The Plant Cell, Vol. 9, 283–296, March 1997 © 1997 American Society of Plant Physiologists

RESEARCH ARTICLE

Nuclear Mutations That Block Group II RNA Splicing in Maize Chloroplasts Reveal Several Intron Classes with Distinct Requirements for Splicing Factors

Bethany D. Jenkins, Doris J. Kulhanek, and Alice Barkan[1]

Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403-1229

To elucidate mechanisms that regulate chloroplast RNA splicing in multicellular plants, we sought nuclear mutations in maize that result in chloroplast splicing defects. Evidence is presented for two nuclear genes whose function is required for the splicing of group II introns in maize chloroplasts. A mutation in the *crs1* (for chloroplast RNA splicing 1) gene blocks the splicing of only the *atpF* intron, whereas a mutation in the *crs2* gene blocks the splicing of many chloroplast introns. In addition, a correlation was observed between the absence of plastid ribosomes and the failure to splice several chloroplast introns. Our results suggest that a chloroplast-encoded factor and a nuclear-encoded factor whose activity requires *crs2* function facilitate the splicing of distinct sets of group II introns. These two genetically defined intron sets also differ with regard to intron structure: one set consists of only subgroup IIA introns and the other of only subgroup IIB introns. Therefore, it is likely that distinct splicing factors recognize subgroup-specific features of intron structure or facilitate subgroup-specific aspects of the splicing reaction. Of the 12 pre-mRNA introns in the maize chloroplast genome, only one is normally spliced in both *crs2* mutants and in mutants lacking plastid ribosomes, indicating that few, if any, of the group II introns in the chloroplast genome undergo autocatalytic splicing in vivo.

INTRODUCTION

RNA splicing, an essential step in mRNA synthesis, is a potential control point in gene expression. Although the splicing rate for most nuclear pre-mRNAs is rapid and is not generally rate limiting for gene expression, unspliced transcripts accumulate to high levels in mitochondria and chloroplasts (Hollingsworth et al., 1984; Collins and Lambowitz, 1985; Koller et al., 1985; Shinozaki et al., 1986; Westhoff and Hermann, 1988; Pel et al., 1992). Therefore, the modulation of organellar splicing rates is likely to impact the abundance of mature transcripts. The degree to which plastid RNAs are spliced varies between cell types in maize (Barkan, 1989), suggesting that the regulation of chloroplast RNA splicing contributes to the characteristic properties of plastids in different tissues.

The majority of organellar introns can be categorized as either group I or group II, based on their primary sequence and predicted structures (reviewed in Saldanha et al., 1993). Although several introns of both types have been shown to self-splice in vitro, genetic evidence indicates that efficient organellar RNA splicing in vivo requires *trans*-acting factors (Saldanha et al., 1993). Characterization of these factors will be important for understanding the regulatory mechanisms influencing organellar splicing and, more generally, the roles of accessory factors in modulating the functions of catalytic RNAs. Mechanistic similarities between group II and nuclear pre-mRNA splicing have led to the suggestion that nuclear pre-mRNA introns evolved from group II–like progenitors (Cech, 1986). A study of factors that facilitate group II splicing may also elucidate how nuclear pre-mRNA splicing evolved from an autocatalytic mechanism to one that is strictly dependent on *trans*-acting protein and RNA factors.

Genetic studies have revealed numerous genes whose products facilitate group I intron splicing in fungal mitochondria (reviewed in Saldanha et al., 1993) and one gene that facilitates the splicing of a group I intron in Chlamydomonas chloroplasts (Herrin et al., 1990). Group II intron splicing, however, has been less amenable to genetic analysis. Only two nuclear gene products in fungi have been reported that seem likely to play direct roles in the splicing of mitochondrial group II introns (Saldanha et al., 1993). Although at least 14 nuclear gene products are involved in the two *trans*-splicing events that generate mature *psaA* mRNA in Chlamydomonas chloroplasts (Choquet et al., 1988; Goldschmidt-Clermont et al., 1990), many of these may mediate functions specific to

[1] To whom correspondence should be addressed. E-mail abarkan@molbio.uoregon.edu; fax 541-346-5891.

the *trans*-splicing process rather than to group II intron splicing in general.

The paucity of genetic data concerning activators of group II splicing may result from the fact that fungal mitochondrial genomes and the Chlamydomonas chloroplast genome contain numerous group I but few group II introns. In contrast, chloroplast genomes of higher plants contain numerous group II introns (Plant and Gray, 1988; Rochaix, 1992), providing more potential targets for *trans*-acting splicing factors. Recent evidence suggests that such splicing factors must exist. For example, attempts to detect in vitro self-splicing of the wheat chloroplast *atpF* intron were not successful (Plant and Gray, 1988), the spinach chloroplast *atpF* intron remained unspliced when expressed in transgenic Chlamydomonas chloroplasts (Deshpande et al., 1995), and several group II introns remained unspliced in barley mutants lacking chloroplast ribosomes (Hess et al., 1994; Hübschmann et al., 1996). This last observation has been cited as evidence that at least one splicing factor is a chloroplast gene product.

To understand the molecular mechanisms responsible for regulated chloroplast RNA splicing and the roles of accessory factors in facilitating the splicing of group II introns, we sought mutations in nuclear genes that block chloroplast RNA splicing. We describe two nuclear genes that behave genetically as activators of group II *cis*-splicing in chloroplasts. A mutation in the *crs1* gene (for chloroplast RNA splicing 1) blocks the splicing of just one chloroplast intron, whereas a mutation in the *crs2* gene blocks the splicing of many but not all group II introns in the chloroplast. Chloroplast RNA splicing defects in mutants lacking plastid ribosomes and in a third splicing mutant with a phenotype similar to but weaker than *crs2* suggest that the splicing of most introns in the chloroplast genome requires either *crs2* gene function or chloroplast translation but not both.

RESULTS

crs1 Mutants Lack the Chloroplast ATP Synthase Due to a Failure to Splice *atpF* mRNA

The *crs1* mutation arose in a maize line with active *Mutator* (*Mu*) transposons and was first detected by virtue of its subtle pale green phenotype (Figure 1). The pale plants subsequently died at a stage characteristic of maize plants that have exhausted seed reserves and are incapable of photosynthesis (Miles, 1994). To determine whether the pale plants lacked components of the thylakoid membrane, we assayed the accumulation of subunits of each thylakoid membrane complex on immunoblots. Figures 2A and 2B show that subunits of the photosystem I core complex, the cytochrome b_6f complex, and the photosystem II core complex accumulated to near normal levels in *crs1* seedlings. However, the abundance of subunits of the chloroplast ATP synthase complex was reduced 10-fold. Therefore, the *crs1* gene is essential for the biogenesis of the ATP synthase complex.

A small reduction (less than twofold) in other thylakoid proteins was also observed in *crs1* mutants. However, these pro-

Figure 1. Pigment Deficiencies Exhibited by the *crs1* and *crs2* Mutants.

Mutant *crs1* and *crs2* seedlings are each shown to the left of a normal sibling. The *crs1* seedling (left) has a pale green phenotype. The *crs2* seedling (right) has an ivory phenotype. Plants were photographed after 12 days of growth in a growth chamber with 14-hr days (28°C) and 10-hr nights (25°C). Small dark green sectors are visible on the *crs2* seedling, and a single large dark green sector is visible on the *crs1* seedling. This somatic instability indicates that the mutations are the result of transposon insertions.

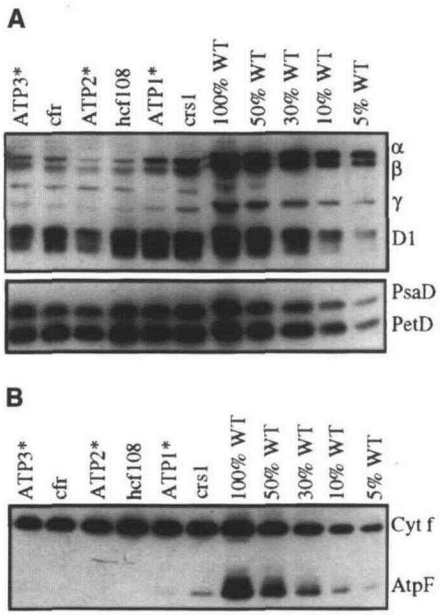

Figure 2. Immunoblot Demonstrating the Loss of Chloroplast ATP Synthase in *crs1* Mutants.

Total leaf proteins (5 μg or the indicated dilutions of the wild-type sample) were fractionated in 13% SDS–polyacrylamide gels, transferred to nitrocellulose, and probed with antiserum cocktails. Five additional ATP synthase mutants (*hcf108*, *cfr*, *ATP1**, *ATP2**, and *ATP3**) were included in this experiment to illustrate the correlation between an ATP synthase deficiency and a minor reduction in other thylakoid proteins and to illustrate the coordinate loss of the CF_1 and CF_0 coupling factor subcomplexes in all such mutants. The level of ribulose-1,5 bisphosphate carboxylase is normal in *crs1* leaves, as indicated by the staining intensity of the small and large subunits after gel fractionation of total leaf proteins (data not shown). Cyt f, cytochrome *f*; WT, wild type.

(A) Loss of the CF_1 complex in *crs1* mutants. The α, β, and γ subunits of CF_1 were detected with an antiserum raised against the entire CF_1 complex. D1 (a reaction center subunit of photosystem II), PsaD (a core subunit of photosystem I), and PetD (a subunit of the cytochrome b_6f complex) were each detected with a monospecific antiserum.

(B) Loss of the AtpF subunit of the CF_0 complex in *crs1* mutants. Cytochrome *f* (a subunit of the cytochrome b_6f complex) and AtpF (subunit I of the CF_0 complex) were each detected with a monospecific antiserum.

teins were reduced to a similar degree in all other ATP synthase mutants examined (Figures 2A and 2B). Therefore, it is likely that this is a consequence of an ATP synthase deficiency rather than a direct consequence of the defect in the *crs1* gene.

To identify the molecular basis for the loss of ATP synthase in *crs1* chloroplasts, we assayed chloroplast mRNAs encoding ATP synthase subunits by RNA gel blot hybridization. The dicistronic transcript spanning the *atpB* and *atpE* genes was of normal size and abundance in *crs1* leaves (data not shown). However, the population of transcripts from the *atpIHFA* gene cluster was aberrant (Figure 3). This aberrant transcript pattern is not a consequence of the loss of ATP synthase because it was not observed in any of the other ATPase-deficient mutants examined (*cfr* and *ATP1** in Figure 3; data not shown).

The population of transcripts in *crs1* suggested a failure to splice the intron that interrupts *atpF* coding sequences: transcripts that comigrate with spliced forms did not accumulate, and transcripts that comigrate with unspliced forms accumulated to increased levels. Hybridization with an intron-specific probe (Figure 3, probe 2) revealed a transcript in wild-type samples whose size was consistent with that of the excised intron. This transcript was not detected in *crs1* but accumulated to normal levels in other ATPase mutants. The loss of spliced *atpF* transcripts, the loss of the excised *atpF* intron, and the increased level of unspliced *atpF* transcripts together provide strong evidence that *crs1* functions in *atpF* RNA splicing rather than in stabilizing spliced *atpF* transcripts.

To test the possibility that the *crs1* mutation blocks the splicing of the *atpF* intron, we examined *atpF* RNA splicing in an RNase protection assay involving a probe that spans the 3′ splice junction. Figure 4 shows that three probe fragments were protected by wild-type RNA, corresponding to

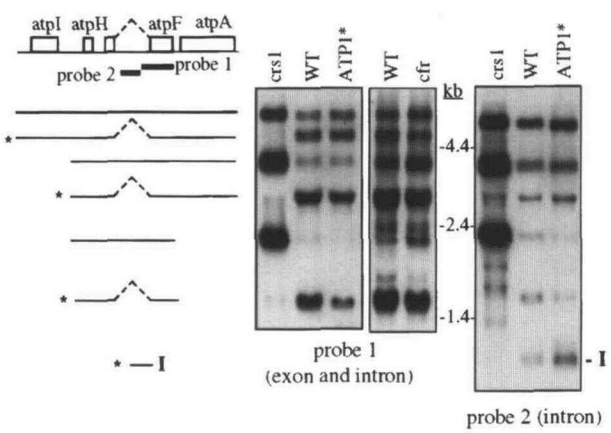

Figure 3. RNA Gel Blot Hybridizations Demonstrating Altered Population of *atpF* Transcripts in *crs1* Mutants.

Total leaf RNA (5 μg) was applied to each lane. The adjacent transcript map shows the approximate structure of each transcript (Barkan, 1989; data not shown), with the dashed lines indicating excised introns. Transcripts missing in *crs1* mutants are marked with an asterisk. Similar RNA gel blots were hybridized with probes specific for *atpA*, *atpI*, and *atpH* (data not shown). In each case, transcripts containing spliced *atpF* sequences were not detected and transcripts containing unspliced *atpF* sequences accumulated to increased levels. The origin of those low-abundance transcripts that are not identified is not known. I, excised intron; kb, positions in the gel of RNA molecules of the indicated number of kilobases; WT, wild type.

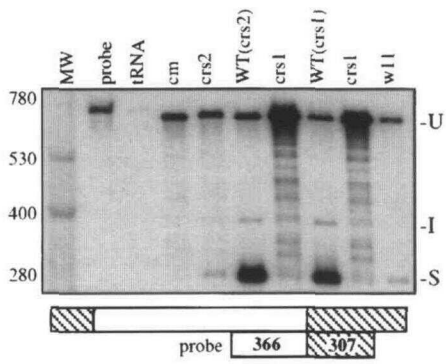

Figure 4. RNase Protection Assay of *atpF* RNA Splicing.

Total leaf RNA (2 μg for wild-type [WT] and *crs1* samples and 3 μg for *chloroplast modifier* [*cm*], *crs2*, and *w11* samples) was analyzed. Hatched and open boxes represent exons and introns, respectively. The lengths in nucleotides of probe sequences corresponding to exon and intron are shown in the map. U, I, and S indicate probe fragments protected by unspliced, intron, and spliced RNA, respectively. The lane designated MW contained radiolabeled RNAs of the indicated number of nucleotides. WT(crs1) and WT(crs2) are wild-type siblings of *crs1* and *crs2* mutants, respectively. *cm* and *w11* are ivory mutants used as controls.

the unspliced RNA, the spliced RNA, and the excised intron. In experiments involving *crs1* RNA, the fragment corresponding to unspliced RNA was increased in abundance, and the fragments corresponding to excised intron and spliced RNA were barely detectable. Therefore, the *crs1* mutation disrupts the splicing of the *atpF* mRNA.

Introns also interrupt maize chloroplast genes encoding cytochrome b_6f subunits, tRNAs, and ribosomal proteins. Splicing defects affecting the *petB* or *petD* mRNAs (which encode cytochrome b_6f subunits) would likely result in a cytochrome b_6f deficiency, and splicing defects affecting tRNAs or ribosomal protein mRNAs would likely result in a global translation defect. The fact that the protein deficiencies in *crs1* mutants were confined largely to the ATP synthase complex (Figure 2) suggested that the splicing defect was specific to the *atpF* mRNA. As demonstrated below, splicing of all 14 of the other introns assayed is normal in *crs1* mutants. These results suggest that the nuclear gene *crs1* functions specifically in the splicing of the *atpF* mRNA.

The *atpF* splicing defect in *crs1* mutants is likely to account for the loss of the entire ATP synthase complex because the failure to synthesize the *atpF* gene product has been shown to destabilize the entire complex in Chlamydomonas chloroplasts (Lemaire and Wollman, 1989). Furthermore, all of the maize mutants described previously as lacking specifically ATP synthase activity (Echt et al., 1987; Miles, 1994) as well as all of the ATP synthase mutants newly isolated in our laboratory are deficient for both membrane intrinsic (CF_0) and membrane extrinsic (CF_1) coupling factor subunits, indicating that these two subcomplexes accumulate coordinately (Figure 2 and data not shown). We cannot eliminate the possibility, however, that *crs1* functions in another aspect of ATP synthase biogenesis in addition to its role in *atpF* RNA splicing.

The *crs2* Gene Product Facilitates the Splicing of Many Group II Introns in Chloroplasts

Several introns in the maize chloroplast genome interrupt genes encoding essential components of the chloroplast translation machinery. A mutation that disrupts the splicing of any of these introns would cause a severe defect in chloroplast translation and, as a consequence, an albino phenotype (Barkan, 1993; Svab and Maliga, 1993). Albinism that results from an early block in chloroplast development is referred to below as ivory to distinguish it from the paper white phenotype resulting from lesions in the carotenoid biosynthetic pathway (Han et al., 1993, 1995).

We have identified an ivory mutant, *crs2* (shown in Figure 1), for which a defect in the splicing of several ribosomal protein mRNAs is likely to be the cause rather than the result of its ivory phenotype. This conclusion is based on comparisons between plastid transcripts in *crs2* seedlings with those in several nonallelic ivory mutants. Most ivory mutants have plastids with few ribosomes (Walbot and Coe, 1979; Han et al., 1993; Hess et al., 1994) and exhibit a variety of similar defects in plastid RNA metabolism (Han et al., 1993, 1995; Hess et al., 1994; Hübschmann et al., 1996). As shown below, the most severe splicing defects observed in *crs2* mutants were not observed in four previously described ivory mutants, including one, *iojap* (*ij*), that has been described as lacking in chloroplast ribosomes (Walbot and Coe, 1979).

The RNase protection assays shown in Figure 5 demonstrate that the splicing of two mRNAs encoding ribosomal proteins, *rps16* and *rpl16*, is dramatically reduced in *crs2* mutants and is relatively unaffected in *crs1* mutants and in the ivory mutants *w11, chloroplast modifier* (*cm*), and *ij* (Figures 5A and 5B). The bacterial homologs of the *rps16* and *rpl16* gene products are thought to be essential for translation in vivo because mutants with defects in these genes have not been obtained (Subramanian et al., 1991). Thus, the failure to splice the *rps16* and *rpl16* mRNAs is likely to result in a loss of plastid ribosome function and in the ivory phenotype of *crs2* mutants.

Similar analyses of other plastid introns revealed numerous additional splicing defects in *crs2* mutants that were not observed in other ivory mutants. Splicing of the *petD*, *petB*, *ndhA*, and *ndhB* introns and the first intron in the *IRF170* gene was nearly undetectable in *crs2* mutants but was near normal in the other ivory mutants examined (Figures 5C to 5G). The decrease in the abundance of the excised *IRF170* intron relative to the unspliced precursor (Figure 5G) provides evidence that *crs2* functions in RNA splicing rather than in

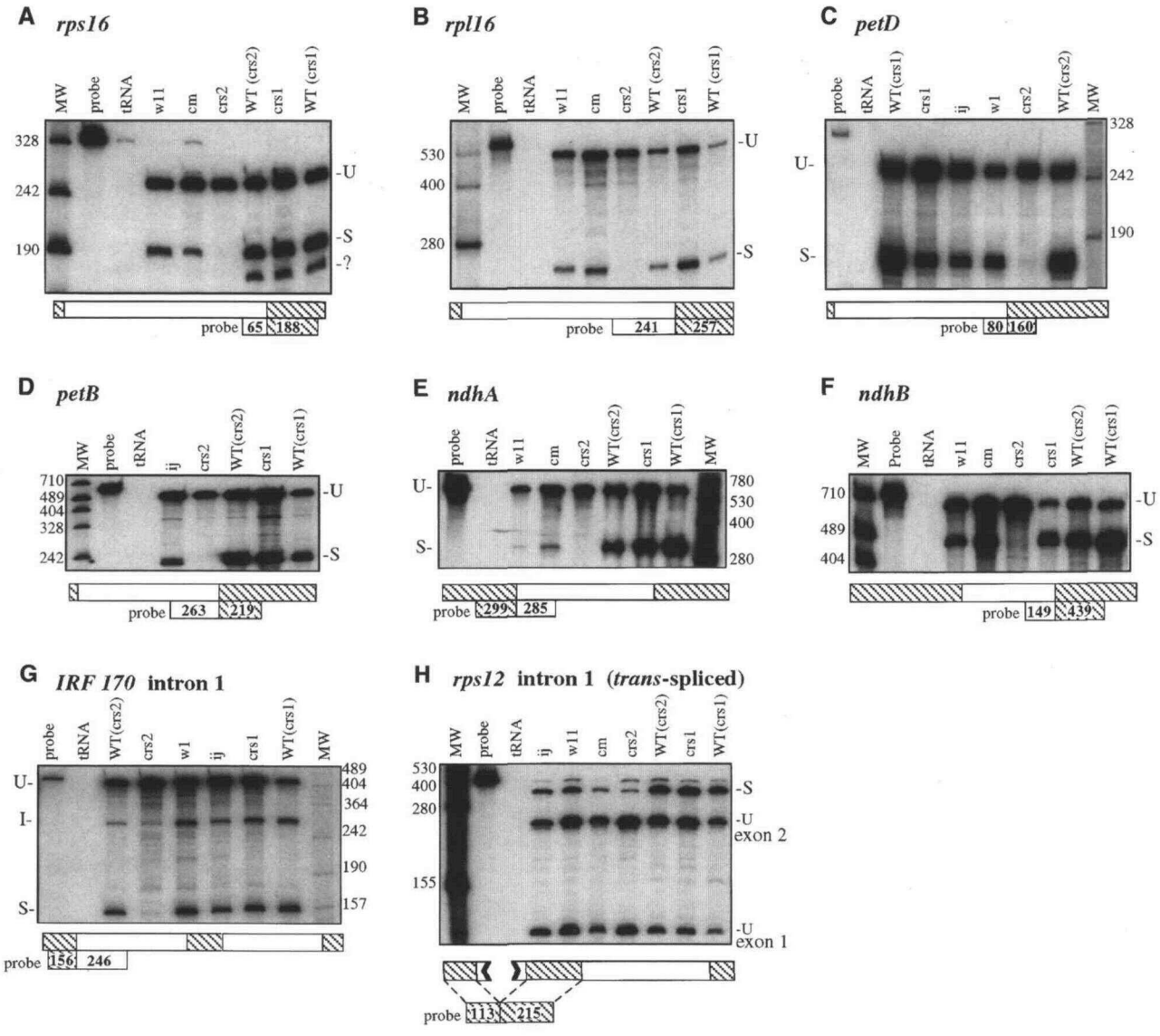

Figure 5. RNase Protection Assays Demonstrating Failure to Splice Group II Introns in *crs2* Mutants.

U, I, and S designate probe fragments protected by unspliced, intron, and spliced RNA, respectively. Lanes labeled MW in **(A)**, **(C)**, **(D)**, **(F)**, and **(G)** contain DNA size standards and in **(B)**, **(E)**, and **(H)** contain RNA size standards of the indicated number of nucleotides. WT(crs1) and WT(crs2) are wild-type siblings of *crs1* and *crs2* mutants, respectively. Hatched and open boxes represent exons and introns, respectively. The lengths in nucleotides of probe sequences corresponding to exon and intron are shown in the maps.

(A) *rps16* intron. Total leaf RNA (3 μg) was analyzed. The results for *ij* and *w1* mutant samples (see Figure 7) were similar to those shown here for *w11* and *cm*. The band marked with a question mark is of unknown origin and appears consistently in samples from green plants but not in samples from ivory plants.

(B) *rpl16* intron. Total leaf RNA (3 μg) was analyzed. The results for *ij* mutants (see Figure 7) were similar to those shown here for *cm* mutants.

(C) *petD* intron. Total leaf RNA (2.5 μg) was analyzed.

(D) *petB* intron. Total leaf RNA (1.5 μg) was analyzed.

(E) *ndhA* intron. Total leaf RNA (8 μg) was analyzed.

(F) *ndhB* intron. Total leaf RNA (4 μg) was analyzed. *ij* mutants spliced the *ndhB* intron normally (data not shown).

(G) *IRF170* intron 1. Total leaf RNA (3 μg) was analyzed. *cm* mutants spliced this intron normally (data not shown).

(H) *trans*-Spliced intron of *rps12*. The probe was transcribed from a cloned cDNA fragment that was derived from the spliced *rps12* mRNA. Total leaf RNA (1.5 μg from *ij*, *w11*, *cm*, and *crs2* samples and 3 μg from *crs1* and wild-type samples) was analyzed.

stabilizing spliced transcripts. Taken together, these results are consistent with a direct role for *crs2* in the activation of these splicing events and argue against the possibility that these splicing defects result simply from plastid ribosome deficiency or from an early block in chloroplast development.

Biogenesis of the *rps12* mRNA involves two splicing events. The first intron is removed in a *trans*-splicing event involving two independently transcribed pre-RNAs (Fromm et al., 1986; Koller et al., 1987; Zaita et al., 1987; Hildebrand et al., 1988). The second intron is a standard group II intron that is spliced in *cis*. The *trans*-splicing of the *rps12* mRNA was assayed in an RNase protection experiment involving a probe generated by transcription of a cDNA corresponding to the spliced mRNA (Figure 5H). In this experiment, unlike the previous ones, spliced transcripts protect a longer probe segment than do unspliced transcripts. The ratio of *trans*-spliced to unspliced transcripts is reduced to a considerable degree in *crs2* mutants, although this splicing defect is less complete than those described above. The ivory mutants used as controls exhibited only a slight reduction in the degree of *trans*-splicing, consistent with findings for a barley mutant lacking plastid ribosomes (Hübschmann et al., 1996). These results suggest that the *crs2* gene product may participate in the *trans*-splicing of the *rps12* mRNA.

Mutants Lacking Plastid Ribosomes Have Splicing Defects That Differ from the Splicing Defects in *crs2* Mutants

Figures 6A and 6B show that the *rpl2* intron and the second intron in *rps12* are not detectably spliced in *ij* mutants. Similar results were obtained with *cm*, another ivory mutant used as a control (data not shown). Analogous observations made with the barley mutant *albostrians*, also described as lacking in plastid ribosomes, led to the suggestion that plastid translation is required for these splicing events (Hess et al., 1994; Hübschmann et al., 1996). Likewise, splicing of the *atpF* intron was undetectable in *cm* (Figure 4) and *ij* mutants (see Figure 9). Each of these three introns is detectably spliced in *crs2* mutants, albeit poorly (Figures 4 and 6). The simplest interpretation of these observations is that the splicing of these three introns requires chloroplast translation and that they are spliced poorly in *crs2* mutants as a consequence of the ribosome deficiency in *crs2* plastids.

An additional mutant, *crs**, was recovered whose phenotype supports our hypothesis that the severe splicing defects in *crs2* mutants (shown in Figure 5) result from the inactivity of a nuclear-encoded splicing factor, whereas the partial splicing defects (shown in Figures 4 and 6) result from the ribosome deficiency in *crs2* plastids. *crs** is a single nuclear mutation that results in ivory seedlings (data not shown). Although its allelic relationship with *crs2* has not yet been established, *crs** behaves as one would expect a leaky allele of *crs2* to behave. Figures 7A to 7E show that like *crs2*, the *crs** mutation is

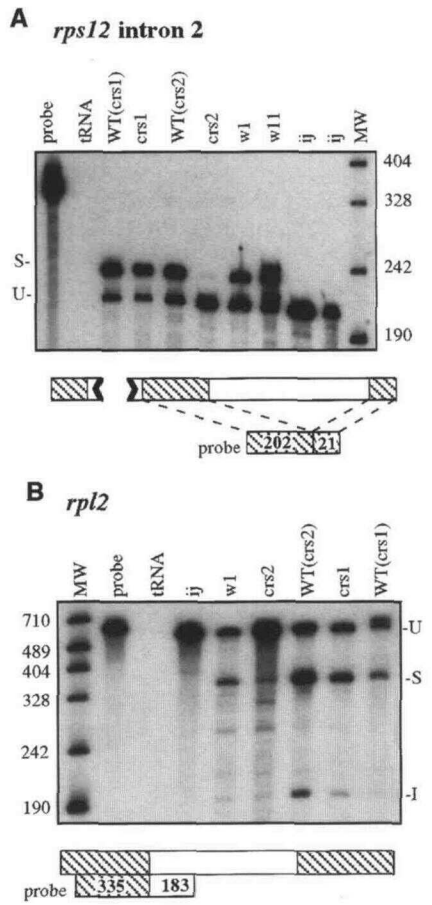

Figure 6. RNase Protection Assays Demonstrating Splicing of Two Chloroplast Transcripts in *crs2* Mutants.

U, I, and S designate probe fragments protected by unspliced, intron, and spliced RNA, respectively. Lanes labeled MW contain DNA size standards of the indicated number of nucleotides. WT(crs1) and WT(crs2) are wild-type siblings of *crs1* and *crs2* mutants, respectively. Hatched and open boxes represent exons and introns, respectively. The lengths in nucleotides of probe sequences corresponding to exon and intron are shown in the maps.

(A) *rps12* intron 2. The probe was transcribed from a cDNA fragment derived from the spliced transcript. Total leaf RNA (1 μg from *w1*, *w11*, *ij*, and *crs2* samples and 2 μg from the remaining samples) was analyzed.

(B) *rpl2* intron. Total leaf RNA (0.6 μg from *ij*, *w1*, and *crs2* samples and 1.5 μg from the remaining samples) was analyzed. To test the possibility that the apparently spliced transcripts detected here were merely cleaved at the 5′ junction but not faithfully spliced, these samples were also assayed in an RNase protection experiment involving a probe derived from the spliced cDNA. The ratio of spliced to unspliced transcripts detected in both assays was similar (data not shown), confirming that those transcripts cleaved at the 5′ splice junction go on to be fully spliced. The results for *w11* mutants were similar to those for *w1* (data not shown).

associated with a defect in the splicing of the *rps16*, *rpl16*, *petD*, and *petB* introns and the first intron in *IRF170*. These splicing defects are slightly less severe in *crs** than in *crs2* mutants.

Because the spliced forms of the ribosomal protein mRNAs *rpl16* and *rps16* accumulate to significantly higher levels in *crs** than in *crs2* mutants, these mRNAs will less severely limit the production of chloroplast ribosomes in *crs** mutants. In fact, *crs** mutants contain significantly more plastid rRNA than do *crs2* or *ij* mutants (Figure 8A), suggesting that *crs** mutants do contain more plastid ribosomes. Furthermore, the chloroplast-encoded protein cytochrome *f* accumulates to higher levels in *crs** than in *crs2* mutants (Figure 8B). These results strongly suggest that the chloroplast translation machinery is significantly more active in *crs** plastids than in *ij* and *crs2* plastids. Therefore, the splicing defects in *crs** mutants are a consequence of the inactivity of a nuclear-encoded splicing factor and are not a consequence of the absence of chloroplast translation.

Figures 9A to 9C show that those splicing events previously hypothesized to be dependent on plastid translation (*atpF*, *rpl2*, and the second intron of *rps12*) occur efficiently in *crs** mutants. Thus, the previously established correlation between plastid translation and the splicing of these introns is extended: *crs** mutants perform significantly more plastid translation than do *ij* and *crs2* mutants; they also splice these introns to a much greater extent.

The results presented above suggest that chloroplast translation is required for the efficient splicing of three chloroplast introns in maize: *atpF*, *rpl2*, and the second intron of *rps12*. Our findings now indicate that a nuclear-encoded factor whose activity requires *crs2* function (and *crs** function if this should prove to be distinct) facilitates the splicing of an entirely different set of mRNAs: *petB*, *petD*, *rpl16*, *rps16*, *IRF170* (intron 1), *ndhB*, and *ndhA*. The poor splicing in *crs2* mutants of *rps12* (intron 2), *atpF*, and *rpl2* mRNAs is likely to be a consequence of the plastid ribosome deficiency that in turn results from the failure to splice the *rps16* and *rpl16* mRNAs.

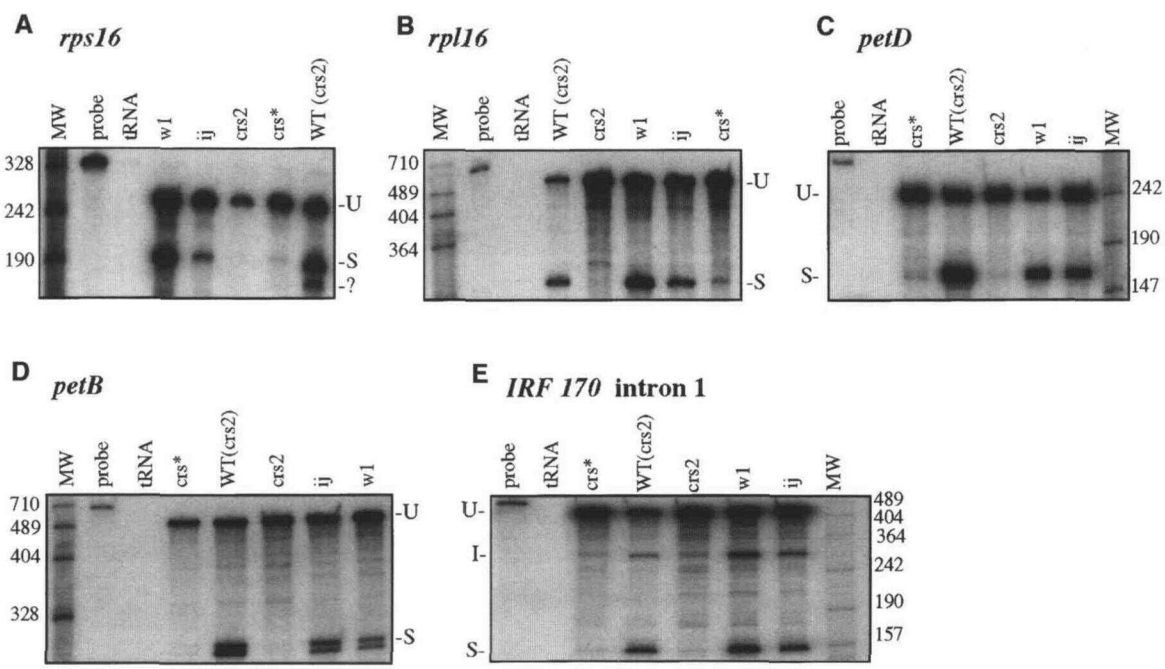

Figure 7. Chloroplast RNA Splicing Defects in *crs** Mutants.

U, I, and S designate probe fragments protected by unspliced, intron, and spliced RNA, respectively. Lanes labeled MW contain DNA size standards of the indicated number of nucleotides. WT(crs2) samples were obtained from wild-type siblings of *crs2* mutants. Probes are the same as those used in Figure 5.

(A) *rps16* intron. Total leaf RNA (3 μg) was analyzed. The band marked with a question mark is of unknown origin and appears consistently in samples from green plants but not in samples from ivory plants.

(B) *rpl16* intron. Total leaf RNA (1.5 μg) was analyzed.

(C) *petD* intron. Total leaf RNA (2.5 μg) was analyzed.

(D) *petB* intron. Total leaf RNA (1.5 μg) was analyzed.

(E) *IRF170* intron 1. Total leaf RNA (3 μg) was analyzed.

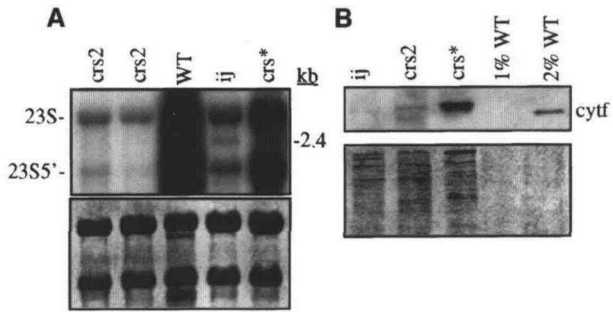

Figure 8. *crs** Mutants Are Active in Chloroplast Translation.

(A) RNA gel blot illustrating the abundance of plastid 23S rRNA. Total leaf RNA (6 μg) was analyzed. Shown at top are the results of hybridization with a probe encoding the 5′ half of maize chloroplast 23S rRNA (Barkan, 1993). The probe was radiolabeled by the random hexamer priming method. Two major bands were detected. These correspond to the intact 23S rRNA (23S) and the breakdown product containing the 5′ half of the molecule (23S5′) (Barkan, 1993). Shown at bottom are the results of staining the RNA bound to the same filter with methylene blue. The prominent bands, corresponding to the 25S and 18S cytosolic rRNAs, are of equal intensity in the different samples, illustrating that equal amounts of leaf RNA were applied to each lane. WT, wild type.

(B) Immunoblot illustrating the accumulation of cytochrome *f* (cytf). Total leaf protein (5 μg from *ij*, *crs2*, and *crs** samples and 0.05 or 0.1 μg of the wild-type sample) was analyzed. Shown at top are the results of probing the membrane with a monospecific antiserum raised against maize cytochrome *f* (the product of the chloroplast *petA* gene). The polypeptide that was detected by the cytochrome *f* antiserum in the ivory mutants corresponds in size to the cytochrome *f* precursor (Voelker and Barkan, 1995) rather than to the mature protein, indicating that cytochrome *f* is inefficiently processed in ivory mutants in general. Shown at bottom are the results of staining the filter with Ponceau S before the antibody incubation. This illustrates the relative amounts of protein applied to each lane.

Normal Splicing of Two Chloroplast Introns in *crs2* Mutants

Just one pre-mRNA intron in the maize chloroplast genome is spliced normally in all mutants examined. Figure 10A shows that the splicing of the second intron in the *IRF170* gene is not affected in *crs2* mutants, in *crs** mutants, or in any of the ivory mutants used as controls. The single group I intron in the maize chloroplast genome, which maps in the *trnL* gene, also appears to be spliced normally in *crs2* and in the control ivory mutants (Figure 10B). Therefore, *crs2* appears not to function in the splicing of the single group I intron in the maize chloroplast genome.

Splicing Defects in *crs2* Mutants Are Not a Consequence of Photooxidative Damage

Photooxidative damage resulting from defects in carotenoid biosynthesis can lead to an albino phenotype and to the degradation of chloroplast proteins and RNAs (reviewed in Taylor, 1989). To address the possibility that the splicing defects in *crs2* mutants result from photooxidative damage to splicing factors and/or to spliced RNAs, we examined the splicing of three *crs2*-dependent introns (*petB*, *petD*, and the first intron of *IRF170*) in *crs2* leaves that had developed in the absence of light. Figures 11A to 11C show that the splicing defects were equally severe in dark-grown and light-grown *crs2* leaves. These results demonstrate that photooxidative damage is not the cause of the splicing defects in *crs2* mutants.

DISCUSSION

We have presented evidence for two nuclear genes, *crs1* and *crs2*, that function in the splicing of group II introns in the maize chloroplast. A mutation in the *crs1* gene causes a

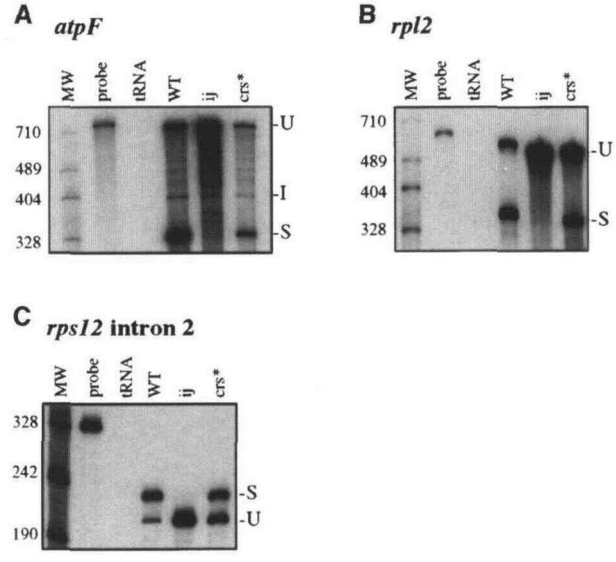

Figure 9. RNase Protection Assays Demonstrating That Introns That Remain Unspliced in Plastid Ribosome-Deficient Mutants Are Spliced Efficiently in *crs** Mutants.

U, I, and S designate probe fragments protected by unspliced, intron, and spliced RNA, respectively. Lanes labeled MW contain DNA size standards of the indicated number of nucleotides. WT samples were wild-type siblings of *crs2* mutants.

(A) *atpF* intron. Total leaf RNA (3 μg of the *ij* and *crs** samples and 2 μg of the wild-type sample) was analyzed. The probe is diagrammed in Figure 4.

(B) *rpl2* intron. Total leaf RNA (0.6 μg of the *ij* and *crs** samples and 1.5 μg of the wild-type sample) was analyzed. The probe is diagrammed in Figure 6B.

(C) *rps12* intron 2. Total leaf RNA (0.6 μg of the *ij* and *crs** samples and 1.5 μg of the wild-type sample) was analyzed. The probe is diagrammed in Figure 6A.

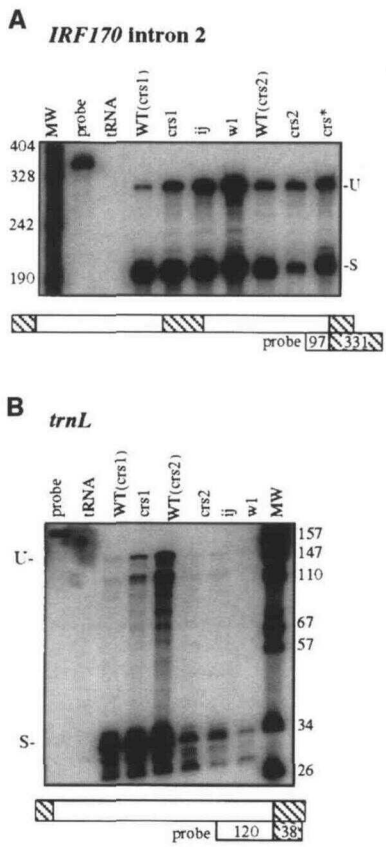

Figure 10. Two Introns Are Spliced Normally in All of the Mutants Examined.

Splicing was assayed by RNase protection. Symbols are as described in Figure 4.

(A) *IRF170* intron 2. The 5′ end of the probe extends ~100 nucleotides past the 3′ end of the *IRF170* transcript. Total leaf RNA (3 μg) was analyzed. This intron is spliced normally in *cm* mutants (data not shown).

(B) *trnL* intron. Total leaf RNA (4 μg of *ij*, *w1*, and *crs2* samples and 2 μg of wild-type and *crs1* samples) was analyzed.

specific defect in the splicing of the *atpF* intron, whereas a mutation in the *crs2* gene disrupts the splicing of many group II introns in the chloroplast. A third nuclear mutation, designated temporarily as *crs**, may be allelic with *crs2* or it may define an additional nuclear gene that functions in the same splicing events as does *crs2*. The mutant phenotypes are consistent with the notion that the *crs1* and *crs2* gene products function (either directly or indirectly) only in the splicing of those introns that remain unspliced in mutant plants. However, if these alleles are leaky or if other genes exist with functions that are in part redundant with the *crs* genes studied here, it is also possible that *crs1* and *crs2* play a more general role in splicing than is reflected by these mutant phenotypes.

Two Classes of Group II Introns in the Maize Chloroplast Genome Are Defined by Distinct Requirements for Splicing Factors

Nuclear mutations that cause the loss of plastid ribosomes in barley are associated with defects in the splicing of several chloroplast introns (Hess et al., 1994; Hübschmann et al., 1996). The ivory phenotype of *crs2* mutants (Figure 1), their very low level of chloroplast rRNAs (Figure 8), and their failure to splice mRNAs encoding ribosomal proteins (Figure 5) suggest that *crs2* mutants have few chloroplast ribosomes. To distinguish between splicing defects caused by the *crs2* mutation and those arising as a consequence of ribosome loss, we assayed splicing in a variety of other maize mutants with ivory leaves and ribosome-deficient plastids. Defects in the splicing of many introns (*petB*, *petD*, *rps16*, *rpl16*, *ndhA*, *ndhB*, and the first intron of *IRF170*) were observed in *crs2* but not in any of the control ivory mutants

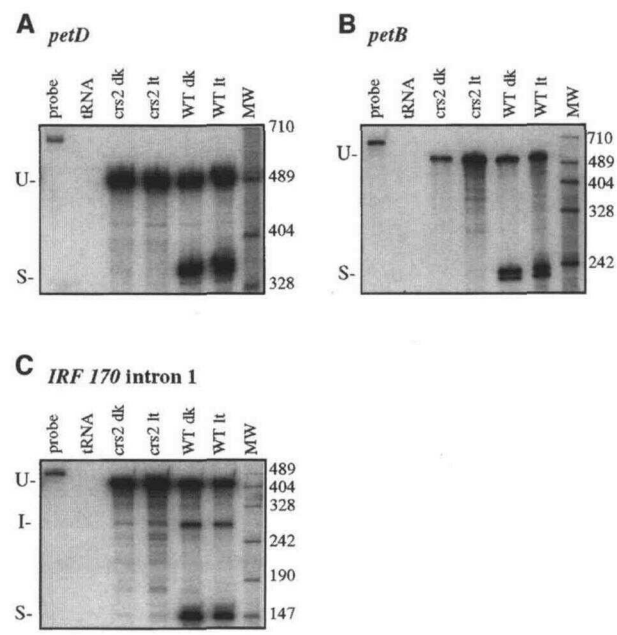

Figure 11. RNase Protection Assays Demonstrating That the Splicing Defects in *crs2* Mutants Are Not a Consequence of Photooxidative Damage.

The progeny resulting from self-pollination of a *crs2*/+ plant were germinated and grown in the complete absence of light. After the expansion of two leaves, individual plants were numbered and leaf tips were harvested with the aid of a green safelight. Harvested tissue was immediately frozen in liquid nitrogen. Plants were then transferred to the light for 24 hr, during which time the wild-type leaves accumulated chlorophyll. RNA was extracted from the tip of a wild-type and *crs2* leaf after 24 hr in the light (WT lt and crs2 lt, respectively) and from leaf material harvested from the same seedlings before their exposure to light (WT dk and crs2 dk, respectively). The probes and amounts of RNA analyzed were as described in Figure 5.

(summarized in Table 1). Therefore, these splicing events are not dependent on chloroplast translation or on chloroplast development, but they do require *crs2* function.

Splicing of an entirely different set of chloroplast introns appears to be dependent on chloroplast ribosomes. Defects in the splicing of *rpl2* and *rps12* (intron 2) mRNAs in *albostrians* and in several other ivory mutants of barley led to the notion that a plastid gene product is essential for these splicing events (Hess et al., 1994; Hübschmann et al., 1996). This plastid gene product has been proposed to be the product of the chloroplast *matK* gene, an open reading frame internal to a group II intron in the *trnK* gene in higher plant chloroplasts (Neuhaus and Link, 1987). The predicted *matK* gene product is similar to fungal maturases that play genetically defined roles in facilitating the splicing of mitochondrial group II introns (reviewed in Costanzo and Fox, 1990). The *matK* gene product is synthesized in vivo (Jardin et al., 1994) and can bind *trnK* transcripts in vitro (Liere and Link, 1995). However, its role in splicing in vivo has not been established. It remains possible that chloroplast ribosomes facilitate one or both of these splicing events not by synthesizing a chloroplast-encoded splicing factor but rather by binding to pre-mRNAs and influencing intron structure.

Our results define two classes of chloroplast introns in maize: those whose splicing is dependent on the nuclear gene *crs2* (and *crs**, if it should prove distinct) and those whose splicing is dependent on plastid translation. It seems reasonable to speculate that the introns within one category share physical features that distinguish them from introns in the other category. Michel et al. (1989) subdivided group II introns into two categories based on conserved sequences and predicted secondary structures. All of the introns that exhibit a strict dependence on *crs2/crs** function fall into group IIB, whereas those whose splicing correlates with

Table 1. Introns in the Maize Chloroplast Genome and Their Splicing in *crs1* and *crs2* Mutants

Intron Type[a]	Gene	Gene Product	Splicing in *crs1*	Splicing in *crs2*	Splicing in Ivory Mutants *ij* and *cm*[b]
Group II Subgroup IIA					
	atpF	ATPase subunit	NO	Reduced	No
	rpl2	Ribosomal protein	+[c]	Reduced	No
	rps12 intron 2	Ribosomal protein	+	Reduced	No
	trnI	tRNA	–[d]	–	–
	trnA	tRNA	–	–	–
	trnV	tRNA	–	–	–
	trnK[e]	tRNA	+	Reduced	Reduced
Group II Subgroup IIB1					
	petB	Cytochrome b_6	+	No	Reduced
	petD	Cytochrome b_6f subunit	+	No	+
	rps16	Ribosomal protein	+	No	Reduced
	IRF170 intron 2	Unknown	+	+	+
Group II Subgroup IIB2					
	rps12 intron 1 (trans)	Ribosomal protein	+	Reduced more than *ij*	Reduced
	rp116	Ribosomal protein	+	No	+
	ndhB	NADH dehydrogenase	+	No	+
	ndhA	NADH dehydrogenase	+	No	Reduced
	IRF170 intron 1	Unknown	+	No	+
	trnG	tRNA	–	–	–
Group I	*trnL*	tRNA	+	+	+

[a] Introns are classified according to Michel et al. (1989).

[b] The ivory *ij* and *cm* mutants analyzed were fully albino progeny of female homozygous mutant plants. These two mutants gave identical results. Two other ivory mutants (*w11* and *w1*) were analyzed as well. All chloroplast introns examined were spliced to some extent in *w1* and *w11* mutants, although in some cases the degree of splicing was reduced.

[c] (+) indicates that the degree of splicing is similar to that in wild-type chloroplasts.

[d] Hyphens indicate not examined.

[e] The intron contains an open reading frame with maturase homology. Splicing of this intron was difficult to assay in ivory plants due to its low abundance. However, the ratio of spliced to unspliced *trnK* RNA was reduced dramatically in *crs2* and *ij* (data not shown).

chloroplast translation fall into group IIA (Table 1). Thus, there may be structural elements common to group IIA introns that are recognized by a chloroplast-encoded factor and different structural elements in group IIB introns that are recognized by a nuclear-encoded factor whose activity requires *crs2/crs** function. The fact that no introns have been identified whose splicing requires both *crs2/crs** function and chloroplast ribosomes is consistent with the notion that the nuclear- and chloroplast-encoded splicing factors have evolved different intron specificities but serve the same mechanistic role in splicing. Alternatively, the different structural features of the two intron subgroups may necessitate accessory splicing factors with distinct biochemical functions.

Few, if Any, of the Group II Introns in the Maize Chloroplast Genome Undergo Self-Splicing in Vivo

Although it is clear that some group II introns are spliced efficiently under physiological conditions only if aided by *trans*-acting factors, it remains plausible that others actually self-splice in vivo. Our results indicate that the splicing of nearly every pre-mRNA intron in the maize chloroplast genome requires either chloroplast ribosomes or *crs2* function (summarized in Table 1). Just one pre-mRNA intron, the second intron in the *IRF170* gene, is spliced normally in all mutants examined. The splicing of this intron may require nuclear gene products not yet identified in our genetic screens. Alternatively, this intron may self-splice in vivo. Several tRNA genes in the maize chloroplast genome also contain group II introns, but their splicing has not been sufficiently studied to draw conclusions regarding their requirements for *trans*-acting factors.

Multiple Factors May Be Involved in *atpF* Splicing

Splicing of the *atpF* intron in higher plant chloroplasts has been particularly well studied. This intron is excised in transgenic tobacco chloroplasts when placed within a reporter gene (Bock and Maliga, 1995) and is excised as a lariat in vivo (Kim and Hollingsworth, 1993). That *trans*-acting factors are involved in its splicing in vivo was deduced from the observations that it could not be made to self-splice in vitro (Plant and Gray, 1988) and that it was not removed when expressed in transgenic Chlamydomonas chloroplasts (Deshpande et al., 1995). The results presented here suggest that multiple *trans*-acting factors may be involved in the splicing of this intron in vivo. At a minimum, *atpF* splicing requires the function of the nuclear gene *crs1*. The failure of this intron to be spliced in the two nonallelic ivory mutants *cm* and *ij* suggests that a chloroplast gene product may be required in addition. This raises the possibility that a multisubunit particle may be involved in the splicing of the *atpF* intron. It is also plausible, however, that the *crs1* gene product is the sole factor required for *atpF* splicing. According to this scenario, the *crs1* gene may be one of a suite of nuclear genes whose expression is increased by a signal emanating from developing chloroplasts (reviewed in Taylor, 1989). The absence of this signal in ivory *ij* and *cm* plants might then, as a secondary effect, cause a loss of *atpF* splicing due to the absence of *crs1* gene expression. This possibility can easily be tested once the *crs1* gene is cloned.

Mechanism of *crs1* and *crs2* Action

The *crs1* and *crs2* genes are as yet the only genes in higher plants whose mutant phenotypes are consistent with their playing a direct role in RNA splicing. The *crs1* and *crs2* gene products may facilitate splicing either directly by interacting with their target introns themselves or indirectly by controlling the function of other nuclear-encoded factors that are in turn directly involved in intron excision. Mechanistic studies of factors that facilitate the splicing of group I introns have revealed roles in promoting the formation and stabilization of the catalytic intron core (Wollenzien et al., 1983; Guo and Lambowitz, 1992; Mohr et al., 1992; Coetzee et al., 1994; Lewin et al., 1995; Shaw and Lewin, 1995; Weeks and Cech, 1995a, 1995b, 1996). Cloning and analysis of the *crs1* and *crs2* genes, now in progress, are essential for establishing their mechanistic roles as well as their participation in the developmental regulation of plastid RNA splicing.

METHODS

Plant Material

crs1, *crs2*, and *crs** arose independently in maize lines with active *Mutator* (*Mu*) transposons. All three mutations are recessive, are inherited through the pollen, and result in seedling lethality at ~3 weeks after germination. Tests for allelism between *crs1* and *crs2* are in progress. However, their very distinct phenotypes suggest that they are not allelic. Tests for allelism between *crs2* and *crs** are also in progress. Four different ivory mutants were used in control experiments: homozygous recessive *w1* and *w11* mutants, and fully ivory progeny resulting from crosses between maternal *ij/ij* or *cm/cm* plants with +/+ pollen.

Leaf material for RNA and protein extraction was obtained from seedlings grown for 10 to 14 days in a growth chamber (14-hr days at 28°C and 10-hr nights at 25°C). The mutant and wild-type samples used in each experiment were siblings obtained from the same planting.

Additional mutants lacking the ATP synthase were used as controls in some experiments. *hcf108* and *cfr* are nuclear mutants that were isolated and described by Echt et al. (1987) and Miles (1994), respectively. These are not allelic to one another or to *crs1* (data not shown). Seed for *hcf108* were provided by D. Miles (University of Missouri, Columbia), and seed for *cfr* were provided by C. Echt (USDA Forest Service, Rhinelander, MN). ATP1*, ATP2*, and ATP3* arose independently in our *Mu* lines. Although these are not allelic to *crs1*, their allelic relationships to one another and to *hcf108* and *cfr* have not been determined.

Preparation and Analysis of Protein and RNA

Leaf proteins were extracted and analyzed on immunoblots by using methods and antibodies described previously (Voelker and Barkan, 1995). The CF_1 antiserum was generously provided by S. Merchant (University of California, Los Angeles). Total leaf RNA was prepared with TRIzol Reagent (Bethesda Research Laboratories), as described by the manufacturer. RNA gel blot hybridizations and RNase protection experiments were performed as described previously (Barkan et al., 1994). Probes for RNase protection experiments included small segments at each end derived from polylinker sequences. Therefore, full-length protection of the chloroplast sequences resulted in fragments somewhat smaller than the full-length probe.

Hybridization Probes

Most probes were derived from cloned fragments of maize chloroplast DNA. The complete sequence of the maize chloroplast genome (Maier et al., 1995) was used to design cloning strategies for many of these plasmids. DNAs were cloned into pBluescript SK+ (Stratagene). The probes used for the RNase protection assays were transcribed from clones of the following DNA fragments: *atpF*, 673-bp SstI-EcoRI fragment of maize chloroplast DNA spanning the 3′ splice junction; *rps16*, 253-bp XhoI-EcoRI fragment of maize chloroplast DNA spanning the 3′ splice junction; *rpl16*, 498-bp BglII-BamHI fragment of maize chloroplast DNA spanning the 3′ splice junction; *petD*, 240-bp Xho-XbaI fragment of maize chloroplast DNA spanning the 3′ splice junction; *petB*, 482-bp HaeIII fragment of maize chloroplast DNA spanning the 3′ splice junction; *ndhA*, 584-bp DraI-EcoRI fragment of maize chloroplast DNA spanning the 5′ splice junction; *ndhB*, 588-bp XmnI-AvrII fragment of maize chloroplast DNA spanning the 3′ splice junction; *IRF170* (intron 1), 402-bp EcoRI fragment of maize chloroplast DNA spanning the 5′ splice junction; *rpl2*, 518-bp AccI-XbaI fragment of maize chloroplast DNA spanning the 5′ splice junction; and *IRF170* (intron 2), 428-bp EcoRI-SspI fragment of maize chloroplast DNA spanning the 3′ splice junction.

Probes corresponding to spliced *rps12* mRNA were generated by reverse transcription polymerase chain reaction (PCR) amplification of leaf RNA. The following primer pairs were chosen based on the primers used to amplify the analogous cDNA fragments from barley (Hess et al., 1994; Hübschmann et al., 1996): *rps12* intron 1 (*trans*-spliced) 5′ primer, ATGCCAACGGTTAAACAACTTAT, and 3′ primer, GCCCTTGTTGACGATTCTTTACT; *rps12* intron 2 5′ primer, CTCTGCCTTACGTAAAGTTGC, and 3′ primer, TTTGGCTTTTTGGCCCCATAT. The 3′ primers were used to prime reverse transcription on a template of chloroplast RNA obtained from seedling leaves of the inbred maize line B73. Each reaction (50 μL) contained 2.5 μg of chloroplast RNA, 50 pmol of primer, 5 μL of 10 × RT buffer (500 mM KCl, 200 mM Tris-HCl, pH 8.3, 25 mM $MgCl_2$, 0.01% gelatin), 5 μL of 10 mM deoxynucleotide triphosphates (dNTPs), 0.5 μL of 1 M DTT, 80 units of RNasin (Boehringer Mannheim), and 50 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim). Reactions were incubated at 65°C for 10 min before the addition of enzyme and dNTPs, cooled to 4°C; enzyme and dNTPs were added, and reactions were incubated for 1 hr at 42°C. After cDNA synthesis, 10 μL of each reaction was used directly in a 50-μL PCR amplification containing 20 pmole of each primer and 4 μL of 10 × PCR buffer (500 mM KCl, 75 mM Tris-HCl, pH 8.3, 12.5 mM $MgCl_2$, 0.01% gelatin). The reactions were incubated at 94°C for 5 min, after which 2.5 units of Taq DNA polymerase (Promega) was added. Amplification was allowed to proceed for 30 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec.

The probe spanning the 3′ splice junction of *trnL* was generated by PCR amplification of a 158-bp fragment from the cloned Bam14 fragment of maize chloroplast DNA (Larrinua et al., 1983; Maier et al., 1995). The following primer pair was used: 5′ primer, CCTTCAATTCATTGTTTTCGAGA; and 3′ primer, GGACTTGAACCCTCACGACT. Plasmid DNA (1 ng) was added to a 50-μL reaction containing 20 pmol of each primer, 5 μL of 10 × PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), and 1 μL of 10 mM dNTPs. The reaction was incubated at 94°C for 5 min after which 2.5 units of Taq DNA polymerase was added. Amplification was allowed to proceed for 30 cycles (94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec).

All PCR products were purified by agarose gel electrophoresis and Qiaex extraction (Qiagen, Inc., Chatsworth, CA), following the manufacturer's instructions. PCR products were resuspended in 10 μL of 1 × T4 polymerase buffer (New England Biolabs, Beverly, MA) and given blunt termini by incubation with 100 μM dNTPs and 3 units of T4 polymerase at 12°C for 20 min. The polymerase was then inactivated by incubation at 75°C for 10 min, and the fragments were ligated into Bluescript SK+ plasmids.

ACKNOWLEDGMENTS

We are grateful to Rainer Maier and Hans Kössel for access to the maize chloroplast DNA sequence before publication, to Laura Roy for help with mutant propagation and pedigree analysis, and to Macie Walker for technical advice. We also thank Sabeeha Merchant for the CF_1 antiserum, Jörg Vogel for providing the *crs** RNA sample, and Wolfgang Hess for communicating unpublished results. Dianna Fisk, Bryan Pickett, Rodger Voelker, Brad Till, and John Davenport provided helpful comments on the manuscript. This work was supported by grants from the U.S. Department of Energy and the National Science Foundation.

Received October 16, 1996; accepted December 20, 1996.

REFERENCES

Barkan, A. (1989). Tissue-dependent plastid RNA splicing in maize: Transcripts from four plastid genes are predominantly unspliced in leaf meristems and roots. Plant Cell **1,** 437–445.

Barkan, A. (1993). Nuclear mutants of maize with defects in chloroplast polysome assembly have altered RNA metabolism. Plant Cell **5,** 389–402.

Barkan, A., Walker, M., Nolasco, M., and Johnson, D. (1994). A nuclear mutation in maize blocks the processing and translation of several chloroplast mRNAs and provides evidence for the differential translation of alternative mRNA forms. EMBO J. **13,** 3170–3181.

Bock, R., and Maliga, P. (1995). Correct splicing of a group II intron from a chimeric reporter gene transcript in tobacco plastids. Nucleic Acids Res. **23,** 2544–2547.

Cech, T.R. (1986). The generality of self-splicing RNA: Relationship to nuclear mRNA splicing. Cell **44,** 207–210.

Choquet, Y., Goldschmidt-Clermont, M., Girard-Bascou, J., Kück, U., Bennoun, P., and Rochaix, J.-D. (1988). Mutant phenotypes support a *trans*-splicing mechanism for the expression of the tripartite *psaA* gene in the C. reinhardtii chloroplast. Cell **52,** 903–913.

Coetzee, T., Herschlag, D., and Belfort, M. (1994). *E. coli* proteins, including ribosomal protein S12, facilitate in vitro splicing of phage T4 introns by acting as RNA chaperones. Genes Dev. **8,** 1575–1588.

Collins, R.A., and Lambowitz, A.M. (1985). RNA splicing in *Neurospora* mitochondria. J. Mol. Biol. **184,** 413–428.

Costanzo, M.C., and Fox, T.D. (1990). Control of mitochondrial gene expression in *Saccharomyces cerevisiae.* Annu. Rev. Genet. **24,** 91–113.

Deshpande, N.N., Hollingsworth, M., and Herrin, D.L. (1995). The *atpF* group II intron-containing gene from spinach chloroplasts is not spliced in transgenic *Chlamydomonas* chloroplasts. Curr. Genet. **28,** 122–127.

Echt, C.S., Polacco, M.L., and Neuffer, M.G. (1987). A nuclear encoded chloroplast ATP synthase mutant of *Zea mays* L. Mol. Gen. Genet. **208,** 230–234.

Fromm, H., Edelman, M., Koller, B., Goloubinoff, P., and Galun, E. (1986). The enigma of the gene coding for ribosomal protein S12 in the chloroplast of *Nicotiana.* Nucleic Acids Res. **14,** 883–898.

Goldschmidt-Clermont, M., Girard-Bascou, J., Choquet, Y., and Rochaix, J.-D. (1990). *trans*-Splicing mutants of *Chlamydomonas reinhardtii.* Mol. Gen. Genet. **223,** 417–425.

Guo, Q., and Lambowitz, A.M. (1992). A tyrosyl-tRNA synthetase binds specifically to the group I intron catalytic core. Genes Dev. **6,** 1357–1372.

Han, C.-D., Patrie, W., Polacco, M., and Coe, E.H., Jr. (1993). Aberrations in plastid transcripts and deficiency of plastid DNA in striped and albino mutants in maize. Planta **191,** 552–563.

Han, C.-D., Derby, R.J., Schnable, P.S., and Martienssen, R.A. (1995). Characterization of the plastids affected by class II albino mutations of maize at the morphological and transcript levels. Maydica **40,** 13–22.

Herrin, D.L., Chen, Y.-F., and Schmidt, G.W. (1990). RNA splicing in *Chlamydomonas* chloroplasts: Self-splicing of 23S preRNA. J. Biol. Chem. **265,** 21134–21140.

Hess, W.R., Hoch, B., Zeltz, P., Hübschmann, T., Kössel, H., and Börner, T. (1994). Inefficient *rpl2* splicing in barley mutants with ribosome-deficient plastids. Plant Cell **6,** 1455–1465.

Hildebrand, M., Hallick, R.B., Passavant, C.W., and Bourque, D.P. (1988). *trans*-Splicing in chloroplasts: The *rps12* loci of *Nicotiana tabacum.* Proc. Natl. Acad. Sci. USA **85,** 372–376.

Hollingsworth, M.J., Johanningmeir, U., Karabin, G.D., Stiegler, G.L., and Hallick, R.B. (1984). Detection of multiple, unspliced precursor mRNA transcripts for the M_r 32,000 thylakoid membrane protein from *Euglena gracilis* chloroplasts. Nucleic Acids Res. **12,** 2001–2017.

Hübschmann, T., Hess, W.R., and Börner, T. (1996). Impaired splicing of the *rps12* transcript in ribosome-deficient plastids. Plant Mol. Biol. **30,** 109–123.

Jardin, P.D., Portetelle, D., Harvengt, L., Dumont, M., and Wathelet, B. (1994). Expression of intron-encoded maturase-like polypeptides in potato chloroplasts. Curr. Genet. **25,** 158–163.

Kim, J.-K., and Hollingsworth, M.J. (1993). Splicing of group II introns in spinach chloroplasts (in vivo): Analysis of lariat formation. Curr. Genet. **23,** 175–180.

Koller, B., Clarke, J., and Delius, H. (1985). The structure of precursor mRNAs and of excised intron RNAs in chloroplasts of *Euglena gracilis.* EMBO J. **4,** 2445–2450.

Koller, B., Fromm, H., Galun, E., and Edelman, M. (1987). Evidence for in vivo *trans* splicing of pre-mRNAs in tobacco chloroplasts. Cell **48,** 111–119.

Larrinua, I.M., Muskavitch, K.M.T., Gubbins, E.J., and Bogorad, L. (1983). A detailed restriction endonuclease site map of the *Zea mays* plastid genome. Plant Mol. Biol. **2,** 129–140.

Lemaire, C., and Wollman, F.-A. (1989). The chloroplast ATP synthase in *Chlamydomonas reinhardtii.* II. Biochemical studies on its biogenesis using mutants defective in photophosphorylation. J. Biol. Chem. **264,** 10235–10242.

Lewin, A.S., Thomas, J., Jr., and Tirupati, H.K. (1995). Cotranscriptional splicing of a group I intron is facilitated by the Cbp2 protein. Mol. Cell. Biol. **15,** 6971–6978.

Liere, K., and Link, G. (1995). RNA-binding activity of the *matK* protein encoded by the chloroplast *trnK* intron from mustard. Nucleic Acids Res. **23,** 917–921.

Maier, R.M., Neckermann, K., Igloi, G.L., and Kössel, H. (1995). Complete sequence of the maize chloroplast genome: Gene content, hotspots of divergence and fine tuning of genetic information by transcript editing. J. Mol. Biol. **251,** 614–628.

Michel, F., Umesono, K., and Ozeki, H. (1989). Comparative and functional anatomy of group II catalytic introns—A review. Gene **82,** 5–30.

Miles, D. (1994). The role of high chlorophyll fluorescence photosynthesis mutants in the analysis of chloroplast thylakoid membrane assembly and function. Maydica **39,** 35–45.

Mohr, G., Zhang, A., Gianelos, J.A., Belfort, M., and Lambowitz, A.M. (1992). The Neurospora CYT-18 protein suppresses defects in the phage T4 *td* intron by stabilizing the catalytically active structure of the intron core. Cell **69,** 483–494.

Neuhaus, H., and Link, G. (1987). The chloroplast tRNA(Lys)(UUU) gene from mustard (*Sinapis alba*) contains a class II intron potentially encoding for a maturase-related polypeptide. Curr. Genet. **11,** 251–257.

Pel, H.J., Tzagoloff, A., and Grivell, L.A. (1992). The identification of 18 nuclear genes required for the expression of the yeast mitochondrial gene encoding cytochrome *c* oxidase subunit 1. Curr. Genet. **21,** 139–146.

Plant, A.L., and Gray, J.C. (1988). Introns in chloroplast protein-coding genes of land plants. Photosynth. Res. **16,** 23–39.

Rochaix, J.-D. (1992). Post-transcriptional steps in the expression of chloroplast genes. Annu. Rev. Cell Biol. **8,** 1–28.

Saldanha, R., Mohr, G., Belfort, M., and Lambowitz, A.M. (1993). Group I and group II introns. FASEB J. **7,** 15–24.

Shaw, L.C., and Lewin, A.S. (1995). Protein-induced folding of a group I intron in cytochrome *b* pre-mRNA. J. Biol. Chem. **270,** 21552–21562.

Shinozaki, K., Deno, H., Sugita, M., Kuramitsu, S., and Sugiura, M. (1986). Intron in the gene for the ribosomal protein S16 of

tobacco chloroplast and its conserved boundary sequences. Mol. Gen. Genet. **202,** 1–5.

Subramanian, A.R., Stahl, D., and Prombona, A. (1991). Ribosomal proteins, ribosomes, and translation in plastids. In The Molecular Biology of Plastids, L. Bogorad and I.K. Vasil, eds (San Diego, CA: Academic Press), pp. 191–215.

Svab, Z., and Maliga, P. (1993). High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. Proc. Natl. Acad. Sci. USA **90,** 913–917.

Taylor, W. (1989). Regulatory interactions between nuclear and plastid genomes. Annu. Rev. Plant Physiol. Plant Mol. Biol. **40,** 211–233.

Voelker, R., and Barkan, A. (1995). Two nuclear mutations disrupt distinct pathways for targeting proteins to the chloroplast thylakoid. EMBO J. **14,** 3905–3914.

Walbot, V., and Coe, E.H., Jr. (1979). Nuclear gene *iojap* conditions a programmed change to ribosome-less plastids in *Zea mays*. Proc. Natl. Acad. Sci. USA **76,** 2760–2764.

Weeks, K.M., and Cech, T.R. (1995a). Efficient protein-facilitated splicing of the yeast mitochondrial bI5 intron. Biochemistry **34,** 7728–7738.

Weeks, K.M., and Cech, T.R. (1995b). Protein facilitation of group I intron splicing by assembly of the catalytic core and the 5′-splice site domain. Cell **82,** 221–230.

Weeks, K.M., and Cech, T.R. (1996). Assembly of a ribonucleoprotein catalyst by tertiary structure capture. Science **271,** 345–348.

Westhoff, P., and Hermann, R.G. (1988). Complex RNA maturation in chloroplasts. Eur. J. Biochem. **171,** 551–564.

Wollenzien, P.L., Cantor, C.R., Grant, D.M., and Lambowitz, A.M. (1983). RNA splicing in Neurospora mitochondria: Structure of the unspliced 35S precursor ribosomal RNA detected by psoralen cross-linking. Cell **32,** 397–407.

Zaita, N., Torazawa, K., Shinozaki, K., and Sugiura, M. (1987). *trans*-Splicing in vivo: Joining of transcripts from the "divided" gene for ribosomal protein S12 in the chloroplasts of tobacco. FEBS Lett. **210,** 153–156.