The *Neurospora crassa* Carotenoid Biosynthetic Gene (Albino 3) Reveals Highly Conserved Regions among Prenyltransferases*

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In the filamentous fungus *Neurospora crassa* the biosynthesis of carotenoids is regulated by blue light. Here we report the characterization of the albino-3 (al-3) gene of *N. crassa*, which encodes the carotenoid biosynthetic enzyme geranylgeranyl-pyrophosphate synthetase. This is the first geranylgeranyl-pyrophosphate synthetase gene isolated. Nucleotide sequence comparison of al-3 genomic and cDNA clones revealed that the al-3 gene is not interrupted by introns. Transcription of the al-3 gene has been examined in dark-grown and light-induced mycelia. The analysis revealed that the al-3 gene is not expressed in the dark and that its transcription is induced by blue light (Nelson, M. A., Morelli, G., Carattoli, A., Romano, N., and Macino, G. (1989) Mol. Cell. Biol. 9, 1271–1276). The al-3 gene encodes a polypeptide of 428 amino acids. Comparison of the deduced amino acid sequence of al-3 with the sequences of prenyltransferases of other species, from bacteria to humans, showed three highly conserved homologous regions. These homologous regions may be involved in the formation of the catalytic site of the prenyltransferases.

Carotenoids are synthesized by bacteria, plants, fungi, and algae (2). While their primary functions are in photoprotection and as accessory pigments in photosynthesis, carotenoids also serve as precursors for vitamin A biosynthesis in animals and for abscissic acid biosynthesis in plants. In *Neurospora crassa* the biosynthesis of carotenoids is regulated by blue light in the mycelium but is constitutive in the asexual spores (3–5). The photoinduction of carotenogenesis in the mycelia requires the *de novo* synthesis of at least three enzymes which have been shown to be the products of the albino (al) genes (5). Three al mutants have been characterized in *N. crassa*, each of which is defective in one step of carotenogenesis.

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Albino 3 (al-3) mutants are defective in GGPP1 synthetase (5), while albino 2 and albino 1 mutants are defective in phytoene synthetase (6) and phytoene dehydrogenase (7), respectively. In previous work we isolated the gene encoding GGPP synthetase from *N. crassa* by complementation of the al-3 mutant; expression studies showed that the transcription of the al-3 gene is controlled by light regulation (1).

The pathway of carotenoid biosynthesis shares some steps with the biosynthetic pathways of other isoprenoid compounds. GGPP synthetase and several other enzymes of these complex pathways are members of the prenyltransferase family. Prenyltransferases catalyze the transfer of an isoprenoid diphosphate to another isoprenoid diphosphate or to a nonisoprenoid compound through a 1'-4 condensation reaction to produce various prenyl compounds that are precursors of such diverse products as steroids, carotenoids, chlorophylls, heme, prenylated proteins, and tRNAs, glycosyl carrier lipids, plant hormones, and the side chains of quinones (8–10). Prenyltransferases produce a wide range of products, from the simple dimer geranyl pyrophosphate to the complex structure of rubber which is thousands of monomers long. GGPP synthetase catalyzes the addition of three molecules of IPP onto DMAPP to form geranylgeranyl pyrophosphate. Here we present the sequence of the al-3 gene and its deduced amino acid sequence. The al-3 protein sequence is compared with those of other known prenyltransferases.

MATERIALS AND METHODS AND RESULTS

DISCUSSION

We have determined the nucleotide sequence of the al-3 gene, which encodes the carotenoid biosynthetic enzyme GGPP synthetase. It is known that blue light induces the biosynthesis of carotenoids in *N. crassa* mycelia and that the activity of GGPP synthetase increases after light treatment (8). The al-3 gene encodes an mRNA of 1683 nucleotides, which is colinear with the al-3 gene, as revealed by cDNA sequence and S1 nuclease mapping analysis. We analyzed the expression of the al-3 gene and found that its mRNA is not present in dark-grown mycelia but is induced by blue light after a short pulse of illumination.
The polypeptide encoded by the al-3 gene has a molecular mass of 47,876 daltons, is weakly basic and hydrophilic, and does not possess any hydrophobic membrane-spanning regions. This is in agreement with the finding that the GGPPS activity, isolated from various sources, is present in the soluble fraction of cellular extracts (8, 36, 37). GGPPS synthetase is a prenyltransferase that catalyzes the 1'-4 condensation of dimethylallyl pyrophosphate with three isopentenyl pyrophosphates. We therefore compared the al-3 polypeptide with other known prenyltransferases. Comparison with the FPP synthetase from Dr. P. Edwards that S. cerevisiae hexaprenyl pyrophosphate synthetase has amino acid sequence homology with domains I and II. We also showed evidence that the cysteine enzyme has a 1'-4 condensation activity.

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**Addendum**—During the preparation of the manuscript, we learned from Dr. P. Edwards that S. cerevisiae hexaprenyl pyrophosphate synthetase has amino acid sequence homology with domains I and II.

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N. crassa strains and plasmids. The following Neurospora crassa wild-type and mutant strains (32) were obtained from the University of Kansas, Kansas City. Kanamycin-resistant transformants were selected in the assay presented in the text. All the other strains listed in Table 1 were obtained from the National Collection of Arthritis and Rheumatism. resistance gene for selection in Neurospora. The plasmid p23 was isolated from this strain and pPHAS3 was made by the use of pBAS70. The pPHAS70 was cloned into pBR322. The pPHAS70 was then cloned into pBR322.

Preparation of DNA. Total DNA was extracted from frozen mycelia powdered mycelia were treated with trisodium citrate as a protein denaturant (31) and RNA was collected by phenol-chloroform extraction. Conditions (73) polyadenylated RNA was isolated by chromatography on oligo(dT)-cellulose II.
Neurospora crassa Geranylgeranyl Pyrophosphate Synthetase Gene

Localization of the al-3 gene in NC12

We had previously cloned the GTP7 synthetase gene by transformation of an al-3 ga-2 ar-9 mutant of N. crassa, with a genomic library in the ph27 plasmid (raw materials and methods) [1]. From this library we had isolated the plasmid pNCS9, with a 7.0 kbp genomic DNA insert containing the intact al-3 gene. The 6.5 kbp of the translines containing this plasmid showed dark orange pigmented colonies, in contrast to the albino phenotype of the al-3 parental strain (table 1). To determine the minimal region of the insert necessary for complementation, we transformed the al-3 ga-2 ar-9 strain with several plasmids containing various subclones of the pNCS9 insert. The pNCS9 plasmid, with an insert of 3.0 kbp (obtained by deletion of the 5.0 kbp Hpa I fragment of the pNCS9 plasmid) was still able to complement the al-3 mutation (showing 54% wild type transformants, table 1). Thus pNCS9HpaI plasmid was chosen for further studies.

| Phenotypes of plasmid transformants | Flasks | %
|------------------------------------|-------|---|
| pNCS9HpaI | 0 | 200 |
| pNCS9 | 42 | 50 |
| pNCS9 HpaI | 54 | 46 |

Table 1: Percentage of wild type and albino 1 phenotypes obtained by al-3 ga-2 ar-9 strain transformation with different plasmids.

The pNCS9 plasmid contains a 7.0 kbp N. crassa genomic insert cloned in the ph27 vector. The pNCS9HpaI plasmid contains a 3.0 kbp DNA insert obtained by deletion of the 5.0 kbp Hpa I fragment of pNCS9.

DNA sequence of the al-3 gene and its predicted gene product

The HindII-SalI, SalI-EcoRI, EcoRI-SalI and SacII-AccI fragments from pNCS9HpaI were subcloned into pUC18 and pUC18 for nucleotide sequencing. Each construct was used as a template for sequencing reactions using the universal primer (21). The complete sequence of the clones was obtained using synthetic primers corresponding to the ends of previously sequenced DNA. Both strands were sequenced with overlaps to ensure any ambiguities. The DNA sequence of the al-3 coding and flanking regions is shown in figure 1. The nomenclature corresponds to the 2.8 kbp HindIII-AccI fragment of NC12. Within the sequence there is only one coding region, which encodes a protein of 439 amino acids. This reading frame is translated above the nucleotide sequence in figure 2. The gene usage of the putative gene product follows the general rules for N. crassa genes, which includes a strong bias towards the use of codons terminating in C (22%) or U (23%).

There are two possible initiation codons at the beginning of the open reading frame. The ATG methionine codon of the al-3 reading frame most likely occurs at position 162 (fig. 2). The nucleotides that precede this codon (nucleotides 92-162) are believed to be important for contact translation and are found in the same position in many N. crassa genes (23). The predicted product is a polypeptide with a molecular weight of about 47,800. The predicted product is hydrophilic and hydrophobic. The hydrophobic profile of trye and Hollobaugh (24) suggested that there are no hydrophobic N-terminal spanning regions (not shown).

The coding portion of the gene has a G-C content of 59%, while the 5′- and 3′- non-translated sequences have G-C contents of 45% and 42%, respectively. This is in agreement with what has been found in other N. crassa genes (25, 26). The 5'-non-translated region contains a putative TATA box about 30 nucleotides upstream of the 5′-transcriptional initiation site. A search for short sequences homology to published promoter sequences of other N. crassa genes failed to turn up any significant homologies. A potential polidamylin recognition element (AAGGAAA) is present 50 bp upstream of the 3′-end of the TATA box.
Neurospora crassa  Geranylgeranyl Pyrophosphate Synthetase Gene

**Fig. 2:** DNA sequence of the al-3 gene and its predicted gene product. Nucleotide regions of the al-3 gene. The putative TATA Sequence and polyadenylation sequence of the 2.0 kbp HindIII-AccI fragment of Fig. 2. Containing the coding WA a8 and determined by SI. nuclease mapping. The Open arrow shows the mRNA 3' end recognition signal are underlined. The arrows indicate the 5' and 3' ends of the determined by SI. DNA sequence. The sequence of the oligonucleotide used for labeling the probe for 5' end studies is also shown.

**Fig. 3:** Northern hybridizations. Total mRNA was prepared from light-induced (L) and from dark-grown (D) mycelia. Samples (4 μg) were separated on a 1% agarose-formamide gel, transferred to nitrocellulose membrane, and hybridized with labeled probes (Fig. 2). Results are shown, respectively, in panels A, B and C.

**Transcription analysis**

In a previous paper (1) we demonstrated that the al-3 mRNA level increases about 15 fold in mycelia, after a short period of illumination, compared to its low level in the dark. Here we present results that confirm the photoregulation of the al-3 mRNA, but on the contrary its level in the dark is undetectable by Northern blotting analysis. This difference is due to the fact that the previous Northern blots were probed with a DNA fragment that contained the al-3 gene plus another gene. This gene is closely linked to al-3 and encodes a messenger RNA of almost identical size (about 1700 nucleotides). The linked gene is transcribed in the opposite direction and expressed in both light and dark regimes (4). Fig. 4A shows that the longer probe a (Fig. 1) that had been used in previous work (1) detected both mRNAs, that of al-3 plus the mRNA of the closely-linked but unrelated gene. Fig. 4B shows that when a probe was used which contains only the al-3 gene [probe b], the mRNA for al-3 was detected only in light-grown mycelia and was undetectable in dark-grown mycelia. Probe c detected only the mRNA of the linked gene; this mRNA comigrated with the al-3 mRNA. Probe c was not photoregulated. These results have been confirmed with more sensitive 5' nuclease experiments (Fig. 4C). Probes a and b were totally degraded when total RNA from dark-grown mycelia was used, while they were completely protected using total or poly(A)^+^ RNA from light-induced mycelia (see below).

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**Fig.3:** DNA sequence of the al-3 gene and its predicted gene product. Nucleotide sequence of the 2.0 kb HindIII-AccI fragment of the al-3 gene. The putative TATA sequence and polyadenylation recognition signals are underlined. The arrows indicate the 5' and 3' ends of the mRNA as determined by S1 nuclease mapping. The open arrow shows the mRNA 3' end determined by clone sequence. The sequence of the oligonucleotide used for labeling the probe for 5' end studies is also shown.

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4- A. Carabelli unpublished results

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Neurospora crassa Geranylgeranyl Pyrophosphate Synthetase Gene

Isolation of cDNA clones of al-3

To confirm that the al-3 gene contains no intervening sequences, we isolated cDNA clones from a library made from light-induced mycelia. Using a 750 bp HindIII-SalI 32P radiolabeled fragment of the al-3 gene (probe b in Fig. 1), 10 positive clones were identified by screening 60,000 colonies of the library. The longest positive cDNA clone was selected for sequence analysis. The complete sequence of the al-3 cDNA clone revealed that the cDNA was shorter, at the 5' end, by about 80 nucleotides than the putative mRNA determined by S1 nuclease mapping (see below). The sequence analysis of the cDNA showed collinearity with the genomic sequence, indicating that there are no introns in this gene.

S1 nuclease mapping

To determine the 5' initiation and 3' termination sites of the al-3 mRNA, S1 protection experiments were performed (Fig. 4). The 5' end of the al-3 mRNA was mapped as shown in Fig. 4a. A DNA probe spanning the 5' end of the al-3 gene (probe 1, 358 bp) was uniformly labeled, hybridized to RNA and treated with S1 nuclease. Fig. 4a shows that S1 nuclease-protected products obtained with total RNA (lane 2) or poly(A)+ RNA (lane 3), corresponded to two groups of closely spaced bands (from 265 to 283 nucleotides in length). These multiple bands most likely represent multiple transcription initiation sites. This is consistent with the finding that, in fungi, multiple initiation sites are quite common (27).

Fig. 4b shows the 3' end mapping of the al-3 mRNA. Probe 2 (520 bp) was protected from S1 nuclease digestion, producing a protected fragment of 200 nucleotides. This result suggested that the 3' end of the al-3 mRNA was at about nucleotide 1745 (Fig. 2). The cDNA sequence revealed, instead, that the 3' end of the al-3 mRNA was 15 nucleotides further downstream (nucleotide 1759, Fig. 2). This discrepancy could be explained by the fact that the region immediately downstream from nucleotide 1745 is very T-rich. This peculiar sequence has a low hybrid stability, and therefore may produce shorter hybrid molecules in S1 nuclease protection experiments. The length of the al-3 mRNA, as revealed by cDNA sequence and S1 nuclease experiments, was 1683 nucleotides.

Fig. 4: S1 nuclease mapping. S1 nuclease mapping of the 5' end (probe 1, panel A) and 3' end (probe 2, panel B) of al-3 mRNA. L indicates RNA extracted from light-induced mycelia and D RNA extracted from dark-grown mycelia. Probes 1 and 2 are shown below the panels.

Panel A: lane 1, sequence ladder; lane 3, probe 1 plus 30 μg of total L RNA, SI-treated; lane 3, probe 1 plus 2 μg of poly(A)+ L RNA, SI-treated; lane 4, probe 1 plus 20 μg of total D RNA, SI-treated.

Panel B: lane 1, probe 2 plus 30 μg of total L RNA, SI-treated; lane 2, probe 2 plus 30 μg of total D RNA, SI-treated; lane 3, sequence ladder. Arrow heads in panels A and B indicate the fragments protected from S1 nuclease digestion.

Homozygous with other prenyltransferases

Comparison of the al-3 sequence with the FPP synthase from Saccharomyces cerevisiae (19) showed a significant homology in three different regions of these two proteins. These regions are also highly conserved between the FPP synthase from human (19) and rat (10) (data not shown). This sequence homology is also evident in the sequence of the crf6 gene, which is part of the carotenoid biosynthetic cluster from Rhodothermus capitis (11), and which contains the same three homologous domains. The relative positions of the homologous domains are the same in these three proteins. Fig. 5 shows the alignment of the three domains of al-3 with the yeast FPP synthase and R. capitis crf6 proteins. When al-3 was compared with FPP synthase and crf6, the first region of homology (domain I in Fig. 5) showed 44% and 51% amino acid identity, or 54% and 65% amino acid similarity, respectively. A closer examination revealed a conserved sequence (LEAEDDEKEDDEK) of highly-charged amino acids. The second region (domain II) showed a weaker homology among the three proteins, but it is still possible to consider it as a conserved region if the al-3 sequence is seen as a mosaic of amino acids of the other two proteins, with the left portion being homologous to FPP synthase, and the right portion to crf6. It is interesting to note that this domain has been indicated to be the active site of prenyltransferases by work of Azem et al. (12). The third region (domain III) had 68% to 70% homology, among the three related proteins, over a span of 13 amino acids. The consensus sequence of this region (FKGKXKXKXK) is also highly charged, as is that of the first domain. It is noteworthy that, at the distance of 12 amino acids from domain III, there is an invariant lysine residue.

The three domains are localized in the proteins with very similar spacing, with 54-59 residues between domain I and domain II; 23-37 residues between domains II and III. Furthermore, the three proteins share other similarities. Domains I and III are flanked by short hydrophobic residues of about 20 amino acids. It is interesting to note that these are the only regions with hydrophobic character in these proteins. Domain II is immersed in a region of strong acidic character. The carboxyl terminus of both OSPP synthase and FPP synthase are highly charged.

There are other enzymes that make use of SMAPP to prenylate non-isoprene substrates whose genes have been isolated. One of these is the E. coli prenyltransferase 445 gene, encoding the protein-modifying enzyme E. coli prenyl pyrophosphate:RNA isopentenyl transferase (33). Comparison of the al-3, FPP synthase and crf6 amino acid sequences with that of mod5 revealed only a very limited homology to the conserved domains I and III. In domain I, the only residues conserved are four contiguous amino acids (NRNR) of the consensus sequence. In the third domain, the homology includes the entire consensus sequence (FKGKXKXKXK). This limited homology should be considered significant, due to the conservation of amino acids whose role could be central in these domains (see Discussion). It is noteworthy that the distance between the mod5 I and III domains is very similar to the respective distances in al-3 and crf6.

Other enzymes utilizing FPP or SMAPP as substrates, such as IPP- SMAPP isomerase from Z. cerevisiae (34) and cytokinin prenyltransferases from Aphelanthaster timulactica and Pseudomonas savastanoi (35), do not share any homology with the prenyl transferase discussed in this paper.