDNAs of germinating conidia and had at least 84,000 inserts. After being plated, the libraries were screened with antiserum to purified native or denatured ODC protein described previously (16). Nonspecific antibodies were removed by cross-absorption of the antisera with a crude extract of a mutant strain carrying the *spe-1* nonsense mutation, LV10 (12), and lacking the ODC protein. Lambda ZAP libraries were plated on *E. coli* BB4^{+}. Two blots of the phage were obtained from each plate, and positive plaques were revealed by Western blotting (immunoblotting) (6) by using 1:500 dilutions of antibody. Plaques positive on both (duplicate) blots were isolated and plaque purified. The cDNA inserts were removed from the phage by coinfection with a f1 helper phage. Infection of bacterial cells with the phage yielded double-stranded phagemids, selected on ampicillin plates according to the Stratagene protocol.

Bluescript plasmids with *N. crassa* ODC sequences are described in Results, and inserts are shown in Fig. 2. The plasmids are (i) pCS1 (in Bluescript I), comprising the entire coding region of the *spe-1* gene, isolated as a phagemid from the lambda ZAP conidial cDNA library above; (ii) pGS1 (in Bluescript II), with a 4.8-kb *Hind*III fragment containing the *spe-1* gene near the 3' end; (iii) pRIS1 (in Bluescript II), containing the *Sac*I-*Hind*III fragment of pGS1 in which the coding sequence of *spe-1* is located; and (iv) pG5' (in Bluescript II), containing the *Sac*I-*Cla*I fragment of pRIS1. Plasmids carrying *N. crassa* genes of interest were pRAL1 (*qa-2*) from A. Lambowitz, pJR2 (*am*, NADP-glutamate dehydrogenase) from J. Kinsey, pβT6 (*BmI*[R]; a benomyl-resistant variant of β-tubulin) from M. Orbach, and pSV50 (*BmI*[R]) from S. Vollmer. The human ODC gene was provided in plasmid pODC10/2H (70) by O. Jänne, and the *S. cerevisiae* ODC gene was provided in plasmid pSPE-12 (21) by W. Fonzi.
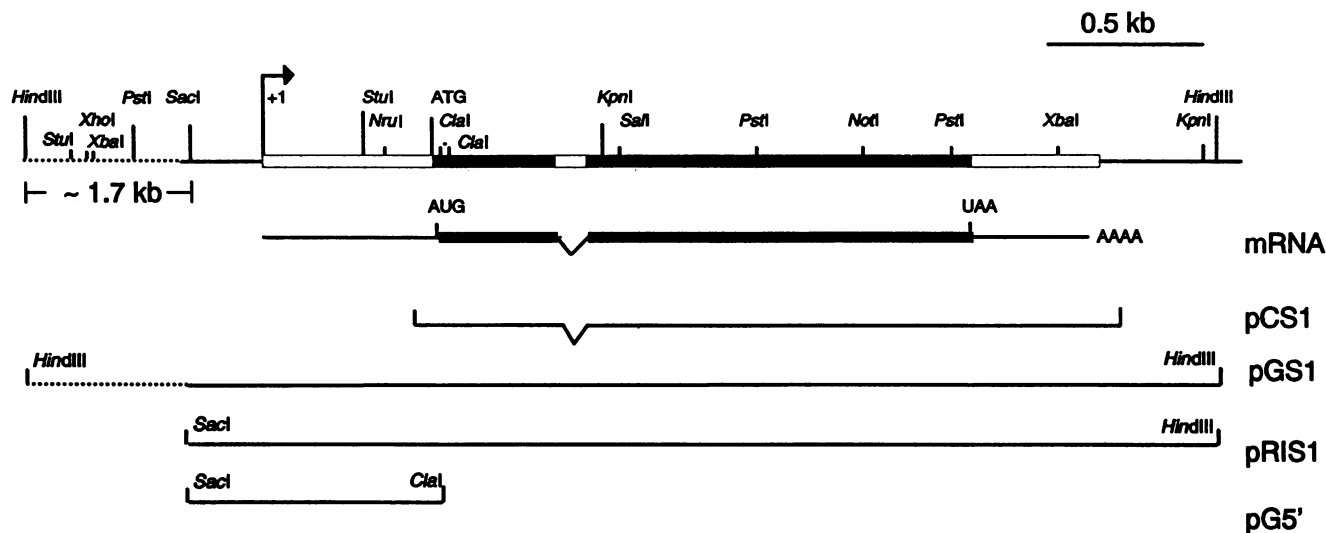
FIG. 2. Restriction map of the *spe-1* gene region of *N. crassa*. The 4.8-kb HindIII fragment is shown, as is the SacI-HindIII portion in detail. The wide box corresponds to the transcribed region, and the start of transcription (+1) is shown with by an arrow. The solid box corresponds to the amino acid-coding region, interrupted by one intron. mRNA is depicted below genomic DNA. The lower four lines show the inserts in the plasmids used: pCS1 contains an incomplete cDNA containing the entire coding region; pGS1, pRIS1, and pG5' genomic fragments are defined by the restriction sites indicated.

DNA isolation and manipulation. *N. crassa* genomic DNA was isolated by two methods. High-molecular-weight nuclear DNA was extracted from nuclei as described by Orbach et al. (45). Genomic DNAs isolated from restriction fragment length polymorphism-mapped strains were isolated by using a moderate-scale DNA preparation protocol reported by Schechtman (59), except that DNA was extracted from acetone powders rather than from frozen powders of mycelia.

Plasmid and cosmid DNAs were isolated by the alkaline lysis method of Birnboim and Doly (4). Small-scale DNA preparations were treated with RNase A (200 μg/ml) at 37°C for 30 min and then extracted with phenol-chloroform-isoamyl alcohol (25:24:1). Large-scale isolation of DNA by the alkaline lysis method was followed by further purification on CsCl-ethidium bromide gradients as described by Maniatis et al. (41). Bacteriophage lambda DNA was isolated from liquid lysates of host *E. coli* (8).

The use of restriction endonucleases and other enzymes in molecular manipulations was performed according to the manufacturers' directions. DNA restriction fragments were isolated from agarose gel slices by using Gene Clean (BIO 101, Inc.) according to the manufacturer's protocol. Gel electrophoresis, nucleic acid blotting techniques, hybridization of DNA and RNA, and autoradiography were done by standard methods (41, 57). Labeling DNA for probes was done by the random-primer method of Feinberg and Vogelstein (18, 19) or by end-labeling with T4 polynucleotide kinase.

Competent cells for transformation of *E. coli* were prepared (41) and stored at −70°C before use. Spheroplasts of *N. crassa* were prepared and transformed by the procedure of Orbach et al. (45). Spheroplasting and transformation techniques were optimized for the selectable markers used.

DNA sequencing was performed by the dideoxynucleotide chain termination method (58), by using the Sequenase kit (version 2.0; United States Biochemical Corporation), with [α-^{32}P]dATP or [α-^{35}S]dATP as the label. Both double- and single-stranded templates were prepared as described for the

Sequenase protocol. The cDNA was originally isolated as the Bluescript BS SK(+) plasmid pCS1 from the lambda ZAP vector. Nested deletions were made from both ends, by using exonuclease III and mung bean nuclease according to the Stratagene protocol. Regions of the cDNA sequence that were ambiguous were clarified by single-stranded sequencing of the plus strand of pCS1 and the plus strand of an inverted insert (plasmid XR1) made by excision with XbaI and EcoRI endonucleases and religation. In these cases, primers based on known sequences were used. All regions of the DNA were sequenced multiply in both directions.

Genomic DNA was sequenced by using a SacI-HindIII insert in a Bluescript II vector. The insert was derived from a HindIII fragment isolated from our EMBL3 lambda phage library. Double-stranded sequencing was carried out with a number of primers based upon the cDNA sequence or, if outside the transcribed region, sequences determined in the process. Oligonucleotide primers were synthesized in the laboratory of Charles Glabe. The 0.8-kb upstream SacI-ClaI fragment overlapping the cDNA was cut at the StuI site within the transcribed region, and both parts were sequenced in both orientations from vector primers.

Sequencing information was stored, compared, and analyzed with the DNANALYZE software package (G. Wernke, National Institutes of Health). PEST regions of the deduced *spe-1* polypeptide amino acid sequence were identified and scored with the PEST program kindly provided by M. Rechsteiner. The Zuker algorithm (71) was used to search for mRNA secondary structure.

Study of the *spe-1* transcript. *N. crassa* RNA was extracted from powdered, acetone-dried mycelia and conidia by the method of Chomczynski and Sacchi (9). Acetone-dried mycelial pads (11) were collected, powdered in cold acetone with a spark-free blender (Omnimixer; Ivan Sorvall, Inc.), and collected by filtration on Whatman no. 1 filter paper. When the poly(A)$^+$ fraction was used, it was isolated from total RNA by fractionation on oligo(dT) spin columns (5'-3', Inc.) as described in the manufacturer's protocol.

S1 endonuclease protection assays were performed as

described by Sambrook et al. (57). Poly(A)$^+$ mRNA (5 μg) of strain IC3, grown in the presence of arginine to starve it for polyamine (49), was hybridized to 320 ng of unlabeled *SacI-ClaI* fragment (0.8 kb) and treated with S1 nuclease. Protected fragments were analyzed by electrophoresis on neutral and alkaline agarose gels, blotted on nylon membrane, and probed with the labeled *SacI-ClaI* fragment. Fine-scale transcript mapping of mRNA of strain IC3, grown on both minimal and arginine-containing media, was performed by hybridizing mRNA to a 560-bp *SacI-StuI* fragment, radiolabeled at the *StuI* end of the noncoding strand (10). Protected fragments were analyzed on strand-separating 6% acrylamide gels by using two sequencing ladders initiated with different oligonucleotide primers of known positions (73 to 108 and 173 to 202 in the sequence described in Results). Both ladders gave the same result.

Regulation of the *spe-1* transcript was measured by probing blots of total RNA, extracted from acetone powders taken at different times during steady-state growth, and separated by electrophoresis, by using labeled ODC cDNA insert of plasmid pCS1 and the β-tubulin DNA sequence of plasmid pβT6 (41). The latter was used uniformly as an internal standard in tests of polyamine-mediated regulation of ODC in vegetative growth. Autoradiographs were quantified with a video densitometer (Bio-Rad model 620), kindly provided by Ben Murray. To control for differences in the specific activities of probes, transfer efficiency, and autoradiography, pairs of mRNA preparations were compared on the same blot, doubly probed with ODC and tubulin probes, and autoradiographed simultaneously. Differences between pairs of preparations (see Table 3) were determined as the ratio of the fold difference in density between ODC mRNA (2.4 kb) and tubulin mRNA (1.8 kb) bands on individual blots. In reconstruction experiments, the relative ratios of ODC and β-tubulin mRNAs remained constant with the amount of RNA loaded and the autoradiographic exposure time within the ranges of the experiments. Standard deviations of the ratios (n = 4 to 8) were based on the variation seen among different samples of the same culture, different cultures of the same pair of strains, and different blots.

Protein sequencing. Pure ODC, derived from strain IC3 derepressed for ODC by polyamine deprivation (16), was denatured, and the peptide of normal molecular weight was repurified on sodium dodecyl sulfate-polyacrylamide gels. The preparation, stored at −70°C for several years, yielded the 53-kDa band and several bands of lower mobilities. The full-length ODC polypeptide (53 kDa) was electroeluted from the appropriate gel slice, and the sequence of the 20 N-terminal amino acids was determined in the laboratory of R. A. Bradshaw, Department of Biological Chemistry, University of California, Irvine.

Assays and metabolite determinations. ODC activity was assayed on duplicate permeabilized samples drawn from exponential cultures (13). Duplicate 100-μl samples were denatured in 1 M NaOH for 36 h at room temperature, and the protein content was determined by the method of Lowry et al. (40). A unit of ODC activity is defined as 1 nmol of product per h at 37°C. Polyamine pool determinations were made by high-performance liquid chromatography of dansylated extracts of samples by methods described previously (13).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been entered in the GenBank database and assigned accession no. M68969 (cDNA) and M68970 (genomic DNA).

RESULTS

Isolation of the ODC cDNA and gene. The *spe-1* gene was first sought by screening the pRAL plasmid and pSV50 cosmid genomic libraries for a clone able to transform strain IC1475-7 to the Spe$^+$ phenotype. Frank Nargang of the University of Alberta, Edmondton, kindly extended the search of the cosmid library by screening additional cosmids made from the ligation mixture used by Vollmer and Yanofsky (67). None of these searches yielded complementing clones. Neither the *S. cerevisiae* genomic (21) nor the human cDNA (70) sequences revealed a homologous gene in either library.

Four monospecific anti-ODC antisera were used to screen cDNA libraries made in lambda ZAP by Orbach et al. (46), one library from mycelial mRNA (324,000 inserts) and one from mRNA of germinating conidia (84,000 inserts). No strong signals from 10^6 plaques from the first library were found. One antiserum revealed two strongly reacting clones from the germinating-conidia cDNA library among 10^5 plaques. Both clones reacted with the other three antisera.

The Bluescript plasmids were excised from the lambda ZAP clones by coinfection with f1 phage. The cDNA inserts, each 1.8 kb in length, had identical restriction patterns (Fig. 2). One insert, in plasmid pCS1, was partially sequenced from both ends. It revealed a poly(A) tract at one end and a sequence beginning at the 66th base at the other end encoding the 20 initial amino acids of the ODC protein, beginning with Pro-Thr-Thr-Val (see below). The sequence determinations indicated that the entire coding region lay within the cDNA insert.

A positive clone was used to map the corresponding genomic sequence with restriction fragment length polymorphisms in 18 recombinant strains designed for such determinations (44) (Fig. 3). Probing the parents of the strains with the cDNA revealed a polymorphism for *HincII* restriction fragments. Probing of the recombinants was carried out in parallel for the presumed *spe-1* sequence and for the *am* gene (in plasmid pJR2), linked tightly (ca. 1 cM) on linkage group V to *spe-1*. The presumed *spe-1* sequence mapped with two differences from the previously mapped, linked *inl* gene, because of recombination in the cross yielding the strains, and with no differences from the *am* sequence. The nucleotide sequence and the genetic data strongly suggested that the insert in plasmid pCS1 represented the *spe-1* gene.

The cDNA of pCS1 hybridized to a lambda EMBL3 clone from a mixed *BamHI-BglII* genomic library. This clone complemented strain IC1894-53, carrying the LV10 nonsense allele of the *spe-1* gene. From the phage, a 4.8-kb *HindIII* fragment (Fig. 2) was isolated and ligated into Bluescript II SK(+), yielding the genomic clone pGS1. *spe-1* strains transformed to Spe$^+$ by pGS1 regulate ODC activity over the range characteristic of ODC in wild-type *N. crassa* (53). We concentrate here on sequence analysis of the cDNA and the *SacI-HindIII* genomic subclone, pRIS1 (Fig. 2), and on the regulation of ODC mRNA.

Gene sequence and transcript mapping. Figure 4 depicts the sequence of the 3.2-kb *SacI-HindIII* genomic fragment and the primary structure of the peptide deduced from regions corresponding to the independently sequenced cDNA.

mRNA of *N. crassa* IC3 grown under different conditions of polyamine supplementation or depletion was subjected to Northern (RNA) blotting using the labeled cDNA insert of plasmid pCS1 as a probe. The blots revealed in all cases a 2.4- to 2.5-kb mRNA which increased greatly in abundance
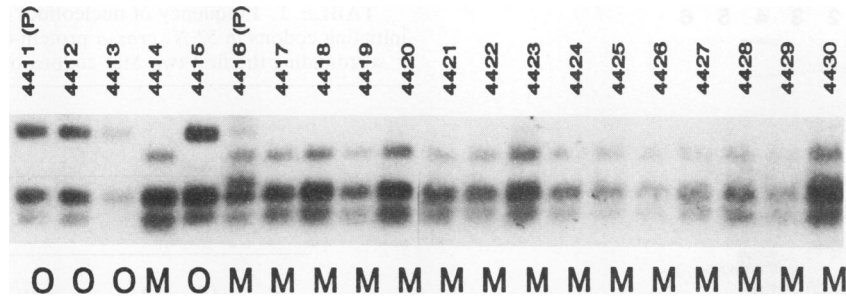
4411 (P) 4412 4413 4414 4415 4416 (P) 4417 4418 4419 4420 4421 4422 4423 4424 4425 4426 4427 4428 4429 4430

O O O M O M M M M M M M M M M M M M M M

FIG. 3. Restriction fragment length polymorphism mapping of the *spe-1* cDNA. Southern blot analysis of *Hinc*II-digested genomic DNAs of the index mapping strains, with radiolabeled cDNA as a probe. Strains 4411 and 4416 are parental (P) strains Oak Ridge and Mauriceville, respectively. The remainder are 18 recombinants of a cross of the parents which had been surveyed for various mutations and sequences (40). The restriction pattern in each recombinant is given below the figure as O (Oak Ridge) or M (Mauriceville). An independent probe of this blot with the *am* gene, closely linked to the *spe-1* locus in genetic tests, yielded the same distribution among strains (results not shown).

in mycelia starved for spermidine (see below). The size of the mRNA, when compared with the 1.8-kb polyadenylated cDNA, suggested that the mRNA had an extremely long 5' leader. All ODC mRNA isolated from cultures was polyadenylated.

Mapping of mRNA start sites was performed by the S1 endonuclease protection method. The mRNA protected a single 0.6-kb piece of the 0.8-kb *Sac*I-*Cla*I fragment, indicating that no introns lay in the transcribed portion of this area of the gene. A 5' ^{32}P-labeled, 0.56-kb *Sac*I-*Stu*I segment

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GAGCTCTTCCATGTGGCAAACCCGTCACGG ACTGCGCAAAAAGGCTGGCTTCAGTTAGCC GACCACCCAAGTTGAAGTCTGCATCCCAGC AGACGACCTACTTCCAGTTTCATCATAGTT -135
^SacI (-254)
GTGGAGGTTTCATCACAGCTCCCAACGCCA AGTCCAACCTACCTCTTCACAAATTTCTCA CCCTTCTCGCACCGCTCTCTCTCTGCCTCT CTACGACTTGGTCACGGTCAAGTTACTAAC -15
ATTGCGCCCATTATCAGGACGGACACGTCG TCGTCATCGCCGTAGCATCCACCTCCCTTG ACTGCCTTTGGTCTTCTCAAAGGCAACCGT ATCTAACACGGCTTCCTCGGCCGTAAAGCT 106
                ^^^transcriptional starts
CGTCTCGGACCAAGATTTCAGCCTCAGAAA GACCTTCCACATCATCAACGTCGTACCTCC CCCATCCGCTCCTGAGGGTCTCTAAGAAGC TCGACGCTCTCTTTTTCGATCCCTCTCCCA 226
ACCAAGGACCTGAACACAGTCCCAACCGTC ACGTGCCGCATAGGACGAGTTCTGCGGTTC TTCCTCTAACGGAGGAAGAGTCGAGGAGGC CTCGTCAGATCGCTCAGGGATCAGTCTCGT 346
ACCGACACCGACCCCCCTAAGACCCGTTCT CAAGAGCATTAGTTGCTTCTTGCTGGCATA TCAACTACACAATAAATCCAAAACCAACCA CAATCAACAAACGCAAACTCCTCCTTACAC 466
CGGGAGTCTCCGAAACCTCGCGAATCTCAC TACCCACTCGATCTTATCACCCCAGTCAAA TCTTGGGATATGGTTATGCCGACTGTTGTC TCCGACCGCATGGGCACTATCGATTTCATC 586
          ^5'end of cDNA                                                  M V M P T V V   S D R M G T I D F I
GATTATACAAACAATCATGTCTTTTCAAAG TGTCAAACCGACAGCTTGAACACCGTCAAC AATGGCTCCTTGAAGCACGATGATTACCTC CACGGCCTTGCCAACGGCAAGTTGGTCGCC 706
 D Y T N N H V F S K   C Q T D S L N T V N   N G S L K H D D Y L   H G L A N G K L V A
AAACAAATGATTGGTGATGCCCTCCGCCAG CGTGTGGAGAGCATCGACTCCGAGTTCTGC GAGCCCGGCGATGAGGACACATTCTTTGTC GCTGACCTCGGCGAGGTCTACCGCCAGCAC 826
 K Q M I G D A L R Q   R V E S I D S E F C   E P G D E D T F F V   A D L G E V Y R Q H
CTCCGCTGGAAGCTGAACCTTCCTCGCGTC AAGCCGTTCTATGGtgagttatcccaatta ctatgcgcttcgtatcgaggattcagtttg ctgacttggatgtttgtcatcagCCGTCAA 946
 L R W K L N L P R V   K P F Y A^intron start                                   intron end^ V K
GTGCCATCCCGACGAAAGGCTCTTGCAGCT CCTGGCCGCTCTCGGTACCGGCTTCGACTG TGCCTCCAAGGCCGAGATCGAGCAAGTCCT CCGCATGGGCGTCGACCCCTCCCGCATTAT 1066
 C H P D E R L L Q L   L A A L G T G F D C   A S K A E I E Q V L   R M G V D P S R I I
CTATGCACAGCCGTGCAAGACCAATTCCTA CTTGCGCTATGTCGCCCAGCAGGGCGTGCG CCAGATGACCTTCGATAATGCCGACGAGCT GCGCAAGATTGCGCGCCTGTATCCCGACGC 1186
 Y A Q P C K T N S Y   L R Y V A Q Q G V R   Q M T F D N A D E L   R K I A R L Y P D A
CGAGCTTTTCCTCCGCATCCTTACCGACGA TTCTTCGTCCCTTTGCCGTTTCAGCATGAA GTTCGGCGCTTCCCTCGACTCGACTGATGG TCTCTTGGGCCTTGCCCGCCAGCTGGGCCT 1306
 E L F L R I L T D D   S S S L C R F S M K   F G A S L D S T D G   L L G L A R Q L G L
CAACGTGGTTGGCGTCAGCTTCCACGTTGG CTCGGGCGCCTCCGATCCTACTGCTTTCTT GAAGGCTGTTCAGGACGCTCATGTCGTCTT CCAGCAGGCCGCTGCTTATGGTTACAGCCT 1426
 N V V G V S F H V G   S G A S D P T A F L   K A V Q D A H V V F   Q Q A A A Y G Y S L
GAAGACCCTCGACGTTGGTGGAGGTTTCTG CAGCGACGATTCTTTCGAACAGATGGCTAA CGTCTTGCGTGCTGCCCTCGATGAGTACTT CCCGGCCCATACCGGTGTCAACCTTATCGC 1546
 K T L D V G G G F C   S D D S F E Q M A N   V L R A A L D E Y F   P A H T G V N L I A
TGAGCCCGGTCGCTACTACGCTTCCTCGGC ATTCACCCTTGCCTGCAACATCATTGCTCG CCGCACTATCCAGGACGGCTCTGCTGTCTC GGTCTCCGATTCCTCTTCTATGAGCGACGA 1666
 E P G R Y Y A S S A   F T L A C N I I A R   R T I Q D G S A V S   V S D S S S M S D D
TGGATCGGTGAACAACGGCGATGCTAGATA TATGGTCTACGTCAACGATGGTCGTACGG AAACTTCAGCAGCATCATGTTCGATCATCA GCACCCTGTGGCCAAGATCCTCCGTGCTGG 1786
 G S V N N G D A R Y   M V Y V N D G L Y G   N F S S I M F D H Q   H P V A K I L R A G
CGGCCGCACCATGTACAACTCTGTTGCTGC CCACGAGTCTTCTGCGGAGGACGCCATTGA GTATAGCATCTGGGGTCCTACTTGCGACGG CATTGATCGCATCACCGAGAGCATCCGCTT 1906
 G R T M Y N S V A A   H E S S A E D A I E   Y S I W G P T C D G   I D R I T E S I R F
CCGCGAGATTCTTGATGTTGGCGACTGGCT GTACTTCGAGGACATGGGTGCCTACACCAA GTGTTCGGCCACCACCTTCAACGGCTTCTC TAACGAGCATGATGTCATCTACGTCTGCAG 2026
 R E I L D V G D W L   Y F E D M G A Y T K   C S A T T F N G F S   N E H D V I Y V C S
CGAGCCTGGTGCTATGGCCGCTCTTGGGATT GTAAGGTGCACGTGCAGCTTTGTACTTGGG AGAGTTGAGACCAGTTTGAGGCTTCTTGGA CAGGGGGAGATGTGTGCTGTTATTTCGTGA 2146
 E P G A M A L L G L   *
TGCGTAGGAGGAAGGTGATACAAAACAATT TTAGGTTTCTTTCGTTGGCTCGTTTGGGTC TCCGCATTATAGAATTTGCTTCGCCTTTTA GACTTTCGAACAAAAAGGGGTTCTAGGAAA 2266
CGATGGTGGGTGGTGTTGGATCAACCTCAG GGTTATAAAACAAAACATGGCTAGAAATAT TGGATGCAAAACAGCAGTTGAGTCTAGACA TCAGACGAATAAAATTGCGTTGGACCCTAT 2386
                                                                                                      end of cDNA^
AAGATATTTGATTTTGAAGTGTCAAAGTGA TGAATGTTGACATAGGGTGGGTGGTGAGGA CGTTAAAGGGATGTGTAATGCAAAAGTGGT TGTGCTTCATTATTTAGGATACAATGAAGT 2506
TGTCACACACGCTCACAAAGTACCTATGAT AGAATACGTAGGTAGTATGCGCGTACGACC AACCAATTTGTACGTCTAGTAGTACGTGTA AGTCACGGCTTCCCCCTATTCATCCCAACA 2626
CCAATATTGTTACGGTTTTGGCAGCATCCA TCCTCTTCCCTTCATGTCTATCTCCCGCAT TCAATCTTCCTTCCCACCCAAGGCTCAGCT CTCCCCGACCCCCTCGGTGATTCGCTGCAC 2746
TTGCCATGTGCTATCACTACCTTACTGACG TGCGCATCATCGTCGCTGGGATTTTGGATC GAGCATCGAGAATGGATGATTCCCCCCGGC AAGCACGTTATAGTGTTAGGGAGAGTCGCG 2866
GTATAACACGTGTGTTACGGTACCACCCAG CATGCGTCCGGTCTTTCTCCCGTCCTCAAA CATCAAGCTT 2956
                                                              ^HindIII
```
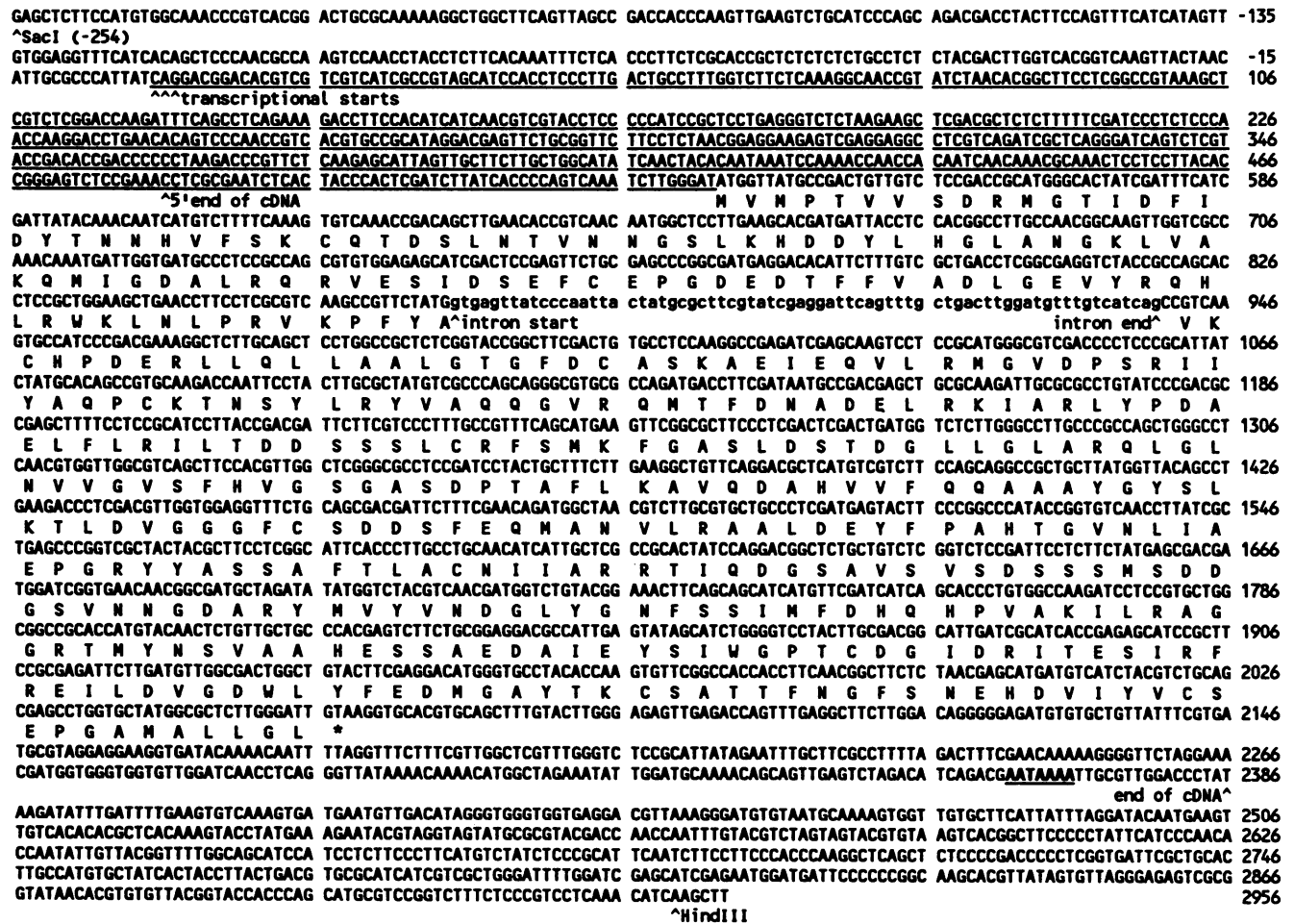
FIG. 4. Nucleotide sequence of the *Sac*I-*Hin*dIII genomic fragment of pRIS1, with the deduced amino acid sequence of the coding region. The sequence is numbered from the first of three consecutive bases defining the start of transcription (labeled as such). The leader of the mRNA is underlined, and the single intron is shown in lowercase letters. The poly(A) addition signal is shown in boldface and is underlined. The beginning and end of the cDNA [which contained a poly(A) tail] are shown at nucleotides 482 and 2386, respectively. The sequence is presented with breaks every 30 nucleotides.
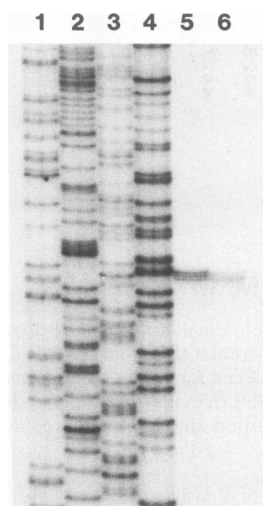
FIG. 5. S1 mapping of the 5' end of ODC mRNA. The unrelated sequence ladder is a standard for length; the three protected fragments are 315, 314, and 313 nucleotides from the *Stu*I site of the protecting fragment (Fig. 1). The 5' end of the leader, therefore, is 535, 534, and 533 nucleotides, respectively, upstream of the first ATG of the transcribed region. Lanes: 1 through 4, sequencing lanes of the standard (A, T, G, and C, respectively); 5 and 6, 30- and 60-min S1 exonuclease treatment, respectively, of the *Sac*I-*Stu*I genomic fragment, in the presence of poly(A) mRNA from the *aga* strain grown in the presence of arginine (derepressed).

TABLE 1. Frequency of nucleotides surrounding the ATG initiating codons in 52 *N. crassa* protein-coding regions and bases surrounding the first two Met codons of the ODC and mRNA

| | Nucleotide position | | | | | | |
Base	-5	-4	-3	-2	-1	A T G	+4
C	15	43	3	24	18	A T G	9
A	12	5	42	24	20	A T G	9
T	22	1	2	3	1	A T G	9
G	3	3	5	1	13	A T G	25
First ATG (ODC)	G	G	G	A	T	A T G	G
Second ATG (ODC)	T	G	G	T	T	A T G	C
Kozak consensus[a]	C	C	A	C	C	A T G	G

[a] From reference 39.

of the same region annealed with mRNA from polyamine-starved cultures revealed fragments differing from one another by one or two nucleotides after S1 digestion (Fig. 5). mRNAs from cultures grown in minimal medium displayed precisely the same pattern. The three fragments had similar intensities on autoradiographs. We arbitrarily designate the first base of the longest transcript as +1 of transcription, which is 535 bases 5' of the first ATG of the sequence and 254 bases downstream from the first base of the *Sac*I site. No obvious TATAA box was found in the sequence upstream from the start of transcription, although a pyrimidine-rich tract at −63 to −43, characteristic of fungal promoters (30), was present.

The unusually long leader has neither the conspicuous secondary structure nor the open reading frame characteristic of the long mammalian ODC mRNA leaders. No stems of more than −14 kcal/mol were found by the Zuker algorithm (71). However, two methionine codons lie one codon apart at the beginning of the coding sequence (Fig. 4). Both (GGGATATGG and TGGTTATGC for the first and second methionine codons, respectively) have relatively rare contexts for translation initiation according to the work of Kozak (39) and Cavener and Ray (7). In the latter compilation, the −321 combinations of GAT and GTT have frequencies among nonvertebrates of 1.7 and 0.6%, compared with more common combinations ranging from 3 to 15%. Both ATGs have extremely unusual base contexts compared with those of 52 translational starts in *N. crassa* compiled by M. Sachs (Table 1). Because the proline following the second methionine is the N terminus of the mature protein, as determined by direct sequencing, we cannot be certain whether the start of translation or posttranslational processing determines the site of the N terminus. The question is further complicated by the fact that the N-terminal amino acids were determined on a pure ODC polypeptide prepara-

tion, which may not have retained its original N-terminal amino acids during purification and storage.

While no AUG codons lie upstream of the first one mentioned above, three upstream open reading frames beginning with CUG, the most efficient non-AUG sequence for initiating protein synthesis in some mammalian systems (43a), lie at 48 to 188 (47 amino acids), 178 to 363 (62 amino acids), and 236 to 292 (19 amino acids). Only the first open reading frame is further qualified by having purines at the −3 and +4 positions in relation to the possible CUG start. The actual significance of these sequences cannot be judged presently.

A 70-bp intron interrupts the coding sequence of the genomic clone at base 870 after the start of transcription. The intron lies within codon 112 of the 484-codon coding sequence. The intron was identified by comparison of genomic and cDNA sequences and has the consensus internal borders (5'-GT and 3'-AG) and an acceptable lariat sequence (TGCTGACT, starting at base 915), characteristic of *N. crassa* genes (30).

The deduced amino acid sequence of the coding region is shown below the nucleotide sequence in Fig. 4. The translation product, starting with the first methionine encoded at positions 536 to 538, has 484 amino acids and a molecular mass of 53,335 Da. Measured from the N-terminal proline (codon 4), the molecular mass is 52,973, equal to the direct estimate of the subunit molecular mass, 53 kDa (16). The codon usage is less biased than genes for abundant proteins such as *am* (glutamate dehydrogenase) and *tub* (β-tubulin), and includes 52 of the 64 codons. Codon usage of *spe-1* resembles those of the *his-3* and *pyr-4* genes, which encode enzymes of amino acid and pyrimidine synthesis, respectively. Although ODC is normally a minor component (0.002%) of the proteins of *N. crassa* (16), it is rapidly turned over (3) and is therefore more rapidly translated than many comparably rare proteins. In addition, derepression of ODC synthetic rates (excluding the variation in turnover) is at least 10-fold (3; results below). Thus, it is not surprising that it displays the codon usage of medium-abundance proteins.

```
Mouse                                              MSSFTKDEFDC--HILDEGFTAKDILDQKINEVSSSDDKDAFYVADLG  46 Mm
N. crassa      MVMPTVVSDRMGTIDFIDYTNNHVFSKCQTDSLNTVNNGSLKHDDYLHGLANGKLVAKQMIGDALRQRVESIDSEFCEPGDEDTFFVADLG  91 Nc
Yeast          MSSTQVGNALSSSTTTLVDLSNSTVTQKKQYYKDGETLHNLLLELKNNQDLELLPHEQ--AHPKIFQALKARIGRINNETCDPGEENSFFICDLG  93 Sc
                                                                                       *  *        *  ***

Mouse          DILKKHLRWLKALPRVTPFYAVKCNDSRAIVSTLAAIGTGFDCASKTEIQLVQGLGVPAERVIYANPCKQVSQIKYAASNGVQMMTFDSEIELMK 141 Mm
N. crassa      EVYRQHLRWKLNLPRVKPFYAVKCHPDERLLQLLAALGTGFDCASKAEIEQVLRMGVDPSRIIYAQPCKTNSYLRYVAQQGVRQMTFDNADELRK 186 Nc
Yeast          EVKRLFNNVVKELPRIKPFYAVKCNPDTKVLSLLAELGVNFDCASKVEIDRVLSMNISPDRIVYANPCKVASFIRYAASKNVMKSTFDNVEELHK 188 Sc
                    *   *** *******   **  *   ****** **  *      *  ** *** *   * *   *   *   *** ** *

Mouse          VARAHPKAKLVLRIATDDSKAVCRLSVKFGATLKTSRLLLERAKELNIDVIGVSFHVGSGCTDPDTFVQAVSDARCVFDMAT-EVGF-SMHLLDI 234 Mm
N. crassa      IARLYPDAELFLRILTDDSSSLCRFSMKFGASLDSTDGLLGLARQLGLNVVGVSFHVGSGASDPTAFLKAVQDAHVVFQQAA-AYGY-SLKTLDV 279 Nc
Yeast          IKKFHPESQLLLRIATDDSTAQCRLSTKYGCEMENVDVLLKAIKELGLNLAGVSFHVGSGASDFTSLYKAVRDARTVFDKAANEYGLPPLKILDV 283 Sc
                  *   * *** ****  ** * * *        **   *        *********  *    ** ** **  *    *     **

Mouse          GGGFPGSEDTKLKFEEITSVINPALDKYFPSDSGVRIIAEPGRYYVASAFTLAVNIIAKKT-------VWKEQPGSDDEDESNEQTF-MYYVND 320 Mm
N. crassa      GGGF-CSDDS---FEQMANVLRAALDEYFPAHTGVNLIAEPGRYYASSAFTLACNIIARRTIQDGSAVSVSDSSSMSDDGSVNNGDARYHVYVND 370 Nc
Yeast          GGGF-----QFESFKESTAVLRLALEEFFPVGCGVDIIAEPGRYFVATAFTLASHVIAKR---KLSENEA-----------------MIYTND 351 Sc
                ****     *        **   **    **  *******     *****  **             * *  **

Mouse          GVYGSFNCILYDHAH-VKALLQKRPKPDEKY----------------YSSSIWGPTCDGLDRIV-ERCNLPE-MHVGDWMLFENMGAYTVAA 393 Mm
N. crassa      GLYGNFSSIMFDHQHPVAKILRAGGRTMY----NSVAAHESSAEDAIEY----SIWGPTCDGIDRIT-ESIRFREILDVGDWLYFEDMGAYTKCS 456 Nc
Yeast          GVYGNMNCILFDHQEPHPRTLYHNLEFHYDDFESTTAVLDSINKTRSEYPYKVSIWGPTCDGLDCIAKEYYMKHDVI-VGDWFYFPALGAYTSSA 445 Sc
                * **   *  **      *                        *********  * *   ****   *   ****

Mouse          ASTFNGF-QRPNIYYVMSRPMWQLMKQIQSHGFPPEVEEQDDGTLPMSCAQESGMDRHPAACASARINV 461 Mm
N. crassa      ATTFNGFSNEHDVIYVCSEPGAMALLGL 484 Nc
Yeast          ATQFNGFEQTADIVYIDSELD 466 Sc
                * ****    *  *
```

Mouse (461)

N. crassa (484)

Yeast (466)

FIG. 6. Comparison of the deduced primary structures of mouse (Mm), S. cerevisiae (Sc), and N. crassa (Nc) ODCs. Asterisks denote the same amino acid residue in all three sequences; pairwise identities are not shown. PEST regions are underlined. Below, sequences are diagrammed roughly to scale. Thin lines indicate gaps in the alignments, and solid rectangles indicate the PEST sequences. The number of amino acid residues in each sequence is shown in parentheses.

Acidic amino acids outnumber basic amino acids, in keeping with the isoelectric point of the enzyme, pH 5.3 to 5.5 (16). There are 10 cysteines, consistent with the high sulfhydril requirement of the enzyme in vitro (16). The primary structure has no conspicuous hydrophobic features (data not shown).

The 3' end of the cDNA has a poly(A) tract, following by about 20 bases the consensus AATAAAA poly(A) addition signal. The 3' HindIII site of the genomic clone lies about 550 bp after the poly(A) addition site. Because only one mRNA species is detected in vivo by Northern blotting, there appears to be only one transcriptional termination site in the N. crassa spe-1 gene, in contrast to the two sites in the same gene in mammals, about 0.3 kb apart (33).

The deduced N. crassa ODC amino acid sequence demonstrates clear homologies to the S. cerevisiae (22) and mouse (29) ODC sequences. As aligned in Fig. 6, the three sequences share 144 identical amino acids (about 30%), scattered throughout. S. cerevisiae and N. crassa ODCs share about 46% identical amino acids, similar to other proteins of these two species (38). The mouse ODC se-

quence shares more identical residues with N. crassa ODC (42%) than with S. cerevisiae ODC (37%).

Substantial gaps of about 19 residues appear in alignments of the S. cerevisiae ODC sequence (466 amino acids) and mouse ODC sequence (461 amino acids) with that of N. crassa (484 amino acids). The gaps in the mouse (after residue 350) and S. cerevisiae (after residue 345) sequences lie at different positions as determined by alignment with the N. crassa sequence; between these positions (365 to 399 of N. crassa ODC), the three sequences have clear similarities. The seven gaps in the N. crassa sequence required for alignment to the others are short (four of one amino acid, one of three amino acids, and two of four amino acids) (Fig. 6).

The two fungal ODCs have N-terminal extensions when aligned with mouse ODC and lack a C-terminal domain found in mouse ODC. The long C-terminal extension of the mouse sequence imparts a high turnover rate to the enzyme in vivo (26, 27). A strong PEST sequence (having high levels of proline, glutamate, aspartate, threonine, and serine), characteristic of proteins with short half-lives (54), lies in this region (Fig. 6). Although N. crassa ODC lacks the C

TABLE 2. Polyamine pools and ODC activity during exponential growth of steady-state cultures

Culture and medium[a]	Mean concn ± SD (nmol/mg, dry wt)		Mean ODC activity ± SD (U/mg of protein)
	Putrescine	Spermidine	
aga + SPD	0.6 ± 0.4	27.5 ± 2.3	27 ± 6
spe-2 + SPD	8.8 ± 1.7	18.5 ± 2.1	27 ± 3
aga + MIN	0.8 ± 0.3	19.2 ± 0.9	57 ± 8
aga + CHA	15.8 ± 1.4	10.8 ± 1.5	316 ± 55
spe-2 + MIN	36.3 ± 3.4	1.8 ± 0.1	817 ± 181
aga + ARG	0.8 ± 0.3	2.2 ± 0.4	2,158 ± 263

[a] Cultures: aga, strain IC3; spe-2, strain IC2568-15. Media: Vogel's minimal medium (MIN) or medium to which 1 mM spermidine (SPD), 10 mM cyclohexylamine (CHA; a spermidine synthase inhibitor), or 1 mM arginine (ARG), which blocks all polyamine synthesis, was added before inoculation.

TABLE 3. Pairwise comparisons of ODC synthetic rates and ODC mRNA contents

Cultures compared[a]	ODC synthetic rate ratio[b]	ODC mRNA ratio[b]	ΔODC synthetic rate/ ΔODC mRNA
aga + MIN vs aga + SPD	2.0 ± 0.6	2.1 ± 0.3	1.0
aga + CHA vs aga + MIN	6.3 ± 1.7	2.3 ± 0.5	2.7
aga + CHA vs aga + SPD	12.4 ± 4.4	4.9 ± 0.9	2.5
aga + CHA vs spe-2 + SPD	12.0 ± 5.0	4.5 ± 1.4	2.7
spe-2 + MIN vs spe-2 + SPD	30.0 ± 7.0	8.9 ± 2.6	3.4
spe-2 + MIN vs aga + CHA	3.0 ± 1.3	2.3 ± 0.7	1.3
aga + ARG vs aga + MIN	4.2 ± 0.7[c]	9.8 ± 4.1	0.4
aga + ARG vs spe-2 + MIN	0.3 ± 0.1[c]	1.7 ± 0.2	0.2

[a] Strain and medium abbreviations are the same as in Table 2.
[b] Quotient of the first strain of the set divided by the second strain.
[c] Value derived from the ratio of specific activities of the two cultures, corrected for the 10-fold stabilization of the enzyme in the aga culture grown in arginine (Barnett et al. [3]).

terminus corresponding to that of mouse ODC, a potential PEST sequence, with a score of 1.88, is found in residues 405 to 427 of the N. crassa sequence, much of which is missing in the mouse (Fig. 6). A less conspicuous PEST sequence (N. crassa score, 0.68) lies at similar positions of both fungal sequences, 68 to 95 of N. crassa and 73 to 96 of S. cerevisiae.

Control of ODC activity. Cultures of aga and of spe-2 strains were grown in steady-state conditions that imposed different rates of polyamine synthesis (Table 2). Because most polyamines are bound to cellular anions (15, 48), impairment of the rate of polyamine synthesis immediately lowers the free polyamine levels to a far greater extent than the extractable cellular polyamine content suggests. The diminished levels of free polyamine provoke a substantial rise in ODC activity (13, 49) (Table 2). The increased ODC activity has two components, (i) an increase in the rate of synthesis of the protein in the absence of spermidine and (ii) the 10-fold stabilization of enzyme in the absence of both putrescine and spermidine (3). Starvation for both polyamines was imposed by the addition of arginine to cultures of the aga strain, because arginine feedback inhibits ornithine synthesis but cannot serve as an alternate source of ornithine in this strain (Fig. 1) (14). The small amount of polyamine in such cultures of the aga strain (Table 2) actually consists of the putrescine analog cadaverine (1,5-diaminopentane, made by the highly derepressed ODC from lysine) and the derived spermidine analog, aminopropylcadaverine. Growth continues indefinitely at one-half the normal rate in such cultures.

In the present experiments, added spermidine repressed ODC activity twofold. Cultures in which spermidine synthesis was partially blocked by cyclohexylamine or fully blocked by the spe-2 mutation led to 6- to 15-fold increases, respectively, of ODC activity over the control culture of aga on minimal medium. In all cultures in which the spermidine pool alone was manipulated (the first five entries of Table 2), variations in activity were due to variations of the rate of enzyme synthesis; maximal turnover (half-life, 55 min) is assured by normal or high concentrations of free spermidine or putrescine. In the steady-state culture of the aga strain grown in arginine, enzyme stabilization (half-life, $t_{1/2}$ 540 min) leads to a greater activity, although synthesis is only fivefold the normal rate (3). The relationship of ODC activity to polyamine pools (Table 2) is consistent with previous findings.

Control of ODC mRNA abundance. ODC mRNA abundance and the rates of ODC synthesis in many pairs of steady-state cultures were compared in order to correlate

changes in the two parameters (Table 3). The rates of ODC synthesis were derived by correcting activity measurements for ODC stabilization in cultures (the aga strain grown with arginine) deprived of both polyamines. The ratios of mRNA and the ratios of enzyme synthetic rates were normalized to cultures of the aga strain grown in minimal medium (Table 4). A Northern blot comparing the ODC mRNA levels in these cultures is shown in Fig. 7.

The relative abundance of ODC mRNA varied by about 20-fold, being lowest in aga and spe-2 strains grown with spermidine (0.5) and highest in the aga strain under prolonged and extreme deprivation of both putrescine and spermidine (9.8) (Table 4). Intermediate relative levels of mRNA were found in conditions of partial or complete starvation for spermidine, with putrescine accumulating to different degrees. These were the aga strain grown with 10 mM CHA (relative mRNA abundance of 2.3) and the spe-2 strain grown in minimal medium (4.5), respectively. The data demonstrate that spermidine starvation led to the derepression of ODC mRNA abundance. This effect was mitigated by putrescine accumulation, suggesting that putrescine was a weak repressor.

Further evidence for the role of putrescine in repression was obtained by monitoring, to high densities, arginine-containing cultures of the aga strain, and of the spe-2 strain

TABLE 4. ODC activity and mRNA accumulation ratios from Table 3, standardized to the aga strain grown in minimal medium

Culture[a]	Relative rate of:		ODC synthesis/ mRNA accumulation
	ODC synthesis	ODC mRNA accumulation	
aga + SPD	0.5	0.5	1.0
aga + MIN[b]	1.0	1.0	1.0
aga + CHA	6.3	2.3	2.7
spe-2 + MIN	15.0	4.5	3.3
aga + ARG (transition)[c]	5.4	3.6	1.5
aga + ARG (long term)	4.2	9.8	0.43

[a] Strain and medium abbreviations are the same as in Table 2.
[b] Control.
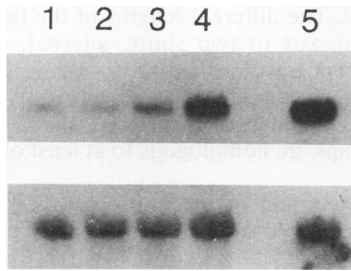[c] Data from results shown in Fig. 8.

FIG. 7. Variation of ODC mRNA in cultures of different poly-amine status. Northern blots of total RNA from strains IC3 (*aga*) and IC2568-15 (*spe-2 aga*) were probed successively with labeled *spe-1* and β-tubulin DNA preparations, revealing the corresponding mRNAs (upper blot, *spe-1* [ODC]; lower blot, *tub-2* [β-tubulin]). Lanes: 1, *aga* strain grown in the presence of 1 mM spermidine (repressed); 2, *aga* strain grown in minimal medium (control); 3, *aga* strain grown in the presence of 10 mM cyclohexylamine (partial depletion of spermidine with putrescine accumulation); 4, *spe-2 aga* strain grown in minimal medium (spermidine depletion with pu-trescine accumulation); 5, *aga* strain grown in the presence of 1 mM arginine (extreme depletion of putrescine and spermidine). The densitometric ratios of the ODC mRNA bands of lanes 1 to 5 of this particular blot, normalized to tubulin and with lane 2 set to 1.0, are 0.86, 1.0, 2.2, 4.3, and 5.8, respectively (Tables 3 and 4).

FIG. 8. Kinetics of the increase in ODC activity and ODC mRNA in the *aga* strain, growing in minimal medium, to which arginine was added (to 1 mM) at time zero. β-Tubulin DNA was used as an internal standard (presumed invariant) for ODC mRNA levels.

growing in minimal medium. Both cultures were unable to make spermidine; the second accumulated more and more putrescine in the period studied (data not shown). As the growth rates of the cultures declined, the ODC mRNA content of the *aga* strain rose slowly and that of the *spe-2* culture declined. The latter culture eventually had fivefold-less ODC mRNA (relative to β-tubulin). Thus putrescine appeared to oppose the accumulation of ODC mRNA brought about by spermidine deprivation.

Enzyme synthesis and mRNA abundance: excess spermidine and short-term polyamine starvation. Repression of ODC mRNA abundance by added spermidine was accompanied by a proportional decrease in the rate of ODC synthesis (Table 4). In order to eliminate the secondary effects of long-term starvation, which will be discussed below, a short-term transition experiment in which arginine was added to a control culture of the *aga* strain growing in minimal medium was performed (Fig. 8; Table 4). As poly-amine depletion due to the exhaustion of ornithine began, ODC mRNA increased 3.6-fold (Fig. 8), and the rate of ODC synthesis increased about 5.4-fold by the time it reached the level of steady-state cultures (a 54-fold increase of activity, corrected for a 10-fold increase in stability). The experiment conforms to our previous direct determination of a four- to fivefold increase in the incorporation of [35S]methionine into the ODC protein immediately upon starvation for poly-amines (3). The roughly proportional relationship of ODC mRNA to the rate of enzyme synthesis in the spermidine-rich and the polyamine-deprived cultures of the *aga* strain reveals little posttranscriptional effect of polyamine excess or deprivation in the short term. As data for other cultures in Table 4 show, however, this conclusion is circumscribed, and we explore a wider range of conditions below.

Posttranscriptional effects of putrescine and spermidine. Spermidine starvation and putrescine accumulation interact in the long term to modify the relationship between ODC mRNA abundance and enzyme synthesis. The *aga* strain grown in the presence of 10 mM cyclohexylamine and the *spe-2* strain grown in minimal medium were partially or
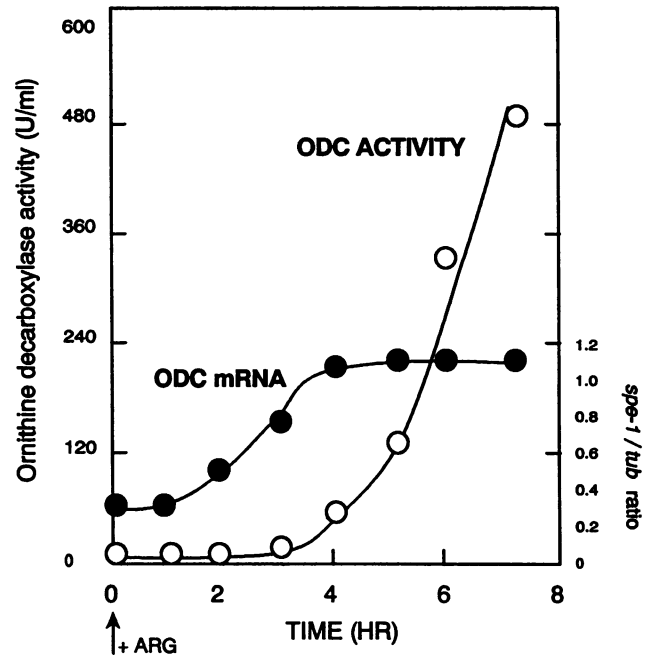
completely starved for spermidine, but both accumulated large amounts of putrescine (Table 2). In these cultures, the elevated enzyme synthetic rates were 2.7- and 3.3-fold higher than the elevation in mRNA levels (Table 4). This suggested that putrescine, even though it repressed the abundance of ODC mRNA, actually improved the translat-ability of the mRNA or the realization of active enzyme, compared with the cultures of the *aga* strain briefly starved by the addition of arginine (Fig. 8; Table 4).

The range of the posttranscriptional effects of both poly-amines was further revealed in cultures of the *aga* strain after prolonged growth in the presence of arginine, resulting in extreme starvation for both putrescine and spermidine. Unlike short-term cultures of this kind (Fig. 8), the ODC synthetic rate for a given amount of ODC mRNA is 43% that of the control and only 18% of the rate measured in the *spe-2* strain growing in minimal medium, which accumulates large amounts of putrescine (Table 3). The data suggest that polyamines have a differential positive effect in *N. crassa* over other mRNAs on the translation of ODC mRNA or the survival of nascent ODC polypeptide and that putrescine can substitute in this process for spermidine. The findings are radically different from those in other organisms, where the polyamines have a profound negative effect on the accumu-lation of ODC protein at a given level of ODC mRNA.

We previously found that cultures of the *aga* strain grown (i) for a brief time after the addition of arginine, (ii) at steady-state with arginine, or (iii) at steady-state with cy-clohexylamine were all about four- to fivefold greater than the control in their ODC enzyme synthetic rates (3). This is consistent with the observations in Table 4, which show that ODC synthetic rates increased 4.2-, 5.4-, and 6.3-fold, re-spectively, in these cultures. Our results as a whole show that the similarity of these results is due to a complex

interplay of three major factors: the repression of mRNA abundance, to different degrees, by spermidine and putrescine, the posttranscriptional enhancement of ODC synthesis caused by excess putrescine, and the posttranscriptional impairment of ODC synthesis or accumulation caused by starvation for both polyamines.

DISCUSSION

Structure of the ODC gene and mRNA. We have determined the sequence of a 3.2-kb SacI-HindIII genomic fragment of N. crassa DNA that includes the coding region of the ODC gene. In parallel, we have sequenced a corresponding 1.8-kb cDNA encoding the entire protein. The sequence maps genetically to the previously described spe-1 gene on linkage group V (17). A comparison of the cDNA and genomic sequences reveals one intron in the coding region; no other introns were found upstream of the coding region of the mRNA in the S1 endonuclease protection experiment. By way of comparison, mammalian ODC genes have 11 introns, some quite large (5, 37); that of S. cerevisiae has none. Transcriptional mapping revealed three mRNA species differing by starts at three consecutive nucleotides, 535, 534, and 533 bases from the first methionine codon. A poly(A) addition signal precedes the poly(A) tail of the cDNA, and there is no evidence of heterogeneous 3' ends of the mRNA.

The long, 535-base, untranslated leader preceding the first methionine has no upstream open reading frame, neglecting for the moment the two methionines separated by a valine at the beginning of the deduced amino acid sequence. The mRNA leader has no obvious strong secondary structure. In N. crassa, long leaders have been found mainly in mRNAs of regulatory genes: qa-1S and qa-1F (407 and 328 nucleotides, respectively [25]), nit-2 (284 nucleotides [23]), and cpc-1 (720 nucleotides [47]). All might be expected to encode relatively low-abundance proteins, although the regulatory cys-3 gene, ostensibly in the same category, has a leader of only 30 nucleotides (24). A long leader per se may have evolved under selective pressure to reduce gene expression (for instance, by diminishing the probability that scanning ribosomes reach the coding sequence) more than transcriptional mechanisms alone would allow. The same pressure for muted expression may have led to the evolution of the poor context for whichever of the two methionine codons initiate the coding sequence. The leader will be discussed further below in connection with ODC regulation in mammals and S. cerevisiae.

ODC protein. The amino terminus of N. crassa ODC must be established with more certainty. The enzyme preparation used for N-terminal analysis yielded proline, corresponding to the fourth codon of the deduced coding sequence, as the first amino acid. If proline is confirmed as the N terminus, the first two codons (Met-Val) would be a subtle hint of an open reading frame in the spe-1 leader, whatever its significance. The question of the true translational start is not resolved by looking at patterns of N-terminal processing in other organisms. Processing of nascent proteins usually calls for removal of the N-terminal methionine if the second amino acid is either valine or proline (2, 64).

The primary sequence of N. crassa ODC has clear homology to the S. cerevisiae and mouse sequences described by others (22, 29). The dissimilarity of S. cerevisiae and N. crassa sequences (almost as great as between mouse and N. crassa) supports the view that S. cerevisiae is taxonomically distant from other ascomycetes, as has been observed by

others (38, 60). The different lengths of the three ODCs can be assigned in part to two short, internal, ca. 19-residue gaps, at different positions in the S. cerevisiae and mouse sequences. Of the three, N. crassa probably has the most primitive ODC, because all regions in the area of and between the gaps are homologous to at least one of the other sequences.

With the exception of trypanosomes (27), the ODC of eucaryotes turns over rapidly in vivo (31). In detailed studies with the mouse enzyme in vitro, turnover is effected by a nonlysosomal, nonubiquitin, ATP-requiring mechanism (55). In most organisms, as in N. crassa, polyamines induce or accelerate turnover. The rapid turnover of the enzyme explains to a great extent the speed with which enzyme activity responds to growth stimuli and to the polyamines. The presence of one or more PEST sequences (54) in ODCs of mammals is correlated with the rapid turnover of ODC. Further evidence that this sequence might impart high turnover is the finding that the loss of part or all of the last 37 amino acids of mouse ODC, containing the PEST region, stabilizes the enzyme (although the issue of polyamine-induced turnover was not addressed [56]) and that the homologous region is missing in the trypanosome ODC (26). In fact, a chimeric protein consisting of the trypanosome sequence attached to the C terminus of the mouse protein is unstable (26). The correlation with the PEST sequence is not perfect; removal of the C-terminal five amino acids of the mouse protein (not overlapping the PEST sequence) stabilizes it (26). If the PEST sequence behaves as a protease target, its use may be conditional upon both nearby sequences and the polyamines.

In N. crassa, ODC is relatively stable in vitro, even in the presence of polyamines (16), and it has a half-life of about 9 h in vivo in polyamine-depleted cells (3). The presence of either free putrescine or free spermidine in cells induces a turnover with a half-life of 55 min (3). The two potential PEST sequences in the N. crassa ODC may endow the protein with a high turnover rate that is conditional on the presence of polyamines. We have found two mutants altered in the polyamine-mediated turnover of their catalytically inactive ODC protein. One is constitutively stable, and one is constitutively labile. Determination of the sequence alterations in these mutants may lead to an unbiased test of the PEST hypothesis, and, with in vitro mutagenesis, to a more refined view of the sequences relevant to turnover.

Regulation of gene expression. In all organisms studied to date, the regulation of ODC synthesis in response to polyamine depletion is generally not accompanied by changes in mRNA abundance, and all ODC mRNAs have a conspicuously long leader. Many have assumed that the leader has a role in the translational control of ODC synthesis. However, in S. cerevisiae, engineered genes have shown that the ca. 200-nucleotide, AT-rich leader (without obvious secondary structure or an open reading frame) had no regulatory role in response to polyamine starvation. Instead, S. cerevisiae appears to control ODC posttranslationally, possibly by the turnover of subunits before the assembly of the active dimer (20). The leaders of most mammalian ODC mRNAs (250 to 320 nucleotides) have strong secondary structure and an open reading frame (5, 68). The secondary structure near the 5' end of the mRNA impedes translation (42, 66), and some researchers have found evidence of a role for the leader (in some cases in combination with the 3' end of the mRNA) in polyamine-mediated translational control (28, 32, 35, 36, 51). This idea has been challenged by the finding that proper regulation of mouse ODC requires only the coding region of

ODC mRNA (65) and that control of ODC appears to be exerted, as in *S. cerevisiae* at a posttranslational step (65). The resolution of this controversy requires more refined study, with attention to possible variation among mammals and experimental systems.

N. crassa is strikingly different from all organisms studied so far in that polyamine-mediated control of ODC synthesis in *N. crassa* is closely related to changes in mRNA abundance. The relationship of the ODC synthetic rate to mRNA abundance is proportional when spermidine is in excess and when cells are briefly starved of both putrescine and spermidine. Putrescine accumulation somewhat diminishes mRNA abundance in spermidine-starved cells, presumably because it serves as an analog for spermidine. It is likely that the effects on mRNA abundance are transcriptional; transformants lacking the *PstI-SacI* segment of the nontranscribed region of the *spe-1* gene are unable to derepress the rate of ODC synthesis (53). Therefore, while *N. crassa* has the longest ODC mRNA leader known to date, its significance, if any, must be sought in aspects of ODC gene expression other than negative translational control mediated by polyamines.

The observations that, at a given level of ODC mRNA, putrescine enhances ODC synthesis and starvation for both polyamines greatly diminishes it may represent trivial differential effects upon the translation of the ODC mRNA. (In both cases, the use of specific activities implicitly shows effects on ODC to be differential ones.) It is well known that polyamines are required for optimal rates of protein synthesis (43), and this is part of the reason that polyamine starvation impairs growth. We have observed a serious impairment of general polysome formation (69) upon starvation for spermidine, which is in keeping with the findings of other workers (34). It is, therefore, possible that ODC mRNA is slightly more affected by starvation than other mRNAs because of its unusual precoding sequence. Conversely, the positive effect of high levels of putrescine may reflect the same factors working in the other direction. This would be consistent with a recent report that putrescine, at high levels, stimulates translation rates in vitro more than do spermidine or spermine (61).

The simplest overall view of ODC regulation in *N. crassa* is that both polyamines repress transcription of the gene and both differentially facilitate translation of ODC mRNA. Future work will test this view, in particular, whether turnover of mRNA and nascent protein subunits also contribute to net mRNA accumulation and new enzyme synthesis, respectively.

ACKNOWLEDGMENTS

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