

A Mating Type-Linked Gene Cluster Expressed in *Chlamydomonas* Zygotes Participates in the Uniparental Inheritance of the Chloroplast Genome

E. Virginia Armbrust, Patrick J. Ferris,
and Ursula W. Goodenough

Department of Biology
Washington University
St. Louis, Missouri 63130

Summary

A characteristic feature of early zygote development in *Chlamydomonas* is the selective degradation of chloroplast DNA from the mating type minus parent. The zygote-specific gene cluster *ezy-1* is linked to the mating type locus and is transcribed almost immediately upon zygote formation. We show here that the acidic Ezy-1 polypeptide is rapidly transported to both the plus and minus chloroplasts, where it interacts with each chloroplast nucleoid. Expression of *ezy-1* is selectively inhibited when plus, but not minus, gametes are briefly ultraviolet irradiated just prior to mating, a treatment known to disrupt the uniparental inheritance of chloroplast traits. We propose that the Ezy-1 polypeptide participates in the destruction of the minus chloroplast DNA in zygotes and thus the uniparental inheritance of chloroplast traits. The *ezy-1* gene represents a valuable molecular probe for dissecting mechanisms underlying organelle inheritance.

Introduction

The non-Mendelian inheritance of chloroplast traits, documented repeatedly since the turn of the century, is a common feature of all photosynthetic eukaryotes (Gillham, 1978). The chloroplast genome is usually inherited from the female parent only; conifers, the main exception to the rule, inherit their chloroplast genome from the male parent (Whatley, 1982). The driving force for the evolution of so common a phenomenon as uniparental inheritance remains unclear. It has been proposed that uniparental inheritance arose as a means of avoiding potential conflict between inherently selfish organelle genomes confined within the newly formed zygote (Cosmides and Tooby, 1981). This hypothesis has recently been expanded to suggest that the occurrence of only two sexes in most species is a direct consequence of the need to minimize organelle warfare (Hurst, 1992; Hurst and Hamilton, 1992).

The genetics of uniparental inheritance have been examined most extensively in the haploid unicellular green alga *Chlamydomonas reinhardtii* (reviewed by Gillham et al., 1991). *C. reinhardtii* cells are of two mating types, plus (*mt⁺*) and minus (*mt⁻*), controlled by a single complex locus on linkage group VI (Smith and Regnery, 1950; Ebersold et al., 1962). Within minutes of mixing, gametes of opposite mating type adhere by their flagella and fuse to form zygotes (reviewed by Goodenough, 1991). The mixing of the gamete cytoplasms initiates an almost immediate transcription of zygote-specific genes (Ferris and Good-

enough, 1987), some of which participate in forming the desiccation-resistant zygotic cell wall (Woessner and Goodenough, 1989, 1992). After a mandatory period of dormancy, the zygote undergoes meiosis and germination to produce four haploid progeny.

Over 90% of the meiotic progeny thus formed inherit chloroplast traits from the *mt⁺* parent, a phenomenon first documented almost 40 years ago by Sager (1954). The 196 kb chloroplast genome of *C. reinhardtii* (Rochaix, 1978), present in about 80 copies per cell (Gillham, 1978), is organized into discrete DNA-protein complexes called nucleoids (Ris and Plaut, 1962). By 1–2 hr after zygote formation, but apparently prior to fusion of the two chloroplasts (Cavalier-Smith, 1970), the chloroplast DNA from the *mt⁺* parent disappears, a process readily monitored by the disappearance of 4,6-diamidino-2-phenylindole (DAPI) fluorescence from half the nucleoids (Kuroiwa et al., 1982; Munaut et al., 1990). The remaining *mt⁺* nucleoids eventually coalesce into two or three nucleoids, and the chloroplast DNA content of the zygote remains constant during the remainder of dormancy; prior to meiosis there is a burst of chloroplast DNA synthesis (Coleman, 1984) such that each meiotic product comes to possess about 80 copies of the *mt⁺* chloroplast genome.

The molecular mechanism by which the *mt⁻* nucleoids are selectively destroyed (reviewed by Gillham et al., 1991) has been proposed to occur in two distinct steps: protection and destruction. In the protection step, the nucleoids of the *mt⁺* parent are somehow modified prior to zygote formation so they are resistant to destruction; destruction then eliminates the *mt⁻* chloroplast genomes in the zygote. In the homothallic species *Chlamydomonas moewusii*, the *mtl-1* locus apparently specifies protector activity: when a mutant *mtl-1* gene is introduced into a cross by the *mt⁺*, but not the *mt⁻*, parent, all the nucleoids in the developing zygote are destroyed, and the zygotes fail to germinate (VanWinkle-Swift and Salinger, 1988). The destruction step has been proposed to be mediated by a nuclease (Sager and Grabowy, 1985) that degrades the unprotected *mt⁻* chloroplast DNA within the first 1–2 hr of zygote development. Importantly, this occurs only in true zygotes, not in cells rendered diploid by other means (Gillham, 1963; Matagne and Hermesse, 1980; Matagne, 1981), underscoring the zygote-specific production of the nuclease. Selective degradation is also prevented when zygotes are formed in the presence of inhibitors of eukaryotic (but not prokaryotic) RNA and protein synthesis (Kuroiwa et al., 1983a, 1983b; Kuroiwa, 1985), indicating that zygote-specific nuclear (but not chloroplast) products are required.

Uniparental inheritance in *C. reinhardtii* can be converted to biparental inheritance in two ways. The *mt⁺*-linked mutation *mat-3* (Gillham et al., 1987), particularly when combined with the ultraviolet (UV)-sensitive nuclear mutation *uvsE1* (Rosen et al., 1991), interferes with the destruction of the *mt⁻* chloroplast nucleoids and hence increases the percentage of progeny that inherit both chlo-

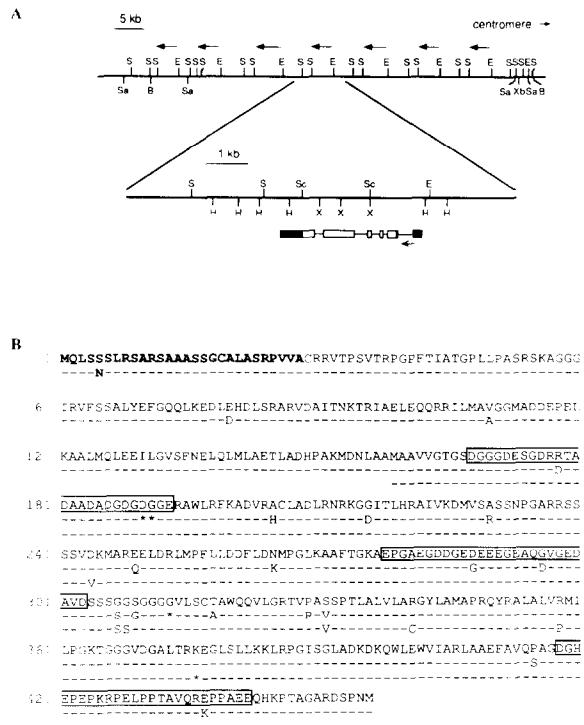


Figure 1. Structure and Sequence of the *ezy-1* Genes

(A) Schematic of genomic organization. Seven copies of the *ezy-1* gene are present in tandem within the *mt*⁻ locus on chromosome VI (top line). The locations of the seven transcription units (arrows) are shown based on the restriction mapping, but it is not known whether all seven are functional. The 70 kb depicted is part of a larger chromosome walk whose orientation relative to the centromere is known. The upper restriction map is drawn with five enzymes: B, BamHI; E, EcoRI; S, SmaI; Sa, SalI; Xb, XbaI. The lower line shows a detailed map of one 9 kb repeat unit (additional enzymes: H, HindIII; Sc, SacI; X, Xhol). The structure of the transcription unit is shown at the bottom. Boxes represent exons; lines represent the five introns. Untranslated RNA is shown as closed boxes. The precise location of the transcription start site was not determined; the start of the mRNA was taken as the end of the 5'-most cDNA clone.

(B) Comparison of deduced amino acid sequences of three *ezy-1* cDNAs. The sequence derived from cDNA clone 16 is presented in its entirety. Sequence differences are presented for cDNA clones 10 and 28; dashes indicate identical amino acid sequence. Deletions in the sequence from cDNA clone 10 are indicated by asterisks. The putative chloroplast transit peptide is shown in bold, and the acidic domains are boxed.

roplast genomes (Munaut et al., 1990; Rosen et al., 1991). Low levels of UV irradiation also increase the percentage of biparental progeny, but only when the *mt*⁺ parent is irradiated prior to mating; irradiation of the *mt*⁻ parent has no effect (Sager and Ramanis, 1967; Gillham et al., 1974). Therefore, in addition to supplying protector function, the *mt*⁺ gamete makes a key contribution to destroyer function that can be compromised by both the *mat-3* mutation and UV exposure.

The present study began with the identification of a zygote-specific cDNA, called Class III, that derives from a *mt*-linked locus, *ezy-1* (early zygote). The *ezy-1* gene is repeated 7–8 times in both genomes, and *ezy-1* message

is detected within 5 min of zygote formation (Ferris and Goodenough, 1987). Here we present evidence that the *ezy-1* gene product participates in the zygote-specific selective destruction of the chloroplast DNA from the *mt*⁻ parent, thus providing a valuable molecular probe for dissecting the mechanism of chloroplast gene transmission.

Results

Multiple Copies of the *ezy-1* Gene Cluster Are Transcribed in the Early Zygote

The *ezy-1* cDNA (clone 28) isolated by Ferris and Goodenough (1987) was used to isolate overlapping genomic clones from a phage library. Restriction mapping of these phage indicated that the *ezy-1* gene is tandemly repeated 7 times in the *mt*⁻ nuclear genome (Figure 1A) and 7–8 times in the *mt*⁺ genome, suggesting that as many as 15 different copies of this gene might be expressed in the developing zygote. The ~1500 bp *ezy-1* cDNA clone 28 recognizes a 2200 nt message. Clone 28 was used to screen a cDNA expression library from 1 hr zygotes in order to isolate full-length cDNAs and to determine whether multiple versions of the *ezy-1* gene are expressed. Two additional cDNAs with unique restriction maps were isolated: clone 10 (1902 bp) and clone 16 (2147 bp). The three cDNAs and a genomic clone were sequenced to completion; the difference in length between cDNAs 10 and 16 was due to alterations in their 3' untranslated regions (data not shown). cDNAs 10 and 16 were assumed to be full length, since genomic probes upstream of the cDNA–genomic overlap failed to hybridize to zygote RNA. The *ezy-1* gene is about 3.5 kb, with one intron of 411 bp in the 5' untranslated region and four introns of 120, 220, 353, and 229 bp in the coding region (Figure 1A). Each copy of the *ezy-1* gene in the chromosome is separated by approximately 5.5 kb. The *ezy-1* gene cluster thus spans about 70 kb in both the *mt*⁺ and *mt*⁻ genomes.

The initiator methionine is assumed to be the first AUG in both full-length cDNA clones, resulting in open reading frames of 1359 and 1368 bp. The predicted molecular mass of the resultant polypeptide is approximately 48 kd. Differences among the three cDNA sequences occur at both the nucleotide and amino acid level (Figure 1B), indicating that at least 3 of the potential 14–15 copies of the *ezy-1* gene are transcribed in the early zygote. The derived amino acid sequence of the genomic clone is identical to cDNA clone 16, although differences are present at the nucleotide level. It is not known whether these transcripts originate from the *mt*⁺ and/or *mt*⁻ genome. It is clear, however, that cDNA clone 10 corresponds to the most centromere-distal copy in both the *mt*⁺ and *mt*⁻ genome, owing to the presence of a unique ApaI site. Interestingly, not all of the amino acid substitutions in the predicted polypeptides are conservative; in fact, a number of substitutions result in changes in charge. The Ezy-1 polypeptide displays no obvious consensus motifs, although it does possess three domains of at least 25 amino acids each that are highly acidic (boxed in Figure 1B), with net negative charges of –2 to –13. The overall charge of the polypeptide is also negative, with theoretical isoelectric points of 5.17

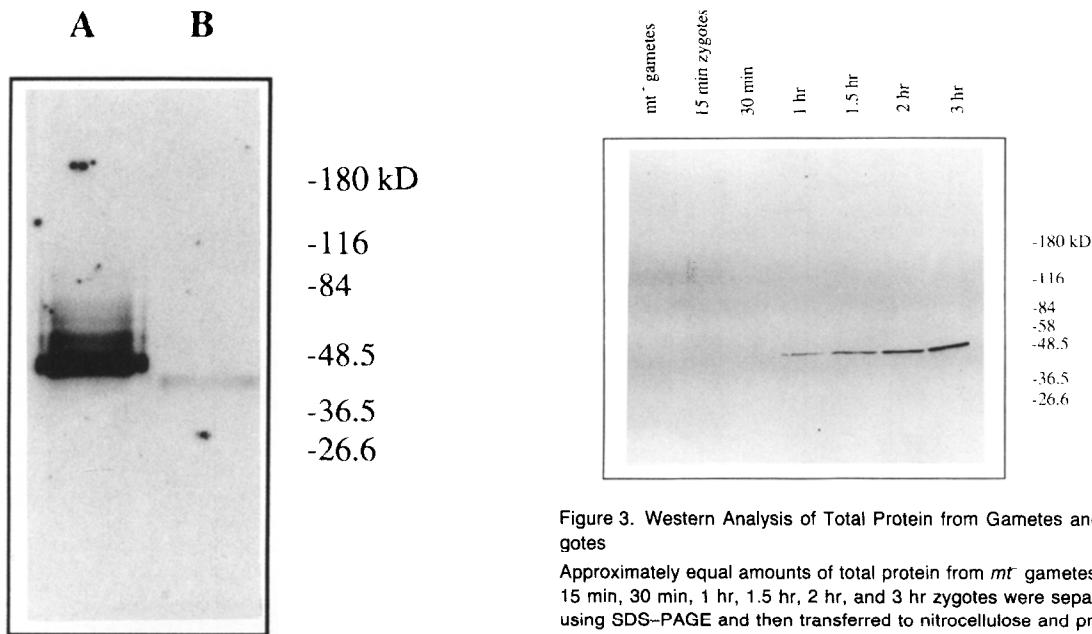


Figure 2. Specificity of α -Ezy-1

α -Ezy-1 was used to immunoprecipitate either [35 S]methionine-labeled in vitro translation products using total RNA from 1 hr zygotes (lane A) or [14 C]acetate in vivo labeled proteins from 1 hr zygotes (lane B). The immunoprecipitated products were analyzed by SDS-PAGE and fluorography.

(cDNA 16) and 5.65 (cDNA 10). Since no homologies to sequences in GenBank have been found, the ezy-1 gene product is assumed to be a novel polypeptide.

The Ezy-1 Polypeptide Localizes to Chloroplast Nucleoids in the Early Zygote

The Ezy-1 polypeptide possesses a putative chloroplast transit sequence (Figure 1B) that displays the expected short, uncharged N-terminal region and a high content of arginine, valine, alanine, and serine in the central region (Franzen et al., 1990). As has been observed with other Chlamydomonas chloroplast transit peptides, the amino acid following the initiator methionine is glutamine (Franzen et al., 1989; Roesler and Ogren, 1990). The cleavage site is predicted to reside between amino acids 28 and 29 based on the presence of Val-X-Ala at positions 26–28 (Franzen et al., 1990).

To ask whether the Ezy-1 polypeptide is indeed directed to zygotic chloroplasts, polyclonal antibodies (α -Ezy-1) were generated against a β -galactosidase-Ezy-1 fusion protein produced in *Escherichia coli*. The specificity of α -Ezy-1 was determined in several ways. Total RNA from 1 hr zygotes was translated in vitro in the presence of [35 S]methionine, and the translation products were immunoprecipitated with α -Ezy-1; in addition, early zygotes were incubated in [14 C]acetate, and labeled proteins were isolated after 1 hr and immunoprecipitated with α -Ezy-1. The immunoprecipitated products were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. In both instances, a single polypeptide was de-

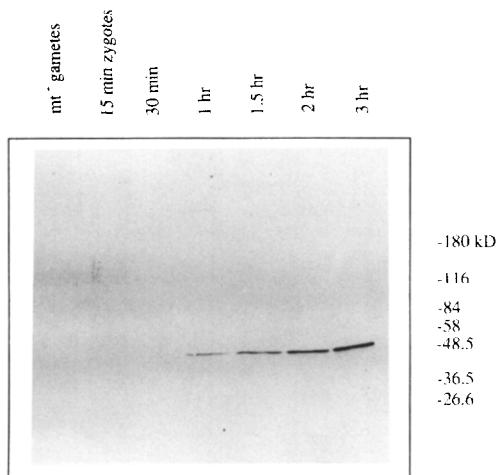


Figure 3. Western Analysis of Total Protein from Gametes and Zygotes

Approximately equal amounts of total protein from mt^+ gametes and 15 min, 30 min, 1 hr, 1.5 hr, 2 hr, and 3 hr zygotes were separated using SDS-PAGE and then transferred to nitrocellulose and probed with α -Ezy-1.

tected (Figure 2). These same bands were not observed when the preimmune serum was used for immunoprecipitation (data not shown). The 48 kd polypeptide immunoprecipitated in vitro (Figure 2, lane A) corresponded in size to the ezy-1 gene product predicted by the sequence data. In contrast, the polypeptide recognized in vivo was approximately 4–5 kd smaller (Figure 2, lane B), presumably owing to the rapid in vivo cleavage of the predicted chloroplast transit peptide. To determine whether α -Ezy-1 recognized a zygote-specific polypeptide, total protein was isolated from different developmental stages and probed on a Western blot with α -Ezy-1 (Figure 3). Beginning about 30 min after gamete fusion, a single polypeptide was detected, which was the same molecular weight as that immunoprecipitated from the in vivo labeling; no polypeptide was recognized in either gametes or 15 min zygotes.

When α -Ezy-1 was used in immunofluorescence studies, a strong punctate staining pattern was detected in early zygotes, first apparent about 30 min after gamete fusion. Neither gametes nor 15 min zygotes displayed any α -Ezy-1 staining (Figures 4A–4C). The localization of the antibodies in early zygotes was completely coincident with the DAPI fluorescence from the chloroplast nucleoids (Figures 4D–4F). There was no labeling of either zygote nucleus, confirming that the Ezy-1 polypeptide is directed to the chloroplasts only. The use of optical sectioning microscopy permitted an analysis of each nucleoid present within the zygote. Very young zygotes, those less than about 1 hr old, still possessed nucleoids in both the mt^+ and mt^- chloroplasts; the α -Ezy-1 antibodies localized to all of these nucleoids. By about 1.5 hr after gamete fusion, the number of DAPI-stained nucleoids rapidly decreased from an average of 14 per zygote to an average of 8 (data not shown), a consequence of the selective destruction of the nucleoids from the mt^+ chloroplast. As the DAPI staining

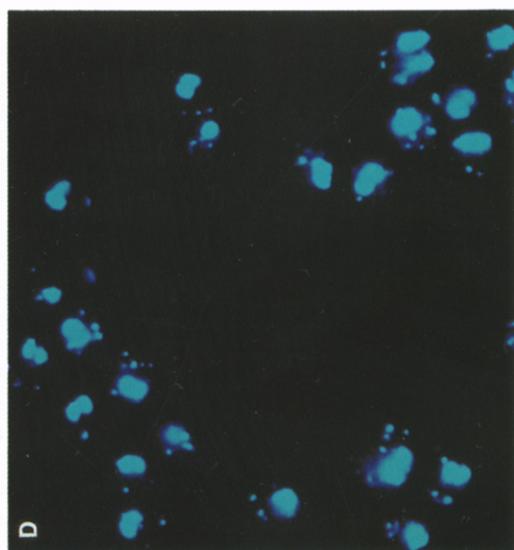
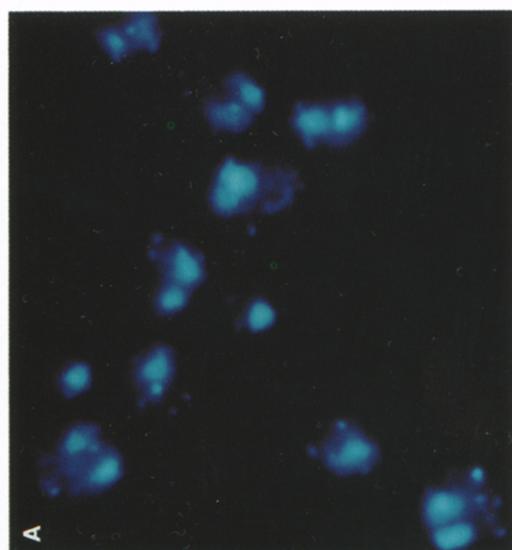
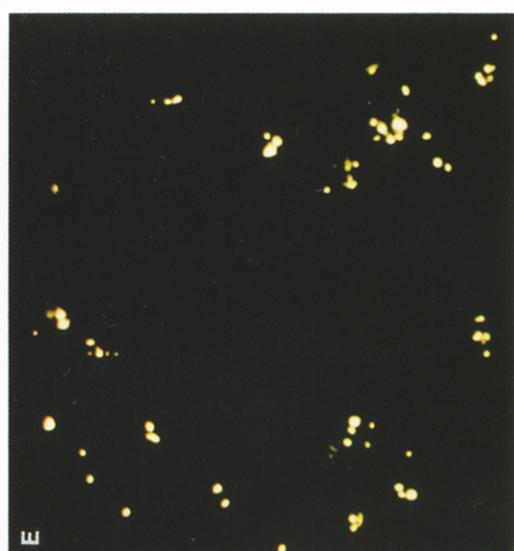
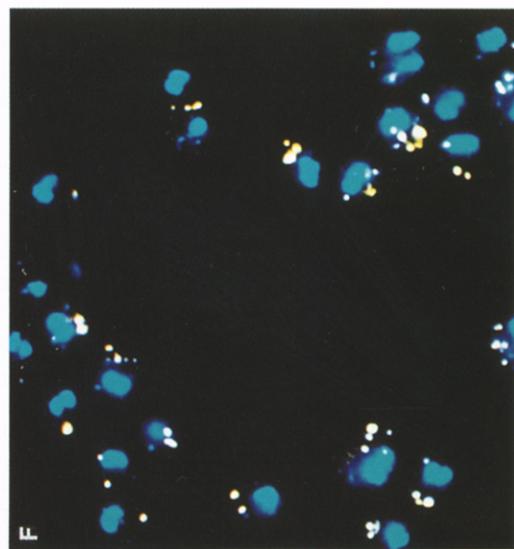
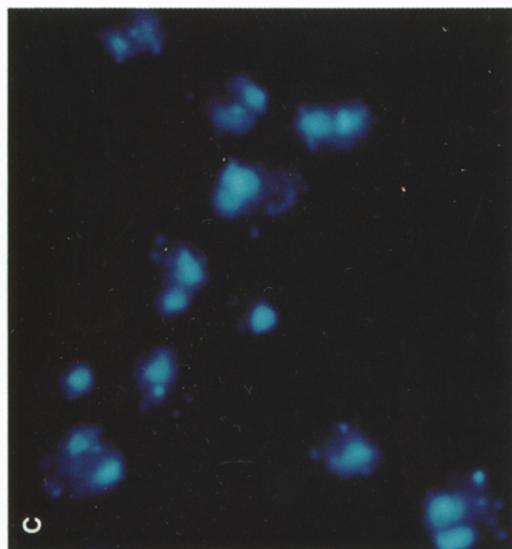


Table 1. Effects of UV Irradiation of *mt*⁺ and *mt*⁻ Gametes on Gamete and Zygote Functions

UV Exposure (s)	% Gamete Survival		% Mating ^a		Timing of Zygotic Wall Insolubilization (hr) ^b		% Inheritance of <i>Kan</i> ^R from the <i>mt</i> Gamete	
	<i>mt</i> ⁻	<i>mt</i> ⁺	<i>mt</i> ⁻	<i>mt</i> ⁺	<i>mt</i> ⁻	<i>mt</i> ⁺	<i>mt</i> ⁻	<i>mt</i> ⁺
0	100	100	52	52	6	6	0	0
5	100	45	40	42	20	6	0	4
10	37	9	50	49	20	6	0	7
20	4	0.6	30	42	NA ^c	6	0	27
30	1.5	0.4	26	53	NA	20	0	73

^a Gametes had been mating in the dark for 1.5 hr when the percent mating was determined.^b Time at which the zygotes were resistant to incubation in 0.1% Nonidet P-40. Measurements were taken at 0, 0.5, 1.5, 3, 4.5, 6, and 20 hr after gametes were mixed.^c NA, not applicable, indicates that the decreased mating efficiency and the fact that gametes do not regenerate walls prevented a determination of the timing of zygote wall insolubilization.

of the *mt*⁻ nucleoids disappeared, the immunofluorescence of these nucleoids also disappeared.

UV Irradiation of *mt*⁺, but Not *mt*⁻, Gametes Disrupt Chloroplast Inheritance Patterns and the Production of the Ezy-1 Polypeptide in the Zygote

Both the localization and the timing of the appearance of the Ezy-1 polypeptide are consistent with a role for this polypeptide in the selective degradation of the *mt*⁻ nucleoids in early zygotes. To address this possibility more directly, we examined the level of *ezy-1* expression in zygotes after irradiation of either the *mt*⁻ or *mt*⁺ gametes with UV, a treatment known to disrupt the normal uniparental inheritance patterns in *C. reinhardtii* (Sager and Ramanis, 1967). When *mt*⁻ gametes possessing the chloroplast marker kanamycin resistance (*kan*^R) were UV irradiated for 0, 5, 10, 20, or 30 s and mated with wild-type *mt*⁺ gametes, none of the resulting zygotes produced *kan*^R progeny, regardless of the duration of the UV exposure (Table 1). In other words, the zygotes behaved normally and transmitted chloroplast traits from the *mt*⁺ parent only. In contrast, when wild-type *mt*⁺ gametes were exposed to the same amount of UV and mated with nonirradiated *kan*^R *mt*⁻ gametes, the percentage of zygotes that transmitted the *mt*⁻ chloroplast marker and produced *kan*^R progeny increased to as much as 73% after 30 s of UV (Table 1). Importantly, UV irradiation of the *mt*⁺ gamete for only 5 s was enough to produce significant biparental inheritance, confirming that UV irradiation of the *mt*⁺ gamete uniquely affects the inheritance of chloroplast traits in a dosage-

dependent manner. Thus, despite the fact that the survivorship and mating efficiency of *mt*⁺ gametes are compromised by increasing UV exposure (Table 1), the selective destruction of the *mt*⁻ nucleoids, a zygote-specific function, is disrupted only when *mt*⁺ gametes are irradiated. Viable progeny are generated after each UV treatment, although zygote development (gauged by the timing of the formation of the detergent-resistant zygote cell wall) is delayed with increasing UV dosage (Table 1).

If the *ezy-1* gene is necessary for the selective degradation of unprotected *mt*⁻ nucleoids, the disruption of uniparental inheritance patterns by UV should be preceded by a comparable disruption of *ezy-1* expression. To test this, gametes were again exposed to increasing UV irradiation and mated in the dark for 2 hr to prevent any potential photoreactivation (Sager and Ramanis, 1967). Total protein was isolated for Western blot analysis 1.5 hr after the gametes were mixed (while the cells were still in the dark) and 3.5 hr after mixing (when the cells had been back in the light for about 1.5 hr). In nonirradiated controls, Ezy-1 polypeptide was present at both time points (Figure 5). Similarly, when the *mt*⁻ gametes were irradiated for 5, 10, or 20 s prior to mating, comparable amounts of Ezy-1 polypeptide were present in both the dark and the light samples (Figure 5). In contrast, when *mt*⁺ gametes were exposed to the same brief amounts of UV irradiation and then mated with nonirradiated *mt*⁻ gametes, the amount of Ezy-1 polypeptide produced by the zygotes was dramatically reduced, regardless of whether the zygotes were in the dark or the light (Figure 5).

Immunofluorescence analysis of the 1.5 hr zygotes re-

Figure 4. DAPI Staining and α -Ezy-1 Immunofluorescence of Early Zygotes

- (A) DAPI staining of a 15 min zygote sample containing both unmated gametes and early zygotes, identified by the presence of one or two lobate nuclei within a given cell. The small punctate structures are the chloroplast nucleoids. Magnification, 1735 \times .
- (B) α -Ezy-1 staining of the same field of cells as in (A), showing the lack of antibody reaction in either gametes or very early zygotes.
- (C) DAPI and α -Ezy-1 staining of the same field of cells as in (A) and (B).
- (D) DAPI staining of a 1 hr zygote sample. Note that the two zygotic nuclei are closer together and have begun to fuse in some cells. Chloroplast nucleoids from both parents are still present in these zygotes. Magnification, 1162 \times .
- (E) α -Ezy-1 staining of the same field of cells as in (D).
- (F) DAPI fluorescence and α -Ezy-1 staining of the same field of cells as in (D) and (E). The localization of α -Ezy-1 is completely coincident with the DAPI fluorescence from the chloroplast nucleoids.

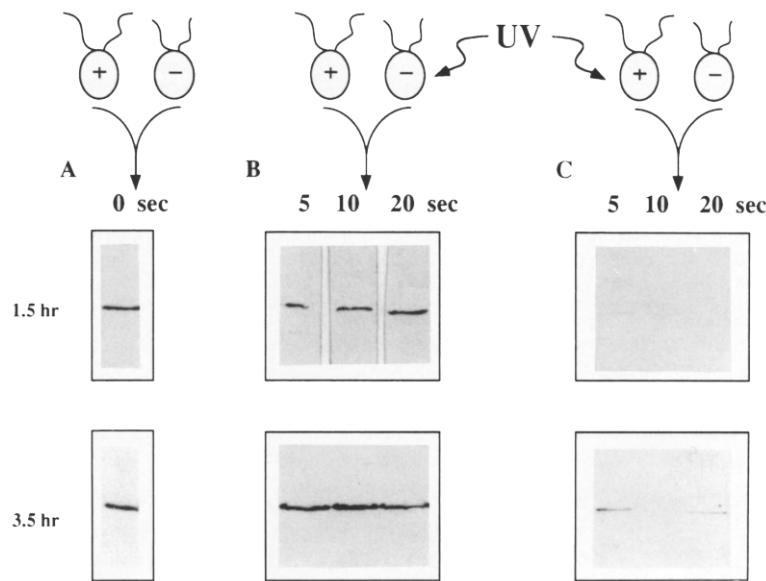


Figure 5. Effects of UV Irradiation on ezy-1 Expression

The expression of ezy-1 was analyzed in zygotes whose parents received no UV irradiation (A), whose mt⁺ parent was UV irradiated for either 5, 10, or 20 s prior to mating (B), or whose mt⁺ parent was UV irradiated for either 5, 10, or 20 s prior to mating (C). Total protein was extracted from each sample either 1.5 hr after mating (when the samples were still in the dark) or 3.5 hr after mating (about 1.5 hr after the samples had been returned to the light). The protein samples were analyzed using SDS-PAGE, transferred to nitrocellulose, and probed with α -Ezy-1. The percent mating that occurred under each condition was used to normalize the protein loading, so approximately the same amount of zygote protein was analyzed for each sample.

vealed a normal localization of α -Ezy-1 to chloroplast nucleoids when the mt⁺ gametes were irradiated (data not shown). In contrast, there was no α -Ezy-1 staining in the 1.5 hr zygote samples following 10 or 20 s of UV irradiation of the mt⁺ parent; there was, however, very weak immunofluorescence from chloroplast nucleoids within some zygotes from the 5 s samples, indicating that, if Ezy-1 polypeptide is made, it is properly transported to the nucleoids (data not shown). After the zygotes were returned to the light, Western blot analysis indicated that the amount of Ezy-1 polypeptide produced in the mt⁺-irradiated samples had increased, but the levels were still much lower than those of the mt⁺-irradiated samples (Figure 5). The immunofluorescence images from these later samples reflected this increase in Ezy-1 polypeptide, but both the number of zygotes stained and the amount of α -Ezy-1 labeling per nucleoid were substantially reduced when the mt⁺ parent was irradiated for 5 or 10 s and almost undetectable when the mt⁺ parent was irradiated for 20 s. Therefore, UV irradiation of the mt⁺, but not the mt⁻, parent prior to mating not only disrupts the normal uniparental inheritance of chloroplast traits but also the expression of the ezy-1 gene in a dosage-dependent manner.

UV Irradiation of mt⁺, but Not mt⁻, Gametes Selectively Disrupts Accumulation of ezy-1 Message

RNAse protection analysis (Melton et al., 1984) was used to determine whether UV irradiation of the mt⁺ gametes prior to mating also selectively disrupts the accumulation of ezy-1 message in early zygotes. Gametes of both mating types were exposed to UV irradiation for 0, 5, 10, or 20 s and mated in the dark with nonirradiated gametes of the opposite mating type; 1.5 hr later, total RNA was extracted from each zygote sample. The resulting RNA was hybridized with two zygote-specific antisense RNA probes, one derived from the ezy-1 gene and a second derived from the Class IV gene, which encodes a zygote wall protein

(Woessner and Goodenough, 1989). The ezy-1 probe protects an mRNA fragment of 118 nt, while the Class IV probe protects an mRNA fragment of 142 nt.

When gametes of either mating type were exposed to increasing amounts of UV irradiation just prior to mating, a general decrease in zygote-specific mRNA levels was observed (Figure 6A), which is consistent with the slowdown in zygote development noted earlier (Table 1). However, for a given UV treatment, comparable amounts of Class IV mRNA were detected regardless of whether the mt⁺ or mt⁻ gametes were irradiated (Figure 6A). In contrast, the ezy-1 message levels decreased dramatically when the mt⁺, but not the mt⁻, gametes were exposed to increasing amounts of UV irradiation just prior to mating (Figure 6A). Because of the apparent general decrease in zygote-specific transcription with increasing exposure of gametes to UV irradiation prior to mating, an important parameter becomes the abundance of ezy-1 message relative to the Class IV message. A decrease in the relative abundance of ezy-1 message was observed when the mt⁺ gametes were exposed to only 5 s of UV; by 20 s of UV irradiation of the mt⁺ gametes, the abundance of ezy-1 mRNA relative to Class IV mRNA had decreased by about 70% (Figure 6B). We have also found, using Northern blot analysis (data not shown), that the abundance of ezy-1 message relative to the message from two other zygote-specific genes, Classes I and VI (Ferris and Goodenough, 1987), decreased when the mt⁺, but not the mt⁻, gametes were exposed to increasing amounts of UV irradiation just prior to mating. Thus, accumulation of ezy-1 message is dramatically and selectively inhibited by UV irradiation of the mt⁺ parent just prior to mating.

Discussion

The selective degradation of the mt⁻ chloroplast genome in Chlamydomonas zygotes, a phenomenon that results in the inheritance of chloroplast genes from the mt⁺ parent

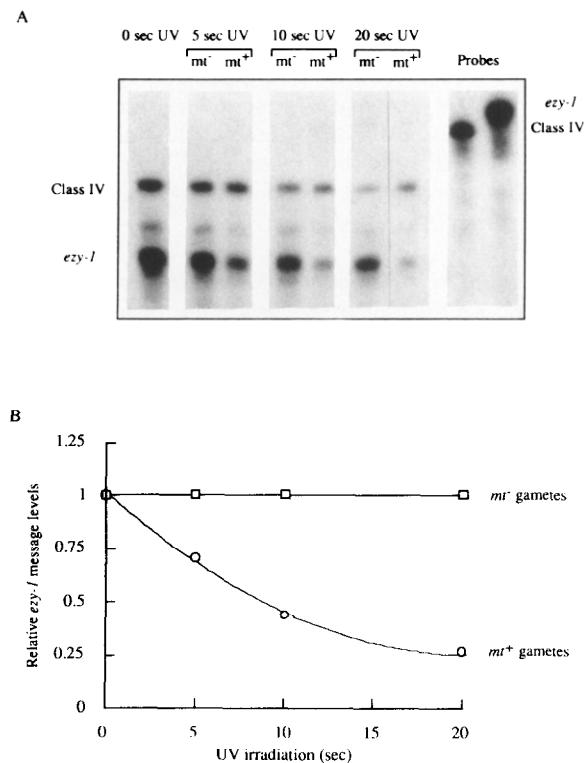


Figure 6. Effect of UV Irradiation on the Accumulation of Zygote-Specific mRNA

(A) RNAase protection analysis of steady-state levels of *ezy-1* and Class IV message in zygotes whose *mt*⁺ or *mt*⁻ parent was UV irradiated for 0, 5, 10, or 20 s. Total RNA was extracted 1.5 hr after mating and hybridized with *ezy-1* and Class IV antisense RNA probes. The *ezy-1* probe is 176 nt, and the protected fragment is 118 nt. The Class IV probe is 174 nt, and the protected fragment is 142 nt. Less RNA was used for hybridization in lane 6; to facilitate visual comparison, lane 6 represents a 2 day rather than 1 day exposure.

(B) The *ezy-1* and Class IV message levels were scanned with a densitometer and, for a given UV exposure, the abundance of *ezy-1* mRNA relative to Class IV mRNA was assumed to be 1 when the *mt*⁻ gametes were irradiated prior to mating. Relative *ezy-1*/Class IV abundances in zygotes in which the *mt*⁺ gametes had been irradiated prior to mating were then scaled accordingly.

only, has been repeatedly documented and genetically analyzed for the past 40 years, but the molecular basis of this process has remained mysterious. We have presented evidence here that the developmentally regulated gene *ezy-1* is involved in the selective degradation of the *mt*⁻ chloroplast genome in early zygotes and hence the uniparental inheritance of chloroplast traits by meiotic progeny.

Zygote-Specific Gene Transcription

At least seven genes, including *ezy-1*, are uniquely expressed in Chlamydomonas zygotes. The almost immediate transcription of these zygote-specific genes occurs long before the nuclei within the zygote fuse and is independent of new protein synthesis; transcription proceeds when gametes are incubated in cycloheximide both prior to mating and during mating and early zygote development (Ferris and Goodenough, 1987). To explain these patterns,

it has been proposed (Goodenough and Ferris, 1987) that gametes contain mating type-specific regulatory proteins, referred to as either P (*mt*⁺-specific) or M (*mt*⁻-specific), that interact in the zygote, perhaps creating a heterodimer similar to $\alpha 1-\alpha 2$ in yeast diploids (Strathern et al., 1981; Sprague, 1990). It is not yet known whether the postulated P-M interaction relieves a repression of zygote-specific gene transcription, as occurs in yeast (Mitchell and Herskowitz, 1986), or whether it instead directly activates transcription. The important point is that the zygote genes are transcribed only when gamete cytoplasms are mixed (Ferris and Goodenough, 1987).

We have shown here that accumulation of *ezy-1* mRNA is selectively inhibited by exposure of *mt*⁺, but not *mt*⁻, gametes to low levels of UV irradiation just prior to mating, a treatment that also prevents the selective degradation of the *mt*⁻ chloroplast DNA. In a previous study, using two-dimensional gel electrophoresis, Nakamura et al. (1988) found that six polypeptides were missing from early zygotes when the *mt*⁺ parent, but not the *mt*⁻ parent, was UV irradiated for 40 s (a relatively long exposure; compare Figure 6) prior to mating. They suggested that one or more of these polypeptides were involved in the selective degradation of the *mt*⁻ nucleoids. Although none of these polypeptides has been directly shown to localize to the chloroplast, their polypeptide 4, with an estimated molecular mass of 52 kd and a pI of 4.9, likely corresponds to the Ezy-1 polypeptide.

A possible explanation for the differential UV effect is that irradiation disables transcription in the *mt*⁺ nucleus, affecting subsequent transcription of *ezy-1* in the zygote; however, this explanation requires that the 8 copies of the *ezy-1* gene contributed to the zygote by the *mt*⁻ gamete be always silent and thus unable to rescue the inhibited *mt*⁺ nucleus, an assumption we have no reason to make, since the *mt*⁺ and *mt*⁻ copies appear to be nearly identical. An alternative explanation is that transcription of the postulated P gene itself is inactivated when *mt*⁺ gametes are irradiated, an explanation contradicted by the fact that cycloheximide does not inhibit the transcription of early zygote genes (Ferris and Goodenough, 1987), meaning that stable P and M proteins must be presynthesized and stored in the gametes. A third possible explanation is that UV irradiation inactivates a factor, supplied by *mt*⁺ gametes, that acts to stabilize *ezy-1* mRNA in the zygote.

A fourth explanation, which we favor, is that UV somehow inactivates P itself and hence blocks P-M formation and *ezy-1* transcription. The inactivation of P might well be an indirect effect of UV. For example, an appealing, but as yet unproven, notion is that UV might activate a protease in Chlamydomonas similar to RecA, which proteolyzes a LexA-like repressor and hence stimulates the expression of genes involved in DNA repair (Walker, 1985); such a protease might also inactivate the P polypeptide. The proteolysis of P might be adventitious; alternatively, this trait may have been selected, perhaps to assure that, when *mt*⁺ gametes are exposed to UV irradiation and their chloroplast genomes potentially damaged, copies of intact *mt*⁻ chloroplast genomes are nonetheless transmitted to the next generation.

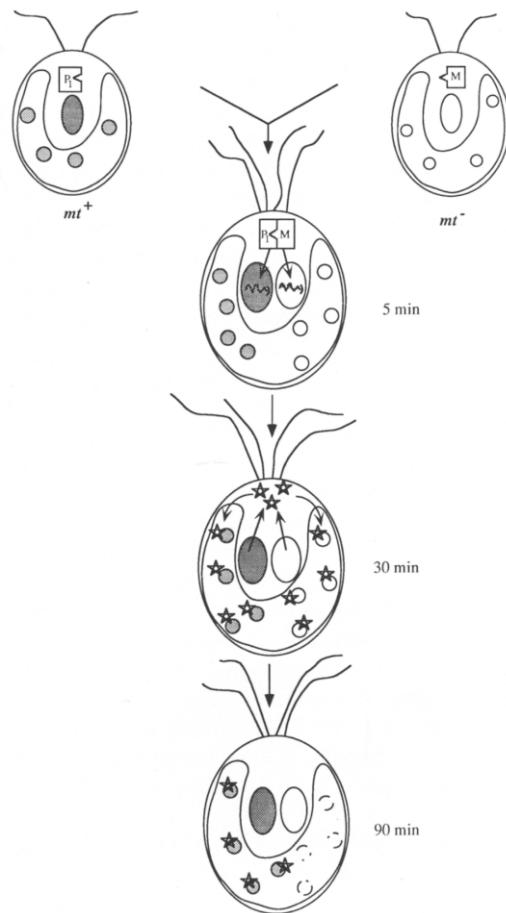


Figure 7. Schematic of the Behavior of the Ezy-1 Polypeptide in Early Zygotes

The mt^+ and mt^- gametes are depicted as possessing a nucleus, a single cup-shaped chloroplast with four nucleoids, and a single regulatory factor, either P₁ or M. The nucleoids of the mt^+ gametes are associated with protector (stippling). (For simplicity, only a single chloroplast is drawn in the early zygote, although some evidence suggests that the two organelles may remain distinct for at least 3 hr [Cavalier-Smith, 1970; Balden et al., 1991].) Upon gamete fusion, P₁ and M interact, and transcription of the ezy-1 gene is initiated within both nuclei (wavy arrows). The Ezy-1 polypeptide, represented by stars, is translated in the cytoplasm and immediately transported to the chloroplast(s), where it interacts with both sets of chloroplast nucleoids but permits the degradation of only the unprotected chloroplast DNA from the mt^- parent.

The fact that transcription of the ezy-1 gene is much more sensitive to UV irradiation than that of other zygote genes can be explained in one of two ways. Expression of the ezy-1 cluster may be particularly sensitive to concentrations of a "universal" P-M heterodimer. Alternatively, several P-M species may activate the zygote program: specifically, a particularly UV-sensitive factor P₁ might be required for ezy-1 transcription, with more resistant P₂-P_n factors involved in the expression of other zygote-specific genes. A transcriptional analysis of the zygote genes will be necessary to differentiate between these two possibilities. For the present we will adopt the notion that a UV-sensitive P₁ factor is necessary for ezy-1 transcription and that P₁-M is the active ezy-1 transcription complex.

As noted in the Introduction, *mat-3 mt⁺* gametes behave like UV-irradiated mt^+ gametes; specifically, about 70%–80% of zygotes from a *mat-3* cross display biparental inheritance of chloroplast genes (Gillham et al., 1987). Unfortunately, the *mat-3* mutation readily undergoes suppression, and a nonsuppressed strain is currently not available; therefore, we have not yet been able to test its effect on ezy-1 expression.

A Model of ezy-1 Function in Chlamydomonas

The two simplest interpretations of our results are that the Ezy-1 polypeptide is either the postulated "destroyer," acting directly as an (endo)nuclease to degrade unprotected chloroplast genomes, or else an activator necessary for the proper functioning of the destroyer, a possibility consistent with earlier models (Kuroiwa, 1985, 1991; Matagne, 1987; Sager and Grabowy, 1985). Since consensus motifs have not been identified for nucleases (R. Roberts, personal communication), nuclease assays with purified Ezy-1 polypeptide will be necessary to determine which interpretation is correct. Regardless of the outcome of these experiments, the key aspect of our model is that the uniparental inheritance of the chloroplast genome will be inhibited when the zygote-specific expression of ezy-1 is disrupted (Figure 7). The Ezy-1 polypeptide is synthesized in the zygote cytoplasm within 30 min of gamete fusion and is rapidly transported to both zygotic chloroplasts, where it interacts with both the mt^+ and mt^- chloroplast nucleoids. Protection of the mt^+ chloroplast DNA somehow prevents the action of the Ezy-1 polypeptide, and only the unprotected mt^- chloroplast genome is degraded during the next hour of zygote development. Thus, only the mt^+ chloroplast DNA will persist until the end of zygote dormancy to be transmitted to the meiotic progeny. Any manipulation that decreases the production of the Ezy-1 polypeptide, such as UV irradiation of the mt^+ gamete, also decreases the extent of degradation of the mt^- chloroplast DNA and therefore compromises uniparental inheritance patterns.

By this model, selective degradation of the mt^- chloroplast genomes occurs only when zygote-specific transcription of ezy-1 is activated by the postulated P₁-M factor. This explains why mt^- chloroplast genomes persist in the occasional vegetative diploids formed in sexual crosses (Gillham, 1963) that forego the zygote program and in somatic fusion diploids between cells of the same mt (Matagne and Hermesse, 1980; Matagne, 1981), which would never form P₁-M. The mt^- chloroplast genome is also transmitted when mt^+/mt^- diploid gametes (phenotypically mt^+) are crossed with mt^+ gametes (Matagne and Mathieu, 1983; Tsubo and Matsuda, 1984), an outcome that can be explained by proposing that the mt^+ -specified protector is synthesized by the diploid and associates with the chloroplast genomes from both parents, hence protecting both from the ensuing action of the Ezy-1 polypeptide.

Once the selective degradation of the mt^- chloroplast DNA is complete, the zygote presumably possesses some way of inactivating the responsible nuclease. We have observed that α -Ezy-1 dissociates from the remaining mt^- chloroplast nucleoids once the mt^- nucleoids have dis-

peared and that this change in α -Ezy-1 staining patterns is coincident with a posttranslational modification that creates a more basic version of the Ezy-1 polypeptide. The fate of the Ezy-1 polypeptide in these later zygotes will be the subject of a separate communication.

Experimental Procedures

Strains and Culture Conditions

All C. reinhardtii strains were maintained in continuous light on Tris-acetate-phosphate (TAP) media (Gorman and Levine, 1965) solidified with 1.5% agar. Wild-type strains CC620 (mt^+) and CC621 (mt^+) were used for all experiments except those that required a determination of the chloroplast inheritance patterns. The mutant strain CC2660 ($kr-u-24-2 ac-29a nic-7 mt^+$) requires 2 μ g/ml nicotinamide and is resistant to 100 μ g/ml kanamycin (kan^r), owing to a single C \rightarrow T change in the chloroplast 16S rRNA gene (Harris et al., 1989). Gametes were obtained by transferring cells maintained on plates for at least 7 days (Martin and Goodenough, 1975) to nitrogen-free high salt minimal (HSM) (Sueoka, 1960) for 1–2 hr. The mating efficiency of a cross is calculated by counting unmated biflagellate gametes (BFC) and mated quadriflagellate zygotes (QFC) and applying the formula: % mating = [2QFC / (BFC + 2QFC)] \times 100.

UV Irradiation of Gametes and Determination of Chloroplast Inheritance Patterns

Gametes of each mating type were diluted to approximately 5×10^6 cells per ml in nitrogen-free HSM, and 5 ml aliquots were transferred to 5.5 cm glass petri dishes with constant stirring. Cells were irradiated as described by Harris (1989) for 5, 10, 20, or 30 s with a germicidal UV lamp (Sylvania G30T8, 30 W, $\lambda = 257$). Gametes were mated in the dark for 2 hr to prevent any potential photoreactivation of the UV effects (Sager and Ramanis, 1967) (during the dark interval, all necessary manipulations were performed in the presence of a sodium safe light). Mating cells and aliquots of unmated irradiated gametes were then returned to the light. To determine survivorship, irradiated mt^+ gametes were plated on TAP, and irradiated mt^+ gametes were plated on TAP supplemented with 2 μ g/ml nicotinamide (TAP + nic). Zygotes were plated on TAP + nic and allowed to mature and germinate as described by Levine and Ebersold (1960). The resulting zygote colonies formed by the meiotic products were replica plated onto permissive plates (TAP + nic) or restrictive plates (TAP + nic + 100 μ g/ml kanamycin). Only colonies including kan^r progeny were able to grow on restrictive plates.

Isolation of Genomic and Full-Length cDNA Clones

A zygote-specific partial cDNA (cDNA 28; Ferris and Goodenough, 1987) was labeled with [α -³²P]dCTP (Du Pont NEN Research Products, Boston), using the random primed labeling kit (Boehringer Mannheim, Indianapolis, Indiana), and used to isolate phage clones from a library containing partially Sau3AI-digested genomic DNA inserted into λ EMBL3, as described by Ferris (1989). The cDNA clone was also used to screen a cDNA expression library in Uni-ZAPXR (Stratagene, La Jolla, California) prepared from 1 hr zygotic poly(A)⁺ RNA (courtesy of J. Woessner). The library was plated on E. coli strain C600 and transferred to nitrocellulose according to Sambrook et al. (1989). The filters were prehybridized and hybridized at 65°C according to Church and Gilbert (1984). Inserts from positive clones were excised as Bluescript SK(+) plasmids with R408 helper phage according to the manufacturer's instructions. Both the genomic clones and the cDNA clones were characterized by restriction mapping.

DNA Sequencing and Analysis

Single-stranded DNA was generated (Ausubel et al., 1989) from either DNA subcloned into pUC118 or pUC119 (Vieira and Messing, 1987) or from the cDNAs in pBluescript SK and used in dideoxy sequencing with the Sequenase kit (US Biochemical, Cleveland). The sequence data were compiled and analyzed using the Genetics Computer Group Sequence Analysis Software Package for VAX/VMS computers (Devereux et al., 1984).

Construction of Fusion Protein and Antibody Preparation

A 1.2 kb Xhol-HindIII fragment from the 3' end of cDNA 28 was ligated in-frame into the expression vector pWR590 (Guo et al., 1984) and used to transform E. coli strain JM109. The resulting fusion protein combined the first 590 amino acids of β -galactosidase with 216 amino acids from the C-terminus of the Ezy-1 polypeptide. The fusion protein was isolated as described by Woessner and Goodenough (1989) and used to generate polyclonal antibodies in a rabbit.

Affinity Purification of Polyclonal Antibodies

Approximately 6 mg of fusion protein was coupled to a 3.5 ml column of cyanogen bromide-activated Sepharose 4B beads (Pharmacia LKB Biotechnology, Piscataway, New Jersey) according to the manufacturer's instructions. The column was washed with 100 ml of phosphate-buffered saline (PBS), the rabbit serum was slowly run through the column at least five times, and the column was again washed with 100 ml of PBS. Bound antibodies were eluted with 8 ml of 0.1 M glycine (pH 3.0). The eluate was collected in 1 ml aliquots and neutralized with 100 μ l of 1 M Tris-HCl (pH 8.0). Aliquots containing protein were pooled, dialyzed against PBS, and concentrated using centricron 30 microconcentrators (Amicon, Beverly, Massachusetts).

Western Blot Analysis

For protein analysis, 1 ml of 1×10^7 to 2×10^7 cells per ml was pelleted in a microcentrifuge, resuspended in about 100 μ l of water, squirted into 1 ml acetone, and stored at -70°C. Each sample was spun for 10 min at 16,000 \times g, and the supernatant was discarded. The pellet was air dried and resuspended in 1 \times SDS loading buffer (0.358 M β -mercaptoethanol, 4 M urea, 0.18 M sucrose, 2% SDS, 0.02% pyronin Y, 50 mM Na₂CO₃) and analyzed by SDS-PAGE on either a 5%–15% or a 10% Laemmli (1970) gel. Protein was transferred to nitrocellulose (Towbin et al., 1979) using a Genie transfer system (Idea Scientific Corporation, Corvallis, Oregon). The nitrocellulose was blocked for 1 hr in 10% milk in PBS-0.1% Tween, incubated for 1 hr in α -Ezy-1 diluted in PBS, washed three times with PBS-0.1% Tween, incubated for 30 min in donkey anti-rabbit immunoglobulin horseradish peroxidase-linked whole antibody (Amersham International, Arlington Heights, Illinois) diluted in PBS-0.1% Tween, and then washed three times with PBS-0.3% Tween and three times with PBS-0.1% Tween. The ECL system was used to visualize reactive proteins (Amersham).

Immunoprecipitation of In Vitro and In Vivo Labeled Products

Total RNA isolated from either 1 or 3 hr zygotes (Woessner and Goodenough, 1989) was translated in vitro using a rabbit reticulocyte lysate system (Promega, Madison, Wisconsin) with [³⁵S]methionine (Du Pont NEN Research Products). Zygote proteins were labeled in vivo by incubating zygotes (~ 2×10^7 cells per ml) for 1 hr with universally labeled [¹⁴C]acetate (Du Pont NEN Research Products) at a final concentration of 10 μ Ci/ml. Zygotes were then pelleted, resuspended in 100 μ l of nitrogen-free HSM, and squirted into 1 ml of acetone. The immunoprecipitations were performed as described by Woessner and Goodenough (1989). The antibody-antigen complexes were released from the protein A-Sepharose CL-4B beads (Pharmacia) by boiling for 5 min in 2 \times SDS loading buffer and were analyzed by SDS-PAGE as described above. The gel was fixed for at least 30 min in 25% isopropanol-10% acetic acid, placed in Enlightning (Du Pont, Boston) for 30 min, vacuum dried, and exposed to Kodak XAR-5 film.

Immunofluorescence

Approximately 2×10^7 to 5×10^7 cells were pelleted briefly at 4000 \times g and washed once with 10 ml of fixation buffer (20 mM HEPES [pH 7.4], 10 mM MgSO₄, 2 mM dithiothreitol, 2 mM EGTA, 50 mM KCl). Washed cells were resuspended in 1 ml of fixation buffer with 20 μ M taxol. An equal volume of 8% paraformaldehyde in fixation buffer was added to the resuspended cells on ice; cells remained in fixative overnight at 4°C. Fixed cells were pelleted at 4°C at 4000 \times g and washed once with 10 ml of fixation buffer. Cells were resuspended in 1 ml of fixation buffer and allowed to settle for 10 min on slides pretreated for 1 min with 1% polyethyleneimine. The slide was washed three times with PBS, and cells were permeabilized for 5 min in -20°C acetone, blocked for 30 min at 37°C in 1.5% bovine serum albumin, 1.5% ovalbumin, and incubated overnight at room temper-

ture with α -Ezy-1. The slide was washed twice with PBS and incubated for 1 hr in the dark with fluorescein-conjugated goat anti-rabbit secondary antibodies (Organon Teknica, Durham, North Carolina), washed twice with PBS, incubated for 1 min with a 1 μ g/ml concentration of the DNA fluorochrome DAPI, washed twice with PBS, and mounted in 90% glycerol containing 5% propylgallate (Kodak, Rochester, New York). For all immunofluorescence analysis, an optical sectioning microscope was used that was equipped with an inverted Olympus IMT-2 microscope (Olympus, Lake Success, New York), a cooled (-45°C) charge-coupled device camera (Photometric, Tucson, Arizona) with a Kodak KAF1400 charge-coupled device chip, and a microslipping motor (Compumotor, Petaluma, California) attached to the focus knob and controlled by a Titan computer (Kubota Pacific Computer, Santa Clara, California). Photographs were taken directly from the computer screen.

RNAase Protection Analysis

Total RNA was isolated from about 1.5×10^6 cells essentially as described by Kirk and Kirk (1985). The ezy-1 RNA probe was generated by subcloning into pBluescript II KS a 600 bp PstI-EcoRI fragment from the 5'-most end of cDNA clone 10. The resulting plasmid was linearized with XbaI, and T7 RNA polymerase was used to in vitro transcribe an antisense probe of 176 nt. The protected ezy-1 probe is 118 nt. The Class IV probe was generated by subcloning into pBluescript II SK a 625 bp Pvull-HincII fragment from near the 5' end of the Class IV cDNA (Woessner and Goodenough, 1989). The resulting plasmid was linearized with NsiI, and T7 polymerase was used to in vitro transcribe a 174 nt antisense probe. The protected Class IV probe is 142 nt. Both probes were gel purified, and 2.3×10^4 cpm of probe was used per sample. The Ambion RPA II Kit (Ambion, Austin, Texas) was used for all RNAase protection assays. Approximately 1.5 μg of total RNA from the 0 s zygote sample was used for hybridization; all other samples were scaled up according to mating efficiencies so that approximately equivalent amounts of zygote RNA were used for each hybridization. The hybridized samples were digested with a mixture of RNAase A and RNAase T1 according to the manufacturer's instructions. The RNA was electrophoresed on a 10% polyacrylamide-8 M urea gel. Preflashed autoradiograms were scanned with a Computing Densitometer model 300A (Molecular Dynamics, Sunnyvale, California).

Acknowledgments

We would like to thank Jeff Woessner, Liz Orr, and Malcolm Campbell for their helpful suggestions and Carol Hwang for her technical assistance. We are also grateful to Richard Keeling, Jim McNally, and Kathy Miller for their help with the optical sectioning microscope (purchased with grant S10-RR04775-01 from the National Institutes of Health). E. V. A. was supported by a National Science Foundation (NSF) postdoctoral fellowship (OCE-9023861); P. J. F. and U. W. G. were supported by grant DMB-8917050 from the NSF.

Received December 21, 1992; revised June 24, 1993.

References

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Siedman, J. G., Smith, J. A., and Struhl, K. (1989). *Current Protocols in Molecular Biology* (New York: John Wiley and Sons).
- Baldan, B., Girard-Bascou, J., Wollman, F.-A., and Olive, J. (1991). Evidence for thylakoid membrane fusion during zygote formation in *Chlamydomonas reinhardtii*. *J. Cell Biol.* 114, 905-915.
- Cavalier-Smith, T. (1970). Electron microscopic evidence for chloroplast fusion in zygotes of *Chlamydomonas reinhardtii*. *Nature* 228, 333-335.
- Church, G. M., and Gilbert, W. (1984). Genomic sequencing. *Proc. Natl. Acad. Sci. USA* 81, 1991-1995.
- Coleman, A. W. (1984). The fate of chloroplast DNA during cell fusion, zygote maturation, and zygote germination in *Chlamydomonas reinhardtii* as revealed by DAPI staining. *Exp. Cell Res.* 152, 528-540.
- Cosmides, L. M., and Tooby, J. (1981). Cytoplasmic inheritance and intragenomic conflict. *J. Theor. Biol.* 89, 83-129.
- Devereux, J., Haeblerli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids Res.* 12, 387-395.
- Ebersold, W. T., Levine, R. P., Levine, E. E., and Olmsted, M. A. (1962). Linkage maps in *Chlamydomonas reinhardtii*. *Genetics* 47, 531-543.
- Ferris, P. J. (1989). Characterization of a *Chlamydomonas* transposon, *Gulliver*, resembling those in higher plants. *Genetics* 122, 363-377.
- Ferris, P. J., and Goodenough, U. W. (1987). Transcription of novel genes, including a gene linked to the mating-type locus, induced by *Chlamydomonas* fertilization. *Mol. Cell. Biol.* 7, 2360-2366.
- Franzen, L.-G., Frank, G., Zuber, H., and Rochaix, J.-D. (1989). Isolation and characterization of cDNA clones encoding the 17.9 and 8.1 kDa subunits of photosystem I from *Chlamydomonas reinhardtii*. *Plant Mol. Biol.* 12, 463-474.
- Franzen, L.-G., Rochaix, J.-D., and von Heijne, G. (1990). Chloroplast transit peptides from the green alga *Chlamydomonas reinhardtii* share features with both mitochondrial and higher plant chloroplast presequences. *FEBS Lett.* 260, 165-168.
- Gillham, N. W. (1963). Transmission and segregation of a non-chromosomal factor controlling streptomycin resistance in diploid *Chlamydomonas*. *Nature* 200, 294.
- Gillham, N. W. (1978). *Organelle Heredity* (New York: Raven Press).
- Gillham, N. W., Boynton, J. E., and Lee, R. W. (1974). Segregation and recombination of non-Mendelian genes in *Chlamydomonas*. *Genetics* 78, 439-457.
- Gillham, N. W., Boynton, J. E., Johnson, A. M., and Burkhardt, B. D. (1987). Mating type linked mutations which disrupt the uniparental transmission of chloroplast genes in *Chlamydomonas*. *Genetics* 115, 677-684.
- Gillham, N. W., Boynton, J. E., and Harris, E. H. (1991). Transmission of plastid genes. In *Cell Culture and Somatic Cell Genetics, Volume 7A*, L. Bogorad and I. K. Vasil, eds. (New York: Academic Press), pp. 55-92.
- Goodenough, U. W. (1991). *Chlamydomonas* mating interactions. In *Microbial Cell-Cell Interactions*, M. Dworkin, ed. (Washington, DC: American Society for Microbiology), pp. 71-112.
- Goodenough, U. W., and Ferris, P. J. (1987). Genetic regulation of development in *Chlamydomonas*. In *Genetic Regulation of Development*, W. F. Loomis, ed. (New York: Alan R. Liss), pp. 171-189.
- Gorman, D. S., and Levine, R. P. (1965). Cytochrome f and plastocyanin; their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* 54, 1665-1669.
- Guo, L.-H., Stepien, P. P., Tso, J. Y., Brousseau, R., Narang, S., Thomas, D. Y., and Wu, R. (1984). Synthesis of human insulin gene. VIII. Construction of expression vectors for fused proinsulin production in *Escherichia coli*. *Gene* 29, 251-254.
- Harris, E. H. (1989). *The Chlamydomonas Sourcebook* (San Diego: Academic Press).
- Harris, E. H., Burkhardt, B. D., Gillham, N. W., and Boynton, J. E. (1989). Antibiotic resistance mutations in the chloroplast 16S and 23S rRNA genes of *Chlamydomonas reinhardtii*: correlation of genetic and physical maps of the chloroplast genome. *Genetics* 123, 281-292.
- Hurst, L. D. (1992). Intrageneric conflict as an evolutionary force. *Proc. R. Soc. Lond. (B)* 248, 135-140.
- Hurst, L. D., and Hamilton, W. D. (1992). Cytoplasmic fusion and the nature of sexes. *Proc. R. Soc. Lond. (B)* 247, 189-194.
- Kirk, M. M., and Kirk, D. L. (1985). Translational regulation of protein synthesis, in response to light, at a critical stage of Volvox development. *Cell* 41, 419-428.
- Kuroiwa, T. (1985). Mechanisms of maternal inheritance of chloroplast DNA: an active digestion hypothesis. *Microbiol. Sci.* 2, 267-270.
- Kuroiwa, T. (1991). The replication, differentiation, and inheritance of plastids with emphasis on the concept of organelle nuclei. *Int. Rev. Cytol.* 128, 1-62.
- Kuroiwa, T., Kawano, S., and Nishibayashi, S. (1982). Epifluorescent microscopic evidence for maternal inheritance of chloroplast DNA. *Nature* 298, 481-483.

- Kuroiwa, T., Kawano, S., and Sato, C. (1983a). Mechanisms of maternal inheritance. I. Protein synthesis involved in preferential destruction of chloroplast DNA of male origin. *Proc. Jpn. Acad. (B)* 59, 177–181.
- Kuroiwa, T., Kawano, S. and Sato, C. (1983b). Mechanisms of maternal inheritance. II. RNA synthesis involved in preferential destruction of chloroplast DNA of male origin. *Proc. Jpn. Acad. (B)* 59, 182–185.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Levine, R. P., and Ebersold, W. T. (1960). The genetics and cytology of *Chlamydomonas*. *Annu. Rev. Microbiol.* 14, 197–216.
- Martin, N. C., and Goodenough, U. W. (1975). Gametic differentiation in *Chlamydomonas reinhardtii*. I. Production of gametes and their fine structure. *J. Cell Biol.* 67, 587–605.
- Matagne, R. F. (1981). Transmission of chloroplast alleles in somatic fusion products obtained from vegetative cells and/or 'gametes' of *Chlamydomonas reinhardtii*. *Curr. Genet.* 3, 31–36.
- Matagne, R. F. (1987). Chloroplast gene transmission in *Chlamydomonas reinhardtii*. A model for its control by the mating-type locus. *Curr. Genet.* 12, 251–256.
- Matagne, R. F., and Hermesse, M.-P. (1980). Chloroplast gene inheritance studied by somatic fusion in *Chlamydomonas reinhardtii*. *Curr. Genet.* 1, 127–131.
- Matagne, R. F., and Mathieu, D. (1983). Transmission of chloroplast genes in triploid and tetraploid zygospores of *Chlamydomonas reinhardtii*: roles of mating-type gene dosage and gametic chloroplast DNA content. *Proc. Natl. Acad. Sci. USA* 80, 4780–4783.
- Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl. Acids Res.* 12, 7035–7056.
- Mitchell, A. P., and Herskowitz, I. (1986). Activation of meiosis and sporulation by repression of RME1 product in yeast. *Nature* 319, 738–742.
- Munaut, C., Dombrowicz, D., and Matagne, R. F. (1990). Detection of chloroplast DNA by using fluorescent monoclonal anti-bromodeoxyuridine antibody and analysis of its fate during zygote formation in *Chlamydomonas reinhardtii*. *Curr. Genet.* 18, 259–263.
- Nakamura, S., Sato, C., and Kuroiwa, T. (1988). Polypeptides related to preferential digestion of male chloroplast nucleoids in *Chlamydomonas*. *Plant Sci.* 56, 129–136.
- Ris, H., and Plaut, W. (1962). Ultrastructure of DNA-containing areas in the chloroplast of *Chlamydomonas*. *J. Cell Biol.* 13, 383–391.
- Rochaix, J. D. (1978). Restriction endonuclease map of the chloroplast DNA of *Chlamydomonas reinhardtii*. *J. Mol. Biol.* 126, 597–617.
- Roesler, K. R., and Ogren, W. L. (1990). Primary structure of *Chlamydomonas reinhardtii* ribulose 1,5-bisphosphate carboxylase/oxygenase activase and evidence for a single polypeptide. *Plant Physiol.* 94, 1837–1841.
- Rosen, H., Newman, S. M., Boynton, J. E., and Gillham, N. W. (1991). A nuclear mutant that exhibits increased sensitivity to UV irradiation, reduced recombination of nuclear genes, and altered transmission of chloroplast genes. *Curr. Genet.* 19, 35–41.
- Sager, R. (1954). Mendelian and non-Mendelian inheritance of streptomycin resistance in *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* 40, 356–363.
- Sager, R., and Grabowy, C. (1985). Sex in *Chlamydomonas*: sex and the single chloroplast. In *The Origin and Evolution of Sex*. MBL Lectures in Biology, Volume 7, H. O. Halvorson and A. Monroy, eds. (New York: Alan R. Liss), pp. 113–121.
- Sager, R., and Ramanis, Z. (1967). Biparental inheritance of nonchromosomal genes induced by ultraviolet irradiation. *Proc. Natl. Acad. Sci. USA* 58, 931–937.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).
- Smith, G. M., and Regnery, D. C. (1950). Inheritance of sexuality in *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* 36, 246–248.
- Sprague, G. F. (1990). Combinatorial associations of regulatory proteins and the control of cell type in yeast. *Adv. Genet.* 27, 33–62.
- Strathern, J., Hicks, J., and Herskowitz, I. (1981). Control of cell type in yeast by the mating type locus: the $\alpha 1$ - $\alpha 2$ hypothesis. *J. Mol. Biol.* 147, 357–372.
- Sueoka, N. (1960). Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* 46, 83–91.
- Towbin, J., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76, 4350–4354.
- Tsubo, Y., and Matsuda, Y. (1984). Transmission of chloroplast genes in crosses between *Chlamydomonas reinhardtii* diploids: correlation with chloroplast nucleoid behavior in young zygotes. *Curr. Genet.* 8, 223–229.
- Van Winkle-Swift, K. P., and Salinger, A. P. (1988). Loss of mt⁻-derived zygotic chloroplast DNA is associated with a lethal allele in *Chlamydomonas moewusii*. *Curr. Genet.* 13, 331–337.
- Vieira, J., and Messing, J. (1987). Production of single-stranded plasmid DNA. *Meth. Enzymol.* 153, 3–11.
- Walker, G. C. (1985). Inducible DNA repair systems. *Annu. Rev. Biochem.* 54, 425–457.
- Whatley, J. M. (1982). Ultrastructure of plastid inheritance: green algae to angiosperms. *Biol. Rev.* 57, 527–569.
- Woessner, J. P., and Goodenough, U. W. (1989). Molecular characterization of a zygote wall protein: an extensin-like molecule in *Chlamydomonas reinhardtii*. *Plant Cell* 1, 901–911.
- Woessner, J. P., and Goodenough, U. W. (1992). Zygote and vegetative cell wall proteins in *Chlamydomonas reinhardtii* share a common epitope. (SerPro). *Plant Sci.* 83, 65–76.

GenBank Accession Numbers

The accession numbers for the ezy-1 sequences reported in this paper are L20945 (19024 bp), L20946 (2147 bp), and L20947 (1452 bp).