Structural Features and Methylation Patterns Associated With Paramutation at the *r1* Locus of *Zea mays*

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ABSTRACT

In paramutation, two alleles of a gene interact and, during the interaction, one of them becomes epigenetically silenced. The various paramutation systems that have been studied to date exhibit intriguing differences in the physical complexity of the loci involved. *B* and *Pl* alleles that participate in paramutation are simple, single genes, while the *R* haplotypes that participate in paramutation contain multiple gene copies and often include rearrangements. The number and arrangement of the sequences in particular complex *R* haplotypes have been correlated with paramutation behavior. Here, the physical structures of 28 additional haplotypes of *R* were examined. A specific set of physical features is associated with paramutability (the ability to be silenced). However, no physical features were strongly correlated with paramutagenicity (the ability to cause silencing) or neutrality (the inability to participate in paramutation). Instead, paramutagenic haplotypes were distinguished by high levels of cytosine methylation over certain regions of the genes while neutral haplotypes were distinguished by lack of C-methylation over these regions. These findings suggest that paramutability of *r1* is determined by the genetic structure of particular haplotypes, while paramutagenicity is determined by the epigenetic state.

EPIGENETIC inheritance—"mitotically and/or meiotically heritable changes in gene expression that cannot be explained by changes in DNA sequence" (Riggs *et al.* 1996)—is a widespread phenomenon that is involved in both normal developmental mechanisms and is a means of coping with repetitive and/or invasive sequences like transposons and viruses. Many epigenetic effects are recognized when expression of a gene is reduced or abolished following introduction of a homologous transgene and are described as "homology dependent gene silencing" (HDGS; reviewed recently by Wolffe and Matzke 1999). HDGS also operates naturally in repeated endogenous sequences such as transposable elements (Fedoroff 1996; Martienssen 1996a). The underlying mechanisms that lead to reduced gene expression are beginning to emerge (Wolffe and Matzke 1999), but the mechanisms that trigger HDGS remain elusive.

Paramutation is a special case of HDGS in which the homologous sequences that interact are alleles. Two key features define the phenomenon of paramutation (Chandler *et al.* 2000). First, two variants of the gene or locus interact in a heterozygote such that one of the variants becomes epigenetically silenced. Second, the silencing is meiotically heritable; *i.e.*, it is maintained in subsequent generations. In paramutation of the *r1* locus of maize, a paramutable haplotype, *e.g.*, *R-r:std*, is made heterozygous with a paramutagenic *r1* haplotype, *e.g.*, *R-st* or *R-mb* (Brink 1956; Brink and Weyers 1957). In the heterozygote, simple dominance of *R-r:std* is observed, producing dark, solid pigmentation of the aleurone layer of the kernel. However, when this heterozygote is subsequently crossed, expression from *R-r:std* is changed so that *R-r:std* confers a lighter, "mottled" pattern of anthocyanin deposition. Thus, paramutant *R-r:std* (designated as *R-r′*) has been partially silenced. Silencing happens in virtually 100% of the kernels into which *R-r:std* is transmitted, but the level of silencing is quite variable, ranging from nearly complete silence to nearly complete expression. The pattern of expression of the paramutagenic (silencing) haplotype is unchanged following the interaction. Not all *r1* variants are paramutable or paramutagenic. Some do not participate in paramutation and are referred to as "neutral."

r1 genes encode nearly identical *myc*-homologous, helix-loop-helix proteins (Ludwig *et al.* 1989; Perrot and Cone 1989; Consonni *et al.* 1992;) that are capable of activating transcription from promoters of structural genes in the anthocyanin biosynthetic pathway (Ludwig *et al.* 1989; Goff *et al.* 1990). Because the *r1* locus often contains multiple *r1* genes, particular variants of the *r1* locus are referred to as distinct *r1* haplotypes with the designation, *R-suffix*, in which the suffix is usually based on a single striking phenotypic characteristic of the haplotype. For example, *R-marbled* (*R-mb*) confers a marbled pattern of pigmentation to the aleurone. A particular

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haplotype will comprise one to several individual *r1* genes. These genes are not all identical; many alleles exist. These alleles are distinguished from one another without use of the *R* prefix. For example, the *R-r:std* haplotype contains four *r1* genes with the distinct allele combination "*P q S1 S2*."

One approach to understanding why certain genes are particularly subject to epigenetic regulation is to examine the structural features that affect their ability to cause silencing or become silenced. Several distinct structural features have been associated with gene silencing: multiple gene repeats (Assad *et al.* 1993; Rossignol and Faugeron 1994; Patterson and Chandler 1995; Sijen *et al.* 1996; Ye and Signer 1996; Bender 1998; Stam *et al.* 1998), the presence of inverted repeats (Que and Jorgensen 1998; Stam *et al.* 1998; Luff *et al.* 1999), the presence of transposon regulatory sequences (Fedoroff 1996; Martienssen 1996b), and the juxtaposition of low and high G/C regions (Schlappi *et al.* 1993, 1994; Jakowitsch *et al.* 1999). Interestingly, *r1* haplotypes that participate in paramutation share many of these features.

The physical structure of a single paramutable haplotype, *R-r:std*, has been the subject of intense investigation for many years (Stadler and Nueffer 1953; Stadler and Emmerling 1956; Dooner 1971; Dooner and Kermicle 1971, 1974; Kermicle 1980; Robbins *et al.* 1991; Walker *et al.* 1995). Four distinct *r1* genes are present in *R-r:std* (see Figure 1A): *P* (*Plant color*), a functional gene that colors coleoptiles, roots, and anthers; *q*, a nonfunctional gene fragment with strong sequence similarity to the promoter region of *P*; and two *S* (*Seed color*) genes, *S1* and *S2*, that color the aleurone of the seed (Robbins *et al.* 1991; Walker *et al.* 1995). The *S1* gene is in an inverted orientation relative to the other *r1* genes of the complex and appears to have arisen as a duplicate copy of the *S2* gene. The *P* gene is located ~190 kb proximal to the rest of the complex (which is referred to as the *S*-subcomplex) and is not strongly affected in paramutation (Brink and Mikula 1958; Brown 1966). Derivative alleles of *R-r:std* that lack *P* are fully paramutable, indicating that instability is a function of the *S*-subcomplex (Brown 1966).

Within the *S*-subcomplex, the *S* genes, *S1* and *S2*, are arranged in a head-to-head (5′ ends together) orientation with only 381 bp separating them. This 381-bp sequence is called σ (sigma) and constitutes the promoter for both of the *S* genes (Walker *et al.* 1995; May and Dellaporta 1998). Thus, the *S1* and *S2* genes of *R-r:std* can be thought of as having a novel promoter that is distinct from the promoters of other *r1* alleles. The majority of σ is derived from the 3′ end of a transposon called *Doppia* (*Dop*) and contains multiple copies of a sequence motif that can be bound by the *Dop*-encoded protein, DOPA (Bercury *et al.* 2001). A second, very small fragment of the *Dop* 3′ end is present just upstream of *S1*. This fragment contains no DOPA binding sites. Between the two *Dop*-derived portions of σ, a short "rearranged" region is present. The origin of the sequences in this region is not clear, but they appear not to be derived from the original *Dop* insertion. The *q* gene fragment contains upstream *r1* sequences that are interrupted by a *Dop* 5′ end (Bercury *et al.* 2001). Thus, *q* is an *r1* promoter with no adjacent coding sequences. Another set of DOPA binding sites is located in the *Dop* 5′ sequences adjacent to *q*.

The simplest way to account for the structure of the *S*-subcomplex is to imagine that a *Dop* element inserted into *r1* and then fractured to generate two entities: a *q* fragment with an adjacent *Dop* 5′ end and an *S2* fragment that included a *Dop* 3′ end upstream of a complete *r1* coding region. A few base pairs of the *Dop* 3′ end, together with the entire coding region of *S2*, were duplicated, and these duplicated sequences inserted in reversed orientation upstream of the original *S2* copy to form *S1* (Walker *et al.* 1995).

Detailed physical structures are available for two paramutagenic haplotypes. The canonical paramutagenic haplotype, *R-st* (see Figure 1B), contains four directly repeated genes designated *Sc* (*Self color*), *Nc1*, *Nc2*, and *Nc3* (*Near colorless*; Eggleston *et al.* 1995; Kermicle *et al.* 1995). *Dop* sequences are located at the 5′ ends of each of the *Nc* genes of *R-st* (Matzke *et al.* 1996). A second paramutagenic haplotype, *R-mb*, comprises three *r1* genes: *Sc*, *Lcm1*, and *Lcm2* (see Figure 1C). These are again arranged as direct repeats (Panavas *et al.* 1999). None of the genes in the *R-mb* haplotype contain *Dop* sequences. This rules out a role for *Dop* sequences in paramutagenicity. The only gene type that is common to both *R-st* and *R-mb* is *Sc*. It should be noted, however, that the *Sc* gene of either haplotype can be replaced by other *r1* genes through unequal crossing over, and the resulting derivatives retain paramutagenicity (Kermicle *et al.* 1995; Panavas *et al.* 1999). In studies of both *R-st* and *R-mb*, the strength of paramutagenicity was directly correlated with gene copy number (Kermicle *et al.* 1995; Panavas *et al.* 1999). Thus, paramutagenicity appears to depend on the presence of multiple gene copies and seems not to be affected by the particular combination of genes found at the locus.

Here we present a survey of the genic compositions of a set of multigenic *r1* haplotypes that includes paramutable, paramutagenic, and neutral types. A combination of PCR, genomic blotting, cloning, and sequencing was used to determine the gene types present in each haplotype. Paramutable haplotypes were found to be structurally very similar to one another, while paramutagenic haplotypes were much more diverse in their genic composition. Surprisingly, neutral haplotypes had no distinct structural features that distinguished them from paramutagenic haplotypes. Neutral haplotypes were, however, distinct from paramutagenic haplotypes in the pattern and level of DNA methylation present at sites in the upstream portions of their genes. These data

suggest that paramutagenicity is dependent on the chromatin conformation of the locus rather than on the presence of particular sequence features. In contrast, the marked structural similarity of the paramutable haplotypes suggests that particular sequence level features may be most important for paramutability. Finally, the structural studies presented here shed light on the evolutionary history of these complex *r1* haplotypes.

MATERIALS AND METHODS

Genetic stocks: All stocks were maintained in the W22 inbred background. All stocks are homozygous dominant for the *a1*, *a2*, *c1*, *c2*, *bz1*, and *bz2* genes necessary for anthocyanin synthesis in aleurone and homozygous recessive for the *pl1* and *b1* genes; see Dooner *et al.* (1991) for descriptions. The *R-n:geographic* (Van Der Walt and Brink 1968), *R-r:std* (Stadler 1948; Walker *et al.* 1995), and *R-mb* (Weyers 1961; Panavas *et al.* 1999) haplotypes used in this study have been described previously. All but *R-mb* confer solid aleurone pigmentation. *R-mb* confers a pattern of aleurone color consisting of large pigmented sectors on a colorless background. The *r*Δ allele confers a colorless phenotype and does not hybridize to *r1* probes on genomic blots nor does it give PCR products with any combination of *r1*-specific primers that we have tested. The W23 strain was used as a colorless aleurone tester for genetic studies that did not include any molecular analyses.

Paramutation test: The *R-n:geographic* haplotypes used in this study were kindly provided to us by Dr. J. Kermicle. Crosses of *R-n:geographic* haplotypes to *r*Δ, *R-r*, and *R-mb* were performed during the summers of 1997 and 1998. Outcrosses to the W23 tester were made in the summer of 1999. The scoring of aleurone color was by visual inspection on whole ears. A minimum of four ears was scored for each haplotype. In cases where weak color changes were suspected, kernels were stripped from the ears, sorted, and scored on a seven-point color scale.

PCR: DNA for PCR reactions was prepared in replicates from two sources: leaves of mature, flowering plants and ungerminated embryos from imbibed kernels (Dellaporta 1994). All PCR reactions were performed using the Expand High Fidelity PCR system (Boehringer Mannheim, Indianapolis) according to manufacturer's instructions. The PCR primers used in this study were as follows:

oR-3′-1 (5′ GGCATGCGTATGCTGGAAAGACGT 3′)
oR-3′-2 (5′ TAGCTCCAGTTGATGCTCCTGGCG 3′)
oR-3′-3 (5′ CCATGCGAAGGGTAGAGAAGAACCG 3′)
oR-5′-2 (5′ AAAAGCAATCAGAAGCTAAAAACACGG 3′)
oLc-5′-1 (5′ CTCCAAAAGGCTCAATTCTCCTCCCC 3′)
oQ-3′ (5′ CACATTTTCGTCGGTCACTCTTGCC 3′)
oSc-5′ (5′ TGCAAAGTATTCCCTTCTCTCCACCTCA 3′)
oS2-5′-1 (5′ AAAATAAGTCGTTTTCGTCGGTACCGA 3′)
oS2-5′-2 (5′ ATAAGTCGTTTTCGTCGGTACCGA 3′)
oS1-5′-1 (5′ CGTGGGCCAAAAACCCACGAAA 3′)
oS1-5′-2 (5′ CGTCGGTACCGACGAAAACGACT 3′)

Inverse PCR: DNA from leaves of the desired *r1* geographic haplotypes was digested with *Hin*dIII and fractionated on a 1% agarose gel. The gel slice corresponding to the MW interval 2.5–4.5 kb was excised and the restriction fragments were purified using the Qiaquick gel extraction procedure (QIAGEN, Valencia, CA). The purified DNA was self-ligated for 2 hr at 22° at a concentration of 2 ng/μl. The self-ligated DNA fragments served as templates in PCR reactions using the Expand High Fidelity PCR system (Boehringer Mannheim) with oR-3′-1 and oLc3676_3702 (5′ GGTGAGGCCCATC CAGATAACATAAGC 3′) primers. The PCR fragments were cloned into the *Sma*I site of the pTZ19U plasmid and sequenced.

Genomic blotting: Preparation of DNA for genomic blots was from leaves of mature plants as described previously (Walker *et al.* 1997). The SAH, σ1011, cDNA-B, and Scm5′ probes have been described previously (Walker *et al.* 1995; Panavas *et al.* 1999). The Lc1013 probe is derived from a PCR product extending from −470 to +71 bp relative to the end of *Lc* cDNA determined by Ludwig *et al.* (1989). The PCR primers to generate the Lc1013 fragment were oR61 (5′ TCCCAACCATCATCAACTCGCTAGCCAAACACA 3′) and oR-3′-3. Southern blotting was performed as described previously (Robbins *et al.* 1989).

RESULTS

***r1* geographic haplotypes:** The set of haplotypes used in this study was first described by Van Der Walt and Brink (1968), who characterized a diverse collection of colored aleurone haplotypes, collected mainly from South, Central, and North America. Van Der Walt and Brink characterized each haplotype as being paramutagenic, paramutable, or neutral. The haplotypes are referred to generically as *R-n:geographic*. The collection, which had been introgressed into standard W22 background, was kindly provided to us by Jerry Kermicle. Paramutability was judged on the basis of reduction in aleurone pigmentation of *R-n:geographic* kernels in outcrosses from *R-n:geographic/R-mb* heterozygotes. Comparison was made to *R-n:geographic* kernels outcrossed from *R-n:geographic/* r_ heterozygotes, in which paramutation does not occur. Paramutagenicity was assessed by the capacity of each *R-n:geographic* haplotype to cause a reduction in pigmentation of *R-r:std* kernels in outcrosses from *R-n:geographic/R-r:std* heterozygotes. *R-r:std* kernels from outcrosses of r_/*R-r:std* heterozygotes were used as controls for full *R-r:std* pigmentation. It should be noted that paramutagenic strength and paramutability were not measured quantitatively for this study. Instead, whole ears were compared to appropriate control ears and scored qualitatively as "full color," "medium," "light," or "colorless." In a few cases, kernels were stripped from the ears, sorted, and scored on a seven-point color scale to resolve potential weak color changes, as described previously (Panavas *et al.* 1999). In every case, the suspected small changes related to haplotypes that were classified as neutral and, in every case, there was no statistically significant difference in color between control and experimental ears. No exceptions to Van Der Walt's previous characterization were noted.

PCR-based analysis of geographic *r1* haplotypes: Five distinct promoter types have been previously identified for particular genes in the *R-r:std*, *R-st*, and *R-mb* haplotypes (Figure 1; Walker *et al.* 1995; Matzke *et al.* 1996; Panavas *et al.* 1999). These five distinct structural features are referred to here as *Lc*-like, *Sc*-like, *q*-like, *S1*-like, and *S2*-like. The 5′ portion of *Lc*-like alleles is similar at

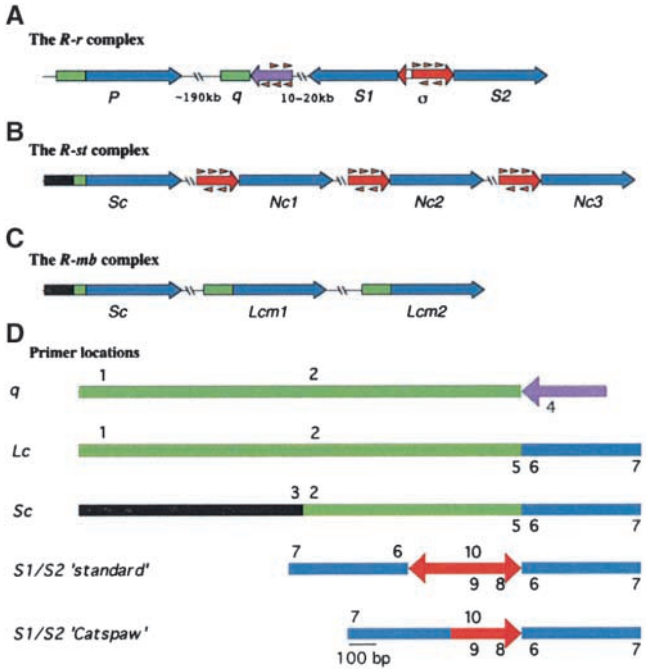

FIGURE 1.—Schematic representations of three *r1* haplotypes of known structure. (A) *R-r:std* (ROBBINS *et al.* 1991; WALKER *et al.* 1995). *r1* sequences situated upstream of the *Dop* insertion site are shown in green; sequences downstream of this site are indicated in blue. Sequences derived from the transposable element *Dop* are indicated in purple and red with 3′ *Dop* sequences in red and 5′ *Dop* sequences in purple. Small arrowheads shown above and below indicate repeat motifs found in *Dop* end sequences. The white portion of σ corresponds to the rearranged region that is not derived directly from *Dop*. (B) *R-st* (EGGLESTON *et al.* 1995; MATZKE *et al.* 1996; CHANDLER *et al.* 2000; E. L. WALKER, unpublished results). The colors are as indicated for A. Additionally, the unique upstream portion of *Sc* is indicated in black. (C) *R-mb* (PANAVAS *et al.* 1999). The colors are as indicated for A and B. (D) Positions of primers used in PCR analysis. The color scheme is identical to that used for A–C. The positions of primers are indicated by numerals. Primers that direct synthesis toward the right are indicated above each gene; primers that direct synthesis toward the left are indicated below each gene. Primer 1, oLc-5′-1; primer 2, oR-5′-2; primer 3, oSc-5′; primer 4, oQ-3′; primer 5, oR-3′-3; primer 6, oR-3′-1, primer 7, oR-3′-2; primer 8, oS1-5′-1; primer 9, oS1-5′-2; primer 10, oS2-5′-1 and oS2-5′-2 (these primers overlap).

the level of sequence to the chromosomally displaced *r1* gene, *Lc*. Members of this group include the *P* gene in *R-r:std* (Figure 1A; WALKER *et al.* 1995), the *Lcm* genes in *R-mb* (Figure 1C; PANAVAS *et al.* 1999), and the chromosomally displaced *Lc* and *Sn* genes (LUDWIG *et al.* 1989; TONELLI *et al.* 1991). *Sc*-like genes are identified on the basis of sequence similarity to the distinct upstream portion of the *Scm* gene of *R-mb* (PANAVAS *et al.* 1999). *Sc*-like genes are found in *R-mb* and *R-st* (Figure 1, B and C). Structures designated as *q*-like are similar to the *q* component of *R-r:std*, which has a *Doppia* transposable element 5′ end located downstream of an *Lc*-like promoter sequence (Figure 1A). The designation *q*-like in this study does not necessarily imply a fractured *Doppia* element like the one found at *q* of *R-r:std*, but could in principle include an intact *Dop* element downstream of the *r1* promoter region. *S2*-like genes are produced coincident with the formation of a *q*-like allele, as they are the right end of *Doppia* juxtaposed to an *r1* coding region. The designation *S2*-like again does not imply a fractured *Dop* element. Examples of *S2*-like alleles include the *S2* gene of *R-r:std* (Figure 1A) and the *Nc* genes of *R-st* (Figure 1B; MATZKE *et al.* 1996). *S1*-like alleles have *r1* coding sequences in a position and orientation relative to a *Doppia* right end that is indicative of an *r1* gene duplication and inversion similar to that found in *R-r:std* (Figure 1A). Thus, the presence of an *S1*-like gene indicates the presence of a gene inversion.

We designed PCR primers that allowed us to amplify each specific promoter. The positions of each of these primers are shown in Figure 1D. For *Lc*-like promoters, the primers were chosen so that the 5′ primer, oLc-5′-1, is complementary to *Lc* in a region that is distinct from the *Sc* upstream sequence. It primes at a position ~1.2 kb upstream of the *Lc* transcription start site. The 3′ primer, oR-3′-1 was taken from the *Lc* sequence downstream of the position where *Doppia* is inserted in *q* and is common to all *r1* genes that have an intact coding region. Thus, the primer combination oLc-5′-1 and oR-3′-1 is specific for *Lc*-like sequences and will not amplify a product from *Sc* or from *q*. Note that the oR-3′-1 primer also primes in both *S1* and *S2*, so, theoretically this primer alone is sufficient to yield a PCR product from the *S1/S2* inversion. In practice, however, such products were never observed using only the oR-3′-1 primer, probably because hairpin formation during the reaction prevents polymerase from successfully synthesizing such fragments. *Sc*-like promoters were amplified using the 5′ primer, oSc-5′, which is specific to *Sc*, in combination with the 3′ primer oR-3′-3, which is complementary to the region common to *Scm*, *Lc*, and *q*. *q*-like structures were detected using the 5′ primer, oR-5′-2, which is common to *q* and *Lc*, in combination with the 3′ primer, oQ-3′, which is specific to *Doppia* sequences adjacent to *q* of *R-r:std*. For both *S1*-like and *S2*-like genes, two redundant primer sets were chosen. The 5′ primers for the sets (oS1-5′-1, oS1-5′-2, oS2-5′-2, and oS2-5′-1) were chosen from the *Dop*-derived portion of σ. The 3′ primers (oR-3′-1 and oR-3′-2) were chosen from *r1* transcribed sequences downstream of the *Doppia* insertion site.

PCR products were analyzed on agarose gels. In almost all cases, either a single band or a blank lane was observed. When more than one band was observed or when a product of unexpected size was observed, the fragments were isolated from the gel and sequenced to confirm their identity.

Table 1 summarizes the results of the PCR analysis. All paramutable haplotypes were positive for *q*, *S1*, and *S2*. Furthermore, because overlapping *S1* and *S2* prod-

TABLE 1

Summary of PCR data

	Sc: oSc-5′ oR-3′-3 (kb)	*Lc*		*q*: oLc-5′-2 oQ-3′ (kb)	*S1*		*S2*		*S1 Catspaw*: oR3′-1 oR3′-2 (kb)
		oLc-5′-1 oR-3′-1 (kb)	*Nde*I digestion (kb)		oS1-5′-1 oR-3′-1 (kb)	oS1-5′-2 oR-3′-2 (kb)	oS2-5′-2 oR-3′-1 (kb)	oS2-5′-1 oR-3′-2 (kb)	
				Paramutable					
R-r:India PI 166163	—	1.5	1.0, 0.4, 0.1	0.85	0.5	0.8	0.2	0.6	—
R-r:India PI 210551	—	1.5	1.0, 0.4, 0.1	0.85	0.5	0.8	0.2	0.6	—
R-r:Kansas PI 222629	—	1.5	1.0, 0.4, 0.1	0.85	0.5	0.8	0.2	0.6	—
R-r:Missouri PI 222889	—	1.5	1.0, 0.4, 0.1	0.85	0.5	0.8	0.2	0.6	—
R-r:Oklahoma PI 213748	—	1.5	1.0, 0.4, 0.1	0.85	0.5	0.8	0.2	0.6	—
R-g:New Mexico PI 218148	—	—		0.85	0.5	0.8	0.2	0.6	—
R-r:Turkey PI 167989	—	—		0.85	0.5	0.8	0.2	0.6	—
R-g:Argentina PI 162573	—	—		0.85	0.5	0.8	0.2	0.6	—
R-g:Arizona PI 213729	—	—		0.85	—	0.4	0.2	0.6	0.5
R-g:Arizona PI 213738	—	—		0.85	—	0.4	0.2	0.6	0.5
R-g:Arizona PI 218175	—	—		0.85	—	0.4	0.2	0.6	0.5
R-g:Arizona PI 218178	—	—		0.85	—	0.4	0.2	0.6	0.5
R-g:Canada PI 214199	—	—		0.85	—	0.4	0.2	0.6	0.5
R-g:N. Dakota PI 213799	—	—		0.85	—	0.4	0.2	0.6	0.5
R-g:New Mexico PI 218170	—	—		0.85	—	0.4	0.2	0.6	0.5
R-g:S. Dakota PI 213779	—	—		0.85	—	0.4	0.2	0.6	0.5
				Paramutagenic					
R-g:Bolivia 724	—	1.5	1.4, 1.0, 0.4, 0.1	—	—	—	0.2	0.6	—
R-g:Bolivia 1520	0.9	—		—	—	—	0.2	0.6	—
R-g:Chile 370	0.9	—		—	—	—	0.2	0.6	—
R-g:Chile 406	—	1.5	1.4, 1.0, 0.4, 0.1	—	—	—	—		—
R-g:Peru 568	—	1.5	1.4, 1.0, 0.4, 0.1	—	—	—	—		—
R-g:Peru 1304-2993	0.9	—		—	—	—	0.2	0.6	—
R-g:Peru San Miguel	0.9	1.5	1.0, 0.4, 0.1	—	—	—	0.2	0.6	—
				Neutral					
R-r:Ecuador 1172	0.9	1.5, 1.3	1.4, 1.2, 0.1	—	—	—	—	—	—
R-g:Peru Corongo ANC150	0.9	1.5	1.0, 0.4, 0.1	—	—	—	—	—	—
R-g:Bolivia 1004	0.9	1.5	1.4, 0.1	—	—	—	—		—
R-g:Bolivia 716-6759	—	1.5	1.0, 0.4, 0.1	—	—	—	—	—	—

Genomic DNA from plants homozygous for each *r1* haplotype was amplified with the indicated primer sets. Sizes of amplified products are indicated. A dash indicates that no product was produced. Products from primer pair oLc-5′-1 and oR-3′-1 were digested with *Nde*I to test for amplification from multiple gene copies.

ucts were amplified in all cases, we conclude that all paramutable haplotypes contain the duplication/inversion characterized by the *S*-subcomplex of *R-r:std*. Five of the paramutable haplotypes (*R-r:India PI 166163*, *R-r: India PI 1210551*, *R-r:Kansas PI 222629*, *R-r:Missouri PI 222889*, and *R-r:Oklahoma PI 213748*) had an *Lc*-like gene in addition to *S*-subcomplex structures. These five haplotypes also gave *S1* and *S2* amplification products identical to those of *R-r:std* (not shown); they appear to be very similar to *R-r:std*. Three other haplotypes (*R-g: New Mexico PI 218148*, *R-r:Turkey PI 167989*, and *R-g: Argentina PI 162573*) had *S1* and *S2* amplification products identical to those of *R-r:std*, but did not appear to contain an *Lc*-like component.

In 8 out of 16 paramutable haplotypes (*R-g:Arizona PI 213729*, *R-g:Arizona PI 213738*, *R-g:Arizona PI 218175*, *R-g:Arizona PI 218178*, *R-g:Canada PI 214199*, *R-g:N. Dakota PI 213799*, *R-g:New Mexico PI 218170*, and *R-g:S. Dakota PI 213779*) no *S1*-specific PCR products were generated using the primer set oS1-5′-1 and oR-3′-1. When the redundant primer set oS1-5′-2 and oR-3′-2 was used, *S1*-specific products were observed, but they were 0.4 kb smaller than expected based on the sequence of the *S1* gene of *R-r:std*. We sequenced two of these smaller PCR products from haplotypes *R-g:Arizona 213738* and *R-g:New Mexico 218170*. The two sequences were identical and had a 469-bp deletion relative to *R-r: std*. At the location of the deletion, a novel portion consisting of 91 bp of rearranged *Doppia* transposable element sequence was present. The deletion involves 166 bp of σ and 303 out of 306 bp of the 5′ nontranslated region of *S1*. A GenBank search revealed a perfect match with the *r1* haplotype *R-d:Catspaw* (GenBank U93178). Thus, we refer to these haplotypes as "Cats-

paw" type. We predicted that Catspaw types, which are missing a portion of the *S1/S2* inversion owing to their truncated *S1* gene, should be amplified by a set of *R* primers (oR3′-1 and oR3′-2). Amplification was predicted from the oR3′-2 site in *S1* to the oR3′-1 site in *S2* (see Figure 1) because, owing to the missing 5′ portion of *S1* in Catspaw types, no hairpin will be formed in such a product. Amplification of this fragment was successful for all Catspaw haplotypes. The amplification of these products also confirms the presence of a gene inversion in these haplotypes, which cannot otherwise be established for Catspaw types owing to the absence of amplification with oS15′-1 and oR3′-1.

None of the paramutagenic or neutral haplotypes contained either *q*- or *S1*-like genes, suggesting the absence of a gene inversion within these haplotypes. All paramutagenic haplotypes contained at least two genes, but there was no strict correlation of gene identity with paramutagenicity. All three of the other gene types (*S2*-like, *Lc*-like, and *Sc*-like) were detected in paramutagenic haplotypes. *Sc*-like genes were found in four of the seven paramutagenic haplotypes. *R-g:Bolivia 724*, *R-g:Bolivia 568*, and *R-g:Chile 406* are the first naturally occurring paramutagenic haplotypes that we are aware of that do not contain an *Sc*-like gene.

To address whether multiple *Lc*-like genes might have been amplified from a single haplotype in which *Lc*-like products were detected, the PCR products were tested for *Nde*I restriction site polymorphisms. Such polymorphisms were detected between the *Lcm1* and *Lcm2* genes of *R-mb* (Panavas *et al.* 1999). When a homogeneous 1.5-kb *Lc*-specific PCR product is digested with *Nde*I, either two fragments (1.4 and 0.1 kb), *e.g.*, *R-g:Bolivia 1004*, or three fragments (1.0, 0.4, and 0.1 kb), *e.g.*, *R-g:Peru San Miguel*, are observed. The presence of four restriction fragments (1.4, 1.0, 0.4, and 0.1 kb) after *Nde*I digestion of *Lc*-specific PCR products indicates heterogeneity of the PCR product and suggests that amplification occurred from at least two different templates. The *Nde*I restriction analysis indicates that haplotypes *R-g:Bolivia 724*, *R-g:Chile 406*, and *R-g:Peru 568* have at least two *Lc*-like genes.

All four neutral haplotypes tested had at least one *Lc*-like gene. One of the *Lc*-like genes of *R-r:Ecuador 1172* has a 214-bp deletion (−265 to −53 relative to the putative transcription start site; Ludwig *et al.* 1989) as revealed by sequence analysis of its unexpectedly small PCR product. This 0.2-kb size difference between two *Lc*-like fragments from *R-r:Ecuador 1172* was confirmed by Southern blot analysis described below. *R-r:Ecuador 1172*, *R-g:Bolivia 1004*, and *R-g:Peru Corongo ANC150* also have an *Sc*-like gene. In terms of gene number, the neutral haplotypes *R-r:Ecuador 1172*, *R-g:Bolivia 1004*, and *R-g:Peru Corongo ANC150* are not strictly different from paramutagenic haplotypes, since they appear by this analysis to contain either three or two genes. Only one neutral haplotype in the collection (*R-g:Bolivia 716-6759*) has a single gene, making it distinct from the paramutagenic haplotypes analyzed here.

Genomic blot analysis of geographic *r1* haplotypes: Equal amounts of genomic DNA from each haplotype were digested with *Hin*dIII for Southern blot analysis. Each blot was hybridized sequentially with four different probes. The Lc1013 probe hybridizes to *q*-, *Lc*-, and *Sc*-like genes but not to *S*-like genes (green in Figure 1). The Scm5′ probe (Panavas *et al.* 1999) hybridizes only to *Sc*-like genes and does not detect *q*-, *Lc*-, or *S*-like genes (black in Figure 1). The SAH probe is derived from transcribed regions and recognizes all *r1* genes that have a coding region (blue in Figure 1). Thus, it recognizes all but *q*-like genes. The σ1011 probe is specific for the rearranged part of σ at *R-r:std* (white in Figure 1). The Catspaw-type *S*-subcomplexes, which are missing this region, do not hybridize to σ1011 probe nor do *Lc*-, *q*-, and *Sc*-like genes.

Sc-like genes are distinguished by hybridization to Scm5′ probe and also are expected to hybridize to Lc1013 and SAH probes, presuming that downstream sequences are also present. All three probes are expected to hybridize to a single *Hin*dIII fragment from a given *Sc*-like gene and, thus, a band of a particular molecular weight that hybridizes to all three of these probes is judged to be *Sc*-like. *Lc*-like genes hybridize to Lc1013 and SAH, but not to the Scm5′ probe. Again, both probes are expected to hybridize to the same *Hin*dIII fragment. *q*-Like structures are expected to hybridize only to Lc1013 probe since they have an *Lc*-like promoter but no coding region immediately downstream. The *S1/S2* inversion is recognized using the σ1011 and SAH probes, which detect a single *Hin*dIII fragment that contains the 5′ ends of both *S*-like genes together with the intervening σ region. Note that if the rearranged part of σ is missing, as in haplotypes with Catspaw-type *S*-subcomplexes, no hybridization to σ1011 will occur. Furthermore, the σ1011 probe does not hybridize to the *Nc* genes of *R-st* (not shown) and thus is not expected to hybridize to any derivatives that lack the *S1/S2* inversion. The *S* genes of Catspaw-type haplotypes should exhibit a *Hin*dIII fragment that is 0.4 kb smaller than that of *R-r:std*, when detected with the SAH probe. Finally, *S2*-like genes that are not part of an inversion complex will hybridize to the SAH probe, but not to any of the other probes used in this analysis.

Table 2 summarizes the Southern blot analyses. For each paramutable haplotype, the Southern analysis confirmed the gene compositions determined by PCR. In the Catspaw-type *S*-subcomplexes (*R-g:Arizona 213729*, *R-g:Arizona 213738*, *R-g:Arizona 218175*, *R-g:Arizona 218178*, *R-g:Canada 214199*, *R-g:N. Dakota 213799*, *R-g:New Mexico 218170*, and *R-g:S. Dakota 213779*) the *S1/S2* fragment (detected by the SAH probe) is 0.4 kb smaller than that of the *R-r:std* types. This result is consistent with the PCR data showing a 0.4-kb deletion in these haplotypes. Furthermore, the Catspaw-type *S*-subcomplexes did not

TABLE 2

Summary of genomic blot data

	Hybridizing fragments (kb)				Inferred gene identities	Gene no.
	SAH	Lc1013	Scm5′	σ1011		
			Paramutable			
R-r India PI 166163	4.0, 5.0	3.5, 4.0	—	5.0	P,[a] q, S1, S2	4
R-r India PI 210551	4.0, 5.0	3.5, 4.0	—	5.0	P, q, S1, S2	4
R-r Kansas PI 222629	4.0, 5.0	3.5, 4.0	—	5.0	P, q, S1, S2	4
R-r Missouri PI 222889	3.7, 5.0	3.5, 3.7	—	5.0	Lc,[b] q, S1, S2	4
R-r Oklahoma PI 213748	4.0, 5.0	3.5, 4.0	—	5.0	P, q, S1, S2	4
R-g New Mexico PI 218148	5.0	3.5	—	5.0	q, S1, S2	3
R-r Turkey PI 167989	5.0	3.5	—	5.0	q, S1, S2	3
R-g Argentina PI 162573	5.0	3.5	—	5.0	q, S1, S2	3
R-g Arizona PI 213729	4.6	3.9	—	—	q, S1(Catspaw),[c] S2	3
R-g Arizona PI 213738	4.6	3.9	—	—	q, S1(Catspaw), S2	3
R-g Arizona PI 218175	4.6	3.9	—	—	q, S1(Catspaw), S2	3
R-g Arizona PI 218178	4.6	3.9	—	—	q, S1(Catspaw), S2	3
R-g Canada PI 214199	4.6	3.9	—	—	q, S1(Catspaw), S2	3
R-g N. Dakota PI 213799	4.6	3.9	—	—	q, S1(Catspaw), S2	3
R-g New Mexico PI 218170	4.6	3.9	—	—	q, S1(Catspaw), S2	3
R-g S. Dakota PI 213779	4.6	3.9	—	—	q, S1(Catspaw), S2	3
			Paramutagenic			
R-g Bolivia 724	3.5, 3.7 × 2[d]	3.7 × 2	—	—	Lc, Lc, S2	3
R-g Bolivia 1520	3.5 × 2, 4.5	4.5	4.5	—	Sc, S2, S2	3
R-g Chile 370	3.5 × 2, 4.5	4.5	4.5	—	Sc, S2, S2	3
R-g Chile 406	3.7 × 2	3.7 × 2	—	—	Lc, Lc	2
R-g Peru 568	3.7 × 2	3.7 × 2	—	—	Lc, Lc	2
R-g Peru 1304-2993	3.5, 4.5	4.5	4.5	—	Sc, S2	2
R-g Peru San Miguel	3.5, 3.7 × 2, 4.0	3.7 × 2, 4.0	3.3, 4.0	—	Sc, Sc (prom. frg.), Lc, Lc, S2	5
			Neutral			
R-r Ecuador 1172	3.5, 3.7, 4.5	3.5, 3.7, 4.5	3.3, 4.5	—	Sc, Sc (prom. frg.), Lc, Lc	4
R-g Peru Corongo ANC150	3.7, 4.5	3.7, 4.5	4.5	—	Sc, Lc	2
R-g Bolivia 1004	3.7, 4.0	3.7, 4.0	4.0	—	Sc, Lc	2
R-g Bolivia 716-6759	3.7	3.7	—	—	Lc	1

Genomic DNA from plants homozygous for the indicated haplotypes was digested with *Hin*dIII, transferred to filters, and hybridized with the probes indicated. The sizes (in kilobases) of the hybridizing fragments are shown. Dashes indicate no hybridization to a probe. The identities of the alleles inferred from these genomic blotting data are shown, and the gene number inferred from both these blotting data and PCR analyses is shown.

[a] *Lc*-like genes having a 4.0-kb *Hin*dIII fragment appear most like the *P* gene of *R-r:std* and so have been assigned as *P*.

[b] *Lc*-like genes having a 3.7-kb *Hin*dIII fragment appear most like the *Lc* gene and so have been assigned as *Lc*.

[c] The S1/S2 genes are similar to those of *R-d:Catspaw*.

[d] The ''× 2'' mark indicates that a particular band was twice as intense as expected, indicating the presence of two genes.

hybridize to the σ1011 probe (as expected), while the standard type *S*-subcomplexes each had a 5.0-kb *Hin*dIII fragment that hybridized to this probe. The *q* fragment in each of these Catspaw-type haplotypes is 0.4 kb larger than that in *R-r:std*, which again indicates their similarity to each other and divergence from the *R-r:std* type of *S*-subcomplex. The *Hin*dIII fragments from *Lc*-like genes migrated either at 4.0 kb (similar to the *P* gene of *R-r:std*) or at 3.7 kb (similar to the *Lcm1* and *Lcm2* genes of *R-mb*). The *S2*-like genes that are not adjacent to an inverted *S1* copy are represented by 3.5-kb *Hin*dIII fragments. The *Sc*-like genes were represented by 4.5-, 4.0-, or 3.3-kb bands. The 3.3 kb band observed in *R-g:Peru San Miguel* and *R-r:Ecuador 1172* hybridized exclusively to Scm5′ but not to the downstream probes Lc1013 and SAH. It is possible, but unlikely, given the lack of hybridization to the Lc1013 probe, that this fragment could represent an *Sc* promoter with a *Dop* 5′ end adja-

cent. This arrangement could come about, for example, through recombination between a *q* gene and an *Sc* gene. (The relevant region of homology is shown in green in Figure 1.) To test this possibility, PCR reactions were performed using the primer combination oSc-5′ and oQ-3′, but no products were observed. This cannot conclusively demonstrate the absence of an *Sc/Dop* configuration, however, since we cannot be certain whether primer sites are present in the genes in question. We can state that *R-g:Peru San Miguel* and *R-r:Ecuador 1172* appear to contain an additional *Sc*-like fragment that lacks downstream promoter and coding sequences and that was not detected using PCR.

In addition to determining gene identity, we evaluated the number of genes present in each haplotype. Since an equal amount of DNA was used in each lane, it was possible to judge the gene copy number even in cases with comigrating fragments. The intensity of the 3.7-kb band hybridizing to SAH and Lc1013 probes indicates that paramutagenic haplotypes *R-g:Bolivia 724*, *R-g:Chile 406*, *R-g:Peru 568*, and *R-g:Peru San Miguel* contained two *Lc*-like genes. We also found that *R-g:Bolivia 1520* and *R-g:Chile 370* have two *S2*-like genes. The presence of at least two *S2*-like genes in *R-g:Chile 370* was confirmed by sequence polymorphism of the cloned *S2* I-PCR fragments from this genotype (see description below).

I-PCR analysis of *S2*-like genes from paramutagenic haplotypes: We used an inverse PCR (I-PCR) approach to investigate the upstream sequences in the *S2* genes of paramutagenic haplotypes. Genomic DNA from *R-g:Bolivia 724*, *R-g:Bolivia 1520*, *R-g:Chile 370*, and *R-g:Peru 1304-2993* was digested with *Hin*dIII and the fragments ranging from 2.5 to 4.5 kb were purified, self-ligated, and used as templates in I-PCR reactions. The primers were chosen so that the unknown 5′ portion of *S2* genes would be amplified. A band of the expected size (1.5 kb) was abundant in the I-PCR reactions from all four genotypes, and direct sequencing of the ends of these PCR products indicated that all four products are essentially identical in sequence. The I-PCR fragments from *R-g:Bolivia 724* and *R-g:Chile 370* were cloned for complete sequencing; however, this analysis was complicated by the presence of an ~150-bp region with strong secondary structure, which was never successfully sequenced. However, 1021 bp of sequence from the 5′ ends and 312 bp of sequence from the 3′ ends were obtained. The *Dop* insertion site in the *S2* genes from *R-g:Bolivia 724* and *R-g:Chile 370* is identical to that of the *S1* and *S2* genes of *R-r:std*. As in *R-r:std*, the terminal inverted repeat of *Dop* is missing its last 5 bp, suggesting that this sequence variation was present in the progenitor of all these haplotypes. Sequence similarity to the *S2* gene of *R-r:std* continues for 221 bp of *Dop*-derived (σ) sequence (Figure 2). The *S2* genes of *R-g:Bolivia 724* and *R-g:Chile 370* contain additional *Dop* sequences that are not present in the *S2* genes of either standard or Catspaw-type

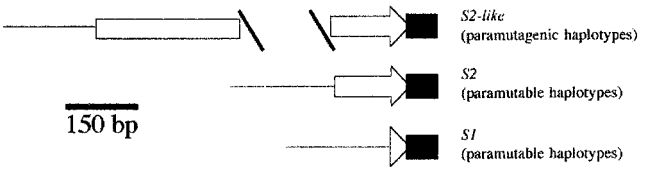

FIGURE 2.—Extent of *Dop* sequences at *S2*-like alleles. *r1* sequences are indicated in black; *Dop* sequences are represented in white; horizontal lines represent DNA that is not apparently derived from either *r1* or *Dop*. Shown are schematic representations of the *S1* and *S2* genes from *R-r:std* and an *S2*-like gene from the paramutagenic haplotype *R-g:Chile 370*. The 5-bp terminal truncation of *Dop* sequences at the *Dop/r1* junction is represented by the imperfect arrowhead. The ~150-bp unsequenced region of the *S2*-like gene of *R-g:Chile 370* is indicated by hash marks.

paramutable haplotypes. Additional *Dop* subterminal repeat element copies are present, and the terminal 269 bp of the transcribed portion of the *Dop*-encoded protein DOPA are also present. Upstream of the *Dop* sequences in *R-g:Bolivia 724* and *R-g:Chile 370*, there is a region that shares similarity to upstream sequences at the maize *adh1* locus (GenBank AF123535; TIKHONOV *et al.* 1999). In all, we estimate that the *S2* genes of *R-g:Bolivia 724* and *R-g:Chile 370* contain ~750 bp of sequence that is derived from the *Dop* 3′ end. Thus, the *S2* genes found in paramutagenic haplotypes have, in their 5′ ends, additional *Dop* sequences not found in the σ regions of paramutable haplotypes, but do not appear to contain intact *Dop* elements.

Structural summary: The overall structural summary derived from combined PCR, Southern blot, and sequencing data is shown in Table 2. There is a strict correlation between paramutability and the presence of two structural features: a *q*-like gene and a gene inversion comprising *S1*- and *S2*-like genes. The presence or absence of an *Lc*-like gene did not correlate with paramutability, which is consistent with previous studies showing that the *P* gene of *R-r:std* is not necessary for its paramutability (BROWN 1966). The *S*-subcomplexes of paramutable haplotypes fall into two categories: some are like *R-r:std* in that they have two intact *S* genes, and others are similar to *R-d:Catspaw* in having a single intact *S* gene (*S2*) and a missing portion of the 5′ noncoding sequences of the *S1* gene.

The structure of paramutagenic haplotypes is more variable. Notably absent are *q*- or *S1*-like genes. Another common feature is that all paramutagenic haplotypes comprise at least two genes. Haplotypes containing up to five repeated components were identified. None of the paramutagenic haplotypes appeared to contain inverted gene copies, nor did any contain *q*-like genes. *S2*-like genes, however, were common.

Three of the four neutral haplotypes analyzed appeared to be structurally similar to paramutagenic haplotypes. These contained two or four genes that were either *Lc*-like or *Sc*-like. One neutral haplotype, *R-g:Boli-*

via 716-6759, contained only a single *Lc*-like gene. None of the four neutral haplotypes had *S2*-like genes, nor did any contain features (gene inversion, *q*-like genes) associated with the *S*-subcomplex.

Cytosine methylation in *r1* geographic haplotypes: Because no physical structures were identified that distinguished paramutagenic haplotypes from neutral haplotypes, we examined whether there are differences in epigenetic features (*i.e.*, chromatin level differences) that distinguish these two classes of haplotypes. To address this issue, we examined the pattern of C-methylation present in each *r1* haplotype. We used the methylation-sensitive restriction enzyme *Hpa*II to assess methylation of cytosine residues in CCGG sites within *r1* genes. *Hpa*II is sensitive to the methylation of either of the C residues within its recognition site. *Msp*I, an isoschizomer of *Hpa*II, was used to judge the presence of restriction sites. *Msp*I is less sensitive to C-methylation and though it does not cut when the first C in its recognition site is methylated, it does cut when the second C in the CCGG site is methylated. *Hpa*II/*Msp*I sites were predicted on the basis of sequences of well-characterized *r1* alleles from the *R-r:std* and *R-mb* haplotypes. The presence of each predicted *Hpa*II/*Msp*I site was confirmed in one of two ways. In many cases, *Msp*I cleavage was detected, confirming the presence of a particular CCGG site. For those cases in which *Msp*I did not cut, or when *Msp*I cutting could not be detected on genomic blots due to the location of the probe, the relevant portion of the gene was amplified by PCR (or I-PCR, in the case of *S2*-like genes from paramutagenic haplotypes, see above) and digested to map these sites. The predicted sites were present in all cases (not shown). Genomic DNA of each geographic haplotype was digested with the methylation-insensitive enzyme *Hin*dIII and also with a combination of *Hin*dIII and *Hpa*II or *Hin*dIII and *Msp*I. The blots were hybridized sequentially with the SAH and Lc1013 probes.

Maps showing the *Hin*dIII and *Hpa*II/*Msp*I sites in the various *r1* genes of the haplotypes are shown in Figures 3A, 4A, and 5A. There are two *Hpa*II/*Msp*I sites present close together in the large second intron of typical *r1* genes. These are referred to as "intronic *Hpa*II sites." In *Lc*-like genes there are two *Hpa*II/*Msp*I sites that lie just upstream (39 and 141 bp, respectively) of the transcription start site (Ludwig *et al.* 1989; Tonelli *et al.* 1991; Consonni *et al.* 1992, 1993). *Sc*-like genes also contain these two sites, but their position relative to the start of transcription is not known, since the transcription start site of *Sc* has not been reported. In *S* genes, only one of the two *Hpa*II/*Msp*I sites is present, because σ sequences have replaced the upstream portion containing one of these two sites. Multiple transcription start sites have been mapped for the *S1* and *S2* genes of *R-r:std* (May and Dellaporta 1998), but because the transcribed portions of *S1* and *S2* are identical in this region, it is not possible to know whether *S1*, *S2*, or both genes initiate transcription at a particular site. These uncertain transcription start sites are indicated as gray arrows in Figure 4A. There is one start site within σ that must be used by the *S2* gene. The *Hpa*II/*Msp*I sites at this position of all genes are referred to as "transcription start *Hpa*II/*Msp*I sites," regardless of whether the precise transcription start is known. Finally, there are *Hpa*II/*Msp*I sites that are present upstream of the transcription start *Hpa*II/*Msp*I sites, which are referred to as "promoter *Hpa*II/*Msp*I sites." It is important to note that, in contrast to the aforementioned *Hpa*II/*Msp*I sites, which are common to all *r1* genes, the promoter *Hpa*II/*Msp*I sites may be at nonhomologous positions in the various *r1* gene types.

Paramutagenic haplotypes generally showed heavy methylation of the *Hin*dIII fragments that include the promoters, the first two exons, the first intron, and 1.7 kb of the large second intron (Figure 3B). Haplotypes *R-mb*, *R-g:Bolivia 724*, *R-g:Bolivia 1520*, *R-g:Chile 370*, *R-g:Peru San Miguel*, and *R-g:Chile 406* were very heavily methylated at all *Hpa*II sites, judging by the presence of a major fraction of undigested 3.7- or 3.5-kb *Hin*dIII fragments that represent either *Lc*- or *S2*-like genes, respectively. The partial digestion observed with *Msp*I demonstrates the presence of *Hpa*II/*Msp*I sites within these fragments. The larger *Hin*dIII fragments from *Sc*-like genes in haplotypes *R-g:Bolivia 1520*, *R-g:Chile 370*, and *R-g:Peru San Miguel* disappear after digestion with *Hpa*II and *Msp*I, indicating that they contain unmethylated sites. However, because complete sequence information is not available for *Sc*-like genes and because many *Sc*-like fragments comigrate with *Lc*- or *S2*-like fragments, it is not possible to assess the methylation status of individual *Hpa*II/*Msp*I sites in *Sc*-like genes.

In *R-mb*, *R-g:Bolivia 724*, *R-g:Bolivia 1520*, *R-g:Chile 370*, *R-g:Peru San Miguel*, and *R-g:Chile 406*, we detected very few or no *Hpa*II-digested fragments using the SAH (Figure 3B) and Lc1013 (not shown) probes, which indicates that *r1* genes in these haplotypes are heavily methylated at promoter, transcription start, and intronic sites. *R-g:Peru 568* and *R-g:Peru 1304-2992* are methylated less heavily. Very little or none of the undigested 3.7- or 3.5-kb *Hin*dIII fragment was observed after digestion with *Hpa*II. However, the absence of 0.9-kb bands and the presence of 2.6- or 2.4-kb fragments following *Hpa*II digestion indicate that the promoter and transcription start *Hpa*II/*Msp*I sites in the *Lc*-like and *S2*-like genes of these haplotypes are methylated. It is the intronic sites that are unmethylated in these two haplotypes. Thus, paramutagenic haplotypes are methylated at sites in their promoters and near the start of transcription. Downstream sites present in the large intron appear to be methylated in many paramutagenic haplotypes, but are clearly unmethylated in others.

Paramutable haplotypes are markedly less methylated than paramutagenic haplotypes (Figure 4B). There were no intact *Hin*dIII fragments following *Hpa*II or *Msp*I

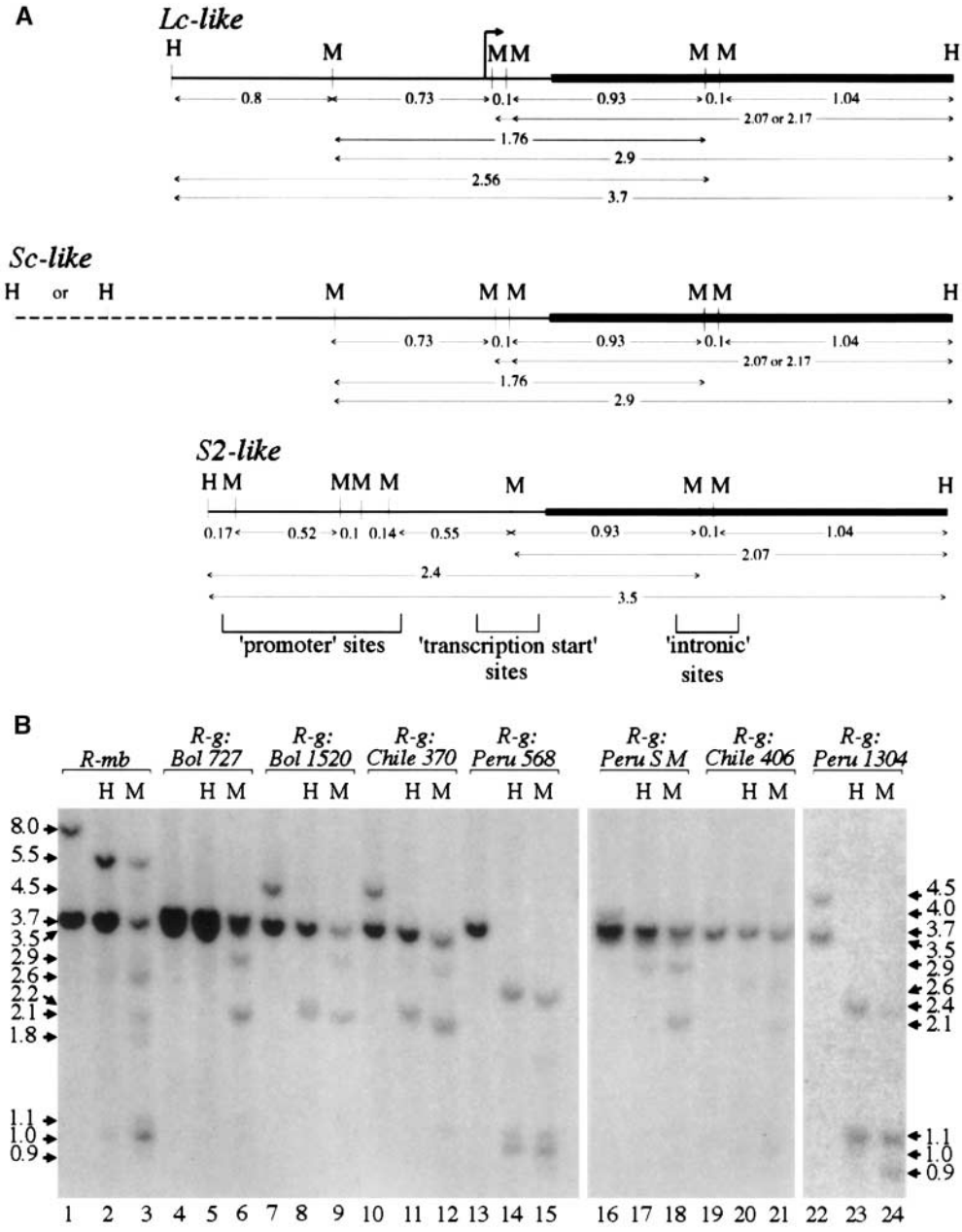

FIGURE 3.—Cytosine methylation tests of the 5′ ends of *r1* genes from paramutagenic haplotypes. (A) Maps of the gene types (*Sc*-like, *Lc*-like, *S2*-like) found in paramutagenic haplotypes. M, *Hpa*II/*Msp*I; H, *Hin*dIII. The position and extent of the SAH probe is represented by black rectangles. Sizes of the predicted restriction fragments that result from complete digestion and sizes of the partial digestion products observed on genomic blots are indicated below the map. In the map of *Sc*-like genes, the solid line represents cloned sequences; the dashed line shows an uncloned region. Three sets of *Hpa*II/*Msp*I sites are indicated: promoter, transcription start, and intronic. (B) Southern blots of paramutagenic haplotypes. The genomic DNA was digested with *Hin*dIII alone (lanes 1, 4, 7, 10, 13, 16, 19, 22, and 25), *Hin*dIII + *Hpa*II (lanes labeled H), or *Hin*dIII + *Msp*I (lanes labeled M); separated on a 1% agarose gel; transferred to a membrane; and hybridized with the SAH probe. The molecular weights in kilobases of the fragments detected are indicated at the left and right. Contents of each lane are as follows: lanes 1–3, *R-mb;* lanes 4–6, *R-g:Bolivia 724;* lanes 7–9, *R-g:Bolivia 1520;* lanes 10–12, *R-g: Chile 370;* lanes 13–15, *R-g: Peru 568;* lanes 16–18, *R-g:Peru San Miguel;* lanes 19–21, *R-g:Chile 406;* lanes 22–24, *R-g:Peru 1304-2993.*

digestion of any of the paramutable haplotypes tested, indicating the absence of full methylation in the region of *r1* detected by the SAH probe. The 0.9-kb and 1.0-kb *Hpa*II fragments expected after complete digestion are present in all paramutable haplotypes except *R-r:std*, showing that the intronic and transcription start *Hpa*II/*Msp*I sites are hypomethylated in all cases. It should be noted that there is a discrepancy between the apparent molecular weight of full-length *S1/S2 Hin*dIII fragments on agarose gels (5.0 and 4.6 kb) and the actual molecular weights according to sequence (4.7 and 4.3 kb). The fragments consistently migrate larger than expected, perhaps because they consist of a large (~2.2 kb) inverted repeat, which may form unusual secondary structures that cause aberrant migration.

Paramutable haplotypes that have *Lc*-like genes (*R-r:std*, *R-r:India 166163*, *R-r:India 1210551*, *and R-r:Oklahoma 213748*) are more methylated than are haplotypes without *Lc*-like genes. The 1.4-kb band observed in these *Lc*-like-containing haplotypes indicates that one of the transcription start *Hpa*II/*Msp*I sites is methylated in the *Hin*dIII fragment containing *S1/S2*. It is not possible to judge whether the methylated site is at *S1* or *S2*. Because the intensity of the 1.4-kb bands in *Hpa*II and *Msp*I lanes of these haplotypes was very similar, the presence of both CCGG sites was confirmed by digesting *S1*- and *S2*-specific PCR products from these haplotypes with *Hpa*II (not shown). The 2.1-kb fragment observed in *R-r:std*, *R-r:India 166163*, and *R-r:Oklahoma 213748* indicates methylation of intronic *Hpa*II sites. This 2.1-kb fragment

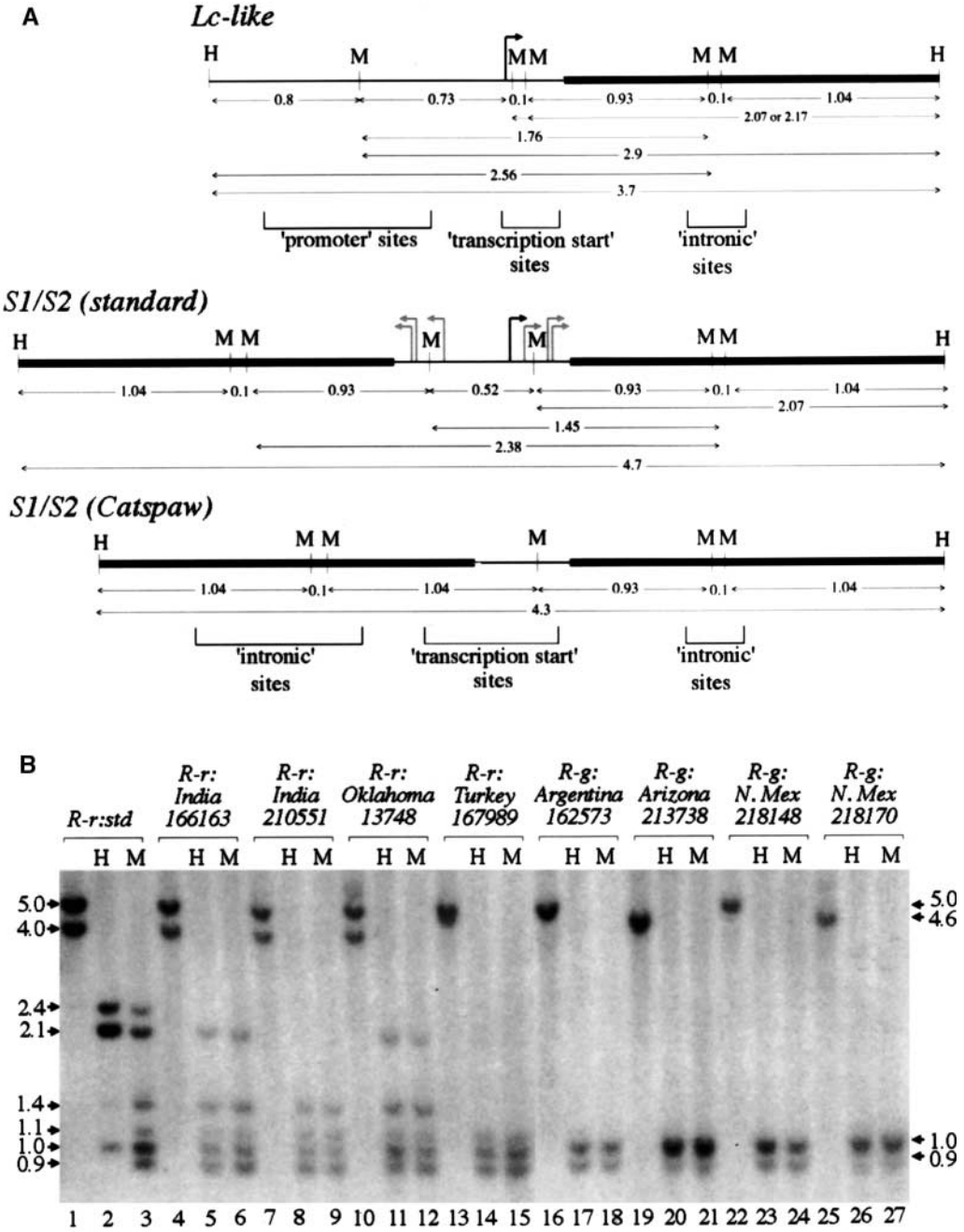

FIGURE 4.—Cytosine methylation tests of the 5′ ends of *r1* genes from paramutable haplotypes. (A) Maps of the gene types (*Lc*-like, *S1/S2*-like from *R-r:std*, and Catspaw-type *S1/S2*-like) found in paramutagenic haplotypes. The *q*-like genes of these haplotypes are not shown, as they are not detected by the SAH probe. M, *Hpa*II/*Msp*I; H, *Hin*dIII. The position and extent of the SAH probe is represented by black rectangles. Sizes of the predicted restriction fragments that result from complete digestion and sizes of the partial digestion products observed on genomic blots are indicated below the map. Three sets of *Hpa*II/*Msp*I sites are indicated: promoter, transcription start, and intronic. (B) Southern blots of paramutable haplotypes. The genomic DNA was digested with *Hin*dIII alone (lanes 1, 4, 7, 10, 13, 16, 19, 22, and 25), *Hin*dIII + *Hpa*II (lanes labeled H), or *Hin* dIII + *Msp*I (lanes labeled M); separated on a 1% agarose gel; transferred to a membrane; and hybridized with the SAH probe. The molecular weights in kilobases of the fragments detected are indicated at the left and right.

could be derived from either *Lc*-like or *S1/S2*-like genes. *R-r:std* was somewhat more methylated than the other paramutable haplotypes, judging by the lack of a 0.9-kb band, increased intensity of the 2.1-kb band, and the presence of a 2.4-kb band. The latter represents an *S1/S2* gene fragment in which methylation occurred at both transcription start *Hpa*II/*Msp*I sites.

All but one of the paramutable haplotypes that lack an *Lc*-like gene (*R-g:Argentina 162573*, *R-g:Arizona 213738*, *R-g: New Mexico 218148*, and *R-g:New Mexico 218170*) showed complete absence of methylation in the region tested. The exception is *R-r:Turkey 167989*, which exhibits a 1.1-kb band indicating methylation of one of the *Hpa*II sites within the second intron. However, the transcription start *Hpa*II/*Msp*I sites in each of these haplotypes are unmethylated.

Absence of full-length *Hin*dIII fragments following *Hpa*II digestion indicates that all four neutral haplotypes are hypomethylated (Figure 5B) relative to paramutagenic haplotypes. Some methylation was present at the intronic sites, as indicated by the presence of 1.1-kb fragments (Figure 5B, lanes 2, 5, and 11) or absence of the 0.9-kb band (Figure 5B, lane 8). The 1.1-kb bands (Figure 5B, lanes 2, 5, 11) could also result from methylation of both *Hpa*II sites defining the 0.93-kb fragment, which would indicate methylation of at least one of the

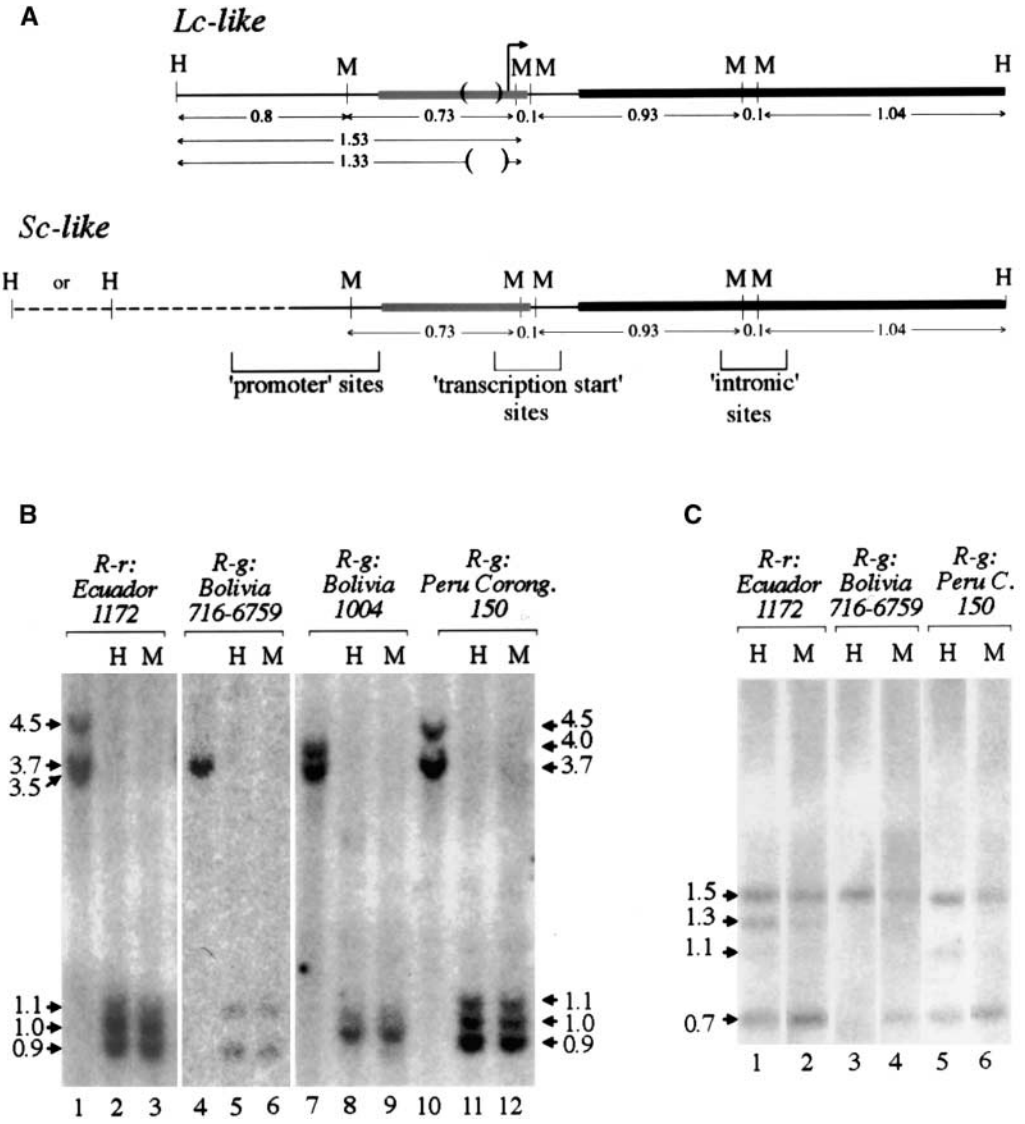

FIGURE 5.—Cytosine methylation tests of the 5′ ends of *r1* genes from neutral haplotypes. (A) Maps of the gene types (*Lc*-like, *Sc*-like) found in neutral haplotypes. M, *Hpa*II/*Msp*I; H, *Hin*dIII. The position and extent of the SAH probe is represented by black rectangles. The position and extent of the Lc1013 probe is represented by gray rectangles. One of the *Lc*-like genes in *R-r:Ecuador 1172* bears a 200-bp deletion. The extent and position of the deleted region are indicated by parentheses. Sizes of the predicted restriction fragments that result from complete digestion and sizes of the partial digestion products observed on genomic blots are indicated below the map. Three sets of *Hpa*II/*Msp*I sites are indicated: promoter, transcription start, and intronic. (B) Southern blots of neutral haplotypes. The genomic DNA was digested with *Hin*dIII alone (lanes 1, 4, 7, and 10), *Hin*dIII + *Hpa*II (lanes labeled H), or *Hin*dIII + *Msp*I (lanes labeled M); separated on 1% agarose gel; transferred to a membrane; and hybridized with the SAH probe. The molecular weights in kilobases of the fragments detected are indicated at the left and right. (C) Southern blots of neutral haplotypes using the Lc1013 probe. DNA was prepared from a new set of individual plants and was digested with either *Hin*dIII + *Hpa*II (lanes labeled H) or *Hin*dIII + *Msp*I (lanes labeled M), separated on 1% agarose gel, transferred to a membrane, and hybridized.

sites near the transcription start. However, when similar blots were probed with the Lc1013 probe (Figure 5C), no evidence for methylation near the transcription start site was observed. In particular, the lack of 0.8- or 1.8-kb bands in any of the lanes in Figure 5C demonstrates the absence of methylation of the transcription start *Hpa*II/*Msp*I sites in these haplotypes.

Blots were probed with the Lc1013 probe (Figure 5C) to indicate methylation of the promoter *Hpa*II/*Msp*I sites of three of the neutral haplotypes. The presence of the 1.5-kb band in *Hpa*II and *Msp*I lanes of all three neutral haplotypes tested shows methylation of promoter *Hpa*II/*Msp*I sites of *Lc*-like genes in these haplotypes. The 1.3-kb fragment in *R-r:Ecuador 1172* represents promoter methylation of the second *Lc*-like gene, which has a 215-bp deletion. The weak 1.1-kb band in *R-r:Ecuador 1172* and *R-g:Peru Corongo ANC150* probably indicates some methylation of the promoter of the *Sc*-like genes in these haplotypes, as it cannot have come from *Lc*-like genes. That the Lc1013 probe does overlap the 100-bp *Hpa*II fragment near the transcription start raises the concern that the 1.1-kb fragment arises from more 3′ portions of the *Lc* gene. However, the overlap is small, 25 bp, and thus is not sufficient to allow detection on maize genomic blots under the stringency conditions employed in this study. The 0.7-kb bands in *R-r: Ecuador 1172* and *R-g:Peru Corongo ANC150* indicate that the promoter sites are unmethylated in a fraction of cells. The fourth neutral haplotype tested, *R-g:Bolivia 1004*, was unmethylated at all promoter sites, as indicated by the presence of a single, 0.7-kb band following hybridization with the Lc1013 probe (not shown).

Summarizing the methylation data for neutral haplotypes makes it clear that both CCGG sites near the putative transcription start sites in each gene are hypomethylated. The *Hpa*II/*Msp*I sites located upstream in the promoters tend to be hypermethylated in these neutral haplotypes, and the intronic sites are likewise frequently methylated. Thus, while the transcription start *Hpa*II/*Msp*I sites are generally hypermethylated in paramutagenic haplotypes, these same sites are always hypomethylated in neutral haplotypes. The methylation status of these sites near the transcription start is the most consistent molecular correlate for paramutagenicity *vs.* neutrality that we have identified.

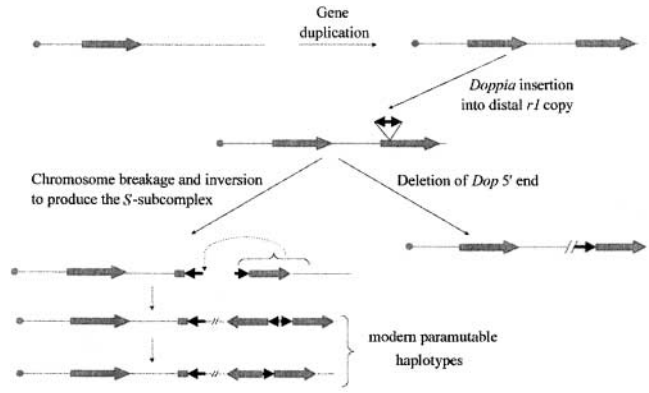

FIGURE 6.—Model of the events that formed the known *r1* genes. Following an initial duplication event, a *Dop* transposable element inserted into the distal copy. Two separate lineages were derived from this haplotype: one in which the 5′ (proximal) end of *Dop* was lost that resulted in the *S2*-like genes in some paramutagenic complexes and a second in which a duplication and inversion occurred that gave rise to the *S*-subcomplex found in all known paramutable haplotypes.

DISCUSSION

We have examined the composition of 26 complex haplotypes and 1 simple haplotype of the *r1* locus of maize. Sixteen of the haplotypes examined are paramutable, and all 16 share a characteristic structure, described as an *S*-subcomplex, that includes the gene fragment *q* and two *S* genes that form a large, head-to-head inverted repeat. No neutral or paramutagenic complex has an *S*-subcomplex. Thus, paramutable *r1* complexes are structurally quite similar to each other and are structurally quite distinct from nonparamutable complexes. Two distinct types of *S*-subcomplex were identified. One of these (the "standard" type) is indistinguishable from that of *R-r:std*. The other (the Catspaw type) is indistinguishable from the *S*-subcomplex of *R-d:Catspaw*. The σ region of Catspaw haplotypes lacks the non-*Dop*-derived "rearranged region" that is present in σ from *R-r:std*. This rearranged region contains a 162-bp segment bearing 85% sequence identity to the promoter regions of simple *Le*-like *r1* genes. Models in which this *r1* promoter-homologous region of σ is critical for paramutability are thus ruled out by these findings.

Other conserved structural features, such as the presence of a large *S1/S2* inversion or *Dop*-derived sequences at *q* or at σ, may be necessary for paramutability, but such an interpretation must be made with caution. The structural similarity of paramutable haplotypes may merely reflect a recent origin for paramutability, which arose in complexes that have this structure. The behavior of a mutant derivative allele of *R-r* called *r-r:N1-3-1* argues against an important role for *q* or the large inverted repeat as determinants of paramutability. This derivative has a nearly precise deletion of σ but leaves *q* and the *S1/S2* inversion intact (WALKER *et al.* 1995). The *r-r:N1-3-1* derivative has lost both *S* gene promotors, which are normally contained within σ. This means that paramutability of this derivative haplotype cannot be judged by a simple color assay, but has instead been assayed by examining two additional changes normally associated with paramutability (KERMICLE 1996; WALKER 1998). The first such change is the acquisition of "secondary paramutagenicity," which refers to the fact that paramutant *r1* haplotypes themselves become weakly paramutagenic. The second change that is typically associated with paramutability is acquisition of cytosine methylation during paramutation. The *r-r:N1-3-1* derivative is only weakly able to acquire secondary paramutagenicity and does not acquire C-methylation during paramutation. This means that loss of the σ region alone changes the response of this haplotype to paramutation. The impaired paramutability of the *r-r:N1-3-1* derivative may result from lack of transcriptional activity of the genes or could reflect a role for transposon-derived σ sequences. Either way, paramutability was partially lost in spite of the presence of both *q* and the large *S1/S2* inversion, indicating that these features alone do not explain paramutability.

The genetic compositions of paramutagenic and neutral haplotypes were much more variable. Paramutagenic haplotypes can include various combinations of three *r1* gene types: *Le*-like, *Sc*-like and *S2*-like. A variety of distinct gene combinations were found among paramutagenic and neutral haplotypes. The gene number in paramutagenic haplotypes was also variable, ranging from two to five. This variability is consistent with previous studies in which the gene number of paramutagenic haplotypes was experimentally manipulated through unequal crossing over (EGGLESTON *et al.* 1995; KERMICLE *et al.* 1995; PANAVAS *et al.* 1999). Both these features are most simply accounted for by the well-known tendency of complex *r1* haplotypes to undergo unequal crossing over that can lead to expansion and contraction of the repeat array and can also lead to shuffling of gene types into and out of complexes (reviewed in ROBBINS *et al.* 1989). The variability of structure for the paramutagenic and neutral classes of *r1* haplotypes suggests that pri-

mary structure is not the most critical feature for explaining their behavior in paramutation.

In contrast to the genic compositions, the epigenetic states, as reflected by the pattern of methylation, were fairly uniform within each class of haplotype. Most (five out of seven) of the paramutagenic haplotypes were hypermethylated at all *Hpa*II sites tested, and the remaining two were both methylated at two *Hpa*II sites near the transcription start, as well as at upstream (promoter) sites. This stands in contrast to the situation for paramutable and neutral haplotypes, which were almost all unmethylated near the transcription start. Methylation at promoter and intronic positions showed no such correlation with behavior in paramutation. These results suggest that the epigenetic signals—probably a particular chromatin conformation or composition—that cause paramutagenicity may lie close to the start of transcription.

The new structural data derived for this study also contribute to our understanding of *r1* complex evolution (illustrated schematically in Figure 6). Structural analysis of *R-r:std* suggested that a *Dop* transposable element inserted into a duplicated copy of an *Lc*-like gene (Walker *et al.* 1995). Formation of *R-r:std* occurred via breakage of this element, to form *q* and *S2*, and duplication of the *S2* gene segment occurred to produce *S1* and form the *S1/S2* inverted duplication. It is now clear that the Catspaw-type haplotypes arose by a subsequent deletion within this original *S*-subcomplex type that removed the 5′ portion of *S1* and the rearranged portion of σ. Further evidence for common ancestry of the Catspaw-type paramutable haplotypes stems from the presence of a restriction fragment length polymorphism associated with *q* that differs between Catspaw and standard types, suggesting that these lineages were distinct prior to the deletion event.

The *S*-subcomplex-containing haplotypes represent only one of two modern lineages from the progenitor haplotype containing *Dop* at *r1*. The second lineage is represented by the *S2*-like genes of paramutagenic haplotypes. These genes cannot have been derived from an *S*-subcomplex, because they contain a far larger fragment of *Dop* than is found within σ. Yet, the *S2*-like genes from paramutagenic haplotypes contain the 3′ end of a *Dop* transposable element inserted at a position identical to that of the *S2* genes in paramutable haplotypes, and this *Dop* end has an identical 5-bp truncation in both. These similarities indicate a common ancestral *r1* gene with a defective *Dop* element inserted. We hypothesize that the *S2*-like genes now found in paramutagenic haplotypes arose by a deletion of the 5′ end of *Dop* together with adjacent *r1* promoter sequences, leaving an *r1* gene that is controlled by *Dop* sequences upstream, but is not part of a duplication/inversion.

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