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Restriction Endonuclease Map of *Euglena gracilis* Chloroplast DNA†

Patrick W. Gray and Richard B. Hallick*

ABSTRACT: A physical map of the *Euglena gracilis* chloroplast genome has been constructed, based on cleavage sites of *Euglena gracilis* chloroplast DNA treated with bacterial restriction endonucleases. Covalently closed, circular chloroplast DNA is cleaved by restriction endonuclease SalI into three fragments and by restriction endonuclease BamHI into six fragments. These nine cleavage sites have been ordered by fragment molecular weight analysis, double digestions, partial digestions, and by digestion studies of isolated DNA fragments. A fragment pattern of the products of EcoRI restriction endonuclease digestion of *Euglena* chloroplast DNA is also described. One of these fragments has been located on the cleavage site map.

Chloroplasts of the unicellular alga *Euglena gracilis* contain double-stranded, covalently closed circular DNA of molecular weight 92 × 10^6 (Manning and Richards, 1972), corresponding to 140 kbp.[1] Depending on the stage of chloroplast development and the conditions of cell culture, there are 500–2000 copies of chloroplast DNA per *Euglena* cell (Rawson and Boerma, 1976a; Chelm et al., 1977). The chloroplast genome is extensively transcribed in vivo, both in light grown and in dark adapted cells. Estimates of the extent of genome transcription of from 12 to 23% (Chelm and Hallick, 1976) and from 23 to 26% (Rawson and Boerma, 1976b) for various stages of light-induced chloroplast development have been reported. Two classes of RNA transcripts are known to be of chloroplastic origin. The most abundant chloroplast transcripts are the 16S and 23S rRNAs (Scott and Smillie, 1967; Stutz and Rawson, 1970), accounting for as much as 26% of *Euglena* cellular RNA (Chelm et al., 1977; Cohen, 1973; Cohen and Schiff, 1976). Chloroplast DNA also contains genes for approximately 25 tRNAs (Schwartzbach et al.,

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[1] Abbreviations used: kbp, kilobasepair; ct DNA, chloroplast DNA; SalI, endonuclease isolated from *Streptomyces albus G*; BamHI, endonuclease isolated from *Bacillus amyloliquefaciens H*; EcoRI, restriction endonuclease isolated from *E. coli RY13*; PstI, endonuclease isolated from *Providencia stuartii*; BS, DNA fragments resulting from a BamHI, SalI double digestion of ct DNA; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

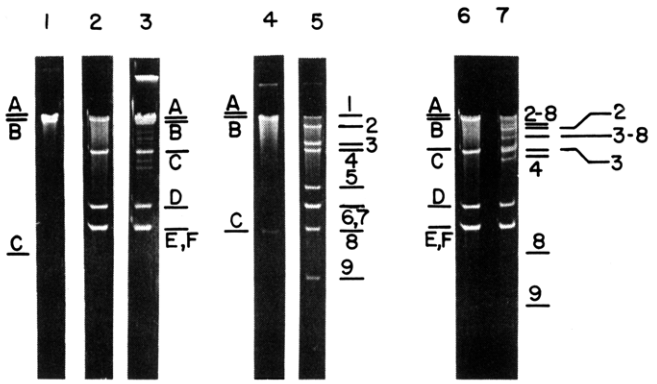

FIGURE 1: Analysis of *Euglena* chloroplast DNA by restriction endonuclease BamHI or SalI cleavage and electrophoresis on 0.7% agarose gels. (1) SalI limit digest; (2) BamHI limit digest; (3) BamHI partial digest; (4) SalI limit digest; (5) SalI and BamHI double digest; (6) BamHI limit digest; (7) BamHI limit digest–SalI partial digest.

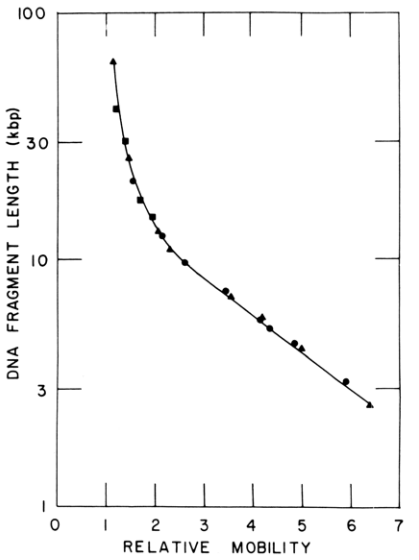

FIGURE 2: Relative electrophoretic mobilities of BamHI–SalI digestion products of *Euglena* chloroplast DNA on 0.7% agarose slab gels. (▲) Products of limit BamHI–SalI double digestion; in order of decreasing length BS 1 (Bam A), BS 2, BS 3, BS 4, BS 5 (Bam D), BS 6 and 7 (Bam E, F), BS 8 (Sal C), BS 9. (■) Products of limit BamHI–partial SalI digestion; in order of decreasing length, BS 2-8-3 (Bam B), BS 2-8, BS 3-8, BS 4-9 (Bam C). (●) Products of limit and partial digestion of λ DNA; in order of decreasing length, using the molecular weights and physical map of Helling et al. (1974), λ1, λ2-3 and 4-2, λ5-4, λ2, λ3, λ4, λ5, λ6.

1976; McCrea and Hershberger, 1976). These two classes of RNA represent a transcript of approximately 4% of the double-strand DNA content of the genome. The remainder of the chloroplast RNA products are unidentified. We have been interested in identifying other chloroplast RNA transcripts, and in studying the temporal regulation of RNA synthesis during light-induced chloroplast development. In the course of our studies, it became apparent that a detailed physical map of the chloroplast genome would be invaluable in studying the control of RNA synthesis since the location of cistrons on the genome could be an important aspect of transcriptional regulation. Our strategy has been to digest intact chloroplast DNA with bacterial restriction endonucleases that make relatively infrequent cleavages in the DNA, and to subsequently order the resulting fragments in a cleavage site physical map. In this report we describe the cleavage site map of *Euglena* chloroplast DNA produced by restriction endonucleases SalI and BamHI. There has been a preliminary report of this work (Gray and Hallick, 1977).

Materials and Methods

Preparation of Chloroplast DNA. For chloroplast DNA isolation, *Euglena gracilis* Klebs, Strain *Z* Pringsheim cells were grown to stationary phase on a photoautotrophic medium, and chloroplasts were isolated as previously described (Hallick et al., 1976). Covalently closed, superhelical *Euglena* chloroplast DNA was isolated by centrifugation of chloroplast lysates in CsCl–ethidium bromide (Chelm et al., 1977).

Restriction Endonucleases. Restriction endonuclease SalI was isolated from *Streptomyces albus G* by the method of Thomas and colleagues (C. A. Thomas, Jr., personal communication). Restriction endonuclease BamHI was purified from *Bacillis amyloliquefaciens H* (Wilson and Young, 1975). EcoRI restriction endonuclease was prepared by the method of Thomas and Davis (1975). Endonuclease reaction mixtures contained 30–200 μg of DNA/mL, 0.05 M NaCl, 0.005 M $MgCl_2$, 0.1 M Tris-HCl, pH 7.5, and the appropriate enzyme(s). Digestion of DNA was achieved by incubation at 37 °C for 10 min to 5 h. Reactions were terminated by heating the mixture at 65 °C for 5 min.

Agarose Gel Electrophoresis. Separation and analysis of restriction endonuclease DNA fragments were achieved by electrophoresis in agarose slab gels. Gels containing 0.5–2.0% agarose 0.05 M Tris-HCl–0.02 M sodium acetate–0.2 mM Na_2EDTA–(pH 8.05)–0.018 M NaCl were cast between glass plates to give a gel 14.5 × 12.5 × 0.3 cm thick. Four to six strips of Whatman 3MM filter paper, 14.5 cm × 0.4 cm, were soaked in glycerol and molded into the bottom of the gel for support. Electrophoresis was performed using the apparatus described by Studier (1973).

Samples of 5–20 μL were mixed with an equal volume of 40% sucrose–0.05% bromphenol blue, and introduced into 0.6-cm wide sample wells. Electrophoresis in 0.05 M Tris-HCl–0.02 M sodium acetate–0.2 mM Na_2EDTA–0.018 M NaCl (pH 8.05) was continued for 5 min at 100 V, followed by 14–16 h at 22.5 V. Following electrophoresis the gel was immersed in a 1 μg/mL solution of ethidium bromide in the electrophoresis buffer for 30 min. DNA bands were visualized during irradiation with an ultraviolet lamp (Ultraviolet Products Model C-51) and photographed using Polaroid Type 105 P/N film and an orange filter.

Isolation of DNA Fragments following Electrophoresis. A modification of the freeze–squeeze procedure (Thuring et al., 1975) was used to elute DNA from agarose gels after electrophoretic separation. Preparative gels with 2.5-cm wide sample wells were used for separation of 5–8 μg of endonuclease-digested DNA. The center 2 cm of each lane was used for sample isolation, while the edges were soaked in ethidium bromide for subsequent fragment localization. DNA-containing regions of the unstained gel were excised, soaked in 0.05 M Tris-HCl–0.05 M NaCl–5 mM $MgCl_2$ (pH 7.5) for 30 min with several changes of buffer and frozen at −20 °C on a sheet of parafilm. DNA was recovered in 50–100 μL of solution when the frozen gel was squeezed between two Parafilm surfaces with the thumb and forefinger.

Results

Preparative Isolation of Euglena Chloroplast DNA by Equilibrium Centrifugation in CsCl–Ethidium Bromide. Following centrifugation of chloroplast lysates in CsCl–ethidium bromide, two DNA bands are seen when the centrifuge tube is illuminated with ultraviolet light. DNA of the lower

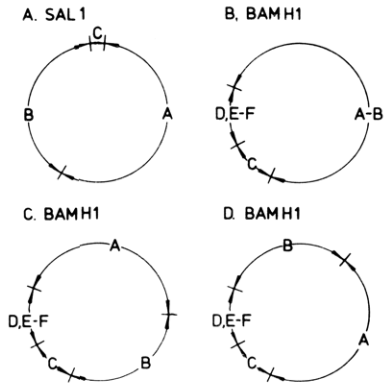

FIGURE 3: SalI and BamHI cleavage site maps of *Euglena gracilis* chloroplast DNA. Letters refer to DNA cleavage fragments described in the text. (A) SalI cleavage site map; (B) BamHI fragment linkage groups deduced from analysis of partial chloroplast DNA digests; (C and D) possible configurations of fragments Bam A, B, and C.

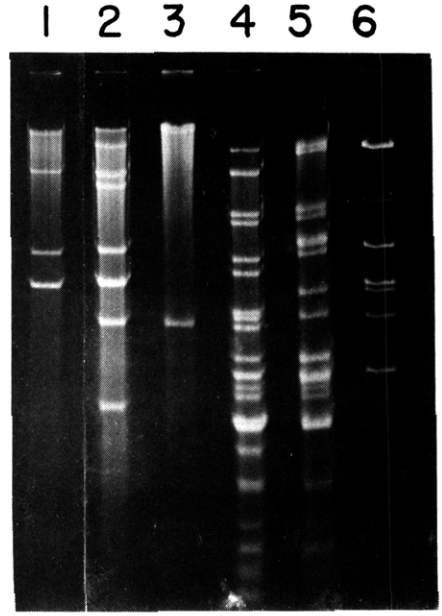

FIGURE 4: Analysis of *Euglena* chloroplast DNA by restriction endonuclease cleavage with BamHI, SalI, and EcoRI. Products were analyzed by electrophoresis on an 0.7% agarose gel as described in the text. From left to right: (1) BamHI, ct DNA; (2) BamHI and SalI, ct DNA; (3) SalI, ct DNA; (4) EcoRI, BamHI, and SalI, ct DNA; (5) EcoRI, ct DNA; (6) EcoRI, λ DNA.

band, which has previously been shown to contain superhelical DNA molecules (Richards and Manning, 1974), was used in the restriction nuclease digestion experiments described below. The upper, main band DNA is also predominately high-molecular-weight chloroplast DNA which is useful for studies with restriction endonucleases such as EcoRI that make numerous cleavages in *Euglena* chloroplast DNA. In a typical DNA preparation, 16 L of cell culture grown to stationary phase in a phototrophic medium yielded approximately 2×10^{10} cells. From these cells, approximately 80 μg of superhelical DNA and 180 μg of main band DNA can be isolated.

SalI Endonuclease Digestion of Euglena Chloroplast DNA. The fragment pattern resulting from the limit digestion of chloroplast DNA with restriction endonuclease SalI is shown in Figure 1. Only three cleavage products are produced, all in equimolar amounts. These are designated Sal A, Sal B, and Sal C. The electrophoretic mobility of Sal C is consistent with a size of 4.4 kbp (Figure 2). It is not possible to make accurate size estimates of Sal A and Sal B based on electrophoretic mobility alone. However, based on this criteria both fragments are larger than intact bacteriophage λ DNA (not shown). If we take 46 kbp as the size of λ DNA (Davidson and Szybalski, 1971), and 140 kbp as the size of *Euglena* chloroplast DNA (Manning and Richards, 1972), then Sal A and Sal B must be in the size range of approximately 50–90 kbp. Sal B is cleaved by restriction endonuclease BamHI into five fragments, whose total fragment size equals approximately 56 kbp (described below). This would suggest that Sal B is approximately 56 kbp and Sal A is approximately 80 kbp. For the three Sal fragments, there is only a single cleavage site map possible for a circular DNA molecule (Figure 3A).

BamHI Digestion of Euglena Chloroplast DNA. When *Euglena* chloroplast DNA is treated with BamHI endonuclease, six fragments are produced designated Bam A, B, C, D, E, and F. Only five of these are resolved on 0.7% agarose gels (Figure 1). The smallest two fragments, Bam E and F, coelectrophorese. Estimates of the size of the four smallest fragments, based on their electrophoretic mobility compared with EcoRI digested λ DNA (Figure 2), are as follows: Bam C, 14 kbp; Bam D, 7.3 kbp; Bam E and F, 5.9 kbp. Since the sum of fragments Bam C, D, E, and F totals 33 kbp, fragments Bam A and B must total approximately 107 kbp. The relative electrophoretic mobilities of the large BamHI and SalI cleavage products are as follows: Sal A < Bam A < Sal B < Bam B. Bam B is cleaved by restriction endonuclease SalI into three fragments whose total fragment size equals approximately 43 kbp (described below). This would suggest that Bam B is approximately 43 kbp and Bam A is approximately 64 kbp.

Partial BamHI Digestion of Euglena Chloroplast DNA. The fragment pattern resulting from partial digestion of *Euglena* chloroplast DNA with BamHI restriction endonuclease is shown in Figure 1. Two partial fragments are apparent with electrophoretic mobilities intermediate between Bam C and Bam D. The sizes of these two partial digestion products are 13.2 kbp and 12 kbp, respectively. The 12-kbp fragment must represent a Bam E–Bam F partial, while the 13.2 kbp fragment must have the structure Bam D–Bam E or Bam D–Bam F. This leads to the conclusion that the linkage group Bam D–(Bam E–Bam F) must exist. Likewise, four partial fragments are apparent (Figure 1) with lengths greater than Bam C but less than Bam B. Such partials can be produced only if Bam C is linked to the Bam D–(Bam E–Bam F) linkage group. The remaining two fragments, Bam A and Bam B must, therefore, be linked and also linked to both ends of the Bam C–(Bam D, Bam E–Bam F) group. This linkage pattern, with unresolved ambiguities in the Bam A–Bam B order, and in the Bam D, Bam E–Bam F linkage, is illustrated in Figure 3B. If the internal order in the 19-kbp (Bam D, Bam E–Bam F) linkage group is unspecified, then the Bam cleavage site map is reduced to two possible configurations, one with Bam C and Bam B adjacent (Figure 3C), and the other with Bam C and Bam A adjacent (Figure 3D).

BamHI and SalI Double Digestion of Euglena Chloroplast DNA. The fragment pattern resulting from a double digestion of chloroplast DNA with restriction endonucleases BamHI and SalI is shown in Figure 1. Nine fragments, designated BS 1–9, are produced, as expected for cleavage of a circular DNA at three SalI and six BamHI sites. The fragments Bam A, D, E, F and Sal C have the same electrophoretic mobilities as fragments BS 1, 5, 6, 7, and 8, respectively (Figure 4). Estimates of the size of the BS fragments based on their electrophoretic

TABLE I: DNA Cleavage Products Resulting from Double Digestion of *Euglena gracilis* Chloroplast DNA with Restriction Endonucleases BamHI and SalI.

Fragment	Length (kbp)	Equivalent Fragment
BS 1	64	Bam A
BS 2	26	
BS 3	13	
BS 4	11	
BS 5	7.3	Bam D
BS 6	5.9	Bam E
BS 7	5.9	Bam F
BS 8	4.4	Sal C
BS 9	2.6	

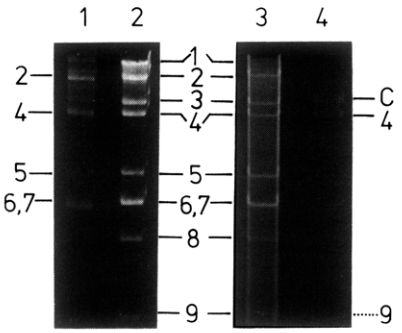

FIGURE 5: Restriction endonuclease analysis of *Euglena* chloroplast DNA. DNA fragments Bam C and Sal B were isolated by the freeze-squeeze procedure following 16 and 72 h of electrophoresis, respectively, as described in the text. The isolated Bam C was digested with endonuclease SalI, while the Sal B was treated with endonuclease BamHI. The products of these reactions were analyzed by electrophoresis on 0.7% agarose gels. From left to right: (1) BamHI digest of Sal B; (2) and (3) BamHI and SalI digest of ct DNA; (4) SalI digest of Bam C. The BS fragment designations (Table I) of the major products are labeled.

mobility compared with EcoRI-digested λ DNA (Figure 2) are listed in Table I. From a comparison of the BamHI and BamHI–SalI products, it is apparent that the three SalI cleavage sites are contained in fragments Bam B and Bam C. Bam B and Bam C are cleaved into the following five products: BS 2, 3, 4, 8 (Sal C), and 9.

Several lines of evidence support the conclusion that Bam B has the structure BS 2–BS 8–BS 3, while Bam C has the structure BS 4–BS 9. First, Bam C is too small to accommodate even the three smallest fragments, BS 4, 8, and 9. Therefore Bam C must contain a single internal SalI site, and Bam B must contain two internal SalI sites. Since BS 8 is the only BS fragment with Sal cleavage sites on each end, BS 8 (Sal C) is the internal fragment in Bam B. Second, when a limit BamHI–partial SalI digest of *Euglena* chloroplast DNA is compared with a limit BamHI digest, the expected partial digestion products for the predicted Bam B and Bam C structures are all observed. In this experiment (illustrated in Figure 1), in addition to the nine BS fragments, fragments Bam B and Bam C, as well as partial digestion products corresponding in electrophoretic mobility (Figure 2) to BS 2–BS 8 and BS 3–BS 8, are observed. Finally, following electrophoresis, fragment Bam C was isolated from an 0.5% agarose gel and subsequently redigested with SalI. As seen in Figure 6, fragments BS 4 and BS 9 are produced. Some undigested Bam C is also present. These two products, BS 4 and BS 9, total 13.6 kbp in size, consistent with the estimated size of Bam C, 14 kbp. As described above, the size of Bam B can be estimated from the sum of the fragments BS 2, BS 8, and BS 3 to be 43 kbp.

BamHI and SalI Restriction Endonuclease Map of Euglena Chloroplast DNA. From the description of the location of SalI cleavage sites in the BamHI DNA fragments, and the knowledge of the approximate size of fragments Sal A and Sal B of 50–90 kbp, it is possible to order the BamHI fragments A, B, and C. Fragments Bam B and Bam C, which contain the three SalI sites, cannot be linked in the *Euglena* genome as shown in Figure 3C. This would lead to the production of a Sal B fragment with a maximum possible size equal to the sum of the largest BS fragments of Bam B and Bam C, BS 2 and BS 4, totaling only 37 kbp. Sal B is larger than Bam B (43 kbp) and intact λ DNA (46 kbp) by the criteria of electrophoretic mobility. Therefore the BamHI fragments A, B, and C of *Euglena* chloroplast DNA are linked as shown in Figure 3D.

Strictly from a molecular weight argument, the composition of Sal B in terms of BS fragments as BS 2-(5,6-7)-4, totaling 56 kbp is most probable. Two other combinations, BS 2-(5,6-7)-9 totaling 47 kbp, and BS 3-(5,6-7)-4 totaling 43 kbp, are less likely since we believe Sal B to be significantly larger than λ DNA, 46 kbp. The final possibility, BS 3-(5,6-7)-9, totaling 35 kbp may be ruled out as too small. To test the first three possibilities, chloroplast DNA was digested with restriction endonuclease SalI. The resulting product mixture was separated by electrophoresis on a 0.5% agarose slab gel. The Sal B fragment was isolated, and redigested with BamHI endonuclease. The analysis of the resulting products is shown in Figure 5. It is apparent that there was some Sal A present in the Sal B preparation since several high-molecular-weight BS fragments are seen. Preparative separation of DNA fragments Sal A and B has proved difficult due to their large size and limited electrophoretic mobility. However, the most prominent bands seen in this experiment, which are believed to be produced from Sal B, are those of fragments BS 2, BS 4 and the double band, BS 6 and 7. This result is consistent with the BS 2-(5,6-7)-4 fragment order for Sal B postulated from molecular weight analysis.

An attempt was made to determine the order of fragments Bam D, E–F (BS 5,6-7) by exonuclease digestion experiments. In fragment Sal B, if BS 2 and BS 5 are adjacent, then BS 5 should be more resistant to digestion by *E. coli* exonuclease III than BS 6 and 7. However, if the BS 6-7 pair is linked to BS 2, then BS 5 would be more readily digested by the exonuclease. *Euglena* chloroplast DNA was digested with restriction endonuclease SalI, and subsequently treated for various lengths of time with *E. coli* exonuclease III. The resulting DNA mixture was treated with restriction endonuclease BamHI. Products were analyzed on a 0.7% agarose slab gel as shown in Figure 6. All nine BS fragments are present at zero time of exonuclease treatment. Some fragments, such as BS 8 and 9 are degraded within 4 min, while BS 6 and 7 are seen as a prominent band after 16 min and are still apparent after 32 min of exonuclease digestion. Fragment BS 5 appears to be less resistant to exonuclease than BS 6 and 7 in this experiment, suggestive of the fragment order BS 2-(6-7)-5-4 for Sal B. We hope to confirm this by isolation of the appropriate BamHI partial digestion products (Figure 1) for redigestion experiments.

The results on the fragment order for SalI, BamHI, and a BamHI–SalI double digestion of *Euglena* chloroplast DNA are summarized as a cleavage site map in Figure 7. The inner map is that of the six BamHI cleavage sites. There is ambiguity

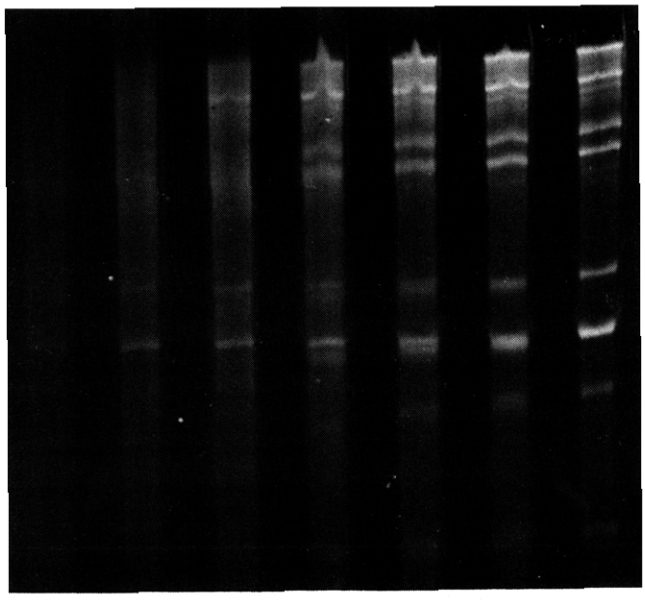

FIGURE 6: Analysis of the BamHI fragments in SalI digested *Euglena* chloroplast DNA by *E. coli* exonuclease III (New England Biolabs) digestions. Chloroplast DNA, 3.6 μg, was digested with SalI endonuclease. The resulting DNA fragments were treated with 8 units of exonuclease III in 0.16 mL of 0.06 M Tris-HCl–0.66 mM $MgCl_2$–1 mM 2-mercaptoethanol (pH 7.9) at 37 °C. Aliquots of 0.02 mL were removed, heated to 65 °C for 15 min, and then treated with BamHI endonuclease. The resulting DNA mixtures were analyzed on a 0.7% agarose gel. From right to left: 0, 1, 2, 3, 8, 16, 32, and 64 min of exonuclease digestion. This experimental plan is based on a modification of a previously published procedure (Thomas et al., 1976).

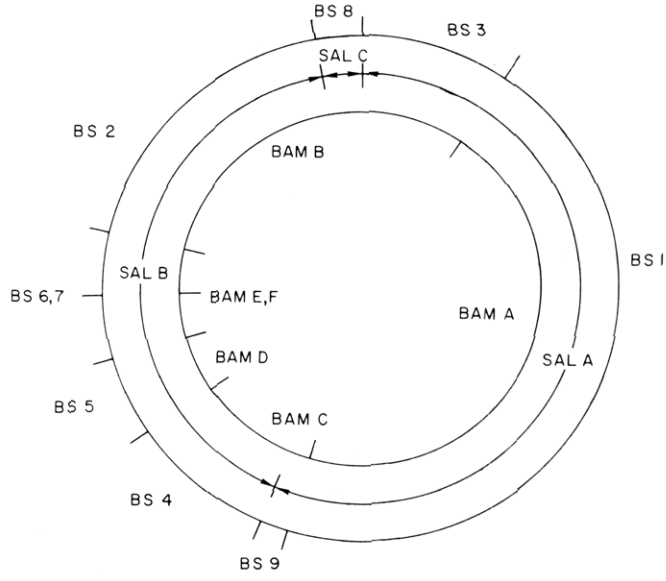

FIGURE 7: BamHI and SalI restriction endonuclease cleavage maps of *Euglena* chloroplast DNA. The letters and numbers refer to cleavage fragments described in the text.

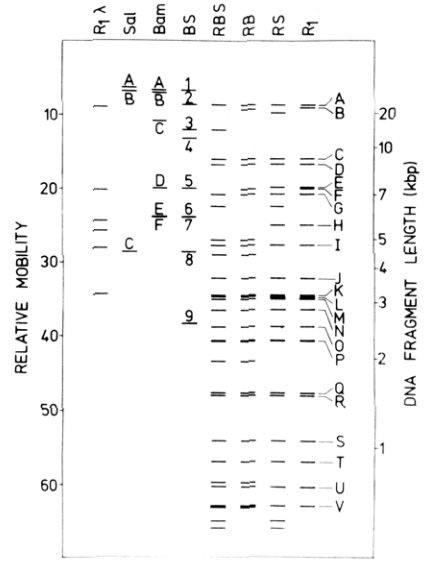

FIGURE 8: Schematic representation of DNA banding pattern of *Euglena* chloroplast DNA digested with restriction endonucleases. Results from a number of experiments are summarized in this figure. Larger fragments (>5 kbp) were analyzed on 0.7% agarose gels, while smaller fragments (0.5–5 kbp) were analyzed on 2.0% agarose gels. Depicted from left to right are: (a) EcoRI, λ DNA; (b) SalI, ct DNA; (c) BamHI, ct DNA; (d) BamHI and SalI, ct DNA; (e) BamHI, SalI, and EcoRI, ct DNA; (f) BamHI and EcoRI, ct DNA; (g) SalI and EcoRI, ct DNA; (h) EcoRI, ct DNA.

in the order of Bam fragments E and F (BS 6,7) which has not yet been resolved. Based on the exonuclease experiment described above, the Bam C–Bam D linkage is indicated. The other two maps are those of the SalI cleavage sites and the BamHI–SalI double digestion sites.

EcoRI Nuclease Digestion of Euglena Chloroplast DNA. When *Euglena* chloroplast DNA is treated with EcoRI endonuclease, at least 22 fragments can be resolved from a limit digestion reaction mixture. The size of the fragments ranges from less than 0.5 kbp to approximately 24 kbp. The larger DNA fragments are resolved well on 0.7% agarose gels, while the smaller pieces were separated on 2% agarose gels. A composite, schematic banding pattern for the 22 bands is shown in Figure 8. Also shown in Figure 8 are schematic representations of the products of *Euglena* chloroplast DNA digested with EcoRI and SalI, with EcoRI and BamHI, and of a triple digestion with all three enzymes. For comparison, the banding patterns with BamHI, SalI, and the BamHI–SalI double digestion of *Euglena* chloroplast DNA are also illustrated. Estimates of the size and stoichiometry of the EcoRI cleavage products are listed in Table II. Summation of the lengths of each fragment yield a value of approximately 125 kbp, somewhat smaller than the estimated size of the genome of 140 kbp. The EcoRI fragment analysis includes several DNA bands not detected on our previously reported gel pattern (Hallick et al., 1976), in which only 17 distinct bands were seen. Previously, EcoRI fragments E and F were an unresolved doublet. These fragments are both separable on 0.7% agarose gels and are further distinguished by internal cleavages sites. One contains a BamHI site, while the other is cleaved with SalI. Additional fragments have also been resolved on 2% agarose gels in the 2.0 to 3.5 kbp size range. What previously appeared as a single broad band at 3.2 kbp is now resolved into fragments K, L, and M, with L present in a stoichiometry of two. Consistent with the assignment of these three fragments was the observation that fragment L is cleaved by BamHI, leaving K, M, and one of the BamHI cleavage products of either EcoRI fragment B, E, or H in this region of the gel. The other darkly staining band in the 2.0–3.5 kbp size range, EcoRI fragment P, 2.3 kbp, is present in a stoichiometry of two or three. Finally, two additional low-molecular-weight fragments, U and V, were not previously seen.

As illustrated in Figure 8, when *Euglena* chloroplast DNA is digested with EcoRI and BamHI endonucleases, EcoRI fragments B, E (or F), H, and L are no longer observed, indicating the presence of an internal BamHI site in these frag-

TABLE II: DNA Cleavage Products Resulting from Digestion of *Euglena gracilis* Chloroplast DNA with Restriction Endonuclease EcoRI.

Fragment	Length (kbp)	Stoichiometry	Internal Cleavage Sites
A	24	1	
B	21	1	SalI, BamHI
C	9.1	1	
D	8.6	1	
E	7.3	1	BamHI (or SalI)
F	7.3	1	SalI (or BamHI)
G	7.0	1	
H	5.5	1	BamHI
I	4.7	1	
J	3.6	1	
K	3.3	1	
L	3.2	2	BamHI
M	3.2	1	
N	2.9	1	
O	2.7	1	
P	2.3	2 or 3	
Q	1.1	1	
R	0.9	1	
S	0.6	1	
T	<0.5	1 or 2	
U	<0.5	1 or 2	
V	<0.5	1	
Totals: 22	125	24–27	

ments. Assuming a stoichiometry of two for fragment L, this accounts for five of the six BamHI sites present. Digestion of *Euglena* chloroplast DNA with EcoRI and SalI leads to internal cleavage of EcoRI fragments B and F (or E), accounting for two of the three SalI sites present. The EcoRI fragments that contain the additional BamHI and SalI sites have not yet been located by double-digestion experiments.

Since EcoRI fragment B contains both an internal BamHI and SalI site, digestion of this fragment with both enzymes should produce one of the Bam–Sal double-digestion products listed in Table I. As illustrated in both Figures 4 and 8, triple digestion of *Euglena* chloroplast DNA with EcoRI, BamHI, and SalI yields BS 3, 13 kbp, as a product. No other BS fragments are produced in this experiment, indicating that the remaining eight BS products all contain internal EcoRI sites. Fragment BS 3 can be unambiguously located within EcoRI fragment B. No other EcoRI fragment except A is large enough to contain a 13-kbp internal fragment, and fragment A is not cleaved by either BamHI or SalI. EcoRI fragment B can therefore be located on the BamHI–SalI cleavage map of Figure 7. Its internal SalI site is the cleavage between BS 8 and BS 3, while its internal BamHI site is the cleavage between BS 3 and BS 1. Work is currently in progress to order the remaining EcoRI fragments with respect to the BamHI–SalI map.

Discussion

We have described a cleavage site map of the chloroplast DNA of *Euglena gracilis*. This analysis is based on the digestion of *Euglena* chloroplast DNA with site specific endonucleases SalI and BamHI. These results represent a structural analysis of one of the largest DNA molecules to be studied in this manner. An important feature of this experimental work was the ability to identify and separate electrophoretically DNA fragments as large as 80 kbp. In order to produce these large DNA fragments, we found it necessary to begin the nuclease digestion studies with intact, superhelical chloroplast DNA. With the availability of preparative amounts of intact chloroplast DNA, these physical mapping studies can in principal be extended to include many of the other previously described site specific endonucleases. For example, during the preparation of this manuscript, we initiated studies on the PstI digestion products of *Euglena* chloroplast DNA. Five cleavage products are produced (P. W. Gray and R. B. Hallick, manuscript in preparation). The size of these DNAs has been tentatively determined as follows: A, 65 kbp; B, 35 kbp; C, 23 kbp; D, 10 kbp; and E, 7 kbp. A preliminary map order of A-(D,E)-B-C and the relation of the PstI cleavage sites to the BamHI and SalI fragments have been elucidated.

Work is currently in progress in other laboratories aimed at characterizing *Euglena* chloroplast DNA by restriction endonuclease cleavage studies. It is of interest to compare some results from these experiments to the present report. Stutz and colleagues have characterized the DNA fragments produced from *Euglena* chloroplast DNA following digestion with endonucleases EcoRI, HindIII, and HaeIII. The EcoRI fragment pattern (Stutz et al., 1977) seems qualitatively identical with the fragment pattern described in this paper. Stutz and colleagues also found that the G + C base content of some EcoRI fragments was clearly higher than the average G + C base content of chloroplast DNA. Four EcoRI fragments which correspond to our fragments B, E, L, and P were found to hybridize with purified *Euglena* chloroplast rRNA. Three of these fragments, E, L, and P, are G + C rich. Two of these DNA fragments, L and P, were estimated to be present in a stoichiometry of three with respect to other fragments. This might indicate that there are three rRNA cistrons per genome. Our best estimate of the stoichiometry of both EcoRI fragments L and P, however, is two, consistent with our earlier estimate of two rRNA cistrons per genome by the criteria of molecular hybridization kinetics (Chelm et al., 1977). Certainly no unequivocal answer to the number of 16S and 23S rRNA cistrons per *Euglena* chloroplast genome is possible until the genes are located on the physical map. Rawson and colleagues have also analyzed *Euglena* chloroplast DNA by digestion studies with EcoRI and BamHI endonucleases. Only three EcoRI fragments, corresponding to our fragments E, L, and P, were found to hybridize with purified chloroplast rRNA (J. R. Y. Rawson, personal communication). An important additional observation was that EcoRI fragment P is located within BamHI fragment D, E, or F. Since Bam D–E, F form a linkage group on our cleavage site map (Figure 7), the approximate location of the rRNA cistron(s) is established. One final observation is that four of the six BamHI cleavage sites are located in the Bam D–E, F linkage group. We find that EcoRI fragment L contains an internal BamHI site, which raises the possibility that fragment L is near P in the Bam D–E, F region.

It is anticipated that more detailed information concerning nuclease cleavage sites and location of chloroplast genes will be forthcoming from this and other laboratories. The map presented in this report is to the best of our knowledge the only physical map of the *Euglena* chloroplast genome available. To facilitate comparison of these results with any future mapping data, we would suggest that a reference point on the genome be designated for orientation purposes. We propose that the center of fragment Sal C be designated as the reference point at 12 o'clock on a circular DNA representation. Sal C is an easily identified fragment, which can be readily analyzed by electrophoresis. Furthermore its location has been determined

with respect to BamHI, SalI, and PstI cleavage sites, as well as to EcoRI fragment B.

The cleavage site map described in this report should provide a useful framework for more detailed studies on the *Euglena* chloroplast genome. The 11 cleavage sites described for the 140-kbp genome are important reference points for any future structural analysis. It is anticipated that structural genes and perhaps control regions will now be located on the DNA with respect to the endonuclease cleavage sites. In principle any of these specific regions of the DNA could be isolated by selection of the appropriate site specific enzymes. A second consequence of this work is the possibility of relating RNA synthesis data to the physical map. Of particular interest will be the orientation of transcribed and nontranscribed segments on the genome. It is hoped that a correlation of RNA transcription data with genome structure may lead to a better understanding of the control of chloroplast gene expression in response to physiologic transitions such as light-induced chloroplast development.

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