Two Independently Inherited Electrophoretic Variants of the Lysine-Rich Histones of Maize (*Zea mays*)

(inbred-line-specific polymorphism/polyacrylamide gel)

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ABSTRACT We report here the first instance of inherited intraspecific histone variation in plants. Maize lysine-rich histone I is composed of four subfractions that are electrophoretically distinct in long polyacrylamide gels. Initial segregation studies indicate that at least one subfraction shows mobility differences inherited as if controlled by a single Mendelian locus. The locus is not on the short arms of chromosomes 2 or 4. The third subfraction also shows variants. Indirect evidence indicates that the genes controlling the variants of this subfraction segregate independently of those of the first. We suggest the symbols *alh-A188* and *alh-W23* to designate two of the alleles we have discovered of the first subfraction (a) of the lysine-rich histones and *clh-A188* and *clh-W23* the alleles of the third subfraction (c) of the lysine-rich histones. Other strains of maize show other differences in their histone I fractions, suggesting that there is inbred-line-specific polymorphism with respect to histone I variants.

The meiotic chromosome mutants, *elongate* and *sticky*, do not appear to condition maize histone alterations that can be detected electrophoretically.

During a study of a maize gene that conditions sticky chromosomes at the meiotic anaphases in pollen mother cells (1), we speculated that the *sticky* (*st*) mutant conditioned an altered form of a chromosomal protein. Our investigations were directed toward the histones, specifically the lysine-rich fraction I that shows electrophoretic and sequence differences between members of even closely-related species (2-5). Apparently, more genetic variability exists among genes coding for this group of histones than among the genes for the others (II, III, and IV). Furthermore, fraction I is speculated to be a nucleohistone fiber crosslinker important in chromosome condensation (6), and so might be involved in the stickiness of chromosomes.

Our initial experiments showed that the polyacrylamide-gel band patterns of the lysine-rich histones from *st/st* plants are distinct from those of histones from a standard line of maize (inbred W23). Subsequent cytogenetic studies, however, showed that the differences were probably not due to the *st* gene but were variations characteristic of the two strains. Further comparisons of histone I gel patterns involving a wide variety of maize inbreds, relatives [*Chalco* teosinte (*Euchlaena mexicana*) and *Tripsacum dactyloides*], and one other chromosome structure mutant (*elongate*), led to our present discovery of inbred-line-specific sets of histone I patterns, each set conditioned by a different group of alleles for

the several genes coding for the histone I subfractions. At least one of these subfraction I genes is inherited as a single Mendelian unit; it is loosely linked to or independent from another subfraction I gene. This is the second demonstration of polymorphism involving variants of a histone fraction or subfraction; the first was of histone V variants in chickens (7).

MATERIALS AND METHODS

Strains. Inbred maize lines A188, A619, A632, and W23 were obtained from the University of Minnesota collection of R. L. Phillips. Lines B14, B37, C103, Oh43, Mo17, N28, and Wf9 were obtained from Clyde Black and Sons, Ames, Iowa, 50010. Chalco-type teosinte was supplied by Dr. George Beadle, University of Chicago, Illinois. *Tripsacum dactyloides* rhizomes were from Dr. Walton Galinat, University of Massachusetts Suburban Experiment Station, Waltham, and Dr. J. M. J. de Wet, University of Illinois, Urbana. The meiotic mutants *st* (chromosome 4S) and *el* were obtained from the Maize Genetics Cooperative, Urbana, Ill.

Isolation of Nuclei. The method used was essentially that of Kuehl (8). Tissues (tassels and leaves from greenhouse-grown plants) were minced with scissors in cold (4°) grinding medium (4 mM 1-octanol-3% acacia, purified according to Kuehl, -0.4 M sucrose-20 mM Tris-2 mM CaCl₂, pH adjusted to 7.6 with glacial acetic acid). About 400 ml of grinding medium was used per 100 g of tissue. Air pressure was briefly reduced over the minced immersed tissue to aid permeation, and the material was stored overnight at 4°.

Nuclei were released from cells by homogenization in a VirTis blender at full speed (45,000 rpm) for 1 min. The homogenate was filtered through cheesecloth and the residue suspended in grinding medium (100 ml/100 g of original wet-weight of tissue) and homogenized as above. The homogenate was filtered through cheesecloth and the filtrates were combined and filtered through nylon monofilament silk-screen cloth (50- μ m openings). This filtrate was centrifuged at 1000 $\times$ g for 20 min at 4°. The pellet was gently suspended in grinding medium (0.1% Triton X-100) in a smooth glass homogenizer with a loose-fitting Teflon pestle. 40 ml of medium per 100 g of original tissue was used. This suspension was centrifuged as above and the pellet suspended in grinding medium containing Triton X-100 to a nuclear concentration of no more than 5 $\times$ 10⁸ nuclei per ml. Nuclei were counted in a hemocytometer with methyl green or acetocarmine stains to differentiate nuclei from any remaining chloroplasts. Nuclei were purified by centrifugation through 2.3 M sucrose in 3 mM

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FIG. 1. Short (10-cm) polyacrylamide-gel electrophoresis comparing (a) maize total histone from inbred line Wf9 tassels and (c) total histone of chicken erythrocytes. Gel (b) is a mixture of equal parts of the two. Roman numerals designate the six major fractions of chicken-erythrocyte histones and the four fractions of maize. Maize III and IV are identified on the basis of their electrophoretic homologies with vertebrate histones III and IV, respectively (13). Maize histone III appears to be composed of two bands in 2.5 M urea gels but only one band in 6.25 M urea gels. Maize histone I was identified by amino-acid composition after it was isolated by P-10 chromatography. Maize fraction II was named by analogy to vertebrate fraction II. Electrophoresis was at 1 mA per gel for 16 hr.

CaCl₂, 10 mM Tris·HCl (pH 7.2). 19 ml of nuclear suspension was layered over 19 ml of 2.3 M sucrose in 38-ml nitrocellulose tubes; the interface was stirred; the tubes were centrifuged in a Beckman SW 27 rotor for 45 min at 17,000 rpm. The purified nuclei pelleted, and the supernatant was discarded.

Chromatin Was Isolated from purified nuclei with a modification of the method of Rizzo and Nooden (9). The pellet of nuclei was suspended in 2 mM EDTA–10 mM Tris·HCl (pH 8.0) at a concentration of 1×10^9 nuclei per 30 ml of lysing medium. This suspension, after incubation for 1 hr at 4°, was homogenized in a VirTis blender at low speed for 30 sec. The homogenate was centrifuged for 10 min at $10,000 \times g$ and the supernatant was saved. The pellet can be treated again with EDTA to obtain a second chromatin extract. The supernatant was made 10 mM in CaCl₂, mixed well, and allowed to stand overnight at 0–4°. Chromatin precipitated and was removed as a pellet by centrifugation at $2000 \times g$ for 20 min.

Preparation of Histone Sulfate. The chromatin pellet was suspended in 0.4 N H₂SO₄ and incubated 1 hr at 4°. The suspension was centrifuged at $2000 \times g$ for 10 min. The supernatant was added to 4 volumes of cold absolute ethanol and allowed to incubate 1–2 days at –20° before the precipitate of histone sulfate was collected by centrifugation at $2000 \times g$ for 15 min. The pellet was washed three times with cold ethanol and dried under reduced pressure.

Dephosphorylation of Histones. A method similar to that of Sherod *et al.* (10) was used to dephosphorylate maize histones; 1.5 units of alkaline phosphatase (*Escherichia coli*, Sigma P04252, in 10 mM Tris·HCl at pH 8.0) was added to 1 mg of histone and the solution was incubated at 32–37° for 20 hr. Incubation was stopped by dialysis of the reaction mixture against 0.9 N acetic acid. For electrophoresis, sucrose was added to the dialyzed incubation mixture to make it 20%.

Histone Fractionation. Maize histones were fractionated on a Bio-Gel P-10 column by a method adapted from Candido and Dixon (11). 100 mg of histone was dissolved in 10 ml of 0.01 N HCl and applied to the top of a 2.8-cm $\times$ 310-cm 2-section P-10 column, equilibrated with 0.01 N HCl. Elution was with 0.01 N HCl at a rate of 1.8 ml/min. 8-ml Fractions were collected after one void volume of 500 ml, and the A_{230} of each fraction was determined.

Electrophoresis. Polyacrylamide gels as formulated by Panyim and Chalkley were used (12). Initial studies were on short gels (7.5-cm long, 0.5 cm in diameter), later ones on medium length (16-cm) and long (27-cm) gels. All gels were 15% acrylamide and were 6 M in urea except where noted. Split-gel electrophoresis was carried out on gels subjected to previous electrophoresis; silicone rubber slats were wedged into the tops of the gels, forming two compartments. After testing of the compartments to make sure they were leak-proof, samples to be compared were loaded in opposite halves of the tubes. The current was turned on for 3–4 min to allow the protein to migrate into the gel a short way; the separating slats were removed; sample buffer was pipetted off the gels; and electrophoresis was resumed.

Chicken-erythrocyte histones used in comparative electrophoresis and Bio-Gel P-10 chromatography with maize histones were supplied by Dr. Elizabeth Freedlender, University of Wisconsin, Madison.

Gels were stained in amido black followed by Coomassie blue. After destaining, they were scanned in a model 2400S Gilford spectrophotometer.

Cosegregation Tests. Maize leaf and tassel tissues yield about 2 to 5×10^7 nuclei per g. This yield made it possible to isolate sufficient histone (0.5–1 mg) from individual plants at a stage when cytological analysis of pollen mother cells could be carried out. From each plant, yellow etiolated sheath leaves and part of the tassel were used for histone analysis while a portion of the tassel was fixed in Farmer's fixative (three parts 95% ethanol–one part glacial acetic acid) for subsequent cytology.

RESULTS AND DISCUSSION

1. Identification of maize histone fractions

Maize histones were compared to chicken-erythrocyte histones by split-gel electrophoresis and electrophoresis of mix-

FIG. 2. Short (7-cm) polyacrylamide split gel comparing *st* (on the upper half) and W23 (lower) histones. The 6.25 M urea gel was run at 2 mA for about 4 hr.

tures of the two. The only obvious electrophoretic homologies were for fractions III and IV (Fig. 1). Fraction III of both species undergoes oxidation to the dimer upon aeration of solutions. Its mobility then is about half that of the monomer that can be formed by reduction of the disulfide bond of the dimer with 2-mercaptoethanol. Panyim and coworkers have reported that for a wide variety of animals, and peas, fraction III is electrophoretically homologous, as is IV (13). The fraction with the lowest mobility (fraction I) is probably maize lysine-rich histone. Chromatography of maize histones on Bio-Gel P-10 in the presence of 0.01 N HCl supports this, since fraction I elutes as the first peak(s) of the chromatographic profile. Previous work by Sung and Dixon showed that histone I occurs in this first peak (20). Further confirmation comes from the amino-acid analysis of maize fraction I. Its three most abundant amino acids are lysine, alanine, and proline, in order of decreasing abundance. Histones I from other plants and animals also have as their three most abundant residues, lysine, alanine, and proline, in order of decreasing abundance (14).

The ratio of lysine to arginine for maize histone I is similar to that of pea and animal lysine-rich histone. The P-10 chromatography of maize inbred W23 histones showed two partially resolved components in the histone I region; the earlier had a ratio of lysine to arginine of 4.7. The lower ratio of lysine to arginine of this first P-10 peak, which was electrophoretically pure histone I, was perhaps due to contamination by a small amount of nonlysine-rich protein. The later component, which by electrophoresis was found to be richer in histone Ia (see below), had a ratio of lysine to arginine of 7.2. In pea and calf-thymus histone I the ratio is 8.4–8.5 (14). Histones other than I have ratios of lysine to arginine of 2.3–0.8.

2. Variations in maize histone I subfractions

Initial comparisons between histones from plants homozygous for the *sticky* mutation and inbred W23 plants revealed slight differences in histone I patterns (Fig. 2). Higher resolution was achieved on 27-cm 2.5 M urea gels run at 4° for 90 hr (Fig. 3). The electrophoretic difference between the two strains with respect to the slowest of the four subfractions (Ia) is obvious. *Sticky* Ia appears to migrate about 1.0% slower than W23 Ia. Treatment of both histones with alkaline phosphatase prior to electrophoresis does not change the relative rates of migration of the two Ia bands. These differences are thus not due to different inbred-specific levels of phosphorylation of identical Ia molecules. Furthermore, the codominant gel pattern seen in the hybrid (Fig. 3, gel c) indicates that the variant patterns are not due to any strain-specific side-chain modifications or degradation of identical molecules. The hybrid is the same in the reciprocal crosses *st* × W23 and W23 × *st*, indicating nuclear inheritance. The second-fastest subfraction (Ic) of the lysine-rich histones of *st/st* plants also differs in its electrophoretic mobility when it is compared to subfraction Ic from inbred-line W23 plants. The subfraction isolated from *st/st* plants migrates about 0.3% slower than W23 Ic. Although both variant bands of this subfraction can be seen in some gels of the hybrid, Fig. 3 does not clearly illustrate this.

3. Mode of inheritance of histone I variants

Variant Ia. An inbred-line W23 plant was crossed with a *st* homozygote to obtain an F₁ hybrid population. The F₁s were backcrossed to W23 to obtain plants of the first backcross

FIG. 3. Photograph of gels (A) and tracings of scans (B) of long (27-cm) 2.5 M urea gels comparing (a) *st*, (b) W23, (c) the hybrid *st* × W23, and (d) an equal-part mixture of *st* and W23 histones. Gels were run at 10 V/cm for 90 hr. The subfractions are designated Ia, Ib, etc., Ia being the slowest of the subfractions in a given parental pattern. All other histone fractions have run off the end of the gel.

generation. Five plants from this backcross generation were self-pollinated to yield five S₁ families. Histones were isolated from four to six individual plants from each of the five families and analyzed on short split gels. Two families were found to have histone I patterns like the W23 parent. The other three families showed segregation of histone I patterns. One of these segregating families was analyzed further. 11 Plants from this family were analyzed by short split gels by which histone I patterns identical to the W23 inbred could be differentiated from those similar to either the *st* parent or the *st* × W23 hybrid. Four plants were definitely W23 histone I homozygotes; the remaining seven were either heterozygotes or *st/st* homozygotes. These numbers are close to the 1:3 ratio expected for a segregating monofactorial character.

22 More plants of the same segregating S₁ family were grown and their histones were analyzed by long gel electrophoresis by which the histone I phenotypes of both parents and the heterozygote could be more clearly differentiated. With respect to histone Ia (Fig. 3), five plants had a W23 pattern, nine had the W23/*st* pattern, and eight had the pattern of the *sticky* parent. No patterns intermediate between these three were observed. The probability of larger than observed deviations from the expected 1:2:1 ratio for a monofactorial character is 0.40 ($\chi^2 = 1.54$, 2 df). These results indicate that the Ia subfraction is inherited as if under the control of a single gene locus.

Variant Ic. Among the same 22 plants segregating for variant Ia, there was no segregation for variant Ic; they were all apparently homozygous for the W23 alternative. The most likely explanation is that the gene loci conditioning the histone Ia and Ic variants are loosely linked or independent. Given that segregation is occurring for the Ia variants, the probabil-

Fig. 4. Approximate relative mobilities and staining intensities of fraction I histones from nine maize inbreds and *Tripsacum dactyloides* analyzed on short, 6.25 M urea, polyacrylamide gels. The relative positions were determined by split-gel electrophoresis of all combinations and by plotting the mobility of each band relative to histone IV in scans of gels of histones from each source run alone. The slowest *Tripsacum* (*Tripsa.*) band has a mobility about 0.51 that of fraction IV; the fastest moves about 0.53 as fast as IV.

ity that variant Ic will be found homozygous in its W23 form is 0.5 for unlinked loci; for closely linked loci it would be close to 0.

4. Nomenclature

At this point we propose the locus and allele designations of the genes controlling the phenotypes of the variant histone I subfractions. For the Ia variant present in W23, the allelic designation we suggest is *alh-W23* (subfraction a, lysine-rich histone, strain W23); similarly *clh-W23* will represent the variant W23 allele of subfraction c of the lysine-rich histone. The alleles coding for the Ia and Ic subfractions of the *st/st* parent will be designated *alh-A188* and *clh-A188*, since comparisons of histone I subfraction patterns of *st/st* homozygotes with those of inbred line A188 in long gels have strongly suggested an electrophoretic homology for these two subfractions between the two strains. The description of a histone allele in terms of a mutant chromosomal phenotype could be misleading, especially since the two are probably not correlated with respect to cause and effect (see section 6 below).

5. Linkage tests

The plant color gene *B* also was segregating in the same 22-plant sample analyzed above. *B* was contributed by the *st/st* parent and its recessive allele *b* by W23. A 1.75:3.50:1.75 ratio of the three histone Ia phenotypes would be expected among the seven *bb* plants if *B* and *alh-A188* (the fraction Ia allele present in the *st/st* parent) were segregating independently. The observed ratio was 1 *alh-W23/alh-W23*:3 *alh-W23/alh-A188*:3 *alh-A188/alh-A188*. The *B* class of plants segregated 4:7:4 for the same respective histone phenotypes. It thus appears that the *alh* locus is not linked to *B* on the short arm of chromosome 2. Also, as indicated below, the mutant *sticky chromosome* (chromosome 4, short arm) and the allele contributed by the *sticky* strain (*alh-A188*) evidently have a high probability of segregation; *alh* is thus not on 4S.

6. Relationship of histone Ia variants to chromosome structure

Using the three segregating *S*₁ families described in section 3, we attempted to determine whether plants having the *sticky chromosome* phenotype also were homozygous for the *alh-A188* allele.

Before outcrossing, *st* plants exhibit high pollen and egg sterility and, cytologically, show bridges of chromatin at the

two anaphases during microsporogenesis. The *st/st* stock we received from the Maize Genetics Cooperative, however, was less like the one originally described by Beadle (1) and more like *st^e*, the allele of *st* described by Schwartz (15), especially in its temperature sensitivity. When *st* is outcrossed to W23 or A188 and the F₁ hybrids self-fertilized, detection of partially pollen- and egg-sterile, putative *st/st*, plants among the F₂ is sporadic and at a frequency much lower than the expected 1 *st/st*:3 *St/-* (16). Cytological examination of some plants of both histone I phenotypes as detected on short gels (W23/-W23 histone I homozygotes with heterozygotes plus *st/st* homozygotes) was performed in order to identify the plants homozygous for the *st* chromosome gene on the short arm of chromosome 4. None of the plants showed the *sticky* phenotype. Experience with other *sticky* lines showed that expression of the *st* phenotype is variable (16). These results cannot contribute evidence for or against independence of *st* and *alh-A188*.

A progeny that was uniformly egg- and pollen-semisterile and presumably homozygous for *st* was derived from a semisterile segregant of an *S*₁ family. (No cytological examination was performed.) It was uniformly W23 in its histone Ia phenotype. Also, all progeny derived from a fertile *S*₁ plant were uniformly egg- and pollen-fertile and so were probably homozygous *St*. All the progeny were apparently homozygous for *alh-A188* with respect to histone I phenotype. These observations suggested that *alh-A188* and *st* were alleles of two loosely linked or independent loci.

While we were investigating the variants of histone I from *st/st* and W23 plants we prepared histones from 11 lines of corn belt maize, two maize relatives, and another chromosomal mutant (*elongate*), and compared the mobilities of their histone I fractions on short gels. Preliminary results showed that each maize inbred line has a distinctive histone I pattern (Fig. 4 and ref. 16). Some inbred lines are related to others; Mo17, A619, and A632 have been derived from C103, Oh43, and B14, respectively. The histone I patterns of each pair of related inbred lines are similar. This suggests that a genetic polymorphism for histone I genes exists in maize. Samples of one inbred line (Wf9) from three sources were analyzed and found to be identical in their histone I phenotypes, further indicating the heritability of this polymorphism.

Histones from plants homozygous for the meiotic mutant *elongate* (22), a Mendelian recessive conditioning the precocious decondensation of anaphase chromosomes in maize microsporocytes, were analyzed and found to have a fraction I pattern very similar to that of histones from inbred line W23.

As mentioned earlier, inbred line A188 has fraction Ia and Ic which are similar in electrophoretic mobility to those of the original *st/st* line. The alleles coding for histones Ia and Ic of the *sticky* strain are designated A188 alleles. We conclude from these observations that there are many genetically determined electrophoretic variants of histone I in maize and that the variants are not manifested in any yet apparent phenotypic manner, such as anomalies of chromosome structure.

Two maize relatives were compared to maize. Chalco teosinte, postulated to be a close relative of corn belt maize, is sympatric and freely interbreeds with maize in Latin America (23). Histones from one strain collected by Dr. G. Beadle were compared to maize histones. The lysine-rich fraction from teosinte was similar in mobility to that of histone I from A188. Histone I from *Tripsacum dactyloides* is signifi-

cantly different from that of maize. It has been proposed that *Tripsacum* is a more distant relative of maize than teosinte (23).

GENERAL COMMENT

Although a great deal of information on histone chemistry, distribution, metabolism, and histone-DNA interactions is known and has been reviewed recently (17-19), little is known of the genetic aspects of histone variability except that histones III and IV have diverged very little since the time when plants and animals had a common ancestor.

Greenaway and Murray (7) were the first to find and utilize variants within a species to elucidate the genetics of histones. Two chromatographically separable variants of histone V of nucleated chicken erythrocytes were found to be inherited in a nonsex-linked manner, since the parent hen (the heterogametic sex in birds) of a testcross had both variants (Va and Vb) and one of the six progeny hens had only one variant (Vb). Unfortunately, this experiment did not allow testing whether the histone V gene is transmitted as a single Mendelian gene.

The evidence presented here, that histone I subfraction alleles behave in genetic manipulations like Mendelian units, does not preclude the possibility that the DNA sequence coding each subfraction is highly reiterated. Genes coding for histone mRNA of sea urchin embryos are repeated 1200 times for each mRNA fraction of one species and 400 times for each fraction of another, and the genes are clustered (21). Although sequence heterogeneity for any of those 9S mRNA fractions could theoretically exist within a species and not affect the primary sequence of the histone for which it codes (due to degeneracy in the genetic code), each mRNA fraction is homogeneous in sequence. The corrective mechanism proposed by Weinberg and coworkers to maintain this homogeneity of reiterated, clustered sequences would also maintain the histone gene as a genetically monofactorially appearing unit, indistinguishable from a single, nonrepeated gene sequence in crosses such as those in the present study.

The evidence from maize that the two factors coding for histone subfractions Ia and Ic segregate independently contrasts with the observation in *Drosophila* that the genes coding for 9S mRNA are all clustered in one region of chromosome II, as detected by *in situ* hybridization (unpublished results, cited in ref. 21).

In summary, two electrophoretic variants of the histone Ia subfraction of the lysine-rich histones in maize behave as if determined by alleles of a gene. In addition, another gene determining lysine-rich subfraction Ic appears to segregate independently of the gene for Ia.

Determining the chromosomal location of the *alh* gene (genes) and examining the inheritance of variants of the three other subfractions (Ib, Ic, and Id) remain to be done.

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