Theor. Appl. Genet. 59, 281-296 (1981)

Restriction Endonuclease Cleavage Site Map of Chloroplast DNA from *Oenothera parviflora* (*Euoenothera* Plastome IV)

K.H.J. Gordon, E.J. Crouse, H.J. Bohnert and R.G. Herrmann
Botanisches Institut der Universität, Düsseldorf (Federal Republic of Germany)

Summary. 1) More than 50 cleavage sites produced by the restriction endonucleases Sal I, Pst I, Kpn I, Sma I and Eco RI have been physically mapped on the 47 μm circular DNA molecule of the *Euoenothera* plastome IV. This plastome (= plastid genome) is considered to be the phylogenetically oldest of the subsection.

2) The DNA molecule is segmentally organized into four regions represented by a large duplicated sequence in inverted orientation whose copies are separated by two single-copy segments. The single-copy regions comprise about 14 and 57 Md in size, respectively.

3) The size of the inverted repeat, about 15 Md, was determined by restriction site mapping, by mapping of genes for ribosomal RNAs and by hybridization of a cRNA transcribed from a homologous part of *Spinacia oleracea* chloroplast DNA which appears to be phylogenetically conserved.

4) Hybridization of radio-iodinated spinach 16S, 23S and 5S chloroplast rRNA species to Southern blots of restricted plastome IV DNA has localized the rDNA to the inverted repeat regions, in the order given. The genes for 16S and 23S rRNA are separated by a 2.4 kbp spacer.

5) The physical map of the plastome IV DNA serves as basis for comparison with the DNA from the four other, closely related *Euoenothera* plastomes.

Key words: *Oenothera* – Chloroplast DNA fragments – Reciprocal digestion – Southern transfers – Chloroplast rRNA hybridization

Abbreviations

bp	base pairs
kbp	kilobase pairs
Md	Megadaltons
rDNA	ribosomal DNA
rRNA	ribosomal RNA
cRNA	complementary RNA
SDS	sodium dodecylsulphate
Sal./Cit.	0.15 M NaCl, 0.015 Na citrate, pH 7.2

Introduction

Chloroplast biogenesis requires the co-operation of the nuclear and the plastid genetic systems (for reviews see Ellis 1977; Herrmann and Possingham 1980). The coding potential of the plastome (= plastid genome) is modest in comparison to that of the genome but it plays an essential part in the organelle's biogenesis. It is contained in a single circular DNA molecule of 120-190 kbp (depending on the plant species; Bedbrook and Kolodner 1979; Herrmann and Possingham 1980) which is highly reiterated. Available biochemical data suggest that this chromosome encodes part of the energetic and translational processes of the organelle. Furthermore, when interspecific genome/plastome hybrids are constructed even from closely related species, serious developmental disturbances may be observed. Such an exchange of genetic compartments can readily be accomplished by interspecific crossing in the genus *Oenothera*.

Interspecific genome/plastome hybrids provide several unique insights for the study of genome/plastome interactions, particularly concerning their specificity and evolution. Consequently, we have chosen to investigate the chloroplast DNAs of *Euoenothera*, a subsection of the section *Oenothera* in the genus *Oenothera* (Stubbe and Raven 1979; Raven, Dietrich and Stubbe 1980) in which the genetics and evolution of the plastomes and genomes have been extensively studied (reviewed in Kutzelnigg and Stubbe 1974; Kirk and Tilney-Bassett 1978). Five wild-type plastomes, designated I to V, and three fundamental nuclear genomes, designated A, B and C have been genetically defined. The genomes occur in phenotypically distinguishable homozygous (AA, BB, CC) or complex heterozygous (AB, AC, BC) species. The five plastomes are genetically distinct, based on their compatibility or incompatibility with these six genotypes as well as by their multiplication rates (Kutzelnigg and Stubbe 1974; Schötz 1954, 1968; Stubbe 1959, 1964).

0040/5752/81/0059/0281/$ 3.20

Using restriction analysis we have recently determined that unique chloroplast DNAs are associated with the five basic *Euoenothera* plastid types (Herrmann 1977; Herrmann and Possingham 1980). In this paper we focus on the DNA of plastome IV which is considered to be the most primitive *Euoenothera* plastome in evolutionary terms. We present a detailed physical map based on restriction endonuclease cleavage sites and localization of rRNA genes. In a subsequent paper the maps of the five basic *Euoenothera* plastome DNAs will be compared (Gordon et al. 1980a). Progress reports on this work have been presented by Herrmann (1977) and Herrmann et al. (1980a).

Materials and Methods

Material

Green plants of *Oenothera* homozygous for genome A (^{h}hookeri Johansen) and containing plastome IV (Drillisch 1975) were grown on soil in a greenhouse with supplementary light during the winter. For the preparation of chloroplast ribosomal RNAs, plants of *Spinacia oleracea* var. 'Monopa' were grown under the same conditions. Chloroplasts and chloroplast DNA from *Oenothera* were isolated as described by Gordon et al. (1980b).

Restriction Endonucleases

Restriction endonuclease nomenclature follows the suggestions of Smith and Nathans (1973). Restriction endonucleases Sal I, Pst I and Kpn I were isolated and used as described in Herrmann et al. (1980). Eco RI, Bam HI and Sma I were purchased from Boehringer, Mannheim. These enzymes were used as specified by the suppliers. The DNA fragments obtained after digestion of total chloroplast DNA or after digestion of isolated fragments were separated by 15-20 hours electrophoresis at 50 mA on gels of Seakem agarose (Marine Colloids Inc., Rockland) at concentrations varying from 0.5 to 2.0% (Herrmann et al. 1980b). The DNA fragments were visualised by UV light after staining with ethidium bromide.

Restriction Endonuclease Cleavage Site Mapping

Cleavage site mapping of the chloroplast DNA was performed by reciprocal digestion of individual DNA fragments with the three endonucleases Sal I, Kpn I and Pst I, according to the method of Herrmann et al. (1980b). This method involves the use of low-gelling-temperature agarose for the initial electrophoretic separation of DNA fragments. It eliminates the need for fragment recovery from gels and permits rapid mapping with relatively little DNA. Molecular weights of DNA fragments were determined by coelectrophoresis of standard markers consisting of λ DNA digested with Eco RI and Hind III from Boehringer, Mannheim, ØX 174 DNA digested with Hae III from Bio Labs, Beverley, Ma. and spinach chloroplast DNA digested with Sal I (cf. Driesel et al. 1979). To yield more accurate values, the molecular weights of primary fragments larger than 8 Md, were generally computed as the sum of secondary fragments obtained upon redigestion. Values derived from replicate digestions may differ by 0.1 to 0.4 Md, depending on fragment size, due to inaccuracies inherent in this method.

Preparation of Spinach Chloroplast Ribosomal RNAs (rRNA)

Spinach chloroplasts were isolated as described by Driesel et al. (1979) and the ribosomes isolated from the chloroplasts as described in Herrmann et al. (1976). Chloroplast ribosomal subunits were separated on sucrose gradients (10 to 35% sucrose in 50 m*M* potassium acetate – acetic acid buffer, pH 5.0, containing 150 m*M* KCl, 1 m*M* EDTA and 0.1% diethyl pyrocarbonate). The small ribosomal subunit yielded the 16S rRNA while the large subunit contained the 23S rRNA and in addition a 5S and 4.5S rRNA species (Bedbrook and Kolodner 1979; Herrmann and Possingham 1980). Both the 16S and 23S rRNA species were purified once in linear sucrose gradients (5 to 25% sucrose in the above buffer). The 5S and 4.5S rRNAs were first separated from each other by electrophoresis in 10% polyacrylamide gels. Thereafter the individual bands were cut out, the gel slices were minced and the rRNAs eluted by vigorously shaking the slurry with 20% phenol in 150 m*M* Tris – HCl, pH 8.3, 150 m*M* NaCl, 1% SDS and 1% sodium dodecyl sarcosinate. The aqueous phase was extracted twice with phenol and the RNA precipitated by cold ethanol. The RNA was then dissolved in a small volume of 0.01 × Sal./Cit., desalted on Sephadex G25, reprecipitated and stored dry. Each isolated rRNA was then radioiodinated with ^{125}I according to the method of Commerford (1971) and freed from unreacted iodine by passing over a Sephadex G25 column (1 × 20 cm) in Sal./Cit.. The peak fractions containing the radioiodinated RNA were combined, filtered through nitrocellulose filters (Millipore, 0.22 μm) and the RNA was precipitated. The washed, dried pellet was dissolved in 3 × Sal./Cit. for use in hybridizations without further purification. Specific activities ranged from 5 to 20 × 10^6 cpm/μg.

Preparation of Complementary RNA (cRNA) Probes

The 6.6. Md Sal I fragment from spinach chloroplast DNA was used for the preparation of cRNA. ^{32}P-labelled cRNA was transcribed from this DNA *in vitro* with *E. coli* RNA polymerase using the method of Wensink et al. (1974).

Hybridization of Chloroplast DNA with Labelled RNAs

Transfer of DNA fragments from agarose gels to nitrocellulose filters was performed according to the protocol of Southern (1975). The filters were incubated with labelled RNA for up to 10 hours in 3 × Sal./Cit. at 61°C for the cRNA or at 68°C for the rRNAs. The hybridization was conducted with DNA in excess. After hybridization the strips were washed in 3 × Sal./Cit. at the hybridization temperature for 30 min, dried and exposed to Kodak XR-5 X-ray film for up to 3 weeks. The autoradiographs of the patterns produced by Sma I, Kpn I, Pst I and Eco RI were scanned with a Joyce-Loebl 'Chromoscan' densitometer, allowing a more precise determination of the cleavage sites within the genes for the 16S and 23S rRNAs.

Results

Cleavage of Chloroplast DNA with Restriction Endonucleases

Six restriction endonucleases were chosen for physical mapping of plastome IV DNA from *Euoenothera*. These

Table 1. Number of cleavage sites recognized by various restriction endonucleases on DNA from *Euoenothera* plastome IV

Enzyme	Sequence recognized[a]	Number of cleavage sites
Sal I	GTCGAC	14
Pst I	CTGCAG	11
Kpn I	GGTACC	12
Sma I	CCCGGG	20[b]
Eco RI	GAATTC	60[c]
Bam HI	GGATCC	60[c]

[a]Recognition sequences are given from 5′ to 3′ and are from Roberts (1978). [b]See Table 2. [c]The actual number in each case is probably over 70

Table 2. Molecular weights (in Md) of fragments of *Euoenothera* plastome IV DNA produced by digestion with the restriction endonuclease Sma I

	20
	11
	10.5 (2x)
	6.8 (2x)
	4.5
	4.2
	4.1
	3.8
	3.6
	3.2
	3.0 (2x)
	1.7
	1.1 (2x)
	0.8
	0.6 (2x)
Total (Md)	101
Total No. of fragments	20

are listed in Table 1, which also summarizes the observed numbers of cleavage sites recognized by each enzyme on the total molecule. It proved necessary to map sites of three endonucleases, in order to produce an unambiguous order of restriction sites on the circular chloroplast DNA molecule. The three endonucleases Sal I, Pst I and Kpn I each recognize a low number of sites on the complete molecule. Analysis of their double digestions showed that the secondary fragments can be reasonably resolved, enabling convenient mapping of these restriction sites. The restriction endonucleases Sma I, Eco RI and Bam HI were employed for refinement of the map.

Sal I cleaves the *Oenothera* chloroplast DNA molecule into 14 fragments ranging in size from 0.45-20 Md (Figs. 1a, b, g, h); Pst I generates 11 DNA fragments from 1.25-23.5 Md (Figs. 1a, b, g, h) and Kpn I generates 12 DNA fragments from 0.55-29 Md (Figs. 1c, d, i, j). Molecular weights of all these fragments, rounded to 0.1 Md for fragments under 20 Md and to 0.5 Md for fragments above 20 Md, are given in Figure 2. Double digestion of total chloroplast DNA using Sal I and Pst I generated 25 fragments ranging from 0.1-18.8 Md (Figs. 1a, b, 2a); with Sal I and Kpn I, 26 fragments from 0.45-13.0 Md (Figs. 1c, d, 2b) and using Kpn I and Pst I, 22 fragments from 0.55-13.0 Md (Figs. 1e, f, 2c). The 25th fragment, which is expected in the Sal I + Pst I double digestion, was only indirectly observed because a Sal I and a Pst I restriction site are very close together (Fig. 2a). Similarly, the virtual overlapping of a Pst I and a Kpn I restriction site (Fig. 2c) means that 22 and not the expected 23 fragments are observed in this double digestion. Of the other restriction endonucleases used, Sma I generates 20 fragments (Table 2; Fig. 4g); Eco RI and Bam HI each produce over 60 fragments (Figs. 4i, j, respectively).

In most chloroplast DNA digests a slight to moderate background contamination is evident. Pycnographic analysis of chloroplast DNA (buoyant density, ρ = 1.696 g cm^{-3}) in an analytical ultracentrifuge confirmed that this was due to contamination with some nuclear DNA (ρ = 1.703 g cm^{-3}; Herrmann et al. 1975). In some cases the contamination was also evident as a zone of high molecular weight material in the gels, but this background contamination did not interfere with either DNA digestion or RNA hybridization. In addition, no distinct bands were observed which were inconsistent with the physical map of the chloroplast DNA. Treatment of chloroplasts with DNAase I to remove contaminating nuclear DNA proved infeasible because *Oenothera* homogenates contain tannins and mucilage. These secondary metabolic components apparently alter plastid envelopes which although morphologically intact do not exclude the enzyme.

Mapping Strategy

The serial order of the cleavage sites (or, equivalently, the DNA fragments) was determined by sequential digestion of fragments with two restriction endonucleases. Individual primary fragments obtained with one endonuclease were treated with the second enzyme, and vice versa. The resulting secondary fragments were compared with those in the double and single digestion patterns of total chloroplast DNA. This comparison is facilitated if digestion of the primary fragments is incomplete. A slight cross-contamination of one of the isolated fragments with another caused, for example, by overloading of the primary gel or by degradation of the larger fragments, usually does not affect the analysis.

The mapping procedure used is based on the following principles. A fragment in the double digest is either a primary fragment obtainable only with one of the two enzymes chosen or it is a secondary fragment resulting from cleavage by both enzymes. Such a secondary fragment is shared by two primary fragments, one from each enzyme. Partial or complete colinearity between the primary fragments can then be deduced in the following ways:

(a) If, for example, a Pst I fragment contains no Sal I cleavage site this primary fragment can be recognized among the secondary fragments obtained by digesting an isolated Sal I fragment with Pst I. This Sal I fragment must therefore centrally contain the Pst I primary fragment, that is, at least two Pst I cleavage sites.

(b) If a Pst I fragment has one Sal I cleavage site two secondary fragments are obtained. These can also be identified among the

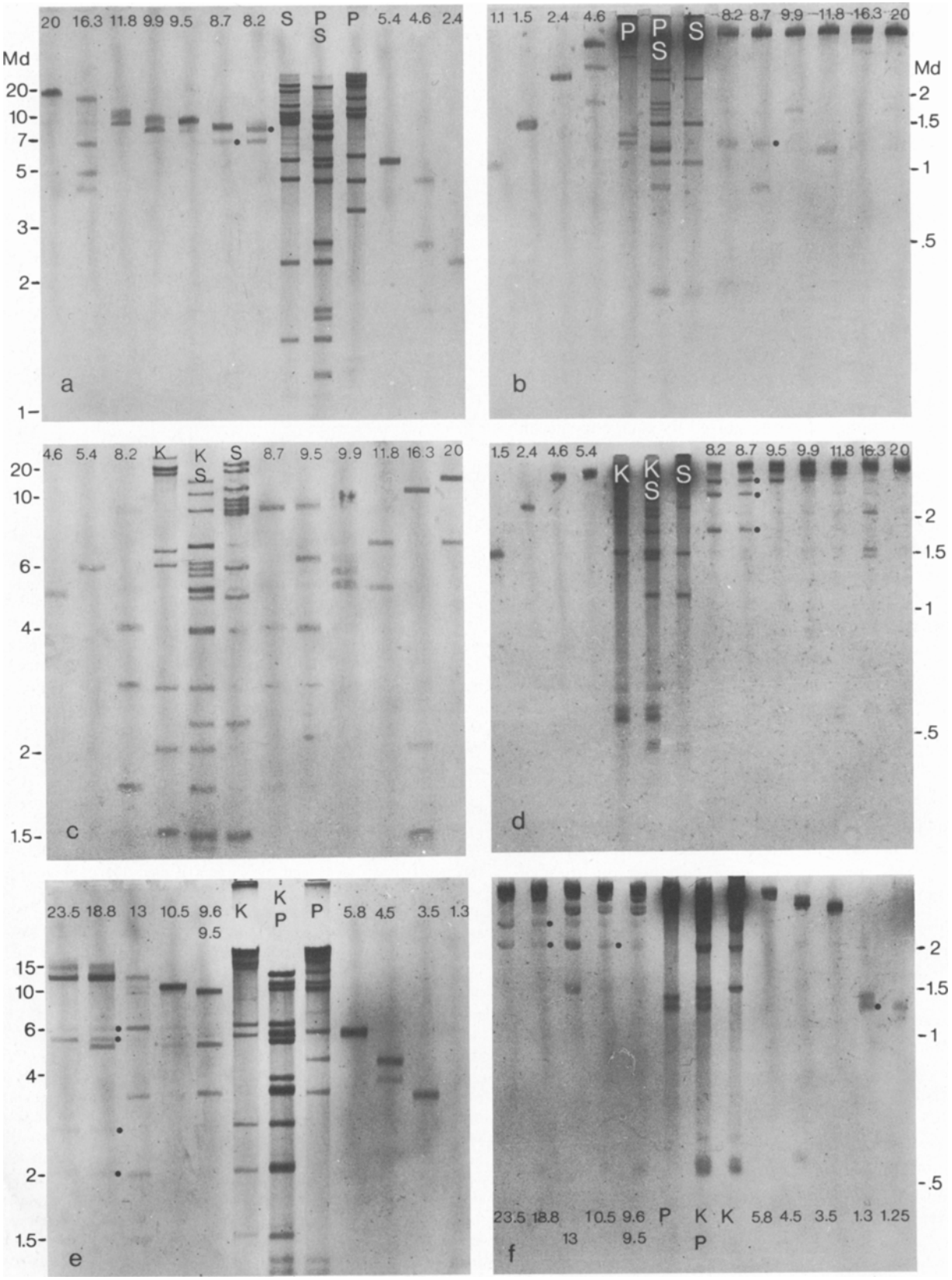
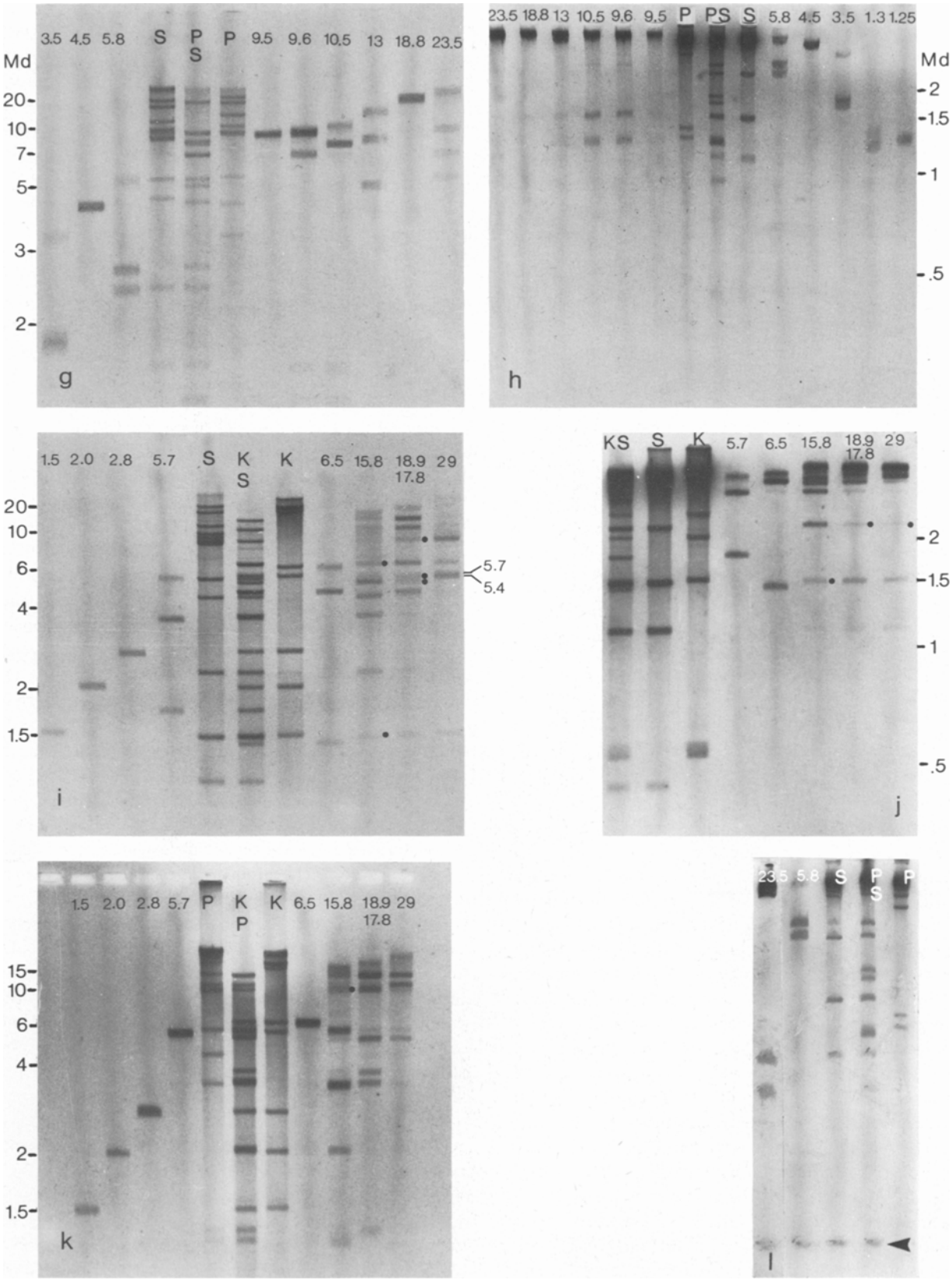


Fig. 1a-l. Sequential digestions of isolated restriction endonuclease fragments of *Euoenothera* plastome IV DNA using two endonucleases. Each gel shows the results of digestion with a second enzyme of a complete (or nearly complete) set of fragments produced by the first enzyme, in addition to single and double digestions of total chloroplast DNA.

Letters in the tracks designate single or double digestions using Sal I (S); Pst I (P) or Kpn I (K). Numbers in the tracks indicate the size in Md of the isolated DNA fragment before redigestion. A molecular weight scale (in Md) is provided for each gel. Dots indicate bands arising



from cross-contamination. **a**, **b** digests of Sal I fragments with Pst I on a 0,6% (**a**) and a 2% (**b**) agarose slab gel. **c**, **d** digests of Sal I fragments with Kpn I on a 0.8% (**c**) and a 2% (**d**) agarose slab gel. **e**, **f** digests of Pst I fragments with Kpn I on a 0.65% (**e**) and a 2% (**f**) agarose slab gel. **g**, **h** digests of Pst I fragments with Sal I on a 0.6% (**g**) and a 2% (**h**) agarose slab gel. **i**, **j** digests of Kpn I fragments with Sal I on a 0.6% (**i**) and a 1.8% (**j**) agarose gel. **k** digests of Kpn I fragments with Pst I on a 0.65% agarose gel. **l** digest of the Pst I primary fragments 23.5 Md and 5.8 Md to demonstrate the origin of the Sal I 0.45 Md primary fragment and 0.45 Md Sal I/Pst I secondary fragment (arrow).

secondary fragments generated by cleaving isolated Sal I primary fragments with Pst I. The two primary Sal I fragments thus identified can be lined up adjacently with the Pst I fragment in the middle, i.e. overlapping them.

(c) If a Pst I fragment has two or more Sal I cleavage sites, upon digestion with Sal I it will yield three or more secondary fragments. The internal fragment or fragments can be identified among the primary fragments of a Sal I digest not cleavable with Pst I. This leaves two terminal fragments, which are also found among the secondary fragments generated by cutting the isolated Sal I fragments with Pst I. The two primary Sal I fragments which overlap the two terminal fragments and bracket the Pst I fragment (and its internal Sal I primary fragment(s)) are mapped in this way. If there are two or more internal (Sal I) fragments, their serial order can be determined with the use of a third enzyme or by partial digestion products.

(d) Ambiguities in arranging fragments will arise if multiple bands exist in the single digestion resulting from primary fragments very similar in size. For example, large fragments of similar size, such as the 17.8 Md and 18.9 Md Kpn I fragments could not normally be separated for the second digestions. Electrophoresis on low-percentage gels for a longer period (over 30 h) confirmed that two different bands were present. The location of these fragments can, however, be solved without their separation since the summation of the sizes of the secondary fragments allows only one arrangement. In other cases, a third enzyme is usually required to determine an unambiguous fragment order. A further limitation in the determination of the serial order of fragments is presented if secondary end fragments of equal or nearly equal molecular weight are generated from more than one primary fragment. Here again, the overlapping primary fragments can sometimes be resolved by mutual mapping with three or more endonucleases. The large inverted sequence duplication of the circular chloroplast DNA molecules presents a special ambiguity which will be discussed below.

Construction of the Sal I + Pst I Map

The reciprocal mapping of Sal I and Pst I (Fig. 2a) cleavage sites will be described in detail, with attention being drawn to ambiguities which could only be settled by comparison with the Kpn I map. Figure 1a shows Sal I fragments which were digested with Pst I and separated on a 0.6% agarose gel in order to clearly distinguish the largest fragments. To locate small secondary fragments, appropriate digests of Sal I fragments were also separated on a 2% agarose gel (Fig. 1b). The results of digestion of Pst I fragments with Sal I are shown in Figs. 1g and 1h.

Digestion of the 20 Md Sal I fragment generates one secondary fragment slightly reduced in size, and corresponding to the 18.8 Md double digestion fragment (Fig. 1a). The 1.2 Md subfragment is visible in Fig. 1b but it is expectedly faint. The 16.3 Md Sal I fragment yields secondary fragments of 6.8 Md, 5.0 Md and 4.5 Md, with some of the undigested fragment remaining (Fig. 1a) and two faint bands considered to be the result of partial digestion. The 11.8 Md Sal I fragment yields a 9.5 Md fragment (Fig. 1a) and a double band at 1.2 Md (Fig. 1b). The 9.9 Md Sal I fragment yields subfragments of 8.2 Md and 1.7 Md (Figs. 1a, b, respectively). The 9.5 Md Sal I fragment is not cleaved by Pst I, which is also the case for the 5.4 Md, 2.4 Md, 1.5 Md and 1.1 Md Sal I fragments. Thus all these fragments in the double digestion pattern are primary Sal I fragments. The 8.7 Md Sal I fragment (contaminated with the 8.2 Md fragment) yields secondary fragments of 7.8 Md and 0.9 Md, whereas the 8.2 Md Sal I fragment yields secondary fragments of 6.8 Md, 1.25 Md and 0.45 Md (Figs. 1a, b). The 4.6 Md Sal I fragment contains one Pst I cleavage site, resulting in two secondary fragments of 2.8 Md and 1.8 Md. All the double digestion fragments have thus been accounted for, with the exception of one 0.45 Md fragment, which is a primary Sal I fragment and thus not cleaved by Pst I.

In the reciprocal experiment with Pst I as the first enzyme and Sal I as the second, digestion of the 23.5 Md Pst I fragment generates fragments of 9.5 Md, 6.8 Md, 5.4 Md (Fig. 1g), 1.1 Md, 0.9 Md and 0.45 Md (Fig. 1 l). The 18.8 Md Pst I fragment lacks a Sal I cleavage site, as do the 9.5 Md, 4.5 Md and 1.25 Md Pst I fragments, so that these fragments in the double digestion pattern are identified as primary Pst I fragments. The 13.0 Md Pst I fragment is cleaved by Sal I into two fragments of 8.2 Md and 5.0 Md, while the 10.5 Md Pst I fragment yields fragments of 7.8 Md, 1.5 Md and 1.2 Md. Although cross-contaminated with the 9.5 Md fragment, the 9.6 Md Pst I fragment clearly is cleaved twice by Sal I, resulting in bands at 6.8 Md, 1.5 Md and 1.2 Md. The 5.8 Md Pst I fragments also contains two Sal I cleavage sites, resulting in fragments at 2.8 Md and 2.4 Md (Fig. 1g) and 0.45 (Figs. 1 h, l). Sal I cleaves the 3.5 Md Pst I fragment once, into fragments of 1.8 Md and 1.7 Md. Finally the 1.3 Md Pst I fragment yields a 1.2 Md secondary fragment upon digestion with Sal I (Fig. 1h) and should also yield a very small fragment of 0.1 Md which, however, is not observable.

From the reciprocal Sal I and Pst I digestions, the serial order of fragments within three segments on the circular molecule of *Oenothera* plastome IV DNA can be established. The determination of serial order is limited by the existence of multiple secondary fragments of 6.8 Md and 1.2 Md which allow alternate arrangements. Table 3 summarizes the relationship between the Pst I and Sal I primary and double digest fragments.

Starting with the 16.3 Md Sal I fragment (Fig. 2a) it is apparent that the 6.8 Md secondary fragment represents the overlap with either the 9.6 Md or the 23.5 Md Pst I fragment. The 4.5 Md fragment is a primary Pst I fragment and therefore must be contained centrally in the 16.3 Md Sal I fragment with the other secondary fragment (5.0 Md) representing the overlap of this Sal I fragment with the 13.0 Md Pst I fragment (Fig. 2a). This Pst I fragment gives rise to only one other secondary fragment, of 8.2 Md, which overlaps with the 9.9 Md Sal I fragment. The

Table 3. Relationships between the Sal I and Pst I primary fragments and the Sal I + Pst I secondary fragments

Secondary fragment (Md)	Primary Sal I fragment from which it is derived (Md)	Primary Pst I fragment from which it is derived (Md)
18.8	20	18.8[a]
9.5 (2x)	11.8, 9,5[a]	23.5, 9.5[a]
8.2	9.9	13.0
7.8	8.7	10.5
6.8 (2x)	16.3, 8.2	23.5, 9.6
5.4	5.4[a]	23.5
5.0	16.3	13.0
4.5	16.3	4.5[a]
2.8	4.6	5.8
2.4	2.4[a]	5.8
1.8	4.6	3.5
1.7	9.9	3.5
1.5 (2x)	1.5 (2x)[a]	10.5, 9.6
1.25	8.2	1.25[a]
1.2 (3x)	20.0, 11.8, 11.8	10.5, 9.6, 1.3
1.1	1.1[a]	23.5
0.9	8.7	23.5
0.45 (2x)	8.2, 0.45[a]	23.5, 5.8
0.1	20	1.3

[a] denotes primary fragments which contain no cleavage site for the other enzyme. Molarity of fragments is given in brackets

next Pst I fragment is the 3.5 Md one, identified by a common 1.7 Md overlap with this primary Sal I fragment. The other secondary fragment from this Pst I fragment is 1.8 Md, and this overlaps with the 4.6 Md Sal I fragment which in turn has a fragment of 2.8 Md at the other end, this being an end segment of the 5.8 Md Pst I fragment. The uncleaved 2.4 Sal I fragment is contained within this Pst I fragment. As mentioned above, one of the two 0.45 Md fragments then forms the overlap between the 5.8 Md Pst I fragment and the 8.2 Md Sal I fragment, which includes the uncleaved 1.25 Md Pst I fragment. The other end segment of the 8.2 Md Sal I fragment is one of the 6.8 Md secondary fragments. The serial order of fragments covering about one-third of the molecule is thus established (Fig. 2a).

The ends of the remaining two-thirds of the molecule are formed by the 9.6 Md and 23.5 Md Pst I fragments. Within this large segment, the serial orders of two groups of fragments may be determined; each comprises about half of the segment. Starting with the 23.5 Md fragment, the end Pst I and Sal I secondary fragment is of 6.8 Md. The Sal I primary fragments 9.5 Md, 5.4 Md, 1.1 Md and 0.45 Md are contained within the 23.5 Md fragment. Their relative order cannot be determined using these two enzymes. The 9.5 Md band in the double digestion of total chloroplast DNA is two-molar. However, the 9.5 Md fragment from the 23.5 Md Pst I fragment is a primary Sal I fragment, and can thus be distinguished from the 9.5 fragment derived from the 11.8 Md Sal I fragment. This latter fragment is a Pst I primary fragment; its mapping will be discussed below. The remaining secondary fragment derived from the 23.5 Md Pst I fragment is of 0.9 Md and represents the overlap with the 8.7 Md Sal I fragment. The other secondary fragment of the latter is of 7.8 Md and shows overlap with the 10.5 Md Pst I fragment, which also includes one of the two 1.5 Md Sal I primary fragments and one of the three 1.2 Md secondary fragments.

The second group of fragments comprises the 11.8 Md and 20 Md Sal I primary fragments, which are directly adjacent, by the following reasoning. The 1.2 Md secondary fragment, from the 10.5 Md Pst I primary fragment, can represent an overlap with either of these large Sal I primary fragments. The 9.6 Md Pst I primary fragment yields a 6.8 Md secondary fragment (the end secondary fragment of the two-thirds of the molecule currently under discussion), a 1.5 Md fragment which is an internally located primary Sal I fragment and a 1.2 Md secondary fragment. This last fragment again may represent an overlap with either the 11.8 Md or the 20 Md Sal I fragment.

Assuming that it is the 11.8 Md Sal I fragment which overlaps the 9.6 Md Pst I fragment (Fig. 2), the 9.5 Md Pst I primary fragment is located entirely therein (see above). The remaining 1.2 Md secondary fragment is the end fragment of the 11.8 Md Sal I fragment and represents an overlap with the 1.3 Md Pst I fragment. Since all Sal I primary fragments have now been accounted for, the 11.8 Md Sal I fragment must link up with the 20 Md Sal I fragment 'coming' from the other direction (Fig. 2a). Mapping using Kpn I fragments has shown that no Kpn I fragment ends colinearly with the 11.8 Md or 20 Md Sal I fragments, nor with the 1.3 Md or 18.8 Md Pst I fragments (Figs. 2b, c, respectively). Thus, although no secondary fragment of approximately 0.1 Md has been directly demonstrated as the overlap between the 1.3 Md Pst I primary fragment and the 20 Md Sal I primary fragment, such a small fragment must exist. Its size is determined by the size reduction of the 1.3 Md Pst I fragment after digestion with Sal I.

The difficulty to identify primary fragments overlapping the multiple 1.5 Md primary and 1.2 Md secondary fragments implies that nearly one third of the DNA molecule comprising the 1.5 Md, 11.8 Md and 20 Md Sal I primary fragments has two possible orientations.

Mapping with Kpn I

The aim of mapping the restriction sites recognized by the third enzyme, Kpn I, was to resolve ambiguities present in the Sal I + Pst I map, to check the maps obtained from

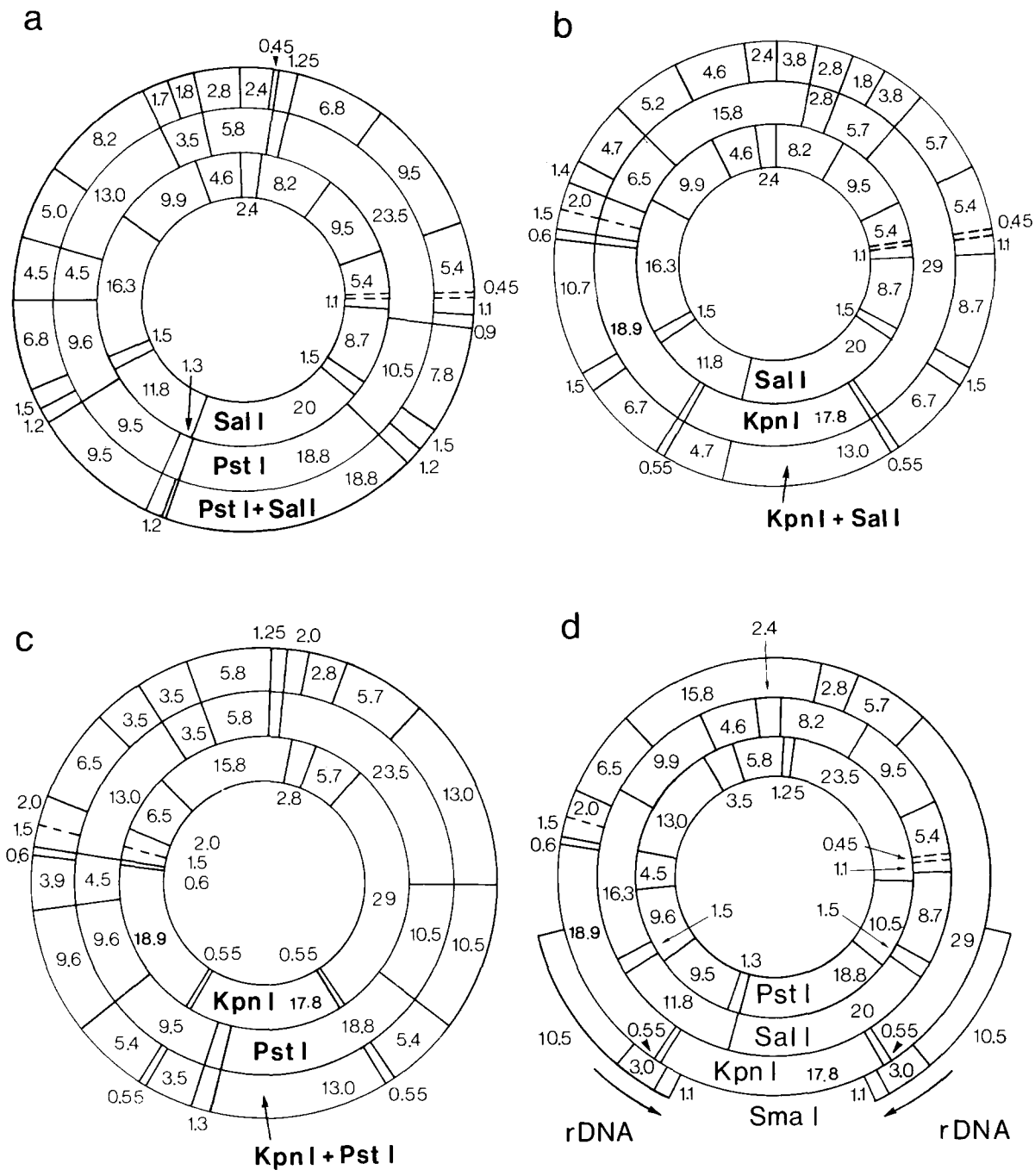


Fig. 2a-d. Restriction endonuclease cleavage site maps of DNA from *Euoenothera* plastome IV. Parts a, b and c show the serial orders of the fragments produced upon single and double digestion with the endonucleases Sal I, Pst I and Kpn I. In each of these diagrams, the two inner rings depict the serial order of cleavage sites arising in the single digestions, while the outer ring shows the serial order of the double digestion fragments. In all cases fragments are identified by their size in Md. The double digestion fragments, contained within the sector corresponding to a particular single digestion fragment, have been verified by redigestion of the fragment in question. Broken lines indicate that the order of the two adjacent fragments is ambiguous (see text).

A composite map (d) shows the positions of the three sets of cleavage sites produced by Pst I, Sal I and Kpn I, respectively. Again, broken lines indicate that the order of the adjacent fragments is ambiguous. The six Sma I fragments shown have been located by RNA hybridizations (see text). They illustrate the location and minimum extent of the two copies of the inverted repeat region. The rDNA units are indicated by arrows giving their location, approximate length and orientation from the 16S rRNA to the 23S rRNA genes

Table 4. Relationship between Sal I and Kpn I primary fragments and the Kpn I + Sal I secondary fragments

Secondary fragment (Md)	Primary Sal I fragment from which it is derived (Md)	Primary Kpn I fragment from which it is derived (Md)
13.0	20	17.8
10.7	16.3	18.9
8.7	8.7[a]	29
6.7 (2x)	11.8, 20	18.9, 29
5.7	9.5	29
5.4	5.4[a]	29
5.2	9.9	15.8
4.7 (2x)	9.9, 11.8	6.5, 17.8
4.6	4.6[a]	15.8
3.8 (2x)	8.2, 9.5	5.7, 15.8
2.8	8.2	2.8[a]
2.4	2.4[a]	15.8
2.0	16.3	2.0[a]
1.8	8.2	5.7
1.5 (3x)	1.5 (2x)[a], 16.3	1.5[a], 18.9, 29
1.4	16.3	6.5
1.1	1.1[a]	29
0.6	16.3	0.6[a]
0.55 (2x)	11.8, 20	0.55 (2x)[a]
0.45	0.45[a]	29

[a] denotes primary fragments which contain no cleavage site for the other enzyme. Molarity of fragments is given in brackets

these endonucleases and to achieve finer mapping of stretches of DNA where few cleavage sites for Sal I and Pst I are located. The mapping of Kpn I cleavage sites was accomplished following the same principles as described for Sal I + Pst I mapping.

The Kpn I + Sal I map: Figs. 1c and d show the redigestions of Sal I primary fragments with Kpn I; the reciprocal experiments are shown in Figs. li and lj. Table 4 summarizes the results of these redigestions. The primary Sal I and Kpn I fragments from which each observed secondary fragment is derived are given. From this analysis of the secondary fragments, it is possible to deduce the serial order and thus construct the Kpn I + Sal I map.

One ambiguity presented in the earlier Sal I + Pst I mapping was the orientation of the segments delimited by the 6.8 Md secondary fragments. Analysis of the Kpn I + Sal I mapping shows that the 8.2 Md and 9.5 Md Sal I fragments must be adjacent as shown in Figure 2b, with the 5.7 Md Kpn I fragment overlapping them.

Since these two Sal I primary fragments are adjacent, another ambiguity, the order of Sal I fragments within the 23.5 Md Pst I primary fragment, is thus partially resolved. The order of the adjacent fragments 5.4 Md, 1.1 Md and 0.45 Md Sal I primary fragments is not known, so they are separated by a dotted line as shown in Figs. 2a, b, d. The order of Sal I primary fragments is thus – (11.8 – 20) – 1.5 – 8.7 – (1.1 – 0.45 – 5.4) – 9.5 – 8.2 – 2.4 – 4.6 – 9.9 – 16.3 – 1.5 –. Fragments whose molecular weights are given in brackets have not been ordered relative to each other.

The only major difficulty remaining with the map, after analysis of Kpn I sites, is that there are effectively two groups of fragments, the relative orientation of which is unknown. The adjacent 11.8 Md and 20 Md Sal I primary fragments may be inserted in the physical map of the circular DNA molecule in inverse orientation to that shown in Figs. 2a and b. This situation is discussed below in the section on the inverted repeat.

Mapping of Kpn I sites from the data in Table 4, allows the simultaneous checking of the order determined for Sal I and Pst I cleavage sites. The established sites in turn will assist in the resolution of ambiguities presented by the Kpn I mapping data resulting in a mutual checking of the maps of the three sets of cleavage sites. Some examples of the location of Kpn I restriction fragments on the map will now be presented, starting with those related to the 16.3 Md Sal I fragment.

Three primary Kpn I fragments, of 2.0 Md, 1.5 Md and 0.6 Md, which are not digested by Sal I, must be contained entirely within the 16.3 Md Sal I fragment, so that the 10.7 Md and 1.4 Md secondary fragments are its end fragments. The only 1.4 Md secondary fragment observed in the double digestion derives from the 6.5 Md Kpn I primary fragment, which therefore overlaps the 16.3 Sal I fragment. The 9.9 Md Sal I primary fragment is known to lie next to the 16.3 Md Sal I fragment (Fig. 2a) and a 4.7 Md secondary fragment does in fact form the overlap between the 6.5 Md Kpn I fragment and the 9.9 Md Sal I fragment (Fig. 2b). The results of double and reciprocal digestions using Kpn I are therefore consistent with the mapping of Sal I and Pst I sites.

The other secondary fragment from the 9.9 Md Sal I fragment is of 5.2 Md, and shows overlap with the 15.8 Md Kpn I fragment (Fig. 2b). This latter Kpn I fragment contains internally the 4.6 Md and 2.4 Md Sal I primary fragments in agreement with the order established by mapping with Pst I (Fig. 2a). The remaining end fragment, of 3.8 Md, could be an overlap with either the 9.5 Md or the 8.2 Md Sal I fragments. The earlier mapping demonstrates that the latter is the correct one. This fragment contains, centrally, the 2.8 Md Kpn I primary fragment. The remaining end fragment, of 1.8 Md, overlaps with the 5.7 Md Kpn I fragment. This Kpn I fragment in turn shares sequences with the 9.5 Md Sal I fragment (the overlap being the other 3.8 Md secondary fragment) and thus links the major segments, obtained by Sal I + Pst I mapping, in a unique orientation, as mentioned above.

The next Kpn I fragment, which overlaps with the 9.5 Md Sal I fragment, is the 29 Md one. Also in agreement with previous mapping this contains five Sal I primary

fragments, of 8.7 Md, 5.4 Md, 1.5 Md, 1.1 Md. and 0.45 Md. The other end fragment from the 29 Md Kpn I fragment is of 6.7 Md and indicates overlap with either the 20 Md or the 11.8 Md Sal I fragments (see section on inverted repeat). Each of these yields, moreover, a 0.55 Md Kpn I primary fragment, which must be centrally located within each of the Sal I fragments. One of the 0.55 Md fragments must therefore be adjacent to the 29 Md Kpn I fragment.

The remaining third of the chloroplast DNA molecule is covered by the 17.8 Md, 18.9 Md and a 0.55 Md Kpn I fragments. Although the two largest of these were not individually isolated the only possible combination of the secondary fragments, compatible with the molecular weights for the primary fragments, is as shown in Fig. 2b. Taking the 20 Md Sal I fragment as that overlapping with the 29 Md Kpn I fragment, an end fragment of 13 Md is obtained. The other secondary fragment from the Kpn I primary fragment, which yields this 13 Md secondary fragment, must be 4.7 Md. The Sal I + Pst I mapping shows that the 11.8 Md Sal I fragment is adjacent, overlapping this 4.7 Md secondary fragment. Its other end fragment is 6.7 Md, which must come from the 18.9 Md Kpn I primary fragment. The latter also includes a 1.5 Md Sal I primary fragment and a 10.7 Md end fragment, which overlaps with the 16.3 Md Sal I primary fragment.

Some ambiguities remain after mapping of Sal I and Kpn I cleavage sites. For example, the 0.6 Md, 1.5 Md and 2.0 Md Kpn I fragments are all within the 16.3 Md Sal I fragment and could be interchanged but the observation of an intermediate in the Kpn I digestion pattern, at about 2.2 Md (Fig. 1d) shows, that the 1.5 Md fragment is adjacent to the 0.6 Md fragment (see also next section). Furthermore, although the two 4.7 Md secondary fragments in the Kpn I + Sal I double digest present ambiguity, the order of Sal I fragments here has already been determined and so the identities of the overlapping Kpn I fragments are as shown in Fig. 2b. Similarly, mapping using Sal I + Pst I resolved the ambiguity arising from the double band at 3.8 Md.

The Kpn I and Pst I map: A similar analysis was performed with Kpn I and Pst I restriction sites, and again confirms the predicted arrangement (Figs. 2a, b). Digestions of Pst I primary restriction fragments with Kpn I are shown in Figs. 1e and f. Fig. 1k shows redigestion of relevant Kpn I fragments on a 0.6% gel. Table 5 summarizes the results of these digestions.

The position of the 4.5 Md Pst I fragment has been established within the 16.3 Md Sal I fragment (Fig. 2a) and it is cleaved once by Kpn I, generating a 3.9 Md secondary fragment and another of 0.6 Md. The former is also yielded by the 18.9 Md Kpn I primary fragment, the latter must be an end segment but is also equivalent to the 0.6 Md Kpn I primary fragment. This means that a Kpn I and a Pst I cleavage site lie so close together that the intervening stretch of a few base pairs cannot be observed. The orientation of the 0.6 Md Kpn I fragment on the map is thus established.

Table 5. Relationship between the Pst I and Kpn I primary fragments and the Pst I + Kpn I secondary fragments

Secondary fragment (Md)	Primary Pst I fragment from which it is derived (Md)	Primary Kpn I fragment from which it is derived (Md)
13.0 (2x)	18.8, 23.5	17.8, 29
10.5	10.5[a]	29
9.6	9.6[a]	18.9
6.5	13.0	6.5[a]
5.8	5.8[a]	15.8
5.7	23.5	5.7[a]
5.4 (2x)	9.5, 18.8	18.9, 29
3.9	4.5	18.9
3.5 (3x)	3.5[a], 9.5, 13.0	15.8 (2x), 17.8
2.8	23.5	2.8[a]
2.0 (2x)	13.0, 23.5	2.0[a], 15.8
1.5	13.0	1.5[a]
1.3	1.3[a]	17.8
1.25	1.25[a]	15.8
0.6	4.5	0.6[a]
0.55 (2x)	9.5, 18.8	0.55 (2x)[a]

[a] denotes primary fragments which contain no cleavage site for the other enzyme. Molarity of fragments is given in brackets

As a further example, the 15.8 Md Kpn I primary fragment contains internally three primary Pst I fragments, of 5.8 Md, 3.5 Md and 1.25 Md (Figs. 2c, d). A 2.0 Md secondary fragment (not the primary Kpn I fragment of this size, whose location was defined in Fig. 2b) forms the overlap with the 23.5 Md Pst I fragment. This confirms that the 1.25 Md and 23.5 Md Pst I fragments are adjacent (Fig. 2c), with the 15.8 Md Kpn I fragment overlapping.

One end fragment from the 29 Md Kpn I primary fragment is 5.4 Md. This secondary band is two-molar and is derived from the 18.8 Md and from one of the mixed 9.6 Md/9.5 Md Pst I primary fragments. This implies that the region from the 9.5 Md Pst I fragment to the 18.8 Md Pst I fragment may be inverted, as found from earlier mapping. The 1.3 Md Pst I fragment is generated as expected upon digestion of the unseparated 18.9 Md/17.8 Md Kpn I fragments.

Whereas the Kpn I + Pst I mapping does not provide resolution of the locations of the adjacent 9.5 Md and 9.6 Md Pst I primary fragments, their digestion data is consistent with the analysis presented for the Sal I + Pst I mapping. Other ambiguities resulting from the double band at 13.0 Md and the triple band at 3.5 Md in the Kpn I /Pst I double digestion, could be resolved. The order shown in Fig. 2c is the only one compatible with the serial order of the Sal I fragments.

Occurrence of an Inverted Repeat; Segmental Organisation of the Circular DNA Molecule

Figure 2d shows the colinear arrangement of the cleavage sites obtained with the three restriction endonucleases. The major ambiguity which has not yet been resolved is the orientation of the 1.5 Md, 11.8 Md and 20 Md Sal I primary fragments (and overlapping Pst I and Kpn I fragments) relative to the rest of the map. In particular the two-molar 6.7 Md Kpn I + Sal I secondary fragment (Fig. 2b), the two-molar 1.2 Md Sal I + Pst I fragment (Fig. 2a) and the two-molar 5.4 Md Kpn I + Pst I secondary fragment (Fig. 2c) cannot be uniquely placed.

These two-molar fragments must be present in two clusters. The identical cleavage maps within the clusters suggest that identical nucleotide sequences cover these DNA segments. Their relative locations on the circular DNA molecule show that they are present in inverted orientation (Fig. 2). Moreover, due to the asymmetry of adjacent cleavage sites, the copies of the duplication must be separated by two regions of single-copy DNA differing in size. Examples of such sites are, in the smaller region, the Pst I cuts between the 9.5 Md and the 1.3 Md fragments and between the 1.3 Md and the 18.8 Md fragments or the Sal I cut between the 11.8 Md and the 20 Md fragments. Therefore the circular DNA molecule is segmentally organized into four regions.

Fig. 3a and b. Hybridization of the cRNA transcribed from the 6.6 Md Sal I fragment of spinach chloroplast DNA to *Euoenothera* plastome IV DNA. In each case, a DNA digestion pattern is shown to the left of the autoradiograph and the molecular weights (in Md) of DNA fragments to which cRNA hybridized are given. Both double digestions were run on the same 0.6% agarose gel. In **b**, faint hybridization to an intermediate band at about 10.5 Md is probably due to undigested Pst I and Sma I primary fragments of this size

The Extent of the Inverted Repeat

A minimum length of 8.7 Md for this inverted repeat can be computed from the above data. That is the 1.5 Md Sal I and 0.55 Md Kpn I primary fragments and the 1.2 Md Pst I + Sal I and 5.4 Md Kpn I + Pst I secondary fragments, within each cluster.

In order to determine the limit of extension of the inverted repeat towards the large single-copy region, the cRNA transcribed from the 6.6 Md Sal I fragment of the inverted duplication in spinach chloroplast DNA was used. This fragment contains 40% of the inverted repeat region nearest the large single-copy region (Driesel et al. 1979; Herrmann et al. 1980b). This cRNA hybridized specifically with the 7.8 Md and 6.8 Md secondary fragments of the Sal I + Pst I double digestion of *Euoenothera* plastome IV DNA, in addition to the two-molar 1.5 Md Sal I band in this double digest (Fig. 3a, cf. Fig. 1a, and see map in Fig. 2a).

The location of Sma I fragments, determined by hybridization of labelled rRNAs (see below), allows more precise definition of the limits of the inverted repeat region towards the large single-copy region. Digestion of the 10.5 Md Sma I fragment and its overlapping Pst I (9.6 Md or 10.5 Md) fragments with Sma I + Pst I generates a 7.5 Md secondary fragment which includes the part of the inverted repeat region nearest the large single-copy region (Figs. 2d, 7). Hybridization of the above mentioned cRNA to a Sma I/Pst I double digest (Fig. 3b) confirms this. There is also faint hybridization to the two-molar 0.6 Md band (Fig. 3b and cf. Fig. 5d, Table 2), which could represent small Sma I primary fragments located next to the 10.5 Md Sma I fragments, towards the large single-copy region.

Since the cRNA did not hybridize to fragments beyond the Pst I cleavage site between the 10.5/23.5 Md Pst I fragments on one side of the molecule, or the 9.6/4.5 Md Pst I fragments on the other, it appears that the Sma I cleavage site at the end of the 10.5 Md Sma I fragment is close to the end of the inverted repeat region. The segment of *Oenothera* chloroplast DNA which is homologous to the 6.6. Md Sal I fragment of spinach chloroplast DNA therefore starts in the 1.5 Md Sal I fragments (since no hybridization is observed to the adjacent 1.2 Md Sal I + Pst I secondary fragments, Fig. 3a), includes a 4.8 Md segment bounded by Sal I and Sma I cleavage sites (i.e. from the 1.5 Md Sal I fragment to the end of the 10.5 Md Sma I fragment nearest the large single-copy region) and proba-

bly an additional 0.6 Md Sma I fragment. The sum of these fragments (6.7 Md) is close to that of the 6.6 Md Sal I fragment in spinach chloroplast DNA.

As for spinach, hybridization of this cRNA to Eco RI partial and complete digestions of total chloroplast DNA from *Oenothera* showed that at least 6 fragments ranging from about 1.7 Md down to about 0.5 Md in size are derived from this segment. This confirms considerable sequence homology between chloroplast DNA of these two higher plant species in this part of the plastid DNA molecule.

In the other direction towards the small single-copy region the inverted repeat is limited by the Pst I cleavage site between the 9.5 Md and 1.3 Md primary fragments and by an equivalent region in the second copy (Fig. 2d). The locations of some Sma I (and Eco RI) sites in this part of the inverted repeat region determined by rRNA hybridizations confirm this (see below).

In conclusion a minimum length of 15 Md can then be calculated for one copy of the inverted duplication.

Mapping of rRNA in the Inverted Repeat

In order to identify the regions of the *Oenothera* chloroplast DNA which code for the ribosomal RNA molecules, hybridization experiments were performed using isolated, radioactively labelled ribosomal RNA from spinach as a probe with restriction endonuclease fragments of *Oenothera* chloroplast DNA. Specific hybridization of individual DNA fragments was obtained with the 16S (Fig. 4), 23S (Fig. 5) and 5S (Fig. 6) ribosomal RNA.

The 16S ribosomal RNA hybridizes with two Sal I fragments, with two Kpn I fragments and with two Pst I fragments (Figs. 4a-c). Since, in the case of Pst I, the hybridizing fragments (18.8 Md and 9.5 Md) are separated by one which does not hybridize (1.3 Md, Fig. 2d) two copies of the gene must be present. This is supported by the Pst I/Sal I double digestion experiment, in which hybridization to these uncleaved primary Pst I fragments was observed (Fig. 4e).

In the Kpn I/Sal I double digestion, the two-molar 6.7 Md band hybrized to the 16S rRNA (Fig. 4d) and in the combination Kpn I + Pst I, hybridization occurred with the two-molar 5.4 Md band (Fig. 4f). The analysis of the distant locations of the hybridizable secondary fragments on the maps (Figs. 2b, c) and of the primary fragments (Fig. 2d) show that the two copies of the gene for the 16S rRNA must be separated by at least 18 Md of DNA. The location of the hybridizable fragments shows one 16S rRNA gene to be located in each copy of the inverted repeat, that is, within the segments defined by the Kpn I cleavage site separating the 0.55 Md from the 29 Md fragment and the Pst I cleavage site separating the 18.8

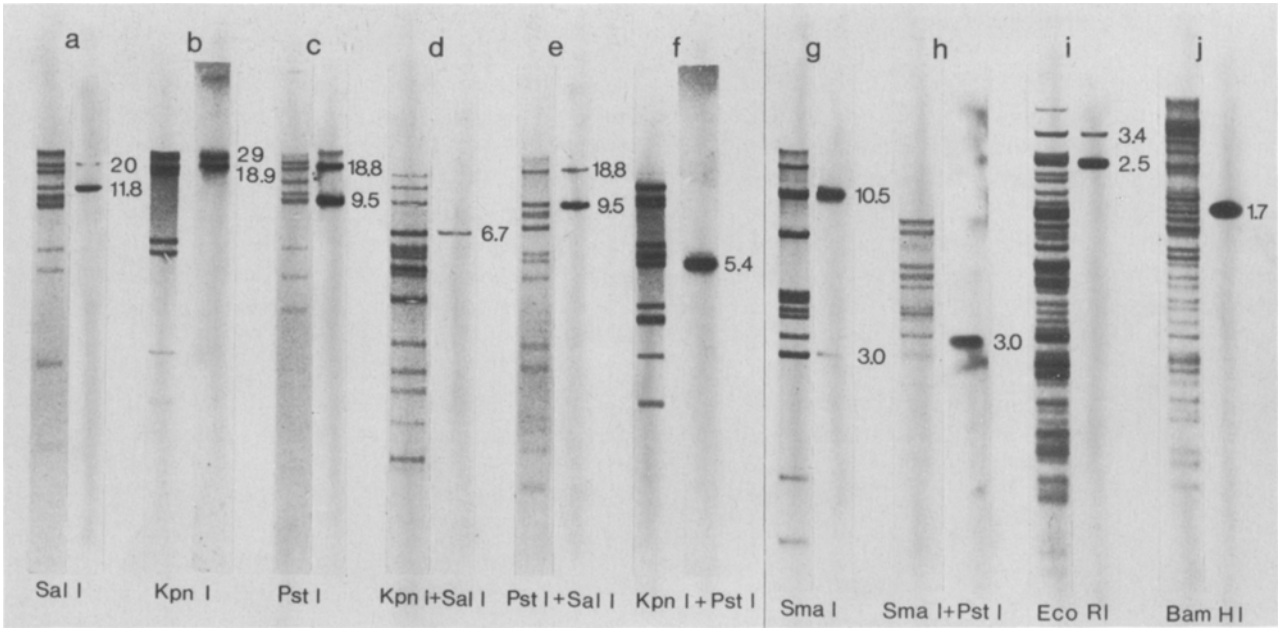


Fig. 4. Hybridization of spinach chloroplast 16S rRNA to restriction endonuclease digests of DNA from *Euoenothera* plastome IV. Two to 4 μg of total chloroplast DNA were used per gel slot. In each case, the digestion pattern of the DNA (left) is shown with the autoradiograph of the 16S rRNA hybridization (right). The sizes of DNA fragments to which the rRNA hybridizes are indicated in Md. The weaker radioactive band in (c) above the 18.8 band is due to incompletely digested material. Electrophoresis of DNA was on 0.6% agarose gels in (a) to (h), on 1.4% in (i) and (j)

Md from the 10.5 Md fragment on the one, and the 0.55 Md from the 18.9 Md Kpn I fragments and the 9.5 Md from the 9.6 Md Pst I fragments on the other.

The 23S ribosomal RNA hybridizes to the same Kpn I and Pst I primary fragments as the 16S ribosomal RNA (Figs. 5a and b). In addition hybridization occurred to the primary 0.55 Md Kpn I fragment of the Kpn I/Pst I digest (Fig. 5c), indicating that the 23S ribosomal RNA gene is located adjacent to the 16S ribosomal RNA gene. Hybridization also occurs to the two-molar 5.4 Md band (cf. Fig. 4f for 16S rRNA) and to the 13.0 Md and 3.5 Md fragments (Fig. 2c). These fragments were previously shown to extend partly into the small single-copy region and are separated by the 1.3 Md Pst I primary fragment. This permits the conclusion that there are also two copies of the 23S ribosomal RNA gene and that they are located between the 16S ribosomal RNA genes and the small single-copy region.

The 5S ribosomal RNA hybridized to the same two Pst I fragments of 18.8 and 9.5 Md (Fig. 6a) as the 16S and 23S ribosomal RNA indicating that there are also at least two copies for this gene and that these are close to the other two ribosomal RNA genes.

Hybridization of the rRNAs to DNA fragments produced by the restriction endonuclease Sma I made it possible to better define the location of the genes. This enzyme cleaves the total chloroplast DNA into 20 fragments the smallest being 0.6 Md in size (Table 2, Figs. 4g and 5d). Of these fragments, 12 are present in six two-molar bands, three of which have sizes of 10.5 Md, 3.0 Md and 1.1 Md. The 16S rRNA hybridizes with the 10.5 Md and 3.0 Md fragments (Fig. 4g), the 23S rRNA with the 3.0 Md and 1.1 Md fragments (Fig. 5d) and the 5S rRNA with the 1.1 Md fragment (Fig. 6b). This would be consistent with an arrangement as depicted in Fig. 7 giving colinearity in the order Sma I 10.5 Md, 3.0 Md, 1.1 Md and the genes for 16S – 23S – 5S rRNAs.

The 16S rRNA was also hybridized to a Sma I/Pst I double digestion of the total chloroplast DNA (Fig. 4h). A strong hybridization occurred only with a 3.0 Md band, which therefore must include a secondary fragment derived from the primary 10.5 Md Sma I fragment and probably the primary 3.0 Md Sma I fragment (Fig. 4g). This locates the Pst I cleavage site in the 10.5 Md Sma fragment 3 Md from the end of this fragment which permits it to be aligned as drawn in Figs. 2d and 7.

The hybridization data indicated that the rRNA genes within each copy of the inverted repeat must be fairly close together. Since the restriction endonucleases Eco RI and Bam HI each cleave the complete chloroplast DNA

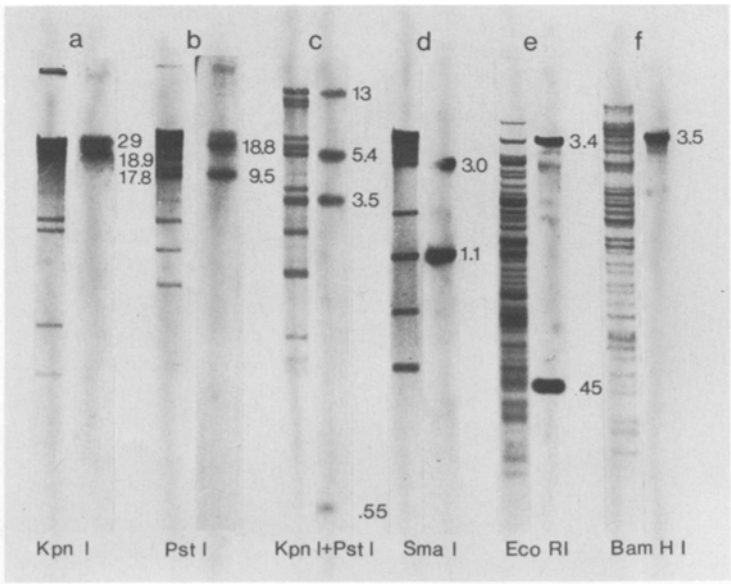


Fig. 5.

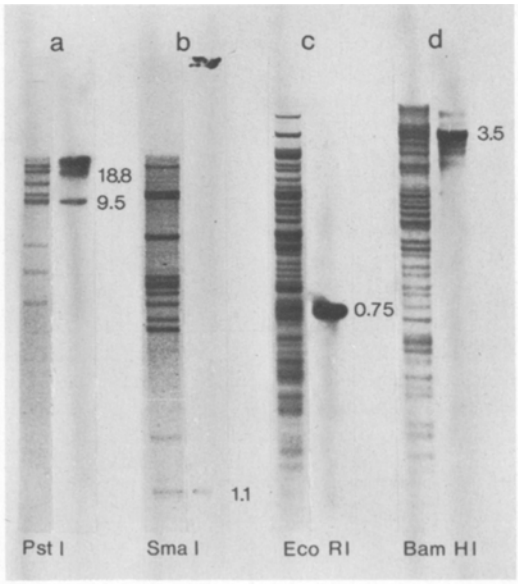


Fig. 6.

Fig. 5. Hybridization of spinach chloroplast 23S rRNA to restriction endonuclease digest patterns of DNA from *Euoenothera* plastome IV. Two to four μg of total chloroplast DNA were used per gel slot. In each case the digestion pattern of the DNA (left) is shown with the autoradiograph of the 23S rRNA hybridization (right). The sizes of the DNA fragments to which the 23S rRNA hybridizes are indicated in Md. In track (a), the 0.55 Md Kpn I fragment has run off the gel and thus no hybridization is visible in contrast to (c). The weaker radioactive band in (b) above the 18.8 band is due to incompletely digested material. Electrophoresis was on 0.6% agarose gels in (a) to (c), 2% agarose in (d) and 1.4% agarose in (e) and (f)

Fig. 6. Hybridization of spinach chloroplast 5S rRNA to digestions of DNA from *Euoenothera* plastome IV. The DNA fragment in the digestion pattern (left) to which the 5S rRNA hybridizes (right) is indicated in Md. Electrophoresis of DNA was on 0.6% agarose gels for (a) and (b) and 1.4% agarose for (c) and (d). The radioactive band above the 18.8 Md fragment in lane (a) is due to hybridization to incompletely digested chloroplast DNA

molecule into over 60 fragments (Figs. 4i, j). Hybridization of rRNAs to fragment patterns produced by these enzymes was therefore employed to map the rDNA region in greater detail.

Hybridization of 16S rRNA occurs with two Eco RI fragments showing that an Eco RI cleavage site lies within the 16S rRNA gene (Fig. 4i). As judged from the intensity of hybridization, the larger part of the gene is located on the 2.5 Md fragment. The remainder of the 16S rRNA gene is on the 3.4 Md fragment which also contains most of the 23S rRNA gene (Fig. 5e). Another part of this latter gene is present on a 0.45 Md fragment. The 5S rRNA gene is located on a 0.75 Md Eco RI fragment (Fig. 6c) which can be deduced from the Sma I hybridization to be adjacent to the 0.45 Md fragment. Hybridization of the 23S and 5S rRNAs to the same 3.5 Md Bam HI fragment (Figs. 5f, 6d) confirms the close positions of those genes, but the 16S rRNA hybridizes to a different Bam HI fragment as shown in Fig. 4j. These results refine the map of the ribosomal RNA genes (Fig. 7).

The similar sizes of the DNA fragments, located in the rDNA region, exclude substantial variation in the transfer rate from gel to filter and in the binding to the filter. Therefore the hybridization intensities derived from the autoradiographs provide an approximate indication of the locations of the cleavage positions within genes. Considering the sizes of DNA fragments and taking the lengths of the 16S and 23S rRNA genes to be about 1.1 Md (1500 bp) and 2.0 Md (2900 bp), respectively, it is evident that both genes must be separated by a spacer segment. This segment is about 1.6 Md (2400 bp) long. The total length of the rDNA unit thus comprises about 35% in each 15 Md copy of the inverted repeat region.

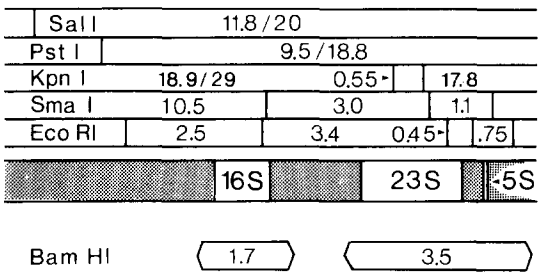


Fig. 7. Map of restriction endonuclease cleavage sites within the part of the inverted repeat region containing the rDNA. The approximate locations of the 16S, 23S and 5S rRNA genes are indicated with their lengths assumed to be the same as in spinach (1.1 Md, 2.0 Md and less than 0.1 Md, respectively). The lengths of DNA fragments to which hybridization occurs are given in Md. For Sal I, Pst I and Kpn I, the two copies of the inverted repeat region are present on fragments of different sizes, as indicated in Md separated by a stroke. Only the Bam HI fragments to which the rRNAs hybridize are indicated because the positions of the Bam HI cleavage sites in relation to those of the other enzymes have not yet been determined

Discussion

The circular restriction endonuclease cleavage site map of *Euoenothera* plastome IV DNA shows an organization similar to that found with other higher plant chloroplast DNAs (Bedbrook and Kolodner 1979; Herrmann and Possingham 1980). It is divided into four regions: two copies of an inverted repeat region are separated by a large and a small single-copy region. The inverted repeat region is about 15 Md large, a size close to that of chloroplast DNA from spinach (Crouse et al. 1978; Herrmann et al. 1980b) and lettuce (Kolodner and Tewari 1979) and a little greater than that reported for chloroplast DNA from corn (Bedbrook et al. 1976). The small single-copy region has a maximum size of less than 15 Md, also very similar to that of spinach.

As with other chloroplast DNAs, the relative orientation of the large and small single-copy regions has not been determined. A restriction endonuclease which does not cleave within the inverted repeat would offer an approach towards solving this problem, but such an enzyme has not yet been found. It remains therefore to be shown whether one or both of the possible orientations exist.

Hybridization of spinach chloroplast rRNAs to *Oenothera* chloroplast DNA shows that two sets of rRNA genes are found which are located within about 35% of each copy of the inverted repeat, near the small single-copy region. The high level of homology among chloroplast rRNAs of different species has been reported by Ingle et al. (1970) and Thomas and Tewari (1974) and was substantiated by fine mapping studies (Bohnert et al. 1980). The present study shows that the homology extends into the part of the inverted repeat near the large single-copy region indicating that the sequence duplication in chloroplast DNAs is evolutionary conserved.

The clustering of the rRNA genes in the order 16S – 23S – 5S, the location of this cluster within each inverted repeat and its orientation with respect to the large and small single-copy regions of the chloroplast DNA molecule are again similar to that found for some other higher plant chloroplast DNAs, e.g. spinach (Crouse et al. 1978; Whitfeld et al. 1978a) and corn (Bedbrook et al. 1976). The 4.5S chloroplast rRNA (Bohnert et al. 1976; Whitfeld et al. 1978b; Bowman and Dyer 1979) was not hybridized.

There is a large spacer of about 2400 bp between the 16S and 23S rRNA genes of *Oenothera* chloroplast DNA. This is about 600 bp longer than the equivalent spacer in spinach chloroplast DNA (Bohnert et al. 1979) and 300 bp longer than that in corn (Bedbrook et al. 1976). Finer mapping is needed to establish whether a spacer exists between the 23S and 5S rRNA genes in *Oenothera* as has been shown in spinach (Crouse et al. 1978) and corn (Dyer and Bedbrook 1979). This spacer could be at most 500 bp long and should be located on the 1.1 Md Sma I and the 0.75 Md Eco RI primary fragments.

It has not been determined for *Oenothera* whether the 16S and 23S rRNA genes are transcribed as a single unit, as in spinach (Bohnert et al. 1977; Hartley 1979) nor whether the spacer DNA between the 16S and 23 rRNA genes contains genes for transfer RNAs as in spinach chloroplast DNA (Bohnert et al. 1979; Driesel et al. 1979).

Acknowledgement

The excellent technical assistance of Miss Christine Michalowski, Miss Barbara Schiller and Miss Monica Streubel is gratefully appreciated. We would also like to thank Prof. W. Stubbe (Düsseldorf) for helpful suggestions, Dr. J.V. Possingham (CSIRO, Adelaide) for reading the paper, Dr. P.R. Whitfeld (CSIRO, Canberra) for making available unpublished data and for reading the paper and Prof. D. von Wettstein (Carlsberg Research Laboratory, Copenhagen) for valuable discussions and critically reading the manuscript. We are very grateful to Lena Kongsrud, Hanne Them Nielsen, Nine Rasmussen, Inge Sommer and Ann-Sofi Steinholtz for their help in the preparation of the manuscript. Endonuclease Pst I was a kind gift of Dr. H. Meyer, Gesellschaft für Biotechnologische Forschung, Braunschweig. The work was supported by the Deutsche Forschungsgemeinschaft (Bo 573 and He 693), the Heinrich-Hertz-Stiftung and Forschungsmittel des Landes Nordrhein-Westfalen. K.H.J.G. was the holder of a Stipendium from the Deutscher Akademischer Austauschdienst.

Literature

Bedbrook, J.R.; Kolodner, R. (1979): The structure of chloroplast DNA. Annu. Rev. Plant Physiol. 30, 593-620

Bedbrook, J.R.; Kolodner, R.; Bogorad, L. (1976): *Zea mays* chloroplast ribosomal RNA genes are part of a 22000 base pair inverted repeat. Cell 11, 739-749

Bedbrook, J.R.; Link, G.; Coen, D.M.; Bogorad, L.; Rich, A. (1978): Maize plastid gene expressed during photoregulated development. Proc. Natl. Acad. Sci. (Wash.) 75, 3060-3064

Bohnert, H.J.; Driesel, A.J.; Crouse, E.J.; Gordon, K.; Herrmann, R.G.; Steinmetz, A.; Mubumbila, M.; Keller, M.; Burkard, G.; Weil, J.H. (1979): Presence of a transfer RNA gene in the spacer sequence between the 16S and 23S rRNA genes of spinach chloroplast DNA. FEBS Lett. 103, 52-56

Bohnert, H.J.; Driesel, A.J.; Herrmann, R.G. (1976): Characterization of the RNA compounds synthesized by isolated chloroplasts. In: Genetics and Biogenesis of Chloroplasts and Mitochondria (eds.: Büchner, Th. et al.), pp. 629-636. North Holland: Elsevier

Bohnert, H.J.; Driesel, A.J.; Herrmann, R.G. (1977): Transcription and processing of transcripts in isolated unbroken chloroplasts. In: Acides Nucléiques et Synthese des Proteines chez les Végétaux. (eds.: Bogorad, L., Weil, J.H.), pp. 213-218. Paris: CNRS

Bohnert, H.J.; Gordon, K.H.J.; Crouse, E.J. (1980): Sequence homologies among chloroplast, mitochondrial and bacterial ribosomal RNA and messenger RNA. Mol. Gen. Genet. (submitted)

Bowman, C.M.; Dyer, T.A. (1979): 4.5S ribonucleic acid, a novel ribosome component in the chloroplasts of flowering plants. Biochem. J. 183, 605-613

Commerford, S.L. (1971): Iodination of nucleic acids in vitro. Biochemistry 10, 1993-1999

Crouse, E.J.; Schmitt, J.M.; Bohnert, H.J.; Gordon, K.; Driesel, A.J.; Herrmann, R.G. (1978): Intramolecular compositional heterogeneity of *Spinacia* and *Euglena* chloroplast DNAs. In: Chloroplast Development (eds.: Akoyunoglou, G. et al.), pp. 565-572. North Holland: Elsevier

Driesel, A.J.; Crouse, E.J.; Gordon, K.; Bohnert, H.J.; Herrmann, R.G.; Steinmetz, A.; Mubumbila, M.; Keller, M.; Burkard, G.; Weil, J.H. (1979): Fractionation and identification of spinach chloroplast transfer RNAs and mapping of their genes on the restriction map of chloroplast DNA. Gene 6, 285-306

Drillisch, M. (1975): Vergleichende Untersuchungen an den 'A-Genotypen' von *Oenothera*. Dissertation, Universität Düsseldorf

Dyer, T.A.; Bedbrook, J.R. (1979): The organization in higher plants of the genes coding for chloroplast ribosomal RNA, p. 47. Genome Organization and Expression in Plants, Edinburgh

Ellis, R.J. (1977): Protein synthesis by isolated chloroplasts. Biochim. Biophys. Acta 463, 185-215

Gordon, K.H.J.; Crouse, E.J.; Bohnert, H.J.; Herrmann, R.G. (1980a): Physical mapping of differences in chloroplast DNA from the five wild-type plastomes in *Oenothera* subsection *Euoenothera*. (in preparation)

Gordon, K.H.J.; Hildebrandt, J.W.; Bohnert, H.J.; Herrmann, R.G.; Schmitt, J.M. (1980b): Analysis of the plastid DNA in an *Oenothera* plastome mutant deficient in ribulosebisphosphate carboxylase. Theor. Appl. Genet. 57, 203-207

Hartley, M.R. (1979): The synthesis and origin of chloroplast low-molecular weight ribosomal ribonucleic acid in spinach. Eur. J. Biochem. 96, 311-320

Herrmann, R.G. (1977): Studies on *Oenothera* plastid DNAs. p. 48. Int. Conf. on Regulation of Developmental Processes in Plants, Halle

Herrmann, R.G.; Bohnert, H.J.; Crouse, E.J.; Driesel, A.J.; Schmitt, J.M.; Gordon, K. (1979): Physical mapping and localization of genes on *Spinacia* and *Oenothera* chloroplast DNA. Hoppe-Seyler's Z. Physiol. Chem. 360, 281

Herrmann, R.G.; Bohnert, H.J.; Driesel, A.; Hobom, G. (1976): The location of rRNA genes on the restriction endonuclease map of the *Spinacia oleracea* chloroplast DNA. In: Genetics and Biogenesis of Chloroplasts and Mitochondria (eds.: Bücher, Th. et al.), pp. 351-359. North Holland: Elsevier

Herrmann, R.G.; Bohnert, H.J.; Kowallik, K.V.; Schmitt, J.M. (1979): Size, conformation and purity of chloroplast DNA of some higher plants. Biochim. Biophys. Acta 378, 305-317

Herrmann, R.G.; Possingham, J.V. (1980): Plastid DNA – the Plastome. In: Results and Problems in Cell Differentiation: The Chloroplast. (ed.: Reinert, J.), pp. 45-96. Berlin, Heidelberg, New York: Springer.

Herrmann, R.G.; Seyer, P.; Schedel, R.; Gordon, K.; Bisanz, C.; Winter, P.; Hildebrandt, J.W.; Wlaschek, M.; Alt, J.; Driesel, A.J.; Sears, B.B. (1980a): The plastid chromosomes of several dicotyledons. In: Biological Chemistry of Organelle Formation (eds.: Bücher, T. et al.), pp. 97-112. Berlin, Heidelberg, New York: Springer

Herrmann, R.G.; Whitfield, P.R.; Bottomley, W. (1980b): Construction of a Sal I/Pst I restriction map of spinach chloroplast DNA using low-gelling-temperature agarose electrophoresis. Gene 8, 179-191

Ingle, J.; Possingham, J.V.; Wells, R.; Leaver, C.J.; Loening, U.E. (1970): The properties of chloroplast ribosomal-RNA. In: Control of Organelle Development. Vol. 24, pp. 303-325. Cambridge: Univers. Press

Kirk, J.T.; Tilney-Bassett, R.A.E. (1978): The Plastids. North Holland: Elsevier

Kolodner, R.; Tewari, K.K. (1979): Inverted repeats in chloroplast DNA from higher plants. Proc. Nat. Acad. Sci. (Wash.) 76, 41-45

Kutzelnigg, H.; Stubbe, W. (1974): Investigations on plastome mutants in *Oenethera*. I. General considerations. Sub-Cell. Biochem. 3, 73-89

Raven, P.H.; Dietrich, W.; Stubbe, W. (1980): An outline of the systematics of *Oenothera* subsect. *Euoenothera* (Onagraceae). System. Bot. (submitted)

Roberts, R.J. (1978): Restriction and modification enzymes and their recognition sequences. Gene 4, 183-193

Schötz, F. (1954): Über Plastidenkonkurrenz bei *Oenothera*. Planta 43, 182-240

Schötz, F. (1968): Über Plastidenkonkurrenz bei *Oenothera* II. Biol. Zbl. 87, 33-61

Smith, H.O.; Nathans, D. (1973): A suggested nomenclature for bacterial host modification and restriction systems and their enzymes. J. Mol. Biol. 81, 419-423

Southern, E.M. (1975): Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517

Stubbe, W. (1959): Genetische Analyse des Zusammenwirkens von Genom und Plastom bei *Oenothera*. Z. Vererbungsl. 90, 288-298

Stubbe, W. (1964): The role of the plastome in evolution of the genus *Oenothera*. Genetica 35, 28-33

Stubbe, W.; Raven, P.H. (1979): A genetic contribution to the taxonomy of *Oenothera* sect. *Oenothera* (including subsections *Euoenothera, emersonia, raimannia* and *munzia*. Plant Syst. Evol. 133, 39-59

Thomas, J.R.; Tewari, K.K. (1974): Ribosomal-RNA genes in the chloroplast DNA of pea leaves. Biochim. Biophys. Acta 361, 73-83

Wensink, P.C.; Finnegan, D.J.; Donelson, J.E.; Hogness, D.S. (1974): A system for mapping DNA sequences in the chromosomes of *Drosophila melanogaster*. Cell 3, 315-325.

Whitfeld, P.R.; Herrmann, R.G.; Bottomley, W. (1978a): Mapping of the ribosomal RNA genes on spinach chloroplast DNA. Nucl. Acids Res. 5, 1741-1751

Whitfeld, P.R.; Leaver, C.J.; Bottomley, W.; Atchison, B.A. (1978b): Low-molecular weight (4.5 S) ribonucleic acid in higher plant chloroplast ribosomes. Biochem. J. 175, 1103-1112

Received July 8, 1980
Communicated by D. von Wettstein

Dr. H.J. Bohnert
Prof. Dr. R.G. Herrmann
Botanisches Institut der Universität Düsseldorf
Universitätsstr. 1.
D – 4000 Düsseldorf (Federal Republic of Germany)

Dr. K.H.J. Gordon*
Department of Biochemistry
University of Adelaide
Adelaide, South Australia 5001 (Australia)

Dr. E.J. Crouse*
Institut de Biologie Moléculaire et Cellulaire, C.N.R.S.
Université Louis Pasteur
15 rue Descartes
F – 67084 Strasbourg Cedex (France)

* Present address