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Polyamine Metabolism and Growth of Neurospora Strains Lacking *Cis*-Acting Control Sites in the Ornithine Decarboxylase Gene[1]

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Ornithine decarboxylase (ODC) initiates the synthesis of polyamines (putrescine, spermidine, and spermine) and is highly regulated. We wished to know the importance of the control of ODC synthesis to the rates of growth and polyamine synthesis in the fungus, *Neurospora crassa*. We identified two control sites of the *spe-1* gene, encoding ODC. One was an upstream activation region (UAR) and the other was the DNA encoding the long ODC mRNA leader, which governs polyamine-mediated repression of enzyme synthesis. Transformants receiving copies of *spe-1* sequences lacking the UAR compensated for the deficiency by derepression or enzyme stabilization; polyamine synthesis was almost normal. A transformant lacking the *spe-1* mRNA leader DNA constitutively expressed ODC mRNA and ODC activity, and synthesized excessive putrescine, especially when provided exogenous ornithine. This transformant grew normally and had only mildly elevated pools of spermidine, the major polyamine of this organism. We conclude that ODC activity normally limits polyamine synthesis, and ornithine becomes limiting in the ODC-constitutive strain. In this strain, however, spermidine synthesis remains rigorously limited by another step of the pathway, as yet unidentified. Thus the control of ODC activity in *Neurospora* is not vital to growth in laboratory culture, and synthesis of toxic levels of spermidine is limited by other mechanisms. © 1994 Academic Press, Inc.

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Ornithine decarboxylase (ODC)[5] initiates the synthesis of polyamines (putrescine, spermidine, and spermine) in *Neurospora crassa* and has a large amplitude of control (ca. 100-fold). *N. crassa* shares some of the unusual regulatory mechanisms seen in other organisms (1–3), such as polyamine-induced turnover of the ODC protein, complex molecular control of enzyme synthesis, and the strong derepression of the enzyme upon polyamine starvation. Allosteric feedback inhibition of ODC has never evolved in any organism, and regulation of ODC enzyme concentration represents the major means of controlling the rate of polyamine synthesis. The high degree of regulation of ODC in most organisms has been taken by many investigators to signify a special significance of ODC control (for review, see Ref. 2).

Polyamines are di-, tri-, or tetravalent cations that bind avidly to polyanions. Consequently, the pathway must supply sufficient polyamines to titrate cellular RNA, DNA, phospholipids, and polyphosphates (inorganic polyphosphate and ATP) while maintaining the small, freely diffusible polyamine pools that serve as metabolic intermediates and control signals (2). This view has been developed in previous work in *N. crassa* (4, 5), in which spermidine is the major polyamine and in which the polyamines, even when present in excess, turn over very little (5, 6).

In this report, we explore the importance of the control of ODC synthesis to normal growth and polyamine synthesis. We describe deletions of upstream untranslated regions of the ODC structural gene, *spe-1*, of *N. crassa* that disturb gene expression. One region activates gene expression fivefold without affecting the degree of repression by polyamines. The other region encodes the 535-nt leader of ODC mRNA which is the target of polyamine repression of ODC synthesis (7). We use transformants
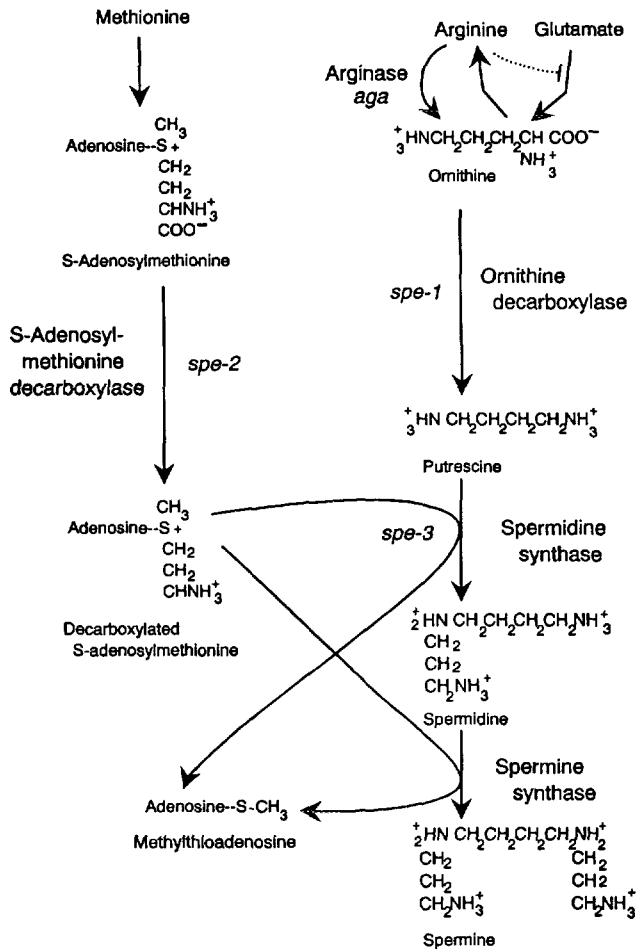
FIG. 1. Polyamine biosynthetic enzymes of *N. crassa*, with the names of corresponding genes (*spe-1-3*). The major polyamine in *N. crassa* is spermidine (18 nmol/mg, dry weight); putrescine and spermine pools are normally 5 and 2% of the spermidine pool, respectively, and spermine is omitted in data reported in tables. The metabolic position of arginase, an enzyme missing in strains carrying the *aga* mutation, is also shown. The dotted line indicates feedback inhibition of ornithine synthesis by arginine.

of *N. crassa* to test the behavior of modified *spe-1* genes in which one or both of these regions are deleted, and their effect upon growth, polyamine synthesis, and the size of polyamine pools in different conditions.

MATERIALS AND METHODS

Strains and growth. The standard *N. crassa* strain used, strain IC3, carries the *aga* (arginase-less) mutation. The recipient for transformation was strain IC2760-3 (*spe-1, aga, his-3*), containing a nonsense mutation (LV10) in the *spe-1* gene (8). Transformants of this strain, PL2, SL7, DP6, and DS4, carried altered *spe-1* constructs borne by a circular plasmid that integrates at the *his-3* gene of the recipient (see below). Standard methods for growth, maintenance and genetics of *N. crassa* were used (7, 9). Histidine (100 µg/ml) and spermidine (250–500 µg/ml) were added to support growth of strains requiring these supplements. *Escherichia coli* strains DH-1, JM101, and XL-1 Blue were used for transformations and amplification of plasmids. *E. coli* strains were grown in Luria–Bertani

(LB) medium (10), and manipulations of cultures and molecular techniques were done according to standard techniques (10).

Polyamine starvation of Spe⁺ strains carrying the *aga* mutation was imposed by growth in 1 mM arginine (Fig. 1). The *aga* mutation eliminates arginase activity, which normally produces ornithine in the presence of excess arginine (11). When grown in arginine, *aga*-carrying strains become ornithine- and polyamine-starved, owing to feedback inhibition of ornithine synthesis by arginine (Fig. 1). Polyamine synthesis ceases except for the formation of a small amount of the analogues cadaverine (1,5-diaminopentane) and aminopropylcadaverine. The analogues originate with the decarboxylation of lysine by the derepressed ODC activity in the absence of ornithine (12), and they support indefinite, slow growth (11).

Molecular methods. Most of the molecular methods used, including RNA analysis and quantification, plasmid screening, and DNA sequencing were standard (10, 13), or have been described previously (7). Oligonucleotide primers were purchased from United States Biochemical or Stratagene or from Dr. Charles Glabe (Department of Molecular Biology and Biochemistry, University of California, Irvine). Sequencing reactions (13) were performed using the Sequenase enzyme kit (United States Biochemical) and [α-^{35}S]thiophosphate dATP. The sequence of the upstream *Hind*III–*Sac*I region, together with the rest of the 4.9-kb *Hind*III insert of plasmid pGS1 has been separately entered into the GenBank database under Accession No. L16920 (ODCHIN). A single correction of the DNA sequence, GC instead of CG at +258 within the ODC mRNA leader region, has been made to the previously deposited sequence (downstream of *Sac*I to the 3′ *Hind*III site), Accession No. M68970.

Plasmid constructions. The plasmid pGS1 (7) contains a 4.98-kb *Hind*III genomic *spe-1* fragment (Fig. 2) in Bluescript-SK⁺ (Stratagene). From this, the *Sac*I–*Hind*III subclone pSP3 was made by introducing the 3′, 3.2-kb *Sac*I–*Hind*III fragment into a *Sac*I–*Hind*III-cut pSP72 (Promega). The 4-kb genomic *Pst*I–*Hind*III subclone, pPH1, was reconstructed from fragments without *Pst*I ends because *Pst*I recognizes two sites in the *spe-1* coding region. First, the 5′, 831-bp *Pst*I–*Sac*I fragment of pGS1 was subcloned into pBluescript-SK⁺ cut with *Pst*I and *Sac*I, to make pUPS2. Second, using the *Eco*RI site of the multiple cloning site, the *Eco*RI–*Sac*I fragment of pUPS2 was removed and inserted into an *Eco*RI–*Sac*I-cut pSP3, yielding a reconstructed 4-kb *Pst*I–*Hind*III *spe-1* gene in the plasmid pPH1.

Two *spe-1* clones lacking all but 62 of the 535 bp encoding the ODC mRNA leader (7) were constructed. The leader contains an *Afl*III site starting at bp +9 after the start of transcription, and, unique to the 5-kb *Hind*III fragment, a *Nru*I site starting at bp +484. There are four
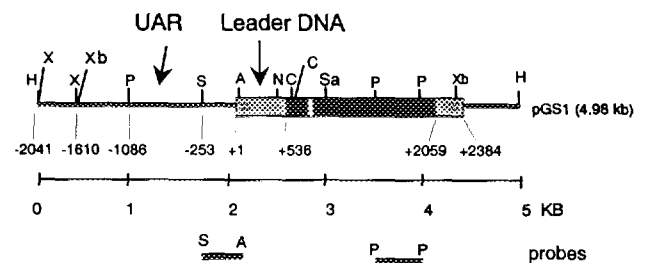

FIG. 2. Restriction map of the *Hind*III fragment of pGS1, containing the *spe-1* gene, with relevant restriction sites marked. In the uppermost part of the figure, the horizontal line represents untranscribed DNA; the wide bar represents the transcribed *spe-1* sequence, with untranslated leader, intron, and 3′ untranslated regions shown by hatching. The *Sac*I–*Afl*III and *Pst*I probes used are shown lowermost. Base pair numbers, counted from the start of transcription (+1), and a scale bar in kilobases (kb) lie beneath. A, *Afl*III (not all *Afl*III sites are shown); C, *Cla*I (two nearby sites); H, *Hind*III; N, *Nru*I; P, *Pst*I; S, *Sac*I; Sa, *Sal*I X, *Xho*I; Xb, *Xba*I; UAR, upstream activation region.

*Afl*III sites in the gene following the *Nru*I site (not shown in Fig. 1). We inserted the 1.4-kb *Sac*I–*Sal*I fragment of the *spe-1* gene into plasmid pSP72 whose single *Afl*III site had been filled in, to form the plasmid pSS1 with unique *Afl*III and *Nru*I sites in the insert. The 473-bp *Afl*III–*Nru*I fragment (most of the leader DNA) was removed and the ends of the *spe-1*–vector fragment were filled in. This was recircularized to form plasmid pSΔS5, having a "leaderless" fragment. The 828-bp, leaderless *Sac*I–*Sal*I portion of this plasmid was used to replace the 1.3-kb *Sac*I–*Sal*I fragments of plasmids pPH1 and pSP3.

The four *spe-1* constructs, differing in their upstream regions, were inserted into plasmid pDE1, carrying a truncated *his-3* gene (Fig 3). The pDE1 *lacZ* fragment was removed by *Bgl*II, *Hin*dIII double digestion and replaced with the *Bgl*II, *Hin*dIII fragment representing the four *spe-1* genomic sequences. The plasmids were designated (using UAR and L for upstream activation region and leader DNA, respectively) pPHL2(+UAR+L), pSHL3 (ΔUAR+L), pDPH1 (+UARΔL), and pDSH1 (ΔUARΔL).

Transformation of N. crassa. The pDE1-based plasmids, carrying a 5'-truncated *his-3* gene, transform recipient *N. crassa* strains containing the 3' Y155M261 mutant allele of the *his-3* gene (14) to a His⁺ phenotype only by homologous recombination at the *his-3* locus (Fig. 3). This provides a common chromosomal context for expression of the *spe-1* inserts. *N. crassa* strains were transformed as spheroplasts using the method of

Case et al. (15, 16). His⁺ transformants were selected; among these, those that were Spe⁺ were purified to the homokaryotic state by three rounds of single conidial isolation, maintaining selection for the His⁺, Spe⁺ phenotype. They were screened then for single-copy transformation by Southern blotting analysis. The transformants used, corresponding to the plasmids described above, were PL2 (+UAR+L), SL7 (ΔUAR+L), DP6 (+UARΔL), and DS4 (ΔUARΔL).

As noted, genomic DNA was isolated (17) and analyzed by Southern blotting (18), using the *his-3* fragment from pDE1 and the *Sac*I–*Afl*III fragment of the *spe-1* gene (Fig. 2) as probes to verify their molecular character. The criteria were: (i) the His⁺ phenotype, assuring the location at the *his-3* locus; (ii) two bands, of similar autoradiographic intensity, of resident and transformed copies of *spe-1* and *his-3* DNA, signifying the single-copy character of the insert and the lack of ectopic integrants; and (iii) detection of terminal restriction sites and expected fragment sizes of the copies introduced, signifying the intactness of the plasmid DNA after integration.

Growth, ODC assay, and metabolite determinations. Cultures were grown at 25°C in exponential culture (9). The cultures were sampled the following day and assayed thereafter for dry weight, ODC specific activity, protein, ornithine, and polyamines in cells and medium by methods described previously (5, 19, 20), including the definitive detection of the diamine cadaverine in polyamine-starved cells. The triamine
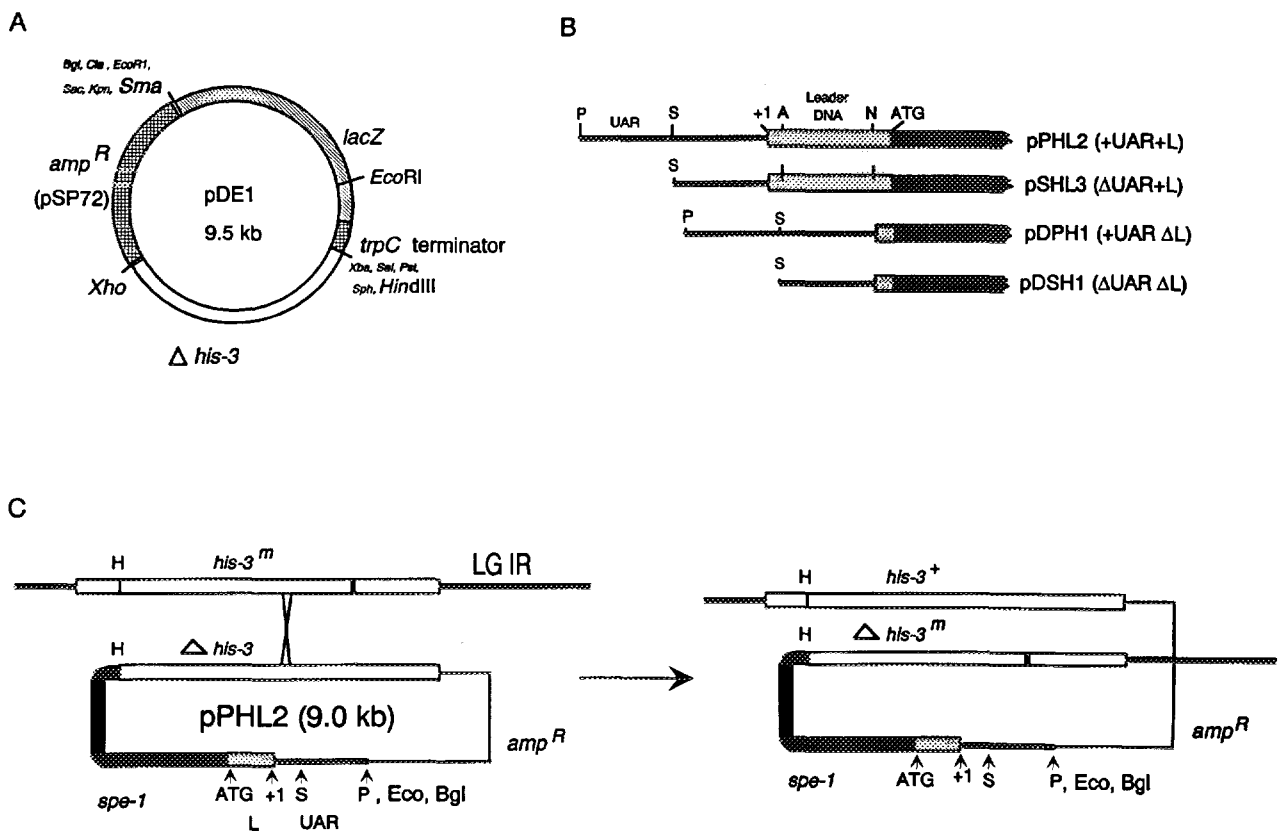

FIG. 3. Transformation of *N. crassa*. (A) pDE1 parent plasmid; (B) *spe-1* inserts replacing the *lacZ* gene of pDE1; all have a 3' *Hin*dIII terminus (Fig. 2). Upstream designations are given at the right. (C) Homologous integration of circular plasmid pPHL2 into the chromosomal *his-3* gene. At left, homologous recombination is shown between the mutant, chromosomal *his-3ᵐ* gene (upper open bar with mutation at right end) with a plasmid-borne homolog (Δ*his-3*) lacking *his-3* DNA 5' of the *Hin*dIII site (H). At right, the resulting integrant shows the vector (thin line) and *spe-1* DNA (hatched and solid bar) flanked by a normal copy of the *his-3⁺* gene and plasmid sequences at the 5' end, and a doubly defective copy (Δ*his-3ᵐ*) of the gene at the other. A plasmid-length fragment is released from transformant DNA by digestion with *Hin*dIII. A, *Afl*III; H, *Hin*dIII, N, *Nru*I, P, *Pst*I; S, *Sac*I; ATG, translational start of the *spe-1* gene; L, ODC leader DNA; UAR, upstream activation region; LGIR, linkage group I, right arm; *amp^R*, ampicillin resistance gene of plasmid vector; other abbreviations as in Fig. 2.

A   *spe-1* probe

*Hind*III   — 9
                                            — 5

*Hind*III,   — 5
*Bgl*II   — 3.2

B   *his-3* probe

*Hind*III   — 9

*Hind*III,   — 5
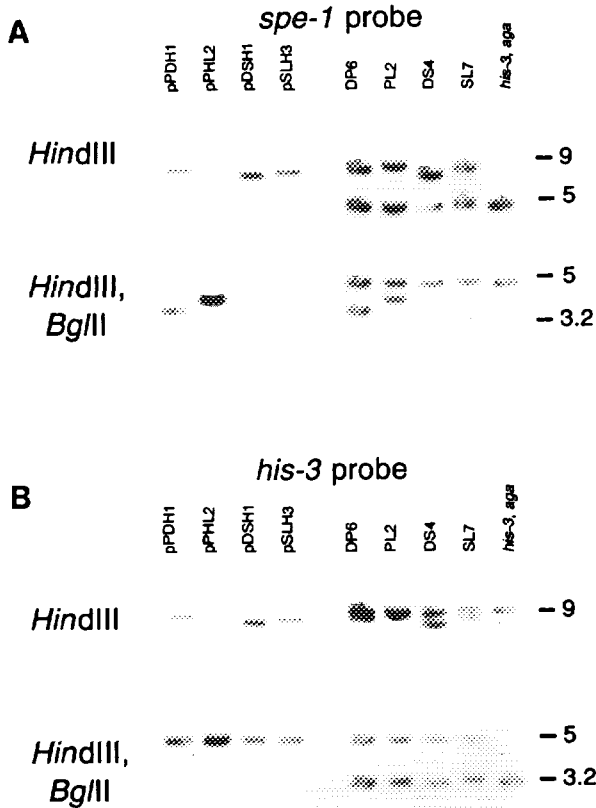*Bgl*II   — 3.2

FIG. 4.   Southern blots of DNA of plasmids and His⁺, Spe⁺ transformants carrying *spe-1* fragments, probed with *spe-1* (A) and *his-3* (B) probes. DNA of an untransformed strain (*his-3, aga*) is shown at the right of each figure. (Top) DNAs cut with *Hind*III; (bottom) DNAs cut with both *Hind*III and *Bgl*II. The singly cut genomic DNAs of strains DP6 (+UARΔL), PL2 (+UAR+L), DS4 (ΔUARΔL), and SL7 (ΔUAR+L) reveal plasmid-length fragments [pDPH1 (8.5 kb), pPHL2 (9.0 kb), pDSH1 (7.7 kb), and pSLH3 (8.2 kb)] with both probes. Resident genomic *his-3* (9.1 kb) and *spe-1* (5.0 kb) fragments are also seen with their respective probes. Doubly cut DNAs reveal 2 *his-3* fragments of constant size and *spe-1* fragments of sizes corresponding to the lengths of their transformed *spe-1* DNA [pDPH1 (3.5 kb), pPHL2 (4.0), pDSH1 (2.7 kb), and pSLH3 (3.2 kb)], as well as the resident copy (5.0 kb).

in cultures having cadaverine and little or no putrescine was assumed to be aminopropylcadaverine on the basis of prior work (12), although it was not rigorously distinguished here from spermidine in high-performance liquid chromatographic profiles.

RESULTS AND DISCUSSION

N. crassa transformants with altered upstream sequences.   The sequence of the entire *Hind*III fragment carrying the *spe-1* gene was completed; a sequence correction and GenBank Accession Nos. are noted under Materials and Methods. Figure 2 shows a partial restriction map. Plasmids carrying the *spe-1* gene lacking one or both controlling regions were constructed and were used to transform a *N. crassa* strain carrying a nonsense *spe-1* mutation (LV10), the *aga* mutation and the counterselection marker, *his-3* (see Materials and Methods).

His⁺, Spe⁺ transformants PL2 (+UAR+L), SL7 (ΔUAR+L), DP6 (+UARΔL), and DS4 (ΔUARΔL) with single copies of the plasmids were isolated and verified by Southern blotting. Probes of DNA cut with *Hind*III revealed plasmid-sized, ca. 9-kb fragments, similar in intensity to endogenous copies of *spe-1* and *his-3* where separately visible (Fig. 4). Double digestion with *Hind*III and *Bgl*II confirmed the presence of the respective sites in the integrated DNA, and demonstrated the intactness of the border of the *spe-1* upstream regions under study. The upstream region of DS4 (ΔUARΔL) displayed a lesser autoradiographic intensity for the introduced copy of the gene owing in part to heterogeneity of band mobility. (This anomaly does not affect the conclusions of this work, since no conclusions are based on its behavior.)

ODC mRNA levels of transformants.   The control strain IC3 and the four transformants were grown in minimal medium, medium containing 1 mM spermidine (repressing), and medium containing 1 mM arginine (derepressing). Total mRNA of the five strains was subjected to electrophoresis, Northern blotting, and autoradiography (Fig. 5, lanes 1–5). The wild type control and transformant PL2 (+UAR+L) (Lanes 1 and 2) behave similarly in derepressing ODC mRNA abundance about 10-fold upon starvation (minimal vs arginine). Deletion of the UAR impairs expression (lanes 3 vs lanes 2), but not regulation (lanes 3, spermidine vs arginine). Deletion of the DNA encoding the ODC mRNA leader renders strains DP6 and DS4 almost constitutive (lanes 4 and 5, all media), although further derepression upon polyamine starvation persists.

Spermidine	Minimal	Arginine	
+ + Δ + Δ	+ + Δ + Δ	+ + Δ + Δ	UAR
+ + + Δ Δ	+ + + Δ Δ	+ + + Δ Δ	Leader

ODC mRNA

β-tub mRNA

1 2 3 4 5   1 2 3 4 5   1 2 3 4 5

★ ★   ★ ★   ★ ★
  ★   ★   ★

★ = wild-type strain IC3

★
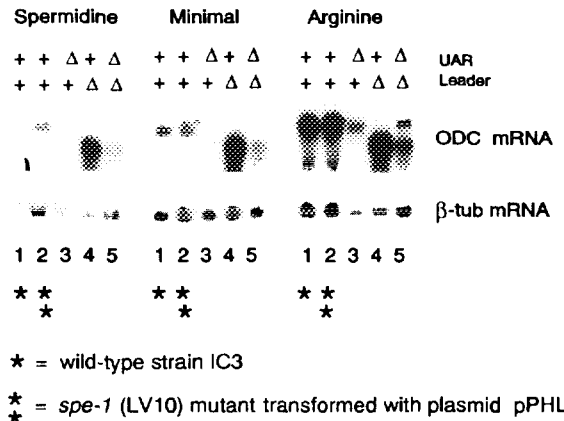★ = *spe-1* (LV10) mutant transformed with plasmid pPHL2

FIG. 5.   Northern blot of total RNA of wild type *N. crassa* and transformants with different upstream regions, after growth on spermidine-containing (repressing), minimal, and arginine-containing (derepressing) media. The lanes are identified with the upstream alterations; lane 1 of each series is the wild type strain (IC3, *aga*), followed by transformants PL2, SL7, DP6, and DS4 (lanes 2–5). (Top) ODC mRNA (normally 2.4 kb), probed with the internal *Pst*I fragment of *spe-1* DNA; (bottom) β-tubulin mRNA (1.8 kb), probed with *Bml*ᴿ (β-tubulin) DNA (16) as a control for mRNA loading.

The *spe-1* allele, LV10, of the recipient strain is a 5′ nonsense mutation (8). Like many 5′ nonsense mutants (21-25), little ODC mRNA accumulates in nonderepressed cultures, and the mRNA derepresses weakly. This is shown in Fig. 5 in the last two lanes of each series, in which the upper ODC mRNA band represents mRNA from the resident allele (LV10), distinct from the shorter ODC mRNA from the *spe-1* genes introduced by transformation. The mRNA from the resident allele compromises visualization of ODC mRNA from transformants PL2 and SL7, which, like the recipient strain, have full-length *spe-1* mRNA. This is particularly true of transformant SL7 (ΔUAR+L), in which ODC mRNA abundance is low. Transformants, equivalent to PL2 and SL7, of a *spe-1* strain having no endogenous ODC mRNA, however, bear out the impression of regulation imparted by Fig. 5.

No conclusions can yet be reached regarding transcriptional or posttranscriptional control mechanisms or specific control sites within the UAR or ODC mRNA leader DNA. (Normal regulation appears to depend not only on the UAR, the leader and the polyamines, but also on the coding region or 3′ untranslated region.) The phenotypes of the transformants, however, allowed us to study the effect of derangements of ODC control upon growth, ODC activity, and polyamine synthesis.

Behavior of transformants in minimal medium. All transformants grew well in minimal medium, showing that the regulation of ODC synthesis was not required for normal growth (Table I). The ODC activities of transformants PL2 (+UAR+L) and SL7 (ΔUAR+L) were normal when they were grown in minimal medium, while, in keeping with their ODC mRNA abundances, those of DP6 (+UARΔL) and DS4 (ΔUARΔL) were high, especially

that of DP6. The putrescine pools of the four strains were consistent with their ODC activities: those of constitutive transformants DP6 and DS4 were high. However, the spermidine pools of all four cultures in minimal medium were much more similar. The spermidine pool was reduced slightly in transformant SL7 in minimal medium in comparison with the control, PL2, while spermidine pools of the constitutive transformants DP6 and DS4 were significantly higher.

Effect of spermidine. Spermidine supplementation (Table I) did not affect the growth of any transformant. The ODC activity of transformant PL2 was repressed twofold by spermidine, while the enzyme activity of the constitutive transformant DP6 was only weakly affected. Significantly, the constitutive strains maintained high putrescine pools even in the presence of abnormally large spermidine pools. This confirms previous data suggesting that excess spermidine does not significantly inhibit the ODC reaction *in vivo* or *in vitro* (19, 26, 27).

When grown in the presence of spermidine, transformant SL7 (ΔUAR+L) had fivefold lower ODC specific activity compared to the SL7 culture grown in minimal medium (Table I). This indicates that ODC activity in the minimal culture was actually slightly derepressed, either by stabilization of the enzyme protein or by an increased rate of ODC synthesis. We attribute the putative derepression in minimal medium to the deficit in the spermidine and putrescine pools (Table I), which are the major signals controlling enzyme synthesis and stability (19, 28). The large effect on enzyme activity of the small polyamine deficit is understandable in light of the demonstration that only the small, freely diffusible fraction (ca. 10-15%) of the polyamine pools participate in regulation (4, 19). Changes in this fraction are not detect-

TABLE I

Growth, Ornithine Decarboxylase Activities, and Polyamine Contents of Transformants Differing
in Their *spe-1* Upstream Regions

Strain	Upstream region	Medium[a]	Doubling time (h)	ODC (sp act)	PUT[a] or CAD* (nmol/mg)	SPD[a] or APC* (nmol/mg)
PL2	+UAR+L	SPD	2.6	35	0	16.3
		MIN	2.9	67	0.6	15.6
		ARG	8.8	2940	<0.1*	0.8*
SL7	ΔUAR+L	SPD	3.0	10	0.4	16.6
		MIN	3.0	58	0.2	12.2
		ARG	7.8	512	<0.1*	0.8*
DP6	+UARΔL	SPD	2.6	1331	15.4	24.2
		MIN	3.0	1679	21.6	21.3
		ARG	5.8	5883	0.9*	2.7*
DS4	ΔUARΔL	SPD	3.0	125	2.2	28.8
		MIN	2.7	241	4.3	29.2
		ARG	8.2	860	<0.1*	0.7*

[a] Abbreviations. ARG, 1 mM Arginine supplement; MIN, minimal medium; SPD, 1 mM spermidine supplement; APC, aminopropylcadaverine; CAD, cadaverine; PUT, putrescine; SPD, spermidine.

TABLE II

Growth, Ornithine Decarboxylase Activities, and Metabolite Pools of Transformants PL2 (Normal) and DP6
(ODC-Constitutive) Grown with 1 mM Ornithine, 1 mM Methionine, or Both[a]

Strain	Medium	Doubling time (h)	ODC (sp act)	ORN (nmol per mg)	Cellular PAs (nmol/mg)		PAs in medium (nmol/ml at end)	
					PUT	SPD	PUT	SPD
PL	MIN	2.6	56	27	0.5	15	<0.1	<0.4
(+UAR+L)	ORN	3.0	60	91	0.5	14	<0.1	<0.4
	MET	1.8	70	21	0.2	14	<0.1	<0.4
	ORN + MET	2.0	75	49	0.2	18	<0.1	<0.4
DP6	MIN	2.5	1770	2	22	22	1.8	<0.4
(+UARΔL)	ORN	2.4	1956	10	58[b]	12	4.7	<0.4
	MET	2.1	2167	1	23	24	1.7	<0.4
	ORN + MET	2.0	2261	1	23	19	2.5	<0.4

[a] Averages of three determinations at intervals during exponential growth, during which few values varied by more than 10–15%. Abbreviations as in Table I.

[b] This figure is an average of values 38, 39, and 97 nmol per mg for the first, second, and third harvest, respectively.

able as such as a part of the extractable polyamines measured (2).

Effect of polyamine starvation. Inhibition of polyamine synthesis by addition of arginine caused all strains to grow more slowly (Table I). In arginine-supplemented medium, sufficient cadaverine is made to support slow growth (see Materials and Methods). The ODC protein is 10-fold more stable in polyamine-starved mycelia than in those grown in minimal or spermidine-supplemented media (26, 28), and the rate of enzyme synthesis, like ODC mRNA abundance, is normally 4- to 5-fold higher than it is in cultures grown in minimal medium (28).

Transformant DP6 (+UARΔL) was conspicuously less inhibited by arginine (Table I). This transformant displayed very high ODC-specific activity, with the appearance of considerable amounts of the lysine derivative, cadaverine. This pool and the triamine pool, presumed to be aminopropylcadaverine (12; see Materials and Methods) were larger than in other, arginine-grown transformants (Table I) and correlated well with the extremely high ODC activity. Accordingly, growth of transformant DP6 in the presence of arginine is faster than that of other strains.

The foregoing data (Table I) show that (i) in normal strains cultured in minimal medium, ODC activity is limiting to putrescine synthesis; (ii) the size of the spermidine pool is only weakly responsive to excess putrescine; (iii) excess spermidine does not block putrescine synthesis in constitutive transformants; and (iv) in terms of ODC activity and polyamine synthesis, the lack of the negatively acting ODC mRNA leader DNA (or the leader itself) more than overcomes the loss of the positively acting UAR (transformant DS4 vs SL7).

Limitations of polyamine synthesis and pools in the ODC-constitutive transformant DP6. The high ODC ac-

tivity of transformant DP6 (+UARΔL) was likely to have rendered another factor in the pathway rate-limiting. We tested the effect of adding methionine, an initial substrates of the pathway (Fig. 1), to cultures of transformants PL2 and DP6. In neither case did the parameters of polyamine metabolism change significantly (Table II). Methionine stimulated the growth of PL2 (for reasons not further investigated here) and slightly reduced the size of the putrescine pool. The data show that endogenous methionine does not normally limit polyamine synthesis *in vivo* in *N. crassa*, even when the putrescine pool is greatly enlarged.

Addition of ornithine (1 mM) to transformant PL2 had little effect. In transformant DP6, it increased the ornithine pool and caused a massive accumulation and excretion of putrescine. At the same time, the spermidine pool actually became lower. The result shows that putrescine synthesis was limited in the ODC-constitutive strain by the availability of ornithine. A more detailed experiment, comparing the DP6 strain on minimal medium and medium containing an even higher level (2.5 mM) of ornithine (Fig. 6) shows that at steady state, putrescine accumulated in the medium in proportion to cell mass. Spermidine was not excreted in this experiment (Fig. 6), and excess spermidine is not normally degraded (5). Therefore, when putrescine is ample, spermidine synthesis appears to be limited by spermidine synthase activity or the substrate levels, enzyme capacity, or negative control of the methionine tributary of the pathway.

The growth of the ornithine-supplemented culture of transformant DP6 is normal despite a putrescine pool of over 100 nmol/mg, dry weight. Earlier studies (29, 30), showed that most of a large putrescine pool (132 nmol/ mg, dry weight) of a polyamine transport mutant was rendered nontoxic by sequestration in vacuoles, although
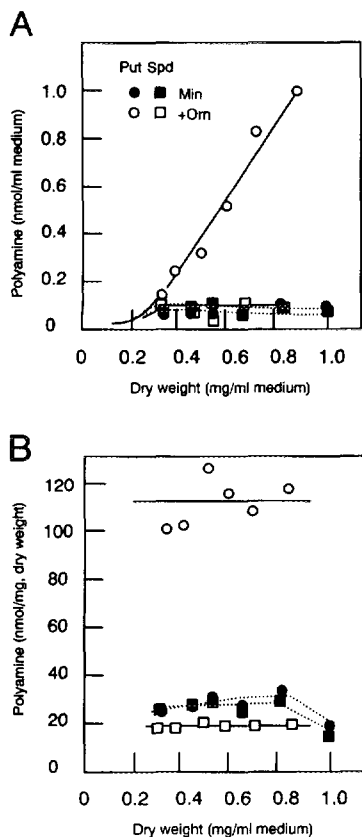
A

B

FIG. 6. Polyamine pools and excretion by transformant DP6 grown in minimal medium or medium containing 2.5 mM ornithine. (A) Polyamines in the medium of the ODC-constitutive transformant, DP6, as a function of growth. (B) Intracellular polyamines of the same cultures. Symbols (key shown in A) are putrescine (circles) and spermidine (squares) in cultures grown in minimal medium (Min, filled symbols) or cultures supplemented with 2.5 mM ornithine (+Orn, open symbols).

higher levels inhibited growth. In the present cultures, most of the putrescine was also vacuolar (data not shown). We have been unable to elevate the spermidine content of *N. crassa* mycelia more than about twofold. We therefore have no definitive evidence regarding whether (and at what concentration) spermidine is toxic to this organism, as it is to mammalian cells (31, 32). Wild-type cultures that reach stationary phase continue to synthesize spermidine, but the excess passively diffuses from the cell (5). That cultures of the constitutive transformant DP6 growing in minimal medium (Table I) did not excrete spermidine may reflect the competition for exit by the high putrescine pool of the same cells.

The role of ODC control mechanisms. Our results show that while ODC is normally limiting to polyamine synthesis, unregulated, high ODC activity has little effect on growth and spermidine pools, even when ornithine exaggerates the putrescine overproduction. The observations bear out findings made previously in *N. crassa* (27), *Escherichia coli*, mammalian cells, and transgenic rats (see

Ref. 2 for review). The high degree of control of ODC activity clearly does not reflect the need to maintain polyamine concentrations within narrow limits (2). We therefore must ask why ODC activity is normally regulated over such a large range (50- to 70-fold in *N. crassa*). No organism displays allosteric feedback inhibition of ODC activity, and rapid control of the amount of enzyme protein is almost universal. It is likely that the ODC reaction is called upon to produce unusually large amounts of putrescine and, thereby, to restore spermidine and spermine pools at the onset of growth. Feedback inhibition would be maladaptive under these conditions (2). The remaining control mechanisms may simply be incapable of fine-tuning the level of the ODC protein, owing to the time lag between regulatory stimuli (e.g., changes in the intracellular putrescine and spermidine levels) and the achievement of the appropriate enzyme specific activity.

Control of spermidine levels. The effect of putrescine overproduction in constitutive variants is limited in *N. crassa,* as it is in most organisms; spermidine pools do not vary greatly. The results highlight the question of what, beyond ODC activity, limits spermidine synthesis, and why.

In recent work with transgenic rodents, Kauppinen *et al.* (33) have shown that tissues with abnormally high spermidine synthase and ODC activities nevertheless have normal levels of spermidine (and spermine). This implicates *S*-adenosylmethionine or *S*-adenosylmethionine decarboxylase as the point of limitation of spermidine synthesis in the presence of excess putrescine. However, the evidence regarding this hypothesis is not conclusive; no experiments in which *S*-AdoMetDC activity has been elevated artificially in suitable conditions have been done. In a study designed to select cells for transgenic *S*-AdoMetDC expression, only those with modest (four- to fivefold) increases were found (34), and spermidine synthesis here may have been limited by low levels of putrescine.

In α-difluoromethylornithine-resistant mouse L1210 cells (32) or rat hepatoma variants (D23a and D23b) (31) that express high levels of ODC activity through gene amplification, spermidine uptake is also greatly enhanced. This allowed the investigators to demonstrate that spermidine added to the medium is toxic to these ODC-constitutive cells, owing to the severe inhibition of protein synthesis by the enlarged spermidine pool (31). (Transformant DP6, by contrast, grows normally in the presence of spermidine and has only a modestly elevated spermidine pool [Table I]).

The D23a and D23b hepatoma variants referred to above depend upon added α-difluoromethylornithine for continued growth, owing to the toxicity of their high, endogenous polyamine content. Using the D23a cells, Tome *et al.* (35) have recently implicated the large putrescine pool as the inhibitory factor, because spermidine levels

did not vary greatly. "Free" spermidine, however, was not excluded as a possible cytotoxic agent. Despite the uncertainties regarding the toxic factor, putrescine appears to be much less toxic than spermidine, and organisms have evolved means to protect themselves from putrescine overproduction (e.g., by excretion [Fig. 6], and sequestration [29]). Assuming that high levels of spermidine are as toxic to *N. crassa* as they are to mammalian cells (31, 32), the restriction of spermidine synthesis in the face of excess putrescine and methionine is clearly an adaptive feature of the control of polyamine biosynthesis.

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