The Neurospora crassa arg-2 Locus

STRUCTURE AND EXPRESSION OF THE GENE ENCODING THE SMALL SUBUNIT OF ARGinine-SPECIFIC CARBAMOYL PHOSPHATE SYNTHETASE

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We have characterized genomic and cDNA clones for arg-2, the gene encoding the small subunit of the Neurospora crassa arginine-specific carbamoyl phosphate synthetase (CPS-A), and examined its transcriptional regulation. The polypeptide’s predicted amino acid sequence (453 residues) is 56% and 36% identical with the sequences of the homologous polypeptides of Saccharomyces cerevisiae and Escherichia coli, respectively. The ARG2 polypeptide has an additional amino-terminal domain with the hallmark features of a mitochondrial signal sequence. The arg-2 mRNA also encodes a 24-residue peptide in the segment upstream of the coding region for the ARG2 polypeptide. This upstream open reading frame (uORF) strongly resembles the uORF in the homologous S. cerevisiae transcript. Northern analyses indicate that arg-2 mRNA levels are reduced by arginine supplementation and increased by amino acid limitation. The large increase in arg-2 mRNA levels that occurs in response to amino acid limitation is not observed in a strain containing the cpc-1 mutation, indicating that the cross-pathway control system participates in arg-2 regulation. Four copies of the sequence TGACTC, the binding site for the CPC1 regulatory protein, are found in the arg-2 genetic region. Two copies are located upstream of the mRNA start sites, and two are present within introns in the arg-2 uORF.

The Neurospora crassa arginine-specific carbamoyl phosphate synthetase (CPS-A) is a nuclear-encoded, mitochondrially localized enzyme subject to multiple levels of regulation (Davis et al., 1986). Synthesis of both the small and large subunits (the products of the arg-2 and arg-3 genes, respectively) is subject to cross-pathway-mediated induction under conditions of amino acid limitation (Davis, 1986). Synthesis of the small subunit, which provides the glutamine amidotransferase function, is in addition subject to arginine-specific regulation; growth of N. crassa in the presence of excess arginine results in a 5- to 10-fold decrease in small subunit levels (Davis and Ristow, 1987).

Synthesis of Saccharomyces cerevisiae CPS-A appears to be regulated in a manner similar to the N. crassa enzyme (Davis, 1986); however, the yeast enzyme is cytoplasmic, not mitochondrial. Levels of both yeast subunits increase under conditions of amino acid limitation; this response is mediated by the general control regulatory circuit (Hinnebusch, 1988a; Kinney and Lusty, 1989). Several TGACTC elements are located upstream of the CPA1 mRNA start site (Werner et al., 1985); in other yeast amino acid biosynthetic genes, these elements are binding sites for GCN4, the DNA binding protein that mediates the S. cerevisiae general control response (Hinnebusch, 1988a). In addition, as in Neurospora, synthesis of the small subunit, CPA1, is specifically regulated by arginine (Hinnebusch, 1988b). With regard to arginine-specific regulation of CPA1 synthesis, both cis and trans regulatory mutations have been obtained (Hinnebusch, 1988b, Werner et al., 1987). Analysis of the cis-acting mutations that abolish arginine-specific regulation demonstrated that CPA1 expression is regulated translationally by a peptide encoded in the upstream region of CPA1 mRNA (Werner et al., 1987).

In this report, we present the sequence of cloned genomic and cDNA copies of the N. crassa gene arg-2, which encodes the small subunit of CPS-A. We also describe our initial analyses of arg-2 regulation.

MATERIALS AND METHODS

Neurospora Strains—Wild-type strain 74-OR23-1A (74A) was obtained from D. Perkins, Stanford University. Strain 43a [arg-2(CD8)] pyr-3(DFC3) ao-2(M246) ao-0(MBC-11) in (90601) A was constructed by W. Schneider (Orbach et al., 1988). The cpc-1(C1B4a) strain was obtained from the Fungal Genetics Stock Center, Dept. of Microbiology, University of Kansas Medical Center, Kansas City, KS 66103.

Plasmids—pAR201 contains a 4.9-kb BamHI fragment of N. crassa DNA that encodes arg-2 (Fig. 1) inserted into the BamHI site of pUC18 (Norlander et al., 1983). pAR23 contains the 4.0-kb SacI fragment of pAR201 inserted in pUC118 (Vieira and Messing, 1987). pAR24 was constructed in two steps to generate a plasmid that has unique restriction sites with 5’ and 3’ protrusions for digestion by, and protection from, EcoRI, respectively. First, the 8.7-kb SacI fragment of pAR201 that contained arg-2 was inserted into pUC18. Then, from an appropriately oriented clone, a 3.0-kb BamHI-SacI fragment (containing the SacI-SacI arg-2 insert) was isolated and inserted into pUC118. Clones for sequencing were generated from plasmids pAR23 and pAR24 using the progressive deletion method described by Henikoff (1984). The plasmid pAN2 contains the BamHI-Psal fragment of pAR201 inserted into BamHI-HindIII-digested Bluescript plasmid pKS (Short et al., 1988).
Plasmid pAU1, which contains an intronless version of arg-2, was constructed in several steps. The 3.1-kb SacI-Post fragment derived from pAR201 was inserted into SacI-Post-digested pDP72 to construct pAE1. pDP72, a modified version of vector pSP72 (Promega Biotec), was constructed by removal of the small Xhol-HindIII fragment to create a vector which contains no PolyII sites. To form pAE1, pAE1 was digested at unique PolyII and Ncol sites to release an 8686-bp fragment which contained all three introns; this fragment was replaced with the intronless 1387-bp Ncol-Intron fragment from cDNA clone pARG228. pARG228 was obtained by probing a λ Zap cDNA library representing the mRNA of fermenting conidia with SP-labeled arg-2 DNA purified from pAR241.

The plasmids used to obtain probes for hybridization analyses were: pAR241 (this work), pHT3 (2; Orbach et al., 1986), and pSRCOX5 (cox-5; Sachs et al., 1988).

Construction of cDNA Libraries—N. crassa poly(A) mRNA was obtained from fermenting conidia, 6-h mycelia, and histidine-starved mycelial cultures (described below). RNA (5 μg) in water was heated to 65 °C for 3 min and chilled on ice. The reaction mixture for first strand cDNA synthesis was incubated for 2 h at 41 °C and contained the following components in a volume of 100 μl: 50 mM Tris-Cl (pH 8.3), 40 mM KC1, 6 mM MgCl2, 1 mM concentration each of dATP, dCTP, dGTP, and dTTP, 100 μg of (Ap3)2TdT, 0.4 mM dithiothreitol, 5 μg of oligo(dT)-15 to 80 units of RNasin (Promega Biotec), and 1 μl of histidine-starved mycelia (Seikagaku). The reaction was stopped by adjusting the EDTA concentration to 12 mM. Nuclear acids were precipitated by adding 20 μg of glycogen, 100 μl of 4 M ammonium acetate, and 400 μl of ethanol, followed by freezing on dry ice. After collection by centrifugation, the nuclear acids were washed with 80% ethanol, dissolved in 10 μl of water, and chromatographed on a 1-ml Bio-Gel A-5m column. Peak fractions were pooled and precipitated with ethanol after the addition of 20 μg of glycogen and dissolved in 10 μl of water.

The reaction mixture for second strand cDNA synthesis contained the following components in a volume of 100 μl: 1–3 μg of first strand cDNA, 100 mM HEPES-KOH (pH 6.9), 70 mM KC1, 6 mM MgCl2, 15 mM d-mercaptoethanol, 0.1 mM concentration each of dATP, dCTP, dGTP, and dTTP, 1.2 units of RNase H (Boehringer Mannheim), and 40 units of DNA polymerase I (New England Biolabs). After 2.5 h at 16 °C, the reaction was stopped by adjusting the EDTA concentration to 12 mM, followed by extraction with phenol/chloroform (1:1) and precipitation with ethanol.

The cDNA products were methylated, made flash-ended with T4 DNA polymerase, and ligated into the EcoRI site of XZap phage DNA (Short et al., 1988; phage DNA was obtained from Stratagene, San Diego, CA) as previously described (Sachs et al., 1986). Recombinant phage DNA was packaged in vitro, and amplified phage libraries were prepared as described (Short et al., 1988). The cDNA library representing mRNA from fermenting conidia was estimated to contain 81,800 different inserts. Plasmid DNA was obtained from XZAP clones by precipitation with ethanol.

The cDNA products were methylated, made flush-ended with T4 DNA polymerase, and ligated into the EcoRI site of XZap phage DNA. The cDNA library represented the mRNA of fermenting conidia with SP-labeled arg-2 DNA purified from pAR241.

RESULTS

DNA Sequence Analysis—The 3681-nt BamHI-PstI region of pAR201 (Fig. 1) was sequenced on both strands using the dideoxy chain termination method of Sanger et al. (1980) with α-[32P]dCTP as a substitute for dGTP to resolve ambiguities. Using this method, the analog deoxy-7-deazaguanosine triphosphate was used as a substitute for dGTP to resolve ambiguities. All cDNA products were methylated, made flush-ended with T4 DNA polymerase, and ligated into the EcoRI site of XZap phage DNA. Cloned DNA was prepared by standard methods (Amersham, 1983).

S1 and Primer Extension Mapping of the arg-2 Transcript—A uniformly labeled 3′-S1 mapping probe was prepared to map the 3′-end of the arg-2 transcript by using a pAR241 deletion template with an end point at base 2610, which is 374 bases downstream of the arg-2 stop codon. The extension reaction was performed in the presence of 5′-[α-32P]dCTP, and 5′-end-labeled 3644-nt S1 nuclease mapping probe obtained from a clone by co-infecting cells with λ and helper phage R406 as described (Short et al., 1988).

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Comparison of the N. crassa ARG2 Polypeptide with Other CPS Polypeptides—An alignment of the deduced amino acid sequence of the ARG2 polypeptide with other CPS polypeptides is shown in Fig. 6. The alignment reveals that the ARG2 polypeptide has a high degree of homology to the CPS polypeptides from other eukaryotes. The alignment also reveals that the ARG2 polypeptide has two regions of high similarity to the CPS polypeptides from other eukaryotes. These regions are likely to be involved in the catalytic activity of the enzyme.

Analysis of mRNA Levels by RNA Blotting—RNA was electrophoresed on 1.5% agarose gels containing 2.2 M formaldehyde, 20 mM Mops (pH 7.0), 5 mM sodium acetate, and 1 mM EDTA, following denaturation by heating at 65 °C for 10 min in the same buffer containing 50% deionized formamide. RNA was transferred to GeneScreen membrane (Du Pont-New England Nuclear) by capillary action using 20 × SSPE as the buffer (1 × SSPE is 10 mM NaCl, 180 mM NaH2PO4, 50 mM NaHPO4, 1 mM EDTA). Blots were incubated in prehybridization solution (50% formamide, 0.2% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.2% Ficoll (molecular weight 400,000), 0.05 M Tris-HCl (pH 7.5), 1 M NaCl, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate, 10% dextran sulfate (molecular weight 500,000), 100 μg/ml denatured salmon sperm DNA). SP-labeled DNA probes (106 rpm), prepared by the random priming method (Feinberg and Vogelstein, 1983), were added to the prehybridization mixtures; RNA blots were hybridized with probes for 14 to 18 h at 42 °C! and then were washed twice for 5–10 min with 2 × SSPE at room temperature and twice for 30 min with 0.2 × SSPE. The blots were exposed at -80 °C using Kodak XAR film and x-ray intensifying screens.

RESULTS

The Sequence of arg-2—We previously described the cloning of arg-2 genomic DNA by direct recovery in Escherichia coli (Orbach et al., 1988). The DNA sequence of a 3681-nt BamHI/PstI fragment containing the arg-2 coding and surrounding regions was obtained by sequencing DNA strands (see "Materials and Methods"). The arg-2 coding region, present in two exons, specifies a polypeptide of 453 amino acid residues (Figs. 1 and 2). Synthesis of the ARG2 polypeptide is predicted to initiate at the second AUG codon in the mRNA (nt 1451–1453, Fig. 2) and proceed to a UAA stop codon (nt 2866–2868). The coding region is interrupted by one 56-nucleotide intron between codons 60 and 61, based on the nucleotide mapping studies and comparison of cDNA and genomic sequences. The codons used to specify the ARG2 polypeptide are biased in a manner similar to other N. crassa genes (Orbach et al., 1986; Sachs et al., 1989).
sequence of the N. crassa ARG2 polypeptide (Nyunoya and Lustig, 1984; Werner et al., 1985) and the E. coli CarA polypeptide (Piette et al., 1984) is shown in Fig. 3A. In this alignment, 56% of the residues are identical between Neurospora and yeast and 36% are identical between Neurospora and E. coli; there are regions of high identity, separated by less conserved segments. The greatest conservation of primary sequence is observed in the regions corresponding to residues 78–126, 171–191, 232–261, and 331–404 of ARG2. The rat CPSI protein (Nyunoya et al., 1986) also shows significant homology to ARG2 in these regions and can be aligned with 35% identical residues (data not shown). The relatedness of the ARG2 polypeptide to other glutamine amidotransferases is discussed in more detail below.

The 45 amino-terminal residues of ARG2 have no counterparts in CPA1 or CarA (Fig. 3A). Furthermore, the predicted size of the ARG2 primary translation product, 48,929 Da, is larger than the measured size of the mature ARG2 polypeptide, 45,000 Da (Davis et al., 1981). Because the N. crassa protein is located in the mitochondrial matrix and the yeast protein in the cytosol, the N. crassa polypeptide would be predicted to contain an amino-terminal signal sequence to target it to the mitochondrion (von Heijne, 1986; von Heijne et al., 1989). Mitochondrial signal sequences are generally rich in serine, leucine, and basic residues, especially arginine, while acidic residues are rare (von Heijne, 1986; von Heijne et al., 1989). The amino-terminal 45-residue domain of ARG2 conforms to these biases. In addition, part of this sequence can be modelled (von Heijne, 1986) as an amphipathic helix with a large hydrophobic moment ($\mu_H = 9.1$ for residues 19–36). The amino-terminal domain of ARG2 thus contains the features of mitochondrial signal sequences.

**Mapping of the Ends of the arg-2 Transcript**—To map the 5' ends of the arg-2 transcript, we used a 5'-labeled DNA fragment containing a spliced copy of nt 634–1718B of the DNA sequence shown in Fig. 2 for S1 nuclease protection experiments. This DNA fragment was annealed to N. crassa total RNA, the products were digested with S1 nuclease, and the protected fragments were analyzed on denaturing gels (Fig. 4). Lanes 3 and 4 show that the 5' ends of arg-2 transcripts are heterogeneous. Two major classes have ends distributed near nt 752 and 809, respectively. The results of primer extension mapping of the arg-2 mRNA 5' ends using a synthetic primer complementary to nt 1359–1375 and poly(A) mRNA were consistent with the S1 mapping results (data not shown). In addition, there were no detectable intron-containing RNA species in the preparations examined. S1 analyses using RNA probes that contained intron sequences accurately placed the 3' boundaries of the second and third introns (data not shown).

**Analysis of Expression of the arg-2 Gene**—Synthesis of the small subunit of CPS-A, like many polypeptides subject to cross-pathway control in N. crassa (Flint and Kemp, 1981), is induced by amino acid limitation (Cybis and Davis, 1975). This induction does not occur when a strain bearing a cpc-1 mutation such as cpc-1(CD15) is subjected to amino acid limitation, indicating that arg-2 is regulated by cross-pathway control (Davis, 1986). To determine whether cross-pathway-mediated regulation of arg-2 is transcriptional, the level of arg-2 mRNA was measured in poly(A) RNA isolated from wild type mycelia grown under four conditions (Fig. 5A, arg-2 panel). The levels of arg-2 transcripts increased significantly when histidine limitation was imposed by growth in the presence of the inhibitor of histidine biosynthesis 3-amino-1,2,4-triazole (+AT). High levels of transcript were also observed in cells grown in the presence of excess tryptophan (+TRP), i.e. under conditions of amino acid imbalance. arg-2 transcript levels were lower in cultures grown in the presence of excess argnine (+ARG) relative to growth in minimal medium (MIN). Reprobing the filters with labeled Neurospora $\beta$-tubulin DNA showed that similar $\beta$-tubulin mRNA levels were present under all growth conditions used (Fig. 5A, tub-2 panel). The transcript levels for two other genes subject to cross-pathway control, trp-1 and his-3, also increased in histidine-starved cultures and in the presence of excess tryptophan (data not shown).

**Total RNA was isolated from wild type** (74A and cpc-1 (CD15)A strains at several intervals following addition of AT
to cultures growing in minimal medium. RNA blot analyses (Fig. 5B, arg-2 panel) indicate that in strain 74A, arg-2 mRNA levels increase within 30 min after AT addition and remain elevated. After 60 min of growth in the presence of AT, the levels of arg-2 mRNA do not increase in the cpc-I(CD15) strain to the same extent as in the 74A strain. 74A urg-2 mRNA levels are reduced by growth in minimal medium supplemented with 1 mM arginine, and they are further reduced by growth in a rich medium (Fig. 5B). RNA samples from these cultures were also probed with ~0x5 DNA (Fig. 5B, ~0x5 panel), which encodes V. crassa cytochrome oxidase subunit V. ~0x5 mRNA levels were similar under all growth conditions, except for a slight decrease after prolonged growth with AT.

The Minimal arg-2 Functional Unit as Defined by DNA-mediated Transformation—In N. crassa, transformation occurs predominantly by nonhomologous integration of introduced DNA (Case, 1986; Dhaule and Marzluff, 1985; Legerton and Yanofsky, 1985; Vollmer and Yanofsky, 1986). Levels of transformation to prototrophy with a truncated gene are typically 1–10% of the levels obtained with the complete gene (Case, 1986; Legerton and Yanofsky, 1985), presumably because transformation to prototrophy mainly occurs by homologous integration or gene conversion. Using...
transformation frequency as an assay, we examined restriction endonuclease-digested DNA to determine the approximate end points of the arg-2 functional unit. The arg-2 strain 43a was transformed (Orbach et al., 1986) with both supercoiled and restriction endonuclease-digested plasmid pAR201, which contains the wild type arg-2 gene on a 4.9-kilobase BamHI fragment (Fig. 1). Plasmid DNA digested with BamHI, SaeI, PvuII, and Psil (Fig. 1) transformed 43a to arginine prototrophy at approximately the same frequency as undigested pAR201 (greater than 10^7 transformants/µg of DNA), whereas DNA digested with NcoI, HindIII, and KpnI gave prototrophic transformants at greatly reduced frequencies (less than 5 transformants/µg of DNA). These results indicate that the arg-2 functional unit is contained within the 2.8-kb PvuII/PstI fragment of pAR201.

In an attempt to determine the extent of the upstream region required for expression of arg-2, relative transformation efficiencies were determined for a series of plasmids with deletions of upstream sequences. High transformation frequencies were observed for 5'-deletions with end points up to position 1310, while low transformation frequencies were observed for 5'-deletions with end points at position 1332 and beyond (Fig. 1). Thus, sequences important for arg-2 expression, but not necessarily its regulation, appear to be located between nt 1311 and 1332. Interestingly, this region lies within the second intron of the uORF. The explanation for high frequency transformation to prototrophy by a presumed promoter-less fragment is not known.

**DISCUSSION**

The Structure of the ARG2 Polypeptide—The arg-2 gene of *N. crassa* encodes the small subunit of arginine-specific carbamoyl phosphate synthetase. The ARG2 polypeptide sequence, deduced from the DNA sequence shown in Fig. 2, is homologous to the sequences of the corresponding yeast and *E. coli* polypeptides (Fig. 3A). These proteins, all of which function as glutamine amidotransferase subunits, contain domains which are homologous to other glutamine amidotransferases. Three of the conserved regions have been shown to be essential for the function of other glutamine amidotransferases (Amuro et al., 1985; Kawamura et al., 1978; Paluh et al., 1985; Rubino et al., 1986; Tso et al., 1980; Weng and Zalkin, 1987). These domains, which are located in the carboxyl-terminal half of the CPS subunits, are indicated in Fig. 3A, and are aligned to some other glutamine amidotransferase polypeptides in Fig. 6.

The amino-terminal halves of the ARG2 polypeptide also show significant sequence conservation (Fig. 3A). The role(s) of these segments are unknown; one possibility is that they are responsible for association of the small and large CPS subunits. Mutations in this portion of the *E. coli* CarA polypeptide have been shown to destabilize complex formation.
Fig. 5. Northern analysis of arg-2 transcription. A, poly(A) mRNA was isolated from midlog phase wild type strain 74A grown in minimal medium (MIN), in minimal medium supplemented with 1 mM arginine (+ARG), in minimal medium with histidine limitation imposed by the addition of 5 mM 3-amino-1,2,4-triazole (+AT) for 3 h, or from cultures that were transferred to fresh minimal medium containing 1 mM tryptophan for 40 min (+TRP) prior to harvesting. Fifteen μg of poly(A) mRNA from each sample was separated on a 1.2% agarose, 6% formaldehyde gel, transferred to nylon membranes, and probed with 32P-labeled DNA representing the N. crassa arg-2 or tub-2 genes, as indicated above the panels. B, Northern analysis of arg-2 transcription in 74A and cpc-1 CD15). Total RNA was isolated from midlog phase cultures of strain 74A grown in minimal medium (MIN), in minimal medium containing either 1% (w/v) NZamine (+NZN), in minimal medium containing either 1% (w/v) MIN plus 1% (w/v) NZamine (+MZN), in minimal medium containing either 1% (w/v) MIN plus 1% (w/v) NZamine (+AT) for varying lengths of time, as indicated (in minutes) below the +AT arrow. Total RNA was isolated from the cpc-1 (CD15) strain grown in minimal medium (MIN) or with histidine limitation imposed by the addition of AT for 60 min. Blots containing 15 μg of total RNA from each sample were prepared and probed with either arg-2 orcox-5 DNA.

Fig. 6. Comparisons of conserved domains among selected glutamine amidotransferases. Shown are domains of CPS small subunits (this work; Nyunoya and Lusty, 1984; Nyunoya et al., 1985; Piette et al., 1984; Werner et al., 1985), anthranilate synthase component II polypeptides (Nichols et al., 1980; Schechtm an and Yanofsky, 1983; Zal kin et al., 1984), p-aminobenzoate synthase (Kaplan and Nichols, 1985), and CTP and GMP synthetases (Weng et al., 1986; Zal kin et al., 1985).

with the large subunit (Guillou et al., 1989), in agreement with this hypothesis.

The arg-2 Upstream Open Reading Frame—Both the N. crassa arg-2 and yeast CPA1 transcripts contain an open reading frame upstream of the CPS-A translational start site. The transcripts of the Neurospora cross-pathway control gene cpc-1 and its yeast general control homolog GCN4 also contain uORFs that have roles in regulating gene expression (Hinnebusch, 1988, a and b; Paluh et al., 1988). However, the uORFs of cpc-1 and GCN4 do not share codon sequence similarity, in contrast to the uORFs of arg-2 and CPA1, which encode homologous peptides (Fig. 3B).

Analysis of the regulation of the yeast CPA1 gene has shown that translation of its uORF is necessary for the arginine-specific regulation of CPS-A polypeptide synthesis, and that single missense mutations in this uORF can abolish this regulation (Werner et al., 1987). Of the two codons in the yeast uORF known to be important for arginine-specific regulation (Werner et al., 1987), Asp-13 codon in the yeast sequence is also present in Neurospora, and the Cys-11 codon is conservatively substituted by a Ser codon in Neurospora. Comparison of the codons of the fungal CPS-A uORFs (data not shown) indicates that most nucleotide differences in their homologous regions are in codon third positions, consistent with the hypothesis that the amino acid sequence of the peptide has functional significance.

Potential Transcriptional Elements Associated with the arg-2 Gene—The sequence TGACTC, or a closely related sequence, is present in the 5'-regions of several N. crassa genes that respond to amino acid limitation or imbalance through the cross-pathway control system (Chow and RajBhandary, 1989; Paluh et al., 1988). The N. crassa CPC1 protein binds to TGACTC elements in vitro.4 In S. cerevisiae, TGACTC sequences are present in the 5' regions of genes regulated by general amino acid control and serve as binding sites for the transcriptional activator GCN4 (Hinnebusch, 1988a). The CPC1 and GCN4 polypeptides have structurally and functionally similar DNA binding domains (Paluh et al., 1988). Therefore, it is likely that, as in yeast general amino acid control, TGACTC is a cross-pathway control regulatory sequence in Neurospora.

Levels of arg-2 mRNA are increased by amino acid starvation by a cpc-1-dependent pathway, as shown in Fig. 5. There are four TGACTC sequences or their complements in the DNA region that we have sequenced (boxed in Fig. 2). Two TGACTC elements are upstream of the mRNA 5'-ends, and two are downstream of the mRNA 5'-ends, one in each of the introns in the uORF. We are currently investigating the role of each of these potential target sequences in the regulation of ARG2 synthesis. Because arg-2 mRNA levels are decreased by growth in the presence of arginine (Fig. 5), arg-2 transcription or mRNA stability may also be subject to arginine-specific regulation.

DNA sequence analyses of genes from N. crassa have shown that they often are not preceded by the conserved elements involved in transcription of other eukaryotic genes, such as the TATA box and the CCAAT box (Ballance, 1986). The arg-2 gene does not contain an obvious TATA box, but it does contain CCAAT-like boxes in a motif repeated three times upstream of the arg-2 mRNA start sites (indicated by heavy underlines in Fig. 2). In other eukaryotes, the CCAAT box binds many different factors (Dorn et al., 1987; Forsburg and Guarente, 1989). Whether CCAAT binding proteins have roles in arg-2 transcription remains to be determined.

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