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Genetics

An extrachromosomal form of the *Mu* transposons of maize

(Mutator/closed circular DNA/shrunken/genome reorganization/*Zea mays*)

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ABSTRACT **Maize lines known as Robertson's Mutator (*Mu*) lines generate unstable recessive mutations at high frequencies. These lines carry actively transposing copies of the transposons (Tn) *Mu1* and *Mu1.7*. Tn*Mu1* and Tn*Mu1.7* are approximately 1400 and 1700 base pairs long, respectively, and they have 210-base-pair terminal inverted repeats. We report here extrachromosomal forms of Tn*Mu1* and Tn*Mu1.7*. The extrachromosomal *Mu1* and *Mu1.7* molecules are resistant to alkaline denaturation and to proteinase treatment and have circular restriction maps; therefore, they are probably covalently closed circular DNA. Further, we show that their occurrence is correlated with *Mu* activity, so they are probably generated during *Mu* transposition as transposition intermediates or as products of *Mu* excision. When the total extrachromosomal supercoiled DNA from immature male flowers of a *Mu* line was examined by electron microscopy, the *Mu* transposons appeared to constitute a significant fraction of the extrachromosomal DNA circles in *Mu* lines.**

The Mutator (*Mu*) system of maize was identified by Robertson in a maize line that generated recessive mutants at high frequencies (1). Molecular analysis of *Mu*-induced mutants has shown that these mutants are associated with the insertion of a 1.4-kilobase (kb) element, *Mu1* (2, 3), or a larger but homologous 1.7-kb element, *Mu1.7* (4). Transposons (Tn) *Mu1* and *Mu1.7* are found in *Mu* lines, usually at 20–30 copies and 0–5 copies per cell, respectively (for review, see ref. 5). Tn *Mu1* has been sequenced and found to have 200-base-pair (bp) terminal inverted repeats, 100-bp internal direct repeats, and four open reading frames of 300–500 bp (6). Although *Mu1* is an unusually active transposon—about 1/4 to 1/2 of the *Mu1* elements are found at new chromosomal locations in the outcross progeny (7)—little is known about the mechanism of transposition, and as yet no mRNA or transposase proteins have been identified. In an attempt to understand the mechanism of *Mu1* transposition we searched for and found extrachromosomal molecules of *Mu1* and *Mu1.7* DNA that are associated with *Mu* activity.

MATERIALS AND METHODS

Isolation and Detection of Extrachromosomal Closed Circular DNA (ccDNA). The procedure used to isolate supercoiled DNA is similar to that of Mossie *et al.* (8) except that the NaOH denaturation step is omitted. Total DNA is prepared from 5 g of tissue by the method of Murray and Thompson (9) and is resuspended in 1 M CsCl following CTAB (hexadecyl trimethylammonium bromide) precipitation. This DNA (50–100 μg; it is important not to overload the gradient) is brought to a concentration of 4.8 M CsCl and 1 mg of ethidium bromide (EtdBr) per ml in a final volume of 5 ml and centrifuged to equilibrium in a vertical Beckman VTi65.2 ultracentrifuge rotor at 60,000 rpm for 12–16 hr. Fractions of 0.25 ml are collected from the bottom of the gradient, extracted with CsCl-saturated isopropanol to remove the EtdBr, then diluted 2-fold and precipitated with ethanol (1–2 μg of tRNA having been added as a carrier for the nonchromosomal fractions). The pellets are washed with 70% ethanol, dried, and resuspended in 20 μl of 40 mM Tris, pH 8/10 mM EDTA.

For detection, 5–10 μl of DNA from each fraction is subjected to electrophoresis in a 1% agarose gel and then is transferred to a nitrocellulose filter by the method of Southern (10). The probe used is ^{32}P-labeled single-stranded RNA synthesized using SP6 polymerase: a 1.1-kb fragment of *Mu1* from a *Not* I site 0.321 kb to the end of the terminal inverted repeat (6) thus containing one inverted repeat as well as most of the internal region was cloned into a pSP65 vector (Promega Biotec, Madison, WI) carrying the SP6 promoter; labeling was accomplished as described by Melton *et al.* (11). Typically 10^8 cpm of RNA probe with a specific activity of 10^9 cpm/μg was used for each filter. Hybridization was done in 50% formamide/0.8 M NaCl/50 mM sodium phosphate, pH 6.5, at 55°C for 16 hr, and the final washes were in 0.1× standard saline citrate (SSC) (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) at 68°C. Under these conditions, we could detect up to 0.1 pg of 1-kb *Mu1* sequence. The *Escherichia coli* plasmids pπvx (800 bp; from B. Seed, Harvard University, Cambridge, MA), pπED1 (2.2 kb, including a 1.4-kb *Mu1* insert), and pπED2-1 (1.5 kb, including a 0.7-kb *Mu1* insert) were used as size markers (this laboratory). These plasmids were also used as controls for estimation of copy number.

A sample calculation is as follows: In the experiment shown in Fig. 1*C*, 100 μg of total DNA was fractionated on a gradient, and 1/3 of the DNA in each fraction was blotted and hybridized following gel electrophoresis. By the intensity of hybridization the total amount of extrachromosomal *Mu1* was estimated to be 8 pg, which is $\approx 5 \times 10^6$ molecules of 1.4-kb *Mu1*. This corresponds to 100 μg of total genomic DNA, which is derived from $\approx 8 \times 10^6$ cells, assuming 12 pg of DNA per diploid genome. Therefore, the concentration of extrachromosomal *Mu1* is estimated to be 0.6 molecules per cell.

Maize Lines. Maize plants with an active Robertson's Mutator genetic background were not inbred but had been crossed a few times to inbred line 1s2p (7). These active lines are abbreviated *Mu*;1s2p, and represent laboratory family numbers MF5407, MF83*5, and MF83*13)]. *Mu*;FkF was obtained by crossing Tn*Mu* into the Funk G4343 hybrid (Funk Seeds International, Johnston, IA). These *Mu*-active families displayed clonal striping of leaves and no modification of the *Hin*fI sites in Tn*Mu1* and Tn*Mu1.7*, which is indicative of actively transposing lines (12), and carried 20–25

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Abbreviations: *Mu*, members of a transposable family in Mutator lines; ccDNA, closed circular DNA; EtdBr, ethidium bromide; EM, electron microscopy; Tn, transposon(s).

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copies of Tn*Mu1*. Samples were either from leaves and stems of 1-month-old plants or from whole tassels in which the average sporocyte was in prophase I.

Other active *Mu* lines examined—standard *Mu* (DR84-1254) and purple aleurone *Mu* (DR79-9027×8028), and two inactive *Mu* lines (DR77-1070 and DR79-9562×8563)—were gifts from D. Robertson (Iowa State University). A third *Mu*-inactive line (VS2567) was recovered in this laboratory as an infrequent, stable progeny in an outcross of a *Mu*-induced *bz1*-mutable line to a *bz1* tester.

Electron Microscopy (EM). To prepare the ccDNA fractions for EM we extracted DNA from ≈80 g of immature tassels of *Mu*-active plants by the described procedure appropriately scaled upward. The DNA was fractionated through three successive CsCl/EtdBr gradients, and each time the fraction containing the chromosomal band material was discarded. After the final fractionation the DNA fractions were concentrated by ethanol precipitation (tRNA carrier amount had been reduced to 0.3 μg) and resuspended in 60 μl of 40 mM Tris·HCl, pH 8/10 mM EDTA. The fractions carrying *Mu1* ccDNA were identified by gel electrophoresis followed by Southern transfer and hybridization to the labeled *Mu1* probe. The concentration of *Mu1* circles in the preparation was estimated by intensity of hybridization to be $\approx 0.2 \times 10^{-6}$ μM. Samples for EM were prepared using the aqueous method of Davis *et al.* (13). Measurements of contour lengths were made from photographic prints using a Numonics model 1224 digitizer. A carbon replica grating of 2160 lines per mm was used for calibration, and 4.1-kb phage strain fd DNA was used as an internal standard.

RESULTS

Detection of an Extrachromosomal Supercoiled Form of *Mu1*. Initially, we verified that our procedure would remove enough main-band maize chromosomal DNA to detect small amounts of extrachromosomal DNA. This was accomplished by adding 10 pg of *E. coli* plasmid DNA carrying a *Mu1* insert to homogenized maize tissue from a nonmutator line in extraction buffer—and subsequently demonstrating recovery of the plasmid DNA in the ccDNA fraction. Having established adequate sensitivity of the technique, we then isolated total DNA from leaf and stem tissue from one-month-old seedlings of our *Mu*;1s2p line. The DNA was fractionated on CsCl/EtdBr gradients, and the fractions were run uncut on 1% agarose gel, blotted, and probed with the *Mu1*-specific RNA probe as described.

As shown in Fig. 1*A*, a faint band was visible in the fractions corresponding to ccDNA, as verified by the migration in these gradients of exogenously added *E. coli* plasmid DNA. Using ccDNA markers, we estimated the size of the DNA in this band at ≈1.4 kb, which suggested that it was *Mu1*. The only hybridization to the linear DNA fractions of the gradient was to the chromosomal DNA near the top of the gel. We detected no extrachromosomal linear *Mu1* DNA; we have verified in reconstruction experiments with exogenously added linear *Mu1* DNA fragments that the chromosomal DNA would not obscure detection of small linear *Mu1* DNA. Occasionally we observed molecules that appeared to be relaxed *Mu1* circles (based on gel migration rate) in the linear fractions. Because the appearance of these molecules is not reproducible, we believe that they are generated by nicking of the supercoiled *Mu1* during isolation. The intensity of hybridization of the band in the ccDNA fractions corresponds to a copy number of about 0.05–0.2 copies of extrachromosomal *Mu1* per cell using copy number controls (data not shown), based on a C-value for maize of 12 pg (14).

Presence of the Extrachromosomal *Mu1* Is Dependent on Mutator Activity. The described extrachromosomal form of *Mu1* was found at similar copy numbers in all active *Mu* lines examined; these include *Mu*;1s2p, *Mu*;FkF, standard *Mu* (Robertson), and purple aleurone *Mu* (Robertson). As might be predicted, it is not detectable in nonmutator lines—such as the 1s2p inbred line.

Mu lines have been found that can spontaneously lose *Mu* activity on outcrossing or inbreeding, a phenomenon called "*Mu*-loss" (15, 16); the plants that have lost *Mu* activity still carry *Mu1* sequences. However, these sequences are modified, presumably methylated, so that they are refractory to several restriction enzymes that normally cut *Mu1* (12, 17). We have examined three independently derived *Mu*-loss lines (see *Materials and Methods*) for the presence of extrachromosomal *Mu1* ccDNA. These *Mu*-loss lines carry ≈20 genomic copies of the *Mu1* sequence, but they show no mutator activity; their *Mu1* sequences exhibit significant modification—e.g., 25–75% of the *Mu1* sequences were resistant to cleavage by *Hin*fI, an enzyme that recognizes sites within the inverted repeats (12). Although this plant carries ≈20 *Mu1* sequences as seen by intense hybridization to the chromosomal bands near the gel top (Fig. 1*B*), no detectable hybridization exists to the ccDNA fractions of the gradient—showing that the existence of the *Mu1* sequences in the chromosome is not sufficient to generate extrachromosomal *Mu1*. We consequently conclude that the

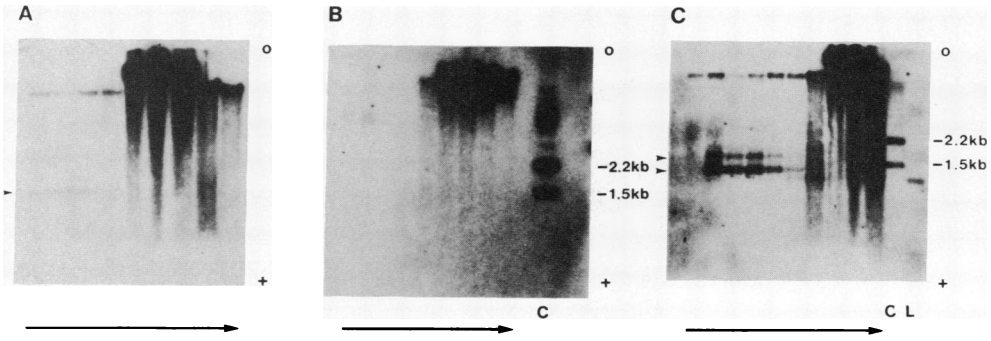

FIG. 1. Autoradiograms of Southern blots of uncut DNA fractions from CsCl/EtdBr equilibrium gradients hybridized to *Mu1*-specific, radiolabeled RNA probe. Long arrows at the bottom indicate concentrations of CsCl from high to low; ccDNA fractions are on the left, and linear DNA fractions are to the right. o, Origin of the gel electrophoresis; +, anode. (*A*) DNA from leaf and stem of a *Mu*;1s2p line (MF83*5). Arrowhead, 1.4-kb band. (*B*) DNA from leaf and stem of an inactive *Mu* line (DR77-1070). (Faint band in the ccDNA fractions at ≈4 kb corresponds to a tracer amount of exogenously added *E. coli* plasmid carrying a *Mu* insertion.) Lane C contains ccDNA size markers, including plasmids pπED1 and pπED2-1 that carry *Mu1* insertions. (*C*) DNA from meiotic tassels of *Mu*;1s2p line (MF83*5). Arrowheads, 1.4- and 1.7-kb bands. Supercoiled DNA markers at 2.2 kb and 1.5 kb in lane C are from plasmids pπED1 and pπED2-1; hybridization to pπED2-1 represents ≈0.4 pg of *Mu1* DNA. Lane L contains linear DNA size markers, including λ DNA digested with *Hin*dIII; this hybridizing band is a 1.1-kb *Mu1* fragment.

extrachromosomal *Mu1* is associated with mutator activity rather than with the presence of *Mu1* sequences.

Genetic data on the timing of *Mu* transpositional activity suggests that Tn*Mu1* is most active late in the development of the plant, in the meiotic and premeiotic stages (18). To look for *Mu1* ccDNA we prepared DNA from immature tassels (at the sporocyting stage) from *Mu*;1s2p plants. Fig. 1*C* shows that these tassels do contain *Mu1* circles. Tassels from three different *Mu*:1s2p plants gave circle/cell estimates of 0.3, 0.6, and 0.7. Three estimates from young *Mu*:1s2p leaves were 0.05, 0.1, and 0.2 circles/cell. Although we have not attempted to separate the germ cells from the bulk of somatic cells that surround them in the tassel, the greater circle copy number seen in the tassel is consistent with the genetic data. We do not detect any *Mu1* ccDNA in immature tassels from *Mu*-loss lines (data not shown).

Most *Mu* lines carry 1–4 copies of a 1.7-kb variant of Tn*Mu1*, called Tn*Mu1.7* (4, 6, 7). The higher concentration of ccDNA from immature tassels permitted the detection of a 1.7-kb molecule hybridizing to *Mu1*; thus both Tn*Mu1* and Tn*Mu1.7* exist as extrachromosomal circles.

Structure of Extrachromosomal *Mu1*. To confirm that the *Mu1*-hybridizing band was, in fact, ccDNA, the relevant fractions were subjected to proteinase K digestion and to hydrolysis with NaOH. As shown in Fig. 2*A*, the band was resistant to proteinase K digestion, which suggests that the ends were not held together by protein. The hybridizing band was also resistant to NaOH hydrolysis, which indicates that the molecule had no nicks and also contained no RNA. Limited restriction mapping of the molecules revealed a circular restriction map (Fig. 2*B*). Comparison with the restriction maps of Tn*Mu1* and Tn*Mu1.7* suggests that these molecules are circularized forms of the *Mu1* and *Mu1.7* transposons and are joined together at the ends of the inverted repeats with no major rearrangements. Within the resolution of our gels (±50 bp) no deletions or insertions appear at this junction.

To examine this junction of inverted repeats more closely, it was first necessary to test whether the ends of *Mu1* insertions between different integration events are well-defined or variable. We compared the border sequences of two different *Mu1* insertions—one into *Adh1* and the other into *sh1*. A *Mu*-induced mutation in *sh1*, *sh-9026*, was isolated by D. Robertson, and the mutant gene was cloned by B. Burr and F. Burr (personal communication). We sequenced the border region of this insertion and compared it with the corresponding sequence of the published *Mu1* insertion into *Adh1* (3). As shown in Fig. 2*C*, in both cases there is a 9-bp duplication of host DNA, and the ends of the *Mu1* element are identical. If these ends were joined (in the simplest case) blunt end-to-blunt end with no intervening host sequence, we would then generate the sequence CTCGAG, which is an *Xho* I restriction site. Therefore, we looked for this site in *Mu1* ccDNA. The fraction carrying *Mu1* ccDNA was digested first with *Bst*EII; this enzyme cuts linear *Mu1* near the center, but, in the case of *Mu1* ccDNA, it generates a linear molecule of 1.4 kb (Fig. 2*A*, lane 2). If the junction sequence were CTCGAG, then further digestion with *Xho* I would generate two approximately equal fragments. In fact, the linearized *Mu1* DNA is resistant to *Xho* I (Fig. 2*A*, lane 1), suggesting this not to be the case. However, we cannot rule out methylation of the internal cytosine in this sequence, making it resistant to cleavage by *Xho* I. To determine the junction sequence it will be necessary to clone the *Mu1* ccDNA and *Mu1.7* ccDNA.

We have examined the ccDNA fractions under the electron microscope with the hope of identifying the *Mu1* ccDNA molecules and also to see whether these fractions contained other species of circular molecules—possibly caused by other unidentified transposons in the maize genome. To do this, we had to remove as much contaminating main-band (chromosomal) DNA as possible; this removal required sequential fractionation through three CsCl equilibrium density gradient centrifugations. The final preparations appeared free of linear chromosomal DNA by hybridization to a *Mu1* probe and by EtdBr staining, but purification resulted in some nicking of the ccDNA so that preparations contained from 25% to 50% relaxed circles of *Mu1* (based on migration

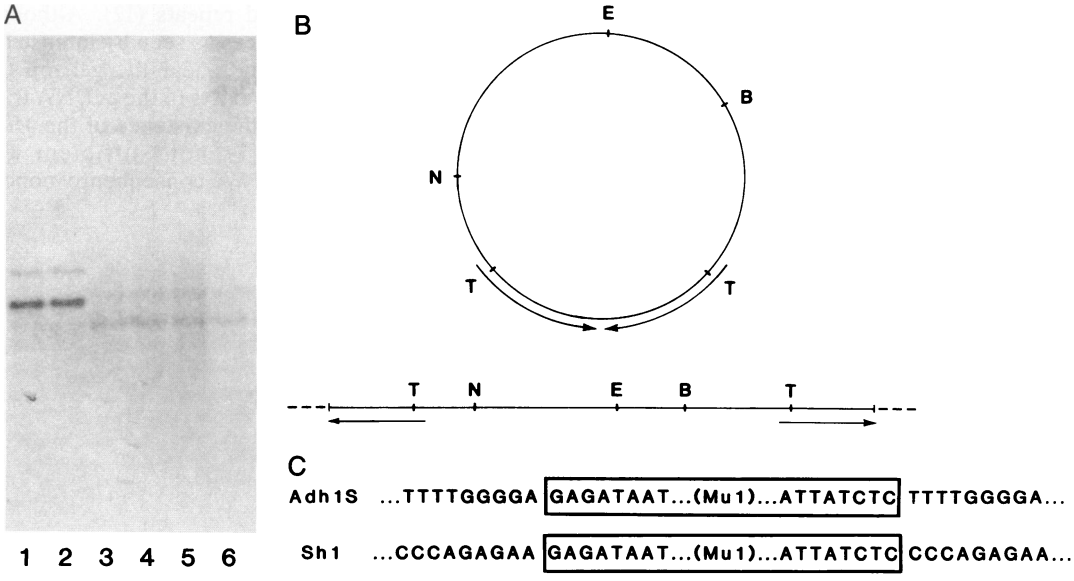

FIG. 2. Characterization of the extrachromosomal *Mu1* DNA. (*A*) Fractions containing extrachromosomal *Mu1* and *Mu1.7* DNA were treated as indicated below, then run on 1% agarose gel, blotted, and hybridized to a *Mu1* probe. Lane 1, *Bst*EII and *Xho* I digest; Lane 2, *Bst*EII digest; Lane 3, untreated control; Lane 4, proteinase K at 200 μg/ml and 37°C for 2 hr; Lane 5, 0.15 M NaOH at 20°C for 1 hr; Lane 6, 0.15 M NaOH at 68°C for 1 hr. The bands near the gel top in lanes 3–6 are caused by contaminating chromosomal cDNA. (*B*) Restriction map of *Mu1* ccDNA with the map of linear integrated *Mu1* shown below for reference. B, *Bst*NI; E, *Bst*EII; N, *Not* I; and T, *Tth*III-1. The *Not* I site is absent in *Mu1.7* ccDNA, as in genomic *Mu1.7* (4). (*C*) Sequence at the junctions of the *Mu1* insertion in *sh-9026* (*Sh1*). The site of the insertion corresponds to one of the major transcription initiation sites identified by Werr *et al.* (19). The sequence of the *Mu1* insertion in *Adh1-S* (3) is included for comparison.

in agarose gels). Preparations were concentrated so that the *Mu1* concentration was about 0.2 pM (judged by the intensity of hybridization). Only a few circular DNA molecules of any size (both relaxed and supercoiled) were observed with EM (Fig. 3 *A* and *B*). We also saw a small number of short linear DNA molecules that we presumed were circles linearized during isolation as well as several long linear molecules ranging in size from 10 kb to >30 kb (probably due to contaminating chromosomal DNA). Measurement of the contour lengths of the circles revealed that the largest size class (17/44) consisted of molecules in the 1.3- to 1.5-kb range, which is within the range of error (±10% using internal standards) for 1.4-kb *Mu1* circles (Fig. 3*C*). The frequency of these circles (≈one per square of 400-mesh grid) was of the order of magnitude predicted by the concentration of *Mu1* ccDNA in the preparation. However, the experiments do not exclude the possibility that some molecules in this size range, though coincidentally of the appropriate size, are unrelated to *Mu1*. In addition, we observed a size class (6/44) of circular molecules of 1.6–1.8 kb that might be *Mu1.7* and another size class (7/44) of 0.8- to 1.0-kb circles of unknown origin.

DISCUSSION

We have shown that active Mutator lines of maize contain a ccDNA species of 1.4 kb that appears identical to *Mu1*. In some cases, a closed circular form of *Mu1.7* could also be detected. Further, these molecules are not present in derivatives of *Mu* lines that have lost mutator activity, even though these derivative *Mu* lines carry *Mu1* sequences. Thus, the presence of these circles is correlated with active transposition. This correlation is strengthened by the observation that the circle copy number is higher in premeiotic tassels as compared with young leaves, as the genetic data would predict.

How can *Mu1* transposition generate this circular species? The *Mu1* ccDNA could be formed by a *Mu1*-specific excision reaction that acts on the ends of the integrated *Mu1* elements. *Mu1* insertions into genes can excise somatically at high

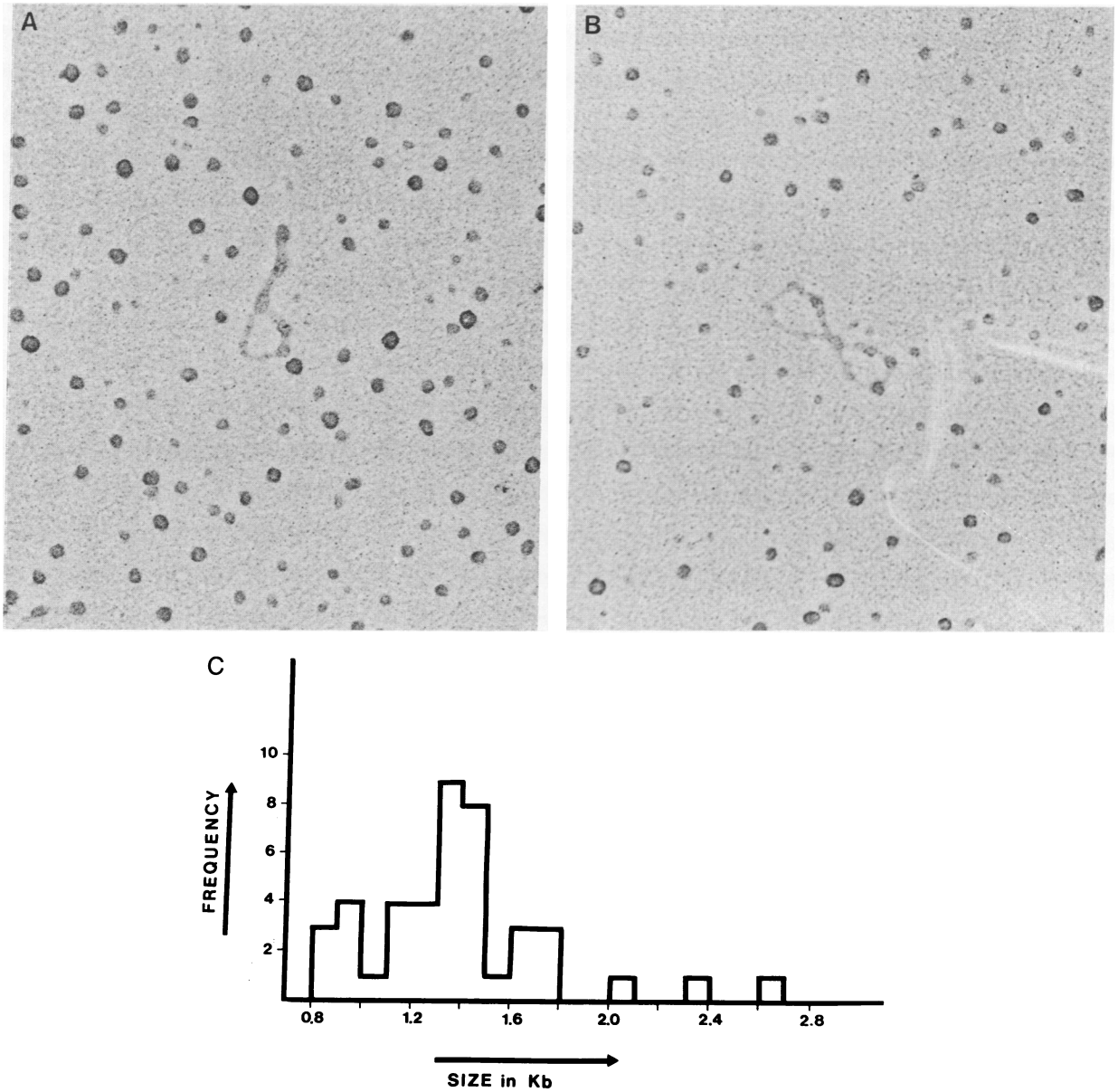

FIG. 3. Electron micrographs of purified ccDNA from an active *Mu* line showing 1.4-kb supercoiled (*A*) and relaxed (*B*) circular molecules. (*C*) Size distribution of the circular molecules in the ccDNA fraction. Each bar represents the number of individual molecules within a 0.1-kb size range.

frequencies as evidenced by spotted kernels, striped leaves, etc. (for review, see ref. 5). The second possibility is that *Mu1* circles are generated by a replicative mechanism, either from integrated *Mu1* elements, or through self-replication as a plasmid-like element. At present we cannot distinguish between these possibilities. Also, our data does not resolve whether these *Mu1* circles reintegrate into the genome—i.e., if they are intermediates in transposition. To answer this question we need to transform isolated *Mu1* circles into non-*Mu* maize lines and test for integration. At present the regeneration of transformed maize plants is not possible, but microinjection of the circles into the nuclei of maize tissue culture cells may be possible. However, the latter strategy may not be workable if *Mu* transposition is developmentally controlled, as has been suggested by Robertson (18).

The mechanism of transposition for prokaryotic transposons is relatively well understood; in most cases (except Tn*10*; ref. 20) the transposition appears to be replicative after the model of Shapiro (21). In this model the transposon always remains attached to host DNA sequences, a model consistent with the failure to detect prokaryotic transposons (except Tn*10*) free of host DNA. The transposition of the eukaryotic, *copia*-like (or retroviral-like) transposons has been studied in detail. These transposons are thought to transpose through circular intermediates following reverse transcription (22, 23). *Mu1* is unlikely to be a retroviral-like element—it lacks long terminal repeats and the terminal structure of a retroviral-like element (24), and it does not encode any protein that has homology to reverse transcriptase. Therefore, the existence of the circles is likely due to a process other than reverse transcription.

Following genetic proof that plant controlling elements can move from one chromosomal locus to another (25, 26), a model of transposition for plant transposable elements was proposed by Saedler and Nevers (27). This model involves physical movement of the transposon through a hypothetical intermediate. A supercoiled circular molecule of the nature that we have identified is a strong candidate for the proposed intermediate. In the circular form of the transposon the terminal inverted repeats are brought next to one another, and supercoiling will result in transient cruciform structures that avail the ends for the excision/integration reactions proposed by Saedler and Nevers. A preliminary search for an extrachromosomal form of *Ac* has not been successful (K. Dawe, this laboratory, unpublished results) but we note that the copy number of *Ac* is lower by factor of 20 than that of *Mu1*.

The only other nonretroviral-like transposon for which an extrachromosomal form has been identified is the transposon Tc*1* of the nematode *Caenorhabditis elegans* (28, 29). Tc*1* shares some other features with *Mu1*—it is small (1.6 kb), has terminal inverted repeats (54 bp), is found as a homogeneous, multicopy family, and appears not to encode any transposase. The extrachromosomal Tc*1* differs from *Mu1* in that 90% of Tc*1* is found as a linear species, and only 10% of it is circular; for *Mu1* we have not detected any linear extrachromosomal species. However, in view of their similarities, Tc*1* and *Mu1* might belong to a class of transposons that can be distinguished from both the retroviral-like elements such as *copia* and *Ty* and the two-element systems such as the *P* element in *Drosophila* and the controlling elements (e.g., *Ac–Ds*) in maize. If so, the common feature of an extrachromosomal form of the transposon could be a clue to a distinctly different mechanism of transposition and, perhaps, transmission.

Extrachromosomal ccDNAs have been found in *Drosophila* tissue culture cells (30) and in *Drosophila* embryos (8); these hybridize to middle-repetitive DNA sequences. Because a large fraction of the maize genome is middle-repetitive DNA, which has been considered to be at least potentially transposable (for reviews, see refs. 31 and 32) and because a *copia*-like transposon has been bound in an insertional mutant in maize (33), we questioned whether a variety of transposons or plasmids were represented in the ccDNA fractions from our gradients. Our EM analysis indicated that most of the molecules, based on size and occurrence frequency within our sample, were *Mu1*. Although it is possible that some circles seen in EM are of mitochondrial origin, the circles seen in mitochondria of male-fertile N-type maize have been reported to be 1.94 kb or larger (34, 35).

In conclusion, we report the existence of extrachromosomal forms of the *Mu* transposons in active Mutator lines. The mechanism by which these extrachromosomal transposons are generated and their connection to *Mu* excision and integration remain to be elucidated.

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