Genetic characterization and molecular cloning of genes involved in kernel development in Zea mays

Michael John Scanlon
Iowa State University

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Genetic characterization and molecular cloning of genes involved in kernel development in *Zea mays*

Scanlon, Michael John, Ph.D.
Iowa State University, 1993
Genetic characterization and molecular cloning of genes involved in kernel development in *Zea mays*

by

Michael John Scanlon

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics
Interdepartmental Major: Genetics

Approved: ... 

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Interdepartmental Major

Signature was redacted for privacy.

For the Major Department

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For the Graduate College

Iowa State University
Ames, Iowa

1993
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GENERAL INTRODUCTION
Explanation of Dissertation Organization

This dissertation is presented as four separate papers discussing the genetic characterization and molecular cloning of genes involved in maize kernel development, and one paper describing the nucleotide sequence and transcript analyses of the maize transposable element \textit{MuA2}. I am the primary author of the four papers discussing kernel developmental genes, and the second author of the paper describing work on \textit{MuA2}. In that paper, I determined the sequence of approximately half the transposon, and performed the northern blot analyses. The papers are preceded by a literature review of these topics, and are followed by a general summary of the dissertation. References cited in the general introduction and the general summary are placed in a section following the general summary. All papers are formatted in accordance with the specific guidelines required for submission of manuscripts to the Journal of Heredity. Paper 1 is submitted to Genetics, paper 2 is submitted to the Journal of Heredity, paper 3 is submitted to Molecular and General Genetics, paper 4 will be submitted in the near future, and paper 5 is published in Plant Molecular Biology.

Morphology and Composition of the Maize Kernel

The maize kernel, or caryopsis, is a single seeded fruit born in ordered rows on the pistillate inflorescence (ear). There are a number of characteristics that make the maize kernel a most tractable model system for analyses of the role of individual genetic loci in plant organ and tissue morphogenesis. The maize kernel is a large, conspicuous structure in which morphology can be easily monitored with little or no magnification. Each ear typically carries several hundred samples, which enables segregating phenotypes to be easily identified. Furthermore, the maize kernel is a
complex system containing both the endosperm and embryo, and is therefore an excellent system in which to study the interactions of these different plant structures during development. Many kernel mutants are known in corn, corresponding to numerous loci which contribute to the development of the embryo, the endosperm, or both. In general, maize is a superior plant for genetic analyses. The physical separation of the male and female flowers in this monoecious species permits controlled pollinations to be performed with relative ease. In addition, the abundance of well described transposable element systems in maize permits the generation and cloning of new mutant alleles affecting kernel development.

This dissertation presents genetic and molecular analyses of particular transposon-tagged, kernel mutants of maize. The majority of the mutants in this study are of the defective kernel \textit{(dek)} variety; the \textit{dek} phenotype is a single gene trait caused by recessive lethal mutations with severe effects on both embryo and endosperm development. These investigations were performed in an attempt to elucidate further the role of specific genetic loci in the complex process of maize kernel development.

The maize kernel contains the endosperm and embryo of the seed, enclosed within the maternally-derived pericarp (Kiesselbach 1949). The pericarp comprises the transformed ovary wall and serves as a protective integument of the seed. The large, triploid endosperm comprises 85\% of the weight of the kernel and functions as the nutrient reserve for the germinating embryo. The cells of the endosperm are filled with starch grains and protein bodies. Maize starch is composed of about 27\% amylose, straight chain polymers of \textit{alpha} 1-4 linked glucose, and 73\% amylopectin, amylose polysaccharides with branches of \textit{alpha} 1-6 linked glucose (French 1984). The major storage proteins of the maize endosperm are known collectively as the
zeins, alcohol soluble prolamines high in glutamine (30%), leucine (20%), alanine (10%) and proline (10%) (Mosse 1966). Zeins contain only trace amounts of lysine and tryptophan, and thus are a poor source of nutritive protein for non-ruminant animals (Nelson et al. 1965). Six different subclasses of zeins have been identified with apparent $M_r$s of 27,000, 22,000, 19,000, 16,000, 15,000 and 10,000 (Gianazza et al. 1977). The 22-kD and 19-kD classes, termed zein-1 proteins, comprise 75-80% of the zeins in the maize endosperm. Of the less abundant zein-2 proteins, the 27-kD and 16-kD classes are higher in cysteine (Gianazza et al. 1977), whereas the 15-kD and 10-kD classes contain higher levels of both cysteine and methionine relative to the zein-1 proteins (Pederson et al. 1986). The concentration of starch and protein is not consistent throughout the expanse of the endosperm (Duvick 1961). In the flint type varieties of maize these compounds are concentrated in the periphery and crown of the kernel, whereas the center is softer and more granular. In contrast, varieties of the dent type exhibit starch and protein body concentrations in a cylinder that partially surrounds the embryo. The crown of dent-variety kernels is the site of the soft granular tissue, which collapses at maturity to generate the characteristic dent morphology.

Specialized conductive cells in the basal portion of the endosperm transport nutrients in the developing kernel (Weatherwax 1930). The outermost layer of the endosperm is differentiated into the single-cell layered aleurone, which furnishes starch digestive enzymes in germinating kernel (Kiesselbach 1949). The aleurone contains no starch grains, and is the only region of the endosperm capable of accumulating anthocyanin pigment, which is responsible for kernel color.

The embryo is embedded in the lower face of the kernel, and is oriented towards the tip of the ear. The mature embryo of a dormant maize kernel contains a tiny
maize plant complete with a primary root (radicle) and shoot (plumule) containing 5 to 6 foliar leaf primordia (Abbe and Stein 1954). The embryonic root is enclosed in a protective covering termed the coleorhiza. The first leaf is modified into a large, spade shaped structure called the scutellum. The most obvious embryonic structure, the scutellum functions in digestion of the endosperm and transmission of nutrients to the germinating embryo (Kiesselbach 1949; Poethig 1982). Enclosing the plumule is the coleoptile, a modified second leaf which protects the embryonic foliar leaves during germination through the soil (Kiesselbach 1949).

Maize Endosperm Development

The endosperm is a triploid tissue that is one product of the double fertilization process; it is a developmental structure unique to angiosperms. No corollary structure exists in animal development, and in gymnosperms the nutrient storage tissue is pre-formed in the maternal gametophyte (Stebbins 1974). The process of double fertilization, however, is not unique to angiosperms. Gymnosperms of the genus Ephedra have been demonstrated to undergo a different type of double fertilization, a process which has been postulated to be an ancestral archetype to angiosperm endosperm development (Friedman 1992).

Morphological aspects of maize endosperm development have been well documented (Kiesselbach 1949; Kowles and Phillips 1988). Approximately 24 hours after pollination, endosperm development commences with the fusion of the sperm nucleus with one of the two polar nuclei. This diploid, fused nucleus then fuses with the second polar nucleus to generate the triploid primary endosperm nucleus (Kowles and Phillips 1988). Rapid mitotic divisions of the primary endosperm nucleus begin 3-5 hours post-fertilization and the free nuclei begin to line the cavity
of the embryo sac. The endosperm first becomes cellular, and the cells uninucleate, with the development of cell walls at about three to four days after fertilization. As cell division continues, the embryo sac is filled and the endosperm becomes solid at about 5 days following fertilization. At this stage the bulk of the ovule is composed of nucellar tissue, which the endosperm begins to digest away and eventually replaces. Also at this stage, basal cells of the endosperm differentiate into the specialized conducting cells that transport nutrients to the developing endosperm. Development continues with unanimous endosperm cell division until about 12 days after pollination (dap). Growth proceeds via both cell division and enlargement, and is most rapid between 8 and 12 dap. The mitotic index reaches a peak value of 10% in developing embryos at 8-10 dap (Kowles and Phillips 1985). At 12 dap, the endosperm usually fills the kernel cavity, whereupon cells in the basal and central region of the endosperm cease mitotic activity (Duvick 1955). Increases in cell and nuclear size continue, however, in these regions of the endosperm. The aleurone layer is formed after 12 dap (Sass 1976) via differentiation of the outermost layer of endosperm cells. Cell divisions after 12 dap are confined to cells in the endosperm periphery. This region is meristematic and periclinal divisions continue until about 20 to 25 dap (Randolph 1936). Endosperm growth after 20-25 dap proceeds solely by cell enlargement, and is completed at about 40 dap. During the process of kernel development, the size of the embryo sac is increased 1400-fold (Randolph 1936).

The haploid antipodal cells are mitotic products of the megaspore nucleus, which lie farthest from the egg cell in the embryo sac (Miller 1919; Cooper 1937). It was believed that the antipodal cells disintegrated soon after fertilization until Weatherwax (1926) first reported the persistence of antipodal cells in the late stages
of endosperm development. These findings were confirmed by Randolph (1936), who reported that antipodal cells undergo mitosis in early kernel development to form 24-48 cells, many of which are multinucleate. This phenomenon is unusual in the plant kingdom and may have gone undetected in early studies since the haploid antipodal cells are very similar in appearance to endosperm cells of the mature kernel.

Clonal analyses of endosperm development have utilized kernels that were genetically mosaic for the starchy \((Wx)\) and waxy \((wx)\) alleles of the \(WX\) locus (McClintock 1978). These studies have generally concluded that the initial division of the primary endosperm nucleus establishes the right and left halves of the maize kernel. Ensuing divisions typically generate clonal patterns that resemble widening, cone shaped sectors, although variations of this pattern are documented.

With the exception of the aleurone layer, all cells of the endosperm accumulate starch granules. Starch begins to accumulate at about 10 dap and continues until the imposition of dormancy around 45-50 dap (Lampe 1931). Although young endosperm cells contain large plastids, little starch can be detected by iodine staining before 12 dap (Sass 1976). Starch accumulation begins in the crown of the kernel and progresses downward, resulting in increased starch deposition toward the crown. Also, new cells formed via peripheral, meristematic division after 12 dap contain less starch than the older, centrally located endosperm cells (Lampe 1931).

Thus, two gradients of starch deposition are found in the mature maize kernel. The temporal and spatial patterns of protein body (zein) accumulation in developing endosperm are similar to those found for starch synthesis (Wilson 1978). The superabundant 19- and 22-kD zein-1 proteins are encoded by multigene families; as many as 100 copies of structural genes for zein-1 proteins are contained in the maize
genome (Burr et al. 1982). The less abundant 27-, 16-, 15- and 10-kD zein-2 proteins are each encoded by only one or two genes (Pederson et al. 1986; Kirihari et al. 1988; Das and Messing 1987). Zein polypeptides are synthesized between 12 and 45 dap on the rough endoplasmic reticulum (Larkins and Hurkman 1978). Following translation, these proteins are imported into the endoplasmic reticulum where they are assembled into dense, membrane bound protein bodies. Zein protein synthesis has also been observed on ribosomes attached to protein bodies (Burr and Burr 1976).

A peculiar characteristic of developing maize endosperm cells is the heterogeneity of nuclear size and DNA content (Kowles and Phillips 1988; Burr and Burr 1976). Increases in both nuclear size and DNA content are initiated between 10-12 dap in centrally located endosperm cells, and reach peak levels between 16-18 dap. The maximum mean DNA content of endosperm cells in the inbred strain A188 was found to be 90 to 100 C (where C is the haploid DNA content), and some cells were found to contain 600 C. Cells with amplified DNA levels are very large, with thin, easily ruptured cell walls. After 20 dap, DNA content of endosperm cells decreases, perhaps due to degradation of accumulated DNA. Comparative hybridization of leaf and endosperm DNA to a wide variety of specific DNA probes has revealed that the entire genome is replicated to increased levels in endosperm cells, a phenomenon known as endoreduplication (Phillips et al. 1983; Phillips et al. 1985). Cytometric analyses demonstrated that levels of DNA amplification are highly variable in different endosperm cell nuclei (Kowles et al. 1990), and the phenomenon is the result of alternating rounds of DNA replication. The cell cycle of endoreduplicating central endosperm cells consists of S and G phases only, and therefore bypasses the mitotic stage.
A number of possibilities have been suggested to explain the role of DNA endoreduplication in cells of the developing endosperm (Kowles et al. 1992). Increased DNA levels may facilitate gene expression, including genes important in endosperm development. The timing of endosperm DNA amplification coincides with tremendous increases in starch grain and protein accumulation. Maybe this huge increase in anabolic activity is facilitated by increases in genome copy number. Another possibility is that very large and fragile endoreduplicated endosperm cells facilitate the translocation of stored nutrients to the embryo. In this view, the nutrients of these labile cells are easily released following small amounts of endosperm cell degradation.

Maize Embryo Development

Embryogenesis in higher plants differs from animal embryo development in several fundamental ways (for review see Meinke 1991). Plant cell walls instill developmental constraints by preventing cellular migration during morphogenesis. In contrast to animals, plant embryo development is not as dependent on mRNA stored in unfertilized egg cells. In addition, the unique process of double fertilization in plants permits interactions between the developing embryo and endosperm, and leads to the elimination of many mutant alleles that render the transcriptionally active male gametophyte nonfunctional.

Early descriptions of maize embryo development were detailed by Randolph (1936) and Kiesselbach (1949). Approximately 24 hours after pollination, the egg and sperm nuclei fuse to form the zygote. The first mitotic division of the zygote is transverse, and produces a two-celled proembryo about 12 hours post-fertilization (Randolph 1936).
Early development of the embryo is much slower than that of the endosperm. At three days after fertilization the endosperm typically comprises 250 or more cells, whereas the embryo usually consists of only 12 cells (Kiesselbach 1949). During the proembryo stage of development, it is impossible to detect cells responsible for particular organ initiation. The basal cells of the spherical proembryo develop into the suspensor at about 6 dap, which orients the developing embryo with respect to the endosperm. Abbe and Stein (1954) described subsequent maize embryo development in terms of shoot apex stages, rather than calendar time. These workers noted that genetic background effects and environmental fluctuations lead to imprecise correlation between morphological stage and time. All chronological times noted in this review with regard to shoot apex stages of embryogenesis are given as general estimates of typical, midwestern corn belt dent varieties.

The transition stage marks the first discernible differentiation of the embryo proper with the formation of a lateral prominence at 9-12 dap. Soon after, the lateral prominence develops into a meristematic ring, the coleoptilar primordium, and the flattened, spade like shape of the scutellum is apparent. This phase, termed the coleoptilar stage, usually occurs at 10-13 dap. Stage 1 marks the appearance of the first foliar leaf primordium, which arises on the side opposite the scutellum at about 12-16 dap. The scutellum and coleoptile increase in size, while the suspensor becomes increasingly less prominent. Subsequent stages marking the development of successive leaf primordia are numbered 2 through 5 or 6, depending on the number of leaf primordia initiated at the time of maturity, about 40 dap. Successive leaves initiate 180 degrees around the axis of, and are enclosed by, the preceding leaf. The suspensor disappears by stage 3 (20-26 dap), and the primary root is recognizable within the coleorhiza. Also at this stage, the enlarging scutellum
begins to enfold the embryonic axis. Growth and development cease about 40-45 dap, at which time embryos become dormant and dehydration begins.

In contrast to developing endosperm tissues, embryos develop solely via increase in cell number and not cell enlargement (Abbe and Stein 1954). New leaves are initiated in the growing embryo at progressively slower rates, as the time of succeeding plastochrons (the length of time between successive meristematic divisions) becomes increasingly greater. Furthermore, studies comparing embryo development of individual kernels from the same ear have demonstrated remarkable uniformity among sibling embryos (Abbe and Stein 1954).

The maize embryo accumulates a wide range of characteristic proteins during development. In particular, large amounts of saline-soluble, water insoluble globulin proteins accumulate in the middle and late embryonic stages (Kriz and Schwartz 1986; Belanger and Kriz 1989). The two most abundant globulin proteins are products of the single copy genes \textit{Glbl} and \textit{Glb2}. Formation of the 63 kD \textit{Glbl} gene product at 19-21 dap requires three post-translational modification steps. The final step in processing of the GLBl protein is regulated by the product of the unlinked locus, \textit{Mep}. In \textit{mep/mep} mutants there is accumulation of the larger molecular weight precursor protein \textit{GLBl'} (Kriz and Schwartz 1986). Expression of the \textit{Glb1} gene is positively regulated by abscisic acid, a plant hormone which plays a major role in regulation of kernel maturation and dormancy (Kriz et al. 1990). Both \textit{GLB1} and the smaller, 45-kD protein \textit{GLB2} are rapidly degraded upon germination. Since the endosperm zeins are mobilized only after the globulin proteins are completely degraded, the \textit{GLB1} and \textit{GLB2} proteins may function as kernel storage proteins, thereby providing the immediate nitrogen source to the germinating embryo.
Mutations Affecting Maize Kernel Development

Development of the maize kernel depends on the expression of multiple genes. Traditionally, studies of kernel developmental loci have involved comparative analyses of wild type versus mutant kernel development. In depth studies have been conducted on numerous mutant alleles that cause a diverse range of abnormal kernel phenotypes. Genetic lesions which disrupt some aspect of kernel development have been mapped to all of the 20 chromosome arms of maize except 8S (Coe et al. 1987). Common practice has separated the wide array of kernel mutants into somewhat arbitrary classes based on similarity of phenotype. In this manuscript, kernel mutants are sorted into four general classes according to both the kernel structure affected, and viability of mature seed. Mutations are designated as lethal if 5% or less of the mutant kernels germinate and produce mature plants (Neuffer and Sheridan 1980). The groupings and designations of kernel mutants described herein are done solely for ease of discussion, and do not necessarily delineate strict biochemical or developmental similarities among members of any one class. Furthermore, the large number, and phenotypic diversity of described kernel mutations frequently leads to problems in classification, as some mutant types display attributes of more than one designated class. In light of these limitations, the four designated kernel mutant classes are as follows.

1) **Embryo-specific (emb) mutants**, in which endosperm development proceeds normally but embryo development is abnormal and viable embryos do not form.

2) **Endosperm mutants**, in which development of the endosperm is abnormal. Embryos are not as affected, such that the kernel are viable.
3) **Viviparous** (*vp*) **mutants**, in which mature embryos fail to become dormant and often grow into plantlets on the ear. Endosperm or aleurone pigmentation is also affected, and many *vp* mutants are lethal.

4) **Defective kernel** (*dek*) **mutants**, in which both endosperm and embryo development is severely altered. The *dek* mutant kernels typically are inviable. Within each mutant class are numerous subdivisions of phenotype as illustrated in the following brief survey.

**Embryo-specific mutants**

Early descriptions of embryo-specific (*emb*) mutations in maize entailed brief descriptions of germless (*gm*) kernels with normal endosperm (Demerec 1923; Wentz 1930). These workers reported that the embryos of *gm* kernel were either absent or arrested early in development, and that the *gm* phenotype was often transmitted in low frequency. Following this early work, this mutant class had been largely ignored for over fifty years, probably because of the difficulties in handling these lethal mutations, for which classification requires shelling of kernel from the ear. More recent investigations by Clark and Sheridan (1986; 1988; 1991) demonstrated that the *emb* mutants correspond to a very large number of loci dispersed throughout the maize genome. Since endosperm development is normal in *emb* mutants, the corresponding loci are shown to be developmentally specific and not general cell function (housekeeping) genes. Mutant phenotypes are diverse and reflect embryogenic arrest at various stages before, during, and after establishment of the embryonic axis. Many *emb* mutations specify disruptions in development of certain embryonic structures (e.g. the scutellum in *emb*8545, the suspensor in *emb*8543).
Careful inspection of numerous *emb* mutant phenotypes has provided important clues in regard to embryo/endosperm interactions, and the regulation of gene expression in the different domains of the developing kernel. For example, it was previously believed that the depression in the lower face of the kernel was formed by digestion of the endosperm by the developing embryo (Smart and Obrien 1983). Because this depression was found even in the endosperm of *emb* mutants with tiny mutant embryos, Clark and Sheridan (1991) concluded that the genetic control of endosperm shape is separate from that of embryo morphogenesis. In addition, the normal endosperm development in *emb* mutant kernels indicates that functional *Emb* loci are not required for endosperm development.

Interestingly, many *emb* mutants that undergo tissue proliferation are also associated with a thickening of the aleurone layer, indicating that this single cell layer may share some aspects of gene expression with the embryo that are absent from the rest of the endosperm. Expression of *Emb* genes in the male gametophyte is indicated by low rates of *emb* mutant allele transmission in some *emb/Emb* heterozygotes (Demerec 1923; Wentz 1930; Clark and Sheridan 1991). Abnormal enlargement and proliferation of the antipodal cells in the embryo sacs of many *emb* mutants indicates that the corresponding wild type genes are normally expressed in the female gametophyte as well (Clark and Sheridan 1988; Clark and Sheridan 1991). Taken together, these results indicate that many *Emb* genes show commonality of expression in the gametophytes, embryo and aleurone. A similar program of expression is documented for a hydroxyproline-rich glycoprotein homologous maize cDNA of unknown function which is expressed exclusively in developing maize embryos and ovules (Jose-Estanyol et al. 1992).
Endosperm mutants

Endosperm-specific mutants are among the best studied in maize genetics because they are viable and relatively easy to distinguish and classify. Enzymatic and molecular analyses have determined the biochemical function of numerous kernel gene products. This brief overview of endosperm developmental loci will utilize the conventional phenotypic groupings used by Coe et al. (1987) with some modifications.

For this discussion, endosperm mutants genes are separated into varieties termed sugary-shrunken, opaque-floury, etched-pitted, and miniature. In sugary-shrunken endosperm mutants the kernel is often collapsed, wrinkled, or glassy in appearance. These mutant phenotypes typically portray altered profiles of starch accumulation. Representative examples include the su1 mutant (common sweet corn), which is characterized by the accumulation of a highly branched polysaccharide, phytoglycogen. Enzymatic studies indicated that recessive su1 mutants are deficient in a starch debranching enzyme which may be the product of the Su1 locus (Pan and Nelson 1984). In contrast, ae mutants accumulate an unbranched amylose starch, and the Ae locus probably codes for an amylose branching enzyme (Hedman and Boyer 1983). Homozygous bt2 and sh2 mutant plants have very similar kernel phenotypes; these genes code for two different subunits of ADP-glucose pyrophosphorylase, a starch biosynthetic enzyme that catalyzes the conversion of glucose-1-phosphate to ADP-glucose (Tsai and Nelson 1966; Hannah and Nelson 1976; Bae et al. 1990; Bhave et al. 1990). Mutants of the Sh1 locus contain 25-30% of normal starch levels. Sh1 is one of two maize loci which code for sucrose synthase (Cssl codes for sucrose synthase activity in the embryo and other tissues) an enzyme that converts available sucrose to UDP-glucose and
fructose (Chourey and Nelson 1976). Although the identity and function of the Btl
gene product has not been established, the gene has been cloned and sequence
similarities suggest the protein product may be involved in amyloplast membrane
transport (Sullivan et al. 1991).

Opaque or floury phenotypes are displayed by many kernel mutants, most of
which show altered protein levels or composition. These mutants often have a dull
or chalky appearance and soft kernel composition. However, the most renowned
member of this class, wx1, affects starch composition and not kernel protein.
Homozygous wx1 kernels are opaque, but are of hard composition and stain
reddish-brown with potassium iodide. The Wx locus encodes the structural gene for
the starch granule-bound NDP-glucosyl transferase, which synthesizes the amylose
component of starch (Nelson and Rines 1962; Shure et al. 1983). Because wx1/wx1
homozygous plants are deficient in glucosyl transferase function, the wx1
endosperm contains only the branched, amylopectin starch component.

Other members of the opaque and floury class often exhibit alterations in zein
accumulation. The two major classes of zein polypeptides (19 kD and 22 kD groups)
found in maize endosperm are affected in specific ways by different opaque-floury
mutants (Tsai 1983; Soave and Salamini 1984). For example, o7 mutants reduce the
amount of the 19-20 kD zeins and corresponding mRNAs, whereas levels of the
larger class of zeins are more reduced in o2 mutants (Soave and Salamini 1984). Mc
and fl2 mutant endosperms exhibit reductions and structural alterations in both the
large and small-sized zein families. Many of the mutants show dosage effects (o2,
fl1, fl2, fl3), and others result in increased endosperm lysine levels (o2, o7 ), a
nutritional benefit. The o2 protein was found to be regulatory (Di Fonzo et al. 1986),
and analysis of the cloned cDNA has revealed a leucine zipper and DNA binding
motifs (Schmidt et al. 1987) characteristic of transcriptional activators. It has been demonstrated that in addition to altered patterns of zein accumulation, f12 mutants also show an overproduction of the b-70 protein. A 70,000 dalton protein found in the maize endoplasmic reticulum, b-70 shows strong homology to the mammalian immunoglobulin binding protein (BiP) (Fontes et al. 1991). BiP has been shown to mediate protein folding and assembly, and it is possible that b-70 may function in the zein secretory pathway. F12 is unlinked to the b-70 locus and is therefore not the structural gene for the b-70 protein. The exact relationship between f12 mutant endosperm and the overproduction of b-70 is unknown.

The etched-pitted class of mutants all display fissures in the endosperm and generate aleurone mosaics. Most mutants of this class show intriguing pleiotropic effects, indicating that the genetic lesions affect basic developmental processes (Coe et al. 1987). The st1 mutation causes chromosomes to stick together, and generates pitted kernels and small, striate-leafed plants. Irregularities in aleurone cell layering and cracked endosperm surfaces characterize the mutants Mscl and Msc2. Small, chlorophyll-deficient plants are produced from the pitted kernel of o12 mutants (Coe et al. 1987), and et1 mutants produce virescent seedlings (Stadler 1940). Further characterization of the et1 phenotype has revealed that amylase levels are reduced in germinating kernel (Sangeetha and Reddy 1988), and chloroplast ultrastructure is reversibly disrupted (Ramesh et al. 1984). In addition, the levels of many photosynthetic enzymes are reversibly affected (Sangeetha and Reddy 1991), implicating the et1 mutation as a fascinating system to study pleiotropism.

The miniature class of kernel mutants are all greatly reduced in size and often show other endosperm abnormalities as well. Despite their defective appearance, all members of this group are viable. Pan and Peterson (1989) described a dominant
miniature kernel mutant putatively tagged with the transposable element \textit{Uq}. This mutation, designated \textit{Mn::Uq}, produces small seeds with small embryos which germinate to generate seedlings with reduced vigor in early stages of growth. Healthy mature plants are produced from \textit{Mn::Uq} seeds, although the pleiotropic effects of the mutation extend to the male gametophyte, which are nonfunctional. The nontransmissibility of male gametes containing the \textit{Mn::Uq} allele is due to failed pollen tube growth. Thus, the wild type allele of the miniature locus encodes a product that contributes to development of the endosperm, embryo, and the male gametophyte.

The \textit{mnl} mutation was first described by Lowe and Nelson (Lowe and Nelson 1946), and has provided information concerning interactions of maternal tissues with the developing kernel. Examination of developing \textit{mnl} kernel revealed that about 9 dap, a gap forms between the conductive basal cells of the endosperm and the degenerating cells of the pedicel, maternal diploid tissue at the base of the kernel (Lowe and Nelson 1946). Soon after this loss of contact with the maternal tissue, \textit{mnl} kernels are nearly arrested in development. Despite the fact that pedicel cells are initially affected in \textit{mnl} mutant plants, the \textit{mnl} kernel phenotype is dependent upon expression of the mutant allele in the endosperm and not in maternal tissues (Miller and Chourey 1992). It was found that \textit{mnl} endosperm shows greatly reduced levels of invertase, which catalyzes the breakdown of sucrose into glucose and fructose (Miller and Chourey 1992). Because sucrose is normally shunted from the ear to the developing endosperm through the pedicel cells, these results indicate that \textit{Mnl} may code for kernel invertase enzyme, and lack of invertase activity in mutant kernels may lead to a buildup of sucrose in the pedicel. This buildup presumably leads to an osmotic imbalance in maternal cells which may result in
their degeneration. Seed development in *mn1* mutants is therewith blocked due to lack of contact with nourishing, maternal tissues of the ear.

**Viviparous mutants**

Viviparous mutants are characterized by an embryo which fails to mature into the dormant state, and may germinate precociously, while the kernel is still on the developing ear. A diverse class which comprises at least nine loci (Robertson 1952; Robertson 1955; Robertson 1961; Robertson 1975), some viviparous mutants have no effect on chlorophyll and carotenoid pigments (*vpl* and *vp8*); some affect chlorophyll and carotenoid levels and are lethal (*ps, vp2, vp5, w3* and *vp9*), and some affect both pigments and are nonlethal (*al* and *y9*). Robertson has demonstrated that the embryo genotype alone determines vivipary, and the effect on kernel pigmentation is likewise determined solely by the endosperm genotype.

Most *vp* mutants are deficient in the biosynthesis of carotenoids and abscisic acid (ABA) (Robichaud et al. 1980; Fong et al. 1983; Moore and Smith 1985; Neill et al. 1986), a plant hormone that functions in the regulation of kernel maturation. The best studied *vp* mutant, *vp1*, is exceptional in that it shows normal levels of carotenoids, reduced levels of anthocyanins, and is viable if germinated embryos are transplanted (Eyster 1933; Robertson 1955). The *vp1* mutation confers reduced ABA sensitivity (Robichaud et al. 1980), but does not affect ABA synthesis (Neill et al. 1986) or ABA metabolism (Robichaud and Sussex 1986). In addition, the embryo storage proteins *GLB1* and *GLB2* (which are positively regulated by ABA) are absent in *vp1* mutants (Belanger and Kriz 1989).

The *Vp1* gene was cloned via transposon-tagging using Robertson's *Mutator*, and was shown to encode a mRNA expressed in both the endosperm and embryo
Biochemical analyses have demonstrated that the \textit{VP1} protein is a novel transcriptional activator, which may play a role in potentiation of kernel response to ABA (McCarty et al. 1991). Subsequent analyses revealed that expression of the anthocyanin regulatory locus, \textit{CI}, is blocked at the mRNA level in \textit{vp1} mutants, indicating \textit{Vp1} may also regulate expression of \textit{CI}. In the \textit{vp1-Mc} allele, anthocyanin expression is blocked whereas embryo dormancy is nearly normal (Robertson 1965). These data indicate that the epistatic effects of \textit{vp1} on the embryo and the aleurone can be separated. Molecular comparisons have revealed that the \textit{vp1-Mc} mutant produces a 3' truncated mRNA, indicating that the C-terminus of the protein may function in regulation of anthocyanin production, whereas regulation of kernel maturation probably involves a different region of the \textit{VPl} protein (McCarty et al. 1989a).

**Defective kernel mutants**

Defective kernel (dek) mutant plants typically produce inviable kernels, and are defective in both endosperm and embryo development. The \textit{dek} phenotype is among the most frequently encountered mutants in maize; a wide variety of phenotypes is observed among \textit{dek} mutants as a class. Because they affect the development of the entire kernel, \textit{dek} mutations are potentially useful tools to study developmental interactions between the embryo and endosperm.

Early studies by Jones (1920) described recessive, lethal mutations of the \textit{De} locus that caused development of defective seeds. Jones reported that most mutations of this locus caused an empty kernel phenotype, although in some instances the defective seeds were partially filled or opaque. Occasionally, defective seeds with some endosperm material germinated to produce slow-growing
seedlings that lacked chlorophyll color. Mistakenly, Jones reported that the empty seeds were the result of an extreme phenotypic variant, and that these, as well as the partially filled and opaque kernel phenotypes all were the result of mutations at the same specific genetic locus. Subsequent studies of the re-named defective kernel mutant class have indicated that the phenotypic diversity reported by Jones was almost certainly the result of mutations at many different loci. Lastly, Jones described the utility of the kernel mutants for use in linkage analyses, as the de phenotype is an easily scored kernel trait. Also noted was the significance of defective kernel in studies of kernel development; "because it is an illustration of defective germ-plasm, which is widely distributed in a cross-fertilized organism and has vital importance in life processes" (Jones 1920).

In spite of the fact that the importance of the defective kernel mutants was recognized in very early investigations of maize genetics, this class was virtually ignored for more than fifty years spanning the time between the published works of Mangelsdorf (1923; 1926) and Neuffer and Sheridan (1980). The inherent difficulty in genetically analyzing mutations causing lethality no doubt contributed to this lack of attention to the defective kernel phenotype.

In a very large analysis of kernel developmental loci, Neuffer and Sheridan (1980) and Sheridan and Neuffer (1980) performed genetic, lethality, morphological and developmental analyses of defective kernel mutants isolated after mutagenesis with ethylmethane sulfonate. Of the 2,457 total heritable mutants induced in their mutagenesis project, Sheridan and Neuffer found that 35% (855) were kernel mutants. These figures illustrate the vast number of maize loci that contribute to kernel development. Of the 855 kernel mutants, 194 were characterized regarding endosperm and embryo phenotype. Ninety mutations were mapped on 17 of the 20
maize chromosome arms. A total of 33 mutations were shown to correspond to novel genetic loci, and were designated dek1 to dek33, in a series (Neuffer and Sheridan 1980; Sheridan and Neuffer 1981; Sheridan et al. 1984; Sheridan et al. 1986; Clark and Sheridan 1986; Sheridan 1991; Neuffer 1992). The phenotypes caused by these dek mutations are diverse, and include all the endosperm mutant types previously described. Most common are the collapsed, empty kernel phenotype (dek 22), but also included are sugary-shrunken (dek5, dek7); floury-opaque (dek28, dek4, pro1); etched-pitted (dek11, dek31); mosaics (dek21) as well as discolored and small seeded mutants. Mutant kernels are usually able to be detected on Missouri-grown segregating ears by 9-14 dap as smaller, whitish kernels (Sheridan and Neuffer 1980). Despite the diverse phenotypes caused by different dek mutations at maturity, most are remarkably uniform in appearance at early developmental stages.

Embryo development in defective kernel mutants is blocked or disrupted at various stages characteristic of different dek mutations. Embryos of dek22 mutant plants are arrested at the transition stage, and embryo development in dek23 mutant plants never proceeds beyond an abnormal coleoptilar stage (Clark and Sheridan 1986). Embryos of other dek mutant plants are blocked over a wide range of stages. For example, fl*1253B embryos are blocked from the coleoptilar stage to stage 2, whereas embryo development in rgh-1210 mutants is variably disrupted from the transition stage to the coleoptilar stage (Clark and Sheridan 1988). As noted in the previous description of embryo specific mutants, mutants in which the embryo undergoes prolific disorganized growth (dek31, rgh*-1210, fl*-1253 and bno*-747B) also exhibit aleurone tissue proliferation and thickening (Clark and Sheridan 1988). The aleurone region most affected is closest to the embryo, which suggests that the mutated embryo may produce a substance that diffuses to and elicits disorganized
growth of the aleurone. Because *dek* mutant plants that do not induce abnormal embryo proliferation are also free from aleurone thickening (including *dek22* and *dek23*), it is probable that the embryo and aleurone share patterns of organization and gene expression in common that are distinct from the rest of the kernel.

The pleiotropic effects of many *dek* mutants also extend to and indicate a physiological link between aleurone integrity and seedling pigmentation. The *sugary-shrunken* mutations *dek5* and *dek7* (Neuffer and Sheridan 1980) cause disruption of the aleurone layer and both mutations produce inviable seedlings that are white with green stripes. Inviable, striated seedlings are also produced from cultured embryos dissected from the pitted endosperm mutation *dek11* (Neuffer and Sheridan 1980). Defective kernels from *dek21* mutant plants are aleurone-mosaics (Sheridan et al. 1986), and produce inviable white seedlings. A physiological explanation of the connection between aleurone defects and seedling pigmentation has not been elucidated.

Studies of embryos from *dek* mutant plants have revealed that the processes of root and shoot morphogenesis can be uncoupled. Mutant embryos bearing *dek23* form a root apex but not a shoot apical meristem (Clark and Sheridan 1986), whereas in *dek1* mutant embryos, shoot development is relatively normal and root development is altered (Sheridan and Neuffer 1980). A surprising result of Sheridan and Neuffer's study is that 80% of *dek* mutant embryos that are lethal at kernel maturity will germinate when placed in tissue culture at immaturity. Moreover, 60% will produce plants greater than 5 cm long. These results indicate that although many *dek* embryos exhibit abnormal development relatively early in development, loss of viability often occurs much later (Sheridan and Neuffer 1980).
In an attempt to explore the complex interactions between embryo and endosperm in developing kernels, aneuploids were used to determine if a normal endosperm helped or hindered *dek* embryo development, and vice versa (Neuffer and Sheridan 1980). It was found that in only four of the nineteen *dek* mutants analyzed, the normal endosperm improved embryo development. In no instances, however, was a normally inviable *dek* mutant embryo rendered viable by a normal endosperm. Normal embryos improved endosperm development in three of nineteen *dek* mutants analyzed. Conversely, in seven cases the endosperm mutation became more extreme in the presence of a normal embryo. These findings suggest that although the endosperm may indeed play a nurturing role in embryo development, this process ultimately is dependent upon embryonic genes and tissues.

The effects of 35 EMS-induced *dek* mutations on mitotic activity and endoreduplication of developing kernels has been investigated (Kowles et al. 1992). All the *dek* mutant plants studied showed a reduction in both mitotic activity and endosperm cell numbers, and all except one displayed reduced levels of endoreduplication. These data demonstrate that mitotic activity and the process of endoreduplication in developing kernels can be separated.

No information is available regarding biochemical or molecular characterization of *Dek* genes or gene products. A possible relationship between the numerous loci identified by defective kernel mutants and quantitative trait loci controlling kernel size has been advanced by Robertson (1985b). Robertson has proposed that many defective kernel mutant genes and kernel size QTL are in fact alleles of the same locus. In this view, minor genic variation at one or more loci affecting kernel development contributes to the diverse spectrum of kernel size phenotypes.
characteristic of quantitatively inherited traits. If, however, a kernel developmental locus were to undergo a more extreme mutation resulting in a null allele, a defective kernel mutant phenotype would result. If this relationship is valid, then it may be possible to isolate QTL affecting kernel size by cloning the corresponding qualitative \textit{dek} mutant alleles. Demonstrating that the cloned \textit{dek} mutation maps to an identified QTL with major effects on kernel size would support the proposed relationship between kernel developmental loci.

\textbf{Robertson's Mutator}

The main focus of this dissertation is to use the Robertson's \textit{Mutator} transposon tagging system to identify and characterize \textit{dek} genes. Therefore, the transposon system and its utility as a genetic tool will be discussed. Transposable elements are mobile DNA that have the capacity to move to different locations in the genome. McClintock (McClintock 1950) used maize to provide the first descriptions and genetic analyses of transposable elements. Many comprehensive reviews of the more than ten families of maize transposable elements which have been genetically defined are available (Federoff 1983; Freeling 1984; Nevers et al. 1986; Chandler and Hardeman 1993). In general, all transposable element systems consist of at least two types of components elements. An autonomous (master) element is a mobile gene which is able to determine its own transposition. Master elements are predicted to encode a transposase function, a protein which can specifically elicit transposition of transposable element family members. Non-autonomous (receptor) elements do not encode a transposase and are therefore only able to transpose when an active autonomous element(s) is also present in the genome. Typically, receptor elements in a given family are structurally heterogeneous, whereas autonomous elements are
structurally conserved. Transposons can generate mutations via inserting into a gene and disrupting its structure. Other ways in which transposons induce mutations include chromosome breakage, deletion and imperfect excision. Insertional mutagenesis often generates null mutations, which are inherently stable in the absence of further tranposition. If, however, transposable element insertion is followed by excision from the locus in question, wild type gene function can often be restored to the locus. The result of such a reversion event is a variegated, or mutable, phenotype, in which wild type revertant cells are present in a background of mutant tissue. Because transposons can potentially disrupt and thereby regulate gene expression at any locus, McClintock (1950; 1951) called them controlling elements.

The Robertson's Mutator (Mu) transposable element system (Chandler and Hardeman 1993) was first characterized in a strain exhibiting a 40 or more fold increase in the spontaneous mutation rate of maize (Robertson 1978). Such strains are referred to as Mu-active stocks. This extremely high mutation rate is due to the presence of multiple copies of the Mu elements and to their transposition, which typically averages 10-15 transpositions per gamete per generation (Alleman and Freeling 1986). Mutator activity has been measured in two different ways. The "forward mutation" assay (Robertson 1978; Robertson 1980) measures the induction of new seedling mutants from Mu-active stocks, whereas the somatic reversion assay (Walbot et al. 1986; Brown et al. 1989; Robertson and Stinard 1989) measures mutability of a Mu transposon-induced allele. Robertson and co-workers (Robertson et al. 1985; Robertson et al. 1988) have found that the two distinct measures of Mutator activity are not always well correlated, and the presence of somatic mutability does not always correspond to high forward mutation rates. It is possible
that Mutator activity is not identical in all maize tissues or for all Mutator elements. Nonetheless, both measures of Mutator activity have equally demonstrated that Mutator does not behave as a simple autonomous element-receptor element system as described for the Ac/Ds (McClintock 1950; McClintock 1951) or Spm/dSpm; En/l (McClintock 1952; McClintock 1956; Peterson 1953; Peterson 1965) systems. Mutator activity was inherited by 90% or more of the offspring of Mutator plants, which indicated that multiple autonomous elements are often present in Mutator lines (Robertson 1978; Bennetzen 1984; Bennetzen 1987). However, subsequent genetic studies by Schnable and Peterson (1989) and Robertson and Stinard (1989) did identify Mutator lines with single autonomous elements which segregated in normal Mendelian ratios. The discovery of a Robertson's Mutator line segregating a single autonomous element (Robertson and Stinard 1989) permitted the initial cloning and molecular characterization of the putative Mu regulatory element (Chomet et al. 1991; Qin et al. 1991). The Cy element was found to control genetic mutability of both the bz-rCy (bz-Mu7) and the a1-Mum2 Mutator-induced alleles (Schnable and Peterson 1989; Schnable et al. 1989). Unlike most Mu-regulatory elements from Robertson's Mutator stocks, Cy usually segregates as a single copy regulator of Mutator activity. The molecular characterization and DNA sequencing of Cy may provide information regarding the differences in genetic behavior of Mu-regulators.

The Mutator transposable element system is a large family of transposable DNA sequences of which at least 10 individual elements have been described. These include at least ten non-autonomous elements: Mu1, Mu1.7, Mu1-del, Mu3, Mu4, Mu5, Mu6, Mu7(rCy), Mu8, MuA; and the sequence identical, putative autonomous elements Mu9, MuA2, MuR1 (Barker et al. 1984; Chandler et al. 1986; Taylor and Walbot 1987; Oishi and Freeling 1987; Varagona et al. 1987; Talbert and Chandler...
Structurally, all Mu elements share inverted terminal repeats of about 210 base pairs in common. The Mutator family exhibits far greater structural heterogeneity than either the Ac/Ds or Spm/dSpm (En/I) transposable element systems (Federoff 1983). The internal regions of most Mu elements are unique, although Mu1, Mu1.7 and Mu1-del show strong homology, as do MuA and the three independently-isolated putative autonomous elements, MuA2, MuR1 and Mu9. In addition, Mu4 and Mu5 both contain an internal extension of the inverted terminal repeats of about 100 base pairs, although the remaining internal regions of these elements are not homologous. Active Mutator lines often contain 30 or more Mu elements, and Mu1 homologous elements are usually the most abundant (10-60 copies) (Bennetzen 1984). Sequences homologous to Mutator transposon termini and middles are found in multiple copies in standard, non-Mutator maize lines and in related species (Talbert and Chandler 1988; Talbert et al. 1989). These findings indicate that Mu termini are ancient and may be capable of transposing independently. Active Mutator lines contain two transcripts with homology to the putative Mu regulator (Chomet et al. 1991; Qin et al. 1991; Hershberger et al. 1991). These transcripts are predicted to encode for the Mu transposase function, and are absent in non-Mu and Mu-inactive lines. None of the ten non-autonomous Mu elements hybridize to a mRNA that corresponds to transposition function.

Mutator activity is complex, and very specifically regulated. Analysis of Mutator activity at nine different loci has revealed that the frequency of Mu-induced mutations differs with respect to loci and genetic background (Robertson 1985a). Host genomic DNA direct repeats 9 base pairs in length are found flanking each
end of inserted *Mu* transposons. These 9 base pair footprints are presumably the result of *Mu* insertion at sites staggered by 9 base pairs. Ingels et al. (1992), have found that *Mu* elements were not randomly distributed throughout the maize genome, but frequently are found in linked clusters. Clustering of *Mu* elements may be the result of transposition to linked loci or into clustered hypomethylated regions of the maize genome.

Somatic *Mutator* activity is restricted to late in development, as indicated by very small sectors of revertant wild type tissue in otherwise mutant tissue. Germline *Mutator* activity is restricted to shortly before, or during meiosis. Although the loss of somatic *Mutator* activity is almost always associated with a concomitant loss of germinal activity, the two activities are not always correlated. In some cases, somatic activity can be retained whereas germline activity is lost, and even intense somatic activity does not insure the presence of germline activity (Robertson 1985a; Robertson 1980). In studies comparing the stability of *Mutator* activity when transmitted through the male and female gametophyte, some researchers (Robertson 1985a; Brown and Sundaresan 1992) have reported no difference in male vs. female transmission whereas others (Walbot 1986; Bennetzen 1987) reported partial female dominance. This discrepancy may be due to differences in the rate of *Mu*-inactivation in the different stocks used.

Genetic mapping studies of the *Mu* regulator (Robertson and Stinard 1992), and molecular analyses of *Mu* copy number (Alleman and Freeling, 1986) have indicated that *Mu* elements may move via replicative transposition. This mode of transposition probably accounts for the high copy number of *Mu* elements in *Mutator* lines.
Interbreeding of active Mutator lines greatly increases the Mu1 copy number (Alleman and Freeling 1986) and eventually inactivates the system (Robertson 1983). Repeated outcrossing of Mu lines has also been effective in inactivating some Mutator lines. Inactivation of Mutator has been shown genetically to be correlated with the loss of the autonomous element function (Schnable and Peterson 1989; Robertson and Stinard 1989). Molecular analysis has demonstrated that the loss of Mutator activity also is correlated with both hypermethylation of Mu1 elements (Chandler and Walbot 1986; Bennetzen 1987) and with the loss of the putative autonomous elements MuR1 (Chomet et al. 1991) or MuA2 (Qin et al. 1991), or Mu9 (Hershberger et al. 1991). Once inactivated, the Mu element copy number is halved upon outcrossing (Alleman and Freeling 1986). Mutator activity can be restored to inactive lines via backcrossing to lines which display germinal Mutator activity (Robertson et al. 1985). Reactivation of Mu-inactive lines has also been reported following gamma-irradiation of seed (Walbot 1988). In both cases, reactivation restores somatic mutability and is accompanied by hypomethylation of Mu1 elements. Suppression of a Mu-induced mutant phenotype has been reported for instances when Mu1 is present in the promoter region of the Hcf106 gene (Martiennsen et al. 1989; Martiennsen et al. 1990). Mutant suppression only occurs in Mu-inactive lines wherein the Mu1 insert is modified by methylation. The transcriptional start sites of Hcf106 transcripts generated from suppressed lines have been mapped to 13 different sites within a 70 bp region of the Mu1 terminus (Barkan and Martiennsen 1991). The transcribed region includes the entire protein encoding region, and transcription is regulated by normal tissue-specific controls as well as the state of Mu1 methylation.
The first reported case of *Mu*-induced deletions was by Taylor and Walbot (1985), who described a stable, 74 base pair deletion derivative of an *ADH1* mutant. Subsequently, Robertson and Stinard (Robertson and Stinard 1987) have detailed several *Mu*-induced deletions of varying length on chromosome 9S. Cytological studies of these stocks have verified the presence of both terminal and subterminal deletions (Robertson, personal communication). Deletions are probably a common consequence of *Mutator* activity, and may be generated by crossing over between *Mu* elements inserted at different sites on homologous chromosomes.

Analyses of transcripts of *Mu*-inserted genes have shown that *Mu1* insertions depress transcription of and induce aberrant processing of primary RNA transcripts (Bennetzen et al. 1984; Strommer and Ortiz 1989). The effect of *Mu1* insertions on RNA transcripts shows great variation in studies of identical mutant alleles in different genetic backgrounds. Studies of *Mu*-excision products have revealed losses of both transposon and host DNA sequences, and inverted duplications of sequences flanking host deletions (Chandler and Hardeman 1993).

The general technique of cloning transposon insertional mutants of maize has gained widespread popularity since first described by Wienand et al. (1982). This strategy allows for cloning of genomic DNA from a locus for which no gene product has been identified. The technique requires the generation of an insertional maize mutation in a transposable element background, and the availability of cloned copies of the transposable element responsible for the mutation. A genomic DNA library obtained from a plant harboring the mutant allele can then be screened using the transposable element fragment as a molecular probe, which permits the isolation of a fragment corresponding to the transposon-tagged locus.
The Mutator family of transposable elements has many peculiar characteristics which are especially advantageous for cloning genes via transposon tagging. Unfortunately, there are also numerous disadvantages to this applied use of the Mutator system. Because Mutator stocks display such a high forward mutation rate, a large number of diverse mutations can be generated in a single mutagenesis project. Also, it is often possible to generate multiple alleles of a single locus which can be used in cross-referencing of alleles, a means of verifying the identity of a genomic DNA clone. Furthermore, the high activity of many Mutator stocks permits the analyses of somatic and/or germinal revertants, both of which are very useful in clone verification. At present, molecular clones from thirteen Mu elements are available for use in screening maize libraries, although Mu1 is most often utilized. Over a dozen different genes have been cloned using Mu tagging, corresponding to a wide array of different mutant phenotypes. These include clones of the anthocyanin biosynthetic mutants a1 (O'Reily et al. 1985) and bz2 (McClughlin and Walbot 1987), the carotenoid biosynthesis mutation y1 (Buckner et al. 1990), the disease resistance gene hml (Johal and Briggs 1992) and the photosynthetic membrane assembly mutation hcf106 (Martiennsen et al. 1989).

The most obvious disadvantage in using the Mutator system for transposon-tagging and cloning is the very high copy number of Mu elements present (often 20 to >80 copies of Mu1) (Bennetzen 1984). High Mu element copy number can generate problems in identification of the specific Mu element insertion causing the particular mutation under study. Additionally, high Mu element copy number can lead to the generation of new mutant alleles which may confuse the analysis. Other problems associated with Mu-tagging include: suppression of the mutant phenotype in Mu-inactive lines (Martiennsen et al. 1989; Martiennsen et al. 1990); the generation
of deletion mutations or imperfect excision products that are not tagged with a Mu element; and putative Mu element-induced rearrangement at a locus far removed from the site of transposon insertion (Thorstenson and Freeling 1989).
PAPER 1. GENETIC CHARACTERIZATION AND MAPPING OF 65 MAIZE KERNEL MUTATIONS FROM ROBERTSON’S MUTATOR STOCKS
Sixty Five Mutations Affecting Maize Kernel Development Isolated from *Mutator* Stocks

M. J. Scanlon, P. S. Stinard, M. G. James, A. M. Myers, and D. S. Robertson

From the Department of Biochemistry and Biophysics (Scanlon, James and Myers), the Department of Agronomy (Stinard), and the Department of Zoology and Genetics (Robertson), Iowa State University, Ames IA 50011. This work was supported by research grants to AMM and DSR from the U.S. Department of Agriculture (88-37234-3316 and 91-37301-6344). Address reprint requests to Dr. Alan M. Myers, Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011.

Running title: Mutations affecting maize kernel development
ABSTRACT

Sixty-five mutations affecting development of the maize kernel were isolated from active Robertson's Mutator (Mu) stocks. At least 15 previously undescribed maize genes were defined by mutations in this collection. Genetic mapping located 54 of these defective kernel (dek) mutations to particular chromosome arms, and more precise map determinations were made for 22 of the mutations. Allelism testing of the putative Mu-induced mutations affecting kernel development identified 21 instances of allelism to previously-described Dek genes and between new dek mutations identified in this study. Viability testing of homozygous mutant kernels identified numerous Dek loci with various pleiotropic effects on seedling and plant development. The mutants in this study presumably arose by insertion of a Mu transposon within a Dek locus. Because these mutant loci are putatively tagged with Mu transposons and are accessible to cloning via transposon-tagging, they represent a potentially important source for future genetic, molecular and biochemical investigations of maize kernel development.
INTRODUCTION

The maize kernel provides a particularly tractable genetic system for analysis of tissue development and differentiation in higher plants. Morphological development of the triploid endosperm tissue and the conspicuous embryo in maize kernels can be monitored easily, and several hundred kernels are available for study on a single ear. Extensive efforts have utilized mutations that affect anthocyanin accumulation in the aleurone, the single-celled outer layer of the endosperm, to analyze tissue-specific gene expression, transposon structure and function, and other biological activities (see reviews in Neuffer et al. 1986; Coe et al. 1987). Another large body of work has centered on the numerous genetic loci that effect composition of starch or protein in the endosperm. Maize kernel mutations have also been useful in characterizing development of the maize embryo (Robertson 1952; McCarty et al. 1989a; McCarty et al. 1991; Clark and Sheridan 1986; Clark and Sheridan 1988; Clark and Sheridan 1991).

An abundant class of maize mutations, later termed defective kernel (dek) mutations, were first characterized by Jones (1920) and Manglesdorf (1923; 1926) based on the kernel phenotype involving profound effects on both endosperm and embryo development. The dek mutations typically are recessive, and in the homozygous state frequently induce development of inviable seed. Probably because of difficulties associated with genetic analyses of recessive-lethal mutations, the Dek genes as a class were largely neglected for the more than fifty years spanning the early studies of Jones and Manglesdorf and the later work of Neuffer and Sheridan (1980), and Sheridan and Neuffer (1980). These latter reports described genetic, morphological, lethality, and embryo rescue studies of numerous
**dek** mutations induced by the mutagen ethylmethane sulfonate (EMS). These workers genetically mapped 89 **dek** mutations to 17 of the 20 maize chromosome arms, and described a diverse array of kernel mutant phenotypes. Subsequent analyses of EMS-induced **dek** mutations identified 33 unique genetic loci (Sheridan and Neuffer 1981; Sheridan et al. 1984; Sheridan et al. 1986; Neuffer 1992). Of 855 recessive kernel mutations recovered, only 147 yielded viable plants, although many produced inviable seedlings with altered pigmentation. Histological examination showed **dek** mutant embryos typically were affected more severely than were the endosperms of the same kernels. Although immature **dek** mutant embryos were developmentally blocked over a wide range of developmental stages, the majority in the study (80%) were able to germinate in tissue culture (Sheridan and Neuffer 1980), indicating that embryo lethality often occurs late in kernel development.

Further investigations utilizing **dek** mutations as a class promise to furnish additional information pursuant to an understanding of the complex process of kernel development. In particular, molecular characterization of the genes, and analyses of the biochemical functions of their gene products, will reveal functions required for embryo and endosperm development. Further genetic investigations also will be useful, for example analyses of background effects, double mutants, and **dek** allelic series which exhibit phenotypic variation.

As a step toward the molecular analysis of Dek gene function, this report describes the genetic characterization of 65 kernel mutant isolates from Robertson's *Mutator (Mu)* (Robertson 1978) stocks. One of the goals of this mutagenesis project was to generate **dek** mutations by insertion of *Mu* transposons for use in molecular cloning studies of genes required for kernel development. Based on the assumption that many of these mutations are caused by *Mu* element insertion, they have been
termed, generically, *dek-Mu*. Viability studies of *dek-Mu* mutant kernels are presented, and chromosome arm placements are reported for 54 mutations. More precise genetic mapping information obtained via linkage determinations are reported for 22 mutants. Fifteen previously undescribed, novel *dek* mutations are described, and the pleiotropic effects of several *dek-Mu* mutations are discussed. The accumulated data presented here will be useful in future genetic, biochemical, and molecular analyses of these various loci that impact development of the maize kernel.
MATERIALS AND METHODS

Stocks

*Mutator* lines are defined as those which been shown to have *Mutator* activity as defined by Robertson (1978). Standard lines, defined as stocks which have never been crossed to *Mutator* lines, are the F1 hybrids inbred Q66 x inbred Q67, or inbred B77 x inbred B79. The complete set of B-A translocations and the *waxy*-marked translocation series were propagated at Iowa State University by D. S. Robertson. A series of 31 EMS-induced mutations, *dek1* through *dek31* (Sheridan and Neuffer 1981; Sheridan et al. 1984; Sheridan et al. 1986), were used to test for allelism to *dek-Mu* mutations. All stocks of marker genes used in linkage determinations were supplied by the Maize Genetics Stock Center, University of Illinois, Urbana, except the *brn1 Lg3* stock which was developed by D. S. Robertson and P. S. Stinard, Iowa State University, Ames.

Isolation and Propagation of Maize Kernel Mutants

The strategy for the isolation of *dek* mutations from a *Mutator* stock is presented in Figure 1. Active *Mutator* plants were outcrossed as males to standard lines, and F1 plants grown from the outcrossed ears were self-pollinated. Self pollinated ears of the F1 plants were then scored for the presence of defective kernels, with the expectation that a recessive *dek* mutation in the heterozygous
Figure 1. Strategy for using the Robertson's *Mutator* system for induction and propagation of maize mutations affecting kernel development.
state in the parent plant would cause 1/4 of the progeny kernels to develop abnormally. Most of the \textit{dek} alleles in this study are recessive-lethal mutations; thus, they were propagated by outcrossing \textit{dek}/\textit{Dek} heterozygotes to \textit{Dek}/\textit{Dek} standard lines. To do so, plants were grown from normal sibling kernels from self-pollinated ears displaying 1/4 defective kernels, and these were outcrossed as males to standard lines. Approximately 10 progeny plants from these crosses were grown to maturity, and both self-pollinated and outcrossed as males to standard lines. The genotype of each plant in the family was assigned by observing self-pollinated ears; the presence of 1/4 defective kernels identified \textit{dek}/\textit{Dek} plants, whereas all normal kernels indicated a \textit{Dek}/\textit{Dek} plant. The outcross progeny from \textit{dek}/\textit{Dek} plants were then used in a continuation of this self-pollination/outcross protocol, to propagate the recessive-lethal \textit{dek} mutations in the heterozygous state in successive generations. As expected, in most instances approximately one half of the progeny plants of each outcross were found to be heterozygous for the \textit{dek} mutation.

\textbf{Viability Tests and Classification of \textit{dek}/\textit{dek} Seedling Phenotypes}

Viability tests were performed on \textit{dek}/\textit{dek} defective kernels that upon inspection at maturity contained some visible embryonic structure, by planting 20 mutant kernels in the field or in pots in the greenhouse. Those \textit{dek} mutations that resulted in kernels with no visible embryonic structure were classified as germless-lethal without further analysis. A germination rate of 5\% or less caused a \textit{dek} mutation to be classified as lethal. Significant germination rates of \textit{dek}/\textit{dek} mutant kernels did occur in some instances, as described below.

The germination products of defective kernels were noted for seedling and/or plant phenotype. In rare instances \textit{dek}/\textit{dek} kernels produced plants with fertile ears
and tassels; these were self-pollinated, and ears were harvested and scored for the corresponding kernel phenotype to verify the homozygous genotype of the parental plants. Whenever putative \textit{dek/dek} homozygous plants produced functional ears but no pollen, they were pollinated by standard \textit{Q60} plants. Kernels from the resulting crosses were planted, and the plants were self-pollinated and scored for kernel mutant phenotype to verify the homozygous genotype of the parental plants. All plants in this outcross are expected to be \textit{dek/Dek} heterozygotes and produce 1/4 defective kernels upon self-pollination.

\textbf{Genetic Mapping}

Three different techniques were used for mapping of \textit{dek} mutations to a specific chromosome arm. Placement was most often accomplished using the B-A translocation method (Roman and Ulstrup 1951) as described by Beckett (1978). In other instances, mutants were placed using either the \textit{waxy}-marked translocation series as described by Anderson (1956) and Robertson (1955), or demonstration of allelism to a previously mapped \textit{dek} mutation (described in the following section). Analyses of linkage of kernel mutants to marker genes of known map location were performed according to standard techniques.

\textbf{Allelism Tests}

Pairs of \textit{dek} mutations that in the homozygous state did not prevent the development of fertile plants were tested for allelism as follows. Plants grown from ten homozygous mutant kernels of each of the two \textit{dek} mutant isolates were reciprocally crossed. Positive tests of allelism were recorded when both ears of this cross produced 100\% mutant kernels. When \textit{dek} mutations to be tested were lethal
in the homozygous state or produce sterile plants, allele tests were usually conducted using plants grown from 20 heterozygous outcross progeny kernel per mutant isolate. One-half of the kernel planted, therefore, are expected to be heterozygous for the particular dek mutation. In some instances, 20 normal kernel were planted from a self-pollinated ear of a heterozygous mutant plant, with the expectation that two thirds of the plants would be heterozygous for the particular dek mutation. Allelism tests were performed by outcrossing pollen of one mutant family to the first ear of the second mutant family, and self pollinating the second ear of the first mutant. This procedure was carried out in reciprocal fashion. Whenever a mutant family did not produce second ears, ears from plants of that mutant family were self-pollinated and the pollen used in an outcross of the first ear of the second mutant family. Allelism was assigned whenever the outcrossed ears of two dek/Dek heterozygous plants from different mutant families (as determined by self pollination) segregated at a 3:1 ratio for normal kernels relative to the mutant kernel phenotype. Independent dek mutations were determined to be non-allelic whenever the outcross ear of such a cross produced all wild type kernels.
RESULTS

Mutant Phenotypic Descriptions and Inheritance
Sixty-five dek mutations were isolated from active Mutator lines as described in Materials and Methods. For each mutation Table 1 lists our initial laboratory designation, the chromosome arm location, a summary description of the kernel phenotype, and an assessment of the viability of dek/dek plants. Table 1 also indicates those mutations that could be assigned as alleles of previously known genes or novel genes identified in this study (see below). Of the mutations listed in Table 1, all are recessive regarding the defective kernel phenotype. These mutations all were identified by self-pollinating Dek/dek heterozygous plants, and propagated by outcrossing such plants onto standard lines as shown in Figure 1.

Two other mutations, originally designated Dap*-1 and Dap*-2, are not inherited as simple recessive Mendelian factors. Both of these mutations cause a defective kernel phenotype in the homozygous condition, but also can cause an aleurone mosaic phenotype termed dappled when combined with a wild type allele in heterozygous kernels. The effects on the maize kernel of Dap*-1 and Dap*-2 in the heterozygous condition are limited to the aleurone in the genetic backgrounds used in this study; there are no other obvious defects in endosperm development, and the embryos produce fully viable plants. The two mutations were shown to be allelic and to define a previously unidentified locus termed Dap1; they have thus been assigned the allele names dap1-1 and dap1-2. Because the aleurone mosaic is most easily observed in kernels with aleurone pigmentation, dap1-1 and dap1-2 were propagated by crossing pollen from plants displaying purple aleurone and.
Table 1. Characterization of *dek* mutations

<table>
<thead>
<tr>
<th>Laboratory Designation</th>
<th>Arm Location^</th>
<th>Placement Method^</th>
<th>Locus</th>
<th>Viability^</th>
<th>Kernel Phenotype^d</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>dek</em>-Mu2115</td>
<td>1S</td>
<td>TB</td>
<td></td>
<td>-</td>
<td>ren to emp, sml gm</td>
</tr>
<tr>
<td><em>dek</em>-Mu2045</td>
<td>1S</td>
<td>TB, WT</td>
<td>Emp1</td>
<td>-</td>
<td>emp, gn</td>
</tr>
<tr>
<td><em>dek</em>-Mu8319</td>
<td>1S</td>
<td>TB</td>
<td></td>
<td>-</td>
<td>ren to emp, gm</td>
</tr>
<tr>
<td><em>dek</em>-MuPIAE0^e</td>
<td>1S</td>
<td>A</td>
<td>Dek1</td>
<td>-</td>
<td>fl, gm, cl</td>
</tr>
<tr>
<td><em>dek</em>-Mu6214</td>
<td>1L</td>
<td>TB</td>
<td></td>
<td>-</td>
<td>emp, gn</td>
</tr>
<tr>
<td><em>dek</em>-Mu15568</td>
<td>1L</td>
<td>TB</td>
<td>Ptd1</td>
<td>(+)</td>
<td>ptd, sml gm</td>
</tr>
<tr>
<td>et*-Mu2352</td>
<td>2S</td>
<td>TB</td>
<td>Et2</td>
<td>+</td>
<td>et</td>
</tr>
<tr>
<td><em>dek</em>-MuPIE3^e</td>
<td>2L</td>
<td>TB</td>
<td></td>
<td>-</td>
<td>ren, gm</td>
</tr>
<tr>
<td><em>dek</em>-MuPIC3^e</td>
<td>2L</td>
<td>TB</td>
<td></td>
<td>-</td>
<td>ren to emp, gm</td>
</tr>
<tr>
<td><em>dek</em>-Mu1047</td>
<td>2L</td>
<td>TB, WT</td>
<td>Emp2</td>
<td>-</td>
<td>emp, gm</td>
</tr>
<tr>
<td><em>dek</em>-Mu2159</td>
<td>2L</td>
<td>TB, WT</td>
<td></td>
<td>-</td>
<td>ren, sml gm to gm</td>
</tr>
<tr>
<td><em>dek</em>-Mu1365</td>
<td>2L</td>
<td>TB</td>
<td></td>
<td>-</td>
<td>ren, ptd, gm</td>
</tr>
<tr>
<td><em>dek</em>-Mu2444</td>
<td>2L</td>
<td>TB</td>
<td>++</td>
<td>ren, sml gm</td>
<td></td>
</tr>
<tr>
<td><em>dek</em>-Mu4160</td>
<td>2L</td>
<td>TB</td>
<td></td>
<td>-</td>
<td>ren, gm, LF</td>
</tr>
<tr>
<td>brn1*-Mu</td>
<td>3S</td>
<td>TB</td>
<td>Brn1</td>
<td>+</td>
<td>brn, brn gm</td>
</tr>
<tr>
<td>brn*-Mu3071</td>
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<td>A</td>
<td>Brn1</td>
<td>-</td>
<td>brn gm</td>
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<tr>
<td><em>dek</em>-Mu1185</td>
<td>3S</td>
<td>TB</td>
<td>Ref1</td>
<td>++++</td>
<td>ren, ptd, fl, sml gm, LF</td>
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<tr>
<td>ae*-Mu33</td>
<td>3S</td>
<td>A</td>
<td>Dek5</td>
<td>+</td>
<td>sh, sml gm, msc, LF</td>
</tr>
<tr>
<td>ae*-Mu25</td>
<td>3S</td>
<td>A</td>
<td>Dek5</td>
<td>+</td>
<td>sh, sml gm, msc</td>
</tr>
<tr>
<td><em>dek</em>-Mu2608</td>
<td>4S</td>
<td>TB, A</td>
<td>Dek7</td>
<td>+</td>
<td>su, sh, msc, sml gm</td>
</tr>
<tr>
<td>su-sh*-Mu5081^f</td>
<td>4S</td>
<td>WT</td>
<td>Su3</td>
<td>++++</td>
<td>su, sh, LF</td>
</tr>
<tr>
<td><em>dek</em>-Mu2058</td>
<td>4S</td>
<td>TB</td>
<td>Dsc1</td>
<td>-</td>
<td>dsc, rgh, gm</td>
</tr>
<tr>
<td><em>dek</em>-Mu3252</td>
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<td>A</td>
<td>Dsc1</td>
<td>-</td>
<td>dsc, rgh, gm</td>
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<tr>
<td><em>dek</em>-Mu2410</td>
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<td>TB, A</td>
<td>Dek25</td>
<td>+</td>
<td>sh, dsc</td>
</tr>
<tr>
<td><em>dek</em>-Mu1566</td>
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<td>A</td>
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<td>-</td>
<td>sh, dsc</td>
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<tr>
<td>su*-Mu2412</td>
<td>4S</td>
<td>A</td>
<td>Su1</td>
<td>++++</td>
<td>su</td>
</tr>
<tr>
<td>su*-Mu8064</td>
<td>4S</td>
<td>A</td>
<td>Su1</td>
<td>++++</td>
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</tr>
<tr>
<td>su*-Mu7110</td>
<td>4S</td>
<td>A</td>
<td>Su1</td>
<td>++++</td>
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</tr>
<tr>
<td>su*-Mu3162</td>
<td>4S</td>
<td>A</td>
<td>Su1</td>
<td>++++</td>
<td>su</td>
</tr>
<tr>
<td>su*-Mu4582</td>
<td>4S</td>
<td>A</td>
<td>Su1</td>
<td>++++</td>
<td>su</td>
</tr>
<tr>
<td><em>dek</em>-Mu2689</td>
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<td>TB, A</td>
<td>Dek31</td>
<td>-</td>
<td>ptd, rgh, gm</td>
</tr>
<tr>
<td><em>dek</em>-MuNS8070^f</td>
<td>5S</td>
<td>TB</td>
<td></td>
<td>-</td>
<td>emp, gm</td>
</tr>
<tr>
<td><em>dek</em>-Mu2146</td>
<td>5L</td>
<td>TB</td>
<td></td>
<td>-</td>
<td>ren, o, sml gm</td>
</tr>
<tr>
<td><em>dek</em>-Mu1182</td>
<td>5L</td>
<td>TB</td>
<td></td>
<td>-</td>
<td>sml o, sometimes gm</td>
</tr>
<tr>
<td><em>dek</em>-Mu5133</td>
<td>5L</td>
<td>TB, A</td>
<td>Sh4</td>
<td>-</td>
<td>sh, fl, gm</td>
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<tr>
<td><em>dek</em>-Mu8186</td>
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<td>TB</td>
<td>Prg1</td>
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<td>ptd, rgh, sml gm</td>
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<tr>
<td><em>dek</em>-MuNS8075^e</td>
<td>5L</td>
<td>TB</td>
<td>Ren1</td>
<td>(++++</td>
<td>ren, o, sml gm</td>
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<tr>
<td><em>dek</em>-MuP0O^e</td>
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<td>TB</td>
<td></td>
<td>-</td>
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</tr>
<tr>
<td>dap1 (D)</td>
<td>5L</td>
<td>TB</td>
<td>Dap1</td>
<td>++++</td>
<td>msc, LF</td>
</tr>
<tr>
<td>dap2 (D)</td>
<td>5L</td>
<td>A</td>
<td>Dap1</td>
<td>++++</td>
<td>msc, LF</td>
</tr>
<tr>
<td><em>dek</em>-Mu1104</td>
<td>6L</td>
<td>TB</td>
<td></td>
<td>+</td>
<td>ren, sml gm, LF</td>
</tr>
</tbody>
</table>
Table 1. (Continued)

<table>
<thead>
<tr>
<th>Dek*</th>
<th>Location</th>
<th>Marker</th>
<th>Phenotype</th>
<th>Notes</th>
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<tbody>
<tr>
<td>dek*-Mu184d</td>
<td>6</td>
<td>L</td>
<td>Mn3</td>
<td>++++</td>
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<tr>
<td>su*</td>
<td>Mu5178</td>
<td>6L</td>
<td>A</td>
<td>Su2</td>
</tr>
<tr>
<td>dek*</td>
<td>Mu208s</td>
<td>7S</td>
<td>TB, WT</td>
<td></td>
</tr>
<tr>
<td>dek*</td>
<td>Mu3193</td>
<td>7L</td>
<td>TB</td>
<td>Ptd2</td>
</tr>
<tr>
<td>dek*</td>
<td>Mu15153</td>
<td>7L</td>
<td>TB</td>
<td></td>
</tr>
<tr>
<td>dek*</td>
<td>MuNS3268</td>
<td>7L</td>
<td>TB</td>
<td>Ren2</td>
</tr>
<tr>
<td>dek*</td>
<td>MuNS4138</td>
<td>7L</td>
<td>TB, A</td>
<td>Ren2</td>
</tr>
<tr>
<td>dek*</td>
<td>MuNS5958</td>
<td>7L</td>
<td>TB, A</td>
<td>Ren2</td>
</tr>
<tr>
<td>dek*</td>
<td>Mu5132</td>
<td>8L</td>
<td>A</td>
<td>Pro1</td>
</tr>
<tr>
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<td>Mu2425</td>
<td>10S</td>
<td>TB</td>
<td>-</td>
</tr>
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<td>TB</td>
<td>-</td>
</tr>
<tr>
<td>dek*</td>
<td>Mu1339</td>
<td>10L</td>
<td>TB, WT</td>
<td>Ren3</td>
</tr>
<tr>
<td>dek*</td>
<td>Mu2221</td>
<td>10L</td>
<td>A</td>
<td>W2/Dek21</td>
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<td>dek*</td>
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<td>N.D.</td>
<td>+</td>
</tr>
<tr>
<td>dek*</td>
<td>Mu4309</td>
<td>N.D.</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>dek*</td>
<td>Mu2080</td>
<td>N.D.</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>dek*</td>
<td>Mu2078</td>
<td>N.D.</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>dek*</td>
<td>MuPS602</td>
<td>N.D.</td>
<td>N.D.</td>
<td>(++)</td>
</tr>
<tr>
<td>dek*</td>
<td>MuNS127683</td>
<td>N.D.</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>dek*</td>
<td>Mu1226</td>
<td>N.D.</td>
<td>N.D.</td>
<td>+++</td>
</tr>
<tr>
<td>dek*</td>
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<td>N.D.</td>
<td>+</td>
</tr>
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<td>N.D.</td>
<td>-</td>
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<tr>
<td>dek*</td>
<td>Mu2192</td>
<td>N.D.</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>dek*</td>
<td>Mu1364</td>
<td>N.D.</td>
<td>N.D.</td>
<td>+++</td>
</tr>
</tbody>
</table>

a All entries for arm locations are included on the basis of one type of positive test. Arm locations confirmed by subsequent criteria are listed in italics. N. D., not determined.

b TB, placement determined using appropriate B-A translocation; A, placement determined using allelism test; WT, placement determined using appropriate waxy-marked translocation (see Table 5 for specific translocation used); L, placement determined via linkage to marker on same chromosome; N. D., not determined.

c Results of lethality tests are for homozygous mutants, except for the semi-dominant Dap1 alleles where results are for heterozygote kernels. -, inviable seed, no germination; +, seedling lethal, die before five leaf stage; ++, plants develop beyond five leaf stage but die before producing flower inflorescence; ++++, plants produce male and/or female flower(s), but one (or both) is sterile; ++++, fertile plants, produce functional male and female flowers; ( ), less than 5% of mutant seed germinate to display indicated phenotype.
Table 1. (Continued)

d See Table 2 for explanation of symbols used to describe kernel phenotypes.
e Mutants kindly provided by Steve Briggs, Pioneer Hi-bred Int'l., Inc.
f May be duplicate factor.
g Mutants kindly provided by Nancy Shepherd, DuPont de Nemours, Inc.
h *dek*-Mu1184 is linked to *y1* on chromosome arm 6L, but is not uncovered by either TB-6Lc, nor TB-6Sa. Therefore it is located proximally to the breakpoints of these B-A translocations.
i Mutant kindly provided by Patrick Schnable, Iowa State University.
homozygous for the wild type Dap1 allele onto ears of plants grown from dappled kernels with the Dap1/dap1 genotype.

The mutations listed in Table 1 display a wide range of phenotypes (described in Table 2); the two most common types are reduced endosperm size (ren) and empty pericarp or papery kernel (emp). Single ears segregating for the ren or emp kernel mutant phenotype often display significant variation in the degree of mutant kernel filling. Although the emp mutant kernels are almost always germless, the ren mutant kernels often contain a small embryo, and larger ren mutant kernels usually contain larger embryos than their smaller sibling mutants. Other commonly observed kernel mutant phenotypes include the sugary-shrunken-brittle varieties, miniature seeds, and numerous etched-pitted, and aleurone mosaic endosperm types (Table 1).

Most of the kernel mutant phenotypes in this study are inherited in the normal Mendelian ratios expected for single factor controlled traits. Ten mutations, however, show a low frequency (LF) of expression on segregating ears of heterozygous plants. In these instances, self-pollinated ears of heterozygous plants displayed less than the expected 25% defective kernels.

Expression of the dappled phenotype caused by the mutations dap1-1 and dap1-2 also is irregular. When plants grown from Dap1/dap1 kernels are outcrossed through the female to purple aleurone tester stocks the frequency of the dappled kernels in the progeny is approximately 38%, instead of the expected 50%. No dappled kernels are observed, however, when Dap1/dap1 heterozygotes are outcrossed as males to the same tester stocks. Even so, the mutant alleles are transmitted through pollen, although also in reduced frequency. Approximately 31% of the plants grown from F1 kernels of the latter cross do produce ears that segregate dappled and purple aleurone kernels when pollinated by the purple aleurone tester plants. Furthermore,
Table 2. Kernel phenotypes

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>bn</td>
<td>brown aleurone—brown color in aleurone cells.</td>
</tr>
<tr>
<td>cl</td>
<td>colorless aleurone—absence of anthocyanin pigments from aleurone cells.</td>
</tr>
<tr>
<td>dek</td>
<td>defective kernel</td>
</tr>
<tr>
<td>dsc</td>
<td>discolored—some variation of normal clear yellow or white color, as though stained reddish or brownish.</td>
</tr>
<tr>
<td>emp</td>
<td>empty pericarp—kernels with little or no endosperm tissue, flattened and papery.</td>
</tr>
<tr>
<td>et</td>
<td>etched—surface of kernel with network of pits; fractures of underlying endosperm.</td>
</tr>
<tr>
<td>fl</td>
<td>floury—endosperm has a soft, chalk-like texture, a generally reduced yellow color and an opaque appearance.</td>
</tr>
<tr>
<td>gls</td>
<td>glassy—endosperm translucent with a hard flinty appearance, like su2.</td>
</tr>
<tr>
<td>gm</td>
<td>germless—embryo very poorly developed or entirely absent.</td>
</tr>
<tr>
<td>LF</td>
<td>mutant kernels on an ear, and/or ears segregating mutant kernels in a family are present in low frequency; non-Mendelian ratios of wild type to mutant phenotype.</td>
</tr>
<tr>
<td>lsp</td>
<td>loose pericarp—a space separates the pericarp from the aleurone, leaving the kernel a grayish appearance over part of the kernel.</td>
</tr>
<tr>
<td>mn</td>
<td>miniature—kernel typical in shape, form and texture, but smaller in size and with slightly loose pericarp.</td>
</tr>
<tr>
<td>msc</td>
<td>mosaic—irregular-shaped patches of color and non-color in the aleurone tissue when appropriate genes for anthocyanin are present; the borders are distinct, as opposed to the mottled effect.</td>
</tr>
<tr>
<td>o</td>
<td>opaque—endosperm is opaque and firm, not chalky and waxy.</td>
</tr>
<tr>
<td>ptd</td>
<td>pitted—surface of kernel strewn with small pits or indentations.</td>
</tr>
<tr>
<td>ren</td>
<td>reduced endosperm—endosperm much reduced in size, often associated with loose pericarp and small embryo.</td>
</tr>
<tr>
<td>rgh</td>
<td>rough—surface of kernel covered with many small irregularities, giving it a rough texture.</td>
</tr>
<tr>
<td>sh</td>
<td>shrunk—two types: (1) a moderately smooth collapse of the crown on both sides of the kernel, like shl, and (2) an extreme collapse of endosperm tissue, often associated with a brittle and/or translucent appearance, like sh2.</td>
</tr>
<tr>
<td>sml</td>
<td>small—used in conjunction with other descriptive terms to designate smaller size.</td>
</tr>
<tr>
<td>su</td>
<td>sugary—kernel is shriveled, hard, and translucent; typical of su1.</td>
</tr>
</tbody>
</table>

a Adapted from Sheridan and Neuffer (1980)
when Dapl/dapl plants are self-pollinated, the ears segregate three kernel types in ratios approaching 2 purple aleurone: 1 dappled: 1 defective kernel. The defective kernels, presumably with the homozygous dap1/dapl genotype, are very small, do not germinate, and display an extreme aleurone disruption. The presence of greater than two purple aleurone kernels for every dappled kernel on self-pollinated ears of Dapl/dapl heterozygous plants, is consistent with the fact that the dappled phenotype is not expressed on F1 ears when the mutation is male transmitted. Thus, with regard to the dappled phenotype, both dap1-1 and dap1-2 appear to be dominant when female-transmitted but seemingly are recessive when male transmitted.

Genetic Mapping of dek Mutations

Classical genetic mapping techniques were used to locate 54 dek mutations on specific chromosome arms (Table 1). Mutants were placed to 15 of the 20 chromosome arms of maize. Subsequent genetic tests, including allelism determination and linkage analyses, were used to confirm the placement of 35 mutations. Although in most instances mutants were placed using the B-A translocation series (Roman and Ulstrup 1951; Beckett 1978) or the waxy-marked translocation series of maize (Anderson 1956; Robertson 1955), some mutations that caused phenotypes reminiscent of those resulting from previously mapped kernel mutations were placed by positive tests of allelism. Chromosome arm locations were not determined for 11 of the mutations. Because most of the mutations are lethal in the homozygous condition and were tested as heterozygotes, and some B-A translocation stocks shed little pollen, some dek mutations were not crossed to all B-A translocation stocks an equal number of times. Also, some of the unlocated
mutant loci may be proximal to the breakpoints of the corresponding B-A translocation, or too far removed from waxy-marked translocation breakpoints to detect linkage, which would prevent their detection (Roman and Ulstrup 1951; Beckett 1978; Anderson 1956; Robertson 1955). Finally, placement of certain kernel mutations can be difficult because some B-A translocations produce small, opaque hypoploid endosperm, a phenotype resembling that of many of the kernel mutants being tested (Roman and Ulstrup 1951).

Viability Tests

The results of viability tests conducted on the kernel mutants in this study are presented in Table 1. Results are reported for homozygous mutant kernel obtained from self-pollinated ears of dek/Dek heterozygotes. An exception is the dominant Dapl alleles, for which the results of mutant dap1/Dapl heterozygous kernels are reported. Results of the lethality screens are reported on a scale from "-", for lethal kernels exhibiting less than 5% germination, up to "++++" for mutant kernel which yield fertile plants as detailed in the legend of Table 2.

Of the 65 putative Mu-induced kernel mutations tested, 39 were lethal in the homozygous condition "-", and an additional 11 caused seedling lethality, "+", in which plants died before reaching the five leaf, adult stage of vegetative development. Four kernel mutations resulted in plants that reached the reproductive stage of development, but the male or female flower, or both, were nonfunctional, "+++". Eleven other mutations were classified as viable, "++++", and produced fertile plants from which self-pollinated ears were obtained.

The healthiest plants were obtained from the small seeded type of mutant kernel. Oftentimes, only those mutant seed with the largest kernels and embryos on
a segregating ear germinated, while the smaller, sibling mutant kernels did not. Therefore, the germination rates of many mutations classified as viable were below 60%. For example, the small seeded, small embryo mutant *dek*-*Mu1185* showed a 30% germination rate, and produced relatively normal, fertile, full size plants that made large ears but shed little pollen. The pitted, small germ mutant *dek*-*Mu3193* exhibited a 15% germination rate and produced small, infertile plants with narrow leaves and frayed, necrotic leaf margins (Figure 2A,B). Other pleiotropic effects of this mutation included rudimentary ear and tassel formation. More than 50% of the small seeded mutant kernels homozygous for *dek*-*Mu2444* germinated, producing small narrow-leaved plants. Numerous aberrations of shoot development also were exhibited homozygous for *dek*-*Mu1364*, a small seeded mutant with a 50% germination rate. Plants homozygous for this mutation were brachythic (short internode) (Figures 3A and 3B), and produced almost twice as many leaves per linear foot of plant height as *dek*-*Mu1364* heterozygotes (Figure 3C). The ligule and auricle domains of *dek*-*Mu1364* homozygous plants were disrupted, and ligular tissue was ectopically expressed in the leaf midrib (Figure 3D). One result of the ligular-auricular disruption was leaves that droop severely (Figure 3A,B). Lastly, the tassels of *dek*-*Mu1364* homozygous plants were barren, although five of the ten plants obtained produced ears that were outcrossed to standard Q60 pollen, and set seed. Several mutations in this study were classified as viable, with a germination rate of 90% or better. Besides the *dap1/Dapl* heterozygotes previously alluded to, these include homozygous mutants of the pitted, miniature *dek*-*Mu1184*, *su-sh*-*Mu5081*, and five other sugary-shrunken mutants found to be alleles of the *su1* locus (see Table 1).
Figure 2. Pleiotropic effects of selected defective kernel mutations. (A) Plants homozygous for the ren2-Mu413 mutation were miniature, and at 75 days post germination produced sterile, rudimentary tassels but no ears. (B) Miniature, sterile plants grown from kernels homozygous for ptd1-Mu1568. The leaves of ptd1/ptd1 plants contained patches of brown, mottled tissue. (C) and (D) Plants grown from kernels homozygous for the ptd2-Mu3193 mutation were small, sterile, and contained narrow leaves with frayed, necrotic tissue in the leaf margins. All plants were photographed at 60 days post-germination. The length of the red tags equals approximately six inches.
Figure 3. Pleiotropic effects of the dek-Mu1364 mutation. Plants grown from small-seeded mutant kernels homozygous for dek-Mu1364 exhibited numerous developmental aberrations. (A) Plants of the genotype dek-Mu1364/dek-Mu1364 at 60 days post-germination. These plants were extremely brachytic (short internode length). (B) Plants of the genotype dek-Mu1364/dek-Mu1364 at anthesis. These plants were male-sterile and contained almost twice as many leaf nodes as wild type plants. The older leaves of mature plants homozygous for dek-Mu1364 droop severely. (C) Wild type dek-Mu1364/Dek-Mu1364 heterozygotes prior to anthesis. (D) ligule disruption in a leaf of a dek-Mu1364/dek-Mu1364 plant. All such leaves exhibited ectopic displacement of ligule tissue into the midrib, which is responsible for the aberrant leaf attitude shown in panel B. (E) Normal ligule development in leaf of a wild type, heterozygous plant of the genotype dek-Mu1364/Dek-Mu1364. The length of the tags equals approximately six inches.
Among the 39 lethal mutations were eight that displayed a germination rate of less than 5%. These included three small, pitted, kernel mutations, dek*-Mu8186, dek*-Mu1568 (Figure 2D) and dek*-MuPS602, for which less than 5% of the homozygous kernel produced seedlings" (+)", small, plants "(++)", and narrow-leaved, infertile plants "(+++)", respectively. Three mutations which caused the reduced endosperm phenotype dek*-MuNS807, dek*-Mu1339 and dek*-MuNS413, also germinated at rates of less than 5%. Relatively normal plants and self-pollinated homozygous ears were obtained from less than 5% of both dek*-MuNS807 and dek*-Mu1339 seed, whereas some dek*-MuNS413 kernels produced small plants with rudimentary tassel formation and no ears (Figure 2C).

One trend observed in the analyses of plants grown from defective kernels is that mutations that affect the aleurone layer of the endosperm, causing phenotypes such as etched, pitted, shrunken, and aleurone mosaic, frequently produced seedlings with reduced chlorophyll content (Sheridan and Neuffer 1980; Coe et al.l987). For example, five mutations found to be alleles of the Dek7, Dek5, Dek25, and Dek21 loci, dek7*-Mu2608, dek5*-Mu25, dek5*-Mu33, dek25*-Mu2410 and dek21*-Mu 2221, produced striated or albino seedlings. Other mutations found to affect seedling greening include dek*-Mu8186 and dek*-Mu5191 (striated leaves), et*-Mu2352 (albino seedlings), brn1*-Mu (pale green seedlings), and dek*-Mu1568 (brown, mottled leaves). Furthermore, alterations in seedling pigmentation were not limited to seedlings grown from kernels with obvious aleurone disruption. An allele of the opaque mutant pro1, pro1*-Mu5132 produced striated seedlings, and the mutation dek*-Mu4198, which caused the small seeded phenotype, produced albino seedlings.
Allelism Tests

Extensive allelism tests were performed as an aid in genetic mapping efforts and in order to distinguish novel kernel mutations from alleles of previously described loci. After genetic mapping to a specific chromosome arm, *dek* mutations were tested for allelism to previously described mutations affecting kernel development located on the same chromosome arm. The results of the allelism testing are given in Table 1, which lists 21 identified cases of allelism among the 65 mutants studied. This list includes 9 putative Mu-induced alleles of *Dek* loci identified by Neuffer and Sheridan (1980), 5 alleles of the *Su1* locus, and 5 allelic pairs of novel *Dek* loci first identified in this study.

In most cases the kernel and seedling phenotypes (where applicable) caused by allelic mutations were identical, although notable exceptions existed. For example, kernels homozygous for *brn1-Mul* were of normal size and contained dark brown pigment in the aleurone and small patches of brown on the embryo. In contrast, kernels homozygous for the allelic mutation *brn1-Mu3071* were relatively small, and the brown coloration in the endosperm was much less severe (Figure 4A). Moreover, whereas kernels homozygous for *brn1-Mu1* produced pale-green, inviable seedlings, those homozygous for *brn1-Mu3071* failed to germinate at all. Heterozygous *brn1-Mu1/*brn1-Mu3071 kernels displayed yet a third distinct phenotype; these kernels were pale brown, of normal size, and produced pale-green, inviable seedlings.

Phenotypic heterogeneity was also present among the two additional alleles of *Dek25* (Neuffer and Sheridan 1980) identified in this study. Although both the original reference allele and *dek25-Mu1566* were lethal mutants, more than 10% of
Figure 4. Kernel-phenotypic variation produced by independently-derived defective kernel mutations. (A) Kernels homozygous for the brn1-Mu mutation (top) are brown-pigmented and produce pale-green, inviable seedlings. In contrast, kernel homozygous for the allelic mutation brn1-Mu3071 (bottom) have mostly yellow endosperm with small patches of brown on the embryo, and do not germinate. (B) Kernels homozygous for the standard sul mutation (top) are translucent and wrinkled throughout the expanse of the endosperm, whereas kernels of the genotype sul-Mu2412/sul (bottom) are wrinkled and translucent only in the kernel crown.
allelic dek25-Mu2410 mutant kernels germinated and produced striated, lethal striated, lethal seedlings (Table 1). No obvious differences in kernel phenotype were detectable among these three alleles.

Kernels homozygous for either of two additional alleles of Dek5 (Neuffer and Sheridan 1980) were phenotypically identical, although the mutations displayed dissimilar inheritance patterns. Specifically, dek5-Mu33 was inherited in low frequency as compared to both dek5-Mu25 (Table 1) and the reference allele dek5-1 (Neuffer and Sheridan 1980). Finally, one allele of the Su1 locus, su1-Mu2412, produced less kernel wrinkling and translucence than the reference su1 allele or any of the other su1 alleles identified in this study (Figure 4B).

**Novel Kernel Developmental Loci**

Of the 65 kernel mutants identified in this study, we have identified 15 as representatives of novel, previously undescribed loci (Table 3). These putative Mu-induced mutations were shown by allelism testing to reside in genes distinct from previously described mutations that caused a similar phenotype. The phenotypes caused by homozygous mutations in these novel loci are presented in Figures 5 and 6.

**Linkage Determinations**

Genetic linkage analysis was used to map more precisely the location of several Dek loci. Tables 4-6 present the results of two point, three point, and four point linkage studies involving 22 different dek mutations. These data are summarized in
Table 3. Novel mutations affecting kernel development

<table>
<thead>
<tr>
<th>Name</th>
<th>Former designation</th>
<th>Map location</th>
<th>Phenotypic attributes and viability</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ptd1</em></td>
<td>dek*-Mu1568</td>
<td>1L</td>
<td>small kernels with pitted, scarred endosperm and small germ; usually lethal, although kernels with larger embryos germinated to produce small, non-flowering plants with large, necrotic, mottled sectors on leaves.</td>
</tr>
<tr>
<td><em>ptd2</em></td>
<td>dek*-Mu3193</td>
<td>7L</td>
<td>pitted, cracked endosperm and small germ; approximately 10% of kernels germinated to produce plants with narrow, frayed leaves with necrotic margins; formed sterile, rudimentary ear with very short silks and sterile unbranched tassel.</td>
</tr>
<tr>
<td><em>ren1</em></td>
<td>dek*-Mu807</td>
<td>5L</td>
<td>small kernels with reduced, opaque endosperm and small germ; usually inviable but kernels with larger embryos germinated to produce fertile plants from which self-pollinated, homozygous ears have been obtained.</td>
</tr>
<tr>
<td><em>ren2</em></td>
<td>dek*-Mu326</td>
<td>7L</td>
<td>endosperm variably reduced in size, often with loose pericarp and small germ; lethal, although larger kernels produced small plants with rudimentary, sterile tassel.</td>
</tr>
<tr>
<td><em>ren3</em></td>
<td>dek*-Mu1339</td>
<td>10L</td>
<td>Reduced endosperm, varying from partially filled to empty pericarp; may have small germ, or germless; often lethal, but larger kernels viable, produced fertile plants; homozygous ear has been obtained.</td>
</tr>
<tr>
<td><em>emp1</em></td>
<td>dek*-Mu2045</td>
<td>1S</td>
<td>papery, empty pericarp, devoid of endosperm material and flattened by compression of surrounding normal seed; germless, lethal.</td>
</tr>
<tr>
<td><em>emp2</em></td>
<td>dek*-Mu1047</td>
<td>2L</td>
<td>papery, empty pericarp with very little or no endosperm material; germless at maturity, lethal.</td>
</tr>
<tr>
<td><em>dsc1</em></td>
<td>dek*-Mu2058</td>
<td>4S</td>
<td>discolored, opaque or brownish seed, with distorted shape and an irregular, rough texture; germless at maturity, inviable.</td>
</tr>
<tr>
<td><em>et2</em></td>
<td>et*-Mu2352</td>
<td>2S</td>
<td>etched endosperm; small germ, yielded mutable, inviable albino seedlings with occasional greening of leaf tips</td>
</tr>
<tr>
<td><em>dap1</em></td>
<td>dap1</td>
<td>5L</td>
<td>dappled aleurone; patches of normal and abnormal cells; shows dosage effect and inherited in reduced frequency; kernels with heterozygous embryos were viable, homozygous kernels were much reduced in size, defective, lethal.</td>
</tr>
</tbody>
</table>
Table 3. (Continued)

<table>
<thead>
<tr>
<th>Name</th>
<th>Former designation</th>
<th>Map location</th>
<th>Phenotypic attributes and viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>bnn1</td>
<td>brn1*-Mu</td>
<td>3S</td>
<td>Kernels homozygous for the <em>brn1-Mu</em> reference allele were brown aleurone, light brown shade in embryo; homozygous kernels germinated and produced pale-green, inviable seedlings; kernels homozygous for the <em>brn1</em>-Mu3071 allele had very light pigment in both endosperm and embryo; seedling lethal.</td>
</tr>
<tr>
<td>mn3</td>
<td>dek*-Mu1184</td>
<td>6</td>
<td>Miniature seed: small kernel, etched/pitted endosperm; viable.</td>
</tr>
<tr>
<td>su3</td>
<td>su-sh*-5081</td>
<td>4S</td>
<td>Sugary endosperm: endosperm glassy, smoother than <em>su1</em>, and shrunken; viable. May be duplicate factor.</td>
</tr>
<tr>
<td>prg1</td>
<td>dek*-Mu8186</td>
<td>5L</td>
<td>Small, pitted, rough endosperm; usually germless, although kernels with larger embryos germinated to produce small, striated seedlings which died at the two to three leaf stage.</td>
</tr>
<tr>
<td>ref1</td>
<td>dek*-Mu1185</td>
<td>3S</td>
<td>Reduced, pitted endosperm with dull, floury appearance; small germs often present; very low frequency of expression on segregating ears; approximately 20% germinated and produced plants with well developed ears, and tassels that shed little pollen.</td>
</tr>
</tbody>
</table>
Figure 5. Ears segregating for mutant kernels were obtained from plants heterozygous for specific *dek* mutations as indicated. All ears were self-pollinated except for (F) which was crossed as female to plants containing purple aleurone. (A) ren1/Ren1. (B) ptd1/PTd1. (C) dsc1/Dsc1. (D) brn1/Brn1. (E) emp2/Emp2. (F) dap1/dap1/Dap1. (G) ref1/Ref1. (H) ren2/Ren2. (I) su3/Su3. (J) ptd2/PTd2. (K) mn3/Mn3. (L) emp1/Emp1. Descriptions of these mutant phenotypes are compiled in Table 3.
Figure 6. Kernel phenotypes of fifteen novel defective kernel mutants. Each panel shows germinal (two left kernels) and abgerminal (two right kernels) views of representative mature wild type (top row) and sibling defective kernels (bottom row) obtained from self-pollinated ears of plants heterozygous for a specific dek mutation as indicated. All samples of defective kernels are homozygous for the particular dek mutation indicated, except for those in (F) which are heterozygous for the semi-dominant kernel mutation dap1 (dap1/dap1/Dap1). (A) ren1. (B) ptd1. (C) dsc1. (D) brn1. (E) emp2. (F) dap1. (G) refl. (H) ren2. (I) su3. (J) ptd2. (K) mn3. (L) emp1. (M) prg1. (N) ren3. (O) et2. Descriptions of these mutant phenotypes are compiled in Table 3.
Table 4. Two-point linkage data

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Genotype of F1</th>
<th>Parental types</th>
<th>Recombinants</th>
<th>Total Plants</th>
<th>Recombinations Total</th>
<th>Percent</th>
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<td>dek*-Mu1568</td>
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<td>19</td>
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<tr>
<td>dek*-Mu1568</td>
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<td>18</td>
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<td>22</td>
<td>17.1 ± 2.0</td>
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<tr>
<td>dek*-Mu1568</td>
<td>bm2 +</td>
<td>21</td>
<td>8</td>
<td>67</td>
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<td>dek*-Mu2410</td>
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Table 4. (Continued)

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<td>8</td>
<td>23</td>
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<tr>
<td></td>
<td>+ dek</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>dek*-Mu3193</td>
<td>+ ijl +</td>
<td>84</td>
<td>90</td>
<td>95</td>
<td>61</td>
<td>156</td>
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<tr>
<td>brn1*-Mu</td>
<td>+ ral</td>
<td>170</td>
<td>181</td>
<td>59</td>
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<tr>
<td>brn1*-Mu</td>
<td>+ g2</td>
<td>282</td>
<td>302</td>
<td>53</td>
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<tr>
<td>brn1*-Mu</td>
<td>+ d1</td>
<td>93</td>
<td>82</td>
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<td>24</td>
<td>223</td>
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<tr>
<td>brn1*-Mu</td>
<td>+ cr1</td>
<td>229</td>
<td>216</td>
<td>16</td>
<td>16</td>
<td>477</td>
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<tr>
<td>brn1*-Mu</td>
<td>+ d1</td>
<td>148</td>
<td>139</td>
<td>82</td>
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<td>447</td>
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<tr>
<td>Dap18</td>
<td>+ T5-9</td>
<td>41</td>
<td>58</td>
<td>37</td>
<td>27</td>
<td>163</td>
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<tr>
<td>Dap1h</td>
<td>+ T5-9</td>
<td>394</td>
<td>367</td>
<td>19</td>
<td>4</td>
<td>794</td>
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<tr>
<td>dek*-Mu5132</td>
<td>+ fi</td>
<td>31</td>
<td>39</td>
<td>21</td>
<td>19</td>
<td>110</td>
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<tr>
<td>dek*-Mu326</td>
<td>+ ijl</td>
<td>60</td>
<td>92</td>
<td>54</td>
<td>23</td>
<td>229</td>
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<tr>
<td>su-sh*-5081i</td>
<td>+ T4-9</td>
<td>28</td>
<td>59</td>
<td>5</td>
<td>1</td>
<td>93</td>
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\[ a \] waxy-marked translocation 2-9d
\[ b \] waxy-marked translocation 1-98918
\[ c \] waxy-marked translocation 3-9c
\[ d \] waxy-marked translocation 5-9a
\[ e \] waxy-marked translocation 9-10b
\[ f \] waxy-marked translocation 7-94363
\[ g \] waxy-marked translocation 5-94790
Table 4. (Continued)

\( h \)  \textit{waxy}-marked translocation 5-9d

\( i \)  \textit{waxy}-marked translocation 4-9g
Table 5. Three point linkage data

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Genotype of F1</th>
<th>Parental types</th>
<th>Recombinants</th>
<th>Total Plants</th>
<th>Percent Recombination</th>
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<tbody>
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<td></td>
<td></td>
<td>Region 1</td>
<td>Region 2</td>
<td>Plants</td>
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<td>+ su1 gl4 +</td>
<td>53 52</td>
<td>6 6 13 15 2 1</td>
<td>148</td>
<td>10.1 ± 2.4 20.9 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>dek +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dek*-Mu2689</td>
<td>+ su1 gl4 +</td>
<td>25 38</td>
<td>10 6 5 12 1 4</td>
<td>101</td>
<td>20.8 ± 4.0 21.8 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>+ + dek</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dek*-Mu1184</td>
<td>+ y1 su2 +</td>
<td>13 26</td>
<td>3 12 12 7 15 8</td>
<td>96</td>
<td>39.6 ± 5.0 43.8 ± 5.0</td>
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<td>+ + dek</td>
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</tr>
<tr>
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<td>su1 + bm3 + dek</td>
<td>108 125</td>
<td>4 4 3 0 0 0</td>
<td>244</td>
<td>3.3 ± 1.1 1.2 ± 0.7</td>
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<td></td>
<td>+ dek +</td>
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<td>dek*-Mu33</td>
<td>brn1 + Lg3 + dek</td>
<td>155 46</td>
<td>57 8 36 31 9 2</td>
<td>344</td>
<td>22.1 ± 2.2 22.6 ± 2.1</td>
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<td>+ dek +</td>
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</tr>
<tr>
<td>dek*-Mu326</td>
<td>+ y8 gl1 +</td>
<td>107 81</td>
<td>20 15 51 50 12 19</td>
<td>355</td>
<td>18.6 ± 2.1 37.2 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>+ + dek</td>
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<td>dek*-Mu807</td>
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<td>92 84</td>
<td>347 21 41 42 10 13</td>
<td>340</td>
<td>23.8 ± 2.3 31.2 ± 2.5</td>
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<td>dek*-Mu1184</td>
<td>+ su15 y1 + dek</td>
<td>161 177</td>
<td>7 8 8 0 0 0 1</td>
<td>362</td>
<td>4.4 ± 1.1 2.5 ± 0.8</td>
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<td>+ dek +</td>
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<tr>
<td>dek*-Mu1184</td>
<td>+ y1 l15 + dek</td>
<td>168 173</td>
<td>5 2 14 14 1 0</td>
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<td>2.1 ± 0.7 7.7 ± 1.4</td>
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<tr>
<td>brn1*-Mu</td>
<td>+ d1 Lg3 + dek</td>
<td>139 127</td>
<td>39 33 41 40 7 1</td>
<td>429</td>
<td>18.7 ± 1.9 20.8 ± 2.0</td>
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<td></td>
<td>+ dek +</td>
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<td></td>
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<tr>
<td>su-sh*-5081</td>
<td>+ su1 gl4 + dek</td>
<td>31 38</td>
<td>25 37 7 11 5 7</td>
<td>161</td>
<td>50.3 ± 3.9 18.6 ± 3.1</td>
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<td>+ dek +</td>
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<tr>
<td>Dapl</td>
<td>pr1 + y2 + dek</td>
<td>107 87</td>
<td>38 53 17 27 2 3</td>
<td>290</td>
<td>28.7 ± 2.5 14.7 ± 1.9</td>
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Table 6. Four point linkage data

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<th>Mutation</th>
<th>F1 Genotype</th>
<th>Parental types</th>
<th>Region 1</th>
<th>Region 2</th>
<th>Region 3</th>
<th>Regions 1 &amp; 2</th>
<th>Regions 1 &amp; 3</th>
<th>Regions 2 &amp; 3</th>
<th>Regions 1,2 &amp; 3</th>
<th>Total plants</th>
<th>Percent Recombination</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
</tr>
<tr>
<td>dek*-Mu807</td>
<td>ac pr gl8 +</td>
<td>96</td>
<td>11</td>
<td>12</td>
<td>7</td>
<td>31</td>
<td>16</td>
<td>2</td>
<td>1</td>
<td>336</td>
<td>9.8 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>+ + dek</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td>18.5 ± 2.1</td>
</tr>
<tr>
<td>dek*-Mu1047</td>
<td>gl1v4 + Ch1</td>
<td>39</td>
<td>49</td>
<td>53</td>
<td>30</td>
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<td>52</td>
<td>33</td>
<td>0</td>
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<tr>
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<td>+ + dek</td>
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<td>7.5 ± 1.4</td>
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<td></td>
<td></td>
<td>47.3 ± 2.7</td>
</tr>
</tbody>
</table>
Table 7, which presents linkage relationships for 22 dek mutations as indicated by the results given in Tables 4-6. Testcrosses were made to marker genes which reside on the particular chromosome arm containing the dek mutation as indicated by the results of TB crosses or other placement methods as described (see Table 1).

In some cases, linkage mapping analyses produced ambiguous results. For example, although multiple positive TB tests located the mutant ptd2 (formerly dek*-Mu3193) to chromosome 7L, this mutant was found to be unlinked to ij1 on 7L. It is possible that ptd2 is located on the extreme distal end of chromosome 7L, or perhaps the initial arm placement was incorrect. The latter explanation seems unlikely, because the unique phenotype of ptd2 mutants is not readily misclassified and does not resemble the small kernel phenotype often expressed in 7L hypoploid endosperms (Roman and Ulstrup 1951).

A similar situation exists for linkage mapping of dek*-Mu1104, which was found to be unlinked to both y1 (6L:17) and su2 (6L:58). The dek*-Mu1104 mutation is closely linked to a mutation producing luteus seedlings, and initial placement was assigned because crosses to TB 6L plants produced both small seeded kernels yielding green seedlings (putative endosperm hypoploids), and large seeded kernels segregating for luteus seedlings (putative embryo hypoploids). If indeed the placement of this mutant to 6L is correct, the locus must be very distal or perhaps the calculated percent recombination value is inaccurate due to the very small sample size (96 individuals). However, despite the small sample size the linkage observed between su2 and y1 is very close to that reported on the 1992 linkage map (41 cM).
Table 7. Summary of linkage data

<table>
<thead>
<tr>
<th>Locus (mutation)</th>
<th>Arm Location</th>
<th>Linkage relationships</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emp1 (dek*-Mu2045)</td>
<td>1L</td>
<td>waxyT1-9b - 34 - emp1</td>
</tr>
<tr>
<td>Emp2 (dek*-Mu1047)</td>
<td>2L</td>
<td>emp2 - 21 - waxyT2-9d</td>
</tr>
<tr>
<td></td>
<td></td>
<td>emp2 - 19 - m3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fl1 - 46 - v4 - 8 - emp2 - 47 - Ch1</td>
</tr>
<tr>
<td>Ptd1 (dek*-Mu1568)</td>
<td>1L</td>
<td>bz2 - 15 - ptd1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ptd1 - 17 - Kn1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ptd1 - 25 - bm2</td>
</tr>
<tr>
<td>Ptd2 (dek*-Mu3193)</td>
<td>7L</td>
<td>ij1 - 47 - ptd2</td>
</tr>
<tr>
<td>Ren1 (dek*-Mu807)</td>
<td>5L</td>
<td>pr1 - 24 - ren1 - 31 - v2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ae1 - 10 - pr1 - 9 - gl8 - 19 - ren1</td>
</tr>
<tr>
<td>Ren2 (dek*-Mu326)</td>
<td>7L</td>
<td>ren2 - 34 - ij1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>y9 - 19 - gi1 - 37 - ren2</td>
</tr>
<tr>
<td>Dsc1 (dek*-Mu2058)</td>
<td>4S</td>
<td>su1 - 3 - dsc1 - 1 - bm3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>or</td>
</tr>
<tr>
<td></td>
<td></td>
<td>su1 - 3 - bm3 - 1 - dsc1</td>
</tr>
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<td>Ren3 (dek*-Mu1339)</td>
<td>10L</td>
<td>r1 - 54 - ren3</td>
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<td>o7 - 32 - ren3</td>
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<td>ren3 - 31 - waxyT9-10b</td>
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<tr>
<td>Ref1 (dek*-Mu1185)</td>
<td>3S</td>
<td>refl - 30 - cl1</td>
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<td></td>
<td>waxyT3-9c - 35 - refl</td>
</tr>
<tr>
<td>Prg1 (dek*-Mu8186)</td>
<td>5L</td>
<td>prg1 - 13 - ae1 or ae1 - 13 - prg1</td>
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<td></td>
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<td>prg1 - 21 - waxyT5-9a</td>
</tr>
<tr>
<td>Dap1 (dap1-1)</td>
<td>5L</td>
<td>dap1 - 39 - waxyT5-94790</td>
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<td>dap1 - 3 - waxyT5-9d</td>
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<td>pr1 - 29 - dap1 - 15 - v2</td>
</tr>
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<td>Brn1 (brn1*-Mu)</td>
<td>3S</td>
<td>brn1 - 7 - cr1</td>
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<td></td>
<td></td>
<td>brn1 - 22 - d1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>brn1 - 27 - m1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>brn1 - 36 - cl1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>g2 - 14 - brn1</td>
</tr>
<tr>
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<td></td>
<td>brn1 - 19 - d1 - 21 - Lg3</td>
</tr>
<tr>
<td>Mn3 (dek*-Mu1184)</td>
<td>6L</td>
<td>mn3 - 4 - w15 - 3 - y1</td>
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<td>mn3 - 2 - y1 - 8 - l15</td>
</tr>
<tr>
<td>Pro1 (dek*-Mu5132)</td>
<td>8L</td>
<td>pro1 - 29 - j1</td>
</tr>
<tr>
<td>Dek5 (dek*-Mu33)</td>
<td>3S</td>
<td>brn1 - 22 - dek5 - 23 - Lg3</td>
</tr>
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</table>
Table 7. (Continued)

<table>
<thead>
<tr>
<th>Locus (mutation)</th>
<th>Arm Location</th>
<th>Linkage relationships</th>
</tr>
</thead>
</table>
| *Dek25 (dek*-Mu2410)* | 4S | *dek25 - 45 - su1*  
| | | *dek25 - 26 - fl2* |
| *Dek7 (dek*-Mu2608)* | 4S | *dek7 - 7 - fl2*  
| | | *dek7 - 21 - su1 - 10 - gl4* |
| *Dek31 (dek*-Mu2689)* | 4L | *dek31 - 20 - gl3*  
| | | *dek31 - 18 - G2*  
| | | *su1 - 21 - gl4 - 22 - dek31* |
| *dek*-Mu2159 | 2L | *dek-2159 - 20 - waxyT2-9d* |
| *dek*-Mu2082 | 7L | *dek-2082 - 35 - waxyT7-94363* |
| *dek*-Mu1104 | 6L | *y1 - 40 - su2 - 44 - dek-Mu1104* |
| *Su3 (su-sh*-Mu5081)* | 4S | *waxyT4-9g - 7 - su3*  
| | | *su3 - 50 - su - 19 - gl4* |

*a* Former designations of novel *dek* mutations given in parentheses.

*b* Data summarized from Tables 4 through 6 of this report.
Linkage mapping of refl (formerly dek*-Mu1185) was hampered by both the small sample size and large deficiencies in the defective kernel phenotypic classes due to the low frequency of mutant expression. Finally, although su3 (formerly su-sh*-Mu5081) was placed to chromosome 4S after observing close linkage to the waxy-marked translocation 4-9g (Table 4), no linkage was subsequently observed with su3 and either sugary-1 (sul, 4S:47) or glossy-4 (gl4, 4L:62) (Table 5). Mutant phenotypes that result from homozygosity for two, unlinked mutations (duplicate factors) are expected to segregate in 15:1 (wild type: mutant) ratios on self-pollinated ears of plants heterozygous for each kernel developmental mutation. Since the su3 mutant phenotype is expressed in low frequency approaching 15:1, it is possible that the mutant phenotype is the result of concomitant homozygosity for duplicate factors, one of which is located on chromosome arm 4S.
DISCUSSION

This study describes kernel phenotypes and associated pleiotropic effects conditioned by 65 dek mutations arising in a Mutator background. The work identified new alleles of 10 known Dek loci, as well as 15 novel Dek genes. An important aspect of this work is the potential to study kernel development by a molecular cloning approach, because many of the mutations probably arose by Mutator transposition.

Several of the mutant phenotypes described in this report are empty pericarp, flattened hulls devoid of both endosperm and embryo tissue. A majority of the emp mutant class may represent mutations of general cell function or "housekeeping" genes that are not expressed specifically in kernel tissue. However, many other dek mutations described in this study may identify more interesting loci that function specifically in the kernel, and are required for its normal development.

Mutator-Induced dek Mutations are Likely to be Useful in Molecular Analyses of Kernel Development

Because these mutations were derived from active Mutator stocks it is likely that many arose from insertion of a Mu element. Thus, the mutations represent a large pool of genetic loci required for kernel development that are potentially accessible to molecular cloning via transposon tagging (Wienand et al. 1982). Although the Mutator transposable element system is a large family of at least thirteen distinct elements (Barker et al. 1984; Chandler et al. 1986; Taylor and Walbot 1987; Oishi and Freeling 1987; Varagona et al. 1987; Talbert and Chandler 1988; Talbert et al. 1989; Schnable et al. 1989; Fleenor et al. 1990; Qin and Ellingboe 1990; Hershberger et al. 1990).
1991; Qin et al. 1991; Chomet et al. 1991), most of the Mu-induced mutations that have been analyzed at the molecular level contain insertions of the 1.4 kbp transposon Mu1 (reviewed in Chandler and Hardeman 1993). The Mutator system has gained widespread use as a means of cloning maize loci for which no protein product has been identified. Numerous genes have been cloned using transposon tagging with Mu, including a1 (O'Reily et al. 1985), vp1 (Kriz and Schwartz 1986), hcf106 (Martiennsen et al. 1989), bz2 (McClaughlin and Walbot 1987), y1 (Buckner et al. 1990), and hm1 (Johal and Briggs 1992). The mutations identified in this study represent excellent candidates for the molecular analyses of genes involved in the complex process of maize kernel development. To date four such loci, dsc1, emp2, ren1, and ren2, have been shown to be tightly linked to specific maize genomic DNA clones isolated based on co-segregation observed between specific Mu1-containing restriction fragments and the mutant phenotype (Scanlon et al. 1993a; Scanlon et al. 1993b) (MGJ unpublished results).

Applications of Genetic Characterization of Putative Mu-Induced Kernel Mutants

The information obtained in this study from genetic mapping and characterization of the dek-Mu mutations has several potential applications in molecular analyses of the corresponding genetic loci. The identification of independently-induced mutant alleles of a specific locus required for kernel development permits the use of cross-referencing as a means for establishing the identity of a cloned gene (O'Reily et al. 1985; Buckner et al. 1990). A frequently encountered problem using Mutator as a transposon tag is that a specific Mu-element may be tightly linked to a mutation, but not be the causative agent of that defect. Cross-referencing attempts to overcome this problem by identifying independent
alleles that also co-segregate with a *Mu*-element insertion. If each of the two (or more) independently-induced allelic mutations contains a *Mu* transposon located within the same genetic element, then the likelihood of that element actually causing the phenotype is very high. Several pairs of putative *Mu*-induced *dek* alleles were identified in this study, which may be applicable to the cross-referencing strategy. The use of cross-referencing of mutant alleles is especially useful in molecular cloning analyses of recessive-lethal mutations such as the *dek* alleles, for which the identification of revertant alleles is not a possible strategy for proving clone identity.

Another possible application of allelic mutations involves those that produce variable kernel mutant phenotypes. For example, the *vpl* mutation blocks anthocyanin production and embryo dormancy in developing kernel (Robertson 1955). In the variant allele *vpl*-Mc, however, anthocyanin pigment accumulation is blocked whereas embryo dormancy is nearly normal (Robertson 1965). The *Vp1* gene was cloned via transposon tagging using Robertson’s *Mutator*, and molecular comparisons revealed that the *vpl*-Mc mutant allele produces a 3' truncated mRNA relative to the wild type *Vp1* allele. These data indicate that the C-terminus of the *VP1* protein may function in the regulation of anthocyanin production, whereas regulation of kernel maturation genes may involve a different region of the *VP1* protein (McCarty et al. 1989b; McCarty et al. 1991). Three examples of variant phenotypes caused by allelic mutations have been observed in this study. The *brnl-Mu* mutation in the homozygous condition produced dark brown endosperm and germ, and caused seedling lethality. In contrast, the putative *Mu*-induced allele *brnl-Mu3071* produced small, mostly yellow endosperms and brown germs, and homozygous mutant kernels failed to germinate. Molecular comparisons of these two alleles may provide information regarding the putative functional domains of
the BRN1 gene product. Similar comparisons are possible for the exceptional allele of the Su1 locus described in this study, su1-Mu2412; kernels homozygous for this mutation were significantly less wrinkled than those homozygous for other Su1 alleles, and the wrinkling was restricted to the crown of the su1*-Mu2412 mutant kernels. Finally, dek25-Mu2410 is the only described mutant allele of the Dek25 locus that when homozygous produced kernels capable of germination.

We have reported linkage mapping data for 23 kernel mutations, which reside on twelve different chromosome arms. Twenty of these mutations produced inviable defective kernels. Because of the widespread genetic map locations of these Dek loci, they are especially useful for the generation of balanced lethal mutation stocks (Muller 1918). Balanced lethal stocks are constructed as a means of selecting plants heterozygous for a lethal mutation under study. When plants containing lethal mutations in two different genes closely linked in repulsion are self-pollinated, the viable progeny will be greatly enriched for double heterozygotes due to lethality of any progeny homozygous for either of the mutations. The detailed genetic mapping of several Dek loci allows for selection of homozygous-lethal dek mutations best suited for the construction of balanced lethal stocks.

Possible Causes of Low Frequency and Altered Patterns of Expression of Some Kernel Mutant Phenotypes

The dap1-1 and dap1-2 mutations were shown to be semi-dominant with regard to the dappled kernel phenotype, i.e. dominant when female-transmitted but recessive when male-transmitted. The most likely explanation for this inheritance pattern is that two doses of the mutant dap1 allele are required in the endosperm to produce the dappled aleurone phenotype. When transmitted through the female
side in outcrosses to purple aleurone tester stocks, two copies of \textit{dap1} are present in the endosperm owing to the two-dose contribution from the female to this triploid tissue. The dappled kernel progeny of this cross would therefore be \textit{dap1/dap1/Dap1} in constitution. Conversely, when \textit{dap1} is male transmitted the endosperm genotype would be \textit{dap1/Dap1/Dap1}. This explanation predicts the observed ratio of kernel phenotypes on self-pollinated ears of \textit{Dapl/dapl} plants; of the four possible triploid endosperm genotypes \textit{dap1/Dap1/Dap1} and \textit{Dap1/Dap1/Dap1} kernels would be purple aleurone, \textit{dap1/dapl/Dap1} kernels would be dappled, and \textit{dap1/dapl/dapl} kernels would be defective and display extreme aleurone disruption. The recessive nature of \textit{dap1-1} and \textit{dap1-2} when male-transmitted also conforms to this explanation, as the expected endosperm genotypes of \textit{Dapl/Dapl/Dapl} or \textit{dap1/Dapl/Dapl} would both produce purple aleurone kernels. Semi-dominance has been observed previously for alleles of several loci affecting endosperm phenotype, for example \textit{floury-2} (\textit{fl2}) (Nelson et al. 1965).

Low frequency of expression of the defective kernel phenotype on self-pollinated ears of heterozygous plants is characteristic of ten mutations described in this study. The phenomenon of low frequency expression has been documented in other studies of \textit{dek} mutations (Jones 1920; Clark and Sheridan 1988), and may be attributed to numerous possible causes. For example, mutant phenotypes resulting from homozygosity at two unlinked, recessive mutations (duplicate factors) are inherited in a 15:1 ratio. One mutant phenotype described in this study, attributed to the novel mutation \textit{su3-Mu5081} exhibits an inheritance pattern approaching this ratio, indicating this mutant phenotype may indeed result from mutations at duplicate factors (Table 4 and Table 5).
Another possible explanation for the low frequency of expression of some of the \textit{dek} mutant phenotypes is that a \textit{dek} mutation affects the development of the male gametophyte, or the functioning of the pollen grain, such that fewer than the expected 50% of the functional pollen grains of \textit{Dek/dek} plants carry the defective allele (Clark and Sheridan 1988). Thus, a reduction in the frequency of mutant kernels on the self-pollinated ears is observed. In an extreme example, the miniature kernel mutation \textit{Mn::Uq} disrupts pollen tube growth in the male gametophyte and is male-nontransmissible (Pan and Peterson 1989).

Suppression of \textit{Mu}-induced inactivation of a locus, as reported by Martienssen et al. (1989; 1990), is another possible explanation for the reduced frequency of mutant kernels on ears of self-pollinated plants. These workers showed that suppression of the \textit{Mu}-induced \textit{hcf106} mutation is correlated with inactivation of \textit{Mutator} stocks and methylation of \textit{Mu1} elements inserted in the 5' promoter region of the \textit{HCF106} locus. In the suppressed state in inactive \textit{Mu} lines, functional mRNA is transcribed from various initiation sites within the terminus of the \textit{Mu1} transposon inserted into the mutant allele \textit{hcf106}, and proceeds into the adjacent \textit{Hcf106} coding region (Barkan and Martienssen 1991). Backcrossing of suppressed \textit{hcf106} mutant lines into active \textit{Mutator} stocks restored the mutant phenotype (Martienssen et al. 1990). Similar genetic behavior has been noted for several other mutations, indicating that suppressible alleles may be a rather common class of \textit{Mu}-induced mutations (M. Freeling, personal communication).

Another possible explanation for the observed low frequency of expression of particular \textit{dek} alleles is that the mutations may result from \textit{Mutator}-induced chromosomal deletions that impair gametophyte development or function. McClintock (1942; 1944) showed that mutations generated as a result of
chromosomal deletions are often transmitted in low frequency through the pollen. Taylor and Walbot (1985) and Robertson and Stinard (1987) showed that Mutator transposons commonly induce deletions of varying length that can generate stable, null mutant alleles. Since the putative Mu-induced dek mutations in this study are most often propagated through the male, it is possible that some mutations that display low frequency of expression may be the result of chromosomal deletions with reduced male transmission. Mutations resulting from transposon-induced deletions are predicted to be stable, because they result from the loss of all or part of a gene sequence at the particular locus, rather than insertion of a mobile genetic element capable of excision and attendant restoration of wild type gene function. In most instances, however, the nature of the kernel phenotypes caused by dek mutations would prevent the differentiation of stable, deletion-induced null mutations from mutable, transposon-induced mutant phenotypes.

Pleiotropic Effects of dek Mutations

Many of the defective kernel phenotypes described in this study and others (Neuffer and Sheridan 1980; Sheridan and Neuffer 1980) indicate a correlation between aleurone disruption in the kernel and altered seedling chlorophyll content. These mutations include alleles of the Dek5, Dek7, Dek21, Etl (Sullivan et al. 1991), Dek25, Prg1, Brn1, Ptd1, and Ei2 loci, and dek*-Mu5191. No physiological or biochemical explanation of the link between the aleurone and seedling phenotypes has been clearly elucidated. It is possible that the altered chlorophyll content of these seedlings may be attributed to nutritional defects caused by altered endosperm storage nutrients and/or improper mutant aleurone functioning. A more intriguing possibility is that some of the mutations affecting kernel development may be
involved in general plastid function and development. In this view, mutant amyloplast development causes disruptions in endosperm structure, whereas mutant chloroplast development leads to discolored, striated, or albino seedlings. Abnormalities in chloroplast development have been documented in plants homozygous for the et1-1 mutation (Sangeetha et al. 1986). Perhaps the Etl locus and other kernel mutant genes are required for amyloplast development as well. These and other possible explanations await further investigation.

A common pleiotropic effect of mutations affecting kernel development is the disruption of embryo development. Many of the mutations in this study generated nonviable embryos in homozygous seed, and still others germinated to produce plants with profound disruptions of various aspects of shoot development. The mutations dek-MuPS602 and ptd2-Mu3193 both affected leaf and flower formation. In plants grown from dek-Mu1364 homozygous seed, ligule formation, internode length and flower development were all disrupted. Detailed analyses of maize leaf development have focused on several dominant, heterochronic leaf mutants which act in the leaf primordium or perhaps earlier, in the shoot apical meristem (Freeling 1992). Curiously, there are no identified recessive mutations corresponding to the seven loci recognized by dominant, heterochronic leaf mutants. Hake (1992) and Freeling (1992) postulated the recessive alleles of these dominant leaf mutant genes may be lethal defective kernel or embryo (emb) mutants. Sheridan and Neuffer (1980) showed that embryos from several inviable dek mutations (dek4, dek12, dek18, dek29, dek30) yield narrow leaf mutant seedlings when rescued in tissue culture. Perhaps several of the inviable kernel mutants described in studies of dek mutations are recessive alleles of dominant leaf mutant loci. More interesting are those kernel mutations which produce plants with abnormal leaf, stem and flower development,
illustrating that some \textit{dek} mutations may disrupt processes fundamental to shoot development.
REFERENCES


PAPER 2. PHENOTYPIC VARIATION CAUSED BY TEN NOVEL MUTATIONS IN THE MAIZE ETCHED-1 LOCUS
Characterization of Ten Novel Mutations Of The Maize *Etched-1 (Et1)* Locus

M. J. Scanlon, P. S. Stinard, M. G. James, A. M. Myers, and D. S. Robertson

From the Department of Biochemistry and Biophysics (Scanlon, James and Myers), the Department of Agronomy (Stinard), and the Department of Zoology and Genetics (Robertson), Iowa State University, Ames IA 50011. This work was supported by research grants to AMM and DSR from the U.S. Department of Agriculture (88-37234-3316 and 91-37301-6344).

Running title: Novel *etched-1 (et1)* mutations in maize
ABSTRACT

The maize gene *Etched-1* (*Et*) is defined by the mutation *etl-1*, which causes both kernel etching and seedling virescence. Ten novel *etl* mutations were identified among the progeny of plants with active Robertson's *Mutator* (*Mu*) transposable element systems. Like *etl-1*, all of the novel *etl* alleles are recessive and affect the phenotype of both kernels and seedlings. Several of the mutations reported here cause unique kernel and seedling phenotypes distinct from those observed in *etl-1* mutant strains. A correlation was observed between the degree of kernel etching and the severity of the seedling virescence caused by individual *etl* alleles. The novel mutations are likely to be the result of *Mu* transposon insertion, and thus may facilitate isolation of the *Et* gene by transposon tagging methods.
INTRODUCTION

The pleiotropic *etched-1 (et1-1)* mutation of maize was first characterized by Stadler (1940) as having unique effects on both kernel and seedling phenotypes. *et1-1/et1-1* mutant kernels are distinctly fissured as the result of cracks and depressions in the endosperm tissue. Furthermore, seedlings grown from *et1-1* homozygous kernels are pale-green, later becoming fully green about 10 days after emergence; this delayed greening phenotype is known as "virescent". Following seedling greening, *et1* homozygous plants are relatively normal and healthy.

Numerous genetic and developmental studies have investigated how the pleiotropic mutation *et1-1* could affect endosperm structure and also reversibly affect the development of the seedling. Genetic studies determined that *et1-1* is a recessive mutation located on chromosome 3L:161 (Stadler 1940). In extensive analyses the kernel and seedling phenotypes were never found to be separable by genetic crossover. A single mutation in the *et1* locus, therefore, most likely is the cause of both the kernel and seedling abnormality.

Etching of *et1-1/et1-1* kernels is first noticeable at ten days after pollination (dap); at maturity, variation in the degree of etching commonly is observed on single ears segregating such mutant kernels (Sangeetha and Reddy 1991). Developing *et1-1/et1-1* mutant kernels accumulate less starch than sibling *Et1/et1-1*, or *Et1/Et1* wild type kernels (Sangeetha and Reddy 1991). Microscopic analyses showed that virescent, *et1-1/et1-1* mutant seedlings undergo reduced rates of chloroplast morphogenesis, and this effect is exacerbated by low temperature (22-25°C) (Sangeetha et al. 1986).

Biochemical analyses revealed a reduction in total amylase enzyme levels in germinating *et1-1/et1-1* mutant seed, which leads to an impairment in starch
hydrolysis (Sangeetha and Reddy 1988). There is no evidence, however, that the Et1 locus codes for, or regulates expression of either alpha-amylose or beta-amylose. Carotenoids, chlorophyll, and chlorophyll-protein complexes accumulate to reduced levels in et1-1/et1-1 mutant seedlings until eight to ten days after emergence, and several stromal chloroplastic proteins are affected reversibly in a similar fashion (Ramesh et al. 1984; Sangeetha and Reddy 1991). Thus, et1-1 has broad effects on chloroplast gene expression and/or biogenesis, which are overcome at later stages in seedling development. Similar effects on endosperm plastids may be responsible for kernel etching. To date the product of the Et1 locus has not been characterized, and the specific physiological role of this gene in kernel and seedling development is not known.

This study describes the use of Mutator transposable element stocks, which generate a 30-50 fold increase in the spontaneous mutation rate of maize (Robertson 1978), to identify 10 independently-induced mutant alleles of the Et1 locus. Several of these alleles cause kernel, seedling, and embryo phenotypes previously undescribed for the reference mutation et1-1. In the et1 mutant strains, a clear correlation was observed between the degree of kernel etching and the severity of the seedling virescence phenotype. Because these ten et1 mutant alleles are derived from Robertson's Mutator stocks, it is likely that many of them arose from insertion of a Mutator transposable element into the Et1 locus. Therefore, these new et1 alleles are potentially accessible to molecular cloning via transposon-tagging (Wienand et al. 1982), and are expected to serve as valuable tools for the further characterization of the pleiotropic Et1 gene.
MATERIALS AND METHODS

Maize Genetic Stocks

*Mutator* lines are maize stocks shown to have active *Mutator* systems as defined by Robertson (1978). Standard lines, which have never been crossed to *Mutator* stocks, are the F1 hybrids inbred Q66 x inbred Q67, or inbred B77 x inbred B79. Both the *Mutator* and standard stocks are homozygous for recessive mutations in R1 and C1, two loci coding for regulatory proteins that affect anthocyanin pigment synthesis in the aleurone see (Clark and Sheridan 1988 for review); in these strains all other genes required for anthocyanin biosynthesis are wild type. Thus, the phenotype of the standard kernel is plump, yellow.

A strain homozygous for the reference allele *etl-1* (Stadler 1940) was obtained from the Maize Genetics Co-operative Stock Center (University of Illinois, Urbana, IL). This stock is homozygous for wild type alleles of all genes required for anthocyanin formation except for *A1*, which codes for an enzyme of this biosynthetic pathway (Schwarz-Sommer et al. 1987). The *etl-1/etl-1* stock is homozygous for *al-m i+Dt),* an unstable, transposon-insertion mutation controlled by the transposable element *Dt* (Nuffer 1961). Self-pollination of this strain, therefore, produces etched, yellow kernels with many small, purple sectors; the purple sectors arise when *A1* function is restored after the transposon within *al-m (+Dt)* is excised from the locus. As described below, the *al-m (+Dt)* allele of the *etl-1* reference stock was used in allele tests to identify contaminating etched kernels arising from self-pollination.

Isolation, Allele Testing, and Propagation of *etl* Mutations

The strategy for induction, identification and propagation of new *etl* mutations using *Mutator* stocks is presented in Figure 1. Active *Mutator* plants were outcrossed
as males to standard lines, and F1 plants grown from the outcrossed ears were self-pollinated. Plants were identified as heterozygous for a mutation causing kernel etching when self-pollinated ears of F1 plants segregated for wild type and etched kernels in the ratio of 3:1. Rare plants that segregated etched kernels were outcrossed to standard plants to propagate the mutation as shown in Figure 1. One half of the plants obtained from the outcross to standard lines are expected to be heterozygous for the mutation and thus segregate for 1/4 etched kernels on self-pollinated ears.

Novel mutations causing kernel etching were tested for allelism with etl-1 as follows. Pollen from plants heterozygous for the novel mutation was crossed onto etl-1/etl-1 tester ears. Allelism was indicated by 1:1 segregation of wild type and etched kernels on the resulting mature ears. Novel mutations meeting this criterion were designated etl-Mu. Etched and wild type kernels from these ears were planted in sand benches at 22-25°C and the resulting seedlings scored for virescence.

Contamination arising from the etl-1 pollen was ruled out in the allele test as follows. Etched kernels in the allele tests are expected to be of the genotype r1/R1, c1/C1, A1/a1-m (Dt), etl-Mu/etl-1 (see above), and thus would be purple owing to the wild type A1, R1 and C1 alleles. In contrast, any etched kernels on the allele test ears that resulted from contaminant self-pollination of the tester stock would be of the genotype R1/R1, C1/C1, a1-m (Dt)/a1-m (Dt), etl-1/etl-1. Such contaminant kernels would be clearly identifiable as etched, yellow kernels with small purple sectors owing to the homozygous mutable allele of the A1 locus.
Figure 1. Strategy for using the Robertson's Mutator system for induction and propagation of et1-Mu mutations.
RESULTS

The *et1* Alleles Obtained from *Mutator* Stocks Display Novel and Diverse Kernel Phenotypes

Ten mutant alleles of the *Et1* locus, designated *et1-Mu*, were isolated as spontaneous mutations in Robertson's *Mutator* stocks as described in Materials and Methods. Allelism was indicated in all instances by observation of both kernel etching and seedling virescence in *et1-Mu/et1-1* heterozygous kernels and seedlings (see Materials and Methods). The kernel phenotypes of representative *et1-Mu* homozygotes and *et1-Mu/et1-1* heterozygotes are shown in Figures 2 and 3; these data, together with descriptions of seedling phenotypes, are summarized in Table 1.

Specific *et1-Mu* mutations caused a kernel phenotype noticeably different than those resulting from the reference allele *et1-1*, as was indicated initially from comparisons of *et1-1/et1-1* kernels to those of the genotype *et1-1/et1-Mu*. The kernel phenotypes differed with regard to the severity of endosperm etching, shrunkenness of the seed, and embryo morphology. For example, the endosperm of *et1-1/et1-1* mutant kernels was moderately fissured (Figure 3A), whereas kernels heterozygous for *et1-1* and either *et1-Mu3328* (Figure 2B; Figure 3C), *et1-Mu2320* or *et1-Mu2162* all showed considerably more severe endosperm etching and cracking. Conversely, comparatively mild endosperm etching was characteristic of *et1-1/et1-Mu24* (Figure 2C; Figure 3D) and *et1-1/et1-Mu43* mutant kernels. Furthermore, *et1-1/et1-Mu* mutant kernels containing one of six separate *et1-Mu* alleles exhibited some degree of kernel shrunkenness in addition to variable degrees of endosperm etching. Kernel shrunkenness was not observed in *et1-1/et1-1* kernels. Specific examples of this novel *et1* mutant phenotype include the mildly shrunken kernel produced by
Figure 2. Phenotypic diversity displayed in mature ears segregating for independently-derived putative Mu-induced alleles of the et1 mutation. (A) Moderately etched kernels segregating on an ear resulting from the cross et1-Mu2424/Et1 x et1-1/et1-1. (B) Severely-etched kernel segregating on an ear of the cross et1-Mu3328/Et1 x et1-1/et1-1. (C) Mildly-shrunken kernel segregating on an ear of the cross et1-Mu24/Et1 x et1-1/et1-1. (D) Severely-shrunken kernel segregating on an ear of the cross et1-Mu2457/Et1/ x et1-1/et1-1. (E) Sugary-defective kernel segregating on the self-pollinated ear of a et1-Mu2320/Et1 plant.
Figure 3. Phenotypic variation among et1 mutant kernels. Each panel shows germinal (two left kernels) and abgerminal (two right kernels) views of kernels displaying mutant phenotypes caused by independently-derived mutations of the Et1 locus. Representative etched mutant kernels (lower row) and wild type sibling kernels (upper row) are shown. (A) Moderately-etched et1-1/et1-1 standard kernels. (B) Moderately etched mutant kernels produced from the cross et1-Mu2424/Et1 x et1-1/et1-1. (C) Severely-etched kernels produced from the cross et1-Mu3328/Et1 x et1-1/et1-1. (D) Mildly-shrunken kernels produced from the cross et1-Mu24/Et1 x et1-1/et1-1. (E) Severely-shrunken kernel produced from the cross et1-Mu2457/Et1 x et1-1/et1-1. (F) Sugary-defective kernel segregate on the self-pollinated ear of a et1-Mu2320/Et1 plant.
Table 1. Summary of kernel and seedling phenotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Kernel phenotype</th>
<th>Degree of etching</th>
<th>Seedling phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>etl-1/etl-1</td>
<td>etched, pitted, plump, normal embryo</td>
<td>moderate</td>
<td>virescent; yellowish-green leaves; gradually green in 7-10 days</td>
</tr>
<tr>
<td>etl-Mu5079/etl-1</td>
<td>etched, pitted, plump, normal embryo</td>
<td>moderate</td>
<td>virescent; similar to seedlings of etl-1/etl-1 standard</td>
</tr>
<tr>
<td>etl-Mu3328/etl-1</td>
<td>severely etched, cracked, plump, normal embryo</td>
<td>extreme</td>
<td>extremely virescent; albino leaves with many small sectors of green (mutable); became green in 7-10 days</td>
</tr>
<tr>
<td>etl-Mu2320/etl-1</td>
<td>severely etched, cracked; plump, normal embryo</td>
<td>extreme</td>
<td>extremely virescent; albino leaves with many small sectors of green (mutable); became green in 7-10 days</td>
</tr>
<tr>
<td>etl-Mu2162/etl-1</td>
<td>severely etched, cracked; plump, normal embryo</td>
<td>extreme</td>
<td>extremely virescent; albino leaves with many small sectors of green (mutable); became green in 7-10 days</td>
</tr>
<tr>
<td>etl-Mu24/etl-1</td>
<td>mildly shrunken, plump, normal embryo</td>
<td>none to slight</td>
<td>mildly virescent; mostly green, with pale green sector near midrib that became all green in 7-10 days</td>
</tr>
<tr>
<td>etl-Mu43/etl-1</td>
<td>mildly shrunken, plump, normal embryo</td>
<td>slight</td>
<td>mildly virescent; mostly green, with pale green sector near midrib that became all green in 7-10 days</td>
</tr>
<tr>
<td>etl-Mu2424/etl-1</td>
<td>variable on sibling ears; some mildly shrunken, etched, normal embryo; others plump, etched, normal embryo</td>
<td>moderate, slightly less etching on shrunken kernels</td>
<td>virescent; similar to seedlings of etl-1/etl-1 standard</td>
</tr>
<tr>
<td>etl-Mu34/etl-1</td>
<td>moderately shrunken, moderate etching, normal embryo</td>
<td>moderate</td>
<td>virescent; similar to seedlings of etl-1/etl-1 standard</td>
</tr>
<tr>
<td>etl-Mu27/etl-1</td>
<td>variable on sibling ears; some plump, etched, others moderately to severely shrunken, moderate etching; embryo normal</td>
<td>moderate</td>
<td>virescent; similar to seedlings of etl-1/etl-1 standard</td>
</tr>
<tr>
<td>etl-Mu2457/etl-1</td>
<td>severely shrunken, with collapsed sides; normal embryo</td>
<td>moderate</td>
<td>virescent; similar to seedlings of etl-1/etl-1 standard</td>
</tr>
</tbody>
</table>
Table 1. (Continued)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Kernel phenotype</th>
<th>Degree of etching</th>
<th>Seedling phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>et1-Mu2320/et1-Mu2320</td>
<td>very small, sugary, defective, germless.</td>
<td>kernel is shriveled</td>
<td>lethal</td>
</tr>
<tr>
<td>et1-Mu2162/et1-Mu2162</td>
<td>very small, sugary, defective, germless.</td>
<td>kernel is shriveled</td>
<td>lethal</td>
</tr>
</tbody>
</table>
et1-1/et1-Mu24 (Figure 2E; Figure 3D), et1-1/et1-Mu2424 and et1-1/et1-Mu43 heterozygous kernels, and the more severely shrunken phenotype of et1-1/et1-Mu2457 kernels (Figure 2D; Figure 3E).

Perhaps the most striking phenotype identified in this study was the small, sugary, defective kernel recovered in 1/4 of the kernels on self-pollinated ears of et1-Mu2162/Etl and et1-Mu2320/Etl heterozygous plants. These kernels, homozygous for et1-Mu2162 or et1-Mu2320 (Figure 2E; Figure 3F), were extremely shriveled, and contained little or no obvious embryo structure. The defective kernel phenotype was observed only in the homozygous condition; as noted above, et1-Mu2320/et1-1 and et1-Mu2162/et1-1 heterozygous kernels were severely etched, but the defective kernel phenotype was not observed.

The specific classes of kernel etching phenotypes described above were obvious despite the fact that phenotypic variation occurred not only among kernels with different et1 mutant alleles, but also among sibling kernels of the same genotype. This report did indeed confirm the findings of Sangeetha and Reddy (1991), who observed phenotypic variation among sibling etched kernels on self-pollinated ears of et1-1/Etl plants. For example, mutant kernels of the genotype et1-1/et1-Mu2424 or et1-1/et1-Mu27 may be plump or shrunken, and et1-1/et1-Mu3328 sibling kernels often showed great variation in the degree of kernel etching. We emphasize, however, that definite phenotypic patterns were observed consistently in specific et1-Mu mutant kernels, as indicated in Table 1 and Figures 2 & 3. For example, et1-1/et1-Mu3328, et1-1/et1-Mu2320 and et1-1/et1-Mu2162 kernels were consistently more etched than other et1-1/et1-Mu or et1-1/et1-1 mutant seed. Likewise, the endosperm of et1-1/et1-Mu2457 mutant kernels was invariably more shrunken and etched than that of et1-1/et1-Mu24, et1-1/et1-Mu43 or et1-1/et1-Mu2424 shrunken kernels. The
small, sugary, defective kernel phenotype of kernel homozygous for et1-Mu2320 or et1-Mu2162 was clearly an effect distinct from any other et1 alleles we have observed. In this instance, however, phenotypic variation was not observed; on self-pollinated ears of both et1-Mu2162/Et1 or et1-Mu2320/Et1 heterozygous plants the defective kernel phenotype was the sole mutant form observed, and all mutant kernels were very similar in appearance.

Variation in Seedling Virescence Resulting From et1-Mu Alleles

As shown in Figure 4 and summarized in Table 1, significant variation was observed in the specific seedling virescence phenotypes resulting from individual et1-Mu mutations. In five instances, et1-Mu/etl-1 heterozygous seedlings displayed a seedling virescence phenotype indistinguishable from that of et1-1/et1-1 plants (Table 1) (Figure 4B, 4C, 4F). Three mutations, et1-Mu3328, etl-Mu2320, and etl-Mu2162, in heterozygous combination with et1-1, resulted in a distinct seedling virescence phenotype. In these instances white, albino-like seedlings were observed (Figure 4D). Despite the extreme lack of coloration in these young seedlings all did become green within 7-10 days, indicative of a virescent phenotype. Extremely mild virescence was exhibited in two other families of et1-1/et1-Mu heterozygous seedlings (Table 1). Mildly virescent seedlings were mostly green at emergence, with small regions of slightly yellow-green tissue surrounding the midrib (Figure 4E). The seedling phenotype resulting from all 10 et1-Mu/et1-1 genotypes was mutable, as demonstrated by numerous small patches of fully green leaf tissue (presumably wild type) on a background of paler, mutant tissue. The mutable phenotype was most easily seen for those alleles that produced white seedlings (Figure D), and may be indicative of restoration of Et1 gene function following excision of a Mu element from the et1-Mu allele. Two alleles, et1-Mu2162 and et1-
Mu2320, in the homozygous condition usually produced inviable embryos that failed to produce seedlings. Infrequently, kernels homozygous for et1-Mu2320 or et1-Mu2162 germinated to produce inviable, albino seedlings that died without becoming green.

Correlation Between The Kernel and Seedling Phenotypes Resulting From Different et1 Alleles

Examination of the kernel and seedling phenotypes caused by specific et1-Mu alleles indicated that the degree of kernel etching was correlated with the severity of the seedling virescence phenotype. Mutant kernels that were extremely etched, with the genotypes et1-1/et1-Mu3328, et1-1/et1-Mu2320 and et1-1/et1-Mu2162, all produced nearly completely white seedlings. In contrast, kernels with comparatively little endosperm etching, of the genotypes et1-1/et1-Mu24 or et1-1/et1-Mu43, resulted in a very mild form of seedling virescence. Moreover, moderately etched kernels resulted in a seedling virescence phenotype similar to that caused by the et1-1 reference allele. The degree of shrunkenness of the et1-1/et1-Mu mutant kernels did not seem to contribute to the degree of seedling virescence. For example, sibling kernels of the genotypes et1-1/et1-Mu2424 or et1-1/et1-Mu27 were either plump or relatively shrunken, with moderate etching in both instances. Virescent seedlings grown from plump, moderately etched kernels were indistinguishable from those grown from sibling kernels that were shrunken and moderately etched. Furthermore, the severely shrunken, moderately etched mutant kernels of et1-1/et1-Mu2457 heterozygotes produced virescent seedling phenotypes comparable to those generated from the plump, moderately etched mutant kernels of et1-1/et1-Mu5079 heterozygotes.
Figure 4. Phenotypic variation among of seedlings homozygous for independently-derived allelic mutations of the Et1 locus. All seedlings were photographed at 7 days post-germination. (A) Normal seedlings grown from Et1/Et1 seed. (B) Virescent seedlings grown from et1-1/et1-1 standard seed. (C) Virescent seedlings grown from et1-Mu2424/et1-1 seed. (D) Severely-virescent seedlings grown from et1-Mu3328/et1-1 seed. (E) Mildly-virescent seedlings grown from et1-Mu24/et1-1 seed. (F) Virescent seedlings grown from et1-Mu2457/et1-1 seed.
DISCUSSION

This report describes 10 novel, independent mutations of the maize *etl* locus that arose spontaneously in Robertson's *Mutator* lines. The high mutation rate in these lines, approximately 50 times that of standard lines, is known to be caused by insertion of *Mu* transposable elements at many sites in the genome (reviewed in Chandler and Hardeman 1993). Thus, the *etl-Mu* alleles are likely to contain *Mu* elements within the *etl* locus. This hypothesis is supported by the observation that the seedling virescence phenotype in *etl-Mu* plants was mutable; i.e. small patches of wild type tissue were present in a background of mutant tissue. Mutable phenotypes of this nature typically are the result of somatic excision of transposons such that gene function is restored (McClintock 1950; McClintock 1951; Robertson, 1978; Robertson 1980). The *etl-Mu* alleles, therefore, are likely to allow molecular cloning of *etl* via transposon tagging (Wienand et al. 1982) using *Mu* transposons as molecular probes.

This collection of *etl-Mu* alleles also is potentially useful for verifying the identity of *etl* clones, and mapping the extent of *etl* in the genome, by the strategy of cross-referencing (O'Reily et al. 1985). Numerous maize genes have been cloned by transposon tagging with *Mu*, and allelic cross-referencing has aided in the verification of the identity of the cloned genes. Examples include *A1* (O'Reily et al. 1985), *Bz2* (McClaughlin and Walbot 1987), *Y1* (Buckner et al. 1990), and *Hm1* (Johal and Briggs 1992). Allelic cross-referencing involves detecting genetic rearrangements at the same locus in multiple, independently-induced mutations. Finding independent insertions at nearby sites in the genome, each of which co-segregates with a specific allele, indicates that specific genomic location contains the
gene of interest. In this way, cross-referencing can help to rule out the possibility that a specific transposon insertion is closely linked to a mutation but is not the causative agent of that genetic defect. Furthermore, mapping the locations of different transposon insertions in a gene will help delineate the extent of that locus.

The et1-Mu mutations described in this study conditioned pronounced kernel and seedling phenotypic variation. Several possibilities exist to explain this phenotypic diversity resulting from alleles of a single locus. Possibly the phenotypic variations are correlated with different relative activities of the Et1 locus resulting from different Mu element insertion sites within the gene in distinct et1-Mu alleles. In this view et1-Mu2162 and et1-Mu2320, the two alleles that in the homozygous condition resulted in inviable, defective kernels, may be completely non-functional mutations of the Et1 locus. Homozygosity for either of these mutations would result in complete loss of Et1 function, which is lethal. In this interpretation, the reference allele et1-1 must be partially functional, because et1-1/et1-Mu2162 and et1-1/et1-Mu2320 heterozygous kernels produced seemingly normal mature plants. Likewise, other et1-Mu alleles which generated relatively mild mutant phenotypes in et1-1/et1-Mu individuals may be relatively more functional than those et1-Mu alleles that corresponded to more extreme mutant phenotypes.

Another possible explanation of the cause of the novel phenotypes exhibited by certain et1-Mu homozygotes is that there are unidentified modifiers of Et1 function present in those genetic stocks that generated the phenotypic variants. The effects of variable genetic backgrounds on expression of et1 alleles can be assessed by crossing mutant plants to different inbred lines for several successive generations and observing expressivity and penetrance of the et1 mutant phenotype in various near-isogenic lines.
The positive correlation between the degree of kernel etching and seedling virescence alluded to in this study may provide clues regarding the physiological role of the \textit{Etl} locus in maize development. Perhaps, as suggested by Sangeetha and Reddy (1988), the deep endosperm cracks in severely etched kernels greatly disrupt the transport of nutrients from the endosperm to the germinating seedling resulting in colorless young seedlings. In this view, because the transport of endosperm nutrients to young seedlings is not so severely disrupted in \textit{etl} mutants that exhibit slight kernel etching, the seedlings produced by these \textit{etl} mutant kernels were mostly green.

A more intriguing possible explanation of the kernel and seedling phenotypic correlation is that the \textit{Etl} locus may function in general plastid development. Sangeetha et al. (1986) showed that chloroplast structure is reversibly altered in \textit{etl-1} mutant seedlings. Perhaps the \textit{Etl} gene product is directly involved with chloroplast biosynthesis or amplification. If so, analogous lesions in amyloplast development or function may also occur in \textit{etl} mutant kernels. In this interpretation, the endosperm cracking exhibited in seed homozygous for \textit{etl} might result from disruptions in amyloplast morphogenesis. It has been noted that starch accumulation is deficient in developing \textit{etl-1} mutant kernels (Sangeetha and Reddy 1991). Aberrations in amyloplast function induced by \textit{etl} mutations may be the direct cause of this mutant phenotype. The ten novel \textit{etl} alleles introduced in this report are likely to prove useful in future investigations of this and other questions regarding the function of the \textit{Etl} locus.
REFERENCES


PAPER 3. CHARACTERIZATION OF EMPTY PERICARP-2 \textit{(Emp2)}, A NOVEL MAIZE GENE REQUIRED FOR KERNEL DEVELOPMENT
Characterization of *Empty pericarp-2 (Emp2)*, a Novel Maize Gene Required for Kernel Development

M. J. Scanlon, D. S. Robertson and A. M. Myers

From the Department of Biochemistry and Biophysics (Scanlon, and Myers), and the Department of Zoology and Genetics (Robertson), Iowa State University, Ames IA 50011. This work was supported by research grants to AMM and DSR from the U.S. Department of Agriculture (88-37234-3316 and 91-37301-6344).

Running title: Maize kernel development gene *Emp2*
ABSTRACT

The recessive maize mutation emp2-1 was identified in progeny of a Robertson's Mutator stock based on a defective kernel phenotype observed in emp2-1/emp2-1 seed. Kernels homozygous for emp2-1 displayed early developmental arrest of both the endosperm and embryo, followed by necrosis and disintegration of kernel structures. At maturity emp2-1/emp2-1 mutant kernels were entirely inviable, and immature mutant embryos could not be rescued in tissue culture. Genetic and physical mapping located emp2-1 at chromosome 2L:91. Because no mutations affecting kernel development mapped to this region are allelic to emp2-1, the mutation defines a previously undescribed maize locus termed Empty pericarp-2 (Emp2). A specific genomic fragment containing the transposon Mu1 was found to be tightly linked to the emp2-1 mutation, and was cloned into a phage lambda vector. Two lines of evidence suggest this Mu element is the causative agent of the emp2-1 mutation and that the cloned genomic fragment comprises the Emp2 locus. First, Emp2/Emp2 siblings of the original emp2-1/Emp2 stock do not contain a Mu element in the genomic fragment that was cloned. Thus, insertion of this specific Mu1 element and the appearance of the emp2-1 allele occurred simultaneously. Second, mRNA is transcribed from the cloned region of the genome specifically in developing kernels, but not in seedling leaves.
INTRODUCTION

The defective kernel (dek) mutations of maize are a frequently encountered class of genetic lesions that prevent kernel development by adversely affecting both embryo and endosperm morphogenesis (Jones 1920; Mangelsdorf 1923; Mangelsdorf 1926; Neuffer and Sheridan 1980; Scanlon et al. 1993). Kernels homozygous for one of the pleiotropic dek mutations usually produce nonviable embryos, and display any of a diverse array of specific endosperm phenotypes including those referred to as papery, reduced endosperm, miniature, etched-pitted, floury-opaque, or sugary-shrunken. Over 250 dek alleles have been mapped genetically to 18 of the 20 chromosome arms of maize (Neuffer and Sheridan 1980; Coe et al. 1984; Clark and Sheridan 1986; Neuffer 1992; Scanlon et al. 1993). Forty-eight of these dek mutations correspond to previously undescribed genetic loci and thus, represent novel genes affecting maize kernel development.

Developmental analyses of embryos dissected from mutant kernels homozygous for specific dek mutations indicated that allele-specific blocks or disruptions in embryogenesis are imposed over a wide range of developmental stages (Sheridan and Neuffer 1980; Clark and Sheridan 1986; Clark and Sheridan 1988). Tissue culture studies of immature embryos dissected from dek mutant kernels demonstrated that although most dek embryos are inviable at kernel maturity, approximately 80% of rescued mutant embryos produce small or large plants in culture (Sheridan and Neuffer 1980). These results indicated that embryo-lethality caused by many dek mutations is imposed relatively late in kernel development. Although these genetic and developmental studies have contributed greatly to an understanding of this intriguing class of maize mutants, no dek
mutations have as yet been characterized at the molecular level and no specific biochemical or physiological function has been assigned to the product of any Dek locus.

Towards the goal of describing molecules that regulate maize kernel development, we have generated a collection of dek mutations from active Robertson's Mutator maize lines. The Robertson's Mutator maize transposable element system, which generates a 30-50 fold increase in the spontaneous mutation rate of maize (Robertson 1978), has proven to be an extremely useful system for both inducing maize kernel developmental mutations (McCarty et al. 1989a; Clark and Sheridan 1991; Scanlon et al. 1993) and cloning of Mu-inserted loci via transposon tagging (O'Reily et al. 1985; McClaughlin and Walbot 1987; McCarty et al. 1989b; Martiennsen et al. 1989; Buckner et al. 1990; Johal and Briggs 1992). This report describes the developmental characterization of kernels homozygous for a mutation in the Empty pericarp-2 (Emp2) locus, a novel defective kernel mutation that was identified from this collection (Scanlon et al. 1993). Using a Mutator transposon as a molecular tag, a specific genomic DNA fragment that is tightly linked to the emp2 mutation was cloned and characterized. The emp2-linked clone was used to isolate a homologous DNA fragment from wild type plants that hybridizes to RNA transcripts in developing kernels, suggesting this fragment codes for a molecule that functions in kernel development.
MATERIALS AND METHODS

Isolation and Propagation of emp2-Mu1047

The strategy for the isolation of the emp2-Mu1047 kernel mutation from Mutator stocks is presented in Figure 1. Mutator lines are those defined by Robertson (1978). Standard lines (stocks which have never been crossed to Mutator stocks) used are the F1 hybrids inbred Q66 x inbred Q67, or inbred B77 x inbred B79. Active Mutator plants were outcrossed as males to standard lines, and F1 plants grown from the outcrossed ears were self-pollinated. A specific F1 plant was found to be heterozygous for a dek mutation, later defined as emp2-Mu1047, by observing normal and defective kernels on the self-pollinated ear segregating in the ratio of 3:1. Because emp2-Mu1047 homozygous kernels are inviable, the mutation was propagated by outcrossing emp2-Mu1047/Emp2 heterozygous plants to standard lines as shown in Figure 1. Approximately one half of the resulting progeny plants were heterozygous for emp2-Mu1047, as indicated by the 3:1 segregation ratio of wild type and defective kernels on corresponding self-pollinated ears.

Developmental Analysis of Homozygous emp2-Mu1047 Mutant Kernels

Portions of self-pollinated ears used for examination of kernel morphological development were collected from emp2-Mu1047/Emp2 heterozygous plants at 3, 6, 9, 12, 14, 16, 20, 21, 24, and 36 days after pollination (dap). The ear segments were collected in a manner such that the development of kernels remaining on the ear was not affected. Emp2/emp2-Mu1047 plants were identified at 14 dap and thereafter by the presence of emp2-Mu1047/emp2-Mu1047 defective kernels on the self-pollinated ears. Self-pollinated ears examined before 13 dap were identified as derived from
Figure 1. Strategy for using the Robertson's Mutator system for induction and propagation of the emp2-Mu1047 mutation.
emp2-Mu1047/Emp2 heterozygous plants by the presence of a specific restriction fragment linked to emp2-Mu1047 in seedling leaf DNA from the same plants (see Results). Confirmation of the emp2-Mu1047/Emp2 genotype of plants examined before 13 dap was made by subsequent re-examination of the remaining, unharvested portions of self-pollinated ears at later stages of development when the emp2-Mu1047 mutant kernel phenotype was distinguishable from wild type kernels. Comparative examinations of sibling wild type (Emp2/Emp2 or emp2-Mu1047/Emp2) and emp2-Mu1047/emp2-Mu1047 mutant specimens of whole kernels, lateral kernel sections, and dissected whole embryos were performed under low level magnification using a dissecting microscope.

Embryo Culture Media and Techniques

The minimum essential medium (MEM) was prepared essentially as described in Sheridan and Neuffer (1980), and contained the mineral salts of Murashige and Skoog (1962), 4% sucrose, 0.8% agar and the following additives: NaCl 85.0 mg/l; (L-amino acids) arginine 126.4 mg/l, histidine-HCl 42.0 mg/l, isoleucine 52.5 mg/l, leucine 52.4 mg/l, lysine-HCl 72.5 mg/l, methionine 15.1 mg/l, phenylalanine 33.0 mg/l, threonine 47.6 mg/l, tryptophan 10.2 mg/l, valine 46.8 mg/l, cysteine-2HCl 24.0 mg/l, glutamine 87.6 mg/l, serine 12.6 mg/l; (vitamins) choline chloride 1.0 mg/l, folic acid 1.0 mg/l, myo-inositol 2.0 mg/l, niacinamide 1.0 mg/l, D-pantothenic acid 1.0 mg/l, pyridoxine-HCl 1.0 mg/l, riboflavin 0.1 mg/l, thiamine-HCl 1.0 mg/l; (nucleic acid bases) adenine 10 mg/l, guanine 10 mg/l, thymine 10 mg/l, cytosine 10 mg/l, uracil 10 mg/l. All amino acids except L-glutamine and L-serine were purchased as a sterile 50X solution (Sigma Product No. M7020) and all vitamins were purchased as a sterile 100X solution (Sigma Product No. M6895). To
prepare the MEM, a solution containing the mineral salts, sucrose and agar and nucleic acid bases was adjusted to pH 5.8, and autoclaved. After cooling to 55°C, the 50X amino acid solution, 100X vitamin solution, and 0.2 mm Nalgene filter-sterilized solutions of L-serine and L-glutamine were added to the indicated final concentrations. The medium was dispensed into 60 x 15 mm plastic culture dishes for primary culturing and also to 50 ml polypropylene tubes for secondary culturing of transferred, cultured plants.

The procedure for dissection of immature embryos was adapted from Sheridan and Neuffer (1980). Ears were harvested at 14, 16, 17 and 20 dap. Husks and silks were removed and whole ears were surfaced sterilized by submersion in 80% ethanol for 5 minutes.

Ears were placed on the surface of a sterile laminar flow hood and the tops of the kernels were removed with a sterile razor blade. Five to ten embryos were dissected from both wild type and mutant sibling kernels at each developmental time point and transferred to culture dishes using a sterile spatula. In some cases, both the embryo and the surrounding endosperm material were dissected and cultured together. The embryo cultures were incubated at 25°C, with a 16-hour light, 8-hour dark cycle, and monitored for embryo enlargement, plumule or radical emergence, and precocious germination. Cultures of responsive embryos were transferred to fresh MEM every 14 days.

Nucleic Acid Hybridization Probes

Nucleic acid manipulations including plasmid constructions were performed using standard methods (Sambrook et al. 1989). Probe M960 is the 960 bp M\(\mu\)I fragment internal to transposon M\(\mu\)I cloned previously (Barker et al. 1984). This
fragment was obtained from plasmid pMJ9 (Bennetzen et al., 1984; gift of J. Bennetzen, Purdue University, West Lafayette, IN), which contains the entire sequence of transposon Mu1 subcloned into pBR322. Probe SacH2.4 is a 2.4 kb HindIII fragment derived from soybean actin cDNA. This fragment was obtained from plasmid pSAC7 (gift of R. Meagher, University of Georgia, Athens, GA). Various maize genomic DNA fragments used as hybridization probes were cloned in pBLUESCRIPT-SK+ (Stratagene Cloning Systems, La Jolla, CA) (pMS2, pMS3, pMS64) or pBLUESCRIPT-KS+ (pMS65). These cloned fragments are described in the Results section. Double stranded DNA restriction fragments used as hybridization probes were purified by agarose gel electrophoresis and radioactively labeled by the random primer extension method (Feinberg and Vogelstein 1983). Single stranded probes were prepared by extension of the universal -40 sequencing primer (New England Biolabs, Inc., Beverly, MA) with DNA polymerase I Klenow fragment using single stranded phagemids pMS64 or pMS65 as the template. Single stranded phagemids were prepared as described (Vieira and Messing 1987). In all instances radioactively labeled DNA probes were separated from free nucleotides in the primer extension reactions by size exclusion chromatography using Sephadex G-50 (Sigma Chemical Co., St. Louis, MO).

Genomic DNA Isolation and Southern Blot Analysis

Maize genomic DNA was isolated from immature ears or 7 day-old seedlings by the method of Dellaporta et al. (1983). Approximately 10 mg of DNA was digested with an excess of restriction enzyme, fractionated by electrophoresis in 0.8% agarose gels and transferred to 0.45 mm nylon membranes (MSI, Westboro, MA) (Southern 1975; Sambrook et al. 1989). Membranes were prehybridized in a buffer containing 6
X SSC (0.9 M NaCl, 90 mM sodium citrate), 1% sodium lauryl sarcosinate, and 50 mg/ml heat denatured salmon sperm DNA for greater than 2 hours at 65°C. Hybridizations were performed overnight at 65°C in the same buffer containing heat denatured, radioactive probe. Membranes were washed two times in 2X SSC, 0.1% SDS at 65°C for twenty minutes each, and final washes were done three times in 5 mM Tris-HCl (pH 8.0), 0.1% SDS at 65°C for 10 minutes each. Washed membranes were exposed to X-ray film with intensifier screens at -70°C for two to four days.

Cloning of Maize Genomic DNA Fragments Linked to emp2-Mu1047

To clone the putative, Mu1-tagged emp2-Mu1047 mutant allele, DNA was isolated as previously described from the immature second ear of a plant of the genotype emp2-Mu1047/Emp2; the presence of the emp2-Mu1047 allele was assigned by observation of 25% defective kernels on the self-pollinated first ear. Approximately 150 mg of genomic DNA was digested with EcoRI, and fragments were fractionated by electrophoresis in 0.8% agarose gels (Gibco ultra grade agarose, BRL, Bethesda, MD). Fragments of the size range 2.5 kbp to 4.0 kbp were isolated by electroelution from the gel, purified by phenol extraction, and concentrated by ethanol precipitation. The size selected fragments of genomic DNA were ligated into EcoRI-digested phage lambda vector NM1149 at an approximate molar ratio of 1:1, packaged into bacteriophage particles (Giga-pack gold II packaging kit, Stratagene Cloning Systems), and used to infect Escherichia coli strain C600 hfl. The library was screened by plaque hybridization (Sambrook et al. 1989) using the radioactive probe MJ960, and a single hybridizing plaque was identified among approximately 6 x 10^4 total plaques. The Mu1-homologous recombinant phage was
purified by two successive rounds of plaque purification. Following isolation of DNA from the *Mu1* hybridizing recombinant bacteriophage isolate (Sambrook et al. 1989), the maize genomic DNA inserts were subcloned into the vector pBR322. This recombinant plasmid is designated pMS1.

The *emp2-Mu1047*-linked genomic clone was used to isolate the corresponding region of the genome from a wild type maize line. A library of 3.5 - 5.0 kbp *EcoRI* fragments from standard line *B70* was prepared in phage lambda vector NM1149 as described above. The library was screened using hybridization probe EG550 obtained from plasmid pMS2 (see Results). A single hybridizing plaque was obtained from approximately $5 \times 10^4$ total plaques screened. After plaque purification the genomic insert of this recombinant phage was subcloned into vector pBLUESCRIPT-KS+ to form plasmid pMS61.

**DNA Sequence Analysis**

The nucleotide sequences of the genomic DNA bordering each end of the cloned *Mu1* transposon were determined by the chain termination method (Sanger et al. 1977). One border was analyzed using oligonucleotide primer SP132 (5'-GCGTACGTCTCTAAA-3'), which is identical in sequence to nucleotides 1302-1316 of *Mu1*. The template was single stranded phagemid pMS3 (see Results). The opposite border was analyzed using a single stranded phagemid pMS4 as a template and the universal sequencing primer. Plasmid pMS4 was derived from pMS2 by unidirectional exonucleolytic digestion of most of the sequence between the binding site for the universal primer and the terminus of *Mu1*. Thus, the sequence obtained extended from within the genomic DNA into the *Mu1* element (see Results).
RNA Isolation and Northern Blot Analysis

For preparation of total RNA, 4 g of maize tissue was ground in liquid nitrogen with a mortar and pestle and thawed in lysis buffer (0.1 M NaCl, 50 mM Tris-HCl pH 7.4, 50 mM EDTA, 2% SDS, 200 mg/ml proteinase K). Following two extractions in phenol/chloroform, the RNA was precipitated with ethanol and resuspended in H₂O. Yields of total RNA isolated by this procedure typically ranged from 500 to 875 mg/g fresh tissue weight. Polyadenylated RNA was selected by affinity chromatography on oligo (dT)-cellulose spun columns (Pharmacia mRNA purification kit).

Procedures for northern blotting and hybridization of maize RNA were adapted from Seeley et al. (1992). Polyadenylated-enriched RNA (3-6 mg) was separated by electrophoresis in 1% agarose/3% formaldehyde gels and transferred to nylon membranes (MSI). Hybridizations were performed for 12-16 hours at 45°C in hybridization buffer (50% deionized formamide, 1.0 M NaCl, 10% dextran sulfate, 1% SDS and 50 mg/ml heat-denatured salmon sperm DNA) containing heat-denatured DNA probes. Posthybridization washes were carried out two times in 2X SSC, 0.1% SDS at 65°C for twenty minutes each, and two final washes were in 0.2X SSC, 0.1% SDS at 65°C for 20 minutes each. Membranes were exposed to X-ray film with intensifier screens at -70°C for one to four days.
RESULTS

Effects of *emp2-Mu1047* on Kernel Morphology and Development

The recessive, embryo-lethal, defective kernel mutation *empty pericarp-2* (*emp2-Mu1047*) was identified on the self-pollinated ear of a single maize plant from Robertson's *Mutator* stocks. The *emp2-Mu1047/Emp2* parent plant and *emp2-Mu1047/Emp2* heterozygotes from successive generations were outcrossed to standard lines (Figure 1). One half the progeny of these crosses carried *emp2-Mu1047*, as shown by the presence of one quarter defective kernels on self-pollinated ears; the other half of the progeny yielded all normal kernels after self-pollination, indicating these plants were *Emp2* homozygotes. These results showed the defective kernel phenotype observed in these families is a single-gene trait caused by the *emp2-Mu1047* mutation.

At maturity, *emp2-Mu1047* mutant kernels were papery, often flattened vestiges with no endosperm material or embryo present (Figure 2). As the maize pericarp is maternal in origin, mature *emp2-Mu1047* mutant kernels were therefore essentially devoid of tissue from the zygotic generation. Genetic mapping located the *emp2-Mu1047* mutation on chromosome 2L, approximately 7.5 cM distal to *virescent-4* (*v4, 2L:83*) (Scanlon et al. 1993). Because *emp2-Mu1047* was not allelic to any previously described kernel mutations identified in this region of the genome (Scanlon et al. 1993), it defined a novel genetic locus in maize termed *Emp2*.

In examinations of *emp2-Mu1047* mutant kernel morphogenesis over a developmental time course (Figure 3) the mutant phenotype could first be observed segregating on self-pollinated ears of *emp2-Mu1047/Emp2* plants at 13-14 dap. At this stage, *emp2-Mu1047* mutant kernels were smaller and more pale in color than
Figure 2. Phenotype of *emp2-1047* mutant kernels at maturity. (A) This panel shows a portion of a mature, self-pollinated ear from a plant heterozygous for the recessive, defective kernel mutation *emp2-Mu1047*. Wild type kernels and sibling, papery, *emp2-Mu1047/emp2-Mu1047* mutant kernels segregate in a 3:1 ratio. (B) This panel shows germinal (two left kernels) and abgerminal (two right kernels) views of wild type (upper row) and sibling *emp2-Mu1047/emp2-Mu1047* mutant kernels (lower row) from the self-pollinated ear of a *emp2-Mu1047/Emp2* heterozygous plant.
Figure 3. The *emp2-Mu1047* mutation causes abnormal endosperm development. Segments of self-pollinated ears of *emp2-Mu1047/Ep2* plants harvested at different timepoints in endosperm development. (A) 20 dap *emp2-Mu1047/emp2-Mu1047* mutant and wild type sibling kernels. (B) Lateral section of kernels from 20 dap ear. (C) 24 dap *emp2-Mu1047/emp2-Mu1047* mutant and wild type sibling kernels. (D) Lateral section of kernels from 24 dap ear. (E) 36 dap *emp2-Mu1047/emp2-Mu1047* mutant and wild type sibling kernels. (F) Lateral section of kernels from 36 dap ear. All samples at 10X magnification.
their wild type siblings (data not shown). In contrast to the empty kernel phenotype seen in mature emp2-Mu1047 mutant kernels, immature emp2-Mu1047 mutant kernels did develop some endosperm tissue. The enlarging endosperm of wild type maize kernels typically fills and solidifies the kernel cavity by 12 dap (Duvick 1955; Randolph 1936). Although emp2-Mu1047 mutant kernels examined at 20 dap were plump and contained some endosperm material, the kernel cavity remained unfilled (Figure 3A, 3B). At 24 dap, wild type kernels had ceased endosperm mitotic divisions, were deep yellow in color, and began to form the characteristic dent in the kernel crown (Figure 3C, 3D). Mutant emp2-Mu1047 sibling kernels were pale in comparison, and the unfilled kernel cavity led to collapsing in of the kernel crown. Furthermore, the lack of appreciable endosperm material in emp2-Mu1047 mutant kernels allowed for the compression of mutant kernels situated between much larger, wild type siblings (Figure 3C, 3D). At later stages in kernel development, the small amount of accumulated endosperm material in emp2-Mu1047 mutants either degraded or was reabsorbed, and the kernels became collapsed, empty hulls at 36 dap and into maturity (Figures 3E, 3F; Figure 2).

Embryos dissected from immature emp2-Mu1047/emp2-Mu1047 homozygous kernels exhibited developmental arrest as compared to those from wild type sibling kernel. Mutant embryos dissected at 14, 16, 20, and 24 dap all appeared to be developmentally arrested at early stage 1 of embryogenesis, according to the morphological descriptions of Abbe and Stein, (1954). As shown in Figure 4A, emp2-Mu1047/emp2-Mu1047 embryos dissected at 20 dap contained an ovate-deltoid shaped scutellum, and the enlarging coleoptile began to enclose the shoot apex that was visible through a small coleoptilar pore. No further elaboration of embryonic structures present in 14 dap emp2-Mu1047/emp2-Mu1047 embryos was noted in
Figure 4. Embryos dissected from emp2-Mu1047/emp2-Mu1047 mutant kernels are developmentally arrested early in embryogenesis. Embryos were dissected from self-pollinated ears of emp2-Mu1047/Emp2 plants. (A) 20 dap emp2-Mu1047/emp2-Mu1047 mutant embryo arrested at abnormal stage 1 showing the ovate-deltoid shaped scutellum, sc; coleoptile, c; and shoot apex, sa. Magnification 42X. (B) 36 dap emp2-Mu1047/emp2-Mu1047 mutant embryo showing disintegration of the scutellum, sc; and shoot apex, sa. Magnification 31X. (C) 20 dap wild type embryo in stage 4 of development showing the elliptical scutellum, sc; scutellar node, sn; and coleoptile, c. Magnification 31X. (D) 36 dap wild type embryo in stage 5 showing scutellum, sc; embryonic root enclosed within the protruding coleorhiza, cz; and coleoepitilar pore, cp. Magnification 13X.
embryos dissected at later times. In contrast, Emp2/Emp2 wild type embryos progressed past stage 1 before 14 dap (data not shown). At 20 dap (Figure 4C), Emp2/Emp2 embryos developed to stage 4, as indicated by the elliptical shaped scutellum and the constriction of the scutellar lips over the scutellar node. Stage 5 embryos were recovered from wild type 36 dap kernels (Figure 4D), whereupon emp2-Mul047 mutant embryos displayed severe necrosis and degradation of the scutellum and embryonic axis (Figure 4B). Further disintegration of emp2-Mu1047 mutant embryos followed, until no embryonic tissue was observed at 40 dap, nor at kernel maturity (Figure 2).

Tissue culturing of immature embryos dissected from emp2-Mu1047 mutant kernels at 16, 18 and 20 dap was unsuccessful in rescuing the inviable mutant phenotype. Cultured mutant embryos showed no enlargement, or plumule or radical emergence, whereas corresponding wild type sibling embryos germinated precociously and formed normal seedlings in culture.

Identification and Cloning of a Mu1-Homologous Restriction Fragment Linked to emp2-Mu1047

The emp2-Mu1047 mutation arose in a Robertson's Mutator stock background and is therefore likely to be tagged with a Mutator transposable element. Mu1 is usually the most abundant Mu element in Mutator stocks, and has been found inserted into most of the mutated genes cloned to date from Mutator lines (Chandler and Hardeman 1993). The strategy used for identification of Mu-tagged genomic DNA linked to the emp2-Mu1047 mutation was to first outcross emp2-Mu1047/Emp2 plants to standard lines for two generations (Figure 1). This procedure reduced the number of segregating Mu1-homologous elements to approximately 10 copies per
genome, as estimated by DNA blot hybridization analysis using the Mu1 internal fragment M960 as probe. Next, DNA was prepared from both emp2-Mu1047/Emp2 and Emp2/Emp2 plants, individually digested with several restriction endonucleases, and analyzed by Southern blot hybridization to identify a particular Mu1-tagged genomic DNA fragment that cosegregated with the emp2-Mu1047 mutant phenotype. Throughout seven successive generations of analysis, an EcoRI fragment of approximately 3 kbp was detected by the Mu1 probe in all 35 emp2-Mu1047/Emp2 plants examined, but was absent in all 44 Emp2/Emp2 sibling plants examined (Figure 5).

To clone the 3.0 kb fragment linked to emp2-Mu1047, a size selected genomic library was constructed from genomic DNA of an emp2-Mu1047/Emp2 plant in the phage lambda vector NM1149. This library was then screened with the Mu1 homologous probe M960. The maize genomic DNA insertion from a single hybridizing recombinant phage plaque was isolated, purified, and subcloned into pBR322 to form pMS1 (Figure 6). Sequence analysis of termini of the Mu element and the adjacent regions of genomic DNA revealed that the nine base pair sequence CGCAACAAC immediately preceding the Mu element insertion is directly repeated in host DNA at the opposite end of the Mu element (data not shown). The sequences of the termini of the Mu element are identical to those of Mu1. Formation of direct repeats of 9 bp at the transposon’s termini is a characteristic consequence of Mu1 element insertion (Barker et al. 1984).

The cloned Mu1 element was confirmed to be linked to emp2-Mu1047 by analyzing the inheritance of genomic sequences flanking the transposon. A 550 bp EcoRI-BgIII fragment, EG550, is contained within the original cloned fragment,
Figure 5. A specific MuI-homologous transposon co-segregates with the emp2-Mu1047 mutation. Genomic DNA obtained from sibling plants of the genotype emp2-Mu1047/Emp2 (lanes A-E) and Emp2/Emp2 (lanes F-L) was digested with EcoRI and analyzed by DNA gel blot hybridization using the M960 probe. The arrow indicates the 3.0 kb MuI-homologous restriction fragment that co-segregated with the emp2-Mu1047/Emp2 genotype.
Figure 6. Restriction map of the cloned genomic fragment that co-segregates with emp2-Mu1047. A partial restriction map is shown of the 3.0 kb EcoRI clone detected by hybridization to the Mu1 probe M960 and found to co-segregate with emp2-Mu1047. The position of the Mu insertion is shown by the heavy line. Subcloned fragments and fragments used as hybridization probes are represented by dashed lines. The restriction sites shown are as follows: E, EcoRI; B, BamHI; N, NotI; C, NcoI; G, BglII.
outside the \textit{Mu1} element (Figure 6). This fragment, prepared from plasmid pMS2, was radioactively labeled and used as a hybridization probe to identify homologous fragments in \textit{EcoRI} digests of genomic DNA from maize plants in a population segregating for the \textit{emp2-Mu1047} (see Figure 1). The inbred lines \textit{B79, B77, Q66,} and \textit{Q67}, which make up the standard lines used in the crossing procedure, also were characterized using the EG550 probe; this analysis identified the sizes of \textit{EcoRI} fragments containing the probe sequences in all wild type alleles present in these strains. Each inbred line was homozygous for an \textit{EcoRI} fragment of distinct size, and all of these wild type alleles were of a different size than the cloned \textit{Mu1}-containing fragment of 3.0 kb (Figure 7, Lanes 1, 2). \textit{Emp2/Emp2} plants in the segregating population contained some combination of the four polymorphic alleles found in the inbred lines, and none of these plants contained a 3.0 kb \textit{EcoRI} fragment homologous to EG550 (Figure 7, Lanes 5, 6). In contrast, all \textit{emp2-Mu1047/Emp2} heterozygous plants contained such a 3.0 kb fragment in addition to one of the four alleles from the standard lines (Figure 7, Lanes 3, 4). This analysis included 35 \textit{emp2-Mu1047/Emp2} heterozygotes and 44 \textit{Emp2/Emp2} siblings collected over seven generations; no exceptions were observed. These results indicated the cloned \textit{Mu1}-homologous fragment is indeed the 3.0 kb \textit{EcoRI} fragment linked to \textit{emp2-Mu1047}. The sizes of the corresponding alleles in the standard lines suggested the 1.4 kb transposon \textit{Mu1} inserted into the 1.6 kb \textit{EcoRI} fragment of the \textit{Q67} allele, to form the 3.0 kb fragment that was later cloned using the transposon as a tag.
**Figure 7.** Southern blot analysis of restriction endonuclease fragments linked to Emp2. DNA gel blot analysis of EcoRI-digested genomic DNA obtained from F1 hybrid B77/B79 (lane A), F1 hybrid Q66/Q67 (lane B), emp2-Mu1047/Emp2 (lanes C and D), and sibling Emp2/Emp2 plants (lanes E and F). Fragments were detected using the EG550 hybridization probe, which flanks the Mu element insertion in the cloned 3.0 kb EcoRI fragment linked to emp2-Mu1047. The fragments derived from the inbred parents of the F1 hybrids were identified in an identical analysis of the parent strains.
Formation of *emp2-Mu1047* and a Specific *Mu1* Transposition Occurred Simultaneously

Co-segregation of the cloned *Mu1* element with *emp2-Mu1047* suggested this specific transposition event was the direct cause of the mutation, and that the cloned genomic DNA is part of the *Emp2* locus. Alternatively, the genetic linkage could be explained by this *Mu1* element being located adjacent to *Emp2* but not within the locus. One means used to distinguish between these possibilities was to characterize the *Mu* elements present in siblings of the plant in which *emp2-Mu1047* was first identified. This strategy determined whether or not the cloned *Mu1*-containing fragment arose by novel transposition in the same generation as that which produced the *emp2-Mu1047* mutation. If the transposition predated the mutation, then it could not be the causative agent.

The *emp2-Mu1047* mutation was detected in the self-pollinated ear of a single individual in a F1 family comprising 20 sibling plants. The mutation arose during gametogenesis in the *Mutator* parent, because only this one specific plant in the family carried *emp2-Mu1047*; if the mutation had been preexistent in the germ line of either the *Mutator* strain or the standard strain that formed the F1 parent plant, then 50% of the plants in the F1 family would have displayed defective kernels in self-pollinated ears. The sibling plants in the F1 family were examined to see whether the 3.0 kb *Mu1*-containing *EcoRI* fragment was present in the line prior to formation of *emp2-Mu1047* (Figure 8). If so, the 3.0 kb fragment would be present in one-half of the F1 progeny seed. Twenty sibling kernels of the single kernel that contained *emp2-Mu1047* were planted, and genomic DNA was prepared from the seedlings.
Figure 8. Southern blot analysis of siblings of the progenitor *emp2-Mu1047/Emp2* plant. EcoRI-digested DNA was analyzed from an *emp2-Mu1047/Emp2* heterozygous plant (lane A), and from seedlings produced by sibling kernels of the original *emp2-Mu1047/Emp2* plant (lanes B-H). The hybridization probe was EG550. Molecular weight estimations identified the genetic source of each EG550-homologous fragment.
These DNA samples were digested with EcoRI and characterized by Southern hybridization analysis using probe EG550. All twenty plants contained EcoRI fragments of the same size as those seen in the F1 hybrid Q66 x Q67; none of them contained a 3.0 kb fragment. Therefore, a wild type, inbred Q67 allele was modified by an insertion of 1.4 kb in the same gamete that produced emp2-Mu1047.

**Molecular Cloning of the Putative Wild Type Emp2 Locus**

EG550 was used to isolate the corresponding wild type allele of emp2-Mu1047 from inbred strain B77. Genomic DNA from hybrid B77 X B79 was digested with EcoRI, and fragments in the approximate size region 3 to 5 kb were collected and cloned in phage lambda vector NM1149. This library was expected to contain the homologous fragment of 4.3 kb derived from inbred B77. The library was screened with probe EG550, and a single hybridizing plaque was observed. After two rounds of plaque purification the 4.3 kb genomic insert in the phage clone was excised and subcloned in pBLUESCRIPT KS+, forming plasmid pMS61. Mapping of restriction endonuclease recognition sites and Southern hybridization analysis of the cloned fragment showed the terminal 1.6 kb region of the wild type EcoRI fragment was homologous to the non-Mu1 portions of the original 3.0 kb clone known be linked to emp2-Mu1047 (Figure 9). As far as could be discerned from this analysis, the non-Mu1 region of the original 3.0 kb clone is identical to the corresponding region of the putative putative wild type Emp2 allele, with the exceptions of the Mutator insertion itself and polymorphism at the EcoRI site at the terminus of the 3.0 fragment, which is not present in the B77 allele.
Figure 9. Restriction map of genomic fragment *Emp2-B77*. The figure shows a partial restriction map of the 4.3 kb *EcoRI* clone derived from inbred strain B77 and identified by hybridization to probe EG550. The region of *Emp2-B77* homologous to *emp2-Mu1047* is indicated by the heavy line. Subcloned fragments and fragments used as hybridization probes are represented by dashed lines. Asterisks show the location of nucleotide sequence polymorphisms that result in restriction sites found in the *emp2-Mu1047* clone that are absent in *Emp2-B77*. The arrow indicates the direction of transcription of RNA homologous to fragment EB1.4.
The Putative *Emp2* Locus is Transcribed in Kernels

Fragments ES1.1, EB2.9, and EB1.4 (Figure 9) of the putative *Emp2* locus cloned from inbred line *B77* were radioactively labeled and used to detect homologous transcripts in polyadenylated RNA from seedling leaves and wild type kernels at various times after pollination. Figure 10A shows probe EB1.4 detected a transcript of approximately 2.5 kb in the RNA from kernel tissue, but not seedling RNA. Neither ES1.1 nor EB2.9 detected this or any other transcript (data not shown). Thus, the *Mul* element insertion in the *Emp2* allele of inbred strain *Q67* is within or adjacent to a transcribed region. The putative *Emp2* transcript was detected in kernels at all developmental stages examined, from 4 to 20 days after pollination.

The direction of transcription of this region was determined using single stranded hybridization probes. Specific strands of probe EB1.4 were radioactively labeled by enzymatic copying of single stranded phagemids. Only when the probe was formed by extension in the 5' to 3' direction from the *BamHI* site towards the *NcoI* site was the 2.5 kb kernel-specific transcript detected (Figure 11.) The gene is transcribed, therefore, from the *NcoI* site towards the *BamHI* site (Figure 9).
Figure 10. Hybridization of the Emp2-B77 clone to kernel RNA. Approximately 3 ug of polyadenylated RNA isolated from wild type Emp2/Emp2 developing maize kernels at the indicated timepoints and from 7-day old Emp2/Emp2 seedlings was analyzed by RNA gel blot hybridization. (A) The EB1.4 fragment derived from Emp2-B77 was used as hybridization probe. (B) The blot used in (A) was stripped of bound radioactivity and hybridized to the SacH2.4 probe derived from soybean actin cDNA to show that each lane contained approximately equal amounts of intact RNA.
Figure 11. Determination of the direction of transcription of *Emp2-B77*. Polyadenylated RNA isolated from 13 dap wild type *Emp2/Emp2* kernels was analyzed by RNA gel blot hybridization. Copies of the phagemids pMS64 (left lane) and pMS65 (right lane) were used as hybridization probes. The arrow indicates the putative *Emp2* transcript.
DISCUSSION

The emp2-Mu1047 Mutation Identifies a Novel Locus Essential for Normal Maize Kernel Development

A novel defective kernel mutation, empty pericarp-2 (emp2-Mu1047), which when homozygous causes papery, inviable kernels, was identified from Robertson's Mutator lines of maize. Mutants displaying the papery phenotype at kernel maturity are among the most abundant class of defective kernel mutants described (Sheridan and Neuffer 1980; Scanlon et al. 1993), and the absence of any endosperm or embryo structure in these mutant kernels indicated that many may result from mutation at so-called "housekeeping genes" required for general cellular functioning. Analyses of endosperm and embryo development in emp2-Mu1047 mutant kernels, however, indicated that the Emp2 gene product may not provide a housekeeping function but instead may fulfill a more specialized role in maize kernel development. Although emp2-Mu1047 mutant kernels were empty at maturity, they exhibited some kernel filling and endosperm morphogenesis, albeit abnormal, early in their development. Indeed, small yet discernible amounts of endosperm material persisted in emp2-Mu1047 mutant kernels beyond 24 dap, and embryos from emp2-Mu1047 mutant kernels progressed to early stage 1 (Abbe and Stein 1954) of embryogeny before developmental arrest and tissue disintegration occurred. These observations indicated that cells homozygous for the emp2-Mu1047 mutation were competent in basic cell functions and could form differentiated tissues. The Emp2 gene product was, however, required for elaboration beyond early programs of both embryo and endosperm development.
Although over 80% of the nonviable defective kernel mutants studied by Sheridan and Neuffer (1980) produced small seedlings or plants when immature mutant embryos were placed in tissue culture, *emp2-Mu1047* mutant embryos were not viable when placed in culture on minimal essential medium. These results indicated that embryos homozygous for the *emp2-Mu1047* mutation were arrested at an abnormal stage 1 of maize embryo development, since wild type stage 1 embryos germinated precociously in tissue culture and formed healthy plants (Sheridan and Neuffer 1980). The necrosis and disintegration of essentially all *emp2-Mu1047* mutant kernel tissue following 25 dap suggested that *emp2-Mu1047* mutant kernels were either too developmentally hindered to achieve dormancy, or the *Emp2* gene product may also function in the imposition of maize kernel quiescence.

**Molecular Cloning of the *Emp2* Locus**

Because the *emp2-Mu1047* mutation arose in plants from a Robertson's *Mutator* transposable element background, it is an attractive candidate for cloning via the technique of transposon-tagging. We have cloned a 3 kbp DNA fragment containing a *Mu1* homologous insertion, and preliminary evidence suggests that this genomic clone may indeed contain the *emp2-Mu1047* mutation. Cosegregation of the cloned 3 kb *EcoRI* fragment containing *Mu1* with the defective kernel phenotype in all 79 plants analyzed in this study proves that this fragment is located in the genome at or near *emp2-Mu1047*. By themselves, however, these data, do not establish the identity of the cloned fragment as part of the *Emp2* locus. The definitive strategy to prove the identity of a genomic clone containing a transposon as a specific mutant allele is to compare the structure of the wild type progenitor allele, the mutant allele, and revertant wild type alleles of the particular locus. This strategy is particularly
feasible for many transposon-induced mutations because they are often unstable; in these instances excision of the transposable element and simultaneous restoration of wild type function would prove the cloned gene's identity. Such a strategy was successfully applied to establish the identity of Mutator-containing clones obtained from the \textit{hm1-mu1} allele (Johal and Briggs 1992), the \textit{y1-mum} allele (Buckner et al. 1990) and the \textit{bz2-mu1} allele (McClaughlin and Walbot 1987). However, because kernels homozygous for \textit{emp2-Mu1047} are inviable, it is not possible to analyze polymorphism of \textit{Emp2} alleles in germinal revertants. Moreover, the phenotype of \textit{emp2} mutant kernels prevents the differentiation of kernels exhibiting revertant sectors of wild type tissue from stable, mutant kernels that sometimes display partial kernel filling.

We used a different strategy to help determine whether the cloned 3.0 kb EcoRI fragment contained the \textit{emp2-Mu1047} mutation. Analyzing siblings of the progenitor \textit{emp2-Mu1047/Emp2} plant indicated that the rare induction of the \textit{emp2} mutation in this population of plants with otherwise wild type kernel development was concomitant with the rare insertion of a \textit{Mu1}-homologous transposon to a locus tightly linked to \textit{Emp2}. Although these data do not rule out the possibility that the rare \textit{Mu1} tranposition event and the rare occurrence of mutation in the \textit{Emp2} locus are separate and coincidental phenomena, we feel the parsimonious interpretation is that the two events are related by cause and effect.

Furthermore, a DNA fragment of the putative \textit{Emp2} locus was used as probe in northern hybridization analyses to identify a 2.5 kbp RNA transcript that accumulated in developing maize kernels. Hybridization of the 1.4 kbp probe was not detected in RNA young leaves. Taken together the data provide strongly suggest the cloned genomic DNA fragments are part of the the \textit{Emp2} locus. Determination of the
direction of transcription of the 1.4 kbp putative Emp2 gene fragment indicated that the remaining 2.9 kb of the cloned, B77 genomic DNA fragment, which does not hybridize to polyadenylated RNA, comprises either a large intron or 3' untranslated sequence. The molecular cloning of a genomic DNA fragment corresponding to sequences upstream of the EB1.4 homologous transcript may be useful in identifying promoter sequences that specify kernel-specific gene transcription in maize. Such kernel-specific gene promoter sequences may allow for bio-engineering of the maize kernel by targeting expression of desirable genes to the developing seed.
REFERENCES


PAPER 4. CHARACTERIZATION AND MOLECULAR CLONING OF
DSCI, A NOVEL LOCUS FUNCTIONING IN MAIZE KERNEL
DEVELOPMENT
Characterization and Molecular Analysis of Dsc1, a Novel Locus Functioning in Maize Kernel Development

M. J. Scanlon, D. S. Robertson and A. M. Myers

From the Departments of Biochemistry and Biophysics (Scanlon, and Myers), and the Department of Zoology and Genetics (Robertson), Iowa State University, Ames IA 50011. This work was supported by research grants to AMM and DSR from the U.S. Department of Agriculture (88-37234-3316 and 91-37301-6344).

Running title: Maize kernel development gene Dsc1
ABSTRACT

The recessive maize mutation *discolored-1 (dsc1-Mu2058)* was identified in progeny of a Robertson's *Mutator* stock based on the phenotype of defective kernel development observed in *dsc1-Mu2058/dsc1-Mu2058* seed. Kernels homozygous for the *dsc1-Mu2058* mutation display retarded and abnormal development of both the endosperm and embryo, followed by necrosis and disintegration of kernel structures. At maturity, *dsc1-Mu2058/dsc1-Mu2058* mutant kernels are entirely inviable, although when placed in tissue culture, immature *dsc1-Mu2058* mutant embryos form small, single leafed plants that die after approximately 30 days in culture. Genetic mapping located *dsc1-Mu2058* on chromosome 4S near the *Bm3* locus; because no mutations affecting kernel development located in this region are allelic to *dsc1-Mu2058*, the mutation identifies a novel genetic locus of maize, termed *Dsc1*. A specific genomic fragment containing the transposon *Mu1* was found to be tightly linked to *dsc1-Mu2058*, and was cloned in a phage lambda vector. Genomic sequences contained in the *dsc1-Mu2058*-linked clone are transcribed in developing kernels in a temporally regulated pattern from 5-9 days after pollination, but not in seedling leaves. The DNA sequence in or near the transcribed region is highly homologous to genomic DNA sequences located upstream of the coding region of maize genes coding for the 27 kD class of zein proteins, which are expressed specifically in endosperm.
INTRODUCTION

The defective kernel (dek) mutations of maize are a frequently encountered class of genetic lesions that prevent kernel development by adversely affecting both embryo and endosperm morphogenesis (Jones, 1920; Mangelsdorf, 1923; Mangelsdorf, 1926; Neuffer and Sheridan, 1980; Scanlon et al. 1993a). Kernels homozygous for one of the pleiotropic dek mutations usually produce nonviable embryos, and display any of a diverse array of specific endosperm phenotypes including those referred to as papery, reduced endosperm, miniature, etched-pitted, floury-opaque, or sugary-shrunken. Over 250 dek alleles have been mapped genetically to 18 of the 20 chromosome arms of maize (Neuffer and Sheridan, 1980; Coe et al. 1984; Clark and Sheridan, 1988; Neuffer, 1992; Scanlon et al. 1993a). Forty-eight of these dek mutations correspond to previously, undescribed genetic loci and thus, represent novel genes affecting maize kernel development.

Developmental analyses of embryos dissected from mutant kernel homozygous for specific dek mutations indicated that allele-specific blocks or disruptions in embryogenesis are imposed over a wide range of developmental stages (Sheridan and Neuffer, 1980; Clark and Sheridan, 1986; Clark and Sheridan, 1988). Tissue culturing of immature embryos dissected from dek mutant kernels have demonstrated that although most dek embryos are inviable at kernel maturity, approximately 80% of rescued mutant embryos produce small or large plants in culture (Sheridan and Neuffer, 1980). These results indicate that the embryo-lethality caused by many dek mutations is imposed relatively late in kernel development. Although these genetic and developmental studies have contributed greatly to an understanding of this intriguing class of maize mutants, no dek
mutations have as yet been characterized at the molecular level and no specific biochemical or physiological function has been assigned to the product of any Dek locus.

Towards the goal of describing molecules that regulate maize kernel development, we have generated a collection of dek mutations from active Robertson’s Mutator maize lines. The Robertson’s Mutator maize transposable element system, which generates a 30-50 fold increase in the spontaneous mutation rate of maize (Robertson, 1978), has proven to be an extremely useful system for both inducing maize kernel developmental mutations (McCarty et al. 1989a; Clark and Sheridan, 1991; Scanlon et al. 1993a) and cloning of Mu-inserted loci via transposon tagging (O’Reily et al. 1985; Mc Claughlin and Walbot, 1987; McCarty et al. 1989b; Martiennsen et al. 1989; Buckner et al. 1990; Johal and Briggs, 1992). This report describes the developmental characterization of kernels homozygous for discolored-1 (dsc1-Mu2058), a novel, defective kernel mutation that was identified from this collection (Scanlon et al. 1993a). Using a Mutator transposon as a transposon tag, a specific genomic DNA fragment that is tightly linked to the dsc1-Mu2058 mutation has been cloned and characterized. Evidence that the cloned sequence is indeed part of the Dsc1 gene is provided by 1) linkage of the cloned fragment with the dsc1-Mu2058 mutation in all 115 plants examined, 2) nucleotide sequence homology of the dsc1-Mu2058-linked sequence to the upstream non-coding region of the genes coding for the 27 kD gamma-zein endosperm storage protein, and 3) expression of the cloned DNA in kernels in a temporally regulated fashion.
MATERIALS AND METHODS

Source, Propagation, and Genetic Analyses of the dsc1-Mu2058 Kernel Mutation

The strategy for the isolation of the dsc1-Mu2058 kernel mutation from Mutator stocks is presented in Figure 1. Mutator lines are those defined by Robertson (Robertson, 1978). Standard lines (stocks which have never been crossed to Mutator stocks) used are the F1 hybrids Q60 (inbred Q66 X inbred Q67) or B70 (inbred B77 X inbred B79). Active Mutator plants were outcrossed as males to standard lines, and F1 plants grown from the outcrossed ears were self-pollinated. One F1 plant was found to be heterozygous for the dsc1-Mu2058 mutation; 1/4 of the kernels on the self-pollinated ear displayed the discolored endosperm, embryo-lethal phenotype. Because dsc1-Mu2058 homozygous kernels were inviable, the dsc1-Mu2058 mutation was propagated by outcrossing dsc1-Mu2058/Dsc1 heterozygous plants to standard lines as shown in Figure 1. Upon outcrossing, approximately one half of the progeny plants are heterozygous for the dsc1-Mu2058 mutation, as indicated by the 3:1 segregation ratio of wild type kernels and defective kernels on self-pollinated ears.

Developmental Analysis of dsc1-Mu2058 Mutant Kernels

Portions of self-pollinated ears used for examination of kernel morphological development were collected from dsc1-Mu2058/Dsc1 heterozygous plants at 2, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 25, 28 and 38 days after pollination (dap). The ear segments were collected in a manner such that the development of kernels remaining on the ear was not affected. Dsc1/dsc1-Mu2058 plants were identified at
Figure 1. Strategy for using the Robertson's Mutator system for induction and propagation of dsc1-Mu2058.
the 8 dap time point and beyond by the presence of \textit{dsc1-Mu2058/dsc1-Mu2058}
defective kernels on the self-pollinated ears. Self-pollinated ears examined before 8
dap were identified as derived from \textit{dsc1-Mu2058/Dsc1} heterozygous plants by the
presence of a specific restriction fragment linked to \textit{dsc1-Mu2058} in seedling leaf
DNA (see Results). Confirmation of the \textit{dsc1-Mu2058/Dsc1} genotype of plants
examined before 8 dap was made by subsequent re-examination of the remaining,
unharvested portions of self-pollinated ears at later stages of development when the
defective kernel phenotype of \textit{dsc1-Mu2058/dsc1-Mu2058} mutant kernels was
distinguishable from wild type. Comparative examinations of sibling wild type
\textit{(Dsc1/Dsc1 or dsc1-Mu2058/Dsc1)} and \textit{dsc1-Mu2058/dsc1-Mu2058} mutant specimens
of whole kernels, lateral kernel sections, and dissected whole embryos were
performed under low level magnification using a dissecting microscope.

\textbf{Embryo Culture Media and Techniques}

The minimum essential medium (MEM) was essentially that of Sheridan and
Neuffer (1980), containing the mineral salts of Murashige and Skoog (1962), 4%
sucrose, 0.8% agar and the following additives: NaCl 85.0 mg/l; (L-amino acids)
arginine 126.4 mg/l, histidine-HCl 42.0 mg/l, isoleucine 52.5 mg/l, leucine 52.4
mg/l, lysine-HCl 72.5 mg/l, methionine 15.1 mg/l, phenylalanine 33.0 mg/l,
threonine 47.6 mg/l, tryptophan 10.2 mg/l, valine 46.8 mg/l, cysteine-2HCl 24.0
mg/l, glutamine 87.6 mg/l, serine 12.6 mg/l; (vitamins) choline chloride 1.0 mg/l,
folic acid 1.0 mg/l, myo-inositol 2.0 mg/l, niacinamide 1.0 mg/l, D-pantothenic acid
1.0 mg/l, pyridoxine-HCl 1.0 mg/l, riboflavin 0.1 mg/l, thiamine-HCl 1.0 mg/l;
(nucleic acid bases) adenine 10 mg/l, guanine 10 mg/l, thymine 10 mg/l, cytosine 10
mg/l, uracil 10 mg/l. All amino acids except L-glutamine and L-serine were
purchased as a sterile 50X solution (Sigma Product No. M7020) and all vitamins
were purchased as a sterile 100X solution (Sigma Product No. M6895). To prepare the MEM, a solution containing the mineral salts, sucrose and agar and nucleic acid bases was adjusted to pH 5.8, and autoclaved. After cooling to 55°C, the 50X amino acid solution, 100X vitamin solution, and 0.2 mm Nalgene filter-sterilized solutions of L-serine and L-glutamine were added to final concentrations. The medium was dispensed into 60 X 15 mm plastic culture dishes for primary culturing and also to 50 ml polypropylene tubes for secondary culturing of transferred, cultured plants.

The procedure for dissection of immature embryos was adapted from Sheridan and Neuffer (1980). Ears were harvested at 15, 16, and 20 dap. Husks and silks were removed and whole ears were surfaced sterilized by submersion in 80% ethanol for 5 minutes. Ears were placed on the surface of a sterile laminar flow hood and the tops of the kernels were removed with a sterile razor blade. Five to ten embryos were dissected from both wild type and mutant sibling kernels at each developmental stage and transferred to culture dishes using a sterile spatula. The embryo cultures were incubated at 25°C, with a 16-hour light, 8-hour dark cycle, and monitored for embryo enlargement, plumule or radical emergence, and precocious germination. Responsive embryos were transferred to fresh MEM every 14 days in culture.

Nucleic Acid Hybridization Probes

Nucleic acid manipulations including plasmid constructions were performed using standard methods (Sambrook et al. 1989). Probe M960 is the 960 bp MluI fragment internal to transposon Mu1 cloned into (Barker et al. 1984). This fragment was obtained from plasmid pMJ9 (Bennetzen et al., 1984; gift of J. Bennetzen, Purdue University, West Lafayette, IN), which contains the entire sequence of transposon Mu1 subcloned into pBR322. Probe SacH2.4 is a 2.4 kb HindIII fragment derived
from soybean actin cDNA. This fragment was obtained from plasmid pSAC7 (gift of R. Meagher, University of Georgia, Athens, GA). Various maize genomic DNA fragments used as hybridization probes were cloned in pBLUESCRIPT-SK+ (pMS47, pMS66) (Stratagene Cloning Systems, La Jolla, CA) or pBLUESCRIPT-KS+ (pMS59, pMS67, pMS68). These cloned fragments are described in the Results section.

Double stranded DNA restriction fragments used as hybridization probes were purified by agarose gel electrophoresis and radioactively labeled by the random primer extension method (Feinberg and Vogelstein, 1983). Single stranded probes were prepared by extension of the universal -40 sequencing primer (New England Biolabs, Inc., Beverly, MA) with DNA polymerase I Klenow fragment using single stranded phagemids pMS66 or pMS67 as the template. Single stranded phagemids were prepared as described (Vieira and Messing, 1987). In all instances, radioactively labeled DNA probes were separated from free nucleotides in the primer extension reactions by size exclusion chromatography using Sephadex G-50 (Sigma Chemical Co., St. Louis, MO).

Genomic DNA Isolation and Southern Hybridization Analysis

Maize genomic DNA was isolated from immature ears or 7 day-old seedlings by the method of Dellaporta et al. (1983). Approximately 10 mg of DNA was digested with an excess of restriction enzyme, fractionated by electrophoresis on 0.8% agarose gels (Gibco ultra grade agarose, BRL) and transferred to 0.45 mm nylon membranes (MSI, Westboro, MA) (Southern, 1975; Sambrook et al. 1989). Membranes were prehybridized in a buffer containing 6X SSC (0.9 M NaCl, 90 mM sodium citrate), 1% sodium lauryl sarcosinate, and 50 mg/ml heat denatured salmon sperm DNA for greater than 2 hours at 65°C. Hybridizations were performed overnight at 65°C in the same buffer containing heat denatured, radioactive probe. Membranes were
washed two times in 2X SSC, 0.1% SDS at 65°C for twenty minutes each, and final washes were done three times in 5 mM Tris-HCl (pH 8.0), 0.1% SDS at 65°C for 10 minutes each. Finally, membranes were exposed to x-ray film with intensifier screens at -70°C for one to four days.

Cloning of Maize Genomic DNA Fragments Linked to *dsc1-Mu2058*

To clone the putative, *Mu1*-tagged *dsc1-Mu2058* mutant allele, DNA was isolated as previously described from the immature second ear of a plant of the genotype *dsc1-Mu2058/Dscl*; the presence of the *dsc1-Mu2058* allele was determined by the observation of 25% defective kernels on the self-pollinated first ear. Approximately 150 ug of genomic DNA was digested with *EcoRI*, and fragments were fractionated by electrophoresis in 0.8% agarose gels (Gibco ultra grade agarose, BRL, Bethesda, MD). Fragments of the size range 3.0 kb to 4.3 kb were isolated by gel electroelution from the gel, purified by phenol extraction, and concentrated by ethanol precipitation. The size selected fragments of genomic DNA were ligated into *EcoRI*-digested phage lambda vector NM1149 at an approximate molar ratio of 1:1, packaged into bacteriophage particles (Giga-pack gold II packaging kit, Stratagene Cloning Systems), and used to infect *Escherichia coli* strain C600 hfl. The library was screened by plaque hybridization (Sambrook et al. 1989) using the radioactive probe MJ960, and four separate hybridizing plaques were identified among approximately 5 x 10^4 total plaques. The *Mu1*-homologous recombinant phage were each purified by two successive rounds of plaque purification. Following isolation of DNA from the *Mu1* hybridizing recombinant bacteriophage isolates (Sambrook et al. 1989), the molecular weights of the cloned DNAs were determined. Maize genomic DNA inserts from a single recombinant phage were
subcloned into the vector pBLUESCRIPT-KS+. This recombinant plasmid is designated pMS47.

DNA Sequence Analysis and Oligonucleotide Primers

The nucleotide sequences of the genomic DNA bordering each end of the cloned Mul transposon were determined by the chain termination method (Sanger et al. 1977). One border was analyzed using oligonucleotide primer SP132 (5'-GCGTACGTCTCTAAA-3'), which is identical in sequence to nucleotides 1302-1316 of Mul. In this instance the template was single stranded phagemid pMS68 (see Results). The opposite border was analyzed using oligonucleotide primer SP131 (5'-GGCTGTCGCGTGCGT-3'), which is identical in sequence to nucleotides 108-94 of Mul (Barker et al. 1984). In this instance the template was single stranded phagemid pMS59. The nucleotide sequence of the single-stranded phagemid pMS67 (described in Results section) was partially determined using the universal sequencing primer.

RNA Isolation and Northern Hybridization Analysis

For preparation of total RNA, 4 g of maize tissue was ground in liquid nitrogen with a mortar and pestle and thawed in lysis buffer (0.1 M NaCl, 50 mM Tris-HCl pH 7.4, 50 mM EDTA, 2% SDS, 200 mg/ml proteinase K). Following two extractions in phenol/chloroform, the RNA was ethanol precipitated and resuspended in H2O. Yields of total RNA isolated by this procedure typically ranged from 500 to 875 mg/g fresh tissue weight. Polyadenylated RNA was selected by affinity chromatography on oligo (dT)-cellulose spun columns (mRNA purification kit, Pharmacia PLK Biochemicals).

Procedures for northern blotting and hybridization of maize RNA were adapted from Seeley et al. (Seely et al. 1992). Polyadenylated-enriched RNA (3-6 mg) was
separated by electrophoresis in 1% agarose/3% formaldehyde gels and transferred to nylon membranes (MSI). Hybridizations were performed for 12-16 hours at 45°C in hybridization buffer (50% deionized formamide, 1.0 M NaCl, 10% dextran sulfate, 1% SDS and 50 mg/ml heat-denatured salmon sperm DNA) containing specific, heat-denatured DNA probes made radioactive as described. Posthybridization washes were carried out two times in 2X SSC, 0.1% SDS at 65°C for 20 minutes each, and two final washes were in 0.2X SSC, 0.1% SDS at 65°C for 20 minutes each. Membranes were exposed to X-ray film with intensifier screens at -70°C for one to four days. Blots used in multiple probings were stripped of bound radioactive probe DNA by boiling for 5 min. Prior to reprobing, stripped blots were exposed to X-ray film for two days to ensure that no detectable radioactive probe DNA remained.
RESULTS

dsc1-Mu2058 Causes Abnormal Endosperm Development and Retarded Embryo Morphogenesis

The dsc1-Mu2058 mutation was identified on the self-pollinated ear of a single maize plant from a Robertson's Mutator stock. The dsc1-Mu2058/Dscl parent plant and dsc1-Mu2058/Dsc1 heterozygotes from successive generations were outcrossed to standard lines (Figure 1). One half the progeny of these crosses carried dsc1-Mu2058, as shown by the presence of one quarter defective kernels on self-pollinated ears; the other half of the progeny yielded all normal kernels on self-pollinated ears, indicating these plants were Dsc1 homozygotes. These results showed the defective kernel phenotype observed in these families to be caused by a mutation at a single genetic locus, termed Dsc1.

At maturity, dsc1-Mu2058 mutant kernels are small, opaque-discolored, irregularly-shaped kernels that contain no embryo tissue (Figure 2). Genetic mapping located the dsc1-Mu2058 mutation on chromosome 4S, approximately 1 cM from Bm3 (Scanlon et al. 1993a). Because dsc1-Mu2058 is not allelic to any previously described kernel mutations identified in this region of the genome (Scanlon et al. 1993a), the dsc1-Mu2058 mutation defines a novel genetic locus in maize.

Examinations of developing dsc1-Mu2058 mutant kernels revealed that the mutant phenotype can be first observed segregating on self-pollinated ears of dsc1-Mu2058/Dsc1 plants at 8 dap. At this time dsc1-Mu2058/dsc1-Mu2058 kernels were smaller and whiter in color than wild type sibling kernel (data not shown). At 16 dap, dsc1-Mu2058 mutant kernels were small and pale, with little solid endosperm material (Figure 3A, 3B). In contrast, wild type kernels normally have filled kernel
Figure 2. Phenotype of $dsc1$-$Mu2058$ mutant kernels at maturity. (A) This panel shows a portion of a mature self-pollinated ear from a plant heterozygous for the $dsc1$-$Mu2058$ mutation. Wild type sibling $Dsc1/Dsc1$ and $dsc1$-$Mu2058$/$Dsc1$ kernels segregate with discolored, abnormally-shaped $dsc1$-$Mu2058$/$dsc1$-$Mu2058$ mutant kernels in a 3:1 ratio. (B) This panel shows germinal (two left kernels) and abgerminal (two right kernels) views of wild type (upper row) and sibling $dsc1$-$Mu2058$/$dsc1$-$Mu2058$ (bottom row) kernels from a self-pollinated ear of a $dsc1$-$Mu2058$/$Dsc1$ heterozygous plant.
Figure 3. The \textit{dsc1-Mu2058} mutation causes abnormal endosperm morphogenesis. Each panel shows a portion of a self-pollinated ear from a \textit{dsc1-Mu2058/Dsc1} heterozygous plant. (A) 16 dap \textit{dsc1-Mu2058/dsc1-Mu2058} mutant and wild type sibling kernels. (B) Lateral sections of kernels from 16 dap ear showing lack of complete cellularization of \textit{dsc1-Mu2058/dsc1-Mu2058} endosperm. (C) 25 dap \textit{dsc1-Mu2058/dsc1-Mu2058} mutant and wild sibling kernels. (D) Lateral sections of kernels from 25 dap ear. (E) 35 dap \textit{dsc1-Mu2058/dsc1-Mu2058} and wild type sibling kernels. Note the discoloration and crown crumpling of the \textit{dsc1-Mu2058/dsc1-Mu2058} kernel. (F) Lateral sections of kernels from 35 dap ear. All samples at 10X magnification.
cavities after 12 dap (Duvick, 1955). Mutant $dsc1-Mu2058/dsc1-Mu2058$ kernels are still not completely filled at 25 dap, at which time the much larger, wild type kernels typically cease mitotic division in the endosperm (Randolph, 1936). The first visible signs of kernel discoloration were noticeable in 28 dap $dsc1-Mu2058$ mutant seed, whereas at 35 dap the mutants displayed kernel crumpling and degradation of endosperm material (Figure 3E, 3F). Mutant $dsc1-Mu2058/dsc1-Mu2058$ kernels on mature ears harvested at 50 dap typically contained small amounts of necrotic endosperm material.

Embryos dissected from developing $dsc1-Mu2058/dsc1-Mu2058$ homozygous kernels exhibited retarded development relative to those of wild type sibling kernels. At 18 dap $dsc1-Mu2058$ mutant embryos reached stage 1 of embryo development (Figure 4A), using the designations of embryo morphology described by Abbe and Stein (1954). Typically, stage 1 embryos have an ovate-deltoid-shaped scutellum, an enlarging coleoptile that begins to cover the shoot apex, and a large suspensor. In contrast, 18 dap wild type sibling embryos progressed past stage 2 (Figure 4B), as indicated by the ovate-shaped scutellum, small coleoptilar pore, and lack of a suspensor. Mutant $dsc1-Mu2058/dsc1-Mu2058$ embryos did not progress to early stage 2 until 20 dap (Figure 4C), whereupon wild type sibling embryos displayed the obovate-shaped scutellum, and constriction of the scutellar lips over the scutellar node characteristic of stage 3 of embryogenesis (Figure 4D). The $dsc1-Mu2058/dsc1-Mu2058$ mutant embryos lagged one to two embryogenic stages behind wild type sibling embryos until after 22 dap, whereupon $dsc1-Mu2058/dsc1-Mu2058$ embryo development ceased at early stage 2 and tissue degradation became noticeable. At 25 dap, wild type embryos progressed past stage 3 (Figure 4F), whereas $dsc1-Mu2058$ mutant, sibling embryos displayed extensive disintegration of
Figure 4. The dsc1-Mu2058 mutation causes abnormal embryo development. Each panel shows embryos dissected from self-pollinated ears of dsc1-Mu2058/Dsc1 plants at different times in embryo development. (A) Wild type embryo dissected at 18 dap, in early stage 3 of embryogenesis. Note the ovate-shaped scutellum, sc; coleoptile, c; and coleoptilar pore, cp. Magnification 44X. (B) Wild type embryo dissected at 20 dap has progressed further into stage 3, as indicated by the obovate-shaped scutellum, sc; coleoptilar pore, cp; and constriction of the scutellar lips near the scutellar node, sn. Magnification 39X. (C) At 25 dap, wild type embryos had the obovate-elliptical-shaped scutellum, sc; coleoptilar pore, cp; and enlarging coleorhiza, cz, of early stage 4 embryos. Magnification 32X. (D) 18 dap dsc1-Mu2058/dsc1-Mu2058 embryo in stage 1 of development. Note the ovate-deltoid-shaped scutellum, sc; large suspensor, s; coleoptile, c; and shoot apex, sa. Magnification 44X. (E) At 20 dap, dsc1-Mu2058/dsc1-Mu2058 embryos progressed to an abnormal stage 2. Note the ovate-shaped scutellum, sc; expanding coleoptile, c; and small coleoptilar pore, cp. Magnification 48X. (F) At 25 dap, dsc1-Mu2058/dsc1-Mu2058 embryos are severely degraded as indicated by the shrinking scutellum, sc; and structural disintegration of the shoot apex, sa. Magnification 50X.
the scutellum and embryonic axis (Figure 4E). Dissections of *dsc1-Mu2058* mutant kernels at 28 dap were unsuccessful at identifying recognizable embryonic structure, and embryos are absent from mature mutant kernels (Figure 2B).

**Immature *dsc1-Mu2058* Mutant Embryos Germinate in Tissue Culture**

Immature embryos dissected from *dsc1-Mu2058/dsc1-Mu2058* homozygous kernels were placed on minimum essential media (MEM) to determine if the embryo-lethal phenotype could be rescued in tissue culture. Ten mutant embryos at late coleoptilar stage to early stage 1 of development were dissected from 15 dap mutant kernels and placed in culture. After seven days, one of the ten 15 dap *dsc1-Mu2058* mutant embryos exhibited a minimal response of plumule emergence (Figure 5A). No further shoot development was displayed by this cultured embryo, whereas wild type 15 dap embryos germinated precociously into plantlets under the same tissue culture conditions (data not shown).

Surprisingly, the majority of 20 dap, early stage 2 *dsc1-Mu2058* mutant embryos germinated precociously after 7 days in culture to form small plants with a single, slender leaf but no visible roots (Figure 5B). The solitary leaves of the *dsc1-Mu2058/dsc1-Mu2058* mutant plantlets achieved a maximum length of about 1 cm before dying after approximately 1 month in culture. No root development was detected in cultured *dsc1-Mu2058/dsc1-Mu2058* mutant plants. In contrast, wild type 20 dap embryos formed large, healthy plantlets with extensive roots and several normal leaves in tissue culture (Figure 5C).
Figure 5. Mutant dsc1-Mu2058/dsc1-Mu2058 embryos form rudimentary shoot structures in tissue culture. (A) 15 dap dsc1-Mu2058/dsc1-Mu2058 mutant embryo, e, after seven days in tissue culture. Despite the emergence of the plumule, no further shoot development was exhibited by this embryo culture. Magnification 31X. (B) 20 dap dsc1-Mu2058/dsc1-Mu2058 embryos, e; produced a single, small leaf but no roots after seven days in tissue culture. Plants died after approximately 30 days in tissue culture, and exhibited no root development. Magnification 11X. (C) Wild type embryos, e; placed in tissue culture at 15 dap germinated precociously and at seven days in culture formed seedlings with large leaves and normal root structure. Magnification 10X.
Identification and Cloning of a Mu1-Homologous Restriction Fragment Linked to dscl-Mu2058

The dscl-Mu2058 mutation arose in a Robertson's Mutator stock background and is therefore likely to be due to insertion of a Mutator transposable element in the Dsc1 locus. Mu1 is usually the most abundant Mu element in Mutator stocks, and has been found inserted into most of the mutated genes as yet cloned from Mutator lines (Chandler and Hardeman, 1993). The strategy used for identification of Mu-tagged genomic DNA linked to the dscl-Mu2058 mutation was to first outcross dscl-Mu2058/Dscl plants to standard lines for two generations (see Figure 1) in order to reduce the number of Mu1-homologous elements in these lines to less than 25 copies per genome, as estimated by DNA blot analysis using the Mu1 internal fragment M960 as probe. Next, DNA was prepared from both dscl-Mu2058/Dscl and Dscl/Dscl plants, individually digested with several restriction endonucleases, and analyzed by Southern blot hybridization (Southern, 1975) to identify a particular Mu1-tagged genomic DNA fragment that cosegregated with the dscl-Mu2058 mutant phenotype.

Throughout eight successive generations of analysis, a Mu1-containing EcoRI fragment of approximately 3.7 kb (Figure 6) and a BamHI fragment of approximately 2.1 kb (data not shown) were present in the genome of all 61 dscl-Mu2058/Dscl plants examined, but were absent in all 54 Dscl/Dscl sibling plants. To clone the 3.7 kb EcoRI fragment, a size-selected library was constructed from genomic DNA of a dscl-Mu2058/Dscl plant, in the phage lambda vector NM1149. This library was screened with the Mu1 homologous probe M960. Four Mu1-hybridizing plaques were isolated and purified, and approximate size determinations identified a single recombinant clone that contained a 3.7 kb EcoRI fragment of maize genomic DNA,
Figure 6. Identification of a $Mu1$-hybridizing fragment that co-segregates with $dsc1$-$Mu2058$. Genomic DNA obtained from sibling plants of the genotype $dsc1$-$Mu2058/Dsc1$ (lanes a-e) or $Dsc1/Dsc1$ (lanes f-j) was digested with EcoRI and analyzed by DNA gel blot hybridization using the M960 hybridization probe. The arrow indicates the 3.7 kb $Mu1$-homologous restriction fragment that co-segregated with the $dsc1$-$2058/Dsc1$ genotype.
which hybridized to the *Mu*1 probe. The maize DNA clone was subcloned into pBLUESCRIPT-SK+ to form pMS47. A partial restriction map of pMS47 is shown in Figure 7, which indicates those DNA restriction fragments that were used as hybridization probes and/or subcloned into pBLUESCRIPT-KS+ or pBLUESCRIPT-SK+ to form pMS59, pMS66, pMS67, and pMS68. Sequence analysis of the *Mu* element-host genomic DNA borders revealed that the nine base pair sequence GGTGGCAAC immediately preceding the *Mu* element insertion is directly repeated in host DNA at the opposite end of the *Mu* element. The sequences of the termini of the *Mu* element are identical to those of *Mu*1. Formation of direct repeats of 9 bp at the transposon’s termini is a characteristic consequence of *Mu*1 element insertion (Barker et al. 1984).

Two lines of evidence confirm that the cloned *Mu*1 element is indeed tightly linked to the *dsc1-Mu2058* mutation. First, restriction enzyme mapping of pMS47 revealed that the *Mu*1-homologous region of the clone is contained within an internal *BamHI* fragment of pMS47 that corresponds in molecular weight to the *Mu*1-hybridizing, 2.1 kb *BamHI* fragment tightly linked to the *dsc1-Mu2058* mutation. Both a 500 bp *EcoRI*/*BamHI* fragment, EB500, and a 1.2 kb *EcoRI*/*BamHI* fragment, EB1.2, are contained within the original 3.7 kb cloned fragment, outside the *Mu*1 element (Figure 7). These fragments were prepared from pMS47, radioactively labeled, and used as hybridization probes to identify homologous fragments in *EcoRI* digests of genomic DNA from maize plants in a population segregating for the *dsc1-Mu2058* mutation (Figure 8). The non-*Mutator* standard lines, hybrid Q60 and hybrid B70 (see Materials and Methods) were similarly characterized. The hybridization patterns revealed that a 3.7 kb fragment with homology to EB500 and EB1.2 was found in all *dsc1-Mu2058/Dsc1* plants and was
Figure 7. Restriction map of the cloned genomic fragment that co-segregates with \textit{dsc1-Mu2058}. A partial restriction map is shown of the 3.7 kb \textit{EcoRI} clone, detected by hybridization to the \textit{Mu} probe M960, that co-segregated with \textit{dsc1-Mu2058}. The position of the \textit{Mu} insertion is shown by the heavy line. Subcloned fragments and fragments used as hybridization probes are represented by dashed lines, and the arrow indicates the direction of transcription of fragment EB1.2. Restriction sites are denoted as follows: E, \textit{EcoRI}; S, \textit{SstI}; B, \textit{BamHI}; N, \textit{NotI}; and X, \textit{XhoI}.
Figure 8. Restriction endonuclease fragments linked to Dsc1. Genomic DNA was analyzed from Dsc1/Dsc1 plants (hybrid strain Q66/Q67) (lanes a), dsc1-Mu2058/Dsc1 plants (lanes b), and sibling Dsc1/Dsc1 plants (lanes c). (A) Probe EB500. (B) Probe EB1.2. Both hybridization probes flank the Mu element insertion in the genomic clone linked to dsc1-Mu2058. The arrows indicate the 3.7 kb EcoRI fragment linked to dsc1-Mu2058.
absent in all Dsc1/Dsc1 plants examined over eight generations. Moreover, these analyses showed that wild type siblings of dsc1-Mu2058/Dsc1 heterozygous plants, and the standard hybrids Q60 and B70 (data not shown) each contained a 2.3 kbp fragment with homology to EB1.2 and EB500. These results indicated the cloned Mu1-homologous fragment is indeed the 3.7 kb EcoRI fragment linked to the dsc1-Mu2058 mutation. The sizes of the genomic DNA fragments that hybridized to the dsc1-Mu2058-linked clone in wild type samples suggested that the 3.7 kb cloned EcoRI fragment was formed by the insertion of a 1.4 kb Mu1 transposon into the 2.3 kb EcoRI fragment identified by the dsc1-Mu2058-linked probes EB1.2 and EB500.

In addition to the 2.3 kb and 3.7 kb dsc1-Mu2058-linked EcoRI restriction fragments presented in Figure 8, these Southern blot analyses revealed several other genomic DNAs with homology to the EB500 and EB1.2 probes. In particular, both probes hybridized to EcoRI restriction fragments of approximately 5 kb and 12 kb; the EB1.2-homologous 5 kb fragment however, is present in significantly increased copy number in the genetic backgrounds used in this analysis. Neither the 12 kb fragment, the single copy 5 kb fragment, nor the highly repetitive 5 kb fragment co-segregated with the dsc1-Mu2058 mutant allele.

The Putative Dsc1 Gene is Transcribed in Early Stages of Kernel Development

The dsc1-Mu2058-linked DNA probes EB500, and EB1.2 (Figure 9) were used to detect homologous transcripts in polyadenylated RNA from seedling leaves and whole, wild type kernels at various times after pollination. Figure 9A (7, 20, L) shows probe EB1.2 detected a transcript of approximately 2.0 kb in RNA isolated from 7 dap kernel tissue, but not 20 dap kernels or 7 day-old maize seedlings. Probe fragment EB500 did not detect this or any other transcript (data not shown). Thus,
Figure 9. The Dscl-linked genomic fragment is transcribed specifically in kernels. Polyadenylated RNA was isolated from wild type maize kernels harvested at different days after pollination (dap), and from 7-day old seedling tissue. Approximately 3 μg of each RNA sample was analyzed by RNA gel blot hybridization. The Dscl-linked fragment EB1.2 was used as hybridization probe in (A) and (C). (B) Ethidium bromide stained gel of RNA samples used in (A), to show that each lane contained approximately equal amounts of RNA. (D) The blot used in panel C was stripped of bound radioactivity and hybridized to probe SacH2.4, derived from soybean actin cDNA, to show that each lane contained approximately equal amounts of intact RNA.
the $Mu1$ element insertion in the putative $dscl-Mu2058$ allele is within, or adjacent to a gene with homology to a mRNA transcript. The putative $Dsc1$ transcript is also detected in RNA isolated from kernels at 5 dap (Figure 10A) and 6 dap (Figure 9B); no 2.0 kb transcript was identified in 4 dap kernel RNA, or in RNA from kernels sampled later than 7 dap (9 dap, 11 dap, 13 dap kernel data not shown).

The direction of transcription of the EB1.2 probe was determined using single stranded hybridization probes. Specific strands of probe EB1.2 were radioactively labeled by enzymatic copying of single stranded phagemids. Only when the probe was formed by extension in the 5' to 3' direction from the $XhoI$ site towards the $BamHI$ site was the 2.5 kb kernel-specific transcript detected (Figure 9B). The gene is transcribed, therefore, from the $BamHI$ site towards the $XhoI$ site.

Maize Genomic DNA Linked to $dscl-Mu2058$ and Transcribed in Developing Kernels Shows Sequence Homology to Seed Storage Genes

The nucleotide sequence of approximately 300 bp of the EB1.2 probe, extending from the $BamHI$ site toward the $Xho1$ in pMS67 (see Figure 7) was determined. Comparison of the nucleotide sequence of this region of EB1.2 to that of the region approximately 1.7 kb upstream of the coding region of the 27 kD gamma-zein (Das et al. 1991) revealed over 76% homology in a 218 base pair overlap (Figure 11). No open reading frames were detected in the sequenced region of pMS67.
Figure 10. Determination of the direction of transcription of the \textit{Dsc1}-linked clone. Copies of the phagemids pMS66 and pMS67 were used as hybridization probes of polyadenylated RNA from wild type \textit{Dsc1/Dsc1} kernels at 6 days after pollination. The arrow indicates the putative \textit{Dsc1} transcript.
Figure 11. Comparison of the nucleotide sequences of EB1.2 and the upstream non-transcribed region of a maize endosperm storage protein. The nucleotide sequence of a 300 bp region of EB1.2, from the BamHI site extending toward the XhoI site, shows 76.1% identity in a 218 bp overlap to the region starting 1756 bp upstream of the transcriptional start site of the 27 kD maize gamma-zein gene. The alignment was generated by the computer program FASTA.
DISCUSSION

Maize kernels homozygous for the novel, recessive, defective kernel mutation \textit{dsc1-Mu2058} (4S:near \textit{bm3}) (Scanlon et al. 1993a) were inviable, showed incomplete and abortive endosperm and embryo development and necrosis of these structures at maturity. A genomic DNA linked to the \textit{dsc1-Mu2058} mutation has been cloned via transposon-tagging using Robertson's \textit{Mutator}. The following evidence supports the hypothesis that the cloned maize DNA is the actual \textit{Dsc1} locus. 1) The cloned region of the genome is tightly linked to the \textit{dsc1-Mu2058} mutation. 2) Expression of a mRNA with homology to the cloned, \textit{dsc1-Mu2058}-linked fragment occurs in maize kernels at early stages of development but not in seedling leaves. The time at which the this mRNA is expressed coincides with the time when abnormalities were first evident in developing \textit{dsc1-Mu2058/dsc1-Mu2058} kernels. 3) A portion of the genomic DNA is highly homologous in nucleotide sequence to sequences upstream of a gene known to be expressed in kernels, namely the 27 kD maize seed storage protein.

Many embryo-lethal defective kernel mutants are expected to result from mutation at general-function loci essential for cell maintenance, rather than genes with roles specific to the development of the maize kernel. Characterization of maize kernels homozygous for the \textit{dsc1-Mu2058} mutation showed that despite the necrotic endosperm and absence of embryonic structure found in mature mutant kernels, embryo and endosperm structural differentiation, albeit abnormal, did take place earlier in kernel development. In particular, the distinguishing discoloration and crumpling of \textit{dsc1-Mu2058/dsc1-Mu2058} endosperm did not occur until after 28 dap, and \textit{dsc1-Mu2058/dsc1-Mu2058} embryos reached an abnormal Abbe and Stein (Abbe and Stein, 1954) stage 2 of embryo morphogenesis before ultimately
disintegrating later in kernel development. Moreover, the putative Dsc1 gene transcript is not expressed in leaf tissues, but is expressed in immature maize kernels at 5, 6 and 7 dap. These results indicate that the Dsc1 gene product does not function in general cell maintenance but rather is essential for the correct implementation of early morphogenetic programs in the maize kernel.

When placed in culture on minimal essential medium, dsc1-Mu2058/dsc1-Mu2058 mutant embryos dissected at 20 dap formed tiny plants comprised of a single leaf and little or no root structure. After about one month in culture, the dsc1-Mu2058 homozygous plants died. These results did not indicate whether the dsc1-Mu2058 mutation affected shoot development directly, or if the sparse shoot development of cultured dsc1-Mu2058/dsc1-Mu2058 embryos was due to the poorly developed root system. Tobacco calli form mainly shoot structure in tissue culture where the ratio of the phytohormones cytokinin/IAA is high (Goodwin and Mercer, 1983). Possibly the dsc1-Mu2058 mutation disrupts the balance of plant hormones, or alters the ability of the plant to utilize or respond to them. The MEM utilized in our study contained no plant hormones, and future experiments should be directed to determine whether the response of 20 dap dsc1-Mu2058 mutant embryos in tissue culture can be improved by the addition of phytohormones to the culture media.

The best strategy to prove that a genomic clone is derived from a transposon-inserted gene is by comparative analysis of the wild type progenitor, mutant and revertant wild type alleles of the particular locus. This strategy is particularly feasible for many transposon-induced mutations because they are often unstable, and excision of the transposable element that caused the mutation can produce a revertant allele of wild type function. Unfortunately, because homozygous dsc1-Mu2058/dsc1-Mu2058 kernels are inviable, it is not possible to identify wild type
revertant kernel on self-pollinated ears of containing otherwise *dsc1-Mu2058/dsc1-Mu2058* mutant kernels. Therefore, reversion analyses are not feasible in this study. However, several independent lines of evidence indicate that we have cloned maize genomic DNA corresponding to the *Dsc1* gene.

Specific restriction fragment length polymorphisms detected by hybridization to the cloned maize DNA cosegregated with the *dsc1-Mu2058* mutant phenotype in all 115 plants examined over eight consecutive generations. Molecular weight determinations of the *Eco*RI restriction fragments hybridizing to maize DNA flanking the *Mu1* insertion in pMS47 showed that all *Dsc1/Dsc1* plants contain a 2.3 kb DNA whereas all *dsc1-Mu2058/Dsc1* heterozygotes contain an additional fragment with a 1.4 kb insertion, which corresponds to the molecular weight of *Mu1* (Barker et al. 1984). Furthermore, the EB1.2 fragment that flanks the *Mu1* element in pMS47 identifies a RNA transcript that is present in immature, whole maize kernels at 5, 6, and 7 days after pollination. The EB1.2-homologous transcript is not detected in RNA from seedlings, nor in earlier or later stages of kernel development. In addition, the expression of the putative *Dsc1* transcript at 5-7 days correlates well with the first appearance of discernibly mutant *dsc1-Mu2058/dsc1-Mu2058* kernels at 8 dap on segregating *Dsc1/dsc1-Mu2058* ears. Because probe EB500 did not hybridize to a mRNA and transcription of the EB1.2 homologous RNA was found to proceed from the *BamHI* site toward the *XhoI* site in EB1.2, the EB500 region of the *dsc*-linked clone is probably either an intron sequence, or alternatively, a region upstream of the transcriptional start site of the putative *Dsc1* gene.

Because the *Dsc1* gene is transcribed very early kernel development, the cloning of a larger fragment containing the entire *Dsc1* gene may permit the analysis and isolation of kernel-specific promoters. The *Dsc1* promoter sequence may prove
useful for targeting desirable genes for expression very early in maize kernel development.

Furthermore, nucleotide sequences contained in the *dsc1-Mu2058*-linked genomic clone that identified a RNA transcript in developing kernels are highly homologous to sequences found in the upstream non-coding region of a 27 kD zein locus of maize (Das et al. 1991). There are two major subclasses of zeins, alcohol-soluble prolamines that comprise 60% of the total protein in the maize endosperm (Scanlon et al. 1993; Heidecker and Messing, 1986; Messing, 1983). The *alpha*-zeins account for 45% of kernel proteins and are encoded by several multigene families, whereas the less-abundant gamma-zeins are encoded by low copy number genes. The 27 kD gamma zein locus is found in one copy in some maize inbred lines; other maize varieties contain two gene copies found on tandemly -linked, 12 kb repeats (Das and Messing, 1987). Clearly, the *dsc1-Mu2058*-linked EB1.2 fragment and the 27 kD zein gene are not alleles of the same locus, because *dsc1-Mu2058* is located on chromosome 4S (Scanlon et al. 1993) and the 27 kD zein is located on chromosome 7S (Burr et al. 1982). Southern blot analyses of maize DNA using the EB1.2 *dsc*-linked fragment as hybridization probe proved that sequences homologous to some portion of the EB1.2 probe are present in multiple copies in the genetic backgrounds used in this study (Figure 8). Although tandemly-linked copies of the 27 kD gamma zein gene are separated by a 2 kb spacer that is repeated approximately 50 times in the maize genome (Das and Messing, 1987), the region of homology between the 27 kD zein and EB1.2 is localized to a low copy sequence about 1.1 kb downstream from the repetitive spacer region of the 27 kD gamma zein genes (Das and Messing, 1987; Das et al. 1991). Since the direction of transcription of the EB1.2 fragment is
known (Figure 11), these data indicate that the repetitive sequence of the EB1.2 clone is located in a downstream region of the \textit{dscl-Mu2058}-linked clone.

Many maize kernel mutations that alter zein composition and accumulation produce the opaque phenotype, including \textit{fl1}, \textit{o1}, and \textit{o2} (Nelson et al. 1965). Thus, the \textit{dscl-Mu2058} mutation, which also causes opaque endosperm, may be involved in zein production. A two-dimensional electrophoretic analysis of \textit{dscl-Mu2058/dscl-Mu2058} kernel endosperm proteins may be useful in identifying alterations in the zein accumulation. Clones corresponding to representatives of each of the two \textit{alpha} zeins and the four gamma zeins have been obtained, (Kirihari et al. 1988; Prat et al. 1985; Geraghty et al. 1981; Pederson et al. 1986; Messing, 1983; Marks and Larkins, 1982) and only the 1.7 kb upstream region of the 27 kD gamma zein was homologous to the sequenced region of EB1.2. Many of the zein gene clones, however, do not contain sequences extending as far as 1.7 kb upstream of the coding region. Perhaps this region of shared homology between the 27 kD zein and EB1.2 is an uncharacterized seed specific promoter or enhancer region located upstream of the coding region of other zein genes. At least one locus encoding an \textit{alpha} zein polypeptide has been located in the vicinity of the \textit{Dsc1} locus on chromosome 4 (Wilson et al. 1989). Analysis of nucleotide sequences located further downstream in the EB1.2 clone may prove useful in characterization of the \textit{dscl-Mu2058}-linked clone.

In addition to the 2.3- and 3.7 kb \textit{dscl-Mu2058}-linked \textit{EcoRI} fragments, EB1.2 hybridizes to multiple \textit{EcoRI} restriction fragments in our genetic backgrounds. The specific type of genetic relationships between the \textit{dscl-linked} fragments and the 12- and 5 kb EB1.2-homologous fragments can not be determined at this time. Perhaps
the EB1.2 clone represents a member of, or shares sequence homology to, a multigene family in maize.
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PAPER 5. DNA SEQUENCE AND TRANSCRIPT ANALYSIS OF \textit{MuA2}, A REGULATOR OF \textit{Mutator} TRANSPOSABLE ELEMENT ACTIVITY IN MAIZE
DNA sequence and transcript analysis of transposon *MuA2*, a regulator of *Mutator* transposable element activity in maize

M. G. James, M. J. Scanlon, M. Qin, D. S. Robertson, and A. M. Myers

From the Department of Biochemistry and Biophysics, Iowa State University, Ames, IA (James, Scanlon and Myers); the Department of Zoology and Genetics, Iowa State University, Ames, IA (Robertson); and the USDA Plant Gene Expression Center, 800 Buchanan, Albany, CA (Qin).

Running title: Characterization of transposon *MuA2*
ABSTRACT

The 4942 base pair DNA sequence of *Zea mays* transposon *MuA2* was determined. Previous evidence indicated *MuA2* controls activity of the *Mu1* transposon located in the mutable allele *a1-mum2*. *MuA2* contains two large, ATG-initiated open reading frames (ORFs) of 612 and 232 codons, respectively, located on opposite strands. *MuA2* produces two transcripts, each containing one of these ORFs. Four different tandem direct repeat sequences are located downstream of the 612 codon ORF. The restriction map of *MuA2* is identical to that of transposon *MuR1*, which also is known to regulate mutability of *a1-mum2*. Furthermore, except for a single nucleotide *MuA2* is identical to the Mutator element *Mu9*. 
RESULTS AND DISCUSSION

This report characterizes MuA2, an autonomous transposon of the maize Mutator (Mu) family known to provide transposition function to an independent Mu element, Mu1, located within the a1-mum2 allele of the A1 gene. Robertson and Stinard (1989) identified a genetic element (termed here MuA2) controlling Mu1 transposition in specific maize lines in which mutable and stable a1-mum2 kernels were produced at a 1:1 ratio in crosses to a1 tester lines. Subsequent genetic analysis of several 1:1 stocks allowed mapping of this controlling element to specific chromosome arms, specifically the long arm of chromosome 2 and both the long and short arms of chromosome 4 (Robertson and Stinard 1992). Furthermore, this genetic analysis demonstrated MuA2 is a mobile locus and that the copy number of MuA2 can increase or decrease in one generation (Robertson and Stinard 1992). Using the same a1-mum2 stocks, other laboratories independently identified genetic loci controlling a1-mum2 mutability; these elements, termed Cy (Schnable and Peterson 1989), and MuR1 (Chomet et al. 1991) are likely to be the same genetic element as MuA2.

The same strain used to define MuA2 genetically was also used to identify and isolate a specific genomic DNA fragment that cosegregated with a1-mum2 mutability (Qin and Ellingboe 1990; Qin et al. 1991). Furthermore, an increase in the copy number of this genomic fragment correlated with an increased degree of mutability. Taken together, these data indicated the cloned fragment contains the MuA2 locus. Nucleotide sequence analysis identified a Mu element within this fragment based on its high degree of homology to other Mu elements in the regions of the terminal inverted repeats (Qin et al. 1991).
The complete 4942 base pair (bp) nucleotide sequence of MuA2 is presented in Figure 1 (EMBL sequence database accession number X62251). This analysis revealed two ATG-initiated open reading frames that could code for a polypeptide of more than 100 amino acids in length (Figure 2). The largest of these is a 612 codon ORF (ORF612, from nt 970 to nt 2805) shown by northern hybridization analysis to be transcribed into a mRNA of approximately 3 kb (denoted transcript "L") (Figs. 2,3). A second ATG-initiated ORF (ORF232, from nt 4477 to nt 3782) is transcribed on the opposite strand from ORF612 into a mRNA of approximately 900 bp (denoted transcript "S") (Figs. 2,3). Both transcripts were present specifically in plants grown from mutable kernels derived from a cross of a MuA2, a1-mum2 stock (90-91-9570-9/8570-5) to a tester stock homozygous for a1 (Figure 3). Transcripts L and S were identified previously by restriction fragments of MuA, a Mutator element homologous to MuA2 but not active as a controlling element (Qin and Ellingboe 1990). Neither ORF612 nor ORF232 specifies a polypeptide similar in amino acid sequence to any entry in the Protein Identification Resource or SwissProt databases. Several short ORFs initiated by ATG codons are located upstream of both ORF612 and ORF232 (Figure 1). RNA splicing, therefore, could extend the 5' ends of the translated sequences comprising either of these two open reading frames.

Two additional ORFs of 114 codons (ORF114, from nt 2804 to nt 3145) and 134 codons (ORF134, from nt 3146 to nt 3547) are located immediately downstream of ORF612 (Figure 1). Considering the large size of transcript L relative to ORF612, as well as the approximate borders of the transcript (Figure 2), it is likely that ORF134 and/or ORF114 are cotranscribed with ORF612. Neither ORF114 nor ORF134 contains an initiation codon; thus, their expression as protein would depend on RNA splicing or a translational mechanism that attached this coding information to
**Figure 1.** Nucleotide sequence of *MuA2*. The single-letter amino acid code of each ORF greater than 100 codons is shown above the first base of each codon. Stop codons at the end of each ORF are denoted by a triple asterisk. Inverted terminal repeat sequences are underlined with a double line. Tandem direct repeat sequences and the interspersed direct repeat are designated by number (with apostrophes indicating imperfect repeats) and are underlined with broken arrows. The single asterisk above nt 4784 indicates the only nucleotide difference between *MuA2* and *Mu9* (Hershberger et al. 1991). Nucleotide numbers are noted on the left of each line, and amino acid numbers of the indicated ORFs are noted on the right.
Figure 1 (continued). Nucleotide sequence of *MuA2*.
Figure 2. The 4942 nt sequence of MuA2 is represented by a solid line with restriction sites indicated for SacI (S), BamHI (B), XbaI (X), XhoI (O), StuI (T), EcoRI (E), and SalI (A). Open polygons show terminal inverted repeats, solid polygons show ORFs beginning with ATG initiation codons, and dotted polygons show ORFs lacking an ATG codon. Numbers under each ORF indicate codon length. Polygons point in the 5’ to 3’ direction of the element they represent. The expanded area comprises 392 nt; arrows represent tandem direct repeats. All elements are drawn to scale. The indicated hybridization probes used in northern analyses are identified by appropriate restriction enzyme letter codes followed by numbers indicating fragment nucleotide length. These were random-primer labeled and hybridized to total maize RNA. The ability of a particular fragment to identify either transcript "L" or transcript "S" is denoted by a "+", and lack of hybridization is denoted by a "-". Representative hybridization data is shown in Fig. 3 for probes H760, BT760, TE555 and ES630. Data for the remaining indicated probes is not shown.
Figure 3. Transcribed regions of *MuA2*. Total RNA isolated from seedlings of mutable or stable *a1-mum2* progeny in a cross showing 1:1 segregation for mutability was separated by electrophoresis in denaturing agarose gels. *MuA2* transcripts were detected using the indicated restriction fragments (see Figure 2) as random-primer labeled probes in northern hybridization analyses. In each case, transcripts were detected only in RNA from mutable plants. Molecular weight standards are the 0.24-9.5 Kb ladder from Gibco-BRL.
ORF612. An interesting feature of ORF134 is the clustering of four different tandem direct repeat (TDR) sequences comprising 55 nt (TDR1), 11 nt (TDR2), 27 nt (TDR3), and 21 nt (TDR4) (Figs. 1, 2). TDR1 is repeated three times, TDR2 is repeated four times, TDR3 is repeated four times, and TDR4 is repeated twice. These repeats are situated so that the 3' nt of ORF114 is the 5' end of the first repeated sequence, TDR1 (Figure 1). In addition, an interspersed direct repeat of 9 nt, termed IDR1, is present three times within a 100 bp region immediately downstream of ORF134 (Figure 1).

None of these repeated sequences are similar to the direct repeats found in Mul, Mul.7, or rq/Mu7.

Comparison of the nucleotide sequence of MuA2 with that of Mu9, a Mu element isolated from the bz2::mu9 allele (Hershberger et al. 1991), showed the two elements to be of exactly the same size and nearly identical in nucleotide sequence. The sole sequence difference is at nt 4784, where MuA2 contains a C and Mu9 contains a G (Figure 1). This difference is located in a terminal inverted repeat region outside of any recognizable ORF, and thus is not expected to affect the amino acid sequence of any MuA2 gene product. Although the autonomous nature of Mu9 thus far has not been demonstrated genetically, the sequence identity with MuA2 implies Mu9 also is capable of providing transposition function to Mu1. Similarly, comparison of the restriction maps of the regulatory elements MuA2 and MuR1, isolated from the same a1-mum2 stock, suggested these two elements are identical (Chomet et al. 1991; Qin et al. 1991). Furthermore, northern analyses of both MuR1 and Mu9 identified transcripts similar in size to those derived from MuA2 (Chomet et al. 1991; Hershberger et al. 1991). Taken together, the data suggest MuA2, MuR1, and Mu9 are nearly identical transposons capable of the autonomous regulation of Mu1. Whether or not these elements control transposition of Mu transposons other
than Mu1 remains to be discovered. However, because the family of Mu elements shares homology in regions of the terminal inverted repeats, it is predicted that the regulatory function of MuA2 will extend to the entire transposon family.
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SUMMARY AND GENERAL CONCLUSIONS

Maize kernel morphogenesis requires the coordinated expression of many genes directing the development of the embryo and endosperm components. In this study, the Robertson's Mutator transposable element system was used to generate over 75 independently-induced mutations affecting maize kernel development. These mutations are useful for genetic, developmental and molecular analysis of genes required for seed development. The majority of the mutations described in this study are dek-Mu mutations, putative Mutator-induced pleiotropic genetic lesions which disrupt both endosperm and embryo development.

Genetic mapping located 64 dek-Mu mutations to 17 of the 20 chromosome arms of maize, including at least fifteen mutations at previously undescribed loci. The dek-Mu mutants analyzed in this study were phenotypically diverse, and therefore probably represent mutations at loci involved in many different aspects of kernel development.

Developmental analyses of mutant kernels corresponding to the novel dek mutations emp2-Mu1047 and dsc1-Mu2058 revealed that although both these dek mutant kernels were germless at maturity and developmental aberrations were encountered very early in development, embryo death did not occur until much later. These data confirm the earlier reports of Sheridan and Neuffer (1980). In addition, these findings indicate that the Dsc1 and Emp2 gene products may be function in the implementation of developmental programs specific to the kernel rather than general cell maintenance. Kernels homozygous for general cell function or 'housekeeping' mutations are not predicted to be competent in tissue and organ differentiation.
The *dek* mutations are pleiotropic by definition, in that they disrupt both embryo and endosperm development. In addition, numerous other pleiotropic effects were identified in seedlings and plants grown from specific *dek-Mu* homozygous kernels. The alterations in leaf pigmentation associated with *dek-Mu* mutations causing aleurone disruption (Neuffer and Sheridan, 1980; Sheridan et al., 1986) were confirmed in this study. Numerous *dek-Mu* homozygous kernels with small, misshapened embryos, however, produced plants with gross developmental abnormalities in the leaves, stem, and flowers. Because the embryo is a tiny maize plant complete with root and shoot structures including 5-6 leaves (Kiesselbach, 1949), the embryo phenotypes of these mutant kernels and the morphological disruptions in plants obtained from them may result from the same defects in shoot development. Such mutants promise to provide more detailed information regarding the specific site(s) of action of the corresponding developmental loci. Those mutants that produced fertile ears and/or tassels are especially useful; homozygous mutants capable of transmitting the associated mutation are much easier to handle in genetic studies than inviable mutants.

Five allelic series of *dek-Mu* mutations were generated in this study that produced variable kernel and/or seedling phenotypes among class members. These allelic series, especially the novel series of ten *etl-Mu* mutations, are potentially useful in biochemical and molecular studies of the putative functional domains of the corresponding gene products.

The genetic analyses presented in this study confirm earlier observations regarding the abundance of defective kernel loci in maize (Jones, 1920; Mangelsdorf, 1923, 1926; Neuffer and Sheridan, 1980). In addition, transposon tagging with
*Mutator* was shown to be an excellent means of generating numerous mutations impacting maize kernel development.

The technique of molecular cloning via transposon-tagging was employed to isolate genomic fragments linked to four *dek-Mu* loci identified in this study, although all 75 *dek-Mu* mutations described in this study were analyzed by cosegregation analyses with *Mu1*-homologous probes for several generations. Molecular analyses were also performed using hybridization probes homologous to other *Mu* elements, including *Mu3* (Oishi and Freeling, 1987), *Mu7* (rCy) (Schnable et al., 1989), *Mu8* (Fleenor et al., 1990) and *MuA2* (Qin et al., 1991). One major obstacle confronted in these attempts at molecular cloning of *dek-Mu* mutations was the high *Mu* element copy number present in many *Mutator* families (Bennetzen, 1984). In many instances, eight generations of outcrossing to non-*Mutator* background were ineffective in reducing the number of *Mu1*-homologous fragments to below 25-30 copies.

This study illustrates the difficulty in establishing the identity of *Mu*-tagged clones linked to lethal mutations for which reversion analyses are not possible. In future attempts at cloning lethal mutations via transposon-tagging, efforts should be made to generate numerous independently-induced allelic mutations that would permit the identity of any putative clones to be assessed by the technique of allelic cross-referencing (O'Reily et al., 1985). The molecular analysis of the *dsc1-Mu2058*--linked clone demonstrated that the identity of clones homologous to multicopy genes is also difficult to establish.

The cloning and analysis of cDNAs corresponding to the putative *emp2-Mu1047* and *dsc1-Mu2058* clones will permit the eventual characterization of the biochemical functions of the genes in the complex process of kernel development.


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ACKNOWLEDGEMENTS

I would like to extend thanks to all the members of my advisory committee; Drs. Alan Myers, Donald Robertson, Michael Lee, Basil Nikolau and Robert Thornburg, for their professional guidance and input. I am grateful also to Dr. Patrick Schnable for the many insightful discussions and suggestions he volunteered regarding this work. Special thanks to my major professor Dr. D. S. Robertson, for introducing me to the maize plant and its genetics, and for the time and effort he generously donated even when times were difficult for him. To my major professor Dr. Alan Myers, I extend my gratitude for the insight, instruction, support, and encouragement you provided throughout my graduate career.

As the considerable professional contributions provided by my research collaborators Philip Stinard and Dr. Martha James are documented in this manuscript, I wish to extend personal thanks to both for their friendship and spirit of teamwork. I would also like to acknowledge the friendship and camaraderie of all the scientists of the Myers lab past and present; including Melissa Blacketer, Joni Johnson, Carla Koehler, Shu-hwa Lee, Julie Peterson, Sarah Coats, Diane Dieringer, Jeff Sturges, and fellow corn field workers, Tom Marsh, John Monnier, Kirsten McDaniel and Jennifer Wanat. Special thanks to Carla, for caring for my pets whenever I was out of town, helping to keep the lab cooler stocked with diet-Coke, and her shared-activism regarding campus artwork!

Ongoing thanks to Don and Charlene Schumann for their continued friendship and encouragement. I doubt this goal would have been realized without their kind support all those years ago.
Finally, I would like to express my sincerest gratitude to my parents, Laura and Paul Scanlon, for their assistance and guidance throughout the expanse of my scholastic endeavors. Their lives are a testament to the value of hard work, sacrifice, and perseverance.