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Chloroplast DNA Sequence Homologies among Vascular Plants[1]

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ABSTRACT

The extent of sequence conservation in the chloroplast genome of higher plants has been investigated. Supercoiled chloroplast DNA, prepared from pea seedlings, was labeled *in vitro* and used as a probe in reassociation experiments with a high concentration of total DNAs extracted from several angiosperms, gymnosperms, and lower vascular plants. In each case the probe reassociation was accelerated, demonstrating that some chloroplast sequences have been highly conserved throughout the evolution of vascular plants. Only among the flowering plants were distinct levels of cross-reaction with the pea chloroplast probe evident; broad bean and barley exhibited the highest and lowest levels, respectively. With the hydroxylapatite assay these levels decreased with a decrease in probe fragment length (from 1,860 to 735 bases), indicating that many conserved sequences in the chloroplast genome are separated by divergent sequences on a rather fine scale. Despite differences observed in levels of homology with the hydroxylapatite assay, S1 nuclease analysis of heteroduplexes showed that outside of the pea family the extent of sequence relatedness between the probe and various heterologous DNAs is approximately the same: 30%. In our interpretation, the fundamental changes in the chloroplast genome during angiosperm evolution involved the rearrangement of this 30% with respect to the more rapidly changing sequences of the genome. These rearrangements may have been more extensive in dicotyledons than in monocotyledons. We have estimated the amount of conserved and divergent DNA interspersed between one another.

From the reassociation experiments, determinations were made of the percentage of chloroplast DNA in total DNA extracts from different higher plants; this value remained relatively constant when compared with the large variation in the diploid genome size of the plants.

Analysis by restriction endonucleases has shown differences in the fine structure of the chloroplast genome in a variety of higher plants (1). However, restriction enzyme maps can be altered by a single nucleotide substitution (29) and do not necessarily reflect the over-all sequence divergence of a particular genome. Hybridization studies employing labeled ribosomal RNA from pea have demonstrated that the chloroplast ribosomal cistrons of monocotyledons and dicotyledons are highly conserved (28). These cistrons comprise only about 8% of the chloroplast genome which ranges in size from 85 to 95 × 10^6 daltons (15); in corn the ribosomal cistrons exist in duplicate within an inverted repeat (3). Denaturation maps of chloroplast DNA from corn and pea show little sequence similarity, but the location of replication initiation (D-loops) may be the same (16).

In the above studies a limited number of loci were used to assess the sequence relatedness of the chloroplast genomes of higher plants. In this paper we have tried to determine the extent of base sequence conservation of the entire chloroplast genome using reassociation kinetics analysis. As pointed out in reviews by Tewari (27) and Kung (17), information of this kind has not been available for chloroplast DNA. Walbot (30) compared *Panicum* and cabbage DNAs and found about 50% homology. Information on sequence conservation should prove useful in aligning the physical and genetic maps of chloroplast DNA from different species. In addition, such information is essential for establishing the functional difference between those sequences that are transcribed and those conserved during evolution. In our studies labeled pea chloroplast DNA was used as a probe to search for homologous sequences in total DNA from a variety of higher plants. The levels of homology we observed were dependent on the fragment length of the probe, an indication that many of the conserved sequences in the chloroplast genome are not arranged in long contiguous blocks, but rather are interrupted by more rapidly evolving segments of DNA. Analysis of short heteroduplexes using S1 nuclease showed a core of conserved sequences among the angiosperms.

From the rate of reassociation of the probe with an excess of heterologous DNA, we estimated the percentage of chloroplast DNA in different higher plants. Although the values are relative, they suggest that the weight ratio of chloroplast DNA to nuclear DNA does not vary dramatically from one species to another despite a great variation in nuclear genome size. However, for pea we have observed a 2.5-fold change in chloroplast content during seedling development.

MATERIALS AND METHODS

Preparation of Chloroplast DNA. Pea chloroplast DNA was prepared following the method of Kolodner and Tewari (14). Isolated chloroplasts were treated with DNase (Worthington, RNase-free), and washed four times with 0.3 M sucrose, 0.05 M Tris, 0.02 M EDTA (pH 8.0) and lysed by incubating at 37 C for 45 min with 2% Sarkosyl, 0.05 M Tris, 0.02 M EDTA (pH 8.0), and 100 μg/ml pronase. The lysate was extracted with phenol, previously neutralized with 0.01 M Tris base. Two volumes of ethanol were added to the aqueous phase and after storage at −20 C overnight the precipitate was collected by centrifugation at 10,000 rpm for 20 min in the Sorvall SS34 rotor and resuspended in 0.05 M Tris, 2 M EDTA (pH 8.0). CsCl (optical grade, Harshaw) was added to a density of 1.58 g/cm^3, ethidium bromide to 210 μg/ml, and the samples were centrifuged for 40 h at 40,000 rpm in the Spinco Ti-65 rotor. The lower UV fluorescent bands from several gradients were combined and recentrifuged. The final lower band, after extraction of ethidium bromide with 0.01 M EDTA-saturated isopentyl alcohol and dialysis against 0.01 M Tris, 1 mM EDTA (pH 8.0), was then used as purified chloroplast DNA. Superhelicity of the DNA was confirmed by electron microscopy; no short linear molecules were seen.

Preparation of Driver DNAs. Total DNA was isolated from shoot tissue of the following: pea (*Pisum sativum* L., cv. Alaska), broad bean (*Vicia faba* L., cv. Broad Windsor), watermelon (*Citrullus vulgaris* Schrad. cv. Dixie Queen), muskmelon (*Cucumis*

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melo L., cv. Iroquois), squash (*Cucurbita moschata* Poir., Cv. Butternut), wakerobin lily (*Trillium ovatum* Pursh), daffodil (*Narcissus pseudonarcissus* L., cv. King Alfred), corn (*Zea mays* L., cv. wf9 × Mo. 17), barley (*Hordeum vulgare* L., cv. Trebi), sitka spruce (*Picea sitchensis* [Bong.] Carr.), western white pine (*Pinus monticola* D. Don), *Equisetum telmateia* Ehrh., *Psilotum nudum* (L.) Griseb., *Tmesipteris billardieri* Endl., and parsley fern (*Cryptogramma crispa* [L.] R. Br.). The method employed has been described by Bendich and Anderson (4, 5). It includes pulverization of frozen tissue, extraction with SDS, ribonuclease and pronase digestion, repeated chloroform extraction, and ethanol precipitation. *Bacillus subtilis* DNA was extracted by the method of Marmur (19). All DNAs were sonicated with a Braunsonic 1510 at a power setting of 120 w for 5 min in 0.02 M NaP,[2] 1 mM EDTA (pH 8.0) in an ice bath. After dialysis against 1 mM Tris, 1 mM EDTA (pH 8.0), samples were stored at −20 C.

Labeling of Probe DNA. Purified chloroplast DNA was labeled with tritiated thymidine *in vitro* by the "nick-translation" procedure of Maniatis *et al.* (18), with the assistance of Dr. D. J. Merlo, to a specific radioactivity of approximately 1×10^6 cpm/μg. The labeled DNA was passed over a Sephadex G-75 column, the excluded counts combined, dialyzed to 1 mM Tris, 1 mM EDTA (pH 8.0) and used as a probe in the reassociation experiments. *B. subtilis* probe DNA was prepared in the same way.

Reassociation of DNA. Replicate points for each reassociation curve were prepared by drying a mixture of probe and driver DNAs, and resuspending in 1 M $NaClO_4$, 0.03 M Tris, 0.1 mM EDTA (pH 8.0), 0.1% Sarkosyl to a final concentration of 150 μg/ml driver DNA unless otherwise indicated in the figure legends. Ten-μl aliquots were sealed in capillary tubes, heated for 3.5 min at 100 C, and incubated at 60 C (chloroplast) or 64 C (*B. subtilis*). T_m values were determined spectrophotometrically for native chloroplast and *B. subtilis* DNAs in 0.14 M NaP buffer (pH 6.8) and used to estimate the appropriate reassociation temperatures in 1 M $NaClO_4$ employing the empirical relationship, $T_{m\ (0.14\ M\ NaP)} + 2.5\ C = T_{m\ (1\ M\ NaClO_4)}$. After reassociation, samples were quickly cooled to 0 C, transferred into 1 ml 0.15 M NaP, and loaded onto hydroxylapatite (Bio-Gel HTP)-cellulose (Whatman CF11) columns at 60 C. Single strands were eluted with 1.5 ml 0.15 M NaP, and double strands were collected with 1.5 ml 0.3 M NaP. Additional washes showed no further elution of radioactivity. After diluting to correct for salt differences, radioactivity was measured by using 4 ml of scintillant (1 volume of Triton X-100 plus 2 volumes of Omnifluor dissolved in toluene) for each 1 ml of aqueous sample.

S1 Nuclease Assay. A 10-μl aliquot of reassociated DNA in 1 M $NaClO_4$ was transferred to a 100-μl reaction mixture containing 100 mM NaCl, 25 mM Na-acetate, 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 5 mM β-mercaptoethanol (pH 4.4), yielding a final sodium ion concentration of approximately 0.2 M. S1 nuclease (provided by M.-D. Chilton) was added and the mixture was incubated for 90 min at 37 C. After raising the volume to 1 ml and addition of 50 μg/ml BSA, S1 resistance was determined by precipitability in 10% trichloroacetic acid at 4 C. These conditions digest 94% of a denatured and quickly cooled sample of probe DNA.

Determination of Fragment Length. Horizontal alkaline gel electrophoresis (22) was used to determine single strand fragment lengths. Eco RI restriction fragments of phage P22 were employed as size markers; corrections were made for deviations from linearity of fragments less than 400 bases. Samples were mixed with an equal volume of 0.02 M NaOH, 4 mM EDTA, 15% glycerol, 0.05% bromphenol blue, and loaded into 7-mm-thick gels of 1% agarose, 0.03 M NaOH, 2 mM EDTA; electrophoresis was for 19 h at 1.4 v/cm. For labeled probe DNA the gel was sliced and each piece was steamed at 100 C in 2 ml water, mixed with 8 ml scintillant and counted. Determinations of weight average length were made using the equation of Hinnebusch *et al.* (10): $L_w = \Sigma CPM_i L_i / \Sigma CPM_i$, where CPM_i equals the radioactivity in a slice, and L_i equals the average length of DNA in each slice. L_i was estimated by adding the largest and smallest fragment per slice, and dividing by 2. The length of unlabeled, sonicated DNA was estimated from a densitometer tracing of a Polaroid negative of an ethidium bromide-stained gel. In the above equation the total number of squares of pen deflection was substituted for CPM_i. The profile of single-stranded sonicated DNA was a symmetrical peak.

Thermal Denaturation Curves. DNA reassociated to 50 to 70%, was loaded onto HAP in 0.15 M NaP at 60 C and washed to remove single strands. Each column was washed at 60 C with 1.5 ml of either 0.06 or 0.08 M KP and then at 5 intervals to 98 C. The eluates at each temperature were counted as described above. The cumulative radioactivity eluted at each temperature was used to construct thermal elution curves.

RESULTS

CHARACTERIZATION OF CHLOROPLAST PROBE

For the kinetic studies of this work, pea chloroplast DNA was labeled *in vitro* by the nick-translation process (18) and used as a probe. The probe was tested for its ability to reassociate using both chloroplast DNA and total DNA from pea plants as drivers. Figure 1 shows that the reassociation of the probe in the presence of a high concentration of unlabeled pea chloroplast DNA followed a second order, single kinetic component curve that terminated at 80% completion measured on HAP. The inset of Figure 1 presents the same data after Scatchard analysis (20); the Scatchard plot was used to estimate the half-reassociation and termination points of the reaction. Reassociation to 80% completion was unexpected for a simple DNA such as chloroplast DNA. The probe was made from supercoiled DNA isolated from DNase-treated chloroplasts and purified by two sequential ethidium bromide (EB)-CsCl gradients, so that nuclear DNA contamination is unlikely. *B. subtilis* DNA, when nick-translated to approximately the same specific activity and reassociated under the same conditions, showed a maximum reassociation of 82%. Native DNA stripped of single strands on HAP yielded 95% duplex DNA when rechromatographed, thus demonstrating the binding efficiency of the HAP columns. We conclude from extensive studies (data not shown) in which we have followed changes in reassociation kinetics due to degradation of the probe, that the failure of the probes to be driven to complete reassociation is mainly due to an inability of a short class of labeled fragments to bind to HAP.

The $C_0t_{1/2}$ values for the *B. subtilis* and chloroplast reassociation reactions are 1.38×10^{-1} and 7.0×10^{-3}, respectively. We have used the equation of Hinnebusch *et al.* (10) to correct these values for the weight average length of each probe. The size of the *B. subtilis* genome has been reported as 1.8 to 2.5×10^9 (12) on the basis of sedimentation, and as 2.0×10^9 by viscoelasticity studies (13). If one accepts an average value of 2.1×10^9 daltons, the complexity of the chloroplast DNA used in these studies was 94 $\times 10^6$. This number is in good agreement with a complexity of 95 $\times 10^6$ daltons determined for pea chloroplast DNA by optical reassociation kinetics (14) and 89 $\times 10^6$ daltons from electron microscopy (15).

Figure 1 also presents the reassociation curve for the chloroplast probe driven by total pea DNA, showing that total DNA from pea tissue produces the same profile as that of purified chloroplast

[2] Abbreviations: C_0t: initial DNA concentration (moles/liter) × time (seconds); $C_0t_{1/2}$: point of half-reassociation of DNA; P_0t: probe concentration × time (same units as C_0t); $P_0t_{1/2}$: point of half-reassociation of probe; HAP: hydroxylapatite; T_e: temperature at which 50% of DNA is eluted from HAP; ΔT_e: T_e of homologous duplexes − T_e of heterologous duplexes; T_m: temperature of melting, 50% of DNA denatured; ΔT_m: T_m of homologous duplexes − T_m of heterologous duplexes; NaP: sodium phosphate; KP: potassium phosphate.

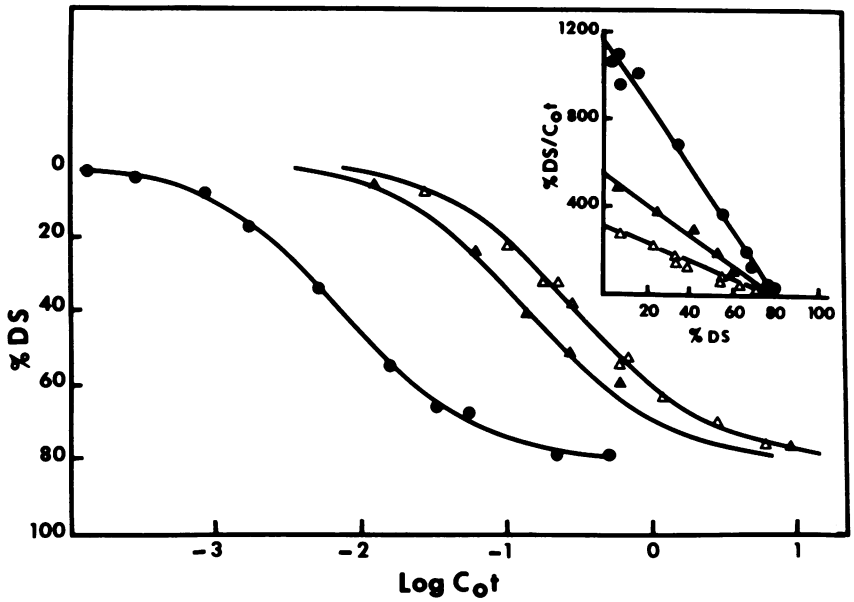

FIG. 1. Reassociation kinetics of chloroplast probe DNA. Tritium-labeled pea chloroplast DNA (0.03 μg/ml) was reassociated at 60 C with sonicated unlabeled chloroplast DNA (1 μg/ml, ●), or total pea DNA (150 μg/ml, △). Tritium-labeled *B. subtilis* DNA was reassociated under the same conditions with unlabeled *B. subtilis* DNA (50 μg/ml, ▲). Per cent double-stranded DNA was determined by HAP-binding. To determine the kinetic complexity of chloroplast DNA adjustments were made for differences in the weight average lengths of the chloroplast and *B. subtilis* probes, using the equation of Hinnebush *et al.* (10). The above curves have been drawn after subtracting the "zero-time" binding from each point. Inset shows Scatchard analysis of the data (20) which was used to draw the curves in the C_0t plot. Correlation coefficients were 0.98. The C_0t values for the unlabeled chloroplast reaction were multiplied by 10 to plot on the same axes.

DNA. The lateral displacement of the curve is expected since the chloroplast DNA accounts for only a portion of the total tissue DNA. The fraction of total DNA contributed by chloroplast DNA will be considered below.

REASSOCIATION OF PEA CHLOROPLAST PROBE DNA WITH TOTAL DNA FROM OTHER PLANTS

HAP Analysis. To assess the extent of sequence relatedness of chloroplast genomes among vascular plants, the pea chloroplast probe was reassociated with a great excess of total DNA from different species. Representative kinetic curves of the plants investigated are shown in Figures 2 and 3. The results are presented in terms of P_0t, after Chilton *et al.* (8), where P_0 equals the initial chloroplast probe concentration. The data for the homologous curve are the same as those in Figure 1, but to facilitate the analysis, data for the homologous reaction was adjusted to a 100% component curve (80% reaction = 100% completion), and the heterologous curves were normalized to this value.

Broad bean, a member of the pea family (Leguminosae), displayed about 90% cross-reaction with the pea chloroplast probe, whereas watermelon, a dicotyledon in another family, exhibited 75% homology (Fig. 2A). Analogous curves for the monocotyledons indicated that *Trillium* and daffodil DNAs were about 65% homologous, while the levels of homology found for the grasses, barley and corn, are 50 to 55% and 40 to 47%, respectively (Fig. 2B). The corn and barley reactions were difficult to analyze because of the onset of the probe self-reaction. Higher concentrations of the driver DNAs (data not shown) were also tested, and the results indicate that the values presented are the maximum homologies obtainable under these conditions. Similar data are given for gymnosperms and several lower vascular plants in Figure 3. The concentration of these heterologous DNAs was 4-fold higher than for the reactions in Figure 2. Only in the case of horsetail was there an indication of a component equal to 25% of the pea chloroplast genome. For gymnosperm and lower vascular plant driver DNAs, although there is apparent chloroplast DNA sequence conservation, the extent of homology could not be accurately ascertained. Calf DNA did not accelerate the probe reassociation.

The reassociation curves in Figures 2 and 3 were constructed when the weight average length of the probe was 1,860 nucleotides (Fig. 4). The driver length throughout was 450 nucleotides; in Figure 3 the horizontal bar represents the distribution of driver fragments. The probe length decreased with time due to radiation damage, and with this change there was a decrease in the levels of homology as assayed on HAP. After 3 months the probe was approximately half its original length, and was then used to reevaluate the various degrees of sequence relatedness. The homologous reaction changed from an 80% single component curve with the 1,860-nucleotide-long probe to a 72% component curve when the probe was 735 nucleotides. Since this change is attributed to a decrease in the fraction of single-stranded DNA capable of forming duplexes of sufficient length to bind to HAP, as discussed in the previous section, the levels of homology were again normalized to the homologous reaction (72% = 100% completion). The relative levels of homology observed for the long and short probes are listed in Table I. In all cases the shorter probe yielded lower levels of cross-reaction than did the longer probe. The ratios of homologies achieved with the short and long probe ranged from 0.89 for broad bean to 0.71 for daffodil. We infer from these ratios that many of the longer probe molecules, which bound to HAP as "duplex" DNA, were not homologous to the driver DNA along their entire length.

S1 Nuclease Analysis. In an attempt to remove nonhomologous regions from the duplex-containing structures, S1 nuclease was used to analyze the heteroduplexes obtained with the short probe. The kinetic data of reassociation for the homologous reaction employing the S1 and HAP assays are presented in Figure 5. At the outset of reassociation about 0.40 of the HAP-bindable DNA was S1-resistant, but as the reaction proceeded this value increased. As indicated by the HAP curve, at 100% reassociation 70 to 75% of the probe DNA was S1-resistant. The S1 kinetic curve, drawn using the equation of Smith *et al.* (26) suggests that the amount of S1-resistant DNA would have gone to 90 to 95%. In the initial part of reassociation the ratio of S1-resistant to HAP-

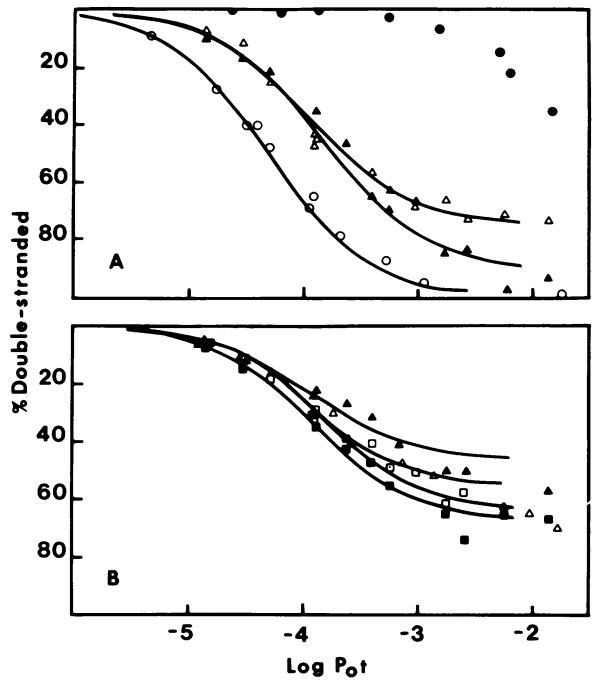

FIG. 2. Levels of homology between pea chloroplast DNA and angiosperm DNAs. Tritium-labeled pea DNA (1,860 bases) was reassociated with about 5,800-fold excess total driver DNA. Pot is the probe concentration (7.8×10^{-8} mol/l) multiplied by time in s. Zero-time binding (6%) to HAP has been subtracted and curves are normalized to that for pea total DNA. The probe self-reassociation is shown as closed circle (●) without connecting lines. Lateral displacement and kinetic component size were determined by Scatchard analysis; correlation coefficients of linear regression analysis are given in Table III, legend. A: pea (○), broad bean (▲), and watermelon (△); B: *Trillium* (■), daffodil (□), corn (△), and barley (▲).

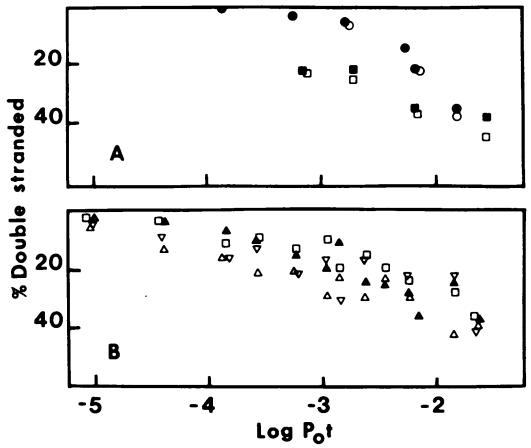

FIG. 3. Levels of homology between pea chloroplast DNA and gymnosperm and lower vascular plant DNAs. A 23,000-fold excess of total driver DNA was used. Other details are described in Figure 2, legend. A: sitka spruce (□), pine (■), calf (○); B: horsetail (△), parsley fern (□), *Psilotum* (▲), and *Tmesipteris* (▽).

bindable DNA (S1/HAP) should equal the extent of overlap between two strands upon collision. This ratio has been estimated by Wetmur (31) as 0.41 for DNA treated with a nonspecific endonuclease; it should increase to 0.85 to 1.0 as the reaction nears completion if the single-stranded tails interact further (26). Since the probe DNA was prepared by DNase and polymerase treatments, and an analysis of probe size (Fig. 4) revealed an extremely broad distribution of fragment lengths, our results are in accord with those expected.

Table II lists the amount of S1-resistant DNA for the interspecies reactions; the assay was made at the termination of reassociation between free strands, as measured by HAP. In this experiment the homologous reaction yielded 70% S1-resistant DNA and broad bean yielded 51%; the values for the other species ranged from 17 to 27% (column 1). Table II, column 2, lists the S1/HAP ratios for these reactions. For pea this ratio was 0.70, for broad bean, 0.65, whereas for the other DNAs it was lower, ranging from 0.31 for watermelon to 0.49 for barley. Although broad bean exhibited one of the slower rates of reassociation with the pea chloroplast probe (discussed below), it showed the highest S1/HAP ratio, indicating that the lower S1/HAP values do not reflect premature measurements of S1-resistant DNA during the reactions. Apparently, there were few secondary nucleation events after the first collision of the probe with most of the heterologous DNAs. The low S1/HAP ratios found for these reactions indicate that the single-stranded tails of the probe were nonhomologous to the driver DNA. For comparison, we have normalized the estimates of S1/HAP found at the termination of reassociation relative to pea (Table II, column 3). By multiplying the per cent homologies given in Table I, column 2, by these normalized values, we can determine the absolute amount of homologous DNA present in the heteroduplexes that bound to HAP. By this approach, the products of reassociation between the probe and pea total DNA remain 100% homologous, and broad bean shows approximately the same level of homology, at 73%; however, all other levels fall into a range of 24 to 38%, with an average of 29 ± 6%. Without referring to the HAP data, these levels of homology can be obtained directly by normalizing the per cent S1-resistant DNA, Table II, column 1, relative to pea.

Further Considerations in Determination of Homology. We have considered the possibility that the levels of homology observed are underestimated due to the probe DNA running out of complementary sequences with which to react because the driver-driver reaction was faster than the probe-driver reaction (31). Two factors could lower the rate of hybrid formation: (*a*) a probe DNA

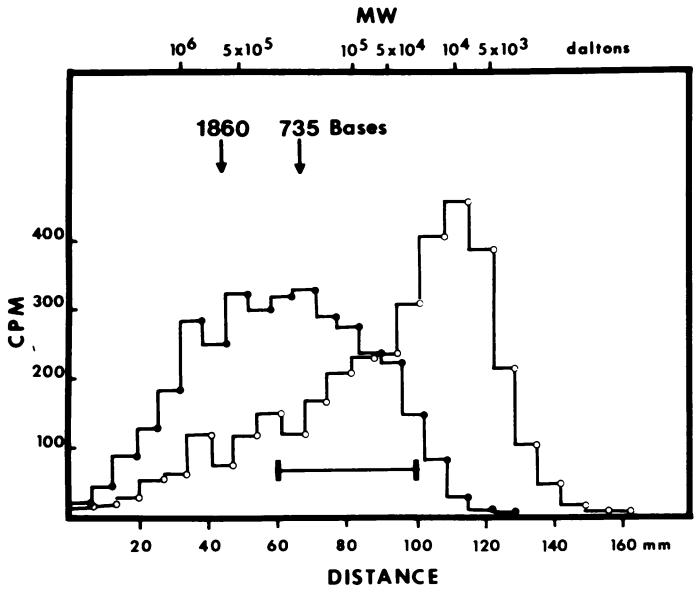

FIG. 4. Length distribution of nick-translated pea chloroplast DNA. After alkaline gel electrophoresis, the gel was sliced and radioactivity determined in each slice. Solid line represents a probe newly labeled by nick-translation; broken line is for a 3-month-old probe. Arrows indicate the weight average length calculated as explained under "Materials and Methods." Bar shows range of sonicated driver DNA fragments which was a normal distribution around a mean of 450 bases as estimated from a densitometer tracing of an ethidium bromide stained alkaline gel containing 2 μg DNA.

TABLE I. Levels of Homology Determined by HAP Chromatography.

Driver DNA	% Homologous DNA Probe Length[a] 1 1860 bases	2 735 bases	3 735/1860[b]
ANGIOSPERMS			
Dicots			
Pea	100[c]	100[c]	1.00
Broad bean	85-90	78	0.89
Watermelon	75	60	0.80
Squash	75		
Muskmelon	74	60	0.81
Monocots			
Trillium	67	57	0.85
Daffodil	63	45	0.71
Corn	50-55	45	0.86
Barley	40-47	37	0.85
GYMNOSPERMS			
Spruce	+[d]		
Pine	+		
LOWER VASCULAR			
Horsetail	+		
Parsley fern	+		
Psilotum	+		
Tmesipteris	+		

Levels of homology were determined as described in Figure 2, legend.

a Weight average length. See Figure 3.

b Ratio of column 2 to column 1; ratio of homology with short and long pea chloroplast probes.

c Absolute levels were 80% and 72% for the 1860-base and 735-base probes, respectively. In column 1 the homologies given for broad bean, barley and corn indicate the range of uncertainty in these determinations. A sonicated probe (450 bases) yielded the following levels of homology for broad bean, watermelon, daffodil, and corn: 72%, 35%, 38%, and 35% respectively.

d + means the probe reassociation was accelerated. Although no clear components were evident (see Figure 3) the gymnosperms and horsetail showed greater than 20% homology with the probe, while the other vascular plants were at least 10% homologous.

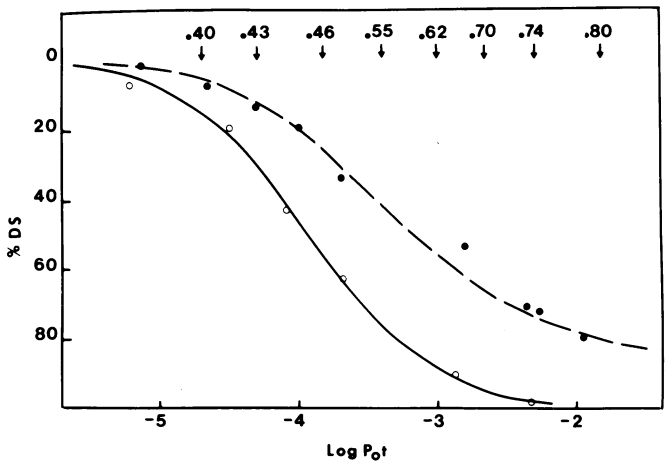

FIG. 5. Comparison of S1 nuclease and HAP reassociation kinetics. Reassociation was performed and data treated as described in Figure 2 using pea total DNA as driver and a short probe (735 bases). In one experiment the reassociated aliquots were passed over HAP (○); in another the per cent S1-resistant probe was determined (●). The S1 curve was plotted using the equation of Smith *et al.* (26): $S/C_0 (1/1 + kC_0t)^n$ where n = 0.45; the best fit was a 90% component curve. Arrows indicate the P_0t values at which S1/HAP ratios were calculated.

considerably shorter than the driver DNA, which we have shown not to be the case by measurement of probe and driver weight average lengths (Fig. 4; see also ref. 9) or (*b*) extensive mismatching. The ΔT_e values in our experiments do not exceed 7 C (see next section) and hence the maximum rate reduction would result in a rate 0.82 times that of the homologous reaction (20). For watermelon, corn, and barley we have increased the driver concentration by a factor of 3; the level of heteroduplex formation did not change appreciably. A more direct test of whether maximum homologies had been achieved was performed by isolating the single-stranded DNA from the watermelon reaction and reassociating it with an additional amount of unlabeled watermelon driver. An average of 4% of this DNA appeared in the duplex fraction upon second incubation, which is the calculated concentration of the probe reacting with itself. We conclude from these considerations that with the HAP assay we have determined the maximum homologies for the various chloroplast genomes.

ESTIMATION OF PERCENTAGE OF CHLOROPLAST DNA IN PEA TOTAL DNA

The ability of a total DNA preparation to drive a chloroplast probe should depend upon the fraction of chloroplast DNA in the total. In principle, we may estimate the amount of chloroplast DNA in a cell. The $P_0t_{1/2}$ values for all of the probe-driver

Table II. Determination of the Amount of Double-stranded DNA in the HAP-bound Fraction Using S1 Nuclease.

Driver DNA	1 % S1-Resistant[a]	2 S1/HAP[b]	3 Relative HAP-bound which is DS[c]	4 Relative % S1-Resistant[d]
Pea	70	0.70	1.00	100
Broad bean	51	0.65	0.93	73
Watermelon	18	0.30	0.43	26
Squash	17	0.28[e]	0.40	24
Muskmelon	24	0.40	0.57	34
Trillium	27	0.47	0.67	38
Daffodil	19	0.42	0.60	27
Corn	20	0.44	0.63	28
Barley	18	0.49	0.70	26
			Average[f] = 29 ± 6%	

a An average of 3 determinations of the percent of input tritium (735-base pea probe) that was S1-resistant at the end of reassociation of free stands (see Text; P_0t values = 2.5, 4.5 and 5.5 x 10^{-3}). The percent S1-resistant DNA found for a sample which was quickly cooled following denaturation has been subtracted in each case as a zero-time point.

b The % S1-resistant DNA divided by HAP-bound DNA, Table I column 2; these determinations were made in separate experiments.

c The relative fraction of HAP-bound DNA which is in double-stranded form. Estimated by normalizing S1/HAP values to the pea reaction.

d These values are obtained either by multiplying column 3 by the percent HAP-bound DNA, Table I column 2, or by directly normalizing the percentages of S1-resistant DNA, column 1 above, relative to pea.

e The percent HAP-bound DNA with the 735-base probe was taken as 60%, which was found for watermelon and muskmelon, members of the same family.

f Includes all driver DNAs outside of the pea family.

reactions performed are given in Table III, as determined with the longer probe. The reaction mixtures were constructed such that the probe-probe reaction (Fig. 2A) was not significant compared to the probe-driver reaction, and hence the rate of appearance of labeled DNA in the duplex fraction depended only on the driver DNA concentration. When the driver concentration was increased 2-fold (tested in the range of 50–350 μg/ml), the $P_0t_{1/2}$ value decreased by a factor of 1.8.

In order to determine the percentage of chloroplast DNA in total DNA from the $P_0t_{1/2}$ values, a model curve employing the pea chloroplast probe with a known amount of pea chloroplast driver was constructed (same data as in Fig. 1). A concentration of 1.03 μg/ml of chloroplast driver DNA yielded a $P_0t_{1/2}$ of 1.75 × 10^{-4}. The $P_0t_{1/2}$ value of 5 × 10^{-5} for the total pea DNA driver is 3.5-fold less, and hence the total pea DNA contains 3.6 μg/ml of chloroplast DNA. Since the concentrations of total DNA used to drive the chloroplast probe was 150 μg/ml, chloroplast DNA represents 2.4% of the DNA extracted from pea shoot tissue.

PERCENTAGE OF CHLOROPLAST DNA IN OTHER SPECIES

Before a similar computation was performed for all of the heterologous DNAs, three factors were considered which might affect the $P_0t_{1/2}$ values: (*a*) the degree of mismatch in the hybrid molecules; (*b*) the possibility that shoot tissue at different stages in development might yield different amounts of chloroplast DNA; and (*c*) there may be fragments of DNA reacting with the probe at different rates, due to differences in sequence complementarity, making the observed rate a composite (8, 24). We have performed experiments bearing on the first two points.

The extent of mismatch in the heteroduplexes was investigated by constructing thermal elution profiles using HAP. Martinson and Wagenaar (21) have carefully documented the pitfalls attendant to the "usual" HAP-melting procedures. In view of these sobering results showing desorption of duplex DNA with increasing temperature, we present the following study. Both native chloroplast and *B. subtilis* probe DNAs were loaded on HAP over a range of phosphate concentrations (0.02–0.1 M KP), and thermally eluted. There was less than a degree change in the temperature required to elute 50% of the labeled DNA (called the T_e point, after Martinson and Wagenaar) from 0.04 to 0.08 M KP. We decided to determine whether the DNA released from HAP was single- or double-stranded at the T_e in 0.06 M and 0.08 M KP for duplexes formed between the chloroplast probe and pea, barley, watermelon, and horsetail driver DNAs; in levels of homology, at least, these species reflect the extent of sequence divergence in chloroplast DNA from higher plants. In 0.08 M KP the DNA released at the T_e from the homoduplex (pea driver) was 25% double-stranded when rechromatographed at 60 C under standard conditions, while a fraction eluted at 98 C was 7% double-stranded (Table IV). For the heteroduplexes the percentage of double-stranded DNA in the T_e fractions was lower, from 14 to 18%, indicating that they contained sufficient mispairing to dissociate into single strands as the T_e was approached. The analogous measurements in 0.06 M KP showed that very little DNA at the T_e was in duplex form; thus the T_e closely approximate the melting point (T_m) of the DNA. The large portion of material eluting at 98 C which subsequently binds to HAP at 60 C (Table IV) may reflect an enrichment for strands carrying an inverted repeat ("foldback" DNA) since DNA should be denatured at 98 C in these buffers, and our HAP columns bind less than 5% of denatured probe DNA from which the foldback material has been removed. The T_e values in 0.08 M KP and 0.06 M KP do not differ by more than a degree, and hence the difference between homol-

TABLE III. Percent Chloroplast DNA in Total DNA.

Driver DNA	$P_o t_{1/2}$	% Chlp DNA	Nuclear Genome Size[a]	ΔT_e 6 mM[b]	ΔT_e 8 mM[c]
CONTROL					
Chloroplast	1.75×10^{-4}				
ANGIOSPERMS					
Dicots					
Pea	5.0×10^{-5}	2.4	10.5	0.0	0.0
Broad bean	1.4×10^{-4}	0.9	24	2.3	1.6
Watermelon	1.0×10^{-5}	1.2	1.4-3.0	5.4,4.5[e]	5.8
Squash	6.8×10^{-5}	1.7	1.4-3.0	5.2	5.8
Muskmelon	3.6×10^{-5}	3.3	1.9	5.6	
Monocots					
Trillium	1.0×10^{-4}	1.2	92-120	5.5	5.4
Daffodil	1.2×10^{-4}	1.0	33	5.5	5.9
Corn	9.0×10^{-5}	1.2	11	5.4	
Barley	1.0×10^{-4}	1.1	11	5.9,7.0[e]	6.7
GYMNOSPERMS					
Spruce	+[d]			4.7	
Pine	+			4.7	
LOWER VASCULAR					
Horsetail	+			6.6,6.0[e]	5.0
Parsley fern	+				
Psilotum	+				
Tmesipteris	+				

$P_o t_{1/2}$ values (M·sec) were determined using the 1860-base probe. Values have been corrected for ΔT_e in 0.06 M KP which was taken as the ΔT_m (see text). Correlation coefficients from least squares regression analysis of Scatchard plots of the reassociation data were from 0.94 to 1.0 for all of the angiosperms except for barley, which was 0.89. The percent chloroplast DNA was estimated relative to the chloroplast reconstruction reaction (control) which contained 1.03 µg/ml DNA. All angiosperm reactions were at 150 µg/ml; others were at 600 µg/ml.

a Diploid amounts in pg are given for pea, broad bean, corn and barley (6), daffodil (23), muskmelon (11); the values given for watermelon and squash show the range found for 5 other species in the same family (11). The genome sizes of Trillium erectum, (6), and T. grandiflorum, (11), are given for Trillium.

b Difference in temperature of elution of 50% of the radioactivity from HAP in 0.06 M KP. The T_e for pea was 79 C.

c Same as b, only in 0.08 M KP. The T_e for pea was 78.1 C.

d + means that no clear component was evident for accurate $P_o t_{1/2}$ estimation.

e Determined by raising aliquots to a given temperature, cooling and separating single from double-stranded DNA at 60 C (see text).

ogous and heterologous T_e values (ΔT_e) are about the same in the two buffers.

An independent method was also employed to assess the ΔT_e values. Aliquots of the reassociation products were held at different temperature from 60 to 98 C for 5 min, quickly cooled, and passed over HAP at 60 C in order to separate single from double-stranded molecules. These ΔT_e values, which should equal the ΔT_m values, were about the same as found by sequential elution of the DNA with increasing temperature. We have used the data obtained in the 0.06 M KP thermal elution to correct the $P_o t_{1/2}$ values to those listed in Table III, using the relationship between ΔT_m and reduction in rate of reassociation established by Marsh and McCarthy (20). The corrections in no case altered the $P_o t_{1/2}$ values by more than 18%.

We have attempted to estimate the change in chloroplast DNA content per µg of total DNA during development by isolating total DNA from young (3 days after planting) and fully green (8 days after planting) pea shoots, and using it to drive the labeled chloroplast probe. The amount of chloroplast DNA in total pea DNA extracted from shoots increased from about 2.4 to 6.0%. The details of this change will be presented elsewhere (manuscript in preparation).

DISCUSSION

A principal finding of our work is that some DNA sequences in the chloroplast genome have been conserved throughout the evolution of land plants, a period of some 400 million years (2). The experiments, using pea chloroplast DNA as a probe in reassociation reactions, show that the phylogenetically most distant species exhibit the greatest sequence divergence from pea. It appears that those sequences that have been conserved are not arranged contiguously, but are separated by divergent sequences on a rather fine scale.

TABLE IV. Assessment of Strandedness of DNA Released from HAP at the Thermal Elution Point.

Driver DNA	Percent Double-Stranded DNA Released			
	0.06 M KP		0.08 M KP	
	at T_e	at 98 C	at T_e	at 98 C
Pea	9	7	25	7
Watermelon	11	7	16	7
Barley	12	17	18	16
Horsetail	5	11	14	13

The pea chloroplast probe (735 bases) was reassociated with each of the above driver DNAs to 50-70% completion (see respective P_0t curves in Figure 2). Reassociated DNA was loaded onto HAP in 0.06 M or 0.08 M KP at 60 C and the temperature was raised to the T_e points determined previously. DNA was eluted from the columns at the T_e and then at 98 C. The T_e and 98 C fractions were reloaded onto columns under standard conditions (60 C) to separate single from double-stranded DNA. The percent double-stranded DNA in each fraction is presented.

With the HAP assay we observed a maximum cross-reaction between the pea chloroplast probe and broad bean, a member of the pea family; all other levels of homology were lower. Among the flowering plants the lowest level of sequence relatedness was found for barley. The DNAs from the gymnosperms, spruce and pine, and lower vascular plants such as *Psilotum*, reacted with the pea chloroplast probe as demonstrated by an acceleration of the probe reassociation, but no distinct levels of homology were evident (Fig. 3). The S1 analysis produced the surprising result that despite large differences in the apparent homology determined by the HAP assay, the actual amount of homologous DNA in most heteroduplexes was nearly the same, as shown in Table II, column 4. For example, the HAP assay indicated that watermelon and barley were 60 and 37% homologous to the pea chloroplast probe, respectively, while the S1 digestion left a core of 26% double-stranded DNA for both. The exception was broad bean, for which the probe exhibited greater than 70% homology in both assays.

In pea the conserved sequences in the circular chloroplast genome appear to be interrupted by divergent regions. Support for this conclusion comes from two lines of evidence. First, a decrease in pea chloroplast probe length resulted in reduced levels of homology. The values of homology achieved with a 735-base probe were 11 to 29% less than those attained with a 1,860-base probe, indicating that the longer probe contained nonhomologous regions scored as "duplex" by HAP because they were linked to conserved stretches of DNA. Somewhat lower levels of homology were found with a 450-base probe (Table I, legends). Second, S1 nuclease analysis of short heteroduplexes (735-base probe) showed lower levels of sequence relatedness than indicated by HAP; thus, even within these duplex-containing structures nonhomologous DNA was interspersed.

At present we cannot describe the physical arrangement of the conserved sequences in the pea chloroplast genome. There may even exist copies of the chloroplast genome in the nucleus (25). The fraction of HAP-bindable DNA in double-stranded form (S1-resistant) relative to that which was single-stranded (Table II, column 3) provides an estimate of the amount of nonhomologous DNA spaced between the conserved regions irrespective of subcellular location. The sequences homologous to watermelon appear to be separated by nonhomologous regions which in total equal them in length (0.43 and 0.57, respectively, of the HAP-bindable DNA), while compared to the monocotyledons the diverged regions are about half the total length of the conserved regions. It is important to note that in the heterologous DNAs the sequences homologous to pea chloroplast DNA may be arranged contiguously, and it is only in the pea genome that they are separated leading to the differences in the HAP and S1 estimates. However, if the same DNA sequences have been conserved in such diverse plants as barley and watermelon, they must be separated in both the watermelon and pea chloroplast genomes to account for the greater discrepancy between the HAP and S1 values observed for watermelon than for barley. Apparently, the major changes in the evolution of the chloroplast genome in angiosperms have not altered the amount of conserved DNA but were events involving rearrangement of this DNA and/or addition-deletion of nonconserved sequences leading to interspersion of conserved and nonconserved regions; these events may have been more extensive in dicotyledons than in monocotyledons.

The amount of chloroplast DNA in a typical preparation of DNA from shoot tissue is relatively constant (Table III, colum 2) despite the great range in the size of the nuclear genome. These values are not considered to represent the absolute amount of chloroplast DNA per cell in a particular plant because we have observed an increase from 2.4 to 6.0% in the amount of chloroplast DNA with development of pea shoots (manuscript in preparation), and comparable patterns may exist in the life cycles of other higher plants. For example, the seemingly high percentage of chloroplast DNA in muskmelon, 3.3% relative to watermelon and squash may reflect a difference in developmental stage when the DNA was extracted. It is not obvious why the amount of chloroplast DNA seems to increase with the size of the nuclear genome. Although it has been shown that there is a correlation between nuclear DNA ploidy and the number of chloroplasts per guard cell (7) there is no evidence that the amount of DNA per chloroplast remains constant as the chloroplast number increases.

From our results we have developed the following working hypothesis. Among the angiosperms only about 30% of the chloroplast genome is strongly conserved by selection, while the remaining 70% is comprised of sequences more tolerant of change. The conserved sequences exhibit a high degree of sequence complementarity with no more than 5 to 7% base change; the same sequence fidelity extends even to the gymnosperms and horsetail. For broad bean the additional 40% of DNA found to be homologous in the S1 assay may not be additional sequences subjected to severe selection, but instead represents that portion of more rapidly changing DNA which has not yet completely diverged or been replaced since recent speciation within the pea family.

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