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Cloning, Sequence, and Photoregulation of *al-1*, a Carotenoid Biosynthetic Gene of *Neurospora crassa*

THOMAS J. SCHMIDHAUSER,[1]† FRANK R. LAUTER,[2] VINCENZO E. A. RUSSO,[2] AND CHARLES YANOFSKY[1]*

Department of Biological Sciences, Stanford University, Stanford, California 94305-5020,[1] and Max-Planck-Institut für Molekulare Genetik, Abteilung Trautner, Ihnestrasse 73, D-1000 Berlin 33, Federal Republic of Germany[2]

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Carotenoid biosynthesis is regulated by blue light during growth of *Neurospora crassa* mycelia. We have cloned the *al-1* gene of *N. crassa* encoding the carotenoid-biosynthetic enzyme phytoene dehydrogenase and present an analysis of its structure and regulation. The gene encodes a 595-residue polypeptide that shows homology to two procaryotic carotenoid dehydrogenases. RNA measurements showed that the level of *al-1* mRNA increased over 70-fold in photoinduced mycelia. Transcription run-on studies indicated that the *al-1* gene was regulated at the level of initiation of transcription in response to photoinduction. The photoinduced increase of *al-1* mRNA levels was not observed in two *Neurospora* mutants defective in all physiological photoresponses. Analysis of a cosmid containing *al-1* and of a translocation strain with a breakpoint within *al-1* indicated that *al-1* transcription proceeds towards the centromere of linkage group I of *N. crassa*.

Carotenoids are the most widespread group of pigments in nature. Most of the over 500 carotenoids identified to date contain a C_{40} carbon backbone consisting of eight C_5 isoprene units. The distinctive yellow to red colors of this group of pigments are derived from the absorption maxima of a polyene chain containing 3 to 15 conjugated double bonds. Carotenoids protect against photooxidative damage and also harvest light in photosynthetic systems (8, 14, 22). Carotenoids are synthesized in all photosynthetic organisms and in many nonphotosynthetic bacteria and fungi (17), and they are the precursors of vitamin A in mammals (3, 31). The composition, accumulation, and localization of carotenoid pigments are subject to regulation by photoinduction, stage of development, formation of chloroplasts, and transition from chloroplasts to chromoplasts (5).

The filamentous ascomycete *Neurospora crassa* is an excellent model organism for the study of regulation of carotenoid biosynthesis. Investigations of carotenoid formation in *N. crassa* have provided a wealth of information on the genes and reactions of this pathway (5, 18, 26, 34). During mycelial growth, carotenoid biosynthesis is regulated in response to photoinduction (18), whereas it can proceed independently of photoinduction during the developmental pathway culminating in the maturation of asexual spores. Mutants carrying mutations in the white collar genes *wc-1* and *wc-2* are not photoinducible (11, 19). Carotenoids are essential for photoprotection of the photosynthetic apparatus of plants (22); however, they are nonessential to the growth of *N. crassa*. The three albino genes *al-1*, *al-2*, and *al-3* encode enzymes that are essential for carotenogenesis (19). In *N. crassa*, the distinctive orange color of carotenoids provides a visual indicator of their function.

The accumulation of the colorless carotenoid phytoene in *al-1* mutants of *N. crassa* suggests that *al-1* encodes the enzyme phytoene dehydrogenase (16). In this report we provide direct support for this view. We describe the isolation of the *al-1* gene and *al-1* cDNA and we present the nucleotide sequence of a 3.1-kilobase (kb) genomic region containing this gene. We also examine photoregulation of *al-1* expression and demonstrate that the response to photoinduction is transcriptional.

MATERIALS AND METHODS

Strains. *N. crassa* T4637 (*al-1*) (FGSC 253), 51504 (*hom*), 34508 (*aur*), and FGSC 5092 (a) were used as recipient strains in transformation experiments. Strain 74-OR23-1 A (FGSC 987) was used as a source of wild-type DNA. These strains were provided by David Perkins (Stanford University). Strains FGSC 4398 (*wc-1* a), FGSC 4396 (*wc-1* a), FGSC 4408 (*wc-2* a), and R251 (*wc-2* a) were used as sources of *wc* RNA (11). The *N. crassa* pSV50 library was used as described before (35). The glutamate dehydrogenase clone (*am*) was provided by J. Kinsey (University of Kansas Medical Center). The *N. crassa* cDNA library was provided by Matthew Sachs (Stanford University).

Medium. Vogel minimal medium (9) was supplemented with 2% sucrose as a carbon source.

Blue light induction. Illumination was carried out as described before with 75-ml cultures in 250-ml flasks (6). Harvested mycelial pads were cut in half; one half was photoinduced as described before (10), and the other half was a dark control. The fluence rate of the blue light was 14 W/m^2, and the fluence rate of the blue part of the white light was 6 W/m^2. In the blue light studies, illumination was for a maximum of 10 min. Mycelia illuminated for 2, 5, and 10 min were immediately frozen in liquid nitrogen. For the 15-min, 20-min, 30-min, and 60-min time points, mycelia were incubated in the dark after 10 min of illumination and prior to freezing. Illumination in the white-light studies was for 30 or 60 min.

DNA excess hybridization and DNA analysis. RNA was extracted from each half-pad of mycelia as described before (7). Then, 5 μg of plasmid DNA containing a specific gene was linearized by restriction enzyme digestion, labeled, denatured, and used to probe RNA samples fixed to nylon membranes in RNA dot blot analysis performed by the protocol of Boll et al. (4). Radiolabeled DNA fragments were

* Corresponding author.
† Present address: Department of Chemistry and Biochemistry, Southern Illinois University, Carbondale, IL 62901-4409.

prepared as described previously (13). DNA sequencing was performed with Sequenase (United States Biochemical Corp.) according to the manufacturer's protocol. Clones used for sequencing were generated by progressive deletion from one end of a cloned DNA fragment with exonuclease as described by Henikoff (20). Southern analysis (33) was performed as described previously (28). Northern (RNA blot) analysis was performed by the protocol of Maniatis et al. (24).

Transcription run-on studies. Five million conidia were used to inoculate 75 ml of medium. Cultures were shaken at 100 rpm for 22 h at 34°C in the dark. Then, 70 ml of fresh warm medium was added, and incubation was continued for an additional 2 h. Mycelial pads were harvested by filtration, wet with 1 ml of medium, and incubated for 30 or 60 min at 34°C in the presence or absence of white light. After light or dark incubation, mycelia were washed once in ice-cold water. Nuclei were isolated by the method of Willmitzer and Wagner (36) as modified by Sommer et al. (32). Transcript labeling and RNA isolation were performed by the procedures of Marzluff and Huang (25) and Sommer et al. (32).

Computer methods. The *al-1* nucleotide sequence and deduced amino acid sequence of phytoene dehydrogenase were analyzed by using the BESTFIT, CODONFREQUENCY, CODONPREFERENCE, and PEPPLOT programs distributed by the University of Wisconsin Genetics Computer Group (12).

Nucleotide sequence accession number. The *al-1*$^+$ sequence was submitted to GenBank under accession no. M33867.

RESULTS

Cloning the *al-1* gene. The *al-1* gene of *N. crassa* is located less than 1 map unit to the left of a selectable marker, *hom* (homoserine requiring), on the right arm of linkage group I (29). Spheroplasts prepared from a *hom al-1* double mutant were transformed to *hom*$^+$ with an ordered cosmid library (35). A single cosmid, designated 3:11:H, was identified that transformed *hom al-1* spheroplasts to prototrophy. Several of the *hom*$^+$ transformants produced carotenoid pigments, indicating that cosmid 3:11:H contains *al-1*$^+$.

To locate the *al-1* gene in the approx. 40 kb of genomic sequence in cosmid 3:11:H, the cosmid was digested with a variety of restriction endonucleases, and the resulting products of single digests were cotransformed with plasmid pSV50 (35) into *al-1* spheroplasts. pSV50 contains a benomyl-resistant β-tubulin gene that can be used as a dominant selectable marker in *Neurospora* (28). Benomyl-resistant transformants were selected and screened for the production of carotenoids. Homologous integration of transforming DNA is infrequent in *Neurospora*. Thus, disruption of the *al-1* expression unit by restriction enzyme digestion decreases transformation to *al-1*$^+$ appreciably. We digested cosmid 3:11:H with a variety of restriction enzymes and examined the effect of transformation efficiency to determine the location of the *al-1* gene. Using this approach, we prepared the functional restriction map of the *al-1*$^+$ region shown in Fig. 1. Appropriate restriction fragments were then isolated and individually cotransformed with pSV50 into *al-1* spheroplasts. We identified a 3.1-kb *Sma*I-*Hin*dIII fragment that efficiently transformed *al-1* spheroplasts to *al-1*$^+$.

The recipient strain used in these studies, T4637 *al-1*, contains a translocation breakpoint within the *al-1*$^+$ gene. ^{32}P-labeled fragments from the putative *al-1*$^+$ gene region were used to probe Southern blots of digests of wild-type and T4637 *al-1* DNA. A *Sac*I fragment probe (Fig. 1) hybridized to a different set of restriction fragments from the translocation strain than wild-type DNA digests for all seven restriction enzymes tested (data not shown). Northern blot analyses with *N. crassa* polyadenylated RNA and *al-1*$^+$-specific probes indicated that the *al-1*$^+$ region encoded a single species of message approximately 2.2 kb in length (data not shown).

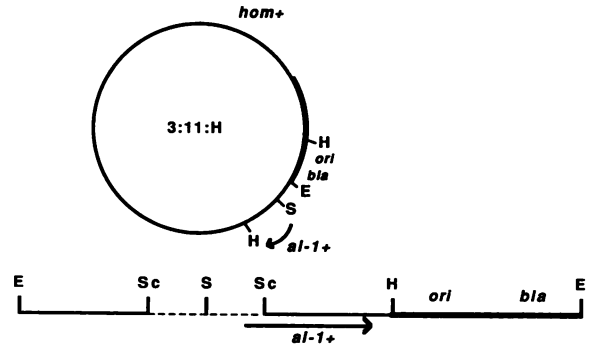


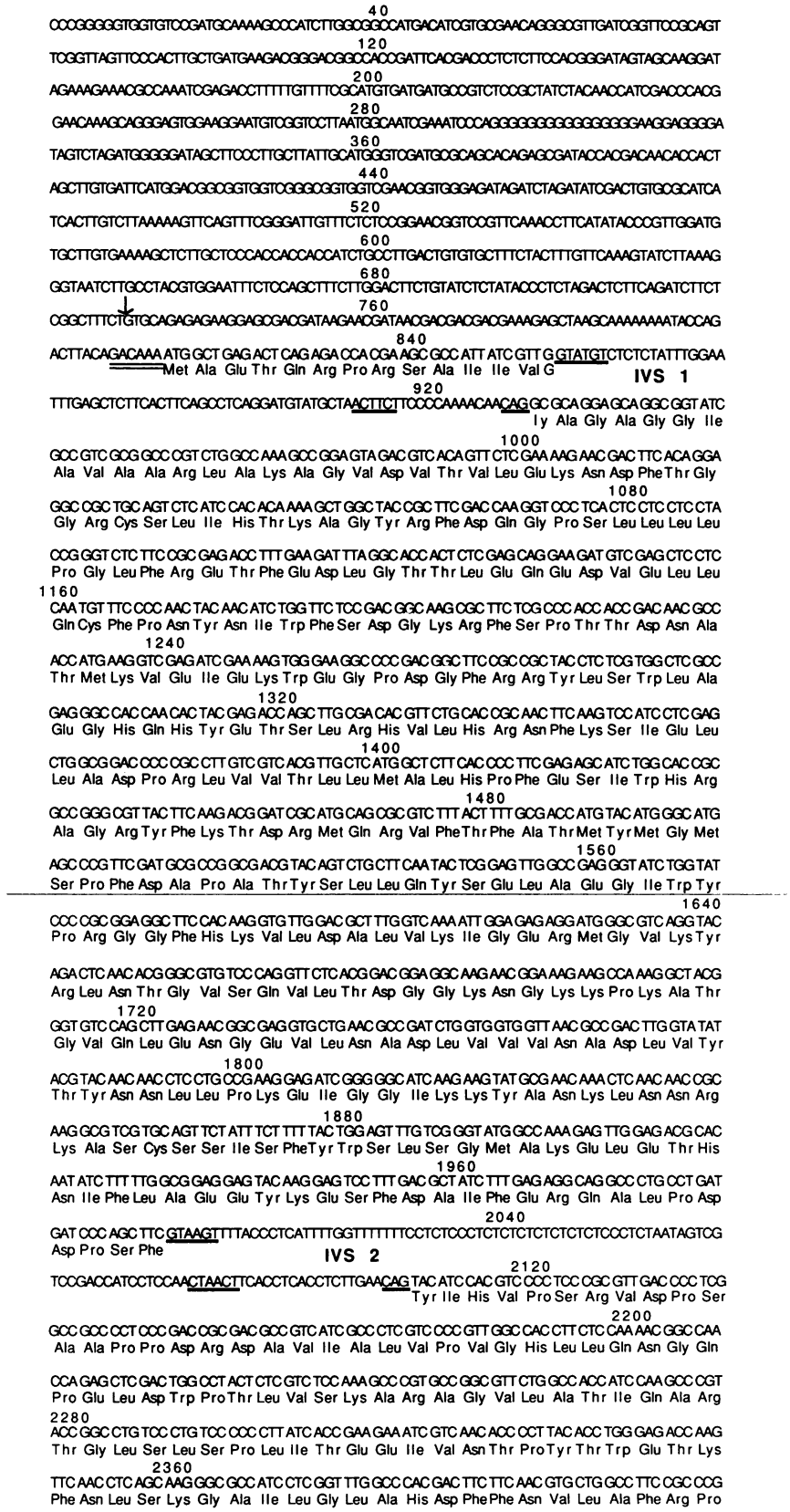
FIG. 1. Physical and genetic map of cosmid 3:11:H and its derivative, the *al-1*$^+$ plasmid pTJS305. Cosmid 3:11:H is drawn to emphasize the locations of *al-1*$^+$, *hom*$^+$, and the sequences contained in pTJS305 and is not to scale. pTJS305 contains approx. 6 kb of *N. crassa* genomic sequences representing one end of the genomic insert cloned in 3:11:H. The plasmid is shown linearized at the unique *Eco*RI site. The orientation and approximate location of the *al-1* transcription unit is indicated by arrows labeled *al-1*$^+$. Vector sequences are indicated by a thick line. The dotted line represents the *Sac*I fragment probe that was used to identify a translocation breakpoint in strain T4637 *al-1*. bla, β-Lactamase gene; ori, origin of replication; E, *Eco*RI; H, *Hin*dIII; S, *Sma*I; Sc, *Sac*I. Only relevant restriction enzyme sites are shown.

Sequence of the *al-1* gene. The complete nucleotide sequence of the *al-1*$^+$ region was determined on both DNA strands by using exonuclease III-generated subclones (Materials and Methods). The sequence shown in Fig. 2 is that of the 3.1-kb *al-1*$^+$-containing fragment extending from a *Sma*I to a *Hin*dIII site (Fig. 1). We also isolated and sequenced a nearly full-length cDNA clone, designated pTJS450, from a *Neurospora* cDNA library prepared from germinating conidia. The nucleotide sequence of this clone was obtained for at least one strand.

Analysis of the genomic and cDNA nucleotide sequences identified a 1,788-nucleotide open reading frame (ORF) consisting of codons that conformed to *N. crassa* codon preferences (28). The ORF encodes a 595-residue polypeptide with a predicted mass of 66 kilodaltons (kDa). Hydrophobicity analyses indicated that the highly hydrophobic C-terminal 18-amino-acid-residue segment of the predicted polypeptide had the potential for membrane association.

Comparison of the genomic and cDNA sequences identified two introns, IVS1 and IVS2 (Fig. 2), 77 and 108 base pairs (bp) in length, respectively. IVS2 was in frame. IVS1 and IVS2 contained 5′, internal, and 3′ splice signal sequences typical of other *N. crassa* genes (23). The putative phytoene dehydrogenase start codon was preceded by six nucleotides, three of which matched the *N. crassa* consensus start codon context sequence compiled by Legerton and Yanofsky (23) (Fig. 2). Sequence analyses with several cDNA isolates indicated that there were at least three polyadenylation sites between bp 2916 and 2976 (Fig. 2).

Orientation of *al-1* transcription. The *hom*$^+$ gene of *N. crassa* has been mapped proximal to the centromere of linkage group I relative to the breakpoint in strain T3647 *al-1*


IVS 1
IVS 2

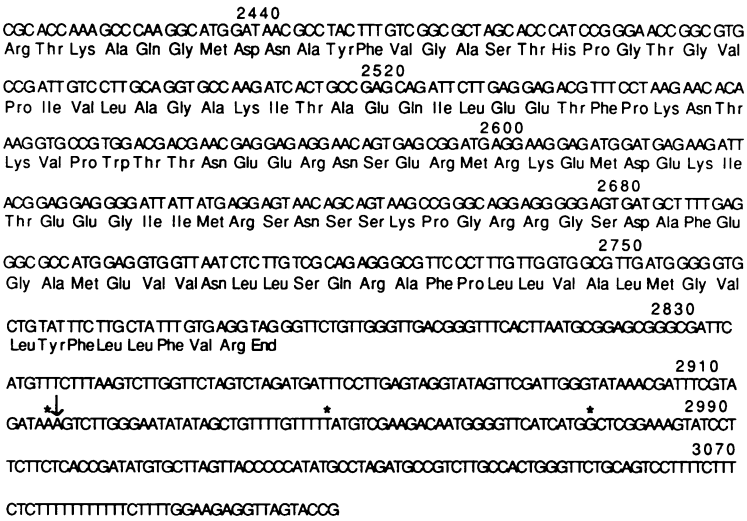


FIG. 2. Nucleotide sequence of *al-1*$^+$ and its flanking regions (GenBank accession number M33867). The nucleotide sequence is numbered from the first nucleotide shown, with the numbers above the nucleotides. The deduced amino acid sequence of *Neurospora* phytoene dehydrogenase is below the DNA sequence. The two introns are indicated. The 5′ and 3′ splice junctions and internal conserved sequences of the two introns are underlined. The six nucleotides preceding the putative start codon are double underlined. Arrows indicate the sequences represented in a *al-1* cDNA insert in pTJS450. Polyadenylation sites determined by sequence analysis of cDNA isolates are marked by asterisks.

(29). The *al-1* gene was mapped to one end of the genomic sequences in cosmid 3:11:H (Fig. 1); we mapped *hom*$^+$ towards the other end of the genomic sequences in 3:11:H (Schmidhauser and Yanofsky, unpublished data). We used radiolabeled *al-1* DNA fragments to map the location of a breakpoint in strain T4637 *al-1* to the 5′ end of the *al-1* gene (Fig. 1). Thus, *al-1*$^+$ transcription proceeds towards *hom*$^+$ and the linkage group I centromere.

Expression of *al-1* in photoinduced and dark-grown mycelia. We determined the level of *al-1* mRNA in mycelia exposed to blue light for up to 10 min relative to that in dark-grown mycelia (Fig. 3). The results of RNA dot blot analyses indicate that actively growing mycelia contain significantly more *al-1* message after 5 or 10 min of photoinduction. Mycelia that were induced with blue light for 10 min and then incubated in the dark maintained an elevated level of *al-1* RNA for at least 20 min. Control hybridizations with a probe from a gene whose expression is known not to be photoinduced, *n-6* (32), showed that similar amounts of this RNA were present in each RNA preparation (Fig. 3). The results of RNA dot blot analyses with mRNA isolated from mycelia illuminated with white light for 30 or 60 min indicated 45-fold (average of five studies) or 82-fold (average of six studies) more *al-1* message, respectively, than in dark-grown mycelia (data not shown).

The photoinduced accumulation of *al-1*-specific message could be the result of increased transcription of the *al-1* gene and/or an increase in *al-1* message stability. We performed run-on transcription studies with isolated nuclei to assess the extent of de novo synthesis of *al-1* message in photoinduced and dark-grown cultures. The results of these experiments are shown in Table 1 and Fig. 4. A 30-min exposure to white light induced *al-1* transcription in comparison to the dark control. After 60 min of white light, de novo synthesis of *al-1* message was reduced relative to that observed at 30 min. The ratios of de novo message synthesis in photoinduced versus dark-grown mycelia for the control genes *am* (21) and *tub-2* (28) indicated no photoinduction (Table 1).

The products of the white collar (*wc*) genes are essential for all blue light-induced physiological effects in *N. crassa* (11, 19). To determine whether the *wc* gene products are necessary for photoinduced *al-1* mRNA accumulation, the expression of *al-1* was examined in two *wc-1* and two *wc-2* strains. The results of RNA dot blot analyses comparing mRNA isolated from dark-grown mycelia and mycelia illuminated for 60 min with white light indicate that *al-1* message did not accumulate in photoinduced *wc* mutant strains (data not shown).

DISCUSSION

In this report we describe the cloning, sequencing, and photoregulation of *al-1*, the *N. crassa* gene encoding phytoene dehydrogenase. The activity of two enzymes in the *N. crassa* carotenoid-biosynthetic pathway, the products of *al-2* and *al-3*, have been shown to increase following photoinduction (19). Nelson et al. (27) cloned the *al-3* gene of *N. crassa* and demonstrated that the steady-state level of *al-3* mRNA increases after photoinduction. Our results establish that *al-1* message levels also increase in response to photoinduction. Transcription run-on studies with isolated *Neurospora* nuclei show that the increased accumulation of *al-1* mRNA resulting from photoinduction is due to increased transcription of *al-1*. The accumulation of *al-1* (this paper) and *al-3* (27) message in photoinduced mycelia versus dark-grown mycelia is significant after 5 and 10 min of induction, respectively. In other studies we have observed a rapid accumulation of *al-2* message in response to photoinduction (Lauter, Schmidhauser, Yanofsky, and Russo, unpublished data).

Neurospora wc mutants are defective in all physiological responses to blue light (11, 19). The *wc* mutants are defective in photoinduced accumulation of mRNA specific to the *al-1* (this paper), *al-2* (Lauter et al., unpublished), and *al-3* (27) genes. The finding that the RNAs of each of the three albino genes accumulate in response to photoinduction suggests that these genes may be controlled by a common blue light response regulatory mechanism. We are currently examining

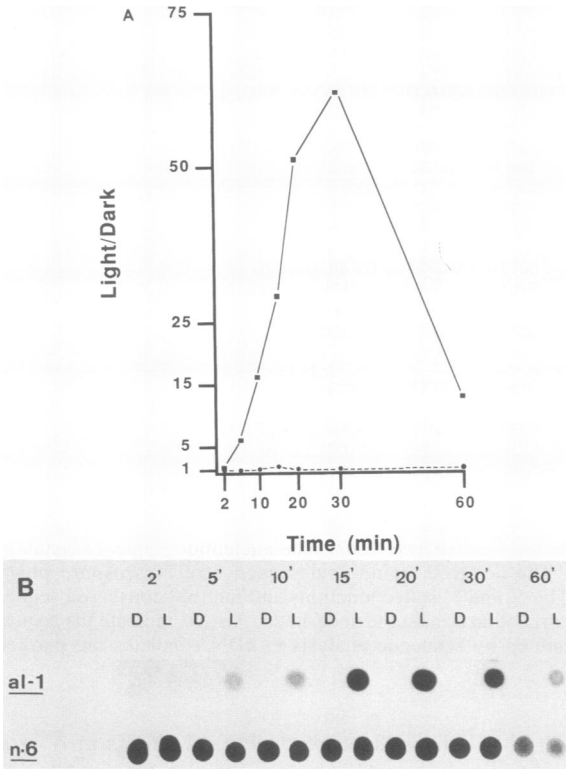


FIG. 3. Kinetics of *al-1* mRNA levels after blue light induction. (A) Plot of RNA dot blot data. Mycelia for RNA isolation were irradiated with blue light for a maximum of 10 min. RNA was extracted at different times after the beginning of illumination. Then, 3 μg of total RNA was immobilized per dot and probed with *al-1* or *n-6*. The extent of hybridization was visualized by autoradiography. The films were analyzed with a 2D LKB Laser Desitometer 2222-010 Ultrascan XL and LKB software. Light/dark values are the ratio of the amount of *al-1* or *n-6* mRNA in blue light-treated versus dark-grown mycelia. Dark values were often at background levels; values for six dark dots that were above background levels were averaged, and this average was taken as the common dark value. Each point is the average of two to five RNA dot blots with independent total RNA preparations. Symbols: ●, *al-1*; ■, *n-6*. (B) Representative dot blots used to calculate the data in panel A. D, Dark; L, light. Time is shown in minutes after initiation of illumination.

the regulation of carotenogenesis during the *N. crassa* asexual cycle. Preliminary results indicate that *al-1* and *al-2* mRNAs accumulate in response to conidiation and during conidial germination (Schmidhauser, Sachs, and Yanofsky, unpublished). We are comparing the 5′ regions of the albino genes for sites responsible for developmental regulation and photoregulation.

Carotenoid dehydrogenase sequence homology. Armstrong et al. (1) and Bartley and Scolnik (2) have determined the nucleotide sequence of carotenoid-biosynthetic genes of the photosynthetic purple bacterium *Rhodobacter capsulatus*. The *R. capsulatus crtI* gene encodes phytoene dehydrogenase and the *crtD* gene encodes neurosporene dehydrogenase. Comparison of the deduced amino acid sequences of the *crtD*, *crtI*, and *al-1* gene products reveals extensive similarities among the three proteins. The polypeptides can be aligned pairwise with 27 to 33% identities by using 11 to 16 gaps in the alignment. Armstrong et al. (1) noted two regions of amino acid homology (>40% identity) in CrtD and CrtI. In Fig. 5 we extend the comparison to the *N. crassa* *al-1* polypeptide, using the corrected initiation codon for Crtl (2). The two regions noted by Armstrong et al. (1) were conserved within the *al-1* polypeptide. We conclude from our observations and on the basis of previous studies of *al-1* (16) that this gene encodes *Neurospora* phytoene dehydrogenase.

TABLE 1. Transcription run-on analysis of *al-1* expression[a]

Photoinduction period and gene	De novo mRNA synthesis ratio (light/dark)	
	Individual prepns	Average
30 min		
al-1	20.7, 35.0, 16.8, 15.9	22.1
am	1.0, 1.3, 0.9, 0.9	1.0
tub-2	1.7, 1.7, 1.3, ND[b]	1.6
60 min		
al-1	2.3, 4.0, 2.6	3.0
am	0.8, 0.9, 0.9	0.9
tub-2	1.2, 0.8, 1.8	1.3

[a] Ratios between the amount of specific labeled RNA produced in transcription run-on analyses with nuclei isolated from mycelia photoinduced with white light for 30 or 60 min and with nuclei isolated from dark-grown mycelia (light/dark). Each value is for an independent RNA preparation and was determined as described in the Fig. 4 legend. Average values for each time point are shown.
[b] ND, Not determined.

Comparison of carotenoid dehydrogenase enzymes is of interest for several reasons. Phytoene is desaturated to produce lycopene, via neurosporene, by a series of four didehydrogenations (5). Mutant analyses suggest that in some organisms, two dehydrogenases catalyze the four enzymatic steps, whereas in other organisms one dehydrogenase is sufficient (5). Studies with *N. crassa* suggest that the *al-1* gene product catalyzes the dehydrogenation of phytoene to lycopene (16). CrtD and Crtl catalyze the analogous four dehydrogenations in *R. capsulatus* (15, 30).

We have demonstrated complementation of a *crtl R. capsulatus* mutant by the *al-1*$^+$ cDNA cloned in plasmid pTJS450 when expressed from a bacterial promoter (G. E. Bartley, T. J. Schmidhauser, C. Yanofsky, and P. A. Scolnik, J. Biol. Chem., in press). We are testing complementation of an *R. capsulatus crtD* mutant with the *al-1* cDNA

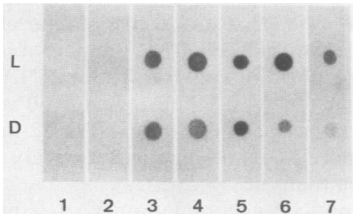


FIG. 4. Transcription run-on analyses with isolated nuclei from dark-grown (D) and light-grown (L) mycelia. Nuclei were isolated from growing mycelium that was either photoinduced (30 min of white light, lanes 1 to 6; 60 min of white light, lane 7) or kept in the dark (lanes 1 to 7). Run-on transcripts were labeled and total RNA was isolated as indicated in Materials and Methods. The labeled RNA was hybridized to an excess of the following DNAs immobilized on filters: 1, pUC18; 2, pBR322; 3, *n-6*; 4, *am*; 5, *tub-2*; 6 and 7, *al-1*. A total of 10^7 cpm of each RNA preparation was used for each hybridization, and the extent of hybridization was visualized by autoradiography. The films were analyzed with a 2D LKB Laser Densitometer 2222-010 Ultrascan XL and LKB software, and the ratios of these values calculated. These ratios are presented in Table 1.

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Al-1  Ile-11 IIVGAGAGGI AVAARLAKAG VDVTVLEKND FTGGRCSLIH
CrtD  Val-8  VVIGARMGGL AAAIGAAAAG LRVTVVEAGD APGGKARAVP
CrtI  Val-12 VVIGAGLGGL AAAMRLGAKG YKVTVVDRLD RPGGRGSSI.
             ***GA--GG* A-A---*--G --VTV**--D --GG*---*-

             TKAGYRFDQG PSLLLLPGLF RETFEDLG  Gly-79  Al-1
             TPGG.PADTG PTVLTMRHVL DALFAACG  Gly-74  CrtD
             TKGGHRFDLG PTIVTVPDRL RELWADCG  Gly-78  CrtI
             T-*G---D-G P-***----* ---*---G

Al-1  Leu-468 LAFRPRTKAQ GMDNAYFVGA STHPGTGVPI VLAGAKITA Ala-506
CrtD  Ala-444 ATFRRPLART GLKGLYLAGG GTHPGAGVPM ALTSGTHAA Ala-482
CrtI  Ala-457 AWFRPHNASE EVDGLYLVGA GTHPGAGVPS VIGSGELVA Ala-495
              --FR------ **---Y*-G* *THPG-GVP- -*-**---A
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FIG. 5. Amino acid homologies in carotenoid dehydrogenases. The two regions of each polypeptide shown below have been aligned to maximize percent homology. The terminal residue in each comparison is numbered to indicate position within each polypeptide. Conserved residues are shown in capitals below the sequences, and asterisks indicate similar residues in all three polypeptides. A period in the amino acid sequence indicates a gap.

construct. Lastly, comparison of the three enzymes indicates a conserved nucleotide-binding fold common to flavoproteins (Bartley et al., in press). Further analysis will increase our knowledge of the genes and proteins of the carotenoid-biosynthetic pathway.

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