Rf8 and *Rf** Mediate Unique T-*urf13*-Transcript Accumulation, Revealing a Conserved Motif Associated With RNA Processing and Restoration of Pollen Fertility in T-Cytoplasm Maize

Carren L. Dill,*,† Roger P. Wise*,†,‡ and Patrick S. Schnable*,§

**Interdepartmental Genetics Program, †Department of Plant Pathology, ‡Corn Insects and Crop Genetics Research, U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS) and §Departments of Agronomy and Zoology and Genetics, Iowa State University, Ames, Iowa 50011*

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ABSTRACT

Rf8 is a newly described nuclear gene that can substitute for *Rf1* to partially restore pollen fertility to male-sterile, T-cytoplasm maize. Families segregating for *Rf8* were used to investigate the mechanism of this fertility restoration and to compare it to the restoration conditioned by *Rf1*. Although *Rf8* is unlinked to the *rf1* locus, it also alters T-*urf13* mitochondrial transcript accumulation and reduces the accumulation of the URF13 protein. Like the 1.6- and 0.6-kilobase (kb) T-*urf13* transcripts that accumulate in T-cytoplasm plants carrying *Rf1*, 1.42- and 0.42-kb transcripts accumulate in plants that are partially restored by *Rf8*. A survey of T-cytoplasm maize lines, inbreds, and F_1 hybrids by mitochondrial RNA gel blot analyses revealed that *Rf8* is rare in maize germplasm. These surveys revealed the presence of another rare, weak restorer factor, *Rf**, which is uniquely associated with the accumulation of 1.4- and 0.4-kb T-*urf13* transcripts. Primer extension analyses position the 5′ termini of the 1.42/0.42-kb and 1.4/0.4-kb transcripts at +137 and +159 nucleotides, respectively, 3′ of the AUG initiation codon of the T-*urf13* reading frame. The conserved motif, 5′-CNACNNU-3′, overlaps the 5′ termini of the *Rf1*-, *Rf8*-, and *Rf**-associated transcripts and the 380 nucleotide, *Rf3*-associated *orf107* transcript from cytoplasmic male sterility sorghum. These results demonstrate that multiple unlinked, nuclear genes can have similar but distinct effects on the expression of the unique T-*urf13* mitochondrial coding sequence to restore pollen fertility to T-cytoplasm maize.

IN eukaryotes the expression of the nuclear and mitochondrial genomes must be coordinated to make a functional cell. Cytoplasmic male sterility (CMS) is a loss of functional pollen in plants that can be attributed to unique mitochondrial genomes. CMS is thought to be due to an incompatibility of these two genomes such that pollen is not properly formed. However, there are nuclear genes designated *Rf* or *Fr* (restorers of fertility) that can correct this incompatibility and allow the development of functional pollen. Thus, CMS offers a system with which to study the mechanisms by which nuclear genes regulate mitochondrial gene expression.

T-cytoplasm maize has been intensively studied due to its role in the 1970 U.S. epidemic of southern corn leaf blight (ULLSTRUP 1972; PRING and LONSDALE 1989). At that time *cms-T* was widely used in hybrid seed production because it eliminated the costly practice of hand detasseling. At the time of the epidemic ~85% of the U.S. maize crop was produced using *cms-T*, which is highly sensitive to host-selective toxins produced by both race T of *Cochliobolus heterostrophus* (asexual stage *Bipolaris maydis*), the causal agent of southern corn leaf blight, and *Phyllosticta maydis*, the causal agent of yellow leaf blight. A unique coding region in T-cytoplasm mitochondria (designated T-*urf13*) is responsible for pathotoxin sensitivity in *cms-T* (DEWEY *et al.* 1986, 1988; ROTTMANN *et al.* 1987; WISE *et al.* 1987a; BRAUN *et al.* 1990). T-*urf13* encodes a 13-kD mitochondrial pore-forming protein (URF13) located in the inner mitochondrial membrane in an oligomeric form (DEWEY *et al.* 1987; WISE *et al.* 1987b; KORTH *et al.* 1991). The URF13 protein binds to the T-toxin produced by *B. maydis* race T (BRAUN *et al.* 1990). The binding of the pathotoxins renders mitochondria incapable of oxidative phosphorylation, ultimately leading to cell death (MATHEWS *et al.* 1979). Male-fertile, toxin-insensitive mutants that possess deletions or a frameshift of T-*urf13* do not synthesize URF13 (DIXON *et al.* 1982; WISE *et al.* 1987b) and, thus, the diseases do not develop.

The male-sterile C, S, and T cytoplasms produce fertile plants only in nuclear backgrounds carrying the appropriate restorer genes. These nuclear-encoded fertility restoring genes compensate for cytoplasmic dysfunctions that are phenotypically expressed during microsporogenesis and/or microgametogenesis. The complementary expression of dominant alleles of both the *rf1* and *rf2* nuclear restorer loci allows normal pollen development in plants carrying T cytoplasm (LAUGHNAN and GABAY-LAUGHNAN 1983). Maize plants

Corresponding author: Roger P. Wise, Department of Plant Pathology, USDA-ARS, 351 Bessey Hall, Iowa State University, Ames, IA 50011. E-mail: rpwise@iastate.edu

TABLE 1

Sequences of complementary oligonucleotides from the T-*urf13* region used in transcript mapping, primer extension, and sequencing

Name	Complementary sequence (5′ → 3′)	TURF 2H3 sequence no.[a]
CD014	CCT TGA TCA AAG GGA GGA AGG	1235–1255
CD1656	AGT AAG ATA GAC GAG GAC TAT TTG ACT CAA	4325–4354[b]
CD1722	TTG AGC CAA ATA GGA ATC ATC CAT TTT ACG	1320–1349
CD0989	GTG GTT GGC TAA CTC GGA GAG TTG AG	1345–1370
CD1721	TCT ATT GTG GTT GGC TAA CTC GGA GAG	1350–1376
CD1536	GGC CAC GTG GCC CGC TTT TGC CGC TTC CAC	1377–1406
CD0380	CAT GAG CTA TCC TTC TCG TGG TTG	1410–1433
CD1537	CAC CCT AAT TTG AAC CCC CCT CAA CCA CGA	1425–1454
CD667	CGC GCA GCG TGC CGC TGG TAA TGA TTC TAC	1961–1990
CD666	GTC CTG AAG ACG GAT GCG ACG GGA AGA AGT	2066–2095
CD565	TTA CAG ATA AGC TCC AGG GTT GTT TCA ACG	2536–2565
CD564	ATA CGG TTG GTG GGC CTC GGT ATC TCT TTT	2716–2745
CD664	CAA GAG AAC ACA GAT AGG TAT TAG GTC ACG	3066–3095

[a] Numbering from DEWEY *et al.* 1986.
[b] Homologous *rrn26* sequence from CD1722 region.

carrying *Rf1* accumulate additional 1.6- and 0.6-kilobase (kb) T-*urf13* transcripts in the mitochondria (DEWEY *et al.* 1987; KENNELL *et al.* 1987; WISE *et al.* 1996). Concurrent with the accumulation of these 1.6- and 0.6-kb transcripts is an ~80% reduction in URF13 accumulation (FORDE and LEAVER 1980; DEWEY *et al.* 1987). Although *Rf2* has no known effects on T-*urf13* transcript accumulation or URF13 synthesis, the recent cloning and sequencing of *Rf2* revealed that the RF2 amino acid sequence is 75% similar and 60% identical to mammalian aldehyde dehydrogenases (ALDH), suggesting a possible mechanism for *Rf2*-mediated restoration (CUI *et al.* 1996).

To investigate alternate mechanisms by which fertility restoration can occur in T-cytoplasm maize, *rf1/rf1 Rf2/Rf2* maize plants that partially suppressed T-*urf13*-mediated pollen sterility were identified. Tassels of these plants exserted variable numbers of anthers that produced viable pollen, suggesting the existence of a third restorer in T-cytoplasm maize (SCHNABLE and WISE 1994). Maize lines that exhibit this trait undergo anthesis 1–2 wk later than near-isogenic siblings and accumulate novel T-*urf13* transcripts distinct from the characteristic *Rf1*-associated transcripts. This initial observation presented two possibilities: that a nuclear gene mediating these effects was a novel allele of *Rf1* or that it was a separate gene, which, like *Rf1*, altered T-*urf13* transcript accumulation. In this article we describe the genetic characterization of two partial-fertility restorers in T-cytoplasm. Maize lines carrying these weak restorers accumulate new 1.42- and 0.42-kb or 1.4- and 0.4-kb T-*urf13* transcripts; the 5′ termini of these two groups of transcripts are just 22 nucleotides apart. In addition, the conserved sequence motif 5′-CNACNNU-3′ overlaps the 5′ termini of all these transcripts. This conserved sequence also overlaps the 5′ termini of the 1.6-kb *Rf1*-associated T-*urf13* transcript and the 380-nucleotide *Rf3*-associated *orf107* mitochondrial transcript from CMS sorghum.

MATERIALS AND METHODS

Plant materials: Plant number 90 8703 (*rf1-m/rf1, Rf2/rf2, Rf8/rf8*) was identified by its sterile phenotype in a 1990 screen for *Rf2* mutants and was fertilized with pollen from our *wx1-m8* stock (*rf1/rf1, Rf2/Rf2, Rf8/rf8*) (SCHNABLE and WISE 1994). Partially-fertile plants (90g 1138-5 and 6; Table 4 in SCHNABLE and WISE 1994) derived from this cross that were homozygous for *Rf2* (*rf1/rf1, Rf2/Rf2, Rf8/rf8*) were self-pollinated; subsequent progeny derived from these plants via self-pollinations or outcrosses are designated as being derived from the 8703 pedigree. The origins of other maize stocks have been described previously (ROCHEFORD *et al.* 1992; SCHNABLE and WISE 1994; WISE and SCHNABLE 1994; WISE *et al.* 1996).

Nucleic acid isolations and analyses: Total DNA was isolated from frozen leaves or husk tissue using a modified CTAB extraction. These DNA extractions, as well as DNA gel blot analyses, were conducted as previously described (WISE and SCHNABLE 1994). Mitochondrial RNA (mtRNA) was isolated as described in WISE *et al.* (1996). Following isolation, the RNA was quantified using 260/280 nM spectral analysis. Four micrograms of mtRNA were denatured with glyoxal and dimethyl sulfoxide (DMSO) for 1 hour at 50°. Samples were then size fractionated through a 1.8% Seakem GTG agarose (FMC, Rockland, ME) gel with 0.01 M iodoacetic acid to inhibit RNAses. Electrophoresis was performed for 16 hr in a 4° cold room with circulation of the 10 mM Na_2HPO_4 (pH 7) running buffer. The RNA was then transferred to Hybond N (Amersham, Arlington Heights, IL) (THOMAS 1980). RNA and DNA marker lanes were stained for 30 min in 300 ml 0.5 M ammonium acetate with 300 μl of 10 mg/ml EtBr. After transfer for a minimum of 14 hr, the membrane was air dried before it was crosslinked with 220 mJ of UV light emitted by 302 nm bulbs in a Stratalinker 2400 (Stratagene, La Jolla, CA) and hybridization was carried out as described previously (WISE *et al.* 1996). Table 1 lists the oligonucleotides used in hybridization and primer extension experiments and their TURF 2H3 (DEWEY *et al.* 1986) sequence designations.

Predictions of RNA secondary structures: RNA secondary structures were predicted with the STAR software for Power

Macintosh (VAN BATENBURG 1993). All three algorithms were used (greedy, stochastic, and genetic), although the genetic algorithm was concluded to be most representative. The calculation was imported into the Loop D Loop software (D. GILBERT, Indiana University) for optimum visualization.

Protein gel blot analyses: Mitochondria were isolated as described previously (MCNAY *et al.* 1984) and lysed in 62.5 mM Tris-HCl pH 6.8, 10% v/v glycerol, 4% SDS, 2.5% 2-mercaptoethanol, 0.1 mM bromophenol blue (LAEMMLI 1970). Samples were quantified according to BRADFORD (1976) and surveyed visually via Coomassie blue staining of SDS-PAGE-separated proteins. One hundred micrograms of isolated protein was size fractionated through a 4% SDS-polyacrylamide stacking gel (0.06 M Tris-HCl pH 6.8, 0.192 M glycine, and 0.1% SDS) and a 15% separating gel (0.025 M Tris-HCl pH 9.1, 0.192 M glycine, 0.1% SDS) with 0.025 M Tris, 0.192 M glycine, and 0.1% SDS running buffer (pH 9.1) (LAEMMLI 1970). Proteins were then transferred to 0.2 μm supported nitrocellulose (Bio-Rad, Hercules, CA) in 25 mM Tris-Cl, 192 mM glycine, and 20% methanol buffer at 14 V for 18 hr or 30 V for 8 hr. Detection of URF13 was performed with an URF13 monoclonal antibody (a gift from C. S. LEVINGS III, North Carolina State University) using an immunoblot goat anti-mouse IgG (H + L) alkaline phosphatase detection kit (Bio-Rad). The nitrocellulose was exposed to the primary antibody for 14–16 hr, followed by the secondary antibody for 2 hr. Final immunodetection was completed in 15 min using the provided color development solution.

Primer extension analyses and DNA sequencing: Precise mapping of the 5′ termini of T-*urf13* RNA transcripts was conducted using primer extension analysis. Oligonucleotides CD0989 and CD0380 (Table 1) were used for primer extension and PCR-based dsDNA sequencing. A GIBCO BRL (Gaithersburg, MD) dsDNA cycle sequencing kit was used to determine the sequence 5′ of the primer using DNA from plasmid T-t221 (WISE *et al.* 1987a). Primer extension was carried out using an AMV Reverse Transcriptase Primer Extension System (Promega, Madison, WI). To optimize the reaction, annealing was carried out at 70° for 20 min followed by gradual cooling of the samples to 42° over 1.5 hr. Following the reverse transcription reaction, the sample was concentrated by ethanol precipitation and 10 μl of loading buffer was used to resuspend the transcribed RNA. Five microliters of the primer extension reaction and 1 μl of the sequencing reaction were size fractionated through a 7% acrylamide gel (Long Ranger, FMC) containing 7.5% UREA in 1× TBE buffer (1× TBE = 0.089 M Tris, 0.089 M boric acid, and 0.002 M Na_2EDTA) for 2 hr at 65 W. The gel was then exposed directly to film. Results were then analyzed by comparing the alignment of primer extension and sequencing reaction products.

RESULTS

Identification of *Rf8*, a single nuclear-, partial-fertility restorer: In the course of analyzing anomalous families derived from an *Rf2* transposon tagging experiment, partially male-fertile plants (90g 1138-5 and 6) were identified in the progeny of a cross between plant number 90 8703 and our *wx1-m8* stock (N *rf1/rf1, Rf2/Rf2*; N and T designate N and T cytoplasms, respectively). These partially fertile 90g plants had the genotype T *rf1/rf1, Rf2/Rf2*, a genotype that normally conditions male sterility. Selfed progeny derived from 90g 1138-5 and 6 consisted of one fertile, 20 partially fertile, and 236 male-sterile plants when grown in our 1991 summer nursery (rows 2150–2152 and 2167–2169). These preliminary results led us to hypothesize that this pedigree was segregating for a partial restorer factor (SCHNABLE and WISE 1994). To test the repeatability of this partial restoration phenotype, remnant seed of the identical 8703-derived progeny family was grown in our 1991–1992 winter nursery, yielding 25 partially fertile and 57 male-sterile plants. The different frequencies at which partially fertile plants arose in the two seasons suggested that this partial-restorer factor (designated *Rf8*) is environmentally sensitive. Based on these hypotheses, a series of genetic tests were performed to fully characterize this new partial-restorer factor(s).

Figure 1 compares the degree of anther exsertion on tassels of *Rf8*-restored, *Rf1*-restored, and nonrestored T-cytoplasm maize. Plants that expressed the partial-fertility phenotype typically exserted a variable (but always reduced) number of anthers several days later than male-fertile, near-isogenic plants. To determine whether this partial restorer phenotype was under nuclear or cytoplasmic control, near-reciprocal crosses were performed. In the first cross, pollen from a partially fertile plant from the 8703 pedigree (*rf1/rf1, Rf2/Rf2, Rf8/−*) was used to fertilize an ear of a T-cytoplasm version of the inbred line W64A (*rf1/rf1, Rf2/Rf2, rf8/rf8*). To make the second cross, pollen from an N-cytoplasm version of W64A was used to fertilize the ear of the same partially fertile (T-cytoplasm) plant in the 8703 pedigree. As shown in Table 2, progeny from both of these reciprocal crosses segregated for partial fertility (in approximately equal frequencies). This result demonstrates that fertility restoration in the 8703 pedigree is nuclear encoded.

To determine if *Rf8* altered the accumulation of T-*urf13* transcripts, mitochondrial RNAs were isolated from immature ears of 50 individual progeny from the 8703 pedigree that were segregating for *Rf8* and subjected to RNA gel blot analyses with T-*urf13* probes (KENNELL *et al.* 1987; WISE *et al.* 1987a; WISE *et al.* 1996). Every partially fertile plant in this analysis accumulated a unique 1.42-kb T-*urf13* transcript as shown in Figure 2. We subsequently demonstrated that all plants that accumulated the 1.42-kb transcript also accumulated a 0.42-kb transcript (shown in Figure 3). Some sterile plants also accumulated these 1.42- and 0.42-kb transcripts. However, as described below, the accumulation of the 1.42- and 0.42-kb transcripts in sterile plants is likely due to the incomplete penetrance of the *Rf8*-induced restoration of male fertility.

To determine the number of genes that control the accumulation of the 1.42- and 0.42-kb transcripts, an additional 79 random progeny from a backcross of plants derived from the 8703 pedigree to *wx1-m8* (*rf1/rf1, Rf2/Rf2, rf8/rf8*) were analyzed for fertility and T-*urf13* transcript accumulation. As presented in Table 3, an approximate 1:1 ratio (42:37) was observed for plants that accumulated the 1.42- and 0.42-kb transcripts to plants that did not. This segregation ratio

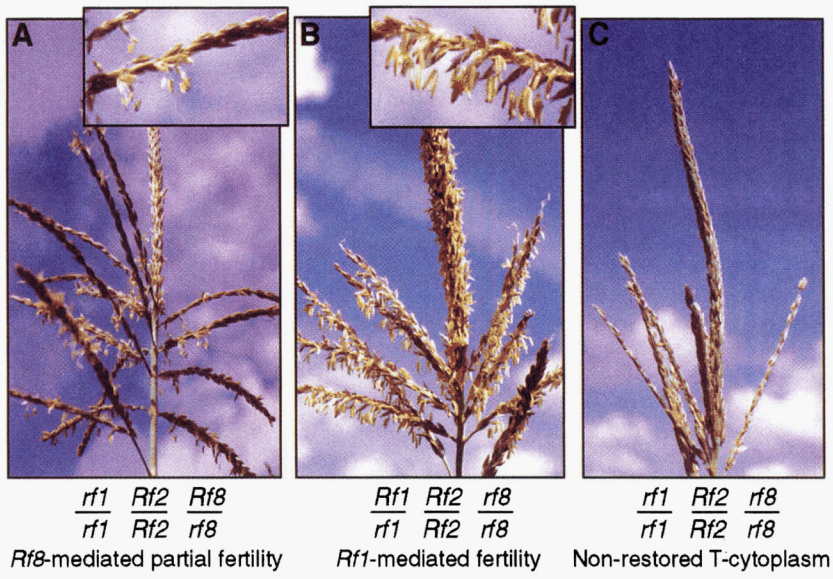

FIGURE 1.—Tassel phenotypes of T-cytoplasm maize plants with defined restorer genotypes. A comparison of a tassel a *Rf8*-restored plant (A) to the tassel of a *Rf1*-restored plant (B) illustrates the partially fertile phenotype. An *Rf8*-restored tassel exserts few anthers but the anthers do produce viable pollen. Anthesis occurs 1–2 wk later in *Rf8* restored T-cytoplasm plants. A sterile plant that is recessive for *rf1* and *rf8* is shown in C.

indicates that the 8703-derived parent was heterozygous for a single gene responsible for the accumulation of these transcripts.

Based on the results of two experiments, it is the dominant allele of the *rf8* gene that is responsible for the accumulation of these transcripts. In the first experiment, pollen from plants in the 8703 pedigree was used as an *Rf8* donor in a backcross to T cytoplasm W64A (*rf1/rf1, Rf2/Rf2, rf8/rf8*). Partially fertile progeny were selected (to maintain the *Rf8* allele) for subsequent backcrosses to W64A. These resulting backcross families consistently segregated for variable numbers of partially fertile individuals, even though T-cytoplasm W64A plants are normally uniformly male sterile. This result demonstrates that restoration is controlled by a dominant allele. In the second experiment, 10 random progeny from a self of a heterozygous *Rf8/rf8* plant from one of the backcross populations described above were analyzed for the presence of the 1.42- and 0.42-kb transcripts. Nine of these 10 progeny accumulated the 1.42- and 0.42-kb *Rf8*-associated transcripts ($\chi^2_{3:1}$ = 0.53; $0.3 < P < 0.5$). Hence, these findings establish that *Rf8* acts in a dominant fashion to control both traits (*i.e.*, fertility restoration and the modification of T-*urf13* transcript accumulation).

Because some sterile progeny accumulated the 1.42- and 0.42-kb transcripts, crosses were performed to verify the incomplete penetrance of *Rf8*-induced restoration of male fertility. Partially fertile and sterile plants that accumulated these transcripts from one of the backcross populations were identified via RNA gel blot analysis. Both classes of plants were crossed as females by an N-cytoplasm version of W64A (*rf1/rf1, Rf2/Rf2, rf8/rf8*). Progeny from both types of crosses segregated for partial fertility as shown in Table 4. A subset of these progeny were assayed for the presence of the 1.42- and 0.42-kb transcripts. As expected, all of the partially fertile plants carried the 1.42- and 0.42-kb transcripts, regardless if the 8703 parent was partially fertile or sterile. These results demonstrate that partial fertility can be recovered from either a fertile or a sterile plant if it carries *Rf8* (indicated by the presence of the 1.42 and 0.42-kb transcripts). In contrast, progeny from the cross of a sterile plant from the 8703 pedigree (that did not accumulate the 1.42- or 0.42-kb transcripts) by W64A did not segregate for partial fertility or the accumulation of these transcripts. Hence, in combination, the tests described above establish that *Rf8* acts as an incompletely penetrant, dominant allele to partially restore fertility to T-cytoplasm maize.

TABLE 2

Male fertility phenotypes of progeny from reciprocal crosses of 8703 and W64A

1994 cross	1994g progeny rows	No. of partially male-fertile plants	No. of male-sterile plants	Total
W64A (T) × [4010-5 (8703 T)]	1175–1178	9	29	38
[4010-5 (8703 T)] × W64A (N)	1179–1182	12	20	32

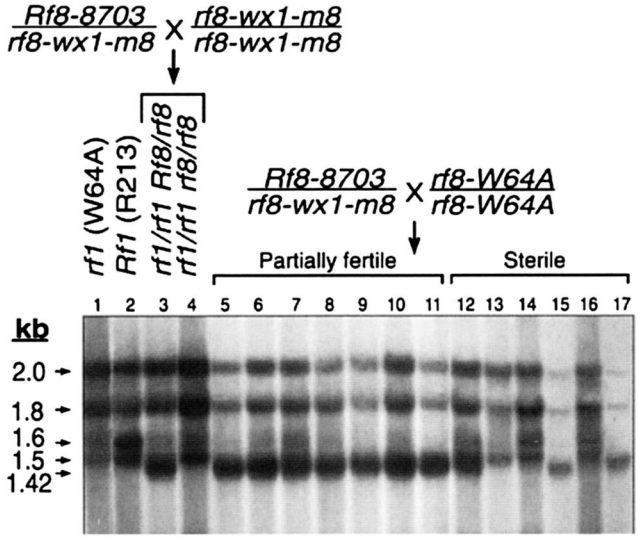

FIGURE 2.—Cosegregation of the 1.42-kb T-*urf13* transcript in *Rf8*-restored plants. RNA gel blots were hybridized with the probe T-a106 (KENNELL *et al.* 1987; WISE *et al.* 1987a). The W64A (*rf1/rf1*) and R213 (*Rf1/Rf1*) control mtRNAs display the transcript patterns normally observed in T-cytoplasm maize. Note the high rate of accumulation of the 1.6-kb transcript associated with the *Rf1-R213* allele in contrast to the lower background accumulation associated with the inbred W64A. Lanes 3 and 4 contain RNA from line 8703 plants a generation prior to the plants in lanes 5–17.

***Rf8* segregates independently of *Rf1*:** The above results could be explained by either of two possibilities. One possibility is that the partial fertility restoration phenotype is controlled by a weak *Rf1* allele, *i.e., Rf1-8703*. The other possibility is that the partial fertility restoration is controlled by a third restorer that is not allelic to the *Rf1* locus but that can mimic *Rf1*. To distinguish between these possibilities, *rf1* and *rf2* genotypes in an 8703 × W64A backcross family were correlated with the accumulation of the *Rf8*-associated 1.42- and 0.42-kb transcripts. Plants within this family segregated for the genotypes *Rf8-8703/rf8-W64A* and *rf8-W64A/rf8-W64A* and were grown in our 1992–1993 and 1993–1994 winter and 1992, 1993, and 1994 summer nurseries. Each plant was scored for male fertility, the presence or absence of the 1.42- and 0.42-kb transcripts, and its allelic constitution at the *rf1* and *rf2* loci. The *rf1* genotypes were scored using the RFLP markers *umc10* and *umc92*, which flank the *rf1* locus (WISE and SCHNABLE 1994). These experiments established that the accumulation of the 1.42- and 0.42-kb transcripts was not correlated with any particular *rf1* genotype, *i.e., Rf8* segregated independently of the *rf1-8703* allele. An example of these data is shown in Table 5 (contingency $\chi^2 = 2.61$; $0.10 < P < 0.25$). Similarly, the *rf2*-linked RFLP marker, *umc153* (WISE and SCHNABLE 1994) was shown to segregate independently of the accumulation of the *Rf8*-associated 1.42- and 0.42-kb transcripts (contingency $\chi^2 = 0.358$; $0.50 < P < 0.75$, data not shown). Since neither the *rf1* nor the *rf2* loci control the presence of the 1.42- and 0.42-kb transcripts, we conclude that their accumulation is controlled by a third nuclear restorer (*i.e., Rf8*).

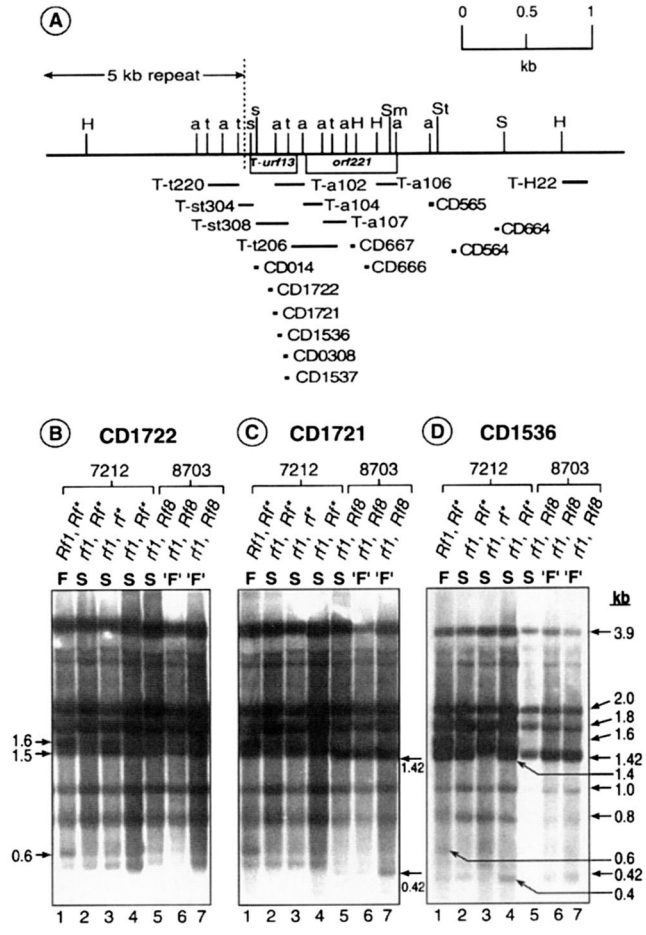

FIGURE 3.—Mapping the 5′ regions of the 1.42- and 0.42-kb (*Rf8* associated) and the 1.4- and 0.4-kb (*Rf**-associated) T-*urf13* transcripts. (A) The T-*urf13/orf221* region and positions of experimental probes. The position of T-*urf13* and *orf221* are shown. Both open reading frames share the same promoter region within the indicated 5-kb repeat. a, *Alu*I; H, *Hin*dIII; s, *Sau*3a; Sm, *Sma*I; St, *Sst*II; t, *Taq*I. (B–D) RNA gel blot hybridizations with the oligonucleotides CD1722, CD1721, and CD1536, respectively. These three oligonucleotides are adjacent, spanning 85 nucleotides of the T-*urf13* reading frame (A). Lanes 1–4 contain RNA from plants that are segregating for the *Rf** allele. Lanes 5–7 contain RNA from plants that contain the *Rf8* allele. The *Rf1*-associated 1.6- and 0.6-kb T-*urf13* transcripts are shown in lane 1 (B–D). CD1721 hybridizes to the *Rf8*-associated 1.42- and 0.42-kb transcripts (C, lanes 5–7), whereas CD1722 does not (B, lanes 5–7). CD1536 hybridizes to the 1.4- and 0.4-kb transcripts present in plants derived from the *rf1-m7212* pedigree (D, lanes 1, 2, and 4), whereas CD1722 and CD1721 do not (B and C, lanes 1, 2, and 4).

***Rf8* is rare in maize germplasm:** To determine if there are other sources of *Rf8* in our germplasm or in other T-cytoplasm lines, RNA gel blots containing mitochondrial RNA from 15 maize lines, inbreds, or F_1 hybrids were hybridized with T-*urf13* probes to assay for the presence of the *Rf8*-associated transcripts (Table 6). In a survey of 20 sources of mtRNAs, only those from a T-cytoplasm version of our *wx1-m8* stock segregated

TABLE 3

Cosegregation of the 1.42-/0.42-kb T-*urf13* transcripts and partial male fertility

T-*urf13* transcript accumulation	No. of partially male-fertile plants	No. of male-sterile plants	Total
1.42-/0.42-kb present	18	24	42
1.42-/0.42-kb absent	0	37	37
Total	18	61	79

Plants were derived from crosses between 8703 [(T) *rf1/rf1, Rf2/Rf2, Rf8/rf8*] and our *wx1-m8* stock (*rf1/rf1, Rf2/Rf2, rf8/rf8*); rows 91g 6207–6208, 92 2083–2086, 94 4008–4010, and 94g 1169–1172. The 1:1 ratio ($\chi^2_{1:1} = 0.32$; $0.50 < P < 0.75$) of plants that accumulated the 1.42-/0.42-kb transcripts to plants that did not indicates that the 1.42-/0.42-kb transcripts are controlled by a single nuclear gene.

for the *Rf8*-dependent, 1.42-kb T-*urf13* transcript. Based on pedigree analyses, this *wx1-m8* stock is the likely donor of the *Rf8* detected in 8703-derived progeny.

In this survey, three maize stocks revealed a T-*urf13* transcript pattern unlike that of *Rf8*- or *Rf1*-restored lines. Inbred 33-16 (ROCHEFORD *et al.* 1992), a family derived from *rf1-m7212* (WISE *et al.* 1996), and our *Rg1* stock no. 1 all had transcripts sized at 1.4- and 0.4-kb (as shown below, these are distinct from the *Rf8*-associated, 1.42- and 0.42-kb T-*urf13* transcripts present in *Rf8*-containing stocks). Inbred 33-16 and a family derived from *rf1-m7212* (both of which have the genotype *rf1/rf1, Rf2/Rf2*) also displayed partial fertility restoration. Tests to correlate *rf1*- and *rf2*-associated RFLP patterns with the presence of the 1.4- and 0.4-kb transcripts demonstrated that the gene controlling their accumulation is not allelic to either *rf1* or *rf2* (data not shown). The allelism of this partial restorer to *rf8* has not been tested. Hence, the 1.4- and 0.4-kb transcripts may be controlled by an allele of *Rf8* or another weak restorer that is not allelic to other T-cytoplasm restorers. Because its allelic identity has not been tested, this restorer has been designated *Rf**.

T-*urf13* transcripts associated with *Rf8* and *Rf:** Previously, it was shown that a unique 1.6-kb T-*urf13* transcript accumulates in T-cytoplasm plants that carry the dominant allele *Rf1*, but not in T-cytoplasm plants that are *rf1/rf1* (DEWEY *et al.* 1987; KENNELL *et al.* 1987). Cosegregation analyses verified this correlation by demonstrating that plants containing four independently derived *rf1-m* alleles did not accumulate the 1.6-kb transcript (WISE *et al.* 1996). It has been hypothesized that the 1.6-kb transcript is a processing derivative of 2.0 or 1.8-kb transcripts that are present in all T-cytoplasm plants and that this processing is under the control of the product of the *Rf1* allele (DEWEY *et al.* 1987; KENNELL *et al.* 1987; KENNELL and PRING 1989; see review by LEVINGS 1993).

To address the question of whether *Rf8*- and *Rf**-associated T-*urf13* transcripts also have features that suggest they arose by processing events, mtRNA was assayed from representative plants with and without *Rf8* or *Rf**. Consecutive hybridizations of mitochondrial RNA gel blots with the series of clones and oligonucleotides from the T-*urf13* region shown in Figure 3A were used to map the T-*urf13* transcripts that accumulate in *Rf8*- and *Rf**-containing plants. Seven major transcripts of 3.9, 2.0, 1.8, 1.5, 1.1, 1.0, and 0.8 kb were detected with various *T-urf13*-associated probes in all T-cytoplasm plants, consistent with previous reports (KENNELL *et al.* 1987; WISE *et al.* 1996).

The 1.42- and 0.42-kb *Rf8*-associated transcripts were mapped using mtRNA from plants within the 8703 pedigree; the 1.4- and 0.4-kb *Rf**-associated transcripts were mapped using mtRNA from inbred 33-16 and a family derived from the *rf1-m7212* pedigree. Figure 3 reveals that probe CD1536, but not CD1722, detects novel transcripts in plants that carry either *Rf8* or *Rf** (Figure 3, D and B, respectively). However, only the *Rf8*-associated transcripts are detected with CD1721 (Figure 3C, lanes 5–7); the *Rf**-associated transcripts are not detected with this oligonucleotide probe (Figure 3C, lanes 1, 2, and 4). Thus, the most 5′ probes that detect the *Rf8*-

TABLE 4

Presence of the partial fertility and the 1.42-/0.42-kb T-*urf13* transcripts in progeny of crosses among 8703-derived plants and W64A

1994 cross	T-*urf13* transcript accumulation in 1994 8703 parent	1994g progeny rows	No. of partially male-fertile plants	No. of male-sterile plants	Total
[4008-4 (8703 T 'F')[a]] × W64A (N)	+1.42-/0.42-kb	1161–1162[b]	12	7	19
[4008-1 (8703 T **S**)] × W64A (N)	+1.42-/0.42-kb	1163–1165[c]	6	9	15
[4008-19 (8703 T **S**)] × W64A (N)	−1.42-/0.42-kb	1173–1174	0	16	16

[a] 'F' and S indicate partially fertile and sterile, respectively.

[b] Presence of the 1.42- and 0.42-kb transcripts were determined by RNA gel blot hybridizations. mtRNA was assayed from three partially fertile plants and two sterile plants. All partially fertile plants carried the 1.42- and 0.42- kb transcripts; one sterile plant carried these transcripts and one did not.

[c] mtRNA was assayed for three partially fertile plants and one sterile plant. All partially fertile plants carried the 1.42- and 0.42-kb transcripts; the one sterile plant did not.

TABLE 5

Independence of *rf1*-flanking RFLPs and the *Rf8*-dependent 1.42-/0.42-kb T-*urf13* transcripts

T-*urf13* transcript accumulation	No. of plants with *rf1-8703*-associated flanking RFLPs[a]	No. of plants with *rf1-W64A*-associated flanking RFLPs	Total
1.42-/0.42-kb present[b]	1	6	7
1.42-/0.42-kb absent	8	8	16
Total	9	14	23

All the progeny in this 1993 planting (rows 5401–5402) were sterile.

[a] Plants were scored for the *umc92* and *umc10 rf1*-flanking RFLPs and the presence of the 1.42-/0.42-kb T-*urf13* mitochondrial transcripts. Plants that were recombinant between the flanking markers were not included in this analyses.

[b] The accumulation of the 1.42-/0.42-kb transcripts indicates the presence of *Rf8*.

associated 1.42- and 0.42-kb and the *Rf**-associated 1.4- and 0.4-kb transcripts are CD1721 and CD1536, repectively. Hence, although we have established conditions to resolve the transcripts accumulated in the presence of *Rf8* or *Rf**, an even more reliable method to distinguish these transcripts from each other is via consecutive hybridizations with the oligonucleotides CD1721 and CD1536. The 1.42- and 0.42-kb *Rf8*-associated transcripts will hybridize to both CD1721 and CD1536, whereas the 1.4- and 0.4-kb *Rf**-associated transcripts will only hybridize to CD1536.

Figure 4 summarizes the mapping of the various *Rf*-associated T-*urf13* transcripts. The 1.42-kb transcript hybridized with each probe between CD1721 and CD564; the 0.42-kb transcript hybridized to all probes from CD1721 through the *orf221* probe T-a107. Similarly, the 1.4-kb transcript hybridized with each probe between CD1536 and CD564 and the 0.4-kb transcript hybridized to all probes from CD1536 to T-a107.

RNA gel blot hybridizations with two related mitochondrial gene probes were conducted to determine if *Rf8* or *Rf** affected other known mitochondrial transcripts. Oligonucleotide CD1656, representing nucleotides 4325–4354 of the *rrn26* gene (Table 1) was selected because it represents a portion of the progenitor sequences of T-*urf13* (DEWEY *et al.* 1986). Similarly, the T-t1 probe encompassing the *atp6* gene (KENNELL and

TABLE 6

Restorer genotypes in T-cytoplasm maize lines

Line	RNA source[a]	T-*urf13* transcripts (kb) 1.6 0.6	1.42 0.42	1.4 0.4	Restorer genotypes[b]		
R213	94 4007-1(IE)	+	–	–	*Rf1/Rf1*	*rf2/rf2*	*rf8/rf8*
B37	95 3126-1(IE)	–	–	–	*rf1/rf1*	*Rf2/Rf2*	*rf8/rf8*
Ky21	95 3042-2(IE)	+	–	–	*Rf1/Rf1*	*Rf2/Rf2*	*rf8/rf8*
Wf9-BG	95 3127-1(IE) 94 4001-13(IE)	+	–	–	*Rf1/Rf1*	*Rf2/Rf2*	*rf8/rf8*
Wf9	94 4002-6(IE)	–	–	–	*rf1/rf1*	*rf2/rf2*	*rf8/rf8*
W64A	95 3109-1(IE) 94 4004-3(IE)	–	–	–	*rf1/rf1*	*Rf2/Rf2*	*rf8/rf8*
wx1-m8	95 3124/3122 (S)	–	+	–	*rf1/rf1*	*Rf2/Rf2*	*Rf8/rf8*[c]
Line C	95 3123/3121 (S)	–	–	–	*rf1/rf1*	*Rf2/Rf2*	*rf8/rf8*
B73	92 2062-2/2072(S)	–	–	–	*rf1/rf1*	*Rf2/Rf2*	*rf8/rf8*
Mo17xB73	92 2063-4/2070(S)	–	–	–	*rf1/rf1*	*Rf2/Rf2*	*rf8/rf8*
8703	92–95 rows(IE)	–	+	–	*rf1/rf1*	*Rf2/Rf2*	*Rf8/rf8*[c]
33-16	95 3148–3150(IE) 95 4519–4522(IE)	–	–	+	*rf1/rf1*	*Rf2/Rf2*	*rf8/rf8*, *Rf*/Rf**
rf1-m7212	95 1913(IE) 95 3217–3218(IE)	–	–	+	*rf1/rf1*	*Rf2/Rf2*	*rf8/rf8*, *Rf*/rf**[c]
*Rg*1 stock no. 1	95 3128(IE) 94 6036(IE) 94 4350(IE)	+	–	+	*Rf1/rf1*	*Rf2/Rf2*	*rf8/rf8*, *Rf*/rf**[c]
Rg1 stock no. 2	94 4149(IE)	+	–	–	*Rf1/rf1*	*Rf2/Rf2*	*rf8/rf8*

[a] Year and row numbers of plants used as a source for RNA extractions are followed by IE or S, designating immature ears or 7-day-old etiolated seedlings, respectively.

[b] Unless otherwise indicated, genotypes of *Rf1* and *Rf2* are based on pedigree or testcross results. Genotypes involving *Rf1* were further confirmed by RNA gel blot analyses. Genotypes involving *Rf8* and *Rf** were inferred based on T-*urf13* transcript accumulation pattern. Unless otherwise indicated, all stocks have the genotype *rf*/rf**.

[c] Line segregates for restorer allele.

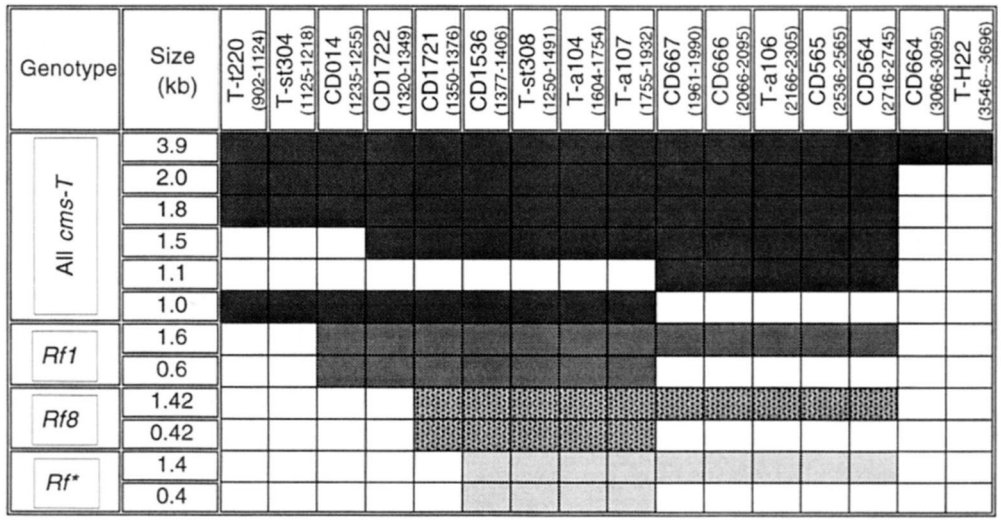

FIGURE 4.—Transcript accumulation in T-*urf13* region. The shaded area represents hybridization of T-*urf13* transcripts by the sequence probes listed across the top of the table. The 1.6- and 0.6-kb transcripts only appear in plants carrying *Rf1*. The 1.42- and 0.42-kb transcripts only appear in plants carrying *Rf8* and the 1.4- and 0.4-kb transcripts appear in plants carrying *Rf** (inbred 33-16 and plants derived from the *rf1-m7212* pedigree). The 3.9-, 2.0-, 1.8-, 1.5-, 1.0-, and 1.1-kb transcripts are present in all T-cytoplasm lines. Numbers in parentheses after each probe indicate its inclusive nucleotides according to the TURF 2H3 sequence (Table 1; DEWEY *et al.* 1986).

PRING 1989; WISE *et al.* 1996) was selected because it shares a common promoter region with T-*urf13* in the 5-kb repeated region (Figure 3). No differences in transcript accumulation were observed among different *Rf1*, *Rf8*, or *Rf** genotypes with these two probes (data not shown).

Determination of the 5′ termini of *Rf*-associated T-*urf13* transcripts by primer extension: Precise mapping of the 5′ termini of the four *Rf8*- and *Rf**-associated T-*urf13* transcripts and other transcripts from the T-*urf13* region was accomplished by primer extension analyses. Lanes 1–4 in Figure 5B contain primer extension reactions performed on mitochondrial RNAs from defined genotypes, each with known combinations of the 1.4- and 0.4-kb (*Rf**-associated) or the 1.42- and 0.42-kb (*Rf8*-associated) transcripts. A labeled extension product that corresponds to nucleotide +137 of the T-*urf13* reading frame is present in lane 2. This extension product is uniquely associated with the 5′ termini of the 1.42- and 0.42-kb transcripts and is not present in lanes 1, 3, or 4, which contain RNAs from *rf8/rf8* genotypes that do not carry these transcripts.

Lanes 3 and 4 show a labeled extension product associated with the 5′ termini of the 1.4- and 0.4-kb transcripts. This extension product corresponds to nucleotide +159 of the T-*urf13* reading frame and is not present in lanes 1 and 2, which contain RNAs from *rf*/rf** genotypes that do not carry the 1.4- and 0.4-kb transcripts. A second but weaker terminus is present at nucleotide +158. However, the appearance of this terminus varied among experiments.

These results are corroborated by the RNA gel blot hybridizations described above, which positioned the 5′ termini of the 1.42-/0.42-kb and 1.4-/0.4-kb transcripts in the vicinity of oligonucleotides CD1721 and CD1536, respectively (Figures 3 and 4). The observed minor 5′ termini may be due to complex secondary structures in the region of CD1721 and CD1536. Indeed, we did not observe this background effect in primer extension reactions targeted at termini upstream of this region, which placed 5′ termini of the *Rf1*-associated 1.6-kb transcript at nucleotides +9 and +12 of the T-*urf13* reading frame (Figure 5A). Additionally, the 5′ terminus of the 1.5-kb transcript (present in all T-cytoplasm) was positioned at nucleotide +41. Although consistent, this result differs slightly from those reported by KENNELL and PRING (1989), which positioned 5′ termini of the 1.6-kb transcript at nucleotides +10 and +14 of the T-*urf13* reading frame and the 5′ terminus of the 1.5-kb transcript at nucleotide +43.

Similarities in sequence motifs associated with fertility restoration and RNA processing in grasses: Analyses of the maize T-*urf13* sequence and the *orf107* CMS-associated mitochondrial sequence from sorghum revealed a small conserved motif in the proximity of the restorer-associated processing sites. Figure 6A shows an alignment of the 5′ termini of the *Rf1*-, *Rf8*-, and *Rf**-associated T-*urf13* mitochondrial transcripts from maize and the 5′ terminus of the *Rf3*-associated *orf107* transcript from CMS sorghum (TANG *et al.* 1996; D. R. PRING, personal communication). The conserved motif 5′-CNACNNU-3′ is centered over or overlapping these 5′ termini.

Interestingly, a sequence encompassing this motif, 5′-CCACAUUA-3′, also overlaps a processing site on the 23S precurser processing stem in the hyperthermophilic archaeon *Sulfoloboluis acidocaldarius* (FIGURE 6B; POT-

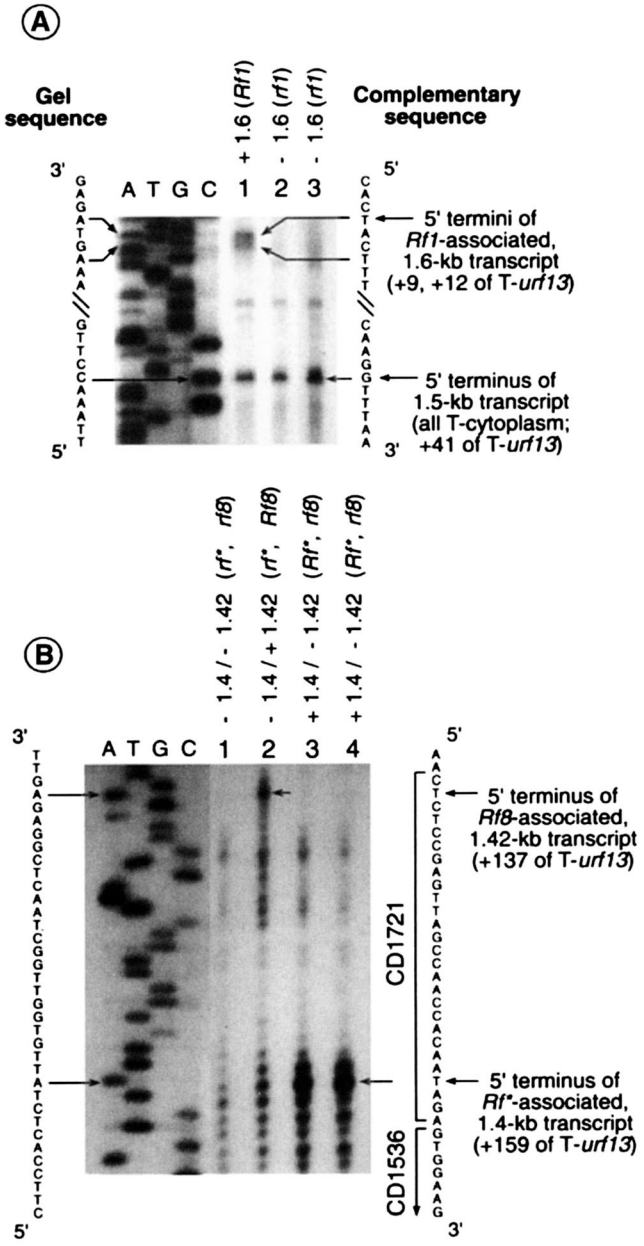

FIGURE 5.—Determination of the 5′ termini of the 1.6-kb *Rf1*-, 1.42-kb *Rf8*-, and 1.4-kb *Rf**-associated transcripts by primer extension. (A) Mitochondrial RNAs derived from plants with (lane 1) and without *Rf1* (lanes 2 and 3) were used as templates to extend from the T-*urf13*-specific oligonucleotide CD0989. The RNA in lane 1 contains the 1.6-kb *Rf1*-associated transcript, whereas RNA in lanes 2 and 3 do not. (B) Mitochondrial RNAs derived from the 8703 pedigree with (lane 2) and without (lane 1) *Rf8* were used as templates to extend from the oligonucleotide CD0380. RNA from the *rf1-m7212* pedigree (lane 3) and inbred 33-16 (lane 4) contain *Rf**. The RNA in lane 1 serves as a negative control as it did not contain either the 1.42-kb *Rf8*-associated or 1.4-kb *Rf**-associated transcripts. The RNA in lane 2 is from a partially fertile individual derived from 8703 and contains the 1.42-kb *Rf8*-associated transcript. Lanes 1 and 2 in A contain the same RNAs as lanes 1 and 2 in B. The RNA samples in lanes 3 and 4 possess the 1.4-kb *Rf**-associated transcript. The associated 5′ termini and their sequence alignment with the T-*urf13* sequence are designated by arrows.

TER *et al.* 1995). This sequence only differs from the *Rf**-associated sequence (5′-CCACAAUA-3′) at the sixth (A/U) nucleotide. Also, the maize mitochondrial *rrn26* gene also contains the motif seven and eight nucleotides upstream of the 5′ termini of its processed transcripts (MALONEY *et al.* 1989). Furthermore, the sequence of the rice mitochondrial *atp9* gene is identical to maize *rrn26* for 26 nucleotides (including the motif) eight nucleotides upstream of the 5′ terminus of the processed *atp9* transcript (KALEIKAU *et al.* 1993). In the maize *rrn26* and rice *atp9* genes, the 5′-CNACNNU-3′ motif begins 20 nucleotides 3′ of the 30 nucleotide conserved sequence block described by TANG *et al.* (1996).

The secondary structure of the T-*urf13* transcript was analyzed via the STAR RNA folding program (VAN BATENBURG *et al.* 1993; GULTYAEV *et al.* 1995) Various algorithms were used in combination with various lengths of T-*urf13* sequence flanking the *Rf1*-, *Rf8*-, and *Rf8*-associated processing sites. Figure 7 depicts the predicted structure from the "genetic" algorithm. This algorithm allows secondary structures to form and disassemble, closely following the folding pathway to reach its final structure, and thus represent the most probable secondary structure. The one common feature from these analyses is that each processing site identified in the primer extension experiments appears to be in or adjacent to an open loop, regardless of the algorithm used to predict the structure. The putative processing site predicted from the *orf107* sequence was positioned near the base of a stem loop as shown in Figure 7. However, this site is at a U weakly paired with a G. Other processing sites, such as those in the *COX*I mitochondrial gene of sorghum and maize, have been shown to be positioned at an unpaired C nucleotide at the base of a predicted stem loop. Interestingly, in the aforementioned example, the CNACNNU motif is positioned 3′ to 5′ (instead of 5′ to 3′) with the 3′ end adjacent to the processing site of *COX*I RNA (BAILEY-SERRES *et al.* 1986).

***Rf8* decreases URF13 accumulation:** Because, like *Rf1*, the action of *Rf8* alters the accumulation of T-*urf13* mitochondrial transcripts, it was possible that it also similarly decreases the accumulation of the URF13 protein. Immunoblot analyses were conducted on enriched mitochondrial proteins isolated from segregating progeny of a cross between a plant from the 8703 pedigree and the inbred line W64A. RNA and proteins were isolated from identical mitochondria preparations from greenhouse- and field-grown plants. Individual plants were genotyped by assaying for the presence (or absence) of the *Rf8*-associated 1.42- and 0.4-kb T-*urf13* transcripts. Protein was quantified by a Bradford assay and Coomassie blue staining. One hundred micrograms of protein from the immature tassel and ear from each plant were separated by SDS-PAGE and transferred to supported nitrocellulose membrane. As a loading and transfer control in these experiments, the

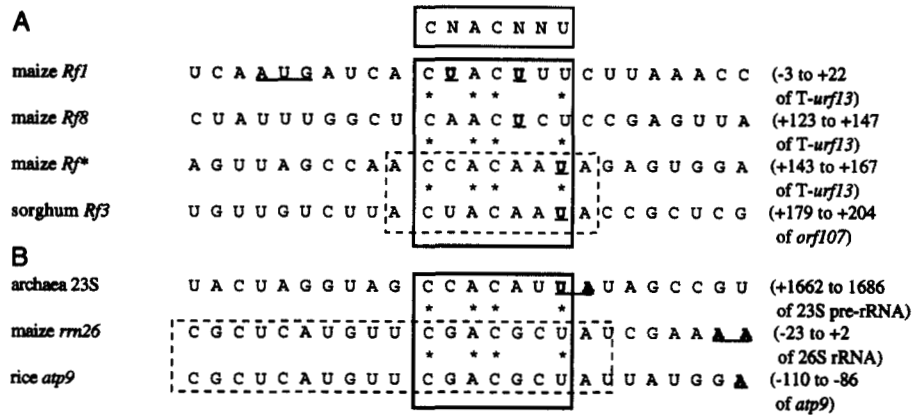

FIGURE 6.—Conserved sequence motif associated with fertility restoration and transcript processing. (A) The sequence 5′-CNACNNU-3′ overlaps the 5′ termini of the 1.6-kb (*Rf1*-associated), 1.42-kb (*Rf8*-associated), the 1.4-kb (*Rf**-associated), and the 380-nucleotide (*Rf3*-associated) transcripts from maize and sorghum. Letters underlined and in bold represent the 5′ termini of mtRNA transcripts associated with fertility restoration as determined by primer extension (present article and TANG *et al.* 1996). The translation initiation codon for T-*urf13* is underlined. (B) The line designated 23S is a partial sequence from the pre-rRNA transcript of the 23S precursor processing stem from *S. acidocaldarius* (POTTER *et al.* 1995). The boldface U and A have been shown to be *in vivo* processing sites. The last two lines represent transcripts that are processed (although not by a known nuclear allele) from the *rrn26* (MALONEY *et al.* 1989) and *atp9* (KALEIKAU *et al.* 1993) mitochondrial genes from maize and rice, respectively. Sequences enclosed in dashed boxes are highly similar or identical (see text).

transferred membrane was stained with Ponceau S prior to immunoblot analysis with an URF13 monoclonal antibody. As shown in Figure 8, plants from families segregating for *Rf1* displayed the expected decrease in URF13 as compared to *rf1*-containing plants, although some variability was observed among genotypes. Similarly, within families segregating for *Rf8*, the three progeny (ear: lanes 1, 4, and 5; tassel: lanes 6, 9, and 10) that carried the *Rf8* allele accumulated less of the URF13 protein than the two siblings (ear: lanes 2 and 3; tassel: lanes 7 and 8) that did not carry *Rf8*, consistent with the partial restoration phenotype. In all plants carrying *Rf8*, *rf8*, (and *Rf**), the accumulation of URF13 was lower in tassels than in ears (data not shown). In

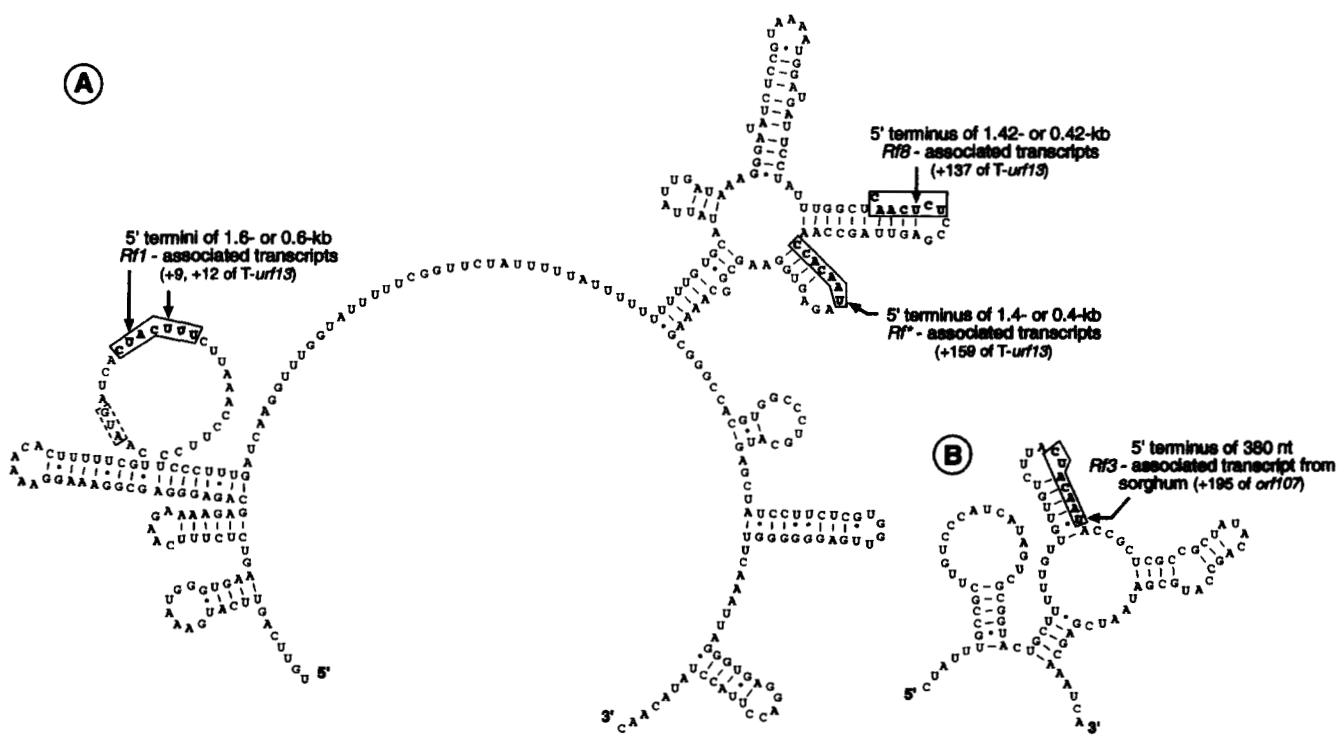

FIGURE 7.—Predicted T-*urf13* and *orf107* transcript secondary structures. RNA secondary structures were predicted with the "Genetic" algorithm of the STAR software. The 5′-CNACNNU-3′ conserved sequence motif that overlaps an RNA processing site are boxed for the *Rf1*-, *Rf8*-, and *Rf**-associated (A) and sorghum restorer-associated sites (B). The T-*urf13* AUG translation initiation codon is boxed by a dashed line (A).

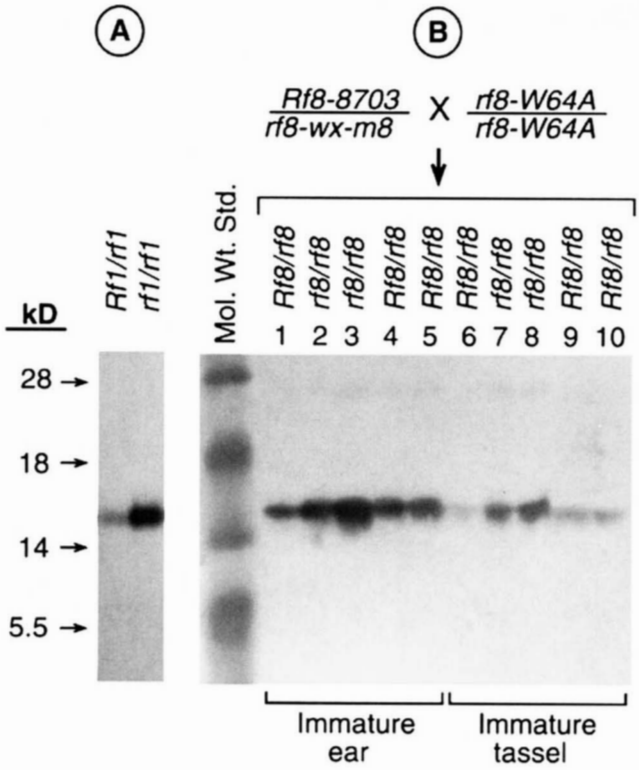

FIGURE 8.—URF13 accumulation in progeny segregating for *Rf8*. The accumulation of URF13 is reduced in plants carrying an *Rf1* allele as shown in A, lane 1. (B) Lanes 1–5 contain equal amounts of mitochondrial protein isolated from immature ears from individual progeny segregating for *Rf8*. Lanes 6–10 contain equivalent amounts of protein isolated from immature tassel from the same plants. Analysis of segregating progeny reveal a decrease in the accumulation of URF13 in the presence of *Rf8*. The accumulation of URF13 in tassel is lower than in ears.

addition, the contrast in URF13 accumulation among plants segregating for *Rf8* was much more evident in tassels as compared to ears. In contrast, plants that carried *Rf1* exhibited a marked decrease of URF13 accumulation in both tassels or ears.

DISCUSSION

Historically, dominant alleles of the *rf1* and *rf2* loci have been shown to be necessary to suppress or compensate for the CMS effects of the T-*urf13* mitochondrial gene in T-cytoplasm maize. This report demonstrates that at least two other nuclear alleles also have a role in the accumulation of unique T-*urf13* transcripts and restoration of pollen fertility. The characterization of these alternate restorer alleles in conjunction with other known restorer factors has revealed a 5′-CNAC NNU-3′ conserved motif overlapping a 5′ terminal U of each of the restorer-associated transcripts. Notably, overlapping the 5′ terminal U of the *Rf**-associated transcripts and the *Rf3*-associated *orf107* transcript from CMS sorghum (TANG *et al.* 1996) is the highly conserved sequence 5′-AC(C/U)ACAAUA-3′, revealing striking similarities among restorer-associated processing sites in these two grasses (Figure 6A). This conserved motif differs from the putative recognition sequence, 5′-UU GUUG-3′ within *orf224/atp6*, 3′ of the 5′ terminus of *Rfp1*-associated transcripts in *Brassica napus* (SINGH *et al.* 1996). However, like the model for *Rfp1/Mmt* in *B. napus*, it appears that the *Rf1*, *Rf8*, and *Rf** restorers may encode functionally similar gene products that enable the modification of transcripts synthesized in T-cytoplasm lines. The length of each of these restorer-associated transcripts can be directly correlated to their individual processing sites, and all of these transcripts appear to be processing derivatives of the common 2.0-, 1.8-, or 1.0-kb transcripts (KENNELL *et al.* 1987; KENNELL and PRING 1989; WISE *et al.* 1996). Although these nuclear restorers may be functionally similar, sequences within and flanking the 5′-CNACNNU-3′ motif are not identical. This might be expected as each independent restorer mediates processing at specific site(s) within the T-*urf13* coding sequence. This again contrasts with the *Rfp1/Mmt* system in *B. napus*, where a single nuclear CMS restorer locus appears to influence transcripts from three separate mitochondrial gene regions (SINGH *et al.* 1996; JEAN *et al.* 1997).

T-*urf13* is a chimeric open reading frame comprised of portions of 3′ flanking sequences of *rrn26* (26S), some unidentified sequences, and *rrn26* coding sequence (DEWEY *et al.* 1986). RNA processing sites have been identified in the *rrn26* sequence, including two confirmed sites seven and eight nucleotides upstream of the 5′-CNACNNU-3′ motif (MALONEY *et al.* 1989). Interestingly, the 26 nucleotides (motif included) that begins five nucleotides 5′ of the maize *rrn26* processing sites exhibit 100% identity with the nucleotides in the same position from the rice *atp9* sequence as shown in Figure 6 (KALEIKAU *et al.* 1993). Additionally, 20 nucleotides upstream of the 5′-CNACNNU-3′ motif in the maize *rrn26* and rice *atp9* genes is the 30 nucleotide conserved mitochondrial sequence described by TANG *et al.* (1996). Similar motifs may play a role in the processing and maturation of mitochondrial transcripts. Yet, how or why rare nuclear (*Rf*) genes evolved to mediate recognition of distinct sites within specific transcripts remains an enigma.

Predictive analyses of the T-*urf13* RNA secondary structure places each of the restorer-associated processing sites within or adjacent to an open loop (Figure 7). Stems may provide resistance to processing endonucleases and a conserved sequence along the stems may provide potential for binding mediated by the respective restorer gene products; the predicted open loops may allow access to the T-*urf13* transcript for *Rf*-mediated processing. The finding that a pre-RNA *in vivo* processing site along the 23S precurser processing stem in archaea fits the 5′-CNACNNU-3′ motif in higher plant mitochondria presents an intriguing hypothesis regarding the origin of these processing complexes and their functions. As was predicted for the *Rf1*-, *Rf8*-, and *Rf**-associated processing sites, the *in vivo* processing

site in archaea is located within a non-base-paired bulge in the 23S precursor processing stem (POTTER *et al.* 1995). The pre-RNA processing endonuclease in archaea does not appear to be a derived eukaryotic function but may predate the divergence of archaea and eukaryotes (POTTER *et al.* 1995). Thus, it may be reasonable that the sequences from which *Rf* genes evolved encoded ribonucleoproteins involved in processing and maturation of transcripts in both prokaryotes and eukaryotes.

A molecular mechanism has yet to be established that explains the accumulation of the various *Rf*-associated T-*urf13* transcripts and the concurrent reduction of the 13-kD URF13 protein. As shown in Figures 2 and 4, mature T-*urf13* transcripts still appear to be available for translation (at least in ear tissue) in the various *Rf* genotypes. We have observed previously that an individual plant can accumulate an abundance of *Rf1*-associated, 1.6- and 0.6-kb transcripts, in addition to *Rf8*- or *Rf**-associated transcripts, with no apparent decrease in the steady-state accumulation of the mature 2.0-, 1.8-, or 1.0-kb transcripts (WISE *et al.* 1996). Therefore, the result of *Rf1* and *Rf8* function is likely posttranscriptional, *i.e.,* inhibition of translation or protein degradation, and not regulation of transcript accumulation. An excess of these processed transcripts might compete with the unprocessed transcript for translation, thereby reducing the accumulation of URF13. An example of such a competitive shut off of translation has been proposed for a plant viral RNA. Sequences in the 3′ untranslated end of the PAV barley yellow dwarf virus (BYDV-PAV) RNA are required in *cis* for translation of the 5′ end and actually inhibit translation if they are present in excess in *trans* (WANG *et al.* 1997). If 3′ *cis* sequences of T-*urf13* transcripts are necessary for translation of URF13, *Rf*-mediated production of truncated T-*urf13* transcripts would allow for the accumulation of excess 3′ sequences in *trans*, which in turn may inhibit translation of URF13.

In summary, both the *Rf8* and *Rf** restorers are nonallelic to, but can mimic, *Rf1* to partially restoring fertility to T-cytoplasm maize in an *Rf2/Rf2* background. Comparative sequence analyses of the putative RNA processing sites mediated by *Rf1*, *Rf8*, *Rf**, and the *Rf3* fertility restorer from CMS sorghum revealed the conserved sequence 5′-CNACNNU-3′. This sequence may represent a binding motif for proteins regulated or expressed by these restorer genes. Further analyses of the primary and secondary structure of these sequences may reveal additional details of the mechanism by which fertility restoration occurs.

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