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D. R. Pring, B. G. Gengenbach and R. P. Wise

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Recombination is associated with polymorphism of the mitochondrial genomes of maize and sorghum

By D. R. Pring[1,2], B. G. Gengenbach[3] and R. P. Wise[2]

[1] *USDA-ARS*, [2] *Plant Pathology Department, University of Florida, Gainesville, Florida* 32611, *U.S.A.*

[3] *Department of Agronomy and Plant Genetics, University of Minnesota, St Paul, Minnesota* 55108, *U.S.A.*

Extensive recombination events characterize higher-plant mitochondrial DNAs. Numerous recombination events resulted in the appearance of an unusual mitochondrial open reading frame, *urf*13-T, which encodes a 13 kDa polypeptide in the male-sterile T cytoplasm of maize. Maize lines with T cytoplasm are unusually susceptible to two fungal pathogens which produce host-selective toxins. Mutants derived from tissue culture expressing male fertility and toxin-insensitivity are characterized by truncation or deletion of *urf*13-T. These events result from a frame-shift associated with a tandem 5 base pair repeat, placing a premature stop codon in frame, or from a recombination event, apparently limited to tissue culture, resulting in the deletion of *urf*13-T. Neither class of mutants produces the 13 kDa gene product. Repeated sequences that participate in recombination in sorghum appear to be randomly distributed among male-fertile or male-sterile cytoplasms. Processes involved in the evolution of mitochondrial DNAs in higher plants therefore include the generation and deletion of configurations through recombination.

1. Introduction

Higher-plant mitochondrial DNAs (mtDNAs) are characterized by extensive polymorphism, as revealed by restriction endonuclease digestion. Some of the variation results from restriction-site polymorphisms; other variation may be a result of inversions, such has been extensively documented for chloroplast DNA (Palmer 1985). Sequence duplication also can contribute to polymorphism; if the resultant repeat is recombinationally active, numerous polymorphisms may result. In simple cases in higher plants, a repeat in the 218 kilobase (kb) turnip and 327 kb spinach mtDNA genomes generates two configurations: a master chromosome and the two subgenomic circles, in equimolar stoichiometry (Palmer & Shields 1984; Stern & Palmer 1986). In a more complex case, the 570 kb mtDNA genome of the maize inbred strain Wf9(N) carries six major repeats; five have been documented to participate in recombination, generating a large number of possible configurations (Lonsdale 1984; Lonsdale *et al.* 1984). We describe sequence duplications and additional recombination events associated with mutation in maize mtDNA, and variation of repeats among sorghum cytoplasms.

2. Mutations induced by tissue culture in T-cytoplasm maize

Cytoplasmic male sterility (CMS) of higher plants is a maternally inherited trait characterized by the inability to produce functional pollen. In maize (*Zea mays* L.) there are three major groups of male-sterile cytoplasms: C (Charrua), S (USDA) and T (Texas). These cytoplasms are distinguished by the genetics of nuclear fertility restoration (Laughnan &

Gabay-Laughnan 1983), mtDNA restriction profiles (Pring & Levings 1978), and characteristic polypeptides synthesized by isolated mitochondria (Forde *et al.* 1978; Forde & Leaver 1980). The T source of CMS was widely used for hybrid production in the U.S.A. until 1970, when epiphytotics of race T of *Cochliobolus heterostrophus* Drechsler (*Helminthosporium maydis*) (southern corn leaf blight) and *Phyllosticta maydis* Arny & Nelson (yellow corn leaf blight) were shown to be associated with this male-sterile cytoplasm (see review by Ullstrup (1972)). Both pathogens produce host-selective toxins, secondary metabolites which are virulence determinants (Comstock *et al.* 1973; Hooker *et al.* 1970; Yoder 1973). The toxins from each fungus are a family of linear β-polyketols (Danko *et al.* 1984; Kono *et al.* 1983; Suzuki *et al.* 1983), which preferentially affect T-cytoplasm mitochondria (Comstock *et al.* 1973; Miller & Koeppe 1971; Yoder 1973).

Although the expression of CMS in T-cytoplasm is stable under normal field conditions, tissue culture of T-cytoplasm maize apparently provides the appropriate conditions to induce or allow genetic changes to occur and be recovered as culture lines and in regenerated plants. Tissue-culture selection for insensitivity to *C. heterostrophus* toxin readily resulted in T-cytoplasm callus cultures that were completely insensitive to the toxin without the need for prior mutagenic treatments (Gengenbach & Green 1975). Plants regenerated from selected cultures were insensitive to the toxin, exhibited lesions typical of the resistant reaction when inoculated with the pathogen, and were often fully male-fertile (Gengenbach *et al.* 1977; Brettell *et al.* 1980; Gengenbach *et al.* 1981). Similar mutant plants also were obtained from cultures of T-cytoplasm maize in the absence of selection for toxin insensitivity. In these experiments the proportion of fertile resistant to sterile susceptible plants varied widely: 35:60 (Brettell *et al.* 1980), 8:169 (Umbeck & Gengenbach 1983), 4:88 (H. J. Jessen & B. G. Gengenbach, unpublished), and 0:67 (B. G. Gengenbach, unpublished). Although the frequency varied, possibly owing to slightly different culture conditions or to non-random sampling of cell lines, one can conclude that tissue culture *per se* is associated with the induction of mtDNA instabilities.

Examination of at least 20 independently derived mutants from the different studies has provided insight into the type of mtDNA alteration associated with the mutant phenotype. Nineteen mutant lines lacked a 6.7 kb *Xho*I fragment characteristic of the parental T-cytoplasm mtDNA. In contrast, this fragment was present in 42 regenerated plants that retained the parental male sterility and susceptibility traits. One fertile resistant mutant, designated T-4, retained the 6.7 kb *Xho*I fragment and was studied in detail.

3. Molecular events associated with mutation to toxin-insensitivity and reversion to male fertility

*urf*13-T *and the* 13 kDa *polypeptide*

Approaches to elucidating the events associated with the mutation included the construction of cosmid libraries of parental T mtDNA, the T-4 (Wise *et al.* 1987*a*, *b*) and V-3 (Fauron *et al.* 1987; Rottmann *et al.* 1987) mutants, and isolation and mapping of cosmids carrying the 6.7 kb *Xho*I fragment. Determination of the genomic configurations associated with the fragment revealed a 5 kb duplication (figure 1) (Fauron *et al.* 1987; Wise *et al.* 1987*a*, *b*) of a single copy region in Wf9(N) mtDNA (Lonsdale *et al.* 1984). The 3′ edge of the repeat is 444 base pairs (bp) from the start codon of *atp*6 (Dewey *et al.* 1985*a*, 1986) in one configuration

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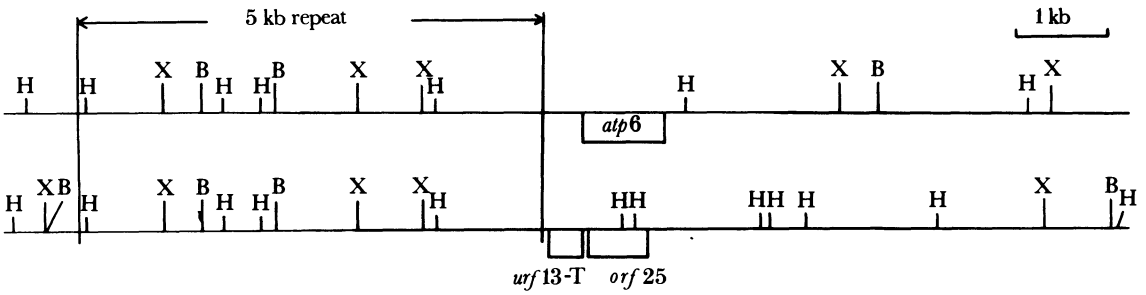

FIGURE 1. Restriction endonuclease map of the *atp*6, *urf*13-T and *orf*25 regions of maize T cytoplasm mitochondrial DNA, including extent of the 5 kb repeat flanking the genes. H, *Hin*dIII; X, *Xho*I; B, *Bam*HI.

and 69 bp from the start codon of a gene designated *urf*13-T in the configuration including the 6.7 kb *Xho*I fragment (Dewey *et al.* 1986) (figure 1).

The 5 kb repeat is active in T and T-4 mtDNA as revealed by isolation of cosmids carrying the four possible genomic configurations (Wise *et al.* 1987*b*). Analyses of the isolated 6.7 kb *Xho*I fragment revealed altered migration of an *Alu*I fragment from the T-4 mutant (Wise *et al.* 1985); sequencing of this fragment revealed a G–A transition as part of a tandem TCTCA repeat (Wise *et al.* 1986).

Dewey *et al.* (1986) isolated and sequenced a 3547 bp region including 1145 bp of the 5 kb repeat and the open reading frames *urf*13-T and *orf*25. This unusual region contained seven recombination points involving the edge of the repeat, sequences homologous to a region 3′ and internal to 26*S* rDNA, and sequences of chloroplast $tRNA^{Arg}$. Both open reading frames are chimeric: *urf*13-T contains 263 bp from the region 3′ to 26*S*, with 33 mismatches, and 50 of 53 bp from the 26*S* rDNA sequence; *orf*25 contains sequences from chloroplast $tRNA^{Arg}$ at its 3′ end.

The *urf*13-T genes in A188(T) and the T-4 mutant were sequenced (Wise *et al.* 1987*b*); the tandem 5 bp repeat of T-4 was internal to *urf*13-T, resulting in a frame-shift (figure 2) and three amino acid replacements followed by a TGA stop codon in frame 4 bp from the repeat, truncating the predicted gene product at 8.3 kb. We have designated this gene *urf* 8.3-T4. Transcription of this region in the T-4 mutant was indistinguishable from parental T mtDNA by northern analyses (Wise *et al.* 1987*b*).

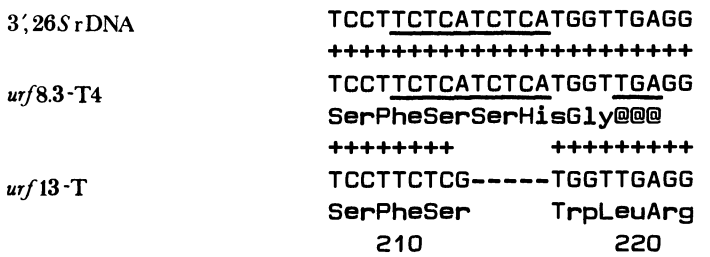

FIGURE 2. DNA sequence and translation of base pairs 205–222 of *urf*13-T, corresponding *urf* 8.3-T4 region, and progenitor sequences, 3′ to 26*S* rDNA. Tandem 5 bp repeat and premature stop codon in *urf* 8.3-T4 are underlined. Coordinates are for *urf*13-T.

Mutants which have lost the 6.7 kb *Xho*I fragment are designated deletion mutants; this class of mutants is characterized by deletion of the *urf*13-T configuration, beginning 3′ to the gene and extending at least 3 kb 5′ (Fauron *et al.* 1987; Rottmann *et al.* 1987; Wise *et al.* 1987*a*, *b*).

Sequencing of parental T and the deletion mutant V3 revealed a 127 bp repeat in T cytoplasm, which carries a 55 bp conserved core with 85% homologous flanking regions (Rottmann *et al.* 1987). This repeat begins 6 bp from the TGA stop codon of *urf*13-T and extends 56 bp into *orf* 25. In the mutants examined, recombination through this repeat is the basis for the deletion event (Rottmann *et al.* 1987), presumably by loss of the resultant subgenomic configuration. The *orf* 25 sequence was left intact in these mutants; *orf* 25 sequences are present in bean, wheat, pea and rice, and are transcribed in N, C and S maize cytoplasms (Dewey *et al.* 1986). Data to date thus implicate *urf*13-T, and not the adjacent *orf* 25, as being involved in the male-sterility and disease-susceptibility traits.

Seven recombination points contributed to the unusual configuration of the region carrying *urf*13-T and *orf* 25. An eighth recombination point can be invoked if we consider the 5′ edge of the 5 kb repeat (Wise *et al.* 1987*b*). Of these eight, six can be considered as contributing to *urf*13-T sequence and expression. Two more recombination points can be added to this myriad, considering the 127 bp repeat involved in deletion of *urf*13-T in the deletion mutants (Rottmann *et al.* 1987).

A pivotal event, probably influencing expression of *urf*13-T and *orf* 25, was the 5 kb duplication of sequences 5′ to the gene *atp*6. Examination of transcripts from clones 5′ to *urf*13-T and *atp*6 (Dewey *et al.* 1985*a*, 1986) suggests the possibility of common transcription-initiation sites within the 5 kb repeat. If so, these patterns of common transcription-initiation sites, resulting from a sequence duplication, would mimic promoters of *cox*I and *cox*III in *Oenothera* mtDNA, which are found in a 657 bp repeat 5′ to the genes (Hiesel *et al.* 1987). Recent transcriptional analyses of the regions 5′ to *atp*6 and *urf*13-T suggest such a possibility (Kennell *et al.* 1987).

A 13 kDa polypeptide is predicted from the 345 bp *urf*13-T open reading frame. Incorporation of [^{35}S]methionine by isolated maize T-cytoplasm mitochondria identified a T-specific 13 kDa polypeptide which is reduced by fertility restoration (Forde & Leaver 1980). [^{35}S]Methionine incorporation by mitochondria isolated from T, T-4 (figure 3A, C) and T-7 (a deletion mutant) revealed abundance of the 13 kDa polypeptide in T cytoplasm, which migrates at *ca.* 15 kDa in our electrophoresis system, and absence of the polypeptide in T-4 and T-7 (Wise *et al.* 1987*c*). A polypeptide of *ca.* 8 kDa was detected at decreased abundance in T-4 (figure 3B), corresponding to the predicted *urf* 8.3-T4 gene product. To determine if the 13 kDa polypeptide is a gene product of *urf*13-T, a synthetic 17 amino acid polypeptide was prepared and used to raise antibody. Immunoprecipitation of native [^{35}S]methionine incorporation products revealed co-precipitation of the 13 kDa polypeptide, and a polypeptide of *ca.* 7 kDa (figure 3D). The latter polypeptide is a major translation product, and has been identified as the DCCD binding protein (Hack & Leaver 1984), which in maize would be the product of the *atp*9 gene (Dewey *et al.* 1985*b*). Immunoprecipitation from T-7 or N cytoplasm mitochondria with the antibody did not precipitate the 7 kDa polypeptide; this result suggests the possibility that the 13 kDa polypeptide is associated with the gene product of *atp*9 in the F_0 ATPase complex.

Tandem repeats

Tandem 4 and 5 bp repeats are common in maize mtDNAs. The frequency of these events is notable in sequence comparisons of recombination products, repeats, and in translocated sequences from the chloroplast genome. In 3547 bp sequenced, including the *urf*13-T and *orf* 25

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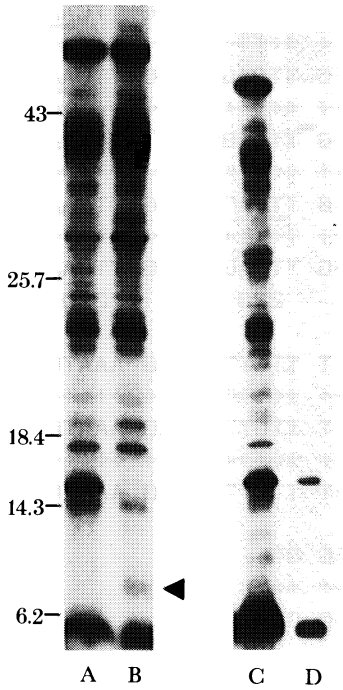

FIGURE 3. [^{35}S]Methionine incorporation by mitochondria isolated from A188(T) (A, C) and A188(T-4) (B); and (D) immunoprecipitation of A188(T) [^{35}S]methionine incorporation products by antibody to a 17 amino acid sequence internal to *urf*13-T. Arrow marks unique polypeptide of T-4. Numbers are kDa values.

regions, Dewey *et al.* (1986) identified three incidences of these small repeats in comparisons with progenitor sequences of these regions. Sequences of the chloroplast *psb*A gene found in the S1 episome (Sederoff *et al.* 1986) have been the sites of a series of tandem repeats which provided nearly all of the deviation from the sequence expected from a number of plants. The 127 bp repeat 3′ to *urf*13-T (Rottmann *et al.* 1987) includes regions 85% homologous in the two copies. Two 5 bp insertions in one copy contribute to the divergence; one insertion has indications of a repeat.

The *urf*13-T progenitor sequences include regions 3′ to 26*S* rDNA and sequence internal to 26*S* rDNA (Dewey *et al.* 1986), when compared with sequences from A188(N) mtDNA (Dale *et al.* 1984). To examine the progenitor sequences in T cytoplasm, clones derived from the region 3′ to 26*S* rDNA in T cytoplasm and the T-4 mutant were compared, including the site of the TCTCA duplication. Such a comparison might allow the discrimination of homologous recombination, gene conversion, or replication errors in the T-4 mutant. The TCTCA tandem repeat is characteristic of T or T-4, and N, 3′ to 26*S* rDNA (figure 4); 16 bp from the edge of the repeat T and T-4 carry a C, like *urf*13-T, whereas N cytoplasm has an A, extending the homology of *urf*13-T to its T progenitor one base pair further than in N. However, T and T-4 diverged from N at this point through the generation of an AAAT tandem repeat in the former; no other deviations of N and T or T-4 3′ to this region were found. If a small gene conversion event occurred during the period of tissue culture, the 3′ limit would have been within 20 bp of the TCTCA repeat. We cannot distinguish gene conversion from the equally likely possibility of misalignment during replication as an explanation for appearance of the repeat.

```
T-4,3′      CTATCCTTCT CATCTCATGG TTGAGGGGGG TTCAAATAAA TGAGGGTGAA GACCTTCCCT
            ++++++++++ ++++++++++ ++++++++++ ++++++++++ ++++++++++ ++++++++++
T,3′        CTATCCTTCT CATCTCATGG TTGAGGGGGG TTCAAATAAA TGAGGGTGAA GACCTTCCCT
            ++++++++++ ++++++++++ ++++++++++ ++ ++++      +++++++++ ++++++++++
N,3′        CTATCCTTCT CATCTCATGG TTGAGGGGGG TTAAAAT--- -GAGGGTGAA GACCTTCCCT
            ++++++++++ ++++++++++ ++++++++++ ++ ++++       +++++++  ++++++ +++
urf 8.3-T4  CTATCCTTCT CATCTCATGG TTGAGGGGGG TTCAAAT--- -TAGGGTGAG GACCTTACCT
            ++++++++++ +       +++ ++++++++++ +++++++      +++++++++ ++++++++++
urf 13-T    CTATCCTTCT CG-----TGG TTGAGGGGGG TTCAAAT--- -TAGGGTGAG GACCTTACCT
                  210               220         230             240         250

T-4,3′      ATACAACGGA AGGGTCCAAT TTGTTTGTAG AAATCGGGTG
            ++++++++++ ++++++++++ ++++++++++ ++++++++++
T,3′        ATACAACGGA AGGGTCCAAT TTGTTTGTAG AAATCGGGTG
            ++++++++++ ++++++++++ ++++++++++ ++++++++++
N,3′        ATACAACGGA AGGGTCCAAT TTGTTTGTAG AAATCGGGTG
            ++++++++++ + +     +
urf 8.3-T4  ATACAACGGA ATGAAGGAGG GG
            ++++++++++ ++++++++++ ++
urf 13-T    ATACAACGGA ATGAAGGAGG GG
                  260         270
```

FIGURE 4. DNA sequences of part of *urf*13-T (202–274 bp), *urf* 8.3-T4 and progenitor regions, sequences 3′ to 26*S* rDNA in A188(N), A188(T) and A188(T-4) mtDNA. Tandem 5 bp repeats of T-4, T and N, 3′ to 26*S* rDNA are underlined, as are tandem 4 bp repeats specific to T-4 and T. The TGA stop codon in *urf* 8.3-T4, resulting from the tandem repeat, is underlined. Coordinates are for *urf*13-T.

Heteroplasmic mitochondrial genomes

Heteroplasmic mtDNA genomes might be defined as the concurrent presence of two or more genotypically variant mitochondria, or of variant mtDNAs of a mitochondrion. Examination of restriction maps of the *urf*13-T region (Dewey *et al.* 1986; Wise *et al.* 1987*a*, *b*) identify 6.5 kb *Bam*HI and 4.5 kb *Xho*I junction fragments associated with the N-like configuration, and corresponding 9.0 kb *Bam*HI and 6.7 kb *Xho*I junction fragments in the T-specific configuration. A series of clones hybridizing to these fragments were used as probes on parental and mutant mtDNAs, which would provide an assay for subliminal parental mtDNA configurations within the mtDNA of the deletion mutants. Subliminal genomic configuration variants carrying the *atp*A gene can be identified among maize cytoplasms (Small *et al.* 1987), demonstrating heteroplasmy and suggesting this possibility for *urf*13-T in T cytoplasm. Prolonged exposure of probes representing part of the 5 kb repeat, the 3′ region of *urf*13-T, and of *orf* 25 hybridized to *Bam*HI and *Xho*I digests of two toxin-selected (R-2, R-5) and two non-selected (T-6, T-7) progeny provided no evidence of the heteroplasmic configuration. The 625 bp probe T-a43 detected the 9.0 and 6.5 kb *Bam*HI junction fragments on T and T-4, but not the 9.0 kb fragment in deletion mutants (figure 5A, B). *Xho*I digests on the same membrane revealed major hybridizing fragments of 6.7 and 4.5 kb and a minor fragment of 9.0 kb in T, and a major 4.5 kb fragment in the deletion mutants; surprisingly, residual homology at 6.7 kb was observed (figure 5C, D). The 6.7 kb fragment is internal to the 9.0 kb fragment; this result could imply that subliminal copies of the intact 6.7 kb fragment are present in the deletion mutants. However, hybridization of the 188 bp probe T-a102, carrying the 3′ region of *urf*13-T, revealed homology to the 9.0 (figure 5E) and 6.7 kb (figure 5G) fragments in T

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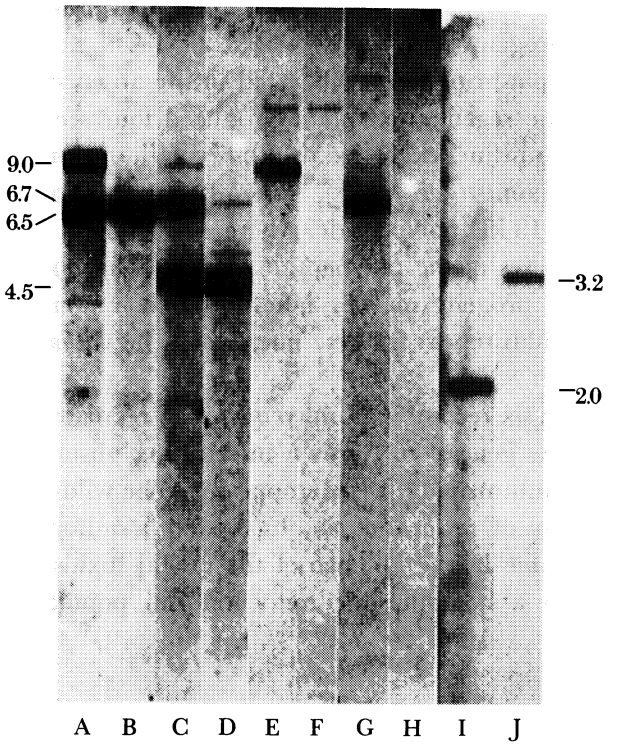

FIGURE 5. Prolonged exposure of probes which would detect heteroplasmy of parental and mutant T cytoplasm mtDNAs. (A–D) Probe T-a43, within the 5 kb repeat, hybridized to *Bam*HI (A, B) digests of T or T-4 (A) and deletion mutants (B) and to *Xho*I (C, D) digests of T or T-4 (C) and deletion mutants (D). (E–H) Probe T-a102, containing the 3′ end of *urf*13-T, hybridized to *Bam*HI (E, F) digests of T or T-4 (E) and deletion mutants (F) and to *Xho*I (G, H) digests of T or T-4 (G) and deletion mutants (H). (I, J) Probe T-a107, internal to *orf* 25, hybridized to *Hin*dIII digests of T or T-4 (I) and the T-7 deletion mutant (J). Numbers are kilobase values of hybridizing fragments; slots (I) and (J) are from a separate gel.

and T-4, with minor homology to a fragment carrying 26*S* rDNA; only the latter homology remained in the deletion mutants (figure 5E, H). We have been unable to discern the location of the subliminal homology of the T-a43 probe to the 6.7 kb *Xho*I fragment. Hybridization of the 178 bp probe T-a107 (figure 5I, J), internal to *orf* 25, revealed only a 2.0 bp *Hin*dIII junction fragment in T and T-4, and a 3.2 kb fragment, resulting from the recombination–deletion event, in the T-7 deletion mutant. These data suggest absence of the mutant configuration in parental T mtDNA. The assays, however, are not rigorous in terms of heteroplasmy at a level of perhaps 1 in 1000. In at least these four mutants, examined and selected for stability, a parental configuration, if present, must occur at a very low copy number.

There is biological evidence of instability among other mutants, including the deletion mutant T-2 (B. G. Gengenbach *et al.*, unpublished data). The instability is manifested by the segregation of sterile susceptible and fertile resistant plants from seed produced by a few plants of these mutant lines. In the T-2 segregants, the fertile resistant plants have the deletion typical of the T-2 mutation, but the sterile susceptible plants have the 6.7 kb *Xho*I fragment characteristic of parental T. The plants which produce segregants also have leaves which are partially to fully susceptible to toxin, but the tassels are partly to fully male-fertile. This suggests that the maternal plant undergoes somatic assortment of mtDNA during plant development

and that sometimes this assortment has not been completed in those cells that give rise to the ear, and subsequently to progeny seed.

Early progeny of T cytoplasm plants regenerated in the absence of toxin also have shown instability of the traits. One regenerated plant produced four seeds; when grown and self-pollinated, progeny of the four lines exhibited instability with one progeny line (32D) showing no male-sterile plants (Dixon *et al.* 1982). Line 32D also showed no synthesis of the characteristic T-cytoplasm 13 kD polypeptide, whereas progeny of lines exhibiting instability synthesized variable quantities of the polypeptide (Dixon *et al.* 1982). These results suggest that at least three of these progeny may be heteroplasmic. Examination for the diagnostic junction fragments of the 5 kb repeat in these lines would be valuable in assessing a possible heteroplasmic condition.

It seems unlikely that the six recombinations required to recreate the *urf*13-T configuration would occur during the few generations grown in selecting unstable mutants, and thus we suggest that the unstable mutants may be heteroplasmic. The wild-type and mutant genomes may exist in a population of mitochondria, or within a mitochondrion. No advantage is conferred on either genome in the absence of the fungal toxins, and somatic segregation might eventually partition a homoplasmic mitochondrial population or a homoplasmic mitochondrion.

4. Recombination in sorghum mitochondrial DNA

We are examining the distribution and roles of repeated sequences in sorghum (*Sorghum bicolor* (L.) Moench.) mtDNA, based on the T cytoplasm maize phenomena. Approximately 300 kb of the IS1112C *Sorghum bicolor* mitochondrial genome was mapped by using coding-region probes (J. E. Ferguson, A. E. Fliss, C. D. Chase & D. R. Pring, unpublished). At least five repeats and their recombination products have been detected by these procedures. These repeats and recombination points were detected by virtue of their presence within families of cosmids hybridizing to gene coding probes; each probe may provide cosmids spanning *ca.* 60 kb. Thus our detection of repeats does not *a priori* indicate that repeats are associated with gene coding regions; our approach limits detection to repeats near gene regions.

Recombination points lie very close to *atp*6, 26*S* rDNA, and 18*S* rDNA. Coding sequences for the gene *atp*6 are found on *Bam*HI junction fragments of 5.0 and 6.3 kb, but this repeat is not universally represented among sorghum cytoplasms. MtDNA of the male-sterile cytoplasm 9E (IS17218, Ghana) and male-fertile cytoplasms kafir, SC223 (IS12684C), and SC420 (IS7064C) carry only the 5.0 kb copy (figure 6*a*), whereas male-sterile cytoplasms milo, IS12662C (Ethiopia), IS1112C (India), KS37 (*Sorghum sudanense*, Sudan), KS39 (*S. niloticum*, Kenya), and male-fertile cytoplasm IS7435C (SC370, Nigeria) carry the 6.3 and 5.0 kb junction fragments (figure 6*b*).

Both 26*S* and 18*S* rDNA are flanked on one side by a repeat, or the two rDNAs, separated by *ca.* 6 kb, are located on a repeat. *Bam*HI junction fragments of 10 and 12.8 kb are found in five male-fertile (two kafir lines, SC370, SC223, and SC420) and six male-sterile (milo, IS12662C, IS1112C, 9E, KS37 and KS39) cytoplasms examined (figure 6*c*, *d*). Four of the six male sterile cytoplasms (milo, IS12662C, KS37 and KS39) carry an additional hybridizing fragment of 16 kb (figure 6E). Interestingly, two of the seven cytoplasms carrying only the 10 and 12.8 kb junction fragments exhibit marked variation in copy number of the two fragments

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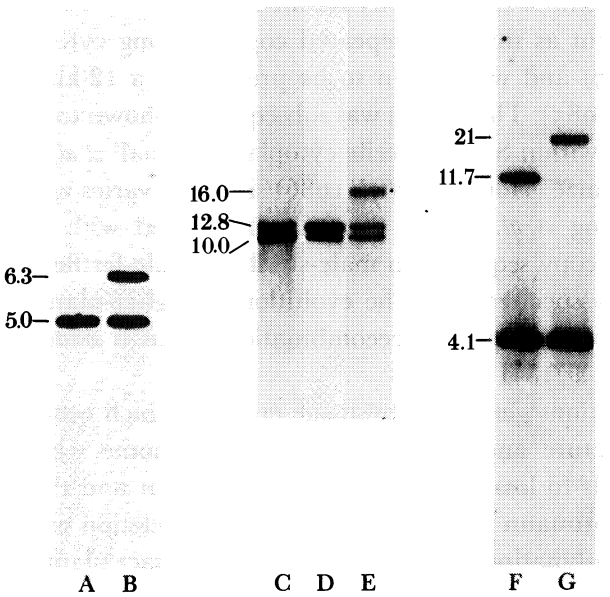

FIGURE 6. Distribution of repeated mtDNA sequences among sorghum cytoplasms. Hybridization of *atp*6 to digests of (A) 9E (IS17218), kafir, SC223 (IS12684C), or SC420 (IS7064C), (B) milo, IS12662C, IS1112C, KS37 (*S. sudanense*), KS39 (*S. niloticum*), or SC370 (IS7435C) mtDNAs. Hybridization of a 26*S* rDNA probe to digests of seven lines, including (C) IS1112C, (D) SC223; and (E) milo, IS12662C, KS37, or KS39 mtDNAs. Hybridization of an 18*S* rDNA probe to digests of (F) IS1112C, 9E, kafir, SC370, SC223, or SC420, and (G) milo, IS12662C, KS37, or KS39 mtDNAs. All digests are *Bam*HI; numbers are kilobase values of hybridizing fragments.

(figure 6C, D); IS1112C carries an enhanced copy number of the 10 kb fragment, and SC223 exhibits a reduced copy number of the 10 kb fragment, or an enhanced 12.8 kb fragment. Densitometry indicates a fivefold variation of these fragments between the two cytoplasms; this variation suggests differential enhancement of putative subgenomic circles resulting from the recombination.

Identification of junction fragments associated with 18*S* rDNA show two patterns which aligned with the 26*S* rDNA patterns. All cytoplasms examined which carried only the 10 and 12.8 kb *Bam*HI 26*S* rDNA junction fragments carried 4.1 and 11.7 kb *Bam*HI 18*S* rDNA junction fragments (figure 6F). Cytoplasms that carried the additional 16 kb fragment hybridizing to 26*S* rDNA had an 18*S* junction fragment of 21 kb (figure 6G). Additional minor homology in all lines was noted at 3.6 kb.

Distribution of the *atp*6-related repeat is thus unrelated to the designation of fertility or sterility of the cytoplasm. Although four of six male sterile cytoplasms carry the additional 26*S* hybridizing fragment and an altered 18*S* rDNA junction fragment, we attach no general significance to this observation in consideration of the limited number of cytoplasms examined.

5. CONCLUSIONS

Polymorphism of higher-plant mtDNA genomes within a species can in part be attributed to probable random recombination, sequence duplication and the generation of resultant recombinational configurations. Deletion events associated with recombination may result in a resultant altered configuration, conferring additional polymorphism. An element of

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randomness of these events is suggested by recent observations of repeat-associated variation. The *atp*A gene is present as single or repeated copies among cytoplasms (Braun & Levings 1985; Isaac *et al.* 1985) and was shown to be present on a 12 kb repeat in N, male-fertile cytoplasm (Isaac *et al.* 1985). This repeat was subsequently shown to vary within normal, male-fertile cytoplasms, and within S male-sterile cytoplasms (Small *et al.* 1987). The 5.2 kb repeat of normal cytoplasm maize (Houchins *et al.* 1986) similarly varies within the C group of male-sterile cytoplasms (Pring *et al.* 1987). A repeat associated with *atp*6 in sorghum may be represented as a single copy sequence in male-sterile or male-fertile cytoplasms. Clearly these events are commonly associated with the evolution of higher-plant mtDNAs, and in the T cytoplasm of maize, a plethora of such recombination events is associated with the generation and deletion of at least *urf*13-T.

The enigma of mutations generated by tissue culture, which occur at high frequency in T cytoplasm, is the structure and conformation of the genome which, in at least 19 of 20 independent cases, leads to loss of the 6.7 kb *Xho*I fragment and thus the *urf*13-T gene. The repeat identified by Rottmann *et al.* (1987), involved in deletion of *urf*13-T, apparently does not participate in recombination at a detectable level in intact plants. Similarly, the extensive homology between over 300 bp of the *urf*13-T and 26*S* rDNA regions is not associated with a high recombination frequency, in that we have not recovered these recombinants from our cosmid libraries. It is unknown how the recombination product carrying *urf*13-T is lost following this event, but one can assume that a disadvantage is conferred on the resultant configuration, resulting in the inability to maintain adequate copy number.

Why is this configuration apparently stable in intact plants, yet readily discarded in tissue culture? The T cytoplasm was used for nearly 90% of all U.S.A. hybrid maize production in the 1960s (Ullstrup 1972), yet no heritable revertants to male fertility have been identified or reported. MtDNA restriction of a series of T mutants indicated several classes of patterns (Gengenbach *et al.* 1981; Umbeck & Gengenbach 1983). Other rearrangements occurred in regenerated plants which remained male-sterile and toxin-sensitive, often including loss of the largest *Bam*HI fragments, of 23.5 kb, with resultant appearance of two hybridizing fragments of 20 and 16 kb (B. G. Gengenbach, H. J. Jessen & K. K. Storey, unpublished). Whether these events are also associated with deletion of a genomic configuration, i.e. if deletions occur in other parts of the genome, is unknown. A line carrying this rearrangement was subjected to a second passage through tissue culture; one of 37 regenerated plants regained the parental T configuration. This indicates that either the original rearrangement was reversible or that the parental T arrangement had not been eliminated from the mtDNAs. Abundant evidence indicates that tissue culture dramatically enhances rearrangement of a mtDNA genome which is stable in intact plants.

If *urf*13-T can be unambiguously shown to play a role in CMS and disease toxin sensitivity, linkage of the traits and the seemingly deleterious, non-obligate gene product implies that CMS is an active function, yet regulated by nuclear fertility restoration genes. The frequency with which *urf*13-T is deleted in callus tissue culture in the absence of toxin implies that no selective advantage is associated with the gene, and, importantly, that the unique configuration carrying the gene is particularly prone to loss through tissue-culture-enhanced recombination events. The tandem 5 bp repeat in *urf* 8.3-T4 is another indication of absence of a selection advantage for *urf*13-T; although tandem repeats are common in non-coding maize mtDNA sequences, this repeat creates a frame-shift and a premature stop codon, ostensibly precluding the resultant truncated polypeptide from a putative role in CMS and toxin-sensitivity.

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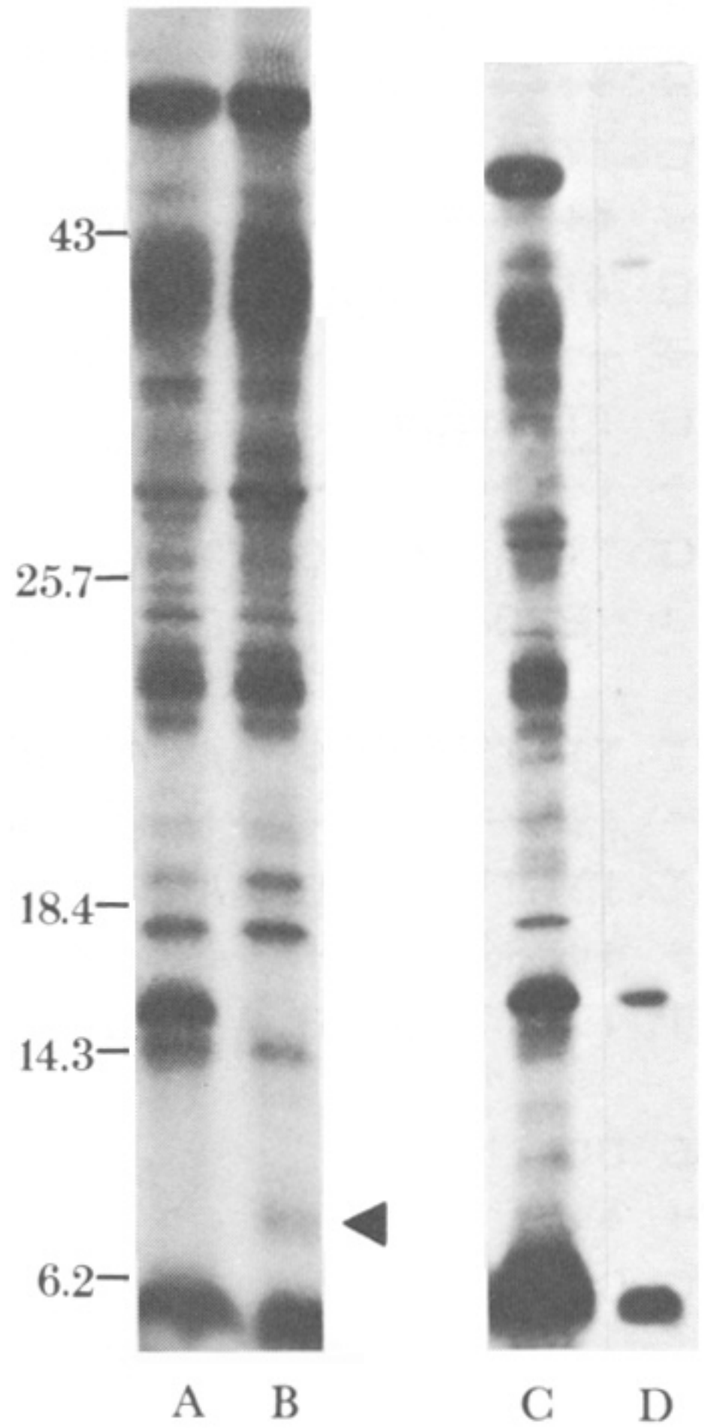

FIGURE 3. [^{35}S]Methionine incorporation by mitochondria isolated from A188(T) (A, C) and A188(T-4) (B); and (D) immunoprecipitation of A188(T) [^{35}S]methionine incorporation products by antibody to a 17 amino acid sequence internal to *urf*13-T. Arrow marks unique polypeptide of T-4. Numbers are kDa values.

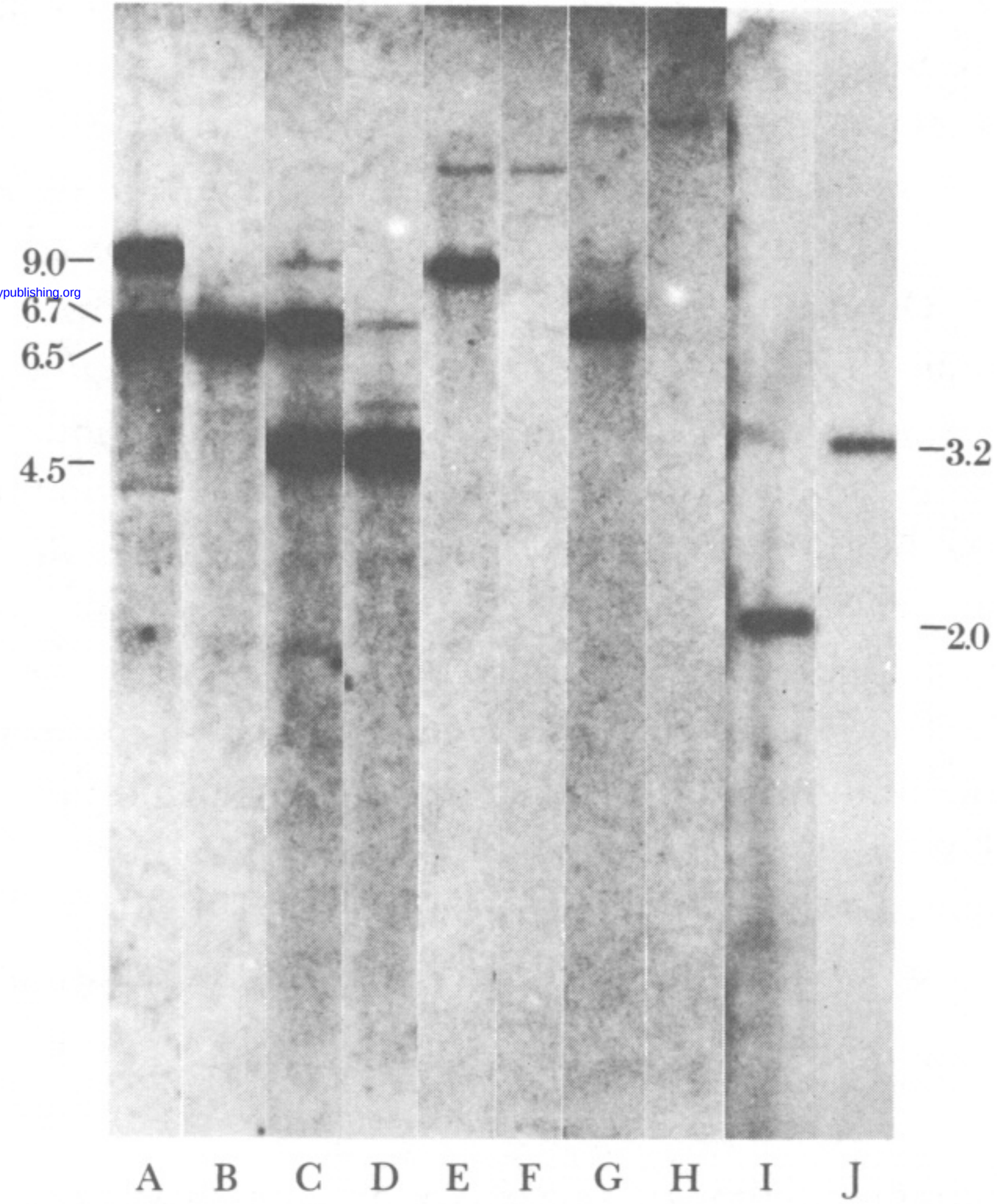

FIGURE 5. Prolonged exposure of probes which would detect heteroplasmy of parental and mutant T cytoplasm mtDNAs. (A–D) Probe T-a43, within the 5 kb repeat, hybridized to *Bam*HI (A, B) digests of T or T-4 (A) and deletion mutants (B) and to *Xho*I (C, D) digests of T or T-4 (C) and deletion mutants (D). (E–H) Probe T-a102, containing the 3′ end of *urf*13-T, hybridized to *Bam*HI (E, F) digests of T or T-4 (E) and deletion mutants (F) and to *Xho*I (G, H) digests of T or T-4 (G) and deletion mutants (H). (I, J) Probe T-a107, internal to *orf* 25, hybridized to *Hin*dIII digests of T or T-4 (I) and the T-7 deletion mutant (J). Numbers are kilobase values of hybridizing fragments; slots (I) and (J) are from a separate gel.

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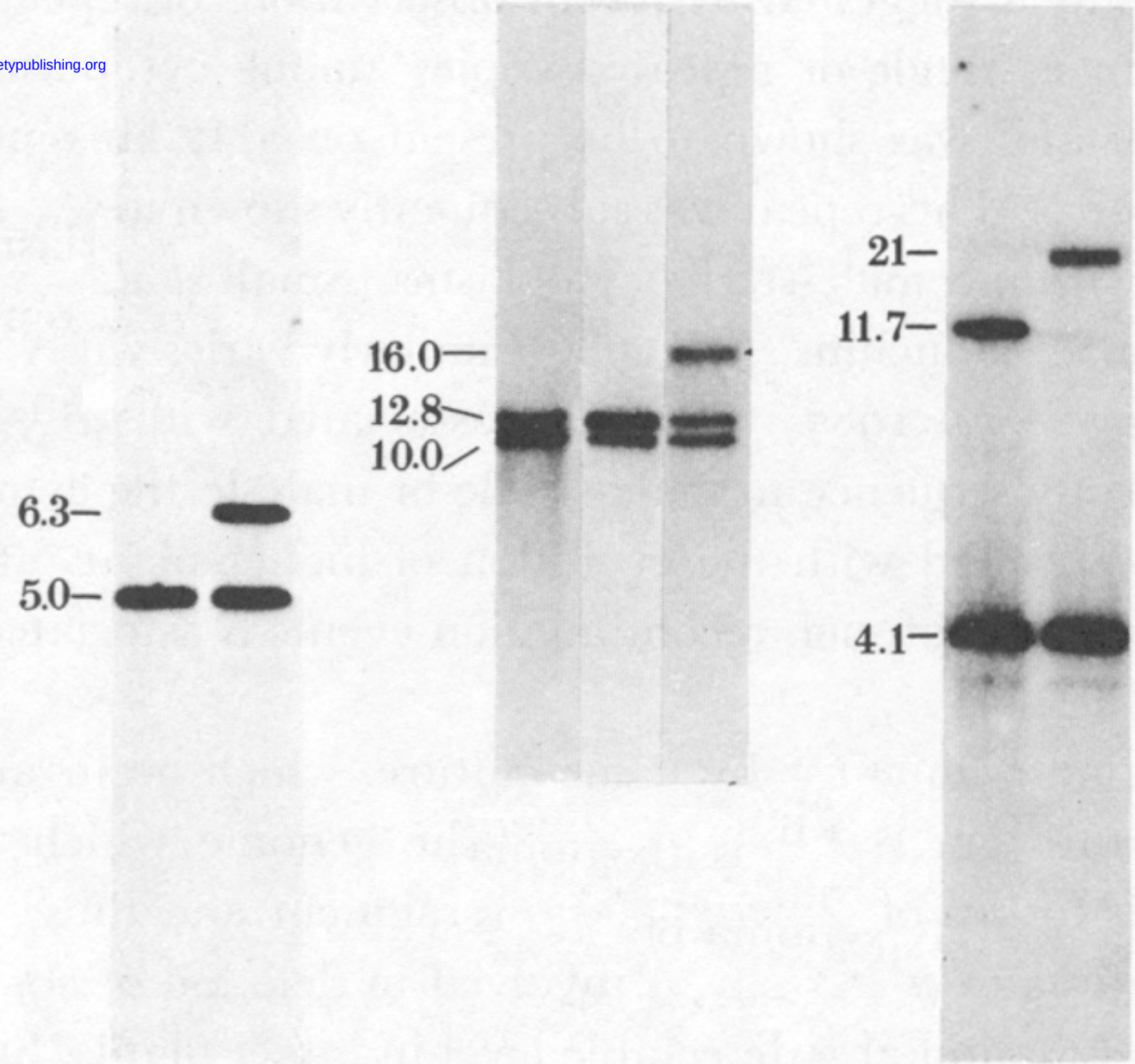

FIGURE 6. Distribution of repeated mtDNA sequences among sorghum cytoplasms. Hybridization of *atp*6 to digests of (A) 9E (IS17218), kafir, SC223 (IS12684C), or SC420 (IS7064C), (B) milo, IS12662C, IS1112C, KS37 (*S. sudanense*), KS39 (*S. niloticum*), or SC370 (IS7435C) mtDNAs. Hybridization of a 26*S* rDNA probe to digests of seven lines, including (C) IS1112C, (D) SC223; and (E) milo, IS12662C, KS37, or KS39 mtDNAs. Hybridization of an 18*S* rDNA probe to digests of (F) IS1112C, 9E, kafir, SC370, SC223, or SC420, and (G) milo, IS12662C, KS37, or KS39 mtDNAs. All digests are *Bam*HI; numbers are kilobase values of hybridizing fragments.