PRESENCE OF A TRANSFER RNA GENE IN THE SPACER SEQUENCE BETWEEN THE 16 S AND 23 S rRNA GENES OF SPINACH CHLOROPLAST DNA

H. J. BOHNERT, A. J. DRIESEL, E. J. CROUSE, K. GORDON and R. G. HERRMANN
Botanisches Institut I, Universität Düsseldorf, Universitätstr. 1, 26.13, D-4000 Düsseldorf, FRG

and

A. STEINMETZ, M. MUBUMBILA, M. KELLER, G. BURKARD and J. H. WEIL
Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France

Received 19 April 1979

1. Introduction

Circular chloroplast (cp) DNA molecules contain as an outstanding feature a repeated region, which in *Zea, Chlamydomonas* and *Spinacia* is an invertedly arranged duplication [1–4], in *Euglena* a tandem triplication [5]. The repeated DNA region in all these species was found to include the genes coding for the plastid ribosomal RNAs [1–5]. It has been shown for *Spinacia* that these genes are transcribed to yield a precursor RNA molecule of which the mature rRNAs comprise about two-thirds, and which contains spacer sequence of hitherto unknown function [6]. A similar arrangement of rRNA genes, yielding a single precursor, has been shown for bacterial rRNA operons [7]. In this case it is known that the RNA spacer between the two largest rRNAs is processed to yield tRNAs for either Ile and Ala or for Glu [7]. In the course of mapping tRNA genes [8,9] on the physical map of *Spinacia* cp DNA [3] we have found that the gene for one of the 22 cp tRNA species localized so far, $tRNA_2^{Ile}$, is located on a *Sma* I DNA fragment (*Sma* I,11) which carries part of the cp rRNA genes. We report here that this rRNA gene is part of the spacer sequence separating the 16 S and 23 S rRNA genes.

Nomenclature: restriction endonuclease notations follow the suggestions of Smith, H. O. and Nathans, D. (1973) J. Mol. Biol. 81, 419–423.

2. Materials and methods

Spinacia oleracea (var. Monopa) plants were grown in soil in a greenhouse with supplementary light during winter. Mature leaves were used for the isolation of cp DNA, ribosomes and tRNAs from structurally intact chloroplasts. For the isolation of cp DNA isolated plastids were treated with DNase and digested by proteinase K in the presence of detergent. The lysate was gently extracted with phenol. The average molecular weight of the cp DNA isolated was $\sim 50 \times 10^6$ which is about half the circle size [9]. Chloroplast ribosomal subunits served as the source for isolation of the 16 S rRNA (small subunit) and the 23 S, 4.5 S and 5 S rRNAs (large subunit). The chloroplast tRNAs were separated by two-dimensional electrophoresis [10]. Most of them have been identified [8] by charging with the specific amino acid, using bacterial or chloroplast aminoacyl-tRNA synthetases [11]. Ribosomal RNAs and individual tRNAs were iodinated [12] and hybridized to filter-immobilized [13] DNA fragments or subfragments from the rDNA region, obtained by cleavage with various restriction endonucleases (Boehringer, Mannheim; New England Bio Labs, Berverly, MA) as stated in the legend to fig.1. DNA bands were resolved on 0.8–2% agarose gels (MCI, Rockville), stained with ethidium-bromide and photographed under ultraviolet light (Polaroid film). Hybrid bands were

Elsevier/North-Holland Biomedical Press

visualized by autoradiography on XR-5 film (Kodak) after exposure for 2 days to 2 weeks.

3. Results

The *Spinacia* chloroplast 4 S RNA preparation can be resolved into 35 spots on the two-dimensional polyacrylamide gel. The DNA sequence coding for 26 of these RNA molecules has been localized on the physical map of the spinach cp DNA molecule [8,9]. Twenty two of these 26 RNA molecules have been identified as tRNAs. Only one of the tRNA genes is yet located on DNA fragments which also carry rDNA sequences (fig.1a,b). This tRNA gene codes for the

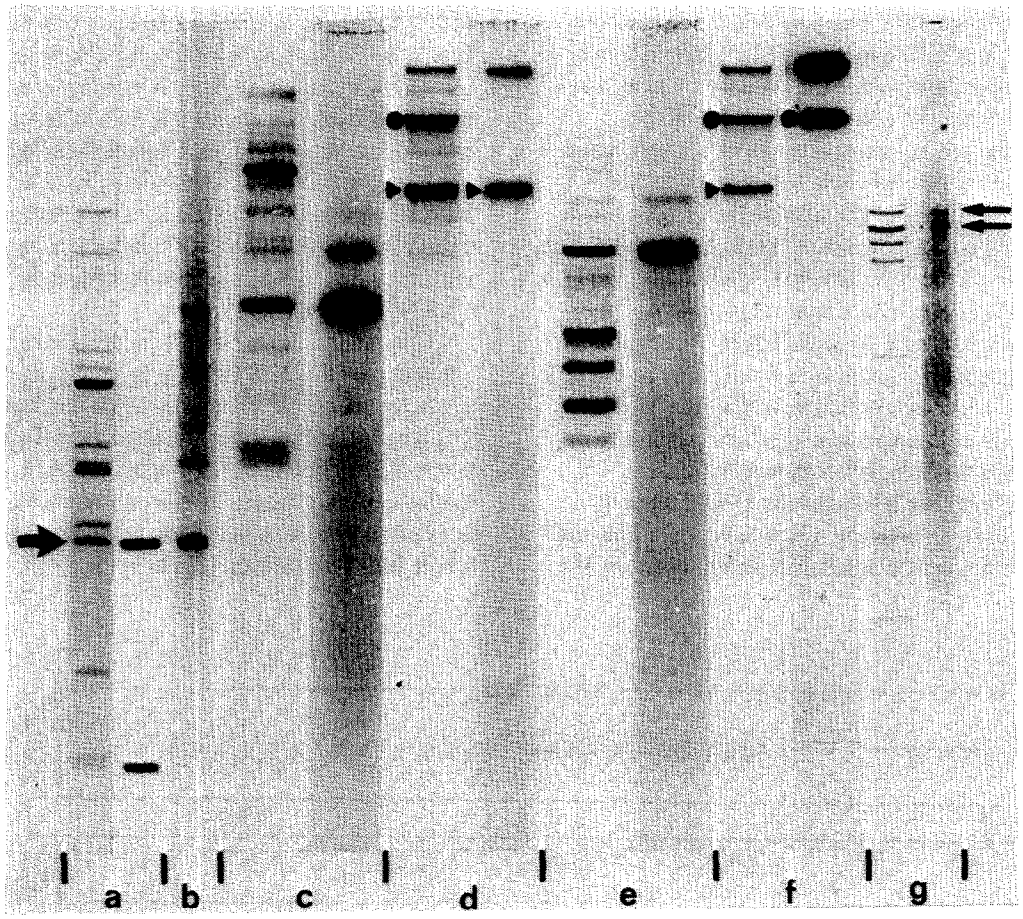

Fig.1. Hybridization of 23 S rRNA or $tRNA_2^{Ile}$ to spinach cp DNA fragments or subfragments produced by restriction endonuclease cleavage. From 2–4 μl of total cp DNA or 0.2–0.5 μg of the isolated *Sma* I,11 DNA fragment was used per gel slot (a, 0.65%; b–f, 1.75%; g, 0.60% agarose). (a) Digestion of total cp DNA using *Sma* I. An autoradiograph of 23 S rRNA hybridization (6 h at 68°C in 6 × 0.5 M NaCl/0.015 Na_3^- citrate is shown adjacent to the gel pattern. The strong hybrid band (thick arrow) indicates the *Sma* I,11 DNA fragment; the weak band indicates the *Sma* I,14 fragment. (b) Autoradiograph of $tRNA_2^{Ile}$ hybridization to the DNA fragments produced by *Sma* I digestion, showing the strongest band at DNA fragment *Sma* I,11. The upper weaker bands result from hybridization to intermediates produced by incomplete digestion. (c) Redigestion (left) of *Sma* I,11 with *Bam* HI and autoradiograph (right) of $tRNA_2^{Ile}$ hybridization. (d) Redigestion (left) of *Sma* I,11 with *Bgl* II and autoradiograph (right) of $tRNA_2^{Ile}$ hybridization (indicated by arrow). The DNA fragment pattern shows additional intermediate bands derived in part from the contaminating fragment *Sma* I,10 (see also f). (e) Redigestion (left) of *Sma* I,11 with *Hpa* II and autoradiograph (right) of $tRNA_2^{Ile}$ hybridization. (f) Redigestion (left) of *Sma* I,11 with *Bgl* II and autoradiograph (right) of 23 S rRNA hybridization (indicated by the solid circles). (g) Digestion (left) of total cp DNA with *Knp* I and autoradiograph (right) of 23 S rRNA hybridization to the two largest DNA fragments (thin arrows).

isoaccepting species $tRNA_2^{Ile}$ hybridizing, for example, to the DNA fragment *Sma* I,11 (fig.1b) which carries part of the rDNA unit only [3,4].

The fragment *Sma* I,11 is 3850 basepairs long. According to the map of the spinach cp rDNA region [3,4] this DNA fragment includes part of the 16 S rRNA sequence (~100 basepairs) and ~1900 basepairs from the presumed 5'-end of the 23 S rRNA gene which is assumed to be 3500 basepairs long. The remaining 1850 basepairs of fragment *Sma* I,11 have, according to mapping and hybridization data, to be placed as a spacer sequence between the two large rRNAs.

Finer mapping of this spacer DNA sequence has been found performed using the restriction endonucleases *Bam* HI, *Bgl* II and *Hpa* II (fig.1c–e) which produce fragments down to ~100 basepairs long. The serial order of the subfragments produced is based on the evaluation of partial digestion products of the DNA fragment *Sma* I,11 as well as by digestion of DNA fragments produced by several other restriction endonucleases which partially overlap the *Sma* I,11 sequence (not shown) using the three endonucleases above. The resulting map is shown in fig.2.

The enzyme *Bgl* II cleaves the DNA fragment *Sma* I,11 only once in the spacer sequence. This site is separated by ~500 basepairs from the start of the 23 S rRNA sequence. As can be seen (fig.1) the *Bgl* II site in *Sma* I,11 separates the 23 S rRNA gene plus a few hundred bases of the spacer (2400 basepairs) from a DNA fragment (1500 basepairs) which consists almost exclusively of spacer sequence.

As with *Bgl* II the enzyme *Bam* HI cleaves fragment *Sma* I,11 in a way that the sequence of the 23 S rRNA gene is contained in one subfragment (2000 basepairs) with a cleavage site just preceding the 23 S rRNA sequence (fig.1c). The spacer region is cleaved more often by *Bam*HI yielding 6 subfragments (fig.1c). The end of the 16 S rRNA sequence, assumed to be in the 3'-end, appears in a 150 basepair DNA fragment which is terminated by a *Sma* I and a *Bam* HI recognition site (fig.2). DNA fragments of ~350, 900, 150, 150 and 300 basepairs long follow with the latter DNA fragment just preceding the 23 S rRNA gene (fig.2).

The endonuclease *Hpa* II, finally, cleaved the sequence of the 23 S rRNA gene more often than the spacer sequence, in contrast to enzyme *Bam* HI (fig.1e, fig.2). The largest of these *Hpa* II subfragments (1200 basepairs) of fragment *Sma* I,11 is derived entirely from the spacer sequence and overlaps partially with a 900 basepair fragment arising upon *Bam* HI digestion (fig.2).

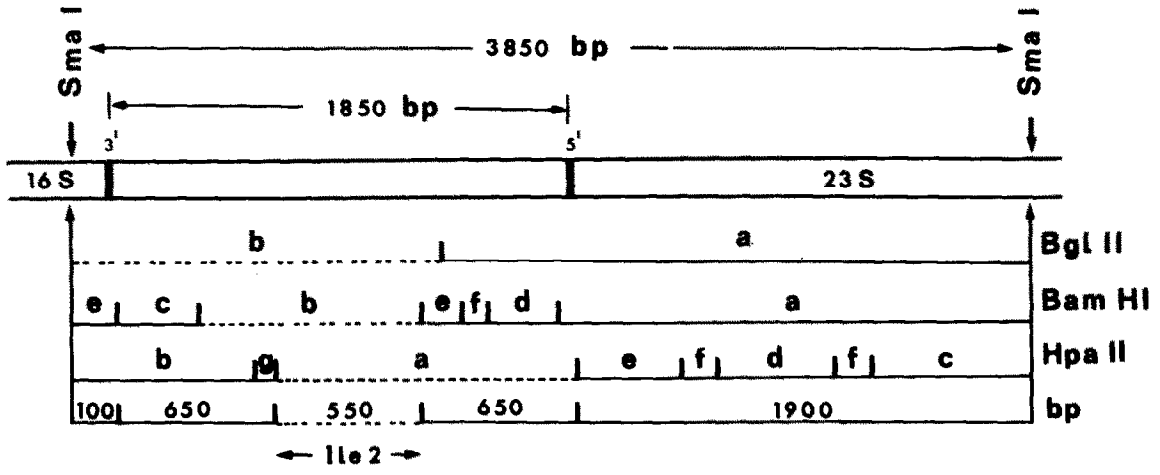

Fig.2. Physical map of the spacer region separating the 16 S and 23 S rRNA genes in *Spinacia* cp DNA. The map shows the arrangement of restriction endonuclease cleavage sites for the enzymes *Bam* HI, *Bgl* II and *Hpa* II on the DNA fragment *Sma* I,11. The ordering of fragments was deduced from analyzing molecular weights of subfragments after incomplete and complete digestion. Phage λ-*Hin*dIII (Boehringer) and φ174-*Hae* III (Bio-Labs) DNA fragments served as length standards. The resolution of the map is ~50 basepairs for DNA fragments 200–1000 basepairs long.

In fig.1 the autoradiograms obtained after hybridization of ^{125}I-labelled $tRNA_2^{Ile}$ (fig.1b–e,g) or 16 S/23 S rRNAs (fig.1a,f) to DNA fragments of the rDNA region are shown adjacent to the corresponding DNA fragment pattern. Whereas the 23 S rRNA hybridizes solely to the largest of the *Bam* HI and *Bgl* II DNA subfragments, the $tRNA_2^{Ile}$ hybridizes to DNA fragments which have now been localized centrally within the spacer sequence. The position of the $tRNA_2^{Ile}$ gene can be placed on an ~600 basepair long DNA sequence which is separated by at least 600 basepairs from the 16 S rRNA and 600 basepairs from the 23 S rRNA sequence (fig.2).

In order to determine whether the $tRNA_2^{Ile}$ gene is present in both rDNA units of the cp DNA molecule it was possible to use hybridization of the tRNA to DNA fragments generated by the endonuclease *Kpn* I. The distribution of recognition sites of this enzyme within the inverted repeat segment and outside the repeat allows the separation of the spacer sequence of the two rDNA units on two large DNA fragments [3]. As is shown in fig.1g, $tRNA_2^{Ile}$ hybridizes to these two large *Kpn* I DNA fragments demonstrating the presence of the tRNA gene in both spacer sequences.

4. Discussion

We have shown by mapping of the spacer region between the 16 S and 23 S rDNA sequence and by hybridization of the isolated $tRNA_2^{Ile}$ to DNA subfragments that the gene coding for this tRNA is part of the spacer in *Spinacia* cp DNA [9]. This finding confirms a suggestion made on the basis of the hybridization of total 4 S cp RNA to restriction endonuclease cleavage fragments [14]. More recently, still unidentified tRNA genes have been found in equivalent positions of *Chlamydomonas* [2] and of *Euglena* [18] cp DNA. From animal mitochondrial DNAs (e.g., [15]) it has also been reported that the spacer between the rRNA genes carries a tRNA gene. Using fine mapping of the spacer sequence in spinach we are now able to place the $tRNA_2^{Ile}$ gene centrally between the 16 S and 23 S rRNA genes.

The arrangement of the rRNA genes in *Spinacia* cp DNA shows a clear similarity to that found in *E. coli* (e.g., [7]) where two types of 'rRNA-operons' are known, one containing the tRNA genes for Ile and Ala, the other only the tRNA gene for Glu, in the spacer between the 16 S and 23 S rRNA genes. Like the bacterial rRNA 'operons' [7] the rDNA unit in spinach cp DNA is transcribed as a single RNA molecule extending from an unknown distance in front of the 16 S rRNA gene to at least the 5 S rRNA gene [6,16]. One of the processing intermediates from this 8 kilobase long transcript is ~1500 bases long and should be the transcribed spacer between the two large plastid rRNAs [6]. We anticipate that this molecule includes the $tRNA_2^{Ile}$. In the case of *Spinacia* cp DNA it will be necessary to determine whether the $tRNA_2^{Ile}$ gene and the rRNA genes do have a common promoter located anterior to the presumed 5′-end of the 16 S rRNA gene as is the case in *E. coli* [17]. At present it cannot be excluded that the $tRNA_2^{Ile}$ gene is, despite its location in the spacer, part of the opposite strand to that coding for the rRNA genes.

On *Spinacia* cp DNA no other tRNA genes have yet been found in the equivalent position (or within the spacer), but there are still several 4 S RNAs whose genes have not yet been located on the physical map [8,9]. The tRNA genes still to be identified include, for example, those for glutamic acid and aspartic acid, which are part of *E. coli* rRNA 'operons' [17], either in the spacer or at the distal end of one of the 'operons'. Even if only the inverted repeat DNA is considered there would be space available for such additional tRNAs distal for the 5 S rRNA gene since the 5 S rRNA gene is separated from the end of the repeat by ~1500 basepairs. Finer mapping studies using cloned DNA, in combination with DNA and tRNA sequencing, might be considered, should the existence of a tRNA gene between the 16 S and 23 S rRNA genes prove to be of physiological or regulatory importance.

Acknowledgements

The technical assistance of Ch. Michalowski, B. Schiller and M. Streubel is gratefully appreciated. These studies were supported by grants from the Deutsche Forschungsgemeinschaft, Heinreich-Hertz-Stiftung, DAAD, EMBO, the Commissariat à l'Energie Atomique and the Fondation pour la Recherche Médicale Française.

References

[1] Bedbrook, J. R., Kolodner, R. and Bogorad, L. (1977) Cell 11, 739–749.

[2] Rochaix, J. D. and Malnoe, P. (1978) Cell 15, 661–670.

[3] Crouse, E. J., Schmitt, J. M., Bohnert, H. J., Gordon, K., Driesel, A. J. and Herrmann, R. G. (1978) in: Chloroplast Development (Akoyunoglou, G. and Argyroudi-Akoyunoglou, J. H. eds) pp. 565–572, Elsevier/North-Holland, Amsterdam, New York.

[4] Whitfeld, P. R., Herrmann, R. G. and Bottomley, W. (1978) Nucl. Acids Res., 5, 1741–1751.

[5] Gray, P. W. and Hallick, R. B. (1978) Biochemistry 17, 284–289.

[6] Bohnert, H. J., Driesel, A. J. and Herrmann, R. G. (1976) Genetics and Biogenesis of Chloroplasts and Mitochondria (Bücher, Th. et al. eds) pp. 629–636, Elsevier/North-Holland, Amsterdam, New York.

[7] Lund, E., Dahlberg, J. E., Lindahl, L., Jaskunas, S. R., Dennis, P. P. and Nomura, M. (1977) Cell 7, 165–177.

[8] Steinmetz, A., Mubumbila, M., Keller, M., Burkard, G., Weil, J. H., Driesel, A. J., Crouse, E. J., Gordon, K., Bohnert, H. J. and Herrmann, R. G. (1978) in: Chloroplast Development (Akoyunoglou, G. and Argyroudi-Akoyunoglou, J. H. eds) pp. 573–580, Elsevier/North-Holland, Amsterdam, New York.

[9] Driesel, A. J., Crouse, E. J., Gordon, K., Bohnert, H. J., Herrmann, R. G., Steinmetz, A., Mubumbila, M., Keller, M., Burkard, G. and Weil, J. H. (1979) Gene 6/4, in press.

[10] Fradin, A., Gruhl, H. and Feldmann, H. (1975) FEBS Lett. 50, 185–189.

[11] Weil, J. H., Burkard, G., Guillemaut, P., Jeannin, G., Martin, R. and Steinmetz, A. (1977) in: Nucleic Acids and Protein Synthesis in Plants (Bogorad, L. and Weil, J. H. eds) pp. 94–120, Plenum Press, New York.

[12] Commerford, S. L. (1971) Biochemistry 10, 1993–1999.

[13] Southern, E. M. (1975) J. Mol. Biol. 98, 503–517.

[14] Whitfeld, P. R., Atchinson, B. A., Bottomley, W. and Leaver, C. J. (1976) in: Genetics and Biogenesis of Chloroplasts and Mitochondria, (Bücher, Th. et al. eds) pp. 361–368, Elsevier/North-Holland, Amsterdam, New York.

[15] Angerer, L., Davidson, N., Murphy, W., Lynch, D. and Attardi, G. (1976) Cell 9, 81–90.

[16] Hartley, M. R. and Head, C. (1979) Eur. J. Biochem. in press.

[17] Morgan, E. A., Ikemura, T., Lindahl, L., Fallon, A. M. and Nomura, M. (1978) Cell 13, 335–344.

[18] Hallick, R. B., Gray, P. W., Chelm, B. K., Rushlow, K. E. and Orozco, E. M. (1978) in: Chloroplast Development (Akoyunoglou, G. and Argyroudi-Akoyunoglou, J. H. eds) pp. 619–622, Elsevier/North-Holland, Amsterdam, New York.