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GENETICS AND REGULATION OF CAROTENE BIOSYNTHESIS

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Abstract - Colour mutants are conspicuous and convenient genetic markers, but there have been few studies on the genetics of carotene biosynthesis. Recent work with the fungus Phycomyces has identified two main structural genes: carB, responsible for phytoene dehydrogenase, and carR, responsible for lycopene cyclase. Four copies of the first enzyme and two of the second constitute an enzyme complex carrying out all reactions from phytoene to β-carotene. The pathway is regulated by a variety of environmental agents with the participation of several genes. Thus, carS mutants overaccumulate β-carotene and carA mutants lack practically all carotenes. Surprisingly, carA and carR are segments of a single, bifunctional gene. The investigations carried out with Neurospora and other microorganisms are briefly reviewed from the viewpoint of the results with Phycomyces.

The genetical aspects of the carotenoid pathways have received only a small fraction of the attention devoted to this class of chemical compounds. This attention has been thinly spread over many organisms, from bacteria to higher plants, synthesizing many diverse carotenoids and allowing a variety of genetic manipulations. There is little reason to suspect that results obtained with one organism may be applied to another. We will consider the situation with the fungus Phycomyces blakesleeanus, before moving on to other microorganisms.

CAROTENE BIOSYNTHESIS IN PHYCOMYCES

β-carotene is responsible for the yellow colour of Phycomyces mycelia and is present in most stages of the life cycle. The concentration of the pigment varies widely depending on environmental conditions, such as nutrients, light, and temperature (1-3).

Colour mutants were first isolated in Phycomyces by M. Heisenberg in 1967. They are easily spotted, at global frequencies of one every few thousand colonies, among the survivors of rather heavy mutagenic treatments. There are three main mutant phenotypes: white, red, and intense yellow.

Carotene analysis of the mutants (4-7) is easily carried out, since any amount of mycelium can be grown under a variety of culture conditions. Complementation analysis, detecting whether two mutations have occurred at the same gene, consists in the study of β-carotene production in heterokaryons, that is, cells containing two kinds of nuclei, each from a different colour mutant (8, 9). Recombination analysis, detecting whether two mutations lie close to each other on a chromosome, involves crosses among colour mutants of opposite sex. Most colour mutants are impotent to complete the sexual process, but special genetic tricks have been devised to overcome this obstacle.

Some of the white mutants accumulate phytoene instead of β-carotene (4-6). The corresponding mutations have all occurred in the same gene, called carB (9). The red mutants accumulate lycopene instead of β-carotene (4). The corresponding mutations have all occurred in gene carR (9). The mere existence of these two kinds of mutants strongly supports the occurrence in Phycomyces of the carotene pathway first proposed by Porter and Lincoln

(10) for tomatoes, in which phytoene and lycopene are essential intermediates.

No mutants accumulating other presumed intermediates of the pathway have been isolated. This may seem a surprising failure. Perhaps the corresponding genes are duplicated in the otherwise haploid genome, but there are more reasonable explanations. Sterols are essential components of cell membranes and thus we expect no mutants blocking sterol biosynthesis. The intermediates up to farnesyl pyrophosphate (C_{15}) are common to both sterol and carotene pathways so that mutants unable to metabolize such intermediates would be inviable. Geranylgeranyl pyrophosphate (C_{20}) synthesis,

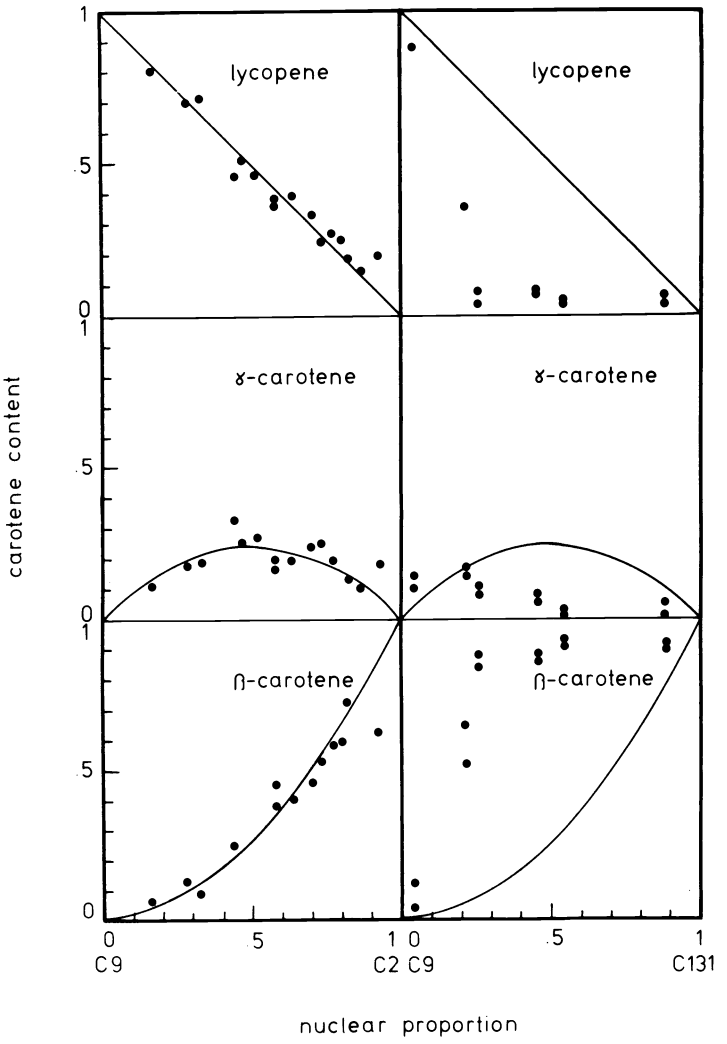

Fig. 1. Proportions of lycopene, γ-carotene, and β-carotene in heterokaryons C2 * C9 (left) and C9 * C131 (right), as functions of the nuclear proportions in the heterokaryons. C2 carries a carA mutation, C9 carries a carR mutation, C131 carries no mutations related to carotenogenesis. The lines represent the predictions from the model presented in Fig. 2.

involving the addition of a prenyl residue, may be carried out by the same prenyl transferase responsible for the two previous reactions of the pathway. Of course there must be a way to regulate the relative flows into the carotene and sterol pathways, but not necessarily through a distinct $C_{15} \rightarrow C_{20}$ enzyme. The enzymes responsible for the steps $C_{20} \rightarrow C_{40}$ (11) may perhaps intervene in the steps $C_{15} \rightarrow C_{30}$ of sterol biosynthesis, particularly if these enzymes are not finicky about the length of their substrates.

All four dehydrogenations from phytoene to lycopene may be accomplished by the same enzyme encoded in gene carB. Leaky carB mutants, accumulating phytoene, phytofluene, ζ-carotene, and neurosporene, provide evidence for this hypothesis (5). The hypothesis is also consistent with the finding of the same intermediates in cells grown in the presence of diphenylamine (12).

The two cyclizations from lycopene to β-carotene may also be accomplished by another enzyme, encoded in gene carR. The finding of lycopene, γ-carotene, and β-carotene in cells grown in the presence of 2-(4-chlorophenylthio) triethylamine or nicotine (13-15) is in keeping with this proposal.

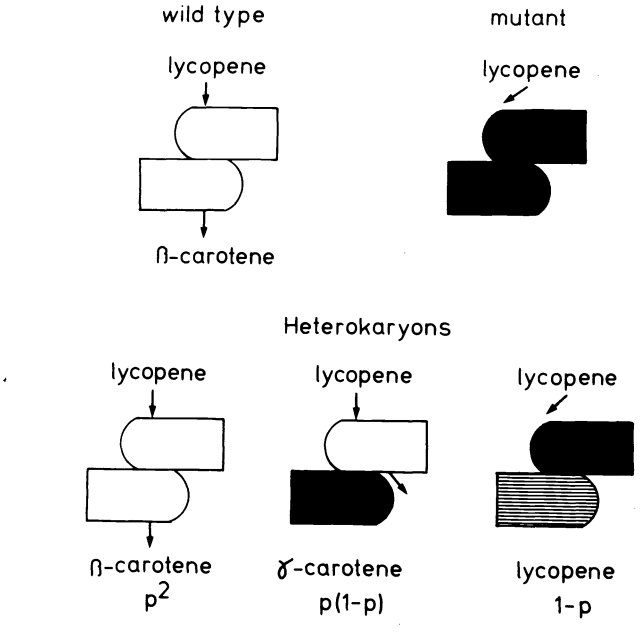

Fig. 2. Two lycopene cyclase molecules successively carry out the reactions lycopene → γ-carotene → β-carotene. White symbols represent active enzymes, the product of wild-type alleles. Black symbols represent the product of a carR mutant, enzymatically inactive but able to aggregate into the complex. The lower part of the figure represents the possible complexes in heterokaryons for carR, their expected products, and their expected frequency, on the assumption of no discrimination at the transcriptional, translational, and aggregational levels.

The best support for the idea that one enzyme is involved in several reactions comes from quantitative complementation analyses. Heterokaryons for mutant and wild-type carB alleles accumulate phytoene, phytofluene, ζ-carotene, neurosporene, and fully-dehydrogenated products (16). Heterokaryons for mutant and wild-type carR alleles accumulate lycopene, γ-carotene and β-carotene (17, and Fig. 1, left). The mathematical relationships between quantitative analyses of carotenes and nuclear proportions in the heterokaryons lead to detailed proposals about the operation of the pathway

(Figs. 2 and 3): phytoene is acted upon by an enzyme complex first involving four copies of phytoene dehydrogenase, all made under the direction of the carB genes present in the cell, and then two copies of lycopene cyclase, both made under the direction of the carR genes present in the cell. Thus, when mutant and wild-type alleles coexist in the same cell, the inactive enzymes may be incorporated into the complex, provoking the ejection of the corresponding untransformed intermediate. Intermediates between phytoene and lycopene are not taken up by neighbouring enzyme complexes, or otherwise transformed, so that they accumulate in the same proportions as they were originally made. The same is true for the intermediates lycopene and γ-carotene if nuclei carrying carA mutations are present (Fig. 1). We have no indication that the specificity to carry out the dehydrogenations in the appropriate order is encoded in additional genes. It is thus likely that the specificity results from the three-dimensional arrangement of the complex. Minor changes in that arrangement may easily explain the formation, under some circunstances, of small amounts of other intermediates, such as β-zeacarotene.

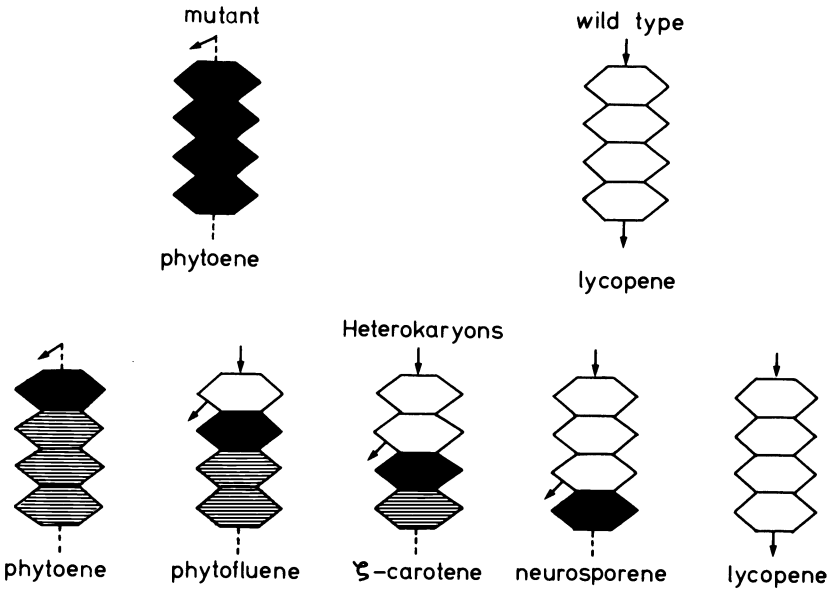

Fig. 3. Four phytoene dehydrogenase molecules successively carry out the reactions phytoene → phytofluene → ζ-carotene → neurosporene → lycopene. White symbols represent active enzymes, the product of the wild type allele. Black symbols represent the product of a carB mutant, enzymatically inactive but able to aggregate into the complex. The lower part of the figure represents the possible complexes in heterokaryons for carB, and their expected products.

REGULATION OF CAROTENE BIOSYNTHESIS IN PHYCOMYCES

The regulation of carotenogenesis is very complex, involving several environmental variables and specialized cellular functions. The carotene contents (μg per g dry weight, or ppm) given below are approximate figures obtained under conditions more adequate for genetical work than for optimum carotene production (7, 18). Under these conditions the unstimulated wild type accumulates about 40 ppm β-carotene in the dark.

Mating is accompanied by increased carotenogenesis (19), mediated, as are the early morphological changes in the sexual process, by the formation of trisporic acids (20, 21). A high level of sexual stimulation is observed

in intersexual heterokaryons, which carry nuclei of opposite sex and accumulate up to 400 ppm β-carotene (7). However, lycopene synthesis is not increased in heterokaryons whose components are carR mutants of opposite sex, unless at least one of the carR mutations is leaky and allows some β-carotene production (18). This fits nicely with the idea of trisporic acids deriving from β-carotene (22).

Mutations in gene carS increase β-carotene content up to about 4000 ppm. These mutations are recessive and formally analogous to the loss of repressor in some bacterial operons (7). They lead to actual overproduction, and not just to slower metabolism of β-carotene, as we have recently shown with "pulse and chase" experiments with radioactive mevalonate.

The carotene pathway is stimulated whenever β-carotene production is hindered, either through carB or carR mutations (7) or through inhibitors (12, 13). This suggests that the pathway is feed-back controlled by β-carotene. Since carS carB double mutants do not accumulate more phytoene than single carB mutants, it is thought that the product of carS plays a role in this apparent feed-back regulation. Sexual interaction and carS mutations act independently on carotenogenesis: heterokaryons composed of carS mutants of opposite sex combine both stimulatory effects and accumulate up to 15000 ppm β-carotene (7).

Many chemicals stimulate carotenogenesis to different extents (6, 23, 24). Particularly interesting is the action of vitamin A. The vitamin itself is not converted into carotenes, but stimulates the usual pathway, thus leading to increased accumulation of β-carotene, lycopene, and phytoene in the wild type, carR mutants, and carB mutants, respectively (6). Vitamin A acts independently of sexual interaction and carS mutations, since intersexual heterokaryons and carS mutants are stimulated by vitamin A to about the same extent as in the wild type. Mutation car-102 renders constitutive the stimulatory action that in the wild type depends on the presence of vitamin A (7).

Thus we have at least three independent ways to stimulate carotenogenesis: sex, carS mutations, and vitamin A (or mutation car-102). These separate actions have been put to work together in an intersexual heterokaryon containing carS and car-102 mutations and stabilized by the presence of balanced lethal mutations. The resulting strain accumulates about 25000 ppm β-carotene, more than 500 times the content of the wild type in the same conditions (18).

Stimulation of carotene biosynthesis by light has been investigated mainly in Fusarium (25, 26), Mycobacterium (27) and Neurospora (28, 29), but seldom with genetic tools (30). Blue light results in increases of more than 10-fold in the β-carotene content of Phycomyces (2, 3), but there is also significant accumulation in the dark, thus obscuring the effect of low light doses. Some of the mutants defective in phototropism (gene madB) are partially defective in photoinduced carotenogenesis (3), suggesting the use of a common photoreceptor. I. López-Díaz and S. Torres-Martínez have recently isolated mutants which are largely defective in photoinduced carotenogenesis, but normal in other responses to light, and in carotenogenesis in the dark. These mutants open up the study of many aspects of the process and, in particular, of the relationships between photoinduction and each of the other ways to stimulate carotenogenesis.

Many white mutants contain no carotenes, even phytoene. There is no complementation among them, but they complement all carB mutants, so they were assigned to another gene, carA (9). Efforts to detect a block in the pathway prior to phytoene formation were fruitless. Most surprising was the frequent isolation, in a single-step search, of white mutants failing to complement both carA and carR mutants. They were considered double mutants which had lost both functions through separate mutations (9).

However, our recent investigations show that carA and carR represent in fact separate segments of a single gene coding for a bifunctional product. Segment A, the larger one, and the closer to the amino terminus of the product, is required for overall operation of the pathway, while segment R is specific for lycopene cyclization. Therefore, we suggest that previous carA mutations are missense mutations and affect segment A, carR mutations affect segment R, and "double" carA carR mutations are single nonsense or

frameshift mutations in segment A, leading to simultaneous loss of both functions. The evidence for this proposal, to be published elsewhere, rests mainly on the facts that (a) carA and carR mutations are very closely linked, (b) base substitutions (the main effect of the mutagen N-methyl-N'-nitro-N-nitrosoguanidine) give rise to all three kinds of mutants, carA being the most frequent class, while frameshifts (the main effect of the mutagen ICR-170) give rise predominantly to carA carR "doubles", and (c) carA carR "doubles" revert to wild type by single mutations which are very closely linked to the original mutation. The gene must be transcribed and translated as a unit and the resulting polypeptide then split into two polypeptides with different functions. This splitting is required for a simple explanation of the analyses of carA * carR heterokaryons (Fig. 1).

OTHER MICROORGANISMS

The characteristic orange colour of Neurospora is due to a mixture of several carotenes, including β-carotene and neurosporaxanthin. Colour mutants have been known for many years (31, 32) and used in complementation and recombination studies, but their phenotypic and genetic analysis has too often remained at a merely qualitative level. With some danger of extrapolation and oversimplification, the situation may be approximated to that of Phycomyces.

Some white mutants, al-2 and al-3, lack carotenoids altogether (33). The rest of the white mutants, al-1, accumulate phytoene, or, if leaky, a mixture of phytoene and its dehydrogenated derivatives, leading to various slightly different phenotypes. The relationship between al-2 and al-1 is reminiscent of the one between carA and carR in Phycomyces, in that al-1 and al-2 are closely linked and in that there is complementation between most, but not all, pairs of al-1 and al-2 mutants (34-36). On the other hand, al-3 lies in a different chromosome and has no analogue in Phycomyces.

Yellow mutants synthesize carotenes, but not the oxidized compound neurosporaxanthin. They form a complementation group defining gene ylo, unlinked to the others.

The phenotype of mutants defective in the cyclization reactions, and thus analogous to carR mutants in Phycomyces, would resemble that of the wild type, were they to contain moderate amounts of pigments. In that case it would be convenient to search for them starting with a yellow mutant.

While quantitative complementation analyses are possible in Neurospora (see Fig. 1 in ref. 37), they have not been carried out to the point where they might give us some insight into the organization of the successive reactions in the pathway. Electrophoretic analysis of the proteins has revealed a one-band difference between the wild type and an al-2 mutant, and this band has been assumed to be a protein complex involved in carotenogenesis (38, 39).

There have been many studies on the carotenoids of different fungi and bacteria and mutant isolation was begun long ago (40, 41), but there have been few reports of genetical analyses. The situation in Ustilago violacea (42) is interesting in that some mutants exhibit synthetic capabilities apparently absent from the wild type. The carotenoid mutants of Rhodopseudomonas capsulata map in five tight clusters, which may correspond to separate genes, all situated within a small chromosome segment. This raises the possibility of coordinated regulation in a "carotenoid operon" (43).

There is still ample space for further genetic analysis, even in the best studied organisms, but major developments in the future are most likely to come from the study of carotene synthesis in cell-free extracts of different mutants and from the characterization of messenger RNAs and proteins involved in carotene biosynthesis and its regulation.

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