The EMBO Journal vol.6 no.6 pp.1571–1579, 1987

Transcription and RNA stability are important determinants of higher plant chloroplast RNA levels

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Communicated by J.H.Weil

Transcription in lysed barley plastids and Northern slot blot analyses were used to determine the relationship between changes in RNA levels and transcription during plastid development. Transcription in plastids of 4.5–9-day-old dark-grown or illuminated barley seedlings declined up to 10-fold as a function of plant age. Decreased transcription of some plastid genes (rbcL, psaA-psaB) was paralleled by decreased levels of mRNA. In other cases (16SrDNA, psbA) the changes in transcription were not followed by proportional changes in RNA levels indicating that RNA stability is important in establishing the amount of plastid RNA for these genes. Further analysis showed that transcription of the plastid rRNA transcription unit is regulated differently than the transcription of protein coding genes such as psbA or rbcL.

Key words: barley/chloroplast/development/RNA/transcription

Introduction

The chloroplast genome in higher plants ranges in size from 120 to 180 kbp (reviewed in Whitfeld and Bottomley, 1983). The complete sequence of a liverwort chloroplast genome (Ohyama *et al.*, 1986) and the tobacco chloroplast genome have been reported (Shinozaki *et al.*, 1986). The tobacco chloroplast genome is predicted to code for more than 120 genes including 31 tRNAs, four ribosomal RNAs, 19 ribosomal proteins and several subunits of the chloroplast RNA polymerase. A large number of chloroplast genes also code for proteins involved in photosynthetic electron transport (Herrmann *et al.*, 1985). Despite a large amount of DNA sequence data, the mechanisms which coordinate the transcription of various classes of plastid genes (tRNA, rRNA or protein genes) or which regulate the expression of specific plastid genes are largely unknown.

Numerous studies have shown that the concentration of specific plastid transcripts can vary with the stage of plastid development and in a tissue-specific manner. For example, psbA mRNA levels have been reported to increase significantly relative to total chloroplast or cellular RNA when dark-grown maize (Bedbrook *et al.*, 1978; Altman *et al.*, 1984), *Spirodela oligorrhiza* (Reisfeld *et al.*, 1978), spinach (Herrmann *et al.*, 1985) or barley (Kreuz *et al.*, 1986) plants were illuminated. In mustard (Link, 1982) and maize (Zhu *et al.*, 1985), activation of the phytochrome system was reported to increase the relative abundance of psbA mRNA. An example of tissue specific differences in plastid mRNA levels can be found in comparisons of plastids of mesophyll and bundle sheath cells of maize (Link *et al.*, 1978; Jolly *et al.*, 1981; Schuster *et al.*, 1986). Jolly *et al.* (1981) reported that atpB transcripts were present at similar levels in chloroplasts of mesophyll and bundle sheath cells whereas rbcL transcripts were much more abundant in bundle sheath chloroplasts. This observation was particularly interesting because the rbcL and atpB genes are adjacent in the plastid genome of maize, and transcribed divergently (Link and Bogorad, 1984). It has also been found that psbA transcripts, in contrast to rbcL mRNA, are deficient in bundle sheath plastids relative to mesophyll cell plastids (Schuster *et al.*, 1986).

Additional examples of differential plastid gene expression during plastid biogenesis have been reported (Klein and Mullet, 1987b; Rodermel and Bogorad, 1985). In maize several genes were distinguished based on the time course of changes in transcript levels during light-induced plastid development (Rodermel and Bogorad, 1985). In barley, RNA levels for several plastid genes (rbcL, psaA-psaB) declined during plastid maturation whereas psbA mRNA levels did not decline (Klein and Mullet, 1987b).

In many of the studies described above, it was suggested that the changes in transcript levels were due to altered transcription of plastid genes. However, in no case has this possibility been directly verified. In fact Altman *et al.* (1984) reported that transcription of psbA in plastids isolated from dark-grown or illuminated plants did not change even though psbA transcript levels increased in illuminated plants relative to total plastid RNA. One way to analyze the regulation of plastid gene transcription involves the development of homologous *in vitro* transcription systems where the role of *cis*- and *trans*-acting elements can be tested (Jolly and Bogorad, 1980; Gruissem *et al.*, 1983; Link, 1984b; Bradley and Gatenby, 1985; Orozco *et al.*, 1985). *In vitro* assays have confirmed that DNA sequences which promote initiation of transcription of plastid genes are similar to those used in prokaryotes (Gruissem and Zurawski, 1985). *In vitro* transcription studies have also revealed an important effect of supercoiling on the transcription of chloroplast genes (Stirdivant *et al.*, 1985). However, the connection between this observation and regulation of gene expression *in vivo* in higher plants is lacking. Futhermore, *in vitro* transcription assays have not provided an explanation for the accumulation of psbA mRNA in illuminated plants relative to levels in dark-grown plants nor for different ratios of rbcL and atpB mRNA in mesophyll versus bundle sheath cells of maize.

In this paper we describe a transcription assay for isolated plastids which is similar to 'run-on' transcription assays used for isolated nuclei (Gallagher and Ellis, 1982; Hagen and Guilfoyle, 1985). We have used the transcription assay and Northern analyses to determine the relationship between changes in transcription and RNA levels in plastids during chloroplast development in barley. Our results provide the first direct evidence that changes in RNA levels for some plastid genes in illuminated plants are due to altered transcription. Furthermore, our data also show that RNA levels for other plastid genes are determined to a large degree post-transcriptionally.

Results

An early consideration in these experiments concerned the manner of expressing changes in plastid RNA levels and plastid transcription during leaf and plastid biogenesis. In most previous studies plastid mRNA levels were expressed relative to total leaf RNA (Thompson *et al.*, 1983; Bennett *et al.*, 1984; Herrmann *et al.*, 1985; Kreuz *et al.*, 1986), total plastid RNA (Altman *et al.*, 1984; Link, 1984a) or plastid ribosomal RNA (Rodermel and Bogorad, 1985). Expression of plastid RNA levels relative to total leaf RNA is problematic because plastid number, size and RNA levels vary greatly during leaf biogenesis (Smith, 1970; Robertson and Laetsch, 1974; Dean and Leech, 1982). The use of total plastid RNA or plastid ribosomal RNA as a reference is also subject to uncertainty because plastid RNA levels and ribosome number per plastid also vary during plastid biogenesis (Dean and Leech, 1982; Fish and Jagendorf, 1982; Klein and Mullet, 1987). Based on these considerations we expressed both plastid RNA levels and plastid transcription activity as a function of plastid number.

Transcription of plastid genes in higher plants has been assayed by incubating plant shoots with $^{32}PO_4$ or radiolabeled uridine (Stummann *et al.*, 1980; for a review, Kirk and Tillney-Basset, 1978). These studies proved difficult due to the long incubation times required (1−3 h) and uncertainties concerning nucleotide pool sizes and compartmentalization of exogenous label. An alternative method to assay RNA synthesis is to use isolated organelles. This approach is based on the assumption that the *in vivo* distribution of RNA polymerase on DNA can be maintained during organelle isolation at low temperature. The distribution is then assayed *in vitro* during a short labeling period which limits new rounds of transcription initiation. This method has been used extensively to analyze changes in plant nuclear gene transcription (Gallagher and Ellis, 1982; Hagen and Guilfoyle, 1985). RNA synthesis has also been examined in isolated intact chloroplasts (reviewed in Ellis and Hartley, 1982). These studies indicated that intact plastids could incorporate [^{3}H]uridine (Hartley and Ellis, 1973), $^{32}PO_4$ (Bennett and Milewska, 1976) or [^{32}P]UTP (Altman *et al.*, 1984) into RNA. However, the rate and extent of labeling of RNA could have been limited by slow uptake of the labeled precursors (Heldt, 1969) or by the rate of incorporation of [^{3}H]uridine or $^{32}PO_4$ into UTP within the plastids. In order to circumvent the potential difficulties with intact plastids, we examined transcription in lysed plastids where exogenous [^{32}P]UTP would be freely accessible to the chloroplast RNA polymerase and variation in nucleotide pool sizes in plastids could be avoided. A previous study by Spencer and Whitfeld (1976) indicated that lysed plastids were capable of RNA synthesis.

Characterization of transcription in lysed chloroplasts

In our initial experiments, intact plastids isolated from dark-grown or illuminated barley plants were added to a hypotonic transcription reaction mixture which caused plastids to lyse (determined by phase-contrast microscopy). The components of the reaction mix (i.e. NTPs, $MgCl_2$, KOAc, DTT) and the concentration of plastids in the reaction were varied to optimize incorporation of UTP into RNA during a 10-min labeling period. The optimized reaction mixture contained 125 μM ATP, CTP and GTP and 10 μM UTP. These concentrations of nucleotide triphosphates exceed the K_m values for these substrates reported for Euglena chloroplast RNA polymerase (ATP = 4.0 μM, CTP = 0.6 μM, GTP = 2.5 μM, UTP = 2.3 μM; Hallick *et al.*, 1976). A plastid concentration of 1 × 10^5 plastids per μl reaction mixture was chosen to ensure that UTP incorporation was proportional to the number of plastids added to the reaction. At plastid concentrations approaching 5 × 10^5 plastids per μl, UTP incorporation was no longer proportional to the number of plastids added (data not shown).

Table I. Characterization of transcription in lysed plastids

Treatment	[^{32}P]UTP incorporation (%)
Control	100
20 μg/ml DNase I[a]	1
25 μg/ml actinomycin D	1
20 μg/ml rifampicin	123
100 μg/ml rifampicin	111
100 μg/ml chloramphenicol	108
100 μM spermidine	98
100 μM spermine	103
Glycerol (4%)	104
10 μg/ml heparin	217
500 μg/ml heparin	291
Exogenous DNA (1 μg)	109
Exogenous DNA (5 μg)	86
Dark	92

Plastids were isolated from 4.5-day-old dark-grown barley seedlings. Transcription in lysed plastids was carried out as described in the experimental procedures with the additions indicated. Exogenous DNA was plasmid pPPBX-10218 containing the plastid psbA promoter (Boyer and Mullet, 1986). Control plastids incorporated 1.9 pmol of UTP per 2.5 × 10^6 plastids during the 10 min assay (100% value).
[a]Lysed plastids were incubated with DNase I for 15 min at 4°C prior to addition to the transcription assay.

The characterization of transcription by lysed plastids is shown in Table I. Incorporation of [^{32}P]UTP into RNA was completely inhibited by DNAse and actinomycin D which is consistent with incorporation dependent on DNA-dependent RNA polymerase. Plastid transcription was insensitive to rifampicin, an inhibitor of bacterial RNA polymerase. Higher plant plastid RNA polymerase has been previously shown to be insensitive to rifampicin (Orozco *et al.*, 1985). Transcription by lysed plastids was not influenced by concomitant protein synthesis since the addition of chloroamphenicol (inhibitor of 70S ribosomes) did not alter UTP incorporation. Furthermore, transcription by lysed plastids did not require exogenous polyamines, such as spermine or spermidine, nor was incorporation stimulated by glycerol. Finally, incorporation of UTP by lysed plastids was light-independent.

Chloroplast RNA polymerase which is not bound to DNA (termed 'free' RNA polymerase) can be detected in plastid extracts by an enhancement of transcription upon addition of an exogenous DNA template containing a chloroplast promoter (Orozco *et al.*, 1985). Furthermore, transcription by 'free' RNA polymerase has been shown to be inhibited by the polysaccharide heparin (Greenberg *et al.*, 1984). In the present study, addition of plasmid DNA containing a plastid gene promoter to lysed plastids did not stimulate incorporation of [^{32}P]UTP into RNA (Table I). Furthermore, transcription in lysed plastids was not inhibited by heparin but instead was significantly enhanced by this compound (Table I). These results show that RNA synthesis in lysed plastids is not due to 'free' plastid RNA polymerase.

The time course of UTP incorporation in plastids isolated from 5-day-old dark-grown barley seedlings or barley grown 4.5 days

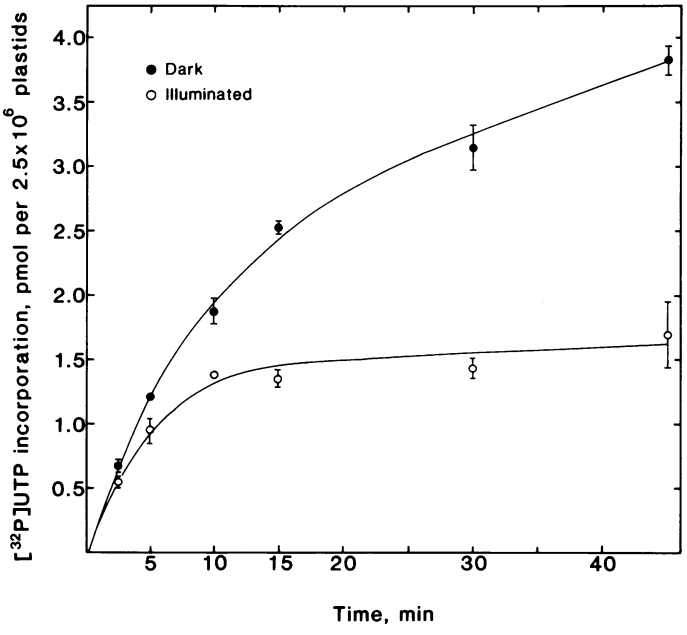

Fig. 1. Time course of incorporation of [^{32}P]UTP into RNA in lysed plastids. Plastids were isolated from 5-day-old dark-grown barley seedlings (●) or barley seedlings which had been grown 4.5 days in darkness then illuminated for 16 h at 10 000 lux (○). Transcription was initiated by the addition of intact plastids to the hypotonic reaction mix as described in the experimental procedures. Duplicate aliquots were removed at the indicated times and incorporation of UTP into RNA was determined. Each data point represents the mean of two observations ± SD.

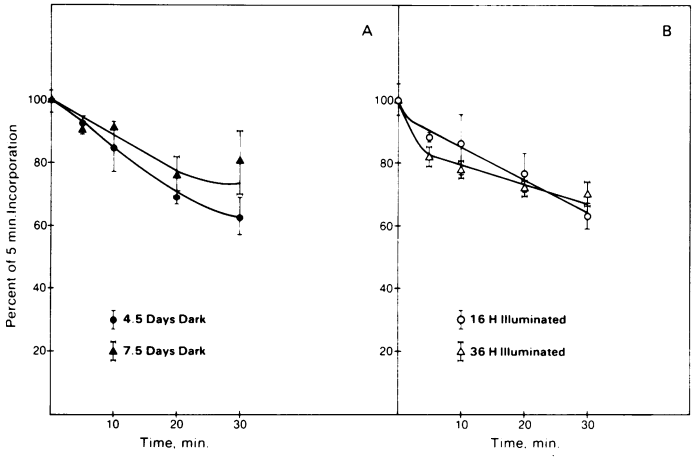

Fig. 2. Measurement of the stability of RNA synthesized in the lysed plastid transcription assay. Plastids were isolated from 4.5 (●)- or 7.5 (▲)-day-old dark-grown barley or barley which had been grown 4.5 days in darkness then illuminated for 16 (○) or 36 h (△). Transcription in lysed plastids was allowed to proceed for 5 min in the presence of [^{32}P]UTP. Incorporation of UTP during the pulse-labeling period for these plastids was similar to the data shown in Figure 3. After the 5-min pulse-labeling period (time 0 in the figure) unlabeled UTP (1 mM) was added to prevent further incorporation of radiolabel and aliquots were removed at the times indicated. Data is expressed as a percentage of [^{32}P]UTP incorporation into RNA during the 5-min labeling period. Each data point represents the mean of two observations ± SD.

in darkness then illuminated for 16 h is shown in Figure 1. Both plastid populations incorporate >0.95 pmol UTP/2.5 × 10^6 plastids during the first 5 min of the transcription assay. Ellis and Hartley (1982) and Altman *et al.* (1984) reported that plastids

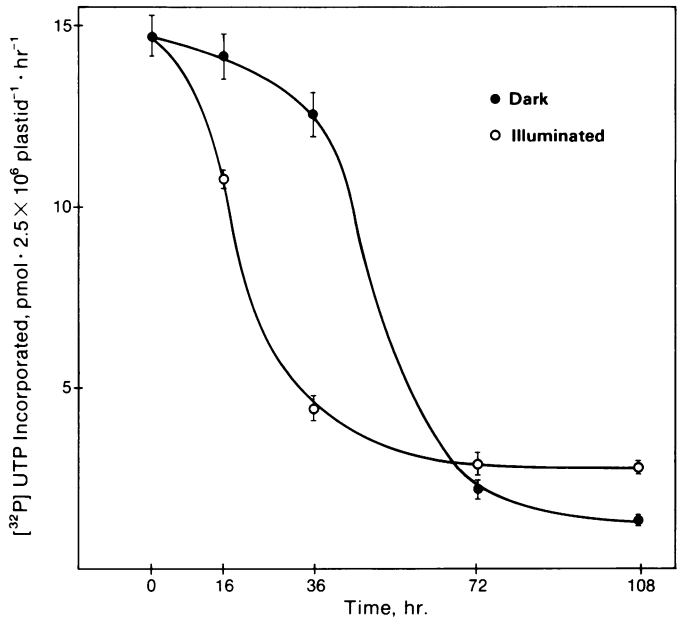

Fig. 3. Transcription activity in plastids isolated from dark-grown and illuminated barley seedlings of different age. Plastids were isolated from the apical 3 cm of 4.5-day-old dark-grown seedlings (0 h) which had been kept in the dark an additional 16, 36, 72 or 108 h (●) or illuminated for an additional 0−108 h (○). Lysed plastids were pulse-labeled for 5 min and UTP incorporation determined. Each data point represents the mean of two observations ± SD.

incorporate 0.1−0.4 pmol UTP/h/2.5 × 10^6 plastids (assuming 2.0 × 10^9 plastids/mg chlorophyll). After the first 5 min transcription in plastids isolated from illuminated plants was reduced significantly compared with plastids from dark-grown plants (Figure 1). Net UTP incorporation by plastids from illuminated plants was nearly complete within 10 min of incubation while UTP incorporation by etioplasts continued for 45 min.

The differences in incorporation of UTP into RNA by plastids of dark-grown and illuminated plants could be due to changes in RNA synthesis or differences in stability of newly synthesized transcripts. Therefore, a pulse−chase experiment was done to explore the possible influence of RNA degradation on [^{32}P]UTP incorporation in the lysed plastid transcription assay (Figure 2). Transcription in lysed plastids was allowed to proceed for 5 min, then unlabeled UTP (1 mM) was added to limit further incorporation of [^{32}P]UTP. Aliquots were removed from the reaction mixture after the 5-min labeling period and at various times during a 30-min chase period. Pulse−chase assays were done using plastids isolated from 4.5- or 7.5-day-old dark-grown plants or plants which had been transferred to light when 4.5 days old and then illuminated for 16 or 36 h. These experiments showed that ^{32}P-labeled RNA in each of the plastid populations examined had a half-life of >30 min (Figure 2). From this result it can be estimated that RNA degradation in lysed plastids would cause a loss of ≤20% of the ^{32}P-labeled RNA synthesized during a 5-min transcription assay. Therefore, a 5-min labeling period was used in all subsequent transcription assays. The time course of RNA degradation was similar in plastids isolated from dark-grown and illuminated plants, although the loss of label during the first 5 min of the chase was somewhat greater in plastids from illuminated plants compared with dark-grown plants. This result indicates that differences in [^{32}P]UTP incorporation detected in lysed plastids of dark-grown or illuminated plants can-

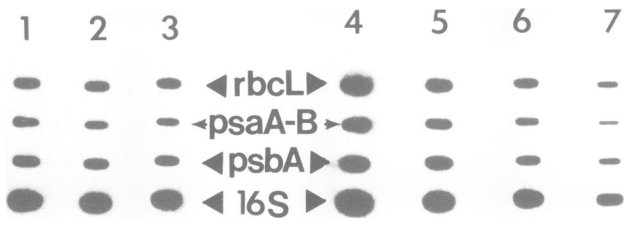

Fig. 4. Determination of reproducibility and responsiveness of the Southern slot-blot assay for quantitating plastid gene transcription. Plasmid DNA (0.25 pmol) containing rbcL, psaA-psaB, psbA or 16SrDNA sequences was restricted, denatured and bound to a nylon membrane in the slots indicated. The strips of DNA in **lanes 1–7** were incubated separately with ^{32}P-labeled RNA from a lysed transcription assay of plastids from 4.5-day-old dark-grown plants. **Lanes 1–3** were incubated with ^{32}P-labeled RNA from 2.0 $\times$ 10^6 plastids (200 000 c.p.m. per blot, X-ray film was exposed for 1 h). **Lanes 4–7** were incubated with ^{32}P-labeled RNA from 2 $\times$ 10^6, 1 $\times$ 10^6, 0.5 $\times$ 10^6 or 0.25 $\times$ 10^6 plastids respectively. After hybridization (48 h, 37°C) the blots were washed and exposed to X-ray film for ~3 h.

Fig. 5. Northern blot analyses of RNA levels in plastids of dark-grown or illuminated barley seedlings. Plastids were isolated from 4.5-day-old dark-grown plants and plants kept in darkness (D) or illuminated (L) for an additional 16, 36, 72 or 108 h (marked at the left of the Figure). RNA was extracted from each plastid population and RNA from 0.25 $\times$ 10^6 plastids was applied to the designated slots. Blots were incubated with rbcL, psaA or psaA-psaB nick-translated probes, then washed and autoradiographed.

not be accounted for by differences in degradation of newly synthesized transcripts.

Changes in plastid transcription activity as a function of plant age

We have previously reported that RNA levels for several plastid genes decline in plastids of 4.5–9-day-old barley seedlings (Klein and Mullet, 1987). The changes in plastid RNA levels could be due to changes in plastid gene transcription. To test this possibility, transcription was assayed in plastids isolated from 4.5-day-old dark-grown barley seedlings (0-h point) which had been kept in darkness or illuminated for up to 108 h (Figure 3). In all plants, plastids were isolated from the apical 3 cm of the barley seedlings as in our previous studies of plastid biogenesis (Klein and Mullet, 1986, 1987). As shown in Figure 3, transcription declined 7- to 10-fold in plastids isolated from dark-grown and illuminated barley seedlings as a function of plant age. The decline in plastid transcription is accelerated when 4.5-day-old plants are illuminated. However, in 9-day-old plants (108-h point), plastid transcription activity is lower in plants kept in continuous darkness compared with illuminated plants.

Transcription from specific plastid genes in lysed plastids

Transcription of psbA, rbcL, psaA-psaB and 16SrDNA in lysed plastids was assayed by Southern blotting. We have previously found that ^{32}P-labeled RNA from lysed plastid transcription assays would hybridize to psbA, rbcL, psaA-psaB and 16SrDNA but not to plasmid DNA (Mullet *et al.*, 1987). To facilitate processing of large numbers of samples, conditions were optimized for slot-blot Southerns. Plasmid DNA was restricted to release chloroplast DNA inserts, blotted and fixed to positively charged nylon membranes by methods similar to those described by Reed and Mann (1985). In one experiment end-labeled DNA was blotted and treated in a trial hybridization to determine DNA retention on the nylon membrane. It was found that ~90% of the blotted DNA remained bound through the duration of the hybridization and washing procedures. The reproducibility of the Southern slot-blot technique was tested by preparing triplicate blots and incubating each with an equal amount of ^{32}P-labeled RNA (Figure 4, lanes 1–3). This test demonstrated that the slot-blot assay was reproducible. Next the response of the assay to different levels of input RNA was tested. ^{32}P-labeled RNA from 0.25 $\times$ 10^6 to 2 $\times$ 10^6 plastids was hybridized to slot blots containing DNA from psbA, psaA-psaB, rbcL and 16SrDNA (Figure 4, lanes 4–7). The amount of radioactivity hybridized was quantitated by excising each slot and scintillation counting. This analysis showed that hybridization of labeled RNA to rbcL, psbA and psaA-psaB DNA increased linearly when RNA from 0.25 $\times$ 10^6 to 2 $\times$ 10^6 plastids was assayed. Increased hybridization of RNA transcribed from 16SrDNA was also observed when ^{32}P-labeled RNA from 0.25 $\times$ 10^6 to 2 $\times$ 10^6 plastids was assayed, although the hybridization was not proportional to plastid number when RNA from 2 $\times$ 10^6 plastids was used. These results indicate that the Southern slot-blot assay could be used to quantitate labeled RNA from 1 $\times$ 10^6 to 2 $\times$ 10^6 plastids although transcription of 16SrDNA would be underestimated to some extent.

The pulse–chase experiment in Figure 2 showed that RNA degradation would not complicate the analysis of overall rates of transcription in the lysed plastid assay. A similar pulse–chase assay was done to determine the time course of loss of label from specific plastid RNAs. This experiment showed that the half-life of RNA transcribed from psbA, rbcL, psaA-psaB and 16SrDNA in lysed plastids of 4.5- or 7.5-day-old dark-grown plants or plants grown 4.5 days in darkness and then illuminated for 16 or 36 h was >15 min (data not shown). The results also indicated that degradation of these transcripts during the 5-min labeling period used in the lysed plastid transcription assay would not be significantly different in the plastid populations analyzed. Therefore, changes in [^{32}P]UTP incorporation into rbcL, psbA, 16SrDNA or psaA-psaB transcripts cannot be accounted for by changes in rates of degradation of newly synthesized RNA.

Relative changes in transcription of plastid genes and plastid RNA levels in dark-grown and illuminated barley seedlings

Our next objective was to quantitate RNA levels and transcription of specific plastid genes during plastid development in dark-grown and illuminated barley seedlings. Plastids were isolated from 4.5–9-day-old dark-grown barley seedlings or seedlings grown 4.5 days in darkness and then illuminated for 16–108 h. RNA was extracted from a known number of the plastids from each time point. This RNA was analyzed by Northern slot blots for psbA, rbcL and psaA-psaB mRNA. Serial dilutions of plastid RNA were analyzed to determine the linear range for the Northern slot-blot assay. Duplicate slot blots were then done using plastid RNA from each time point. A typical result from this assay is shown in Figure 5 and the data from the series of RNA determinations is plotted in Figures 6–8. RNA levels are ex-

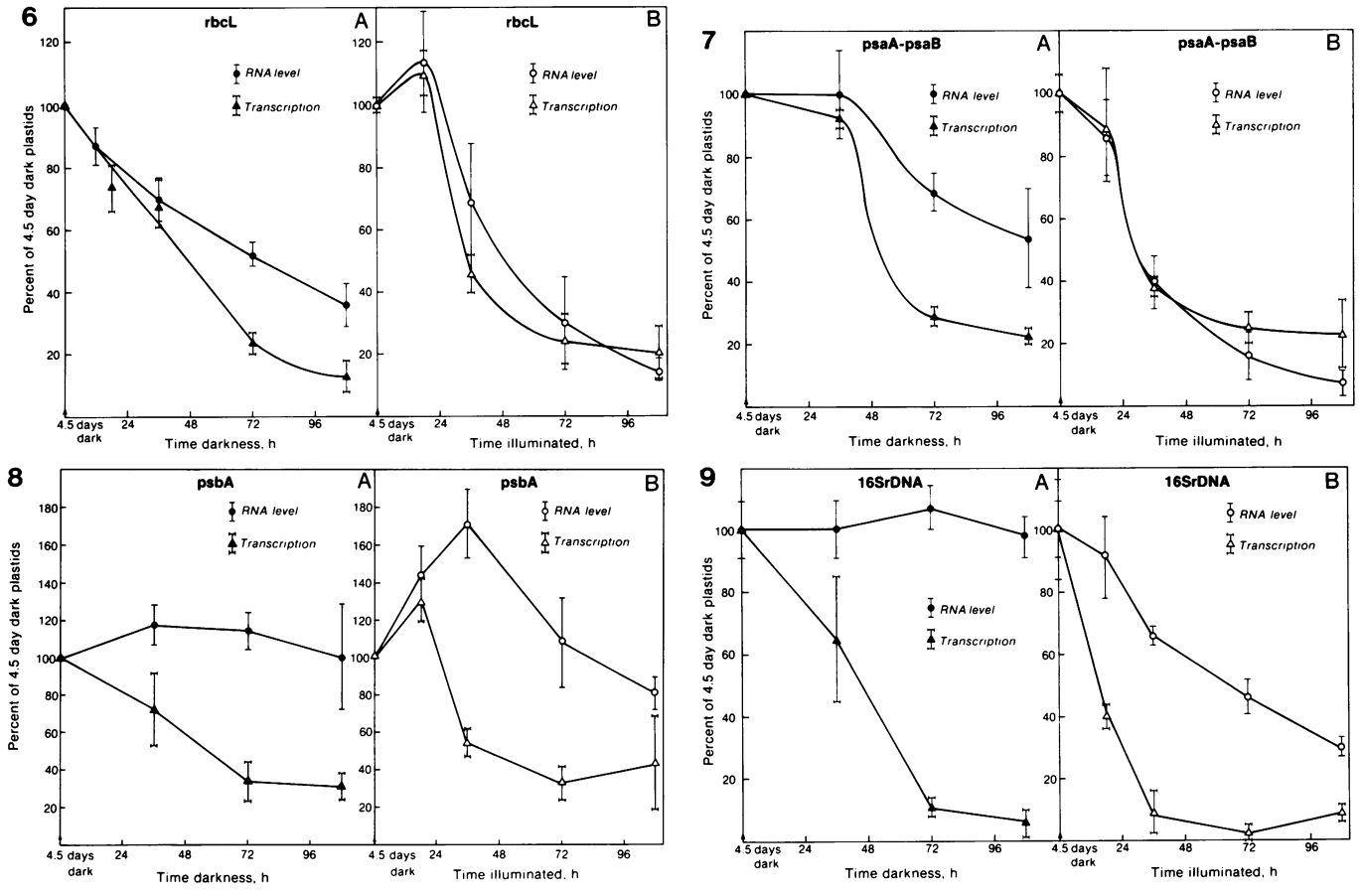

Fig. 6–9. Quantitation of RNA levels and transcription of plastid genes during plastid development in dark-grown and illuminated barley seedlings. RNA levels (○,●) and transcription (△,▲) of four plastid genes, rbcL (**Fig. 6**), psaA-psaB (**Fig. 7**), psaA (**Fig. 8**) and the 16SrDNA gene (**Fig. 9**) were determined. Plastids were isolated from 4.5–9-day-old dark-grown barley (**A**, closed symbols) or from seedlings which had been grown for 4.5 days in darkness and then illuminated for 16–108 h (**B**, open symbols). Error bars represent ± SE.

pressed as a percentage of the value determined for plastids of 4.5-day-old dark-grown barley seedlings.

Plastids isolated from each time point were also used in lysed plastid transcription assays. RNA synthesis was allowed to continue for 5 min in the presence of [^{32}P]UTP then RNA was extracted and transcription of psbA, rbcL, psaA-psaB and 16SrDNA was analyzed using Southern slot blots. The transcription data for psbA, rbcL,psaA-psaB and 16SrDNA for the time course is shown in Figures 6–9. Transcription was expressed as a percentage of the value determined for plastids isolated from 4.5-day-old dark-grown barley seedlings.

Discussion

Characterization of transcription in lysed plastids

Plastids isolated from 4.5-day-old dark-grown barley seedlings showed high rates of UTP incorporation when plastids were lysed in an optimized transcription reaction mix. UTP incorporation during the first 5 min of incubation (0.95–1.21 pmol UTP/2.5 × 10^6 plastids) was greater than rates of transcription previously reported for isolated plastids (Ellis and Hartley, 1982). The high rate of incorporation could be partly due to the fact that the chloroplast RNA polymerase is freely accessible to exogenous nucleotide triphosphates in lysed plastids in contrast to intact plastids (Heldt, 1969). This situation also obviates the need for illumination during transcription which is required in intact plastids for nucleotide triphosphate generation. Thus, the lysed plastid transcription assay can be used to measure transcription of plastid genes in the dark and in organelles isolated from non-photosynthetic tissue.

In previous studies, light-independent incorporation of UTP in plastid preparations was often due to bacterial contamination (reviewed in Ellis and Hartley, 1982). This potential problem was avoided in the present study by washing plant tissue extensively in an ice-cold solution of Tween 20, NaCl and Chlorox prior to plastid isolation (Klein and Mullet, 1986). The effectiveness of this procedure is shown by the fact that transcription in lysed plastid preparations is insensitive to rifampicin, an inhibitor of bacterial RNA polymerase. The hybridization of ^{32}P-labeled RNA transcribed in lysed plastids to chloroplast DNA provides additional evidence that the [^{32}P]UTP incorporation being measured is plastid dependent.

Transcription in lysed plastids was not inhibited by heparin nor stimulated by addition of exogeneous DNA containing a chloroplast promoter. These results argue that most of the transcriptionally active plastid RNA polymerase is bound to DNA in the lysed-plastid transcription assay. Therefore, it is likely that most of the [^{32}P]UTP incorporation in lysed plastids is due to pre-initiated RNA polymerase. However, it has been reported that heparin-insensitive initiation of transcription can occur in DNA–RNA polymerase complexes isolated from chloroplasts (Hallick *et al.*, 1976; Briat and Mache, 1980). The lack of a

detectable pool of 'free' RNA polymerase in isolated plastids could indicate that most of the chloroplast RNA polymerase is bound to DNA *in vivo* and that RNA polymerase interactions with DNA have been maintained during plastid isolation at low temperature. However, in the absence of a method to pulse-label plastids in intact plants, this assumption is difficult to validate.

Pulse–chase experiments showed that RNA synthesized in lysed plastids from dark-grown or illuminated plants had a half-life of >15 min. Furthermore, RNA half-lives were similar for all plastid populations examined. Based on this experiment, it is concluded that the amount of degradation of newly synthesized psbA, rbcL, psaA-psaB or 16SrDNA transcripts which occurs during a 5-min transcription assay is relatively small and is similar in all plastids examined. Therefore if we assume that the chain elongation rates for RNA are similar in these plastid populations then changes in [^{32}P]UTP incorporation can be attributed primarily to changes in transcription initiation.

Overall changes in transcription and transcript levels in plastids of dark-grown and illuminated barley seedlings

Previous studies have shown that barley leaf growth occurs primarily from a basal meristem (Robertson and Laetsch, 1974). There are fewer than 10 plastids/cell in the leaf base and these plastids are 1–2 μm in diameter. Development of mesophyll cells during barley leaf maturation occurs first in apical cells of the leaf and is accompanied by an increase in plastid number per cell (up to 65) and plastid enlargement to a final diameter of 6–8 μm. The rapid build-up of the plastid compartment which occurs during barley leaf cell development is correlated with high rates of plastid protein synthesis and high levels of RNA per plastid (Klein and Mullet, 1987). However, once plastid volume and plastid number per cell approach values found in mature leaves, the rate of plastid protein synthesis and plastid RNA levels decline. We have previously determined that RNA levels of several plastid genes are high in 4.5-day-old barley seedlings and that these levels decrease with increasing plant age (Klein and Mullet, 1987). In this paper, transcription was found to be highest in plastids of 4.5-day-old plants and thereafter declined. Transcription of specific plastid genes (psbA, psaA-psaB, rbcL and 16SrDNA) also decreased over the same period although the time course and extent of the decline varied depending on the gene examined (Figures 6–9). These results indicate that plastid transcription activity is high during the rapid build-up of plastids in leaf cells and declines as this phase of plastid development is completed.

Illumination of 4.5-day-old dark-grown barley caused transcription in plastids to decline rapidly compared with plastid transcription in dark-grown plants of the same age. Light-induced events such as chlorophyll biosynthesis which lead to plastid maturation may cause the rapid decline in transcription activity of plastids of illuminated plants. The decline in plastid transcription activity could be due to decreased levels of RNA polymerase. However, the time course of UTP incorporation in plastids of dark-grown and illuminated plants is remarkably different (Figure 1). This suggests that transcription initiation or termination may be different in plastids of illuminated plants compared with dark-grown plants.

Changes in transcription and transcript levels of specific plastid genes during plastid development

In most previous studies of this type, plastid RNA levels were evaluated as a function of total cellular RNA, total plastid RNA or plastid ribosomal RNA (Altman *et al.*, 1984; Link, 1984a; Rodermel and Bogorad, 1985; Herrmann *et al.*, 1985; Kreuz *et al.*, 1986). In this paper RNA levels and transcription of specific plastid genes were analyzed as a function of plastid number. This was done because RNA levels per plastid as well as plastid number, size and RNA content per cell vary considerably during leaf biogenesis (Robertson and Laetsch, 1974). Data expressed on a per plastid basis allows separate analysis of plastid events and cellular events. We also confined our studies to the top 3 cm of the primary leaf of 4.5–9-day-old barley seedlings. Plastid number per leaf cell in this tissue is not expected to vary significantly and is close to levels found in mature leaves (Robertson and Laetsch, 1974). Therefore the changes in RNA levels and transcription reported here occur in a defined plastid population during the transition from the phase of rapid plastid biogenesis to the establishment of mature chloroplasts. In the discussion below changes in 16SrDNA, rbcL, psaA-psaB and psbA RNA level and transcription during this transition will be considered.

Protein coding genes: RbcL, PsaA-PsaB and PsbA

The large subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) is encoded by the plastid rbcL gene. The large subunit of Rubisco accumulates in plastids of dark-grown and illuminated barley plants (Kleinkopf *et al.*, 1970; Klein and Mullet, 1986). Transcription of rbcL and rbcL mRNA levels declined in parallel in plastids of 4.5- to 6-day-old dark-grown barley plants (Figure 6A). However, in older dark-grown plants rbcL mRNA levels did not decline as rapidly as transcription of the rbcL gene perhaps due to increased stability of rbcL mRNA in older plants. In illuminated plants rbcL mRNA levels and transcription of rbcL showed a small increase during the first 16 h of illumination then a rapid decline with increasing time of illumination (Figure 6B). The changes in rbcL transcription paralleled changes in RNA levels during chloroplast maturation indicating that the decline in rbcL mRNA levels in illuminated plants was primarily due to decreased transcription.

PsaA-psaB are cotranscribed in barley plastids (Berends *et al.*, 1986) and code for two chlorophyll-binding proteins of photosystem I (Fish *et al.*, 1985). PsaA-psaB mRNA is found in plastids of dark-grown barley seedlings, although the mRNA is not translated until plants are illuminated (Klein and Mullet, 1986). PsaA-psaB mRNA levels gradually decline in plastids of dark-grown plants as a function of plant age although not as rapidly as transcription of the psaA-psaB gene (Figure 7A). This indicates that psaA-psaB mRNA levels in dark-grown plants are determined in part post-transcriptionally. This could be related to the fact that psaA-psaB mRNA is not translated in dark-grown plants and may be sequestered in a way which stabilizes the mRNA.

In illuminated plants, psaA-psaB mRNA levels and transcription decline in parallel (Figure 7B). The transcription of psaA-psaB decreases 10–20% during the first 16 h of illumination of 4.5-day-old dark-grown barley seedlings and then declines rapidly to a low level after 108 h of illumination. This shift-down in psaA-psaB transcription and mRNA level in illuminated plants is similar to the decline in psaA-psaB gene product translation during chloroplast maturation (Klein and Mullet, 1987). Therefore, in illuminated plants, psaA-psaB mRNA levels and ultimately the synthesis of the psaA-psaB gene products appear to be determined primarily by the rate of psaA-psaB transcription.

The third protein-coding plastid gene examined in this paper is psbA, a gene which encodes a quinone-binding protein of photosystem II (Vermaas *et al.*, 1983). PsbA mRNA is not translated in plastids of dark-grown barley seedlings but the psbA gene

product is the major translation product labeled when plastids from mature light-grown barley leaves are incubated with [^{35}S]methionine (Klein and Mullet, 1987). It has been reported that psbA mRNA levels increase relative to total leaf RNA, total plastid RNA or plastid ribosomal RNA when dark-grown plants are illuminated (Bedbrook *et al.*, 1978; Link, 1982; Rodermel and Bogorad, 1985; Herrmann *et al.*, 1985; Kreuz *et al.*, 1986). In the present study, psbA mRNA levels per plastid increased 1.7-fold during the first 36 h of illumination then declined to levels close to those found in plastids of 4.5-day-old dark-grown plants (Figure 8B). This time course is significantly different than previously reported for psbA mRNA in illuminated plants (Rodermel and Bogorad, 1985; Kreuz *et al.*, 1986). This is due to the fact that our data is expressed on a per plastid basis rather than relative to total leaf RNA or total chloroplast RNA. If psbA mRNA levels measured in this paper were expressed relative to plastid rRNA or total chloroplast RNA then psbA mRNA levels would show much larger apparent increases and a different time course due to the fact that plastid rRNA levels decrease 4-fold during illumination of barley plants for 108 h (Figure 9B).

The transcription of psbA increased during the first 16 h of illumination of 4.5-day-old dark-grown barley seedlings, then declined to a lower level after longer times of illumination (Figure 8b). Therefore, part of the increase in psbA mRNA in plastids of barley plants illuminated for 36 h is probably due to increased transcription. However, psbA transcription decreased between 16 and 36 h of plant illumination, whereas psbA mRNA levels increased during this same period of time. This result shows that under some circumstances, changes in psbA mRNA levels are not due to altered transcription. In dark-grown plants, psbA mRNA levels were approximately the same in plastids of 4.5- and 9-day-old seedlings, although transcription of psbA declined with increasing plant age (Figure 8A). This result indicates that in dark-grown plants, psbA mRNA levels are determined in part post-transcriptionally.

In summary, the three protein-coding genes examined all show high levels of transcription during the rapid phase of plastid biogenesis followed by decreased transcription with increasing leaf age. The basis of these changes in plastid transcription activity could be due to changes in RNA polymerase level and/or plastid DNA copy number. Our present research is directed toward obtaining estimates of DNA copy number and RNA polymerase levels in plastids during barley leaf development in order to address this question. Although overall trends were similar, careful analysis of the transcription of psbA, rbcL and psaA-psaB during leaf development revealed that changes in the transcription of these genes is not always coordinate. For example, transcription of psbA increased transiently when 4.5-day-old dark-grown plants are illuminated whereas transcription of psaA-psaB declined. This may be due to a general change in RNA polymerase−template interaction (or ratio) which leads to preferential transcription of genes with strong promoters. Alternatively specific factors could be involved in activating transcription of genes such as psbA.

Transcription and RNA levels of 16SrDNA

16SrRNA levels do not change significantly in plastids of 4.5−9-day-old dark-grown plastids. In contrast, the transcription of the 16SrDNA gene declined >10-fold in the same plastid populations (Figure 9A). This result suggests that the level of 16SrRNA is determined post-transcriptionally in dark-grown plants. Ribosomal RNA could be stabilized when associated with ribosomal proteins in the 70S chloroplast ribosome. If this is the case, 16SrRNA levels would not change in dark-grown plants until ribosome stability and the number of ribosomes per plastid is altered. This hypothesis suggests that rRNA which does not associate with ribosomal proteins would be less stable than rRNA in ribosomes. This situation has been observed in *Escherichia coli* at low growth rates (Gausing, 1977). In illuminated plants 16SrRNA levels declined 3- to 4-fold during a 108-h illumination period. A similar decrease in plastid ribosome content was previously reported during plastid development in light-grown peas (Fish and Jagendorf, 1982). The decrease in 16SrRNA level in illuminated tissue could have been due in part to decreased transcription of the 16SrDNA gene. However, transcription of the 16SrDNA gene declined much more rapidly than 16SrRNA levels (Figure 9B). Therefore, 16SrRNA levels may be determined in part by additional processes which alter the stability of chloroplast ribosomes or which directly degrades 16SrRNA.

Our analysis also revealed an interesting difference between the influence of light on the transcription of 16SrDNA and psbA or rbcL. Illumination of 4.5-day-old dark-grown barley plants for 16 h caused a 60% decrease in 16SrDNA transcription (Figure 9B). In contrast, the transcription of rbcL and psbA changed only a small extent during the same illumination period. This result suggests that different mechanisms control the rates of initiation of transcription of 16SrDNA and the psbA and rbcL genes. It has been proposed that chloroplasts have two RNA polymerases, one involved in transcribing the rDNA transcription unit and another for tRNA and protein-coding genes (Greenberg *et al.*, 1984). This proposal is based on the ability to isolate a DNA−RNA polymerase complex which transcribes primarily rDNA (Briat *et al.*, 1979; Rushlow *et al.*, 1980). The protein composition of the polymerase which transcribes rDNA is different from RNA polymerases isolated from high-salt-treated plastids and the transcription properties of the soluble and rDNA polymerase differ (Narita *et al.*, 1985). However, in other studies the differences between the two polymerase activities are less distinct (Reiss and Link, 1985). It could be that the difference in transcription of the 16SrDNA and the psbA and rbcL genes is due to independent regulation of two chloroplast RNA polymerases. Alternatively, as in *E. coli*, a single RNA polymerase may transcribe both types of genes and a small molecule, such as ppGpp, may bind to RNA polymerase to alter its interaction with certain promoters (Gallant, 1979).

Materials and methods

Plant growth

Barley (*Hordeum vulgare* L.var Morex) seedlings were grown and maintained for all experiments in controlled environment chambers at 23°C. Seed were planted in vermiculite and watered with full-strength Hoaglands nutrient solution. For developmental studies, seeds were germinated and grown for 4.5 days in a dark chamber located in a light-tight room. After 4.5 days, seedlings were either transferred to an illuminated chamber with a light intensity of 10 000 lux (fluorescent plus incandescent bulbs) or were maintained in the dark for an additional 16, 36, 72 or 108 h. All manipulations of dark-grown plants were performed when possible in complete darkness. However, when required, light was provided by a dim green safelight (Klein and Mullet, 1986).

Plastid isolation

Intact plastids were isolated on percoll gradients as described previously (Klein and Mullet, 1986). Plastid preparations yielded 90−95% intact plastids determined by phase-contrast microscopy. All manipulations were performed in a light-tight cold room (2−4°C) in the presence of a dim safelight with wavelength emission of 515−560 nm.

Transcription in lysed plastids

Plastids, isolated from percoll gradients, were resuspended at ~2 × 10^9 plastids/ml in 0.33 M sorbitol, 50 mM Hepes[KOH], pH 8.0 at 4°C. Plastid

number (plastid number per μl suspension volume) was determined by counting in a hemocytometer. Microfuge tubes containing the transcription reaction mix were warmed to 23°C and transcription was initiated by adding 2.5 × 10^6 plastids (1–2.5 μl of the plastid suspension) to the reaction mix (final volume 25 μl). The final reaction mix was 50 mM Hepes[KOH], pH 8.0, 10 mM $MgCl_2$, 25 mM potassium acetate, 10 mM dithiothreitol, 125 μM CTP, GTP, ATP, 10 μM unlabeled UTP plus varying amounts of [α-^{32}P]UTP (i.e. 10 μCi to 100 μCi per reaction, sp. act. 800 Ci/mmol). For standard reactions, plastid transcription was carried out for 5 min in the dark (plastids from dark-grown seedlings) or in light (plastids from illuminated seedlings). Following the labeling period, reactions were terminated by (i) addition of RNA-extraction buffer and phenol (Orozco *et al.*, 1985), (ii) by spotting aliquots on DE-81 paper (Hallick *et al.*, 1976) or (iii) by placing samples on ice. For pulse–chase assays, plastids were labeled for 5 min, then unlabeled UTP was added to a final concentrations 1 mM. Incorporation of [^{32}P]UTP into RNA was quantitated by spotting aliquots on DE-81 paper, washing extensively in 5% Na_2HPO_4 followed by water, ethanol and ether (Hallick *et al.*, 1976). Filters were air dried and counted in a scintillation counter. In one experiment plasmid pPPBX-10218 containing the plastid psbA promoter was used. The plasmid is pUC18 with a 709-bp *Xba*I–*Pst*I DNA insert from pea chloroplast DNA (Boyer and Mullet, 1986).

Isolation and quantitation of plastid RNA

Chloroplast RNA was isolated by phenol extraction as previously described (Klein and Mullet, 1986). RNA samples were treated with RQ1-DNase I (Promega Biotech), phenol extracted and precipitated twice. Quantitation of *in vivo* levels of plastid RNA was done by blotting RNA extracted from 0.25 × 10^6 plastids onto a nylon membrane (Gene Screen, NEN) using a slot-blot apparatus (Biorad). Blots were dried and baked for 2 h at 80°C prior to hybridization. Blots were prehybridized for 12–24 h at 37°C [50% formamide, 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS, 2 × Denhardt's, 100 μg/ml salmon sperm DNA[and hybridized for 48–72 h at 37°C in the same conditions (Berends *et al.*, 1986). After hybridization, the blots were washed 60 min at 23°C in 2 × SSC then 60 min at 50–55°C in 0.1 × SSC. Blots were analyzed by autoradiography or by excising radioactive spots and counting in a scintillation counter. Methods used for DNA labeling were as described previously (Klein and Mullet, 1986). The DNA probe for rbcL mRNA was a 1.3-kbp *Pst*I–*Hin*dIII internal fragment from barley rbcL (Zurawski *et al.*, 1984). The DNA probe for psaA-psaB mRNA was either a 1.4-kbp *Bam*HI–*Bam*HI DNA fragment from spinach which contains a portion of the open reading frames of psaA and psaB (Berends *et al.*, 1986) or a 1.8-kbp *Bam*HI–*Bam*HI probe from the psaA-psaB transcription unit of barley (T.Berends, P.E.Gamble and J.E.Mullet, in preparation). The DNA probe for psbA mRNA was a 0.55-kbp *Eco*RI–*Pst*I DNA fragment which is from within the pea psbA open reading frame (Boyer and Mullet, 1986). The 16S rRNA probe for transcription assays was a 1.2-kbp *Hin*dII–*Hin*dII DNA fragment from within the maize 16SrDNA gene (Schwarz and Kössel, 1980). 16SrRNA levels in plastids of different developmental stages were examined by separating total plastid RNA on agarose gels followed by staining with ethidium bromide. In all plastid populations examined a very large percentage of plastid RNA was ribosomal RNA (Klein and Mullet, 1987). Therefore, changes in 16SrRNA were estimated by monitoring changes in plastid RNA at A_{260}.

Quantitation of [^{32}P]RNA synthesized in lysed plastids

Southern blots were used to quantitate ^{32}P-labeled RNA synthesized by lysed plastids. Plasmid DNA containing inserts from specific chloroplast genes (see RNA probes described above) was restricted, separated on agarose gels, blotted to Gene Screen Plus (NEN) and baked for 2 h at 80°C. When DNA was directly blotted, 0.25–0.5 pmol of plasmid DNA was restricted, boiled for 10 min, cooled on ice and applied to individual slots by vacuum filtration. The blots were than placed on paper towels saturated with 0.4 M NaOH for 10 min, followed by 2 × SSC for 10 min, dried and baked for 2 h at 80°C. Prehybridization and hybridization of ^{32}P-labeled RNA was performed as described above for Northern blots.

Acknowledgements

We would like to thank Dr Emil Orozco for sending a clone containing the 16SrDNA from maize from which a subclone was prepared and Melanie Martin for technical assistance. This work was supported by grant no. pCM-8316431 from the National Science Foundation to J.E.M.

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Received on February 25, 1987