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Expression and subcellular location of the tetrapyrrole synthesis enzyme porphobilinogen deaminase in light-grown *Euglena gracilis* and three nonchlorophyllous cell lines

(hydroxymethylbilane synthase/porphyrin synthesis/*Astasia longa*/bleached *Euglena* mutant)

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ABSTRACT **The expression and subcellular location of porphobilinogen deaminase (PBGD, also known as hydroxymethylbilane synthase; EC 4.3.1.8), one of the early enzymes of porphyrin synthesis, was investigated in light-grown *Euglena* and in three cell lines that do not contain chlorophyll: dark-grown *Euglena*, a streptomycin-bleached mutant, and *Astasia longa*. In wild-type *Euglena*, immunogold electron microscopy demonstrated that all the immunodetectable enzyme protein was in the chloroplast. PBGD was shown to be photoregulated, and like many other nuclear-encoded proteins in *Euglena*, the regulation was at the posttranscriptional level. In the three nonchlorophyllous cell lines, as in light-grown *Euglena*, a single protein of 40 kDa was detected with antiserum to PBGD. This same antiserum immunoprecipitated a larger precursor protein from the total translation products of poly(A)$^+$ RNA, and a single transcript, which was large enough to encode the precursor, was detected on Northern blots of all four cell types. Therefore, in cells that make chlorophyll and those that do not (or cannot), PBGD is located in the plastid. No evidence was obtained for another form of the enzyme, which suggests that in *Euglena* there is only one pathway for the synthesis of the tetrapyrrole moiety of chlorophyll and heme.**

The cellular porphyrins chlorophyll and heme share a common biosynthetic pathway from the first committed precursor, 5-aminolaevulinic acid (ALA), to the tetrapyrrole protoporphyrin IX before the pathways diverge with the insertion of either Fe or Mg, leading to heme or chlorophyll, respectively (1). In photosynthetic eukaryotes, the chloroplasts contain all the enzymes for both the C_5 pathway of ALA synthesis from glutamate (2) and for the synthesis of chlorophyll and heme (3, 4). In animals and yeast, ALA for heme synthesis is produced from succinyl-CoA and glycine by the mitochondrial enzyme ALA synthase, the so-called Shemin pathway (5), but there is no convincing evidence for this enzyme in plants (1). In contrast, the phytoflagellate *Euglena gracilis* has been shown to possess ALA synthase as well as the C_5 pathway of ALA synthesis (6). Furthermore, Weinstein and Beale (7) found that [^{14}C]glutamate is the most efficient precursor for the tetrapyrrole ring of chlorophyll, whereas heme *a*, which is confined to mitochondria, was specifically labeled by [^{14}C]glycine. Protoheme was labeled equally well by either precursor. These results imply that there are two separate pathways for the synthesis of protoporphyrin IX in *Euglena*. A similar situation prevails for fatty acid synthesis, where in dark-grown cells, there is a single animal-like (type I) fatty acid synthetase, while cells growing phototrophically also possess a type II fatty acid synthetase, which is chloroplast located (8).

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Although the evidence for two pathways for ALA synthesis in *Euglena* is well established, nothing is known about the possible duplication of the subsequent enzymes for protoporphyrin IX synthesis. In our laboratory, we have isolated a *Euglena* cDNA clone for one of these enzymes, porphobilinogen deaminase (PBGD, also known as hydroxymethylbilane synthase: EC 4.3.1.8) (9) by using antibodies raised against the enzyme purified from light-grown *Euglena* (10). This clone encodes the mature protein and an N-terminal extension of 139 amino acids, which has the characteristics of a chloroplast transit peptide, so the protein is presumably located in the chloroplast. In view of the findings of Weinstein and Beale (7), we were interested in investigating the possibility of another form of PBGD involved in the synthesis of mitochondrial heme. To date, no evidence has been obtained for a second PBGD isozyme (9), but all these studies were carried out with wild-type *Euglena* cells growing phototrophically in the light, where it would be expected that the plastid pathway of tetrapyrrole synthesis would predominate. We therefore decided to investigate the location and synthesis of PBGD in three cell lines that would be more likely to operate an extraplastidic pathway—that is, those that did not, or could not, make chlorophyll. The cell lines chosen were dark-grown wild-type *Euglena*, where chlorophyll synthesis is repressed, while ALA synthase activity is stimulated (11); *Astasia longa*, which is a colorless relative of *Euglena* that contains no visible proplastid-like bodies (12) but has been shown to contain a circular plastome (13); and a "bleached" mutant, which was induced from wild-type cells by growth on streptomycin in the light. This treatment has been shown to give rise to mutants with various degrees of plastid loss (14), which are deficient in chloroplast DNA but are likely to retain at least some of the plastome (15). Since none of these three lines make chlorophyll or plastid heme, any tetrapyrrole synthesis would be for mitochondria and other organelles. Thus, if there is a PBGD other than that in the plastids, it should be detected in these cell lines. The results of this investigation are presented in this paper.

MATERIALS AND METHODS

Growth of *Euglena*. *E. gracilis* strain Z and *A. longa* (from Sammlung von Algenkulturen, Gottingen, F.R.G.) cells were grown as described (16) either in complete darkness or under continuous illumination (12.4 μmol per m^2 per sec). For the greening experiment, wild-type cells of *Euglena* were continuously subcultured in heterotrophic medium in the dark for 3 weeks. An exponential culture of these dark-adapted cells (4 days of growth) was then transferred to continuous light, and samples were removed at various time intervals for estimation of chlorophyll and PBGD activity and for protein

Abbreviations: ALA, 5-aminolevulinic acid; PBGD, porphobilinogen deaminase (hydroxymethylbilane synthase).

and RNA measurements. Intact chloroplasts were isolated from *Euglena* as described by Price and Reardon (17), with a yield of 3–4% of total chlorophyll. Mutant *Euglena* cells lacking chlorophyll were generated by growing the normal green cells on nutrient agar containing 0.05% streptomycin (14). After 5 days several white colonies were observed, which remained white even on transfer to agar plates without streptomycin. A single white colony was picked from the plate and transferred to heterotrophic medium in the light, where the cells remained colorless. A crude organellar fraction from the various cell lines was prepared as described by Siemeister and Hachtel (13).

Chlorophyll Estimation and Protein Analysis. Chlorophyll was estimated by the method of Arnon (18) and PBGD activity was assayed essentially as described (10). Protein was determined with the Bio-Rad protein assay. Western blot analysis was carried out as described by Sharif *et al.* (9). For dot blot analysis of protein from the greening experiment, a Bio-Dot microfiltration apparatus (Bio-Rad) was used according to the manufacturer's instructions.

Isolation of Total RNA and Northern Analysis. Total RNA was extracted from *Euglena* and *Astasia* cells as described by Sharif *et al.* (9). Glyoxalated RNA gels (19) were run to check the quality and purity of RNA samples, and Northern blots were prepared as described (19), except that the RNA was fixed to the membrane by exposing it to UV light for 2 min followed by baking at 80°C for 30 min. For dot blot analysis of RNA from the greening experiment, samples were glyoxalated (19), except that dimethyl sulfoxide was excluded, and spotted onto a Biodyne nylon membrane mounted on a Bio-Dot apparatus. Northern and dot blots were then probed with a ^{32}P-labeled cDNA clone for PBGD as described (9).

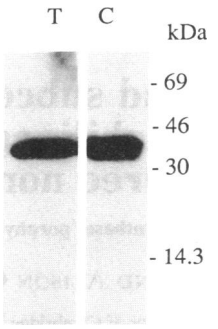

FIG. 1. Autoradiograph of Western blot of PBGD in *E. gracilis*. Twenty micrograms of total soluble protein (T) or 20 μg of chloroplast protein (C) was challenged with antiserum to PBGD as described in *Materials and Methods*.

***In Vitro* Translation and Immunoprecipitation.** Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (19) and translated *in vitro* in a wheat germ system (Promega Biotec) according to the supplier's instructions. Products of the *in vitro* translation were immunoprecipitated and analyzed as described (9).

Immunogold Electron Microscopy. To facilitate processing, *Euglena* cells were first embedded in 1% agar. The agar blocks were fixed in 0.1% (vol/vol) glutaraldehyde in 50 mM Pipes (pH 7.2) at 0°C for 12 hr and washed thoroughly in the same buffer. After dehydration the blocks were infiltrated

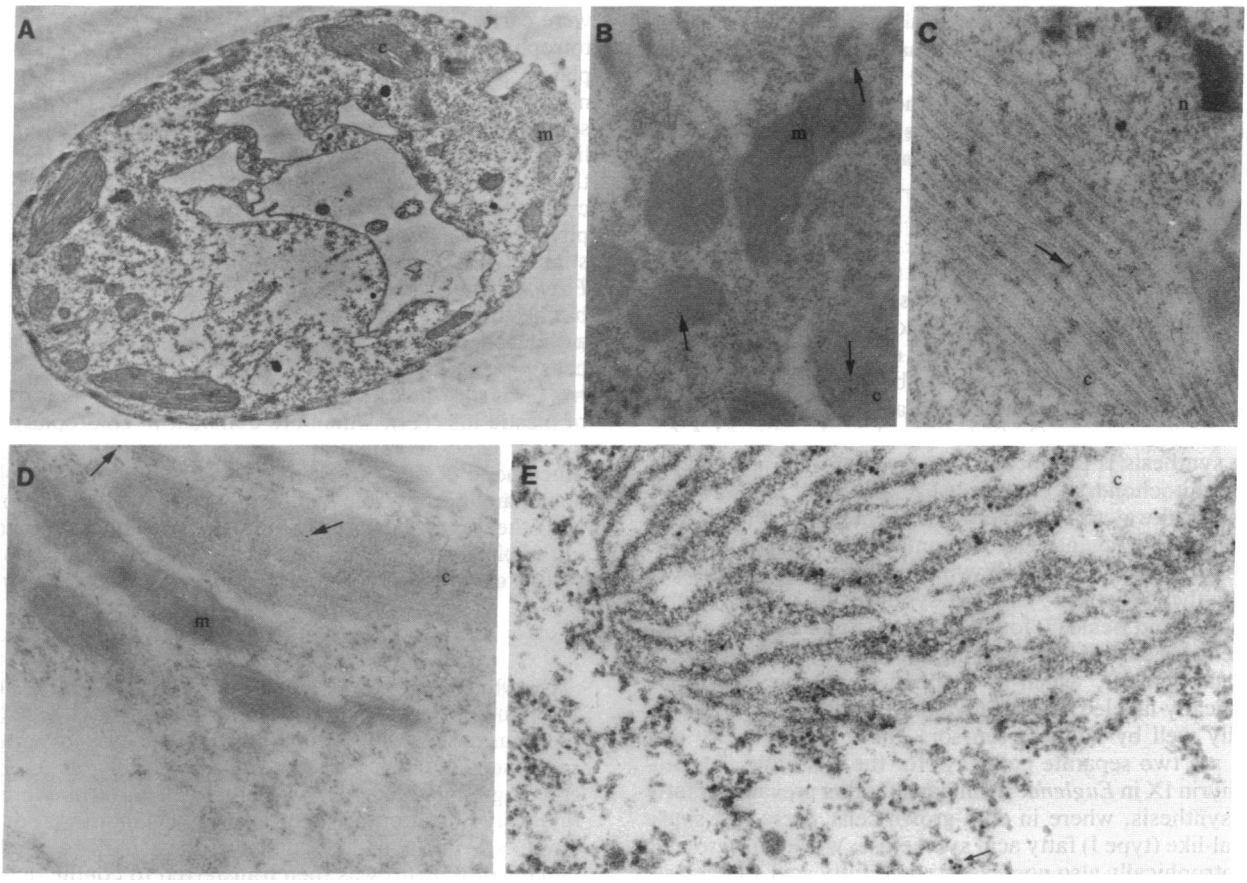

FIG. 2. Localization of PBGD in light-grown *Euglena* cells by immunogold electron microscopy. Sections of cells were either viewed immediately after staining (*A*) or after being challenged with serum, followed by gold-conjugated second antibody (*B–E*). (*A*) Whole cell. (×8400.) (*B* and *C*) Sections treated with PBGD antiserum. (×23,800.) (*D*) Part of a cell treated with preimmune serum. (×29,050.) (*E*) Higher magnification of a chloroplast treated with PBGD antiserum. (×47,600.) c, Chloroplast; m, mitochondrion; n, nucleus. Arrows indicate representative gold particles.

Table 1. Immunogold labeling of PBGD in different compartments of light-grown *Euglena* cells

Serum	Whole cell	Chloroplast	Mitochondria	Cytoplasm
Preimmune	0.51 ± 0.11	0.88 ± 0.30	1.12 ± 0.40	0.49 ± 0.09
PBGD antibodies	3.11 ± 0.55*	17.97 ± 2.22*	2.05 ± 0.60	0.83 ± 0.17

Sections of *Euglena* cells were challenged with either PBGD antiserum or preimmune serum followed by gold (10 nm)-conjugated goat anti-rabbit IgG. The values given are the number of gold particles per μm^2 ± SE, from the measurement of 12 independent cells.
*One percent level of significance.

with L R Gold (Agar Scientific, Stansted, U.K.) for 48 hr. Polymerization was carried out in an oxygen-free atmosphere under illumination by using Benzil (0.1% wt/vol) as the initiator of polymerization. Silver-gold sections were cut with an Ultrotome-Nova (LKB–Produkter) and were mounted on 400-mesh gold grids (Agar Scientific). Sections were challenged with serum (1:500) followed by goat anti-rabbit IgG (1:25) conjugated with 10-nm gold particles (Biocell, Cardiff, U.K.), essentially as described by Dehesh *et al.* (20). After washing, the sections were stained with uranyl acetate for 2 min and then poststained for a further 3 min with lead citrate. The sections were examined with a Philips EM301 electron microscope at 60 kV.

RESULTS

Subcellular Localization of PBGD in Light-Grown *Euglena* Cells. The polyclonal antibodies raised against PBGD protein purified from light-grown *Euglena* (9) were used to challenge a Western blot of total soluble protein and protein from isolated intact chloroplasts (Fig. 1). As can be seen, a single protein band of 40 kDa is detected in both lanes. This confirms the presence of the enzyme in *Euglena* chloroplasts but does not exclude the presence of an isozyme of the same molecular mass elsewhere in the cell. We therefore decided to use immunogold electron microscopy to examine the subcellular location of the enzyme in more detail. To obtain good ultrastructural preservation of the *Euglena* cells, while retaining antigenicity, L R Gold resin was used in conjunction with much lower glutaraldehyde concentrations than normal (0.1% rather than 2%), and postfixation with osmium tetroxide was avoided. Fig. 2*A* shows a typical micrograph of a whole *Euglena* cell, in which mitochondria, chloroplasts, and a number of other organelles are apparent, as is the structure of the proteinaceous pellicle. For immunodetection, sections were challenged either with preimmune serum (Fig. 2*D*) or PBGD antiserum (Fig. 2 *B*, *C*, and *E*), followed by gold-conjugated goat anti-rabbit IgG. The number of gold particles per unit area was estimated for the whole cell and for the various compartments (Table 1). It can be seen that, compared with the sections treated with preimmune serum, there was much more gold labeling of the experimental sections, and most of the gold particles were concentrated on chloroplasts (17.97/μm^2). Although there was some labeling of the mitochondria and cytoplasm (arrows in Fig. 2*B*), this was not significantly different from that found with preimmune serum. Interestingly, particles were sometimes detected in the nucleus. This has been observed previously (e.g., ref. 21), although its significance is uncertain. The data obtained here suggest that, within the limits of detection of the technique, the antibodies recognize only a chloroplast-located PBGD. However, it is still possible that there is another form of the enzyme, which does not cross-react with the antibodies.

Expression of PBGD During Greening of Dark-Adapted *Euglena* Cells. Since a chloroplast enzyme might be expected to be photoregulated, we followed the amount of PBGD activity, enzyme protein, and transcript levels during the greening of dark-grown *Euglena*. The results of a representative experiment are shown in Fig. 3. As expected, although chlorophyll synthesis started immediately upon illumination, there was a lag of some 36 hr before an exponential rate of increase occurred. In contrast to the absence of chlorophyll, there was measurable PBGD activity in dark-grown cells, although it was only about 20% of that in the culture at 96 hr. Furthermore, there was an immediate and substantial rise in PBGD activity after the onset of illumination.

To determine the point at which PBGD is photoregulated, protein and RNA dot blots were carried out on an optimized greening time course of 0–72 hr. In common with the enzyme activity, there was a distinct increase in the amount of PBGD protein during greening (Fig. 4). Conversely, no marked differences in the levels of PBGD transcript were observed on a dot blot of total RNA from the same time course (Fig. 5), although there was a slight increase at 72 hr. Thus, the considerable increases in PBGD protein and activity in the absence of any appreciable alteration in the amount of PBGD transcript imply that the photoregulation of PBGD in *Euglena* is at the posttranscriptional level. Similar observations have been made for several other nuclear-encoded proteins in *Euglena*, including light-harvesting chlorophyll protein II, citrate synthase, and fumarase (for a review, see ref. 22).

Expression of PBGD in Nonchlorophyllous Cell Types. The increase in PBGD activity prior to an increase in chlorophyll

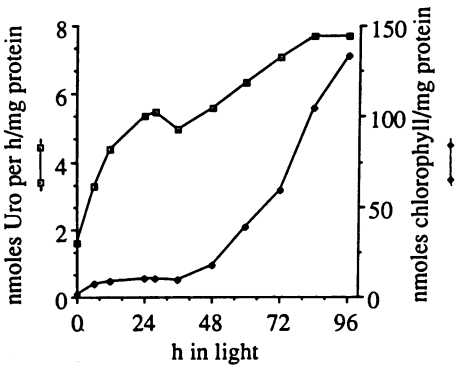

FIG. 3. Chlorophyll content and PBGD activity during the greening of dark-adapted *Euglena* cells. Uro, uroporphyrin.

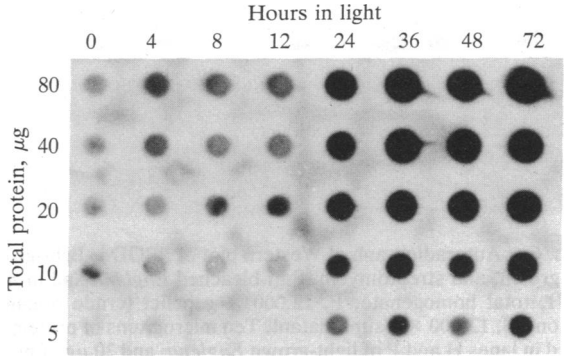

FIG. 4. Autoradiograph of dot blot of total soluble proteins extracted at different time intervals during greening of dark-adapted *Euglena*. Five to 80 μg of protein was challenged with antiserum to PBGD as described in *Materials and Methods*.

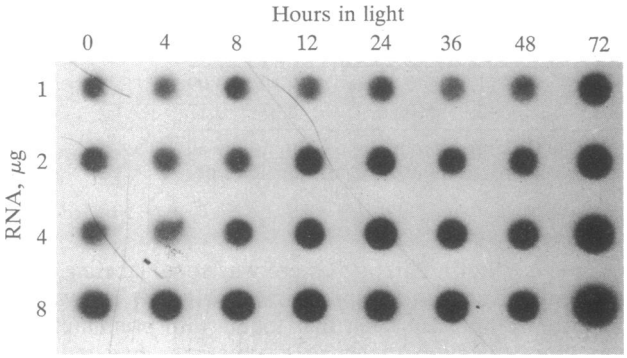

FIG. 5. Autoradiograph of dot blot of total RNA extracted at different time intervals during greening of dark-adapted *Euglena*. One to 8 μg of total RNA was probed with a cDNA clone for PBGD as described in *Materials and Methods*.

content suggests that the newly synthesized enzyme is required for chlorophyll synthesis and is thus in the chloroplast. Further, dark-grown cells, which contain measurable enzyme activity, also contain a protein that cross-reacts with antiserum shown to detect a chloroplast-located PBGD. However, since these cells have the potential to make chlorophyll, a nonplastid form of the enzyme may be repressed or obscured by the chloroplast one. We therefore decided to compare the expression of PBGD in wild-type *Euglena* with that in two cell lines that cannot make chlorophyll—a streptomycin-bleached mutant and *A. longa*, a colorless relative of *Euglena*—where a nonplastid pathway of porphyrin synthesis should be apparent.

Astasia and the streptomycin-bleached mutant contained a similar level of PBGD activity to that found in dark-grown cells (1–1.6 nmol of uroporphyrin per hr per mg of protein), compared with 11 nmol of uroporphyrin per hr per mg of protein in light-grown *Euglena* (see legend to Fig. 6). However, for each of the four cell lines, most of this activity could be recovered in a 12,000 × *g* pellet of total cell homogenate, which could be considered as a crude organellar fraction (13), while none could be detected in the supernatant. On a Western blot of these different fractions (Fig. 6), no immunodetectable protein was found in the supernatant fractions,

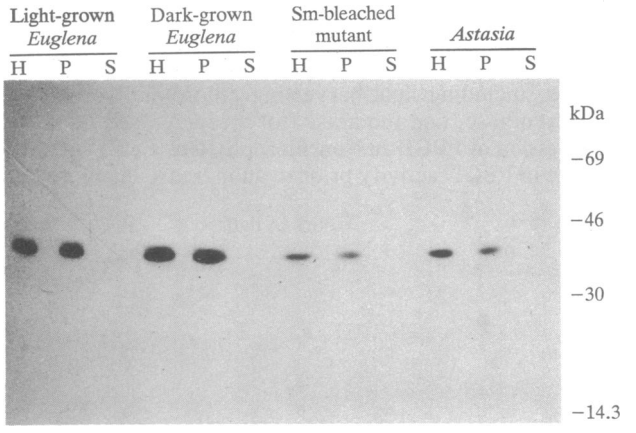

FIG. 6. Autoradiograph of Western blot of PBGD in light-grown, dark-grown, and streptomycin (Sm)-bleached *Euglena* and in *Astasia*. H, total homogenate; P, 12,000 × *g* pellet (crude organellar fraction); S, 12,000 × *g* supernatant. Ten micrograms of protein was loaded in lanes H and P of light-grown *Euglena*, and 30 μg of protein was loaded in all other lanes. The average specific activity of PBGD in nmol of uroporphyrin per hr per mg of protein, estimated from several separate preparations, was 10.98 ± 0.95 for light-grown *Euglena*, 1.68 ± 0.30 for dark-grown *Euglena*, 1.45 ± 0.27 for streptomycin-bleached *Euglena*, and 1.07 ± 0.21 for *Astasia*.

even when, for the light-grown cells, 3 times as much protein was loaded as for the homogenate and pellet. It is also clear that the band in each lane has a molecular mass of 40 kDa, which is identical to PBGD purified from light-grown *Euglena* (10).

On Northern blots of total RNA probed with the cDNA for PBGD, only one transcript was seen in the nonchlorophyllous cells (Fig. 7*A*), which was the same size (1.8 kilobases) as that in light-grown *Euglena* cells and also as the cDNA for the plastid-located PBGD (9). Since the control of expression of PBGD is at the posttranscriptional level, it remains possible that this message is not translated *in vivo* and the PBGD protein and activity detected in the nonchlorophyllous cells is encoded by a different message, which does not hybridize to the cDNA for the plastid enzyme. However, when poly(A)$^+$ RNA was translated *in vitro*, only one major polypeptide of 49,000 Da was immunoprecipitated with PBGD antiserum (Fig. 7*B*). The lower molecular mass bands, which are frequently seen in *in vitro* translation products, probably represent premature termination products. Because the protein present *in vivo* is 40 kDa (Fig. 6), these results imply that PBGD is synthesized in all four cell types as a higher molecular mass precursor, which is subsequently processed to the mature form.

DISCUSSION

Isolated *Euglena* chloroplasts are capable of the synthesis of chlorophyll from exogenous ALA (4), so they must contain all of the enzymes of the pathway, including PBGD. The studies of Weinstein and Beale (7) suggest the presence of a duplicate pathway for nonplastid tetrapyrrole synthesis and thus a second form of PBGD. By using antiserum raised against the enzyme purified from light-grown *Euglena* (10) and a cDNA clone isolated with this antiserum (9), we examined the possibility of the existence of this other PBGD. During the greening of dark-adapted *Euglena* cells, there was a 5-fold increase in enzymic activity (Fig. 3) and a similar increase in immunodetectable protein (Fig. 4). Interestingly, as for many other nuclear-encoded organellar enzymes in *Euglena* that are photoregulated (22), the control of expression is posttranscriptional. The increase in PBGD activity before the exponential increase in chlorophyll synthesis is consistent with the involvement of the newly synthesized enzyme in chlorophyll synthesis. A chloroplast location for

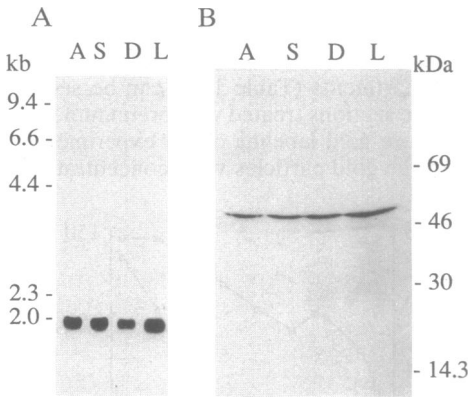

FIG. 7. (*A*) Autoradiograph of Northern blot of PBGD transcripts. A Northern blot of 20 μg of total RNA from each cell type was probed with a cDNA clone for PBGD as described in *Materials and Methods*. (*B*) Immunoprecipitation of PBGD from translation products of poly(A)$^+$ RNA. One microgram of poly(A)$^+$ RNA from each of the cell lines was translated *in vitro* and challenged with PBGD antiserum as described in *Materials and Methods*. A, *Astasia*; L, light-grown *Euglena*; D, dark-grown *Euglena*; S, streptomycin-bleached *Euglena*. kb, Kilobases.

PBGD in light-grown *Euglena* was confirmed by subcellular fractionation (Figs. 1 and 6) and immunogold electron microscopy (Fig. 2 and Table 1). It might be argued that this latter technique is not sufficiently sensitive to identify an extraplastidic PBGD if it is present in very low amounts compared to that in the chloroplast. Weinstein and Beale (7) found that in light-grown cells there was almost 1000 times more chlorophyll than heme *a*, and since the cytoplasmic compartment is much larger than that of the chloroplasts, the concentration of an extraplastidic enzyme would be much lower than in the chloroplasts. However, in dark-grown cells there is 15–20% of the PBGD activity of light-grown cells (Fig. 3), and so unless this were turned over rapidly during greening, it would be expected to be detectable by immunogold. Indeed, there was a significant gold labeling when sections of dark-grown *Euglena* cells were treated with PBGD antibodies (unpublished observations). Because of the reduced resolution caused by the need to retain antigenicity, it was not possible to distinguish plastids from other vesicles, so no conclusions could be drawn about the compartment in which the enzyme was located.

An alternative explanation for the apparent absence of an extraplastidic form of the enzyme is that the antibodies do not recognize it. For instance, antibodies against the cytosolic isozyme of fructose-1,6-bisphosphatase (21) do not cross-react with the chloroplastic form and vice versa. Nevertheless, our antibodies do recognize PBGD protein in dark-grown *Euglena* cells (Figs. 4 and 6) and in a streptomycin-bleached mutant and *Astasia* (Fig. 6), and the immunodetectable protein was in a crude organellar fraction in all cell types (Fig. 6). Furthermore, only one message, which was long enough to encode the transit peptide, is present (Fig. 7*A*), and a single polypeptide, larger than the mature protein, is immunoprecipitated from the translation products (Fig. 7*B*), suggesting that in wild type cells and in cells that cannot make chlorophyll, PBGD is synthesized as a higher molecular weight precursor, which is translocated to the plastid and processed to the mature size. Although *Astasia* and bleached mutants have both been described as aplastidic, this is probably not the case. Plastid remnants have been identified in most artificially bleached mutants by electron microscopy (12), and although there are some strains, including *Astasia*, in which these proplastid-like bodies appear to be completely absent, this may simply reflect the difficulty in distinguishing vestigial plastids from other vesicles in the cell. Furthermore, the presence of chloroplast DNA fragments has been demonstrated in all bleached mutants (15) and in *Astasia* (13).

There are a number of explanations that could reconcile our conclusions with the findings of Weinstein and Beale (7). First, there may be two separate pathways for porphyrin synthesis in each plastid, and the intermediates are somehow compartmentalized from one another. A similar situation has been proposed to exist in cucumber chloroplasts, where inhibition of ALA synthesis by gabaculine, 4-amino-5-hexynoic acid, or protoheme had little effect on the synthesis of the chlorophyll precursor protochlorophyllide (23). The authors propose a model in which there are two pools of ALA, one for chlorophyll and one for protoheme synthesis, which can be distinguished from one another by their differential sensitivity to the inhibitors. Second, there may be two populations of plastid in the *Euglena* cell, one of which develops into chloroplasts, while the other remains as proplastids, responsible for the synthesis of nonplastid porphyrin. Third, there could be another PBGD enzyme in the cytoplasm, which we are unable to identify, and the plastid-located PBGD is not functional in the nonchlorophyllous cell types. Recently, Lutzow and Kleinig (24) described a preparation of chromoplasts from daffodil that had no chlorophyll and yet contained most of the enzymes for chlorophyll synthesis in a highly active form. If the last explanation is correct, then this means that the pathways for the synthesis of mitochondrial heme and chlorophyll have diverged considerably during evolution.

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