Quantitative Analysis of Transcription and RNA Levels of 15 Barley Chloroplast Genes

TRANSCRIPTION RATES AND mRNA LEVELS VARY OVER 300-FOLD; PREDICTED mRNA STABILITIES VARY 30-FOLD*

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Higher plant plastid genomes encode rRNAs, tRNAs, and protein subunits of the RNA polymerase, ribosomes, and the photosynthetic apparatus which vary over 1000-fold in abundance. Quantitative analysis of transcription and RNA levels was carried out on 15 plastid genes which are located in 14 different transcription units covering 50% of the barley plastid genome. Transcription of 16S rRNA, trnF-tnrnG, and trnK was high relative to most other plastid genes. Transcription of trnFM-trnG was 5 times greater than trnK indicating that differences in tRNA levels in plastids could be due, in part, to differences in transcription. Among the protein coding genes, mRNA levels varied over 900-fold and transcription over 300-fold. The gene showing the lowest transcription rate and mRNA level, rpoB, is located in a gene cluster which encodes subunits of the plastid RNA polymerase (rpoB-rpoC1-rpoC2). RpoA, which encodes the α subunit of the RNA polymerase, was located in a gene cluster encoding ribosomal proteins (rpl23, rps19, rpl16) and infA. RNA from this gene cluster is 30-fold more abundant than rpoB mRNA, suggesting that expression of rpoA is regulated at the level of translation or protein stability. Polycistronic operons encoding subunits of the photosynthetic apparatus (psbB-psbH-petB-petD; psbK-psbI-psbD-psbC; atpB-atpE; psaA-psaB) had higher transcription rates and correspondingly higher mRNA levels than genes which encode ribosomal proteins (rp123, rps19, rp116) and infA. RNA from this gene cluster is 30-fold more abundant than rpoB mRNA, suggesting that expression of rpoA is regulated at the level of translation or protein stability. Polycistronic operons encoding subunits of the photosynthetic apparatus (psbB-psbH-petB-petD; psbK-psbI-psbD-psbC; atpB-atpE; psaA-psaB) had higher transcription rates and correspondingly higher mRNA levels than genes which encode ribosomal proteins or RNA polymerase subunits. RbcL and psbA, which are located in separate transcription units, exhibited the highest transcription rates and mRNA levels. Correspondence between transcription rate, mRNA level, and protein abundance indicates that transcription is a primary determinant of barley plastid gene expression. In addition, a 30-fold variation in predicted mRNA stability was observed which further increases the dynamic range of plastid mRNA abundance.

Plastids of most higher plants contain multiple copies of a circular DNA molecule which ranges in size from 120 to 217 kbp, depending on plant species (for review, see Palmer (1990)). Variation in plastid genome size is due primarily to differences in the size of an inverted DNA repeat and small differences in gene content (Palmer, 1990; Baldauf and Palmer, 1990; Gantt et al., 1991; Shinozaki et al., 1986; Hiratsuka et al., 1989). Plastid DNA contains over 120 genes which encode tRNAs, rRNAs, and proteins. Plastid rRNAs (16S, 23S, 4.5S, 5S) are co-transcribed from an operon located in an inverted DNA repeat in those genomes having this DNA structure (Strittmatter and Kössel, 1984; Keus et al., 1984). Genes encoding tRNAs are distributed throughout the genome. Some tRNA genes are transcribed individually, others are located within; tRNA gene clusters, two are located within the trnM transcription unit, and some are located within or at the end of larger transcription units which contain other genes. The plastid-encoded proteins include subunits of an RNA polymerase (encoded by rpoA, B,C2), plastid ribosomes (12 rps, 9 rpl genes in rice), a putative NADH oxido-reductase complex (ndhA, B,C, D,E, F,G), and proteins involved in photosynthesis. This latter group of genes encodes the large subunit of Rbu-P2 carboxylase (rbcL), subunits of photosystem I (PSI) (psaA, B,C), photosystem II (PSII) (psbA, B,C, D,E, F, G, H, I, K, L), the ATP synthase (atpA, B,E, F, H, I), and the cytochrome b-f complex (petA, B,D) (for review, see Umesono and Ozeki (1987) and Ohyama et al. (1988)). Plastid protein complexes accumulate to dramatically different levels. Chloroplasts contain approximately 107 Rbu-P2 carboxylase complexes (Dean and Leech, 1982), 106 PSI, PSII, ATP synthase, and cytochrome b-f complexes (Mullet et al., 1990), 105 to 106 ribosomes (Klein and Mullet, 1987), and less than 106 RNA polymerase complexes per organelle. Each of these complexes contains proteins which are nuclear-encoded. Therefore, plastid and nuclear genes encoding subunits of a common protein complex need to be expressed at similar levels, while genes encoding subunits of different complexes are often needed at very different levels.

Many plastid genes are organized into polycistronic transcription units which are conserved between different plant species (Palmer, 1985). For example, the trnF transcription unit encodes 16S, 23S, 4.5S, and 5S rRNA plus trnL and trnRNA (Strittmatter and Kössel, 1984; Keus et al., 1984). Other conserved gene clusters include rpl23-rpl2-rps19-rpl22-rps3-rpl16-rpl14-rps8-infA-rps6-rps11-rpoA, rpoB-rpoC1-rpoC2, psbB-psbH-petB-petD, atpA-atpB-atpF-atpA, psbD-psbC, psaA-trnH, atpB-atpE, and trnFM-trnG (Palmer, 1990; Umesono and Ozeki, 1987; Ohyama et al., 1988; Hiratsuka et al., 1989). The abbreviations used are: kbp, kilobase pair; Rbu-P2 carboxylase; ribulose-P2 carboxylase; PS, photosystem; PCR, polymerase chain reaction; kb, kilobase; MOPS, 4-morpholineethanesulfonic acid.

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1989; Shinozaki et al., 1986). Some of the plastid transcription units contain genes encoding proteins exclusively for a single protein complex such as the plastid RNA polymerase (rpoB-rpoC1-rpoC2), or genes involved in transcription and translation but not photosynthesis (rrn transcription unit, rpl23—rpoA gene cluster). Others exclusively encode proteins for photosynthetic electron transport complexes (atpB-atpE; psbB-psbH-petB-petD). The organization of genes encoding proteins related with functions into transcription units may facilitate the coordinated production of subunits of a single protein complex and/or allow groups of related genes to be co-regulated. However, plastid genes for different functions are not entirely segregated. Transcription units which encode proteins for photosynthesis sometimes terminate with genes encoding tRNAs which may facilitate transcription termination or stabilize primary transcripts (psbK-psbI-psbD-psbC-orf62; aL, 1990b; Bennett et al., 1990) and extensive RNA processing of primary transcripts is often observed (Westhoff and Herrmann, 1988; Tanaka et al., 1987). Therefore, the expression of genes within gene clusters can be selectively modulated through the use of different promoters or at the levels of RNA stability and translation.

Previous studies have shown qualitatively that the transcription activity and RNA levels of different plastid genes varies considerably (i.e. Deng and Gruissem, 1987; Mullet and Klein, 1987). However, quantitative data on these parameters has been collected only for psbA and rbcL (Klein and Mullet, 1990). Both of these genes are highly expressed in plastids and encode proteins involved in photosynthesis. In this paper, we have quantitated the transcription activity and RNA levels of 15 plastid genes at one stage of chloroplast development in barley. This analysis revealed over 500-fold variation in transcription activity and more than 900-fold variation in RNA level for different plastid genes. In general, variation in transcription activity was paralleled by transcript abundance in all cases except that at this developmental stage transcription activity is a primary determinant of RNA level for most of the genes examined. In addition, among the RNAAs analyzed, a 1.5-fold variation in predicted mRNA stability was observed.

**MATERIALS AND METHODS**

**Plant Growth, Plastid, and RNA Isolation**—Barley seedlings were grown in controlled environmental chambers as described by Baumgartner et al. (1989). Plants were isolated from apical 3 cm sections of primary leaves of 4-day-old dark-grown seedlings as previously described (Baumgartner et al., 1989). RNA was extracted from isolated plastids as described in Orozco et al. (1989). To ensure maximum recovery of plastid RNA, no more than 2 x 10^9 plastids were used per 400 µl of extraction buffer.

**Polymerase Chain Reaction Procedures**—DNA fragments containing portions of barley chloroplast genes were prepared by the polymerase chain reaction procedure. For known barley genes, oligonucleotide primer sequences were derived from published barley plastid DNA sequence data ( Sexton et al., 1990b; Boyer and Mullet, 1988; Zurawski et al., 1984; Reverdatto et al., 1989; Oliver and Poulsen, 1984; Neumann, 1988). Genes for which the sequence from barley was unknown, 5'-3' orientation of a rice, maize, and tobacco chloroplast DNA were aligned, and regions of 100% homology (12-18 nucleotides) were chosen for use as PCR primers (Shinozaki et al., 1986; Hiratsuka et al., 1989). Additional nucleotides which encoded restriction endonuclease cleavage sites were added to the ends of the primers to facilitate cloning of the PCR amplified fragments. Thirty PCR cycles were used for amplification with a 1-min denaturation at 94 °C, 2 min annealing at 48 °C, and polymerization for 45 s at 72 °C.

**Cloning of Chloroplast DNA Gene Fragments**—DNA Sequence Analysis, Gene Organization—DNA restriction fragments containing portions of barley chloroplast genes, or PCR-generated fragments digested with the appropriate restriction endonucleases were cloned into pBluescript vectors (Samboorok et al., 1989). DNA sequence analysis was carried out using the dideoxy chain-termination procedure with the Sequenase Kit (U. S. Biochemicals, Cleveland) and using progressions from Intelligenetics. RpoA, rpoB, rpoC1, rpoC2, ndhA, rpl16, infA, rpl16, and rpl23 were localized by Southern blotting and PCR amplification reactions. Probes for rpoA, rpoB, rpl16, and ndhA were generated by PCR amplification (Table I). Southern blotting and PCR amplification reactions were carried out as described by Mullet and Klein (1987), with Southern blotting and PCR amplification reactions located rpoB, rpoC1, and rpoC2 on Ps1 fragment Hw204 (2.4 kb), ndhA on Hw192 (a 4.2-kbp Ps1 fragment), and rpl16, infA, and rpoA on Hw47 (a 7.8-kbp HindIII fragment) (Oliver, 1984; Poulsen, 1983). PCR amplification reactions showed that rpoB primer 5'-GGTATTGGACCGCA-3' was 1.76 kb from rpoC1 primer 5'-TCATAGATGGTCTT-3' and the inverse complementary rpoC1 primer 5'-TGAACAGACATT-3'. In a similar way, PCR amplification produced a 2.1-kbp product between rpl23 primer 5'-TTCACCGGGTTAT-3' and rpl19 primer 5'-TAAACACAGCA- GAT-3'; a 2.5-kbp PCR product between the inverse complement of the rpoB primer and rpl16 primer 5'-4ATTTCTCCTCTCTATG-3', a 2.8-kbp between the inverse complement of the rpl16 primer and rpoA primer 5'-TGAAGGGTCGTCG-3'.

**Analysis of Plastid RNA Transcript Abundance**—RNA extracted from isolated plastids was fractionated on formaldehyde-agarose gels as previously described (Klein and Mullet, 1989). After electrophoresis, RNA was transferred to GeneScreen Plus nylon filters in 0.4 M NaOH via vacuum (Bio-Rad) or capillary transfer. RNA gel blots were hybridized with in vitro generated antisense RNA probes. RNA probes radiolabeled with [α-32P]UTP were synthesized from recombinant pBluescript expression vectors with T7 or T3 RNA polymerase (Stratagene Co.). Frehybridization, hybridization, and wash conditions were as previously described for RNA probes (Sexton et al., 1990a).

Linearized CaCl2-purified plasmid DNA (10-20 µg/100 µl reaction) was used for in vitro transcription reactions (Strategene Co.). After 2-h reactions, samples were treated with 5 units of RNase I (Promega) for 40 min at 37 °C. RNA was extracted with phenol:chloroform:isoamyl alcohol (1:1:0.4), the aqueous phase was adjusted to 0.3 M sodium acetate and 2.5 volumes of ethanol was added to precipitate the RNA. RNA was resuspended by centrifuging 15 min in a microcentrifuge, washed with 70% ethanol, and dried 2 min in a speed vac. The dried RNA pellet was dissolved in 100 µl of TE (made with diethylpyrocarbonate-treated water) and 300 µl of a 6.33 M CaCl2 solution was added. To obtain purified in vitro RNA, the samples were transferred to Beckman LA100.2 polycarbonate centrifuge tubes and centrifuged at 100,000 rpm, 15 °C for 4 h in a Beckman TL-100 topset ultracentrifuge. After centrifugation the supernatant was discarded, the RNA was washed with 70% ethanol, and dried in a speed vac. The RNA was resuspended in 50-100 µl of TE and residual CaCl2 removed using a Sephadex G-50-80 spin column. RNA was separated on formaldehyde-agarose gels, stained with ethidium bromide, and RNA integrity visualized using a UV transilluminator. The concentration of RNA was determined using the absorbance at 260 nm (1 absorbance unit equal to 40 µg of RNA m-1).

RNA isolated from a known number of plastids was dissolved in RNA loading buffer (0.04 M MOPS, pH 7.0, 50% formamide, 6% formaldehyde, 0.001 M EDTA, 0.01 M Na acetate), heated 10 min at 65 °C, and applied in duplicate to a GeneScreen Plus membrane with a Schleicher and Schuell Minifold 1 (Deltin) dot blot apparatus. Known amounts of in vitro synthesized RNA standards were applied to the same membrane. Membranes were baked for 2-4 h at 80 °C. The blots were then cut into strips, each containing plastid RNA and a different RNA standard dilution series. Individual strips were hybridized to RNA probes complementary to the RNA standard, hybridization and wash conditions were the same as for Northern analysis. The amount of radioabeled probe hybridized to plastid RNA and the standards was quantitated with a Betagen Betascope blot analyzer or by scintillation counting of excised dots.

**Plastid Run-on Transcription Assays**—Plastid run-on transcription assays were carried out as described by Mullet and Klein (1987), with some modifications. ATP, GTP, and CTP were used at 0.5 µM, 2 x 10^9 plastids were used in 200-µl reactions, and 250 µCi of [α-32P]UTP per reaction in 5-min assays. Radiolabeled transcripts from 5-min plastid run-on reactions were extracted and hybridized to dot blots containing 1 µg of in vitro synthesized antisense RNA transcripts for each of the 14 genes being analyzed. Antisense RNAs were applied in duplicate for 16 S RNA, rbcL, psaA, trnK, trnM-
Barley Plastid Genes Selected for Analysis—Fifteen barley plastid genes were selected for analysis of transcription and RNA level. The group of genes to be analyzed encode representative rRNA, tRNA, and proteins found in the plastid RNA polymerase, ribosomes, the putative ndh complex and each of 5 protein complexes involved in photosynthesis (Rub-P, carboxylase, PSI, PSII, ATP synthase, cytochrome b-f). Eleven of the genes to be studied had previously been located on the barley plastid genome (psbA, trnK, rps16, psbD, trnM, trnG, psaA, atpB, rbcL, petB, 16S rRNA). Probes for the previously characterized barley plastid genes were obtained from existing subclones by amplifying selected regions of the genes using PCR or subcloning restriction fragments (Table I). All gene-specific probes were cloned into Bluescript vectors and verified by DNA sequencing. Four genes selected for study had not been previously located on the barley plastid genome (rpoA, rpoB, ndhA, rpl16) although they are present in the plastid genomes of other higher plants (i.e. Shinozaki et al., 1986; Hirotaka et al., 1989). Sequences of these genes from rice, tobacco, and maize were aligned and 12-18-base pairs with 100% identity were used to design PCR primers for amplification of barley plastid DNA (Table I). The resulting amplified DNA was cloned and a minimum of 150 base pairs of each insert was sequenced. The sequences of the inserts showed greater than 90% sequence homology with the corresponding genes in rice (data not shown). In order to map the four genes, insert DNA was radiolabeled and used to probe an ordered bank of PsI DNA fragments which spans the barley plastid genome (Poulsen, 1983). In some cases, PCR primers were tested for their ability to amplify DNA from clones containing specific PsI subclones of the barley genome. The organization of rpoA, rpoB, and rpl16 relative to several other genes in the plastid genome was next determined by a series of genome walking experiments (using PCR amplification of overlapping regions, see "Materials and Methods"). In other higher plant plastid genomes, rpoB is located in the gene cluster rpoB-rpoC1-rpoC2. Likewise, rpl16 and rpoA are part of a large conserved gene cluster found in other plastid genomes (rpl23-rpl2-rps19-rpl22-rps4-rpl16-rpl14-psaG-psaJ-rp126-rps11-rpoA) (Shinozaki et al., 1986; Hirotaka et al., 1989). To test if these genes were also clustered in barley, PCR primers were used to probe homologous regions in rpoB, rpoC1, and rpoC2 and rpl16, rpoA, rps23, and rpl19. In barley, these regions were found to hybridize to antisense RNA dot blots as described above. Following hybridization and washing (including two additional 23°C, 5-min washes with 2X SSC, 0.5% SDS. Filters were air-dried and rehybridized to antisense RNA fixed on nylon filters. Following hybridization and washing (including two additional 23°C, 5-min washes with 2X SSC, 0.5% SDS. Filters were air-dried and rehybridized to antisense RNA fixed on nylon filters. Estimation of Hybridization Efficiency—Percent hybridization of DNAs to run-on transcription assays to antisense RNA fixed on nylon filters was estimated as follows. A known amount of radiolabeled in vitro synthesized sense RNA was hybridized, in the presence of unlabeled plastid RNA from 2×106 plasids, to dot blots containing in vitro synthesized antisense RNA transcripts (1 pmol/dot, 2 or 4 dots/gene). The same hybridization conditions were employed (equal time and volume) as those used for the hybridization of run-on transcription assays. After washing, radioactivity hybridized to antisense RNA was determined by cutting out the dots and counting in a scintillation spectrometer.

| Gene    | Clone | Insert size | PCR primer coordinates | Source of clone or sequence |
|---------|-------|-------------|------------------------|---------------------------|
| 16S rRNA| pBSS24| 0.86        | X15901                 |                           |
| trnM-trnG|pBHP15| 0.9        | X15901                 |                           |
| trnK-ORF| pBHP9 | 2.3        | X15901                 |                           |
| rbcL    | pBH13 | 1.3        | X15901                 |                           |
| psbA    | pBH13 | 1.4        | X15901                 |                           |
| psbD    | pBE58 | 0.87       | X15901                 |                           |
| psaA    | pTV1  | 1.2        | X15901                 |                           |
| atpB    | pBHP12| 1.5        | X15901                 |                           |
| petB    | pBHP14| 0.68       | X15901                 |                           |
| ndhA    | pBHP20| 0.47       | X15901                 |                           |
| rps16   | pBHP3 | 1.0        | X15901                 |                           |
| rpl16   | pBHP3 | 0.35       | X15901                 |                           |
| rpoA    | pBHP10| 1.0        | X15901                 |                           |
| rpoB    | pBHP10| 2.3        | X15901                 |                           |
different lengths of time so that RNAs from all the genes could be detected.

The 16S rRNA probe detected a predominant 1.6-kb RNA which corresponds to mature 16S rRNA (Fig. 1, lane 1). In contrast, the probe for rnrM-trnrG hybridized to RNAs of 1.0 kb and less than 0.1 kb in size (Fig. 1, lane 2) which correspond to an unspliced precursor and tRNA size RNAs as previously described (Oliver and Poulsen, 1984). The trnK gene contains an intron which encodes an open reading frame of 504 amino acids (Sexton et al., 1990b; Boyer and Mullet, 1988). The trnK probe detects the unspliced precursor (2.6 kb) RNA but not spliced tRNA(Lys) (Fig. 1, lane 3). Six genes encoding proteins involved in photosynthesis were analyzed. The probe for rbcL, the gene encoding the large subunit of RbcP2 carboxylase, hybridized to a 1.6-kb primary transcript of minor abundance and a 1.4-kb RNA (Fig. 1, lane 4) (Mullet et al., 1985; Poulsen, 1984). PsbA encodes protein D1, a reaction center subunit of photosystem II. Northern analysis showed that the psbA RNA hybridized to a 1.2-kb RNA as previously described (Boyer and Mullet, 1988). PsbD, like psbA, encodes a photosystem II reaction center protein (D2). This gene is located in a complex operon which includes psbK-psbL-psbD-psbC-orf62-trnG (Berends et al., 1990). The Northern blot obtained with the psbD probe reflects this complexity (Fig. 1, lane 6). PsbA encodes a subunit of the photosystem I reaction center. This gene is co-transcribed with psbB in barley to yield a 5.3-kb RNA (Fig. 1, lane 7) (Berends et al., 1987).

Genes encoding subunits of the ATP synthase are located in 2 gene clusters (atpB-atpE; atp1-atpH-atpF-atpA). The atpB-atpE gene cluster has been shown to be co-transcribed in several plants and in barley, the RNA hybridizing to the atpB probe (2.2 kb) is sufficiently large to include atpE as well (Fig. 1, lane 8). Two of the 3 plastid genes encoding subunits of the cytochrome b–f complex are located in the psbB-psbH-petB-petD gene cluster. In other higher plants these genes are co-transcribed and complex RNA processing events yield a heterogeneous population of RNA (Westhoff and Herrmann, 1988; Tanaka et al., 1987). Not surprisingly, the petB RNA hybridized to numerous RNAs ranging in size from 4.8 to 1.0 kb (Fig. 1, lane 9). Seven genes encoding subunits homologous to the proteins of the mitochondrial NADH oxidoreductase are located in tobacco and rice plastid genomes (Shinozaki et al., 1986; Hiratsuka et al., 1989), although the function of this complex in higher plant plastids is at present unknown. One representative of this group of genes (ndhA), which contains an intron in rice (Matsubayashi et al., 1987), was chosen for analysis. Northern analysis using the ndhA probe revealed RNAs of 4.0, 2.2, and 1.3 kb (Fig. 1, lane 10). Two genes encoding ribosomal proteins were analyzed (rps16, rpl16). Rps16 is located upstream from trnK, is independently transcribed, and contains an intron (Sexton et al., 1990b). Northern blots using the rps16 probe detected unspliced precursor RNAs of approximately 1.2 kb and spliced RNA of 0.6 kb (Fig. 1, lane 11). Rpl16 in contrast, is located in a conserved gene cluster with several other ribosomal protein encoding genes, infA, and rpoA (Fig. 1, lanes 12 and 13). The rpoB probe hybridized to RNAs ranging from 0.8 to 3.0 kb and the rpoA probe hybridized to RNAs ranging in size from 1.2 to 5.1 kb. Finally, RNAs hybridizing to rplB are shown in Fig. 1, lane 14. The rpoB RNA is 6.0 kb suggesting that in barley, rpoB may be co-transcribed with rpoC1. In spinach, co-transcription of rpoB, rpoC1, and rpoC2 was detected (Hudson et al., 1988).

The 15 genes selected for study are located in 14 different transcription units. With the exception of the 16S rRNA gene, all are single copy genes. While the 15 genes represent only about 10% of the total number of plastid genes, we estimate that the transcription units encoding these genes represent approximately 70 kbp of transcribed DNA or about 50% of the barley plastid genome.

Quantitation of Plastid RNA Levels—The abundance of the RNAs corresponding to each selected gene was determined using Northern dot blots prepared under the same conditions used for the Northern blots in Fig. 1. In preliminary experiments, RNA from 10⁷ to 10⁸ plastids was applied to nylon membranes and blots were probed with radiolabeled antisense RNA from each of the genes. The blots were analyzed to determine the plastid RNA concentration where hybridization signals were proportional to input RNA. Control experiments were carried out with known amounts of sense RNA synthesized in vitro from each of the selected genes to determine the concentration range needed for the standard curves (data not shown). Linear regression analysis showed that hybridization of the probes to the RNA standards increased linearly with increasing input of RNA with correlation coefficients greater than 0.98. Next, plastid RNA and a set of RNA standards for one gene was applied to a blot. The blot was hybridized with the corresponding gene-specific RNA probe and the resulting hybridization signals quantitated so that the level of hybridizing RNA could be calculated. The data from this analysis is shown in Table II.

Quantitation of Plastid Gene Transcription—Variation in plastid RNA levels for different genes could be due to differences in transcription or RNA stability. Therefore, the transcription activity of the 15 genes under study was assayed using a quantitative run-on transcription assay previously developed for barley (Mullet and Klein, 1987; Klein and Mullet, 1990). To carry out the assay, plastids were isolated from chilled leaves to maintain the in situ distribution of RNA polymerase on plastid DNA. A known number of plastids were lysed in a transcription buffer which contained nucleotide triphosphates, including [³²P]UTP, and transcription was allowed to proceed for 5 min. Heparin was added to the transcription reactions to prevent soluble RNA polymerase from reinitiating on the DNA and to block RNA degra-
TABLE II

RNA abundance, transcription rate, and predicted relative RNA stability of selected plastid genes

| Gene     | RNA level fmol/5 × 10^6 plasts^-1 | Transcription rate fmol UMP incorporated/5 × 10^6 plasts^-1.5 min^-1 kb^-1 | Relative RNA level-transcription rate^-1 |
|----------|-----------------------------------|--------------------------------------------------------------------------|-----------------------------------------|
| rRNA, tRNAs          |                                   |                                                                          |                                         |
| 16S rRNA              | 1183 ± 136                        | 98 ± 8                                                                  | 12 ± 2                                  |
| trn/M-trnG            | 516.5 ± 6.9                       | 174 ± 27                                                                | 0.3 ± 0.03                              |
| trn-K-ORF 5S0         | 3.7 ± 0.1                         | 305.5 ± 4                                                               | 0.1 ± 0.02                              |
| Photosynthesis        |                                   |                                                                          |                                         |
| rbcL                  | 45.1 ± 2.5                        | 25.8 ± 1.6                                                               | 1.7 ± 0.1                               |
| psbA                  | 38.1 ± 2.8                        | 153 ± 25                                                                | 0.2 ± 0.04                              |
| psbD                  | 13.0 ± 1.6                        | 13.5 ± 3.5                                                              | 1.0 ± 0.2                               |
| psaA                  | 8.5 ± 1.0                         | 15.6 ± 2.8                                                              | 0.5 ± 0.1                               |
| atpB                  | 3.9 ± 0.5                         | 14.3 ± 4.0                                                               | 0.3 ± 0.08                              |
| petB                  | 12.5 ± 4.2                        | 4.2 ± 0.5                                                                | 2.8 ± 1.0                               |
| ndh complex           | 0.3 ± 0.04                        | 2.4 ± 0.3                                                                | 0.1 ± 0.02                              |
| Ribosomal proteins    |                                   |                                                                          |                                         |
| rps16                 | 0.3 ± 0.8                         |                                                                          |                                         |
| rpl16                 | 2.4 ± 0.5                         | 2.4 ± 0.3                                                                | 1.0 ± 0.3                               |
| RNA polymerase        |                                   |                                                                          |                                         |
| rpoB                  | 1.6 ± 0.2                         | 1.2 ± 0.2                                                                | 1.3 ± 0.2                               |
| rpoA                  | 0.06 ± 0.01                       | 0.5 ± 0.1                                                                | 0.1 ± 0.02                              |

Plastid transcription units are often arranged in tandem with little nontranscribed DNA separating each transcription unit. This situation introduces two potential sources of error into the determination of transcription activity. First, it is possible that transcription of the gene being assayed could be influenced by transcription from an upstream gene if normal termination events are disrupted in vivo. This source of error could be significant if the upstream gene is transcribed at a much higher rate than the gene being analyzed. We tried to minimize this source of error by keeping the assay time to a minimum, but in one case (rps16) we believe that transcription rates were underestimated due to read-through from trnQ upstream of rps16. A second source of error could occur if transcription begins within the region of the gene being assayed and continues downstream of the probe for a significant distance during the assay. This would result in hybridization to the probe with a substantial single stranded region which extend beyond the probe. These latter single stranded RNAs would be susceptible to mung bean nuclease. To test the magnitude of this potential error, run-on transcripts hybridized to the 14 antisense RNAs were treated with mung bean nuclease. The amount of radioactivity removed by mung bean nuclease treatment ranged from 33% (psbA, psbD) to less than 10% (data not shown). In mock experiments using completely homologous radiolabeled sense probes with no overlap, approximately 10% of the radiolabel was removed from the blots. This experiment indicates that read-through transcription influences the run-on assay results to only a small extent.

DISCUSSION

Barley Plastid Gene Organization—The barley plastid genome is approximately 133 kbp in size and contains an inverted repeat similar to most other higher plant plastid genomes. Approximately 25% of the barley plastid genome has been sequenced and over 40 genes identified (Chakhmakhcheva et al., 1989; Neumann, 1988; Reverdatto et al., 1989, Sexton et al., 1990b; Sogaard and Wettstein-Knowles, 1987). During the course of this study, 4 additional genes, rpoB, rpoA, rpl16, and ndhA, were identified and located on the barley plastid genome. Subclones of each of these genes were obtained using PCR primers and partial sequence analysis used to verify gene identity. In addition, rpoB was located in the rpoB-rpoC1-rpoC2 gene cluster and rpoA and rpl16 located within a gene cluster which also contains infA, rps19, and rpl23 as has been found in other higher plant genomes (Shinozaki et al., 1986; Hirasaka et al., 1989; Hudson et al., 1988). Interestingly, the arrangement of identified genes in the plastid genome of barley is nearly identical to rice (Hiratsuka et al., 1989). This may not be too surprising because rice and barley are both C3 monocots which diverged from C4 monocots within the last 40 million years (Wolfe et al., 1989). In contrast, monocots and dicots diverged about 100–200 million years ago and flowering plants from liverwort about 400 million years ago (Wolfe et al., 1989). Even so, dicot, monocot, and liverwort plastid genomes contain many similar co-transcribed gene clusters (for review, see Bauda and Palmer, 1990 and Palmer (1985)). Even more remarkable are plastid gene clusters which appear to be remnants of operons found in Escherichia coli (i.e. S10-spec-α). The plastid gene clusters often encode proteins exclusively for photosynthesis or transcription/translation suggesting that segregation of functionally related genes into common transcription units is advantageous to the plant. In this paper we report that expression of different gene clusters varies dramatically at a single stage of barley chloroplast development.

Expression of Genes Encoding rRNA and tRNA—The transcription and accumulation of 16S rRNA was analyzed during this study because the production of rRNAs is a key regulatory point in the production of ribosomes, and ribosome level can potentially limit translation capacity, growth, and development (Nomura et al., 1984; Bendich, 1987). Plastid ribosomes contain stoichiometric amounts of 16S, 23S, 4.5S, and 5S rRNAs which are co-transcribed from an operon which also
encodes trnI and trnA. In barley, these genes are duplicated because they are located in the inverted DNA repeat. Analysis of 16S rRNA levels in chloroplasts of 4-day-old dark-grown plants showed the 16S rRNA to be the most abundant RNA analyzed. If one 16S rRNA is present in each ribosome, then plastids at this stage of development contain approximately 150,000 ribosomes. Previously it was estimated that barley plastids contain 20,000 to 200,000 ribosomes depending on developmental stage (Klein and Mullet, 1987). Transcription of the genes encoding the 16S rRNA was very active relative to other genes consistent with our previous observations (Klein and Mullet, 1990). A high capacity for rRNA synthesis is important if rRNA synthesis limits ribosome accumulation and the rate of chloroplast biogenesis as proposed by Bendich (1987).

The transcription of three genes encoding tRNAs was directly examined (trnK, trnFM, trnG). Information on the expression of several other genes encoding tRNAs was obtained because they are co-transcribed with other genes being analyzed (16S-trnI-trnA-23S-5.5S-5S; psbA-psbK-psbD-psbC-orf62-trnG; possibly psbA-trnH). The trnFM-trnG gene cluster was one of the most actively transcribed DNA regions analyzed. A similar level of transcription may occur for trnH if this gene is co-transcribed with psbA in barley as found for spinach (Thomas et al., 1988; Gruissem and Zurawski, 1985b). Transcription of trnI and trnA which are located within the rRNA transcription unit, was about 1.5-fold less active than trnFM-trnG and transcription of trnK 6-fold lower. Transcription of the psbD-psbC operon is similar to trnK suggesting that genes encoding tRNAs within this operon (trnG) may also be synthesized at rates lower than trnFM-trnG. It is possible that the different rates of tRNA synthesis result in differential accumulation of the tRNAs. In bean plastids, tRNA abundance varied 5-fold and was correlated with codon usage (Pfitzinger et al., 1987). Unfortunately, the Northern blots used for RNA analysis in this study are not suitable for tRNA quantitation. The trnK probe hybridized primarily to unspliced precursor RNAs and the trnFM-trnG probe to precursor and tRNA sized RNAs. Further analysis will be required to determine the relationship between transcription of the genes encoding tRNAs and the relative abundance of the corresponding tRNAs.

Overall Transcription and RNA Abundance of Protein Coding Genes—The transcription and RNA levels of 11 plastid genes which encode proteins were analyzed. These genes encode subunits of the RNA polymerase (rpoA, rpoB), ribosomes (rps16, rpl16), the putative NADH oxidoreductase (ndhA), and the photosynthetic complexes (Rbu-P carboxylase (rbcL), PSII (psbA, psbD), PSI (psaA), cytochrome b_{6f} (petB), ATP synthase (atpB)). The abundance of these protein complexes in plastids varies approximately 1000-fold. Analysis of RNA abundance revealed over a 900-fold range of mRNA level from a high of 45 fmol/5 x 10^{6} plastids (for rbcL to 0.05 fmol for rpoB). Similarly, transcription of the 11 protein coding genes varied over 300-fold with psbA the most actively transcribed and rpoB the least actively transcribed. Overall, there was a strong correlation between gene transcription activity and RNA abundance. Transcription and mRNA levels of genes encoding proteins for photosynthesis was greater than genes encoding proteins for ribosomes or the RNA polymerase. The difference in transcription activity among transcription units could help explain the maintenance of gene clusters which encode proteins of related function. For example, if a gene which encodes a protein involved in photosynthesis was moved into the rpoB-rpoC1-rpoC2 transcription unit, the transcription rate in this region might be insufficient to support normal biosynthesis of the protein unless unusual transcript stability or translation efficiency were developed.

In vitro studies using a limited set of spinach genes showed that the relative strength of the psbA, rbcL, and atpB promoters was 1.6/1/0.07 (Gruissem and Zurawski, 1985a). The run-on transcription assays described here demonstrate that transcription of psbA was 6-fold greater than rbcL and 11-fold greater than atpB. In other words, the run-on assay revealed a 12-fold variation in transcription activity between these genes, whereas in vitro transcription assays revealed only a 2-fold difference. This result could be due to differences between barley and spinach promoters for these genes. Alternatively, the DNA templates or protein extracts used for in vitro studies may have lacked important elements which specify relative promoter activity. Genes with the highest transcription activity (psbA, rbcL) have transcription promoter elements located -10 and -35 base pairs from the transcription initiation site which resemble prokaryotic a-70 transcription elements (for review, see Hanley-Bowdoin and Chua (1987)). Promoter elements for plastid genes transcribed at very low rates, such as rpoB, have not been characterized. It is possible that variation in promoter sequences or spacing between elements could account for the observed differences. Alternatively, the differences in transcription could be due to the presence of different classes of promoters in the plastid genome which require specific a-factors (Tiller et al., 1991) or different RNA polymerases (Greenberg et al., 1984).

Expression of Genes Encoding Proteins Involved in Photosynthesis—The abundance of mRNAs which encode proteins involved in photosynthesis varied 12-fold. The most abundant mRNA of this group corresponded to rbcL. This is consistent with greater abundance of the large subunit of Rbu-P carboxylase which is encoded by rbcL compared to the electron transport units (10^{3} versus 10^{6} units/plastid). The next most abundant mRNA analyzed corresponds to psbA. This gene encodes protein D1, one of two reaction center protein subunits of photosystem II (Mattoo et al., 1989). D1 is one of the least stable proteins of the thylakoid membrane because it is damaged and turned over during the course of normal photosynthetic activity (for review, see Mattoo et al. (1989)). Therefore, it is not surprising that psbA mRNA levels are higher than mRNAs which encode more stable proteins (i.e. subunits of PSI encoded by psaA-psaB). There are small differences in the abundance of psbD, petB, psaK, and atpB mRNAs. Although the protein complexes in which these proteins are localized are usually present in stoichiometric amounts (for exceptions, see Chow et al. (1990)), the number of subunits per complex varies (psbD = 1; petB = 4; atpB = 3; atpE = 1; psaA = 1). This indicates that the final accumulation of each protein subunit is regulated by the abundance of mRNA as well as at other levels of expression.

Several plastid genes encode proteins with homology to subunits of the mitochondrial NADH oxidoreductase (ndh genes) (Shinozaki et al., 1986; Hiratsuka et al., 1989; Matsubayashi et al., 1987). At present, activity of this putative complex has not been reported in higher plant plastids. The abundance of transcripts hybridizing to ndhA was lower than the genes encoding photosynthetic proteins. This does not discount a role for the NADH oxidoreductase in photosynthesis, but suggests that this complex may be present at lower levels than the other photosynthetic electron transport complexes.

Expression of Genes Encoding Ribosomal Proteins and RNA Polymerase Subunits—The abundance of rps16 and rpl16 mRNA differed 10-fold even though the ribosomal proteins encoded by these genes probably accumulate in stoichiometric amounts.
amounts in ribosomes as in E. coli (Lindahl and Zengel, 1982). The difference in abundance of translatable mRNA for these two genes may be even greater because a significant amount of unspliced rps16 mRNA was detected. This suggests that a difference in translation efficiency or protein stability accounts for equal end-product formation. The gene with higher mRNA level, rpl16, is co-transcribed with several other ribosomal protein genes in a gene cluster which resembles the 5S-10-spc-a operon of E. coli (Zhou et al., 1989; Ruf and Kössel, 1988). Synthesis of ribosomal proteins from this gene cluster in E. coli is autoregulated translationally by free ribosomal proteins subunits (for review, see Lindahl and Zengel (1982)).

Perhaps a similar type of regulation occurs in plastids. Similarly, rpoA mRNA levels are 30-fold greater than rpoB mRNA levels, yet these subunits probably accumulate in a 2:1 ratio as in E. coli (Burgess, 1969; Rajasekhar et al., 1991). RpoA is the terminal gene in the 5S-10-spc-a operon in E. coli and is also autogenously regulated at the translational level (Lindahl and Zengel, 1982). Therefore, it is possible that the rpoB-rpoC1-rpoC2 mRNA and corresponding RNA polymerase subunits are produced in limiting amounts and rpoA translation is modulated by a feedback mechanism.

**Predicted RNA Stability—**Quantitative analysis of RNA levels and transcription rates provides a method to predict relative mRNA stability by comparing the ratio of RNA abundance/transcription rate for different genes. Using this approach, the relative stabilities of RNAs hybridizing to 14 of the genes analyzed in this study were calculated (Table II). As expected, 16S rRNA had the highest predicted relative stability consistent with this RNA being a component of the ribosome, a relatively stable structure. In contrast, the predicted stability of RNAs hybridizing to trnFM-trnG and trnK was much lower. This is consistent with the observation that the probes used for these genes hybridized primarily to precursor RNAs and not mature tRNAs. Among the mRNAs there was a 30-fold difference in predicted RNA stability as compared to a 300-fold variation in transcription rate. If only the genes encoding proteins involved in photosynthesis are considered, a 9-fold variation in predicted RNA stability was observed compared to a 35-fold range in transcription rate. Stability did not correlate with level of gene expression because abundant mRNAs (rbcL) and those which accumulate at lower levels (rpoA) had similar predicted stabilities. In general, while significant variation in RNA stability was predicted, the influence of RNA stability on mRNA levels at this stage of barley chloroplast development appears to be a less important determinant of plastid mRNA levels than transcription. This conclusion of course must be qualified by noting that for many of the genes analyzed, several different RNAs were detected. Therefore, our analysis represents the sum of these RNAs and individual RNAs within the complex populations detected could have different stabilities.

Recent discussions have emphasized the importance of RNA stability in determining plastid RNA levels (Klaff and Gruissem, 1989; Mullet and Klein, 1987; Stern et al., 1991; Krupinska and Apel, 1989) which may appear at variance with our results. A role for modulated RNA stability has been invoked in several studies, including our own (Mullet and Klein, 1987), where light induced changes in plastid transcription and RNA levels were analyzed. In these cases, transcription rates for specific genes changed to a greater extent than RNA levels. For example, illumination of 8-day-old dark-grown barley for 4 h caused a 4-fold increase in psbD-psbC transcription but only a 1.5-fold change in mRNA level (Sexton et al., 1990a). Likewise, a 3-fold decrease in psbA transcription during light-induced chloroplast maturation is ac-

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