Organization of Paramutagenicity in *R-stippled* Maize

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Manuscript received October 24, 1995
Accepted for publication June 5, 1995

ABSTRACT

In heterozygotes, *R-stippled* (*R-st*) reduces the pigmenting potential of sensitive *r* alleles heritably (paramutation). *R-st* is comprised of four *r* genes arranged in direct orientation. Unequal crossing over within *R-st* generates deletion products retaining from one to three *r* genes. Paramutagenic strength decreased in parallel with copy number, both among internal and distal deletions. Single-gene *R-st* derivatives were nonparamutagenic. This was so whether or not the single gene retained the transposable element (*I-R*) responsible for seed spotting. Adding back *r* genes by intragenic recombination increased paramutagenicity in proportion to total gene number. Each member of a set of overlapping deletions retained moderately strong activity, showing that no single *r* gene or intragenic region is required for paramutagenicity. Proximal and distal loss *R-st* derivatives, each retaining two *r* genes, were less paramutagenic in *trans* than the corresponding four copy *cis* combination, indicating *R-st*'s paramutagenic determinants function as a *cis*-interdependent unit in bringing about modification of a sensitive allele.

A tenet of classical genetics holds that alleles do not affect one another heritably during passage through heterozygotes. Exceptions to this rule have been observed in a number of plant species and collectively termed paramutation. In reviewing these phenomena R. A. BRINK (1973) defined paramutation as "an interaction between alleles that leads to directed, heritable change at the locus with high frequency, and sometimes invariably, within the time span of a generation". Two of the extensively studied paramutation systems involve the *r* and *b* loci of the *R* gene family in *Zea mays* (reviewed in COE 1966; BRINK *et al.* 1968; BRINK 1973). These loci encode proteins of the *myc* class of basic, helix-loop-helix, transcriptional activators (LUDWIG *et al.* 1989; GOFF *et al.* 1990; RADICELLA *et al.* 1991); alleles of each regulate anthocyanin production in vegetative, floral and seed tissues (STYLES *et al.* 1973). Although *r* and *b* share many similarities in DNA structure and protein function, paramutation phenomena at these two loci appear to differ in terms of time of occurrence and paramutant stability (reviewed in DOONER *et al.* 1991; PATTERSON and CHANDLER 1995).

Paramutation at the *r* locus is expressed as a difference in anthocyanin pigmenting action of alleles derived from homozygous strains compared with the same allele derived from certain heterozygotes. Thus seed pigmentation conferred by *R-r:standard* (*R-r*) segregating from heterozygotes with *R-stippled* (*R-st*) is markedly reduced in testcrosses on *r-g/r-g* (colorless) relative to testcrosses involving *R-r/R-r* homozygotes or *R-r/r-g* heterozygotes. Alleles sensitive to alteration, such as *R-r*, are termed paramutable; those inciting the change, such as *R-st*, are paramutagenic.

R pigmenting and paramutational components are tightly linked in that an allele's paramutational properties cosegregate with its pigmenting phenotype. Although cosegregation is the rule, exceptions occur as intragenic recombinants. For example, ASHMAN (1965a) identified intralocus crossovers involving *R-st* and *R-r*. Analysis of the recombinants for paramutagenicity (ASHMAN 1965b) and a reinvestigation of such products based on knowledge of the order of pigmenting components in *R-st* (SATYANARAYANA 1970; reviewed in BRINK 1973) showed that paramutagenicity could be fractionated and mapped. The pigmenting components of *R-st* that served as landmarks in these studies are *Sc*, *I-R*, and (Nc), ordered centromere to telomere. *Sc* is a potentially strong seed-pigmenting gene whose action is inhibited irregularly by the transposable element *I-R* to give kernel spotting (WILLIAMS *et al.* 1984). (Nc) is an incompletely penetrant, near-colorless seed phenotype (ASHMAN 1965a). In particular, the retention of paramutagenicity and (Nc) by crossovers that had lost *Sc* and *I-R* placed a region necessary for strong paramutagenicity distal to *I-R*.

Previously, MCWHIRTER and BRINK (1962) found self-colored (*R-sc*'s) alleles derived from *R-st* homozygotes by loss of *I-R* to range, seemingly continuously, from strongly paramutagenic (unchanged relative to *R-st*) to completely nonparamutagenic. Approximately one-third of such *R-sc* revertants are associated with recombination of flanking markers (KERMICLE 1970), sug-

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gesting that fractionation of paramutagenicity among *R-sc* alleles may result from displaced synapsis leading to unequal recombination at different sites within the *R-st* complex. Together, the above genetic studies indicate that multiple paramutagenic determinants are present in the *R-st* complex.

Molecular analysis of recombinant products revealed that *R-st* is comprised of four *r* genes arranged in direct orientation, *Sc, Nc1, Nc2* and *Nc3* in proximal to distal order (EGGLESTON *et al.* 1995). *R-st*'s spotting is correlated with the presence of a 3.3-kb insertion in the 3′ end of *Sc* that has been identified as the mobile element *I-R*. In the absence of this insert, *Sc* confers full-color expression (*R-sc* derivatives). The near-colorless phenotype reflects collective expression of the *Nc1, Nc2* and *Nc3* genes. Analysis of *R-sc* derivatives from *R-st* homozygotes confirmed that genetic instability of *R-st* results primarily from unequal recombination between various sites in the complex. Nearly three-fourths of the *R-sc* derivatives involved exchange between *Sc* and one of the *Nc* genes, resulting in loss of *I-R*. These alleles form a set of internal deletions of the *R-st* complex anchored at *Sc* and retaining 4, 3, 2 and 1 *r* genes.

A parallel set of distal truncations in *R-st* was generated through recombination with the displaced *r* duplication marked by *leaf color* (EGGLESTON *et al.* 1995). *Lc* maps two centimorgans distal to *r* and recombines with it, deleting the intervening region (DOONER and KERMICLE 1976). In *R-st Lc* homozygotes, unequal recombination between the *Lc* duplication and sites in the *R-st* complex were detected as losses of *Lc* phenotype. Such exchanges produced a set of distal deletions retaining one to four *r* genes.

The present report relates changes in *R-st* structure to changes in level of paramutagenic action. Sets of *R-st* deletions were evaluated for paramutagenicity directly, were recombined to increase *r* gene number then evaluated for paramutagenicity, and were also combined in *trans* for comparison with the *cis* arrangement in parental *R-st*.

MATERIALS AND METHODS

r allele stocks: Table 1 gives the designations and distinguishing phenotypic effects of the *r* alleles used in this study. *R-st, R-r, Lc* and various *r-g* accessions were collected from diverse sources and incorporated by backcrossing into the background of inbred W22. Other alleles were derived from these primary sources by mutation or intralocus recombination. The derivation and molecular organization of *r* genes in *R-st Lc* and in 41 *Lc*-loss derivatives (*R-scΔlc*'s and *R-stΔlc*'s) recovered from *R-st Lc/R-st Lc* homozygotes is described in EGGLESTON *et al.* (1995). This report also details the molecular organization of *R-st* and of 80 *R-sc* derivatives recovered from *R-st/R-st* homozygotes by ASHMAN (1960) and tested for paramutagenicity by MCWHIRTER (1961).

Separate nomenclature is used to distinguish *r* allelic complexes from the genes that make up the complexes (STADLER 1954). For example, the designation *R-r* (colored seed, red plant) denotes the phenotype of an allele class in a collective sense. In the particular *R-r* accession used here, termed *standard,* plant and seed color mutate independently and constitute separate complementation groups. These components (putative genes) have been designated *R(P)* and *R(S),* or simply *P* and *S*. Molecular analysis has clarified the status of *P* and *S* as genes. *P* is a single transcription unit whereas *S* is composed of two transcription units under the control of a common promoter (WALKER *et al.* 1995). Recombinant alleles are designated to reflect particular combinations of parental features.

Linked markers: Homozygous *golden-1* seedlings and mature plants are yellowish green. The *g1* locus lies 20 maps units proximal to *r*.

M-st is located 5.6 crossover units distal to *r*. This marker enhances *R-st* instability to give more densely spotted kernels (ASHMAN 1960).

isr designates *inhibitor of striate,* a modifier of chlorophyll striping mutations. The locus lies a fraction of a centimorgan distal to *R* and is included within the *Lc* duplication. *Isr-st,* the allele associated with *R-st,* inhibits striping more strongly than *Isr-lc,* the allele associated with *Lc*.

Synthesis of trisomic-*10* genotypes: Plants trisomic for chromosome *10* were utilized to test the combined effect of two *r* alleles, #1 and #2 for example, on an allele that is sensitive to paramutation, #3. To obtain plants of genotype 1/2/3, trisomic plants in an established trisomic-*10 R-r* strain were pollinated for two generations with strain 1 or 2, then with the other and finally with strain 3. The final crosses were either 1/1/2 × 3/3 or 1/2/2 × 3/3. All trisomic offspring in representative progenies together with a sample of the majority disomic class, respectively 1/3 and 2/3 for the two final crosses, were testcrossed on *r-g/r-g* to evaluate paramutation. Trisomic plants were identified phenotypically (BRINK 1959), and genotypes were confirmed by testcross phenotypes.

Evaluation of paramutagenicity: *r* alleles to be evaluated for paramutagenicity were made heterozygous with a sensitive allele, either *R-r* or *R-g:1,* and testcrossed using inbred W23 *r-g/r-g* as female. This inbred tester produces kernels of relatively uniform size having transparent pericarp. With the exception of *R-sc* derivatives of *R-st,* pigmentation of the resulting *r-g/R-r* or *r-g/R-g* kernels was measured with an Agtron reflectometer as before (ALLEMAN and KERMICLE 1993). Because reflectance is related inversely to pigmentation, the scale was reversed by subtracting the reading from 100 to give the relative pigmentation values reported. Although the reflectometer was calibrated using the same set of standards throughout, nonpigmented reference kernels produced in different experiments (often different years) produced relative pigmentation scores ranging from 32.3 to 39.9. Comparable scores of solid-colored reference kernels (*r-g/R-sc*) ranged from 88.6 to 96.8.

Paramutagenicity data for the 80 *R-sc* derivatives of *R-st* homozygotes studied are from MCWHIRTER (1961). In these tests, matings similar to those described above were made but aleurone pigmentation was assayed by individually comparing random samples of 100 kernels from four to 11 testcross ears against a series of six graded kernels, defining seven classes of pigmentation ranging from 1 (completely colorless) to 7 (full color).

Nucleic acid purification and analysis: Molecular analyses were performed using materials and methods as described previously (EGGLESTON *et al.* 1995).

RESULTS

Fractionation of *R-stippled* paramutagenicity by unequal crossing over with *leaf color* (*Lc*): *R-stippled* under-

TABLE 1

Component constitution, pigmenting effects, paramutagenic action and source of *r* alleles used

Allele	Genetic composition	Pigmentation: Seed (one dose aleurone)	Pigmentation: Roots and anthers	Pigmentation: Nodes and pericarp	Paramutagenicity[a]	Source/description
R-sc	*Sc* ± (Nc)[b]	Solid	−	−	N-ST	*R-st/R-st* (ASHMAN 1960; MCWHIRTER 1961; MCWHIRTER and BRINK 1962)
R-r:standard	*P q S*[c]	Mottled	+	−	PB	BRINK (1958); ROBBINS *et al.* (1991)
R-g:1	*q S*	Mottled	−	−	PB	*R-r:standard*
r-r	*P*	Colorless	+	−	N	*R-r:standard*
r-g	*p* or *s*	Colorless	−	−	N	Various accessions
Lc[d]	*Lc*	Colorless	±	+	N	*R-r:Ecuador1172 Lc*
R-st Lc[e]	*Sc I-R* (Nc) *Lc*	Spotted	±	+	ST	*R-st/R-r:Ecuador1172 Lc*
R-scΔlc	*Sc Isr-lc*	Solid	−	−	N	*R-st* Lc/*R-st Lc* (EGGLESTON *et al.* 1995)
R-stΔlc (I)	*Sc I-R Isr-lc*	Spotted	−	−	N	*R-st* Lc/*R-st Lc* (EGGLESTON *et al.* 1995)
R-stΔlc (II)	*Sc I-R* (Nc) *Isr-lc*	Spotted	−	−	W to ST	*R-st* Lc/*R-st Lc* (EGGLESTON *et al.* 1995)
R-stΔlc (III)	*Sc I-R* (Nc) *Isr-st*	Spotted	−	−	ST	*R-st Lc/R-st Lc* (EGGLESTON *et al.* 1995)
r-r:nc-v210	*P* (Nc)	Faint	+	−	W	*R-r:standard/R-st* (SATYANARAYANA 1970)
r-r:nc-v210 Lc	*P* (Nc) *Lc*	Faint	+	+	W	*r-r:nc/R-g:1 Lc* (this report)
re[f] *R-st Lc*	*Sc I-R* (Nc) *Lc*	Spotted	±	+	W to ST	*R-stΔlc (II)/r-r:nc-v210 Lc* (this report)

[a] ST, strong; W, weak; N, nonparamutagenic; PB, paramutable.

[b] Nonitalicized (Nc) designates presence or potential for near-colorless phenotype. Different combinations of the genes *Nc1*, *Nc2* and *Nc3* are possible.

[c] The *q* sequence is a 3′-truncated *P* gene that can recombine with *P* but is phenotypically null (ROBBINS *et al.* 1991). *S* is a complex of two *r*-coding regions joined head to head in an inverted duplication (WALKER *et al.* 1995).

[d] *Lc* is the *r* gene present in a displaced duplication located ~2 map units distal or *r*; absence of the duplication is designated *lc* (DOONER and KERMICLE 1976).

[e] This chromosome carries one copy of the *inhibitor of striate-2* (*isr*) locus distal to *R-st* (*Isr-st*) and another copy distal to *Lc* (*Isr-lc*).

[f] re., resynthesized.

goes unequal crossing over with the displaced duplication *Lc*, leading to the loss of *Lc* phenotype together with various components of *R-st*. Thus 41 *Lc* loss derivatives from *R-st Lc* homozygotes were classified into four phenotypic classes based on whether the transposable element *I-R* and near-colorless phenotype (Nc) were present, and on whether the allele of *inhibitor of striate* associated with *R-st* or that from *Lc* was retained (EGGLESTON *et al.* 1995). The potential of parental *R-st Lc* and these 41 derivatives to reduce the pigmenting action of *R-r* in heterozygotes is presented in Figure 1 as a deletion analysis.

The *Lc* loss derivatives were isolated as heterozygotes with nonparamutagenic *r-g*. Crosses of these 41 plants and seven parental *R-st Lc* counterparts with *R-r/R-r* produced two classes of sib plants carrying *R-r*: one heterozygous with nonparamutagenic *r-g*, the other heterozygous with *R-st Lc* or a derivative allele. Final testcrosses on *r-g/r-g* of *r-g/R-r* plants in the resulting 48 progenies gave *r-g/R-r* pigmentation values averaging from 79.8 to 84.3, all close to the 82.1 obtained from testcrosses of 11 homozygous *R-r/R-r* plants. These control values, based on reflectometer readings, compare with typical averages of 30 for nonpigmented reference kernels and 90 for full-color kernels (see MATERIALS AND METHODS). Also encompassed within the range of nonparamutagenic *r-g* controls are the seven *R-scΔlc* isolates and the sole *R-stΔlc* class (*I*) isolate, *g327*, which retained neither (Nc) nor *Isr-st*. The 29 *R-stΔlc* class (*II*) isolates, which retained (Nc) but had lost *Isr-st*, are bimodal. Four are weakly paramutagenic, with scores ranging from 68.2 to 76.5, whereas 25 are moderately to strongly paramutagenic, with scores ranging from 38.2 through 55.4. The four *R-stΔlc* class (*III*) alleles, which retained (Nc) and *Isr-st*, scored from 41.8 to 47.2, well within the range of the 25 strongly paramutagenic *R-stΔlc* (*II*) derivatives. They overlap the *R-st Lc* parental control class, but as a group are significantly less paramutagenic ($P < 0.01$).

In the above experiment a complete loss of paramutagenicity accompanied the loss of near-colorless phenotype. However, the bimodal nature of paramutagenicity of (Nc)-retaining derivatives suggests that two or more regions in (Nc) are contributing to paramutagenic strength. Since previous work indicated that *Lc* loss derivatives retaining (Nc) phenotype, *i.e.*, *R-stΔlc* classes

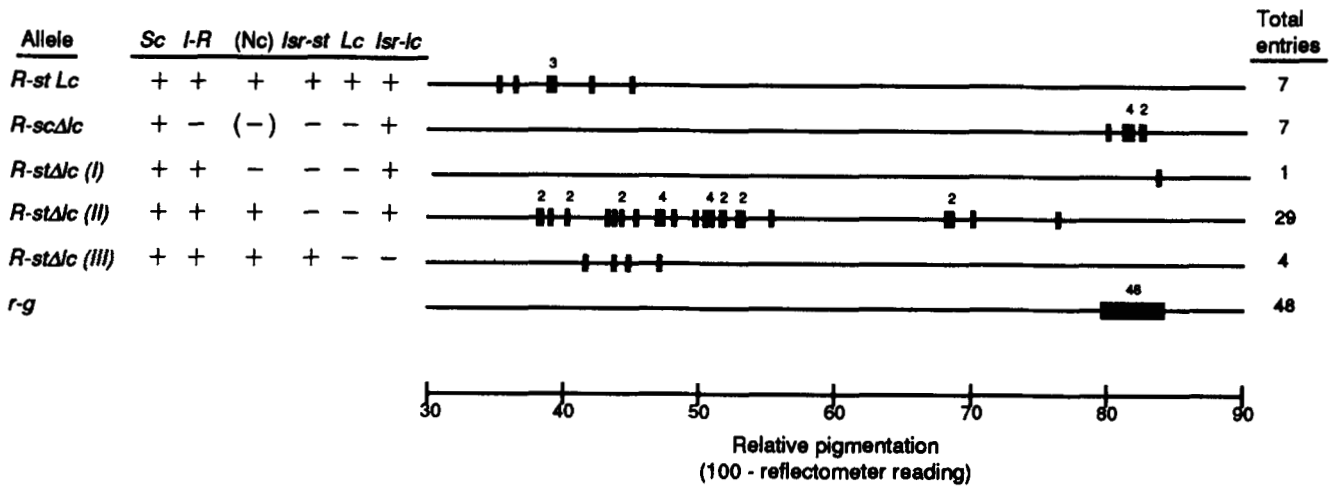

FIGURE 1.—Deletion analysis of *R-st Lc* paramutagenicity. Pigmentation of *r-g/R-r* kernels obtained from testcrosses of plants heterozygous for paramutable *R-r* and the allele class listed on the left. Each vertical line is the average of 4–15 testcross ears representing a subline of parental *R-st Lc* or one of its derivatives. Numbers above vertical lines indicate alleles having similar values. In the case of *r-g*, ■ represents the range of 48 control tests involving 5–16 plants each. An absence of (Nc) in the *R-scΔlc* class is inferred from loss of the flanking components *I-R* and *Isr:st*. For the arrangement of *R-st Lc* components relative to *r* duplicate sequences see Figure 7B.

(*II*) and (*III*), contained from one to three of the *r* genes associated with (Nc) (EGGLESTON *et al.* 1995), it was plausible that the variation in paramutagenic strength detected among (Nc)-retaining alleles could be accounted for by differences in the number of *r* genes present following unequal recombination.

To examine paramutagenic strength in relation to number of *r* genes in *R-st Lc* and its *Lc* loss derivatives, molecular data from the accompanying study was related to the paramutagenicity data by plotting the number of *r* genes in *R-st Lc* and the *Lc* loss alleles versus the relative pigmentation of *R-r* following heterozygosity with each (Figure 2). This figure shows that as *r* gene number decreases, paramutagenic strength decreased

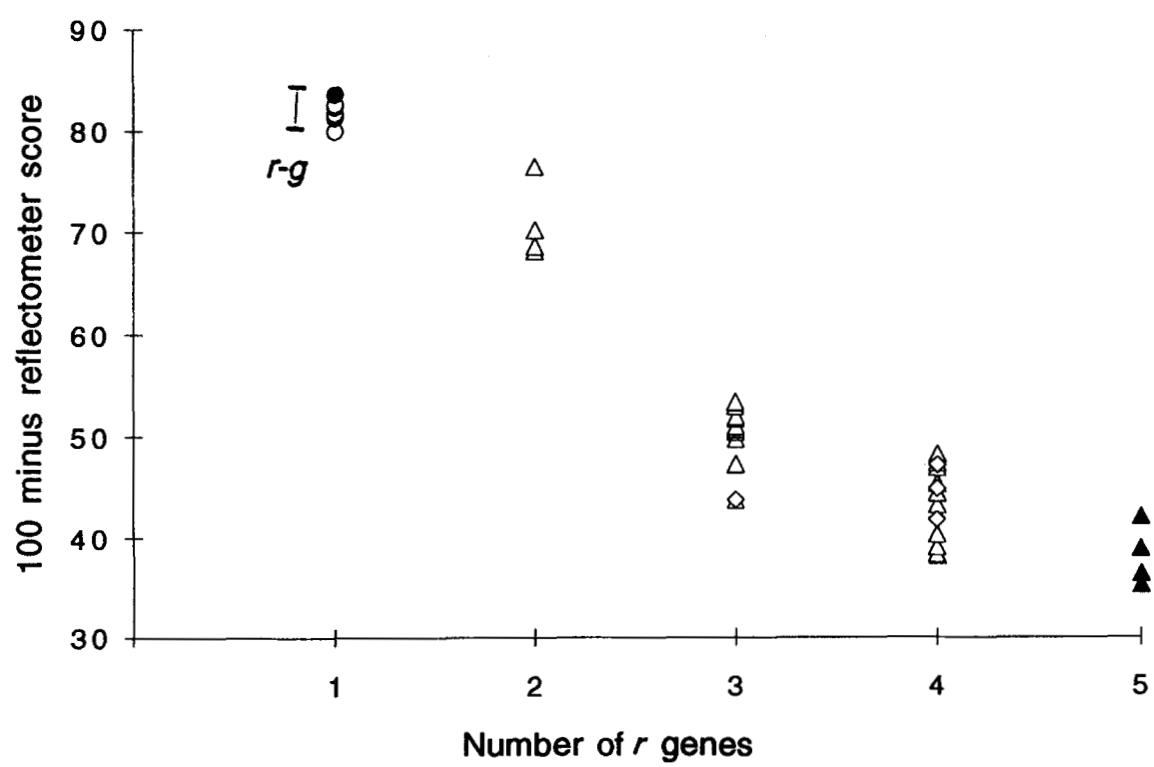

FIGURE 2.—Relationship between paramutagenic strength (Figure 1) and *r* gene number (EGGLESTON *et al.* 1995) among *R-st Lc* and its 41 *Lc*-loss derivatives. The bar labeled *r-g* represents the range for 48 nonparamutagenic *r-g/R-r* sibling control groups. ○, *R-scΔlc;* ●, *R-stΔlc* (*I*); △, *R-stΔlc* (*II*); ◇, *R-stΔlc* (*III*); ▲, *R-st Lc.*

TABLE 2

Relative pigmentation of *r-g/R-r* kernels produced in crosses on *r-g/r-g* of *R-r lc/R-st Lc* and *R-r lc/R-st lc*, testing the effect of *Lc* on paramutation

	Relative pigmentation[a]	
R-st lc crossover	*R-r lc/R-st Lc*	*R-r lc/R-st lc*
N1-1583-1	37.4 ± 0.82	41.8 ± 1.06
N1-1584-2	37.9 ± 0.52	40.5 ± 1.34
N1-1584-3	37.2 ± 0.77	38.6 ± 1.37
Average[b]	37.5	40.4

[a] Values are the average of from six to nine test plants.
[b] $P < 0.01$.

among the distal deletion series of alleles formed by *R-st Lc* and its *Lc* loss derivatives. Paramutagenic strength decreased progressively in the order 5 > 4 > 3 > 2 > 1 with respect to *r* gene number.

Influence of *Lc* on paramutation: The slight reduction in paramutagenicity of *R-stΔlc* (*III*) alleles, which contain four *r* genes, relative to parental *R-st Lc*, which contains five, suggests the presence of a weak determinant of paramutagenicity located distal to the flanking marker *Isr-st*. To test whether the *Lc* region modifies paramutation, three *R-st lc* crossovers were isolated from *R-st Lc/r-g lc* heterozygotes. The *R-st lc* class in this circumstance results from exchange occurring anywhere within the two-map-unit interval between *r* and *Lc*. It need not have involved unequal crossing over between *r* sequences within *R-st* and *Lc*. The data of Table 2 show the three parental *R-st Lc* lines to be slightly but consistently more paramutagenic than the three *R-st lc* crossovers ($P < 0.01$). The regularity of the difference suggests that loss of *Lc* or possibly *Isr-lc* or other linked sequences contributes to the small reduction in paramutagenicity observed in *R-stΔlc Isr-st* unequal crossovers relative to parental *R-st Lc*.

To determine whether *Lc* by itself is paramutagenic, *Lc* from the original source strain, *R-r:Ecuador 1172 Lc*, was coupled with nonparamutagenic *r-g*. Two independent *r-g Lc* crossovers were crossed with *r-g lc* and then to *R-r lc/R-r lc* to compare *R-r* recovered from *R-r lc/r-g lc* and *R-r lc/r-g Lc* sib classes. In neither case did the two testcross values differ appreciably (Table 3), indicating that *Lc* in combination with phenotypically null *r-g* was not detectably paramutagenic.

Fractionation of *R-st* paramutagenicity by internal deletions: A majority of *R-sc* derivatives recovered from *R-st* homozygotes lose one or more *r* genes, forming a series of stepwise internal deletions of the complex. All retain the promoter region of *Sc* but lose *Nc* genes in reverse order relative to the *Lc* loss deletion series (EGGLESTON *et al.* 1995). A minority of *R-sc* reversions from *R-st/R-st* parents retain all four *r* genes, the outcome expected to result from *I-R* transposition.

To determine whether internal truncations of *R-st* had an effect on paramutagenic strength similar to distal truncations, the *r* gene number of 80 *R-sc* derivatives determined in EGGLESTON *et al.* (1995) is plotted in Figure 3 *vs.* the paramutagenic strength reported for each allele by MCWHIRTER (1961). The 22 *R-sc* revertants retaining four *r* genes did not differ from *R-st*, verifying that *I-R* does not effect *R-st*'s paramutagenic strength. The 58 *R-sc*'s with fewer *r* genes showed a regular reduction in paramutagenic strength, with relative strength in the order 4 > 3 > 2 > 1 with respect to *r*-gene number. Together with the *Lc*-loss experiments, these results indicate that sequential loss of *r* genes, beginning either distally or internally, progressively reduces the paramutagenic strength of the *R-st* complex.

TABLE 3

Relative pigmentation scores of *r-g/R-r* kernels produced in crosses of sib *R-r lc/r-g lc* and *R-r lc/r-g Lc* plants to *r-g/r-g*, comparing the paramutagenic effects of *Lc* and *lc*

	Relative pigmentation[a]	
r-g Lc crossover	*R-r lc/r-g lc*	*R-r lc/r-g Lc*
N1-2374-1	91.1 ± 1.45	92.3 ± 0.89
N1-2376-4	93.5 ± 0.86	91.6 ± 0.73

[a] Values are averages of nine or more tests plants. The difference between the two genotypes is not statistically significant.

Effect on paramutagenicity of increasing *r*-gene number: Based on the above findings, it was expected that unequal recombination between weakly paramutagenic alleles might generate derivatives with increased copy number and paramutagenicity. To test this possibility, weakly paramutagenic *R-stΔlc* class (*II*) isolates *g301* and *g305* were chosen as one parent containing two *r* genes (EGGLESTON *et al.* 1995). The second weakly paramuta-

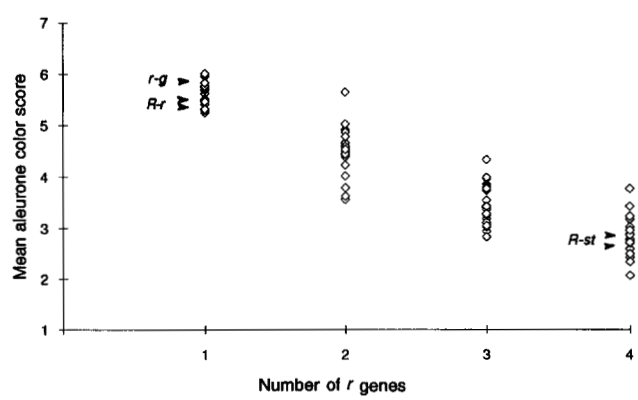

FIGURE 3.—Relationship between paramutagenic strength and *r* gene number in 80 *R-sc* derivatives of *R-st*. Relative pigmentation values are from MCWHIRTER (1961) and *r* gene numbers are from EGGLESTON *et al.* (1995). Arrows denote values for *R-r* following transmission from homozygotes (labeled *R-r*) and *r-g* heterozygotes (labeled *r-g*), which serve as nonparamutagenic controls, and from heterozygosity with the parental *R-st* allele (see MCWHIRTER and BRINK 1962 for details). ◇, mean values for tests with individual *R-sc* alleles.

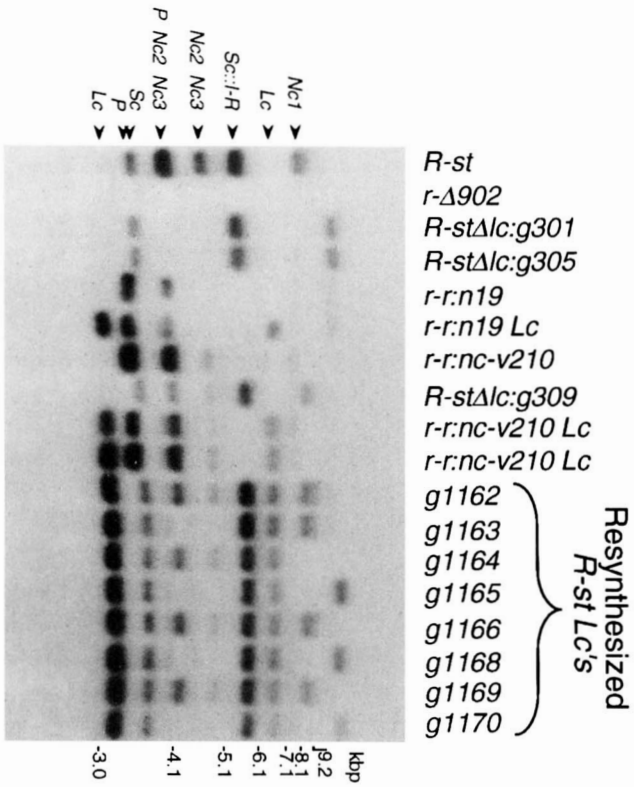

FIGURE 4.—Hybridization of pR-nj:1 to *Bam*HI plus *Eco*RV digests of genomic DNA from resynthesized *R-st Lc*'s and their progenitors. The pR-nj:1 probe is from the *r* promoter/transcription start region (DELLAPORTA *et al.* 1988). Identity of fragments released from *R-st* is from EGGLESTON *et al.* (1995). The allele *r-Δ902* is an *r* locus deletion (ALLEMAN and KERMICLE 1993). Isolate *n19* of *r-r* contains an intact *P* gene from *R-r* (ROBBINS *et al.* 1991). Allele *r-r:nc-v210,* recovered from an *R-st/R-r* heterozygote, contains *P‖nc2* and *Nc3* genes (see text for details). *R-stΔlc:g309* is included for comparison because it has only one of the indistinguishable *Nc2/Nc3* genes present in *R-st* (EGGLESTON *et al.* 1995).

genic parent was *r-r:nc-v210.* It arose via intragenic recombination between *R-st* and *R-r:standard* and contains *P* from the proximal end of *R-r* and (Nc) from the distal end of *R-st* (SATYANARAYANA 1970). Molecular analysis determined that *r-r:nc-v210* contains two *r* genes, one containing the 5′ end of *P* fused to the 3′ end of *Nc2,* denoted *P‖nc2,* the other, intact *Nc3* (Figure 4).

Lc was incorporated into *r-r:nc-v210* to detect recombination with *R-stΔlc.* Instances of stippled kernels showing *Lc* seedling phenotype were then selected among progeny of *r-r:nc Lc/R-stΔlc* testcrossed by *r-g lc/r-g lc.* Because the *R-stΔlc* alleles are deficient for the entire *r* to *lc* region, such products are expected to originate by recombination within or between *r* genes in *r-r:nc* and *R-stΔlc.* Eight *R-st Lc* isolates were obtained among 21,130 progeny, a frequency of 3.8×10^{-4}. All carried the proximal flanking marker *golden-1* introduced with the *R-stΔlc* (*II*) parent and the distal flanking marker *M-st* of *r-r:nc Lc.* Through recombination analysis with *R-r* (per ASHMAN 1965a), each *R-st Lc* was verified as carrying near-colorless.

TABLE 4

Structures of resynthesized *R-st Lc*'s and progenitors

Allele	Structure[a]
Progenitors	
r-r	*P*
r-r:nc-v210	*P‖nc2 Nc3*
r-r:nc-v210 Lc	*P‖nc2 Nc3 Lc*
R-stΔlc:g301, R-stΔlc:g305	*Sc::I-R Nc1‖lc*
Resynthesized *R-st Lc*	
g1162, g1166, g1169	*Sc::I-R Nc1‖nc2 Nc3 Lc*
g1163, g1164, g1165, g1168	*Sc::I-R Nc1‖nc3 Lc*
g1170	*Sc::I-R Nc1‖lc Lc*

[a] Exact exchange points between genes have not been determined. Hybrid genes identified by Southern blot analysis are denoted by capitalized promoter region and lowercase 3′ region following the double vertical lines. Because hybrid genes in the resynthesized alleles have undergone two cycles of exchange, they may be more complex than indicated. The simplest structure consistent with the available Southern blot data is given.

Figure 4 shows a molecular analysis of the resynthesized *R-st Lc* derivatives and their progenitors after digestion of genomic DNA with *Bam*HI plus *Eco*RV and hybridization with the 5′ *r* locus clone pR-nj:1 (DELLAPORTA *et al.* 1988). Not shown are hybridizations of the same filter with the 3′ *r* locus probes pSc323:I14 and pSc323:J20 (ALLEMAN and KERMICLE 1993). Organization of the eight resynthesized *R-st Lc*'s was determined by comparing them to their direct progenitors and to *r-r, r-r Lc, r-r:nc-v210* and *R-st* controls. *R-stΔlc:g309* is included to illustrate the difference between the presence of *Nc2* or *Nc3* or both of these similar *r* genes. This allele has only a single *Nc2/Nc3*-like gene, which is illustrated by the lower relative intensities of fragments released from *Nc2/Nc3* in this allele compared to *R-st.*

All eight resynthesized *R-st Lc*'s lost the fragment specific for the 5′ end of *P* as would be expected if each arose by unequal recombination. All eight retained the *Sc::I-R* and *Lc* specific fragments as anticipated from their phenotypes. Each allele retained one or two additional *r* genes for a total of three or four. The structures of the progenitors and resynthesized *R-st Lc* alleles are summarized in Table 4. Note that due to their origin by successive rounds of intragenic exchange, the eight resynthesized *R-st Lc* alleles are structurally distinct from the five *r*-gene *R-st Lc* stocks used in the distal truncation analysis described above.

Figure 5 shows a plot of *r* gene number *vs.* relative pigmentation of *R-g:1* after heterozygosity with control *R-st* and *R-st Lc* stocks, resynthesized *R-st Lc* alleles, and the *R-stΔlc* and *r-r:nc* progenitors. All eight resynthesized *R-st Lc* alleles reduced pigmentation of *R-g:1* more effectively than either of the two-gene progenitor alleles. Resynthesized *R-st Lc* alleles with four *r* genes reduced pigmentation more strongly than corresponding alleles with three *r* genes and as strongly as control *R-*

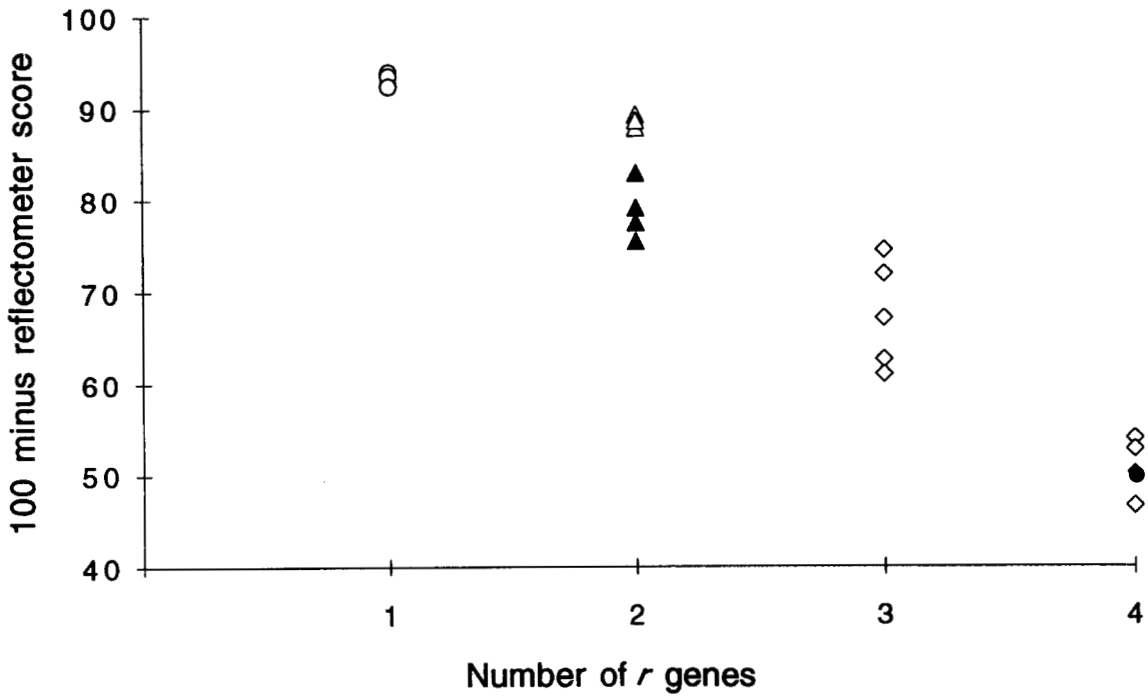

FIGURE 5.—Relationship between paramutagenic strength and *r*-gene number in eight resynthesized *R-st Lc* alleles and their progenitors. Paramutagenic strength measured as in Figure 2 except that *R-g:1* rather than *R-r* was used as the paramutable allele. Alleles range in paramutagenic strength from nonparamutagenic *r-r* to strongly paramutagenic parental *R-st*. ○, *r-r*; △, *r-r:nc*; ▲, *R-stΔlc*; ◇, resynthesized *R-st Lc*; ◆, *R-st*.

st, which has four *r* genes. Therefore, substitution of an *Nc* gene by *Lc, i.e., R-st vs.* the four-gene resynthesized *R-st Lc*'s, did not alter paramutagenic strength appreciably. Nevertheless, the difference in paramutagenic strength between the two types of alleles with two *r* genes, namely *R-stΔlc (II)* and *r-r:nc-v210*, indicates that alleles with the same number of *r* genes do not necessarily have equal paramutagenic strengths.

Complementation of paramutagenicity in proximal-loss and distal-loss combinations: Need *r* genes be associated in *cis* for strong paramutation? To test the *trans* arrangement, trisomic-*10* plants were constructed having three different *r* alleles: either of the two distally truncated, weakly paramutagenic *R-stΔlc (II)* isolates described in the preceding section, the proximal *R-st* truncation product *r-r:nc-v210* also described above, and third, the paramutable allele *R-g:1*. Combining parental *R-st* and nonparamutagenic *r-r* together with *R-g:1* furnished parallel trisomic controls for the *cis* combination. Use of the green plant allele *R-g:1*, which is equally sensitive to paramutation as *R-r:standard* (BROWN and BRINK 1960), facilitated the identification of plant genotypes in this experiment.

The upper portion of Figure 6 gives the paramutation results for the disomic segregants in the trisomic test. Both *r-r:nc* and the two *R-stΔlc* isolates reduce the pigmentation level of *R-g:1* relative to nonparamutagenic *r-r*. Although consistent, the effect is small compared with *R-st* where pigmentation is reduced from 93.1 to 48.2. The effect of *R-st* is not changed appreciably by adding *r-r* in trisomic plants (difference between lines 1 and 5, bottom portion). This evidence, like that obtained earlier using paramutagenically neutral *r-g* (BRINK 1959), indicates that chromosome-*10* trisomy *per se* does not alter the level of paramutation.

Central to the comparisons among trisomic genotypes is the *trans* combination of *r-r:nc-v210* with an *R-stΔlc (II)* allele. If there is no effect of distribution of *R-st* components among homologues, this combination is expected to be as paramutagenic as *R-st*, since a total of four *r* genes are present in each. Although moderately paramutagenic, the *trans* combination is significantly less so than the *cis* combinations (Figure 6, bottom). To examine whether the *r-r:nc-v210* and *R-stΔlc (II)* alleles contribute different functions to paramutagenicity, the *trans* combination can be compared with two doses of *r-r:nc-v210* and two doses of *R-stΔlc (II)*. That the *trans* combination is intermediate between the two dose combinations indicates an absence of detectable complementation. Two doses of *r-r:nc-v210* and of *R-stΔlc (II)* are used for comparison with the *trans* combination since all the paramutagenic alleles, including parental *R-st*, showed a positive dosage effect. The positive effect of dosage indicates that even in *trans* increased *r* gene number is correlated with increased paramutagenicity.

DISCUSSION

Crossovers within *R-st*, identified on the basis of recombined pigmenting components, lose more or less

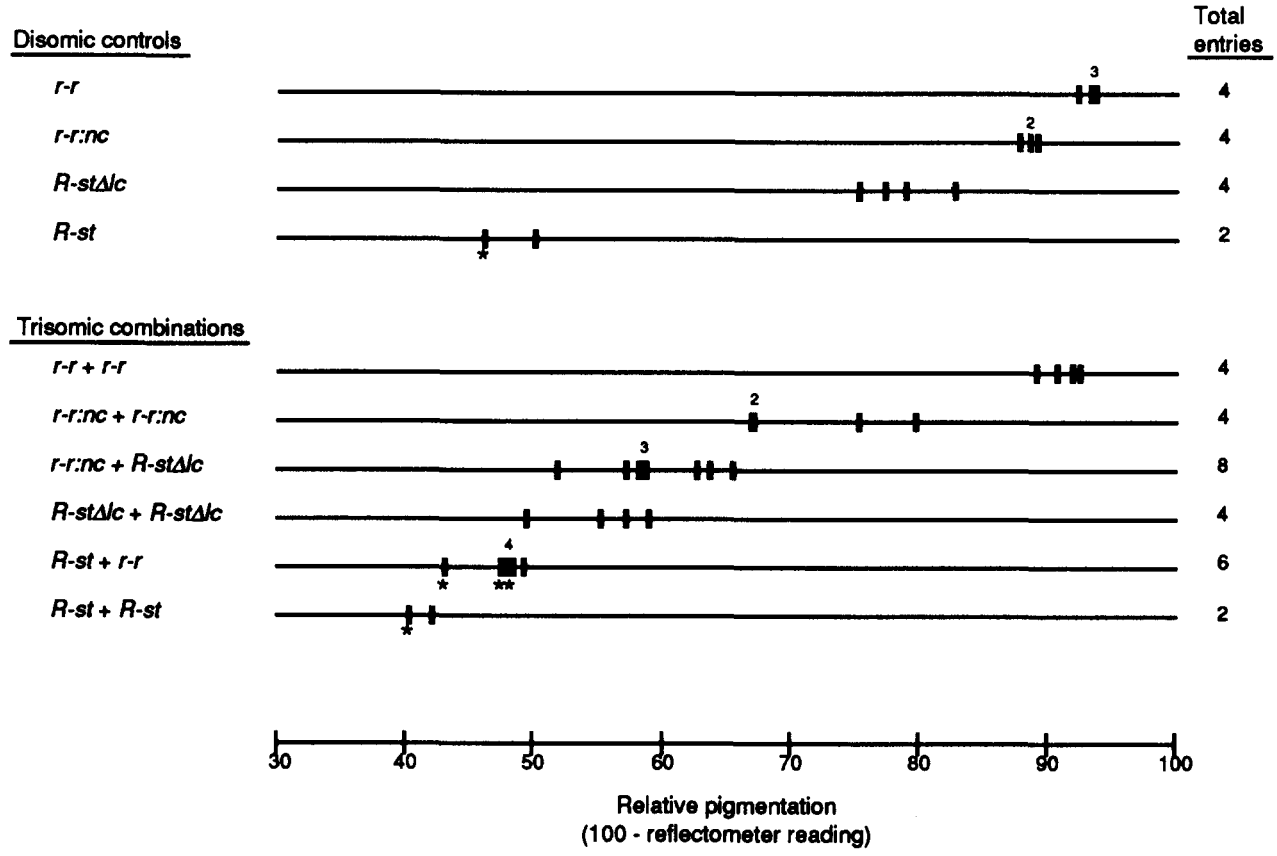

FIGURE 6.—*R-g:1* paramutation as indicated by pigmentation level of *r-g/R-g:1* kernels produced in testcrosses on *r-g/r-g* of *R-g:1* combined in heterozygotes with alleles listed on the left. Top: disomic combinations; bottom: trisomic combinations. ★ designates *R-st* alleles carrying *Lc*.

of this allele's potential to alter certain other alleles in heterozygotes (paramutation). Previous recombination strategies served to truncate *R-st* from the proximal end (ASHMAN 1965a,b; SATYANARAYANA 1970). Present strategies provide complementary materials by truncating distally and by deleting internally (EGGLESTON *et al.* 1995). To simplify the initial interpretation of these experiments, the paramutagenic determinants themselves are assumed not to be fundamentally altered in the recombinant products. Consistent with this assumption, strongly paramutagenic derivatives of the expected strength could be resynthesized from weakly paramutagenic alleles by crossing over. This relative constancy of paramutagenic determinants permits regions containing them to be characterized by conventional genetic approaches.

Organization of paramutagenic determinants in *R-st*: Sets of unequal crossover deletions of *R-st* and *R-st Lc* ranged in paramutagenicity from unchanged relative to the parent stock to nonparamutagenic. Variation among 80 *R-sc* derivatives from *R-st* appears to be continuous (MCWHIRTER and BRINK 1962). Similarly, the distribution among 41 *Lc* loss derivatives of *R-st Lc* reported here showed only one discontinuity. When categorized by *r*-gene number, however, both sets separated into tightly clustered subgroups (Figures 2 and 3) with stepwise decreases in paramutagenicity associated with decreased *r* gene number. Corresponding increases occurred when *r* gene number was built up from weakly paramutagenic alleles (Figure 5). In contrast, *R-sc* revertants originating with no change in *r* gene number were unchanged from *R-st* in paramutagenicity. Earlier, insertion of *I-R* at linked sites after excision from *R-st* was proposed as a basis for variation among *R-sc* revertants (MCWHIRTER and BRINK 1962). Frequent intragenic transposition of maize transposable elements recently has been demonstrated (ATHMA *et al.* 1992; MORENO *et al.* 1992; WEIL *et al.* 1992). However, among the 80 *R-sc* derivatives characterized molecularly, no instance of *I-R* presence within an *r* gene was detected (EGGLESTON *et al.* 1995).

Change in paramutagenicity occurred in relatively uniform increments within and between experiments, even those conducted at different times and using different means of quantifying pigmentation. Importantly, the order in which *r* genes were lost or gained in these experiments differed. It is as though each region marked by an *r* gene, when present beyond a threshold value of one, contributed a given increment to paramutation. Some regions undoubtedly contribute more than others. The distinct discontinuity among the *Lc*-loss derivatives from *R-st Lc* is associated with the differ-

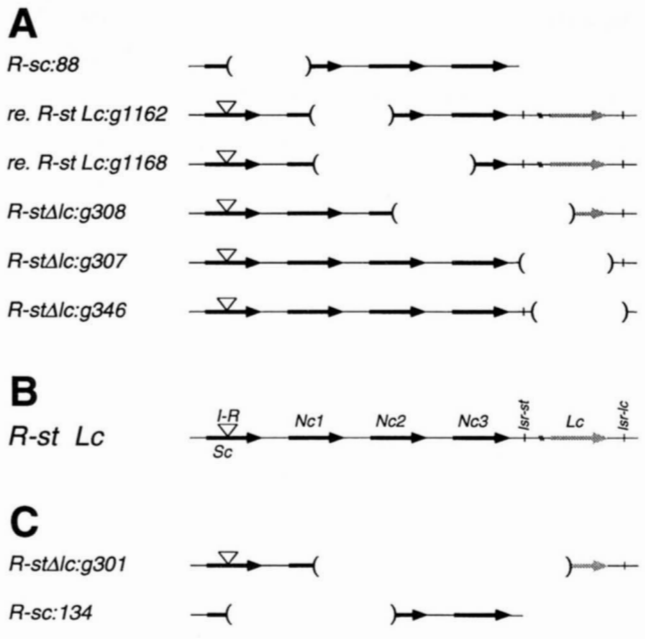

FIGURE 7.—Paramutagenic classes among *R-st* deletion products. The deleted regions are indicated by open parentheses. (A) Strongly paramutagenic derivatives. (B) Parental *R-st* carrying *Lc*. (C) Weakly paramutagenic derivatives. All alleles derive from an *Lc*-carrying chromosome except *R-sc:88* and *R-sc:134*.

ence between three and two *r* genes, specifically with removal of the 5′ portion of *Nc2*, the intervening *Nc1* to *Nc2* region, and the 3′ region of *Nc1*. The difference could be due to these particular *r* segments or to the intervening region, including the possibility of other *r* sequences oriented opposite to *Lc*. Such a marked difference between two and three copy derivatives was not evident in the *R-st* resynthesis experiment, where the segment in question was absent in both parental alleles.

An *r* copy number threshold has been observed in a number of paramutation contexts. The plant color gene *P* is sensitive to paramutation when coupled with the seed-color complex *S*, but insensitive when present alone (BRINK and MIKULA 1958; BROWN 1966). When separated from the *Nc* complex, *Sc* was not detectably paramutagenic. Adding *Lc* to *R-st* strengthened paramutagenicity, although this gene was not detectably paramutagenic in combination with phenotypically null *r-g*. To date, no single copy *r* allele having paramutational properties has been identified.

The regional distribution of paramutagenicity, together with the triplicate basis of the near-colorless phenotype, confound attempts to localize paramutagenicity to particular sites by conventional mapping. Alternatively, deletion mapping can be employed utilizing the segmental losses generated by unequal crossing over. Figure 7A pictures six classes of deletions that retain at least moderately strong paramutagenic action. The six are seen to comprise a set of overlapping deficiencies extending on the proximal side from the transposable element *I-R*, through *Isr-lc* distally. Only the 5′ end of *Sc* is untested. Concerning it, the 5′ end of *P* from *R-r* was shown to substitute for the corresponding region of *Sc* with no (ASHMAN 1965b) or little (SATYANARAYANA 1970) reduction in paramutagenicity. Therefore no particular region in *R-st Lc* is essential for paramutation. What the moderately paramutagenic alleles have in common is the presence of three *r* genes and two intergenic regions.

The above consideration does not exclude the possibility that some particular region might be sufficient for strong paramutagenicity. This question is addressed by considering which regions are retained by deletion products that lose strong paramutagenicity. Derivatives represented by *R-stΔlc:g301* and *R-sc:134* in Figure 7C test all but a medial segment of *R-st* and the *R-st* to *Lc* interval. The latter is present in stocks such as *r-g Lc*, which is nonparamutagenic. Therefore, no single region of *R-st* is sufficient for strong paramutagenicity with the possible exception of the medial region where the two deletions overlap, which has yet to be tested.

Two *r* genes present in each of two chromosomes proved less paramutagenic than four genes in one chromosome, *i.e.*, the *R-st* proximal and distal region deletions failed to complement in *trans*. The nature of the effect of position is speculative. Dependence on transcriptional read through from one *r* gene to the next is a formal possibility. Alternatively, paramutation may involve a copy-counting feature that functions more efficiently intrachromosomally than interchromosomally. In the two plus two situation, each chromosome exceeds a one-copy threshold by only one, whereas in intact *R-st* this threshold is exceeded by three.

Dependence of *R-st* paramutagenic strength on the number and distribution of *r* genes may be related to level of near-colorless phenotype. (Nc) penetrance varied inversely with number of *cis* copies (EGGLESTON *et al.* 1995). Earlier, SATYANARAYANA (1970) compared near-colorless phenotype and paramutagenicity among proximal truncations of *R-st* produced by recombination with *R-r*. Among the resulting *P* plus *Nc* products, near-colorless phenotype was inversely related to paramutagenic strength. He inferred that near-colorless was paramutable and capable of reverting to stronger pigmentation when separated from other *r* genes. Extending this view, *r* paramutation could be considered a transference of *cis* inactivation among DNA sequences in one molecule to homologous sequences in another molecule.

The ready fractionation of paramutagenic components in *R-st* contrasts with the difficulty in partitioning determinants that confer sensitivity to paramutation. In *R-r*, the order of components (proximal to distal) is a plant color gene, *P*, a truncated 5′ *r* segment, *q*, and the seed color genes *S1* and *S2* arranged as a reverse repeat (WALKER *et al.* 1995). Unequal crossing over be-

tween *P* and *q*, detected as loss of *P* function, deletes the intervening region (ROBBINS *et al.* 1991). None of eight such *R-g* isolates of this class was found to be altered in paramutability (BROWN 1966). In connection with the present experiments, 13 instances of *Lc*-loss from *R-g Lc* homozygotes were established by the same procedures used in the isolation of *R-stΔlc* derivatives. Recombination in this case should involve *S2*, the only gene of *R-g* having a coding sequence in the same orientation as *Lc*. Although there was significant heterogeneity in paramutability among the 13 isolates, they did not differ significantly in average level of response from four parental *R-g:1 Lc* control lines (data not given). Taken together, evidence from the two experiments suggests that the region between *q* and the coding portion of *S2*, which includes *S1* and the promoter for the *S* genes, is essential for sensitivity to paramutation.

Mobile elements and *r* locus paramutation: Mobile element effects on gene expression are well documented in maize, as are negative effects on expression between copies of some elements (reviewed in FEDOROFF 1989). Additionally, mobile elements have been postulated to be responsible for a form of paramutation at the *nivea* locus in *Antirrhinum majus* (UPADHYAYA *et al.* 1985; KREBBERS *et al.* 1987). In contrast, the mobile element *I-R* appears to play no role in the maintenance of *R-st*'s paramutagenicity since the simplex allele *R-stΔlc:g327*, which retains *I-R*, is nonparamutagenic. Furthermore, *R-sc* alleles that retain all four *r* genes but lose *I-R* have unchanged paramutagenic strengths relative to *R-st*. It is possible of course that *I-R* played a role in introducing paramutagenicity to the complex but is not required for its maintenance.

A recent report on structure of the paramutable allele *R-r:standard* raises the question of involvement of mobile elements other than *I-R* in paramutation (WALKER *et al.* 1995). Located at the rearrangement junction between the *S1* and *S2* genes are sequences homologous with the CACTA family of transposable elements. It will be interesting to learn whether this or other repetitive sequences are present in or around *r* genes of other paramutationally active alleles.

Relationship of *r* locus paramutation to paramutation at the maize *b* locus: Current molecular data suggest that *r* locus paramutation may be mechanistically distinct from paramutation at its displaced homologue, the *b* locus. First, the genetic phenomenology at *r* and at *b* differ significantly (reviewed in PATTERSON and CHANDLER 1995). Paramutation at *b* occurs spontaneously in homozygotes at significant rates and is not reversible. *r* paramutants are metastable, capable of full reversion, and have not been observed to arise spontaneously (BRINK *et al.* 1968). Whereas newly paramutant *b* alleles are strongly paramutagenic (COE 1966), newly paramutant *r* alleles are only weakly so (BROWN and BRINK 1960). At *b*, expression of sensitive alleles is strongly suppressed in the initial heterozygote (COE 1966), whereas, at *r*, suppression of a sensitive allele is weak in the initial heterozygote (MCWHIRTER and BRINK 1963).

Significant differences also are present at the molecular level. PATTERSON *et al.* (1993) have shown that interacting *b* alleles are simplex, that no detectable change in methylation patterns accompany paramutation and that transcription and steady-state mRNA levels are reduced in heterozygotes and in subsequent generations. In contrast, both paramutable and paramutagenic *r* alleles contain multiple *r* genes. The cytosine residues in promoter, but not coding, regions of the *r* genes involved in paramutation are methylated (EGGLESTON *et al.* 1995; M. ALLEMAN and J. KERMICLE, unpublished observations). Transcription rates and steady-state mRNA levels of *r* have not been tested. Although the differences in genetic and molecular phenomenology at *r* and *b* are numerous, they do not rule out similarities at other levels, such as modified chromatin confirmation, altered transcription complexes, etc.

Relationship of *r* locus paramutation to allelic and ectopic gene interactions in other plants and fungi: Paramutation at *r* bears similarities to reports of dosage-dependent interactions in other plants and in fungi. In at least six plant species, addition of multiple copies of endogenous or exogenous genes has led, with varying efficiency, to silencing of all copies (reviewed in JORGENSEN 1993; MATZKE and MATZKE 1993; FLAVELL 1994). Where tested, silencing generally is correlated with increased copy number and with increased cytosine methylation in promoter regions (*e.g.*, JOHN and AMASINO 1989; MATZKE *et al.* 1989; GORING *et al.* 1991), but exceptions are known (*e.g.*, SCHEID *et al.* 1991; MEYER *et al.* 1993). In a minority of cases, copy number-dependent silencing is partially heritable following reduction in copy number by meiotic segregation (*e.g.*, GORING *et al.* 1991; MATZKE and MATZKE 1991). In fungi, duplicated sequences are recognized and methylated/inactivated with high specificity and efficiency both in Neurospora (SELKER *et al.* 1987; FOSS *et al.* 1991) and in Ascobolus (FAUGERON *et al.* 1990; BARRY *et al.* 1993).

Unequal crossing over also has been used to analyze transgene silencing. ASSAAD *et al.* (1993) transformed Arabidopsis with a multiple copy construct and then selected reduced copy number derivatives. In this way copy number was changed without varying chromosome position. Changes in copy number were correlated with changes in transgene expression and promoter region methylation. Single copy genes had higher expression and much lower promoter region cytosine methylation than their multiple-copy progenitor. Like *r* paramutation, level of silencing was progressive over generations and the effects potentially reversible.

There is now abundant evidence that in some fungi and higher plants, repeated sequences are recognized and inactivated. The mechanism and significance of this inactivation remain to be elucidated. Silencing may

exist to prevent expression of foreign DNA such as mobile elements or to prevent overexpression of endogenous genes. It also has been suggested that these mechanisms act to prevent recombination between repeats at ectopic sites in genomes (*e.g.*, KRICKER *et al.* 1992). Further analysis of the roles of paramutation and related phenomena may enlighten our understanding of interactions involving the genome as a whole.

This work is paper no. 3432 from the Laboratory of Genetics. We thank WARREN WILLIAMS and HUGO DOONER for discussions during the formative stages of the experiments and BEVERLY OASHGAR and DAVID HELLER for technical assistance. We were supported by U.S. Department of Energy grant FG02–86ER13539.

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Communicating editor: J. A. BIRCHLER