Radiation-induced *alcohol dehydrogenase* mutants in maize following allyl alcohol selection of pollen

BY MICHAEL FREELING AND DAVID S. K. CHENG

*Genetics Department, University of California, Berkeley,*

*Berkeley, California 94720*

*(Received 15 August 1977)*

**SUMMARY**

The *alcohol dehydrogenase-1* gene in maize presents advantages for mutational analysis. Foremost among these is the ability to chemically select ADH-negative and ADH-low gametophytes owing to their resistance to allyl alcohol vapour. Immature tassels were irradiated with either 220 kV X-rays or 400 MeV/amu accelerated neon-ions; spontaneous mutants were also selected and recovered. RBE for neon-20 was about 5. A total of 70 presumptive mutants were placed into one of four classes on the basis of allozyme profiles following electrophoresis and ADH staining: (A) dysfunction, (B) underproducer, (C) overproducer, and (D) wp-Adh2 gene. Mutants have been recovered and confirmed in the first three classes. These include two male-transmissible deletion-type lesions induced by X-rays, five underproducer transpositions and one overproducer transposition induced by neon-20. Certain of the neon-induced alleles are unstable in their expression. All 70 mutants are chromosomal aberrations; no intragenic lesions were recovered although our experimental design would have preferentially recovered them if they had occurred.

The Discussion considers the mutagenic action of ionizing radiation, and especially the well-documented differences between maize and Drosophila data. In particular, the effect of these chromosome derangements on the ‘programmable’ component(s) of the *Adh1* cistron is discussed.

1. INTRODUCTION

The nature of the gene in higher organisms is not understood. The phenomena of determination (or presetting), competence and somatic heredity must surely have correlates at the level of the specific gene. We call this correlate a ‘programmable’ cis-acting gene component(s) whose function is to affect transcription of its adjacent structural gene component(s). A ‘program’ mutant would alter the rate, timing, balance, cell specificity or gene circuitry of transcription without altering the primary structure of the message. *Induced* mutants affecting programmable components of genes or altering cell-specific gene circuits are very rare (possible examples: McClintock (1951) for gene instabilities generated by breaks in chromosomes; Mottinger (1973) for unstable *Bronze-1*; and cf. Baker, 1968 review of position effect variegation in Drosophila). All of the above cases appear to involve...
chromosomal breakpoints near a target gene, and none have proven biochemically accessible. Naturally occurring variants have proven more encouraging. Several presumptive 'programming' variants of specific genes have been identified (examples include: Schwartz (1962) for maize esterases and Schwartz (1971) for alcohol dehydrogenase-1; Swank, Paigen & Ganschow (1973) for murine β-glucuronidase; Dickinson (1975) for Drosophila aldehyde oxidase; Freeling (1975) for both maize alcohol dehydrogenase genes; Chovnick et al. (1976) for Drosophila rosy; cf. Brink, Styles & Axtell (1968) for maize R; and cf. McClintock (1956) for transposable elements). Unfortunately, naturally occurring variants are especially difficult to interpret because their evolutionary origins are obscure. The overall aim of these initial studies is to artificially induce and recover mutants in 'programmable' components of the alcohol dehydrogenase-1 (Adh1; ADH enzyme, EC 1.1.1.1.) cistron in maize.

Text-fig. 1. Chemical selection, recovery and classification of Adh1-mutants. The paternal or target Adh1 allele Adh1-S, is abbreviated 1', etc. The electrophoretograms depict semi-quantitative, banding patterns and intensities after native starch gel electrophoresis. O denotes origin; + denotes the anode. The ADH activities reflected in these diagrams are from the wild-type F1 (wt), Adh1-negative heterozygote (A) and Adh1-underproducer heterozygote (B) extracts obtained from F1 scutellar slivers. The subunit composition(s) of each band is indicated (Freeling & Schwartz, 1973) where subunit 1⁰ is specified by Adh1-S, etc. The dashed and dotted bands in the marker isozyme region of gel denote low-activity and usually-not-present, respectively.
The Adh1 complementation group (i.e. cistron) in maize offers many advantages for mutational analysis. First, ADH catalyses dispensable functions under aerobic conditions (Schwartz, 1969). Secondly, much is known about the maize ADH allozymes and isozymes themselves and the rules underlying their organ-specific expression (Schwartz, 1966, 1971; Freeling, 1975). Thirdly, our laboratory has developed recently an in situ ADH staining procedure for maize pollen permitting quantification of intragenic recombination and reversion frequencies below 10^{-7} (Freeling, 1976). Fourthly, recent results (Schwartz & Osterman, 1976; Cheng & Freeling, 1976) demonstrated that allyl alcohol (C = C − C − OH) selectively kills ADH^+ gametophytes allowing for preferential fertilization by rare, mutant ADH^- pollen. For these reasons, maize Adh1 is among the few specific genes in higher organisms which bring together high genetic resolution, a mutation selection scheme and biochemical accessibility. Text-fig. 1 diagrams the first steps in Adh mutant induction, selection, recovery and confirmation by allozyme analyses. Table 1 lists further tests to which each single mutant heterozygote (F1) is subjected by way of preliminary characterization.

Table 1. Eight steps for initial mutant characterizations

| I. | F1 scutellar allozyme/isozyme profiles and check for paternal marker. (Class A, B, C, or D.) |
| II. | Viability of F1 seed. |
| III. | F1 plant morphology and behaviour. |
| IV. | F1 chromosomal counts from root tips. |
| V. | Allozyme profile from pollen shed from F1 plants (F2 gametophytic population). Especially check for hybrid allozyme indicating hyperploidy for Adh1 region of 1L. |
| VI. | Pattern of pollen abortion and ADH staining (F2 gametophytes). |
| VII. | Transmissibility of mutant allele through male and/or female from allozyme analyses of F2 or backcross progeny scutella. |
| VIII. | Instability or variegation after transmission? |

Intuitively, accelerated heavy ions (high Z) should generate a unique class of multi-hit, relatively small (0-1-100 kilobases) chromosomal aberrations with single-hit kinetics. Given idealized conditions, Chatterjee et al. (1971) and Christensen, Tobias & Taylor (1972) have used LET, energy per amu and the angular dependence of secondary electrons (δ rays) to estimate the length of such single-hit deletions. What actually occurs within the cell depends on repair/degradation processes, the shape of the gene and the like. We employ neon-20 accelerated to 400 MeV/amu at the Bevatron, Lawrence Berkeley Laboratory, as well as X-rays as our ionizing radiations.

Previous attempts to generate intragenic forward mutants in maize and barley using ionizing radiation have failed (Stadler, 1928; Stadler & Roman, 1948; Amano, 1968, and especially results and further citations of Mottinger, 1970). Possible exceptions to this generality are some of the C-series waxy mutants (cf. Nelson, 1968), and our Adh1-FunkFγ25 (unpublished ‘point’ mutant), both of which were recovered following gamma irradiation of maize seeds. Other organisms do not give this result (Muller, 1955 for review). Certain mutants in Drosophila...
melanogaster revert with X-rays (Patterson & Muller, 1930; cf. Green, 1961) although the nature of the original mutants (inserts?) remain unknown. The genetic fine structural analysis of XDH structural gene component of rosy by Chovnick and co-workers (Gelbart et al. 1973; see Chovnick et al. 1976) indicated clearly that X-ray induced mutants often behave recombinationally as points, but not necessarily base-substitutions. In microbes, ionizing radiation may induce intragenic lesions including base-substitutions as well as breakage-type lesions (e.g. Bridges, Dennis & Munson, 1967 in E. coli; Weber & deSerres, 1965; Mailing & de Serres, 1967 in Neurospora). As a partial explanation for the discrepancy in data between plants and all other tested organisms, we will advance the notion that two-strand breaks may be ripped-out by exonucleases before reunion. Such chromosomes would not transmit through the gametophytes. We call this suggestion ‘chromosome suicide’.

Our system has been designed carefully in order to diminish the preponderance of gross chromosomal aberrations recovered by previous maize geneticists. (1) Pollen mother cells were irradiated prior to the first microspore mitosis (however, tassels contain some germ cells at all stages from premeiotic to mature pollen: a distinct disadvantage of our system). (2) A fertile pollen grain must be ADH− in tube nucleus in order to survive allyl alcohol vapour selection, and must also transmit an Adh-altered gene in order to be identified as a mutant in the F1 allozyme screen. (3) An electrophoretically detectable marker, Adh2-P on chromosome 4 was used to rule out contamination and gynogenetic haploids. Given these three considerations, we should have filtered out most macrolesions and chromosomal derangements because they abort at the male gametophyte, or pass through with difficulty. However, a reciprocal translocation resulting from the union of clean breaks, one of which bisects Adh1, should pass through our screen via alternate segregation and be recovered.

There are two unlinked Adh genes in maize (Schwartz, 1966; Freeling & Schwartz, 1973): Adh1 (within 2 mu from Kn and Lw on chromosome 1L; Schwartz, unpublished) and Adh2 (on chromosome 4S; 20 mu from Su1; Dlouhy and Freeling, unpublished collaboration). In total, there are three isoforms of ADH: Set I (ADH1·ADH1 dimer), Set II (ADH1·ADH2 heterodimer) and Set III (ADH2·ADH2 dimer). The scutellum of the quiescent embryo and mature pollen expresses ADH1 subunits in great preponderance. Figure 1 includes subunit compositions of our F1 allozyme/isozyme profile.

This report gives our first data on mutant recovery via pollen selection, and must necessarily cover our methods and their controls in detail. Initial characterization of our first 69 radiation-induced Adh lesions is presented, along with cursory information on three spontaneous point mutants. Relative biological effectiveness (RBE) for neon-ion compared to X-rays is estimated. Among these mutants, and particularly among the neon-induced underproducer class, we have evidence for alterations of Adh1 programming and stability of expression. None of our 69 radiation-induced mutants are confined to the structural gene component of the Adh1 cistron.
2. METHODS AND MATERIALS

(i) Inbred lines, Adh1 alleles and nomenclature

The single cross hybrid, *Funk-G4343*, generously supplied by Funk Seeds International, was used consistently as the detasselled, female tester. This vigorous line carries *Adh1* alleles which specify ADH1 subunits conferring a relatively fast electrophoretic migration rate to dimers containing these subunits. These alleles are called *Adh1-F*. At one point, the allele designation *Adh1-F(Std)* is used; this ‘fast’ allele is not the same as the *Adh1-F* alleles in *Funk-G4343*. The mutational target alleles were *Adh1-S*, *Adh2-P* in a line inbred for four generations. The *Adh1-S* allele specifies an electrophoretically slower product than *Adh1-F*, and *Adh2-P* specifies an electrophoretically slower product than the wild-type *Adh2-N* (Freeling & Schwartz, 1973). In one section of results, the naturally occurring *Adh1* alleles *Adh1-C* and *Adh1-FCm* are used. These alleles have been described previously (Schwartz, 1966).

The nomenclature we use is changed slightly from that used by Schwartz. For example, one of our underproducer mutants is designated *Adh1-S1945*. The ‘*Adh1*’ denotes the gene involved, the ‘*S*’ indicates the wild-type mutant progenitor allele and ‘1945’ is the arbitrarily chosen family number of the F₁ presumptive mutant seed. This allele would be abbreviated 1945. No component of this revised nomenclature reflects the type of mutational lesion or the mutagen. This nomenclature differs from that used by Schwartz in one important respect: the arabic allelic designation is used by Schwartz to denote the electrophoretic behaviour of the mutant’s product rather than the product of the progenitor allele of the mutant.

(ii) Treatment with X-rays and accelerated neon, and dosimetry

Plants to be irradiated were grown in 10 in. pots. When the immature tassel was at or just before the stage usually chosen for collecting germ cells in pachytene – the ‘sporocyting’ stage – the potted plant was moved to the radiation source. An immature maize tassel at the stage of irradiation is not composed of a single type of germ cell; premeiotic, meiotic and, in some cases, postmeiotic gametophytic stages were present as mutational targets.

The X-ray source was a Philips 250 kV. We used a 220 kV, 15 mA beam with 0.25 mm Cu and 1.0 mm Al filters. A 20 cm length of stem was irradiated, including the entire immature tassel. The typical dose rate was about 58 rads/min as measured by a Victoreen Condensor 250 kV R meter after the beam had passed through the plant.

400 MeV/amu neon 20 was accelerated at the Bevatron, Lawrence Berkeley Laboratory, on 1 September 1976. Dose rate and beam level is constantly monitored using an on-line parallel-plate ionization chamber. Our target area was a 7 cm diameter circle; certain immature tassels were too large, and the tip of the main tassel stalk was not irradiated. The Bragg peak was placed just past the
target such that plateau LET values prevailed. Mean dose rates were about 50 rads/min.

(iii) Pollen collection, storage, allyl alcohol treatment and the test-cross

After mutagenic treatment, potted plants were grown outdoors during the summer months. Shortly before anthesis, plants were transported to a greenhouse isolated three miles away from any Adh1-deficient pollen; contamination by mutants already in our collection was eliminated. On days 2 and 3 of shedding, the heaviest days, Lawson 400 pollination bags were closed at 9 a.m. and opened again between 10 and 12 noon for pollen collection; $5 \times 10^5$ grains is a reasonable sample since these plants have relatively small tassels. Using Millipore pollen counting techniques (Freeling, 1976), 1 mg of Adh1-S pollen was found to reflect about $2 \times 10^3$ grains. Collected pollen was separated from anthers using a folded glassine bag and stored at 4 °C for 0.5–4 h. Control experiments (D.S.K.C., unpublished) show that this cold treatment increases germination in vitro by about 10%. After cold treatment, pollen was dispensed in about 500 mg lots ($10^6$ grains) onto glassine paper for allyl alcohol vapour treatment. A 500 ml Mason jar with sealed lid containing 20 ml granular CaSO$_4$ desiccant was used; unless specified, we used between 1 and 6 $\mu$l allyl alcohol/500 ml chamber for a total treatment time of 20–30 min; 1 $\mu$l allyl alcohol vapour/500 ml equals 75 $\mu$m. After treatment, the seal is broken, the jar is cooled in an ice bucket and transported to the field of detasselled plants (Adh1-F, Adh2-N) whose silks had been cut back the day before. Tester ears were pollinated with about $5-8 \times 10^4$ pollen grains/ear using a camel hair brush; there are about 400 silks, each leading to an ovum. Each ear was then covered until harvest about 2 months later.

(iv) David’s Bread Loaf

The in vitro pollen germination medium and conditions developed by Cook & Walden (1965) have been revised. Our overall procedure is called ‘David’s Bread Loaf’ because the most important revision involves gametophyte germination on a newly cut solid agar surface. The medium is 17% w/v sucrose, 300 mg/l CaCl$_2\cdot$H$_2$O, 100 mg/l H$_3$BO$_3$ and 0.7% w/v Difco Bactoagar, adjusted to pH 6.4 after the addition of agar but before heating to 120 °C for 8 min. The medium was solidified in a Griffin beaker of the desired diameter and stored at 4 °C until use. Slices, 2 mm thick, were placed onto opaque grids; pollen was dusted onto the newly cut surface using a camel’s hair brush. Consistent percentage germinations of 75–95% are obtained, and there is little or no density dependence and minimal differences in the performance of pollen from various inbred and exotic lines (unpublished).

After germination, gametophytes may be cytochemically stained for the presence of ADH enzyme activity. These methods are detailed in Freeling (1977).
Electrophoretic analyses of ADHs in scutellum and pollen

The scutellum is a diploid nutritive organ which encloses the embryonic axis: the scutellum plus axis comprise the 'embryo'. The removal of a small (4 x 4 x 1 mm) sliver of scutellum does not impede germination of the seed but does allow for electrophoretic analyses of ADH enzyme constitution. Slivers were macerated in 100 µl of 50 mM Trisma-HCl, pH 8.0 containing 10 mM dithiothreitol (Calbiochem). If many seeds were involved in a genotype, their order was maintained by writing numbers directly on the seed and by taping seeds to file cards. The extract was absorbed into Whatman No. 3 filter paper rectangles and subjected to electrophoresis in starch gel according to the methods of Schwartz & Endo (1966); cutting and staining of gels specifically for ADH enzyme activity was according to the modifications of Freeling (1974).

Pollen was extracted and analysed as above, with the following exceptions. A mortar and pestle were used to grind the pollen in extraction buffer, and a small piece of Miracloth (Calbiochem) intervened between the slurry and the filter paper rectangles; the latter procedure presumably removes some lipids. All procedures were conducted at 4 °C using precooled equipment.

Our choice of native starch gel electrophoresis over any of various polyacrylamide gel procedures was made after careful comparisons. Even after pre-running with running buffer (Tris-glycine, Tris/citrate, or borate), native slabs of polyacrylamide separating gel are not as sensitive to ADH activity as starch gel (NBT_red band intensity X time^-1 X ADH units loaded^-1). The polyacrylamide system leads to the inactivation of various mutant-specified allozymes which remain active under our conditions of starch gel electrophoresis (M. Sachs, this laboratory). In addition, we routinely analyse 288 scutellar slivers in one 8 h man-day using a total of 1 l. of gelled starch matrix. Polyacrylamide, while offering superior resolution of protein bands, is inferior to starch in staining sensitivity, gentleness to active ADH enzymes and application to large numbers of samples.

Chromosomal counts from root tips

Plants to be assessed for mitotic chromosome number were grown in Jiffy Pots until several roots pierced the peat bottom. About five root tips (1 cm) were removed, covered with about 2 cm of 0.2% (w/v) 8-hydroxyquinoline for 3 h at 18 °C or overnight at 6 °C. Roots were then fixed for over 0.5 h in Carnoy's, hydrolysed, and stained by Feulgin procedures (basic fuchsin from Gurr, lot No. 22397). At least three root tips were prepared, and at least five unambiguous cells per root were counted. Our data are presented as a modal chromosome number unless dramatic deviations from the mode occurred. It should be noted that root tip ploidy may sometimes not reflect the ploidy of the apical meristem, especially when chromosomally unstable situations were generated via radiation-induced derangements.
(vii) **Plant morphology and vigour**

Whole plant morphology was photographically recorded on 35 mm plus X film after 3 weeks of growth in Jiffy Pots just after transplantation to 10 in. pots. These photographs show early growth rate, leaf shape, symmetry of leaf blades, yellow/white striping, blotching, tillering and the like. Careful observations were recorded in writing at the time of flowering, especially concerning sterility, haploid syndrome (Chase, 1952) and timing of male and female flower maturity.

(viii) **Transmission of Adh-mutants through pollen and egg**

A few milligrams of pollen were collected as shed from mutant F₁ plants, dispersed onto the surface of isotonic phosphate buffer, frozen, thawed and stained for the presence or absence of ADH enzyme activity as described previously (Freeling, 1976). We made two important modifications: (1) the surface of the ice block was not washed because aborted (shrivelled, hollow) pollen grains tend to float, and (2) the dialysate was not decanted since aborted grains would be preferentially removed. Instead, the dialysate was fortified with an equal volume of 2× concentrated stain. After 2–3 h of staining at 25 °C, at least four types of gametophytes resulted: plump and blue (wild-type, ADH⁺), plump and yellow, shrivelled and blue (aborted, ADH⁺) and shrivelled and yellow. For example, a pollen sample which was 50% aborted, but of the plump grains, 50% were ADH⁺ and 50% were ADH⁻, would fit the interpretation that the mutant F₁ was a reciprocal translocation heterozygote where one of the breakpoints inactivated Adh₁-S function. Clearly, allozyme analysis of pollen adds much to these sorts of preliminary interpretations. In reality, many more than these four classes of pollen often occur, including huge grains, gradations in ADH activity content and small rather than aborted grains.

F₁ mutant plants were self-pollinated when possible, and often backcrossed to one parental line (Funk-G4363 containing an Adh₁-F and Adh₂-N alleles) or the other (Standard S, containing Adh₁-S allele with or without the Adh₂-P allele). The resulting seeds were genotyped using scutellar slivers and electrophoretic procedures. In addition, F₁ ears which did develop were assessed for seed abortion.

3. **RESULTS AND CONCLUSIONS**

(i) **Controls on the reliability of allyl alcohol selection and pollen viability**

Pollen was collected as described from an Adh₁-deficient mutant heterozygote – Adh₁-(Std)F/Adh₁-(Funk)Fγ25 where γ25 is a CRM⁻ intragenic mutant. This pollen was cold-treated, subjected to allyl alcohol treatment, germinated on fresh slices of David’s Bread Loaf solid medium and then stained for the presence of ADH; these methods have been detailed. There are four possible phenotypes: blue grain with tube, blue grain without tube, yellow grain with tube, and yellow grain without tube. In the absence of chemical treatment, pollen viability is about 80% with half of the gametophytes staining ADH⁺ (blue) and half ADH⁻. Plate I shows...
Mutants from pollen

Mutants from pollen

this result. Allyl aclohol preferentially kills ADH+ gametophytes, presumably because the relatively innocuous alcohol is oxidized to deadly acrolien in a reaction catalysed by ADH1 activity (Megnet, 1967). Table 2 shows a typical set of comparative kill data for sibling ADH+ and ADH− gametophytes assayed after germination in vitro. It is clear that concentrations of allyl alcohol and treatment durations exist which should permit mutant selection with few or no ADH+ escapers.

Table 2. Allyl alcohol selection of ADH− pollen quantified using germination and cytochemical staining

<table>
<thead>
<tr>
<th>Allyl alcohol (mM)</th>
<th>Mean relative percentage pollen germination*</th>
<th>ADH+ (blue)</th>
<th>ADH− (yellow)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 (78.5%)</td>
<td>100 (80.7%)</td>
<td></td>
</tr>
<tr>
<td>0.07</td>
<td>40</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>9</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>0.29</td>
<td>Zero</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>0.74</td>
<td>Zero</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>1.47</td>
<td>Zero</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>2.21</td>
<td>Zero</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>2.94</td>
<td>Zero</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>3.68</td>
<td>Zero</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The results of four independent experiments are averaged for each data point; mean absolute control was 79%, as indicated parenthetically; allyl alcohol was diluted with methanol such that 50 μl of foreign vapour were present; treated for 40 min. Otherwise, as detailed in Methods and Materials.

According to the data in Table 2, treatment under these conditions with about 0.3 mM allyl alcohol vapour should let just a few ADH+ gametophytes germinate. Does a positive in vitro germination assay indicate ability to fertilize an ovum successfully? In order to approximate an answer to this question, progeny tests were done on a pollen sample divided into three Mason jars and treated with 0.3, 0.22, and 0.0 mM allyl alcohol, respectively. Pollen treatment conditions are given under Table 2. After treatment, the pollen was brushed onto silks which had been cut back the previous day. The female testers were homozygous for an electrophoretically unique Adh1 allele so that test-crossed seeds resulting from fertilization by Adh1+ or Adh1− pollen could be distinguished easily from scutellar allozyme profiles. All seed sets were 99–100%. The 0.3 mM treatment gave zero Adh1+ alleles transmitted/142 tries, 0.22 mM gave 3 transmissions/65 and the control was 42/80; the latter fraction fits the Mendelian expectation. We conclude that 0.22 mM allyl alcohol vapour is just borderline-selective both in the in vitro germination test and in progeny tests. However, the small sample sizes underlying these data can only give an approximate direct correlation between ability to germinate and ability to fertilize an ovum.
(ii) *Spontaneous mutant recovery: a control*

The best test of any mutant recovery system is its ability to deliver heritable mutants. Spontaneous forward mutant frequency in $ADH^+ \rightarrow ADH^-$/viable gametophyte for the $Adh1-S$ and $Adh1-F$ (Standard) isoalleles is below $2 \times 10^{-7}$ (Freeling, 1977). It was therefore not surprising that we recovered only one spontaneous $Adh1-S$ mutant out of an estimated $2.6 \times 10^7$ viable pollen grains (Table 3, column 1). This mutant, 1561, will be described further in subsequent sections. Other naturally occurring $Adh1$ isoalleles -- $Adh1-C^t$ and $Adh-FC^m$ -- have yielded spontaneous mutants. Two independent $Adh1$-deficient alleles have been recovered and confirmed out of a total of $1.4 \times 10^6$ viable pollen grains; both may be intragenic lesions because transmission through pollen is not affected (mutants $Adh1-C^t1522$ and $Adh1-FC^m1543$; unpublished). Our methods work.

(iii) *Four classes of presumptive Adh1-S or Adh-system mutants identified by aberrant $F_1$ scutellar allozyme profiles*

With the exceptions of the spontaneous mutants mentioned briefly in the preceding Results and Conclusions section, the $Adh1-S$ allele was the mutational target. $Adh1-S$ specifies polypeptide subunits which migrate to the anode (+) relatively slowly during native gel electrophoresis. Figure 1 diagrammed the standard test-cross $Adh1-F/Adh1-F \times Adh1-S/Adh1-S$ (3). Since maize ADHs are dimers, the routine $F_1$ allozyme profile contains three bands of enzyme activity: $1S-1S$, $1S-1F$ and $1F-1F$ using the subunit abbreviations of Fig. 1. In addition, the male (target) plants carried an electrophoretically distinguishable $Adh2$ allele, $Adh2-P$, which marks chromosome 4 (see Fig. 1 and Methods and Materials).

Plants to be used as males were irradiated with X-rays or accelerated neon-20 at the sporocyte stage, as described in Methods and Materials. Pollen shed from these plants were tested for abortion, ability to germinate in vitro, treated with just enough allyl alcohol to kill not quite all $ADH^+$ gametophytes (74–296 $\mu$m for 20–30 min) and then crossed onto tester ears at a mean of 57800 pollen grains per ear. Borderline concentrations of allyl alcohol were used in hopes of recovering mutants via pollen with lowered levels of ADH but not total absence. The infrequent $F_1$ seeds were removed from the ear and subjected to a scutellar biopsy which in no way affected their ability to germinate, and were stored for future use. The scutellar slivers were extracted, and their pattern of ADH allozymes and isozymes were assessed, as described, using starch gel electrophoresis. Our choice of starch over polyacrylamide gel for this particular assay is deliberate and is discussed in Methods and Materials. Plate II shows our nonmutant profile and our classes of aberrant profiles.

Each small slab of starch shown in Plate II analyses three profiles: two non-mutant (marked 'wt') and one presumptive mutant (A, B, C or D). These classes are (A) $Adh1-S$ dysfunction, (B) underproducer, which is balanced toward $Adh1-F$ product, (C) overproducer, which is balanced toward $Adh1-S$ product and (D) up-$Adh2$, where the $Adh2$ gene is switched on in scutellum. At least super-
Pollen shed from an Adh1+/Adh1− plant after in vitro germination and in situ staining for ADH activity. The dark (ADH+) grains are actually blue and opaque; the light (ADH−) grains are actually yellow and translucent. Our kill-curves were obtained by quantifying deviations from controls such as this.
Four classes of Adh mutants detected in F1 seeds by abnormal allozyme ratios. Each electrophoretogram analyses three extracts, each reflecting a single scutellar sliver. The nonmutant profile is designated 'wt'. Class A is a total Adh1-S dysfunction; Class B is an allozyme balance skewed toward Adh1-F product; Class C is an allozyme ratio skewed toward Adh1-S product; and Class D includes apparent elevation of Adh2 gene products. The arrow marks the fuzzy ADH2. ADH2 isozyme, which is actually composed of three bands (see Text-fig. 1).

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Officially, Classes B, C, and D fit expectations for regulatory mutants not involving the Adh1-S structural gene and Classes C and D are difficult to explain in any other way. The weakly staining bands which move anodally are the result of intergenic hybrid dimers between Adh1 and Adh2 gene product; the subunit composition for each band is diagrammed in Fig. 1. Our point in identifying these faint isozymes is that all of our presumptive mutants carried the chromosome 4 marker, Adh2-P; therefore, we were not misled by gynogenetic haploids or contaminants, although they did occur infrequently (unpublished).

In the absence of our molecular level assay system, where allelic expression could be easily compared and quantified, the most interesting Classes B, C and D would have gone unnoticed.

(iv) Recovery and RBE data

Table 3 condenses our F1 recovery data for the first 69 Adh mutants arising after treatment with ionizing radiation as compared with the single spontaneous mutant. Note that we are measuring recovery frequency and not mutant frequency or mutation rate. The overall relative biological effectiveness (RBE) of 400 MeV/amu plateau Ne10+ to 220 kV X-rays (in mutants recovered × viable gametophyte−1 × rad−1) is about 5. This RBE is a mere approximation since there is much variation in the exact premeiotic, meiotic or postmeiotic stage when the target germ cell was

Table 3. Presumptive Adh mutant recovery data with neon-20 RBE to X-ray approximations

<table>
<thead>
<tr>
<th>Mutagen:</th>
<th>None</th>
<th>X-ray</th>
<th>Ne10+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean dose in rads at c. 50/rads/min., weighted†</td>
<td></td>
<td>327</td>
<td>307</td>
</tr>
<tr>
<td>A. Dysfunction</td>
<td>0</td>
<td>13</td>
<td>35</td>
</tr>
<tr>
<td>B. Underproducer</td>
<td>1</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>C. Overproducer</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>D. Up-Adh2</td>
<td>0</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Total No. A + B + C + D</td>
<td>1</td>
<td>18</td>
<td>51</td>
</tr>
<tr>
<td>% ears with at least one seed*</td>
<td>17.2</td>
<td>28.9</td>
<td>31.1</td>
</tr>
<tr>
<td>No. ears with seeds analysed electrophoretically</td>
<td>153</td>
<td>92</td>
<td>33</td>
</tr>
<tr>
<td>Total no. of ears analysed</td>
<td>890</td>
<td>318</td>
<td>106</td>
</tr>
<tr>
<td>Est. no. pollen grains at 5-8 × 10^4/ear (× 10^6)</td>
<td>51.6</td>
<td>18.4</td>
<td>6.1</td>
</tr>
<tr>
<td>Est. % pollen viability‡</td>
<td>50</td>
<td>45</td>
<td>60</td>
</tr>
<tr>
<td>No. viable pollen (× 10^6)</td>
<td>25.8</td>
<td>8.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Mutants/viable pollen (× 10^-4)§</td>
<td>3.9</td>
<td>212</td>
<td>1378</td>
</tr>
<tr>
<td>Mutants/viable pollen/rad (× 10^-4)</td>
<td>—</td>
<td>0.7</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* Allyl alcohol concentrations were about equivalent for all mutagenic treatments. X-ray data includes all ears from all 42 tassels irradiated with 300–500 rads; neon-20 data from two irradiated tassels only.

† Each ear given the rad value of its irradiated pollen parent.

‡ Pollen viability determined empirically. Aborted pollen was estimated to weigh 10% of a plumb grain. The unmutagenized pollen has such a low viability because it, unlike other pollen samples, was field collected.

§ Note that these are mutant recovery frequencies, not mutation frequencies.
### Table 4. Summary Adh-mutant characterizations in $F_1$ plants and $F_2$ gametophytes: initial data

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mutant class (No. $F_1$ mutants)</th>
<th>Fraction surviving</th>
<th>'Mutant' designation</th>
<th>Sporophyte characters</th>
<th>Root tip ploidy</th>
<th>Pollen characters Pollen (allozymes)*</th>
<th>Fraction transmission†</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>— —</td>
<td>Adhl-S</td>
<td>Normal</td>
<td>20</td>
<td>2% abort; 98% ADH-blue; size constant = normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-ray</td>
<td>A (18)</td>
<td>8/10 8 mutants</td>
<td>1999</td>
<td>1/1</td>
<td>Usually 50% abort; usually constant size</td>
<td>0, &lt; 1/18 or low/no seed set</td>
<td>Non-mutant $F_1$</td>
<td></td>
</tr>
<tr>
<td>(12)</td>
<td>0/36 SP 4/9 BC</td>
<td>0/5 BC through ♀</td>
<td>0/50 BC through ♀</td>
<td>0/4 SP</td>
<td>Severe chromosomal aberrations with little chance of transmission</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Small semi-lethal deletion or equivalent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1/1 1 mutant</td>
<td>Abnormal, nectotic</td>
<td>1999</td>
<td>1/1</td>
<td>35% abort, 18% ADH-pink, 50% plump, ADH-blue (mutant allele expressed)</td>
<td>0, &lt; 1/18 or low/no seed set</td>
<td>As for 1999 above, except the structural gene may be intact; organ-specific differences</td>
<td></td>
</tr>
<tr>
<td>(1)</td>
<td></td>
<td>0/5 BC through ♀</td>
<td>0/50 BC through ♀</td>
<td>0/4 SP</td>
<td>Severe chromosomal aberration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1/2 1 mutant</td>
<td>Normal</td>
<td>1999</td>
<td>1/1</td>
<td>37% abort; size and ADH-colour variable</td>
<td>0, &lt; 1/18 or low/no seed set</td>
<td>Apparently Adhl-S lost</td>
<td></td>
</tr>
<tr>
<td>(2)</td>
<td></td>
<td>0/5 BC through ♀</td>
<td>0/50 BC through ♀</td>
<td>0/4 SP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>3/3 3 mutants</td>
<td>Normal</td>
<td>1999</td>
<td>1/1</td>
<td>35% abort, 18% ADH-pink, 50% plump, ADH-blue (mutant allele expressed)</td>
<td>0, &lt; 1/18 or low/no seed set</td>
<td>Normal; Adh2-up mutants not confirmed</td>
<td></td>
</tr>
<tr>
<td>(3)</td>
<td></td>
<td>0/5 BC through ♀</td>
<td>0/50 BC through ♀</td>
<td>0/4 SP</td>
<td>Severe aberrations; a few may transmit based on pollen characterizations, but none have</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>20/35 20 mutants</td>
<td>Usually very short; usually sterile</td>
<td>1999</td>
<td>1/1</td>
<td>50% abort</td>
<td>0, &lt; 1/18 or low/no seed set</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(35)</td>
<td></td>
<td>0/5 BC through ♀</td>
<td>0/50 BC through ♀</td>
<td>0/4 SP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4. (cont.)

<table>
<thead>
<tr>
<th>Treatment (No. F₁ mutants)</th>
<th>Mutant class (No. F₁ mutants)</th>
<th>Fraction surviving</th>
<th>'Mutant' designation</th>
<th>Sporophyte characters</th>
<th>Root tip ploidy</th>
<th>Pollen characters*</th>
<th>Fraction transmission†</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neon ion (51) B (6)</td>
<td>5/6</td>
<td>1945</td>
<td>Normal</td>
<td>20–30</td>
<td>14% abort; 18% ADH-pink; ~ 60% ADH-blue; size not variable; allozymes prove hyperploidy</td>
<td>3/20 SP</td>
<td>31/181 BC through ♀</td>
<td>8 of the 31 transmitted Adh₁-S alleles were clearly underproduced. Adh₁-S is transposed to a new centromere; hyperploid gametes result. Adh₁-S is unstable in its expression</td>
</tr>
<tr>
<td>1946 Normal, late 10–30</td>
<td>Approximately as above</td>
<td>11/36 SP (also hyperploid pollen)</td>
<td>41/123 BC through ♀ and ♀</td>
<td>7 of the 52 transmitted Adh₁-S alleles were clearly under-produced; 3 over-produced. As above, but more unstable</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1948 Normal, barren tassel 10–18</td>
<td>40% abort, ADH⁺; 40% abort, ADH⁻; 10% large, ADH⁻; 10% large, ADH⁻ (no allozyme data)</td>
<td>13/52 BC through ♀; male sterile</td>
<td>No low-producers confirmed of the 13 Adh₁-S alleles transmitted. Probably as above</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1951 Normal N.D.</td>
<td>7% abort; 50% ADH⁺, ~ 40% var. ADH⁺ colour (also hyperploid pollen)</td>
<td>75/138 BC through ♀ or ♀</td>
<td>Only one of the 75 transmitted Adh₁-S alleles were low-balance. Otherwise, as above</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1952 Normal, late 11–30</td>
<td>15% abort; var. ADH⁻ colour and size (also hyperploid pollen)</td>
<td>15/23 BC through ♀ and ♀</td>
<td>Two of the 15 are confirmed low-balances. As above</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment (No. F&lt;sub&gt;1&lt;/sub&gt; mutants)</td>
<td>Mutant class (No. F&lt;sub&gt;1&lt;/sub&gt; mutants)</td>
<td>Fraction surviving</td>
<td>'Mutant' designation</td>
<td>Sporophyte characters</td>
<td>Root tip ploidy</td>
<td>Pollen characters*</td>
<td>Fraction transmission†</td>
<td>Comments</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>----------------------------------------</td>
<td>--------------------</td>
<td>----------------------</td>
<td>----------------------</td>
<td>----------------</td>
<td>-------------------</td>
<td>----------------------</td>
<td>----------</td>
</tr>
<tr>
<td>C</td>
<td>(2)</td>
<td>2/2</td>
<td>1950</td>
<td>Normal</td>
<td>20</td>
<td>40% abort; 60% ADH-blue; no var. size</td>
<td>0/92 BC through ♂ or SP</td>
<td>Overproducer probably lost, but Adh1-S was over-expressed in pollen grains. Not confirmed</td>
</tr>
<tr>
<td>D</td>
<td>(8)</td>
<td>5/8</td>
<td>1955 Normal, late</td>
<td>20</td>
<td>50% abort; var. ADH&lt;sup&gt;+&lt;/sup&gt; colour and size</td>
<td>18/32 SP</td>
<td>Only one of the 18 Adh1-S alleles transmitted clearly over-produced its product. Confirmed, but unstable</td>
<td></td>
</tr>
<tr>
<td>Spontaneous</td>
<td>B (1)</td>
<td>1/1</td>
<td>1561</td>
<td>Usually normal</td>
<td>N.D.</td>
<td>Normal when tested</td>
<td>Normal when tested</td>
<td>Adh2-up mutants not confirmed. Epigenetic? Artifact?</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>B (1)</td>
<td>1/1</td>
<td>1561</td>
<td>Normal</td>
<td>N.D.</td>
<td>Normal; but, all are Adh1-F&lt;sup&gt;*&lt;/sup&gt;-specified al alozyme</td>
<td>10/22 SP and F&lt;sup&gt;*&lt;/sup&gt; expressed</td>
<td>Data seems internally inconsistent; tests are being repeated</td>
</tr>
</tbody>
</table>

N.D. denotes 'no data'.

* The neon ion-induced class B mutants are distinctive in displaying some interallelic hybrid al alozyme in pollen extracts, a condition indicating Adh1-S/Adh1-F hyperploid gametophytes; see text.
† This fraction is in the following units: Adh1-S alleles detected in single scutellar extracts/total Adh1 alleles transmitted from the F<sub>1</sub> plant. SP indicates transmission data from self-pollinated ears. BC denotes a backcross where the mutant F<sub>1</sub> was used as either ♂ ♀ for any single cross.
Mutants from pollen

irradiated. The 18 X-ray induced mutants represent 9 different tassels; the 51 mutants generated by heavy-ions represent only 2 irradiated tassels. Given the general lack of clustering of mutants from the same class, the late stage of irradiation, and the unique behaviour of most mutants (Table 4), mutant duplication due to somatic cloning (jackpot effect) is not likely, but might have happened in a few cases. From these data, only Class B mutants appear preferentially with NeIM+, and even this correlation is not statistically significant based on these quantitative data alone. None of our four classes are induced exclusively by either X-rays or neon-ions.

(v) Three control experiments for the mutant recovery results

Proper interpretation of the previous data on radiation-induced allyl alcohol selected mutant recovery require three controls: (1) unirradiated and unselected; (2) unirradiated and selected; and (3) irradiated and unselected.

Control 1: $1^S \cdot 1^S$: $1^S \cdot 1^F$: $1^F \cdot 1^F$ dimer (Set I) allosemce ratios from 4000 $Adh1-F/Adh1-S$ individuals were electrophoretically analysed as described, as a part of another project in this laboratory. There was no mutagen or allyl alcohol selection involved. Zero mutant electrophoretic profiles were found; the allosemce ratio is remarkably constant. On the other hand, the 69 presumptive induced mutants reported in Table 3 reflect the analysis of only 1722 seeds. The allosemce ratio character appears to be free of spontaneously generated noise.

Control 2: The first column of Table 3 gives the spontaneous mutant recovery data where the allyl alcohol selection system was used. All 69 or the radiation-induced mutants are probably the result of radiation, as would be expected on the basis of previous determinations (Freeling, 1977) of spontaneous mutation rate for $Adh1-S$.

Control 3: The irradiated but unselected control is the most crucial because the irradiated immature tassels might well have contained some proportion of binucleate or trinucleate postmeiotic gametophytes. Since we intentionally chose our allyl alcohol concentrations at borderline concentrations to permit the transmission of ADH-low pollen, the majority (ca. 95%) of our surviving $F_1$s proved to carry an electrophoretically normal $Adh1-S$ allele. If, for example, a sperm nucleus in a mature pollen grain were to lose the entire long arm of chromosome 1, including $Adh1-S$, it might well show up among our presumptive mutants as an unselected volunteer. To test for this possibility, 1094 $F_1$ seeds were analysed where the pollen mother cells had been irradiated with various doses of X-rays (300–600 rads; 440 rads weighted mean) during the sporocytling stage and crossed to testers without allyl alcohol treatment. We found 8 mutants, all of which belonged to Class A, the $Adh1-S$ dysfunction class. Although 6 of these mutants came from one pollen sample, we did not correct these data. When the appropriate normalization per rad is done, the unselected volunteer frequency in mutants $\times$ seed$^{-1} \times$ rad$^{-1}$ for X-rays is $1.7 \times 10^{-7}$. The frequency for selected presumptive mutant recovery (mutants $\times$ seed$^{-1} \times$ rad$^{-1}$) was $9.8 \times 10^{-5}$. Note that these recovery units are not those used in Table 3. On the basis of these data, we estimate that about
17% of our presumptive mutants recovered in Table 3 would have occurred without allyl alcohol selection. Since unselected volunteers should rarely transmit through the gametophytes, and since all unselected mutants were Class A (dys-function), we expected considerable nontransmission (50% abortion) from Class A lesions. Data on unselected neon-20-induced mutants are not available.

(vi) Mutant characterization and preliminary interpretations

Each of the 70 mutants recovered in F₁ seeds and classified in Table 3 were subjected to preliminary characterization (see Table 1). Since we irradiated immature germ cells and employed a gametophytic selection scheme, several transmissible lesions in or affecting Adh1 occurred. These data are condensed and interpreted in Table 4, and are summarized as follows:

(a) At least one mutant in each of Class A (dysfunction), Class B (underproducer), and Class C (overproducer) transmitted through self-pollinations (SP) and/or backcrosses (BC) of the original F₁ mutant; Class D (up-Adh2) ‘mutants’ did not transmit and the F₁ plants appeared normal in phenotype.

(b) Of the surviving Class A (dysfunction) mutant F₁s, 2 of 9 from X-ray and 0 of 20 from neon proved transmissible deletion-type chromosomal aberrations (Table 4, items 3 and 4). For mutants 1999 and 2008, we tentatively conclude that at least one breakpoint lies outside the Adh1 cistron (as delineated by ethyl methanesulfonate-induced point mutants, cf. Freeling, 1976) since (1) pollen abortion is associated with both 1999 (ca. 15%) and 2008 (ca. 35%); (2) pollen abortion is linked to ADH-negativity in pollen from these two mutants stained in situ for ADH activity; and (3) both transmissible mutants displayed dominant sporophytic phenotypes characteristic of small chromosomal derangements in maize, but not of whole-arm aneuploidy (as with a 1L hypoploid generated by a Tb-1a translocation). Furthermore, pollen shed from the Adh1-F/Adh1-S2008 plant contained electrophoretically detectable amounts of ADH1-S subunits. This result suggests that the Adh1-S structural gene component remained intact, but was turned off in the scutellum. In the absence of genetic fine structure or pachytene analysis data, these deletion-type lesions could be local inversions, deletions, or insertions. Transpositions involving centromeres other than that of chromosome one are not likely, given the low levels of pollen abortion.

(c) Class B mutants (underproducers) were so classified because the F₁ scutellum expressed an abnormally low I₁S·I₁S; I₁S·I₁F; I₁F·I₁F allozyme dimer ratio. That is, the binomial distribution of allozyme activity was shifted toward Adh1-F product. The single X-ray induced Class B mutant was a severe aberration (Table 4, item 5). However, each of the five surviving neon ion Class B’s (Table 4, items 9-13) displayed characteristics in common with one another: variable root-tip ploidy, less than 50% pollen abortion, some fraction of hyperploid pollen containing functional Adh1-S and Adh1-F alleles with both types of haploid and hyperploid genomes passing through male and female gametophytes. In addition, 1945, 1946 and perhaps all five of these mutant lesions (Table 4, items 9-13) confer instability onto Adh1-S expression in the scutellum. ‘Instability’ is deduced from an
extremely variable $1^S \cdot 1^S; 1^S \cdot 1^F; 1^F \cdot 1^F$ scutellar allozyme ratio among different Adh1-S//mutant/Adh1-F siblings of the back-cross Adh1-F/Adh1-F x Adh1-F/Adh1-S//mutant. Similar cases of breakpoint-associated instability have been reported by Mottinger (1973) for Bz2 in the aleurone. The last section of Discussion treats this interesting class of neon-20-induced mutants in greater depth.

(d) Only one of the Class C mutants (overproducers) clearly transmits: 1955 induced by neon-20 (Table 4, item 15). As with the neon-induced underproducers, the transmission of mutant Adh1-S' behaviour is infrequent as compared with transmission of apparently normal Adh1-S alleles. This seems to be another case of breakpoint-associated instability of gene expression; definitive experiments are in progress.

(e) The most mysterious mutant characterized is the single spontaneous Class B (underproducer): 1561 (Table 4, last item). Pollen extracts contained only Adh1-F product, but Adh1-S was transmitted normally through the male, and was expressed normally in backcross scutella. Further, the pollen from the F1 plant was apparently plump and ADH+. These data do not appear internally consistent. We have not yet completed a repetition of these data.

(f) Except for the deletion-type mutants 1999 and 2008, all other transmissible mutants could conceivably map away from Adh1. Therefore, we use the full designation (e.g. Adh1-S1999) for a chromosomal aberration which includes at least a part of the Adh1 cistron. For other mutants, like 1561, we leave the mutant number temporarily unattached to a particular gene pending continued investigation.

4. DISCUSSION

(i) Chemical selection of Adh mutants in pollen

There have been previous reports of allyl alcohol selection of ADH− gametophytes in maize (Schwartz & Osterman, 1976; Cheng & Freeling, 1976; Freeling, 1977). This report shows that confirmed mutants may be selected via a surviving gametophyte and recovered in F1 seeds. Our detailed methods of pollen collection, storage, germination on slices from David’s Bread Loaf and in situ staining of germinated pollen for ADH enzyme activity permitted accurate and reproducible allyl alcohol dose-response data. On the basis of these kill-curves, we were able to choose borderline concentrations and treatment conditions which permitted the escape of a few ADH+ gametophytes. Thus low ADH as well as ADH-negative gametophytes survive our treatment. Since we chose allyl alcohol concentrations which were not 100% restrictive to ADH+ gametophytes, and since the immature tassel does contain some germ cells past the microspore stage, it was possible that certain of our mutants were unselected volunteers whose occurrence was independent of allyl alcohol treatment. We have performed the appropriate control for our X-ray recovery data (control experiment No. 3, Results and Conclusions): selected recovery frequency was $9.8 \times 10^{-6}$ mutants $\times$ seed$^{-1}$ $\times$ rad$^{-1}$ while unselected volunteers occurred at $1.7 \times 10^{-7}$ mutants $\times$ seed$^{-1}$ $\times$ rad$^{-1}$. Therefore, approximately 83% of our 17 X-ray induced mutants were allyl alcohol selected.
A similar control experiment for our neon-ion data has not yet been done. In any case, these early mutant recovery data prove that allyl alcohol can be used to select mutants on the basis of gametophytic survival. Our detailed methods should largely extrapolate to other genes in other plants.

(ii) Use of allozyme ratios for mutant confirmation and classification

Most specific gene systems in Drosophila, maize and mice which have been used routinely as mutational targets for ionizing radiations share one unfortunate attribute: they involve the controlled read-out of a hypothetical polypeptide which is unknown or very difficult to assay. Rosy in Drosophila is an exception. Similarly, maize ADHs are well characterized at the allozyme/isozyme level owing largely to the work of Drew Schwartz and associates. Our choice of using electrophoretically distinguishable $Adh1$ alleles for the treated male and tester female was influenced by Schwartz's (e.g. 1969) general scheme for recognizing ethyl-methanesulphonate-induced $Adh1$ structural gene mutants. As a result of our nondestructive assaying of each presumptive mutant $F_1$ for an altered ADH allozyme profile, we recognized classes of mutants in addition to $Adh1$-dysfunction (Plate II). Our classes B, C and D, recognized on the basis of allozyme/isozyme ratio, probably occurred but were undetected by previous investigators working with biochemically inaccessible genes.

We have recovered and transmitted mutational lesions from all classes but D, the $up-Adh2$. Abnormally high ADH2 activity level in the scutellum is often associated with vivipary or defective seeds, although the Class D 'mutant' kernels we included in Tables 3 and 4 appeared normal. We do not as yet have a satisfactory explanation for our failure to confirm Class D mutants; perhaps the endosperm's genotype affects $Adh1-Adh2$ balance via the physiological milieu.

We did not find any mutants which specify alterations in the electrophoretic mobility of ADH1-S. As will be discussed, none of our mutants appear to be confined within the $Adh1$ gene. Here, the $Adh1$ 'gene' is taken to be a complementation group composed of an uninterrupted stretch of dispensable DNA: the $Adh1$ structural gene plus adjacent cis-acting components. Of course, it is conceivable that lethal or semi-lethal stretches of DNA also act in cis to regulate the expression of other less dispensable functions as well as $Adh1$.

(iii) Recovery frequencies and RBE data: this system's weakness

A major weakness in irradiating immature germ cells is the extreme heterogeneity of the target cells. Our RBE data on 400 MeV/amu plateau neon-ion vs. 220 kV X-rays for the recovery of $Adh$ mutants is certainly accurate, but not complete or rigorously controlled; see Results and Conclusions.

Our experimental design should be modified if further RBE data were desired at the $Adh1$ system: (1) reversions could be assayed as rare blue-staining pollen (Freeling, 1976) or (2) forward mutations could be identified by rare allyl alcohol resistant, ADH− gametophytes germinating on an artificial medium (Freeling, 1977).
Mutants from pollen

However, quantifying the ratio of mutants among Classes A, B, C and D may be accomplished reliably. Our high-LET radiation generated a particular class (B; underproducers) of mutants originally identified in the F$_1$ by lowered expression of irradiated $Adh1$-$S$ as compared to the tester allele, $Adh1$-$F$. All five surviving members of this class showed some but less than 50% pollen abortion, some $Adh1$-$S/Adh1$-$F$ hyperploid (partially disomic for the $Adh1$ region of 1L) pollen, unstable ploidy in root tips, lowered male transmission frequencies, and instability of $Adh1$-$S$ after expression. The presence of such hyperploid pollen suggests that $Adh1$-$S$ has been relocated to a new centromere. Although these five surviving members of this class did come from the same irradiated, immature tassel, each F$_1$ showed phenotypes which suggest independent mutational origins. Class B transpositions appear specific to the high-LET treatment. The instability of these $Adh1$-$S$ alleles may be particularly important, and is evaluated further in the last section of this Discussion.

(iv) On the absence of intragenic mutants in maize

There can be no doubt that most nonplant organisms are capable of confining the damage caused by a chromosomal break to within the complementation group. Thus, ionizing radiations have been shown to generate lesions ranging from multi-break chromosomal abnormalities to base-substitutions. The Introduction cites studies in Drosophila, Neurospora, mice and E. coli which substantiate this 'a little bit of everything' conclusion. The fact remains: there are no cases of dominant 'point' mutants or intragenic lesions in forward mutation tests in plants, although scores of small derangement lesions which do transmit through the sensitive male gametophyte were recovered (see Introduction and especially Stadler, 1941; and Mottinger, 1970). The intergenic nature of these mutants was established in a most straightforward way: lowered transmission frequencies through male, lethal phenotype of homozygous plant where spontaneous mutants at the gene delineated a fully dispensable function, and often lowered recombination between markers flanking the region. Our X-ray mutants 1999 and 2008 may now be added to the long list of small deletion-type specific gene events induced in male germ cells by sparsely ionizing radiation. All of our other transmissible mutants are blatantly associated with breakpoints even though our allyl alcohol screen and meiotic irradiation were designed to augment for intragenic mutants which behave recombinationally as points.

Stadler (1944) said of the totality of maize data: The (radiation-)induced mutations observed may be merely gene losses tolerated by the gametophyte. Based on a body of evidence indicating clearly that intragenic lesions do happen in Drosophila, Muller (cf. 1955) dismissed the maize data as reflecting special repair properties of the pollen grain and advanced his argument that, being intragenic, X-rays produced 'progressive' mutants: '... we must admit that at bottom evolution has been built up out of intragenic mutations, together with some super-structure of intergenic structural changes that worked only by utilization of
the gene differences brought about by the intragenic mutations’ (Muller, 1955, p. 134). Muller concerned himself with the unitary gene (or structural gene) and progressive evolution. In contrast, Stadler stressed the experimental fact of his system: X-rays induce chromosomal aberrations. It seems important to ask whether or not Drosophila’s intragenic lesions are merely microscopic relatives of maize’s intergenic aberrations; unfortunately the data are equivocal. All of the radiation-induced reversion studies cited in the Introduction gave ambiguous results because the molecular nature of the original mutant lesions was not understood. Green (cf. 1977) has demonstrated that insertional-type aberrations (mutants) both should and do revert as a response to chromosome breakage. Chovnick and coworkers (Chovnick et al. 1964; Gelbart, McCarron & Chovnick, 1976) tested 41 radiation-induced xanthine dehydrogenase-negative (ry−) for their ability to complement known complementing alleles: three of 41 tested positive. XDH is a dimer. 6 of 24 EMS-induced ry− mutants complemented in some inter se combination. Unfortunately, complementation might be accomplished even by a polypeptide which is missing amino acids. Indeed, it is now clear that two grossly truncated polypeptides can, in some cases, complement (Morrison, Zipser & Goldschmidt, 1971). Rigorous analyses of the complementing proteins is needed; there is no clear evidence for a base substitution being induced in higher organisms by ionizing radiations. It seems likely that the intragenic mutants Muller used to explain progressive evolution were composed largely of short (point) intragenic aberrations. If such aberrations fell within the structural gene component of the cistron, they would be the least likely candidates for contributing to selectively meaningful change. Ironically, a chromosomal rearrangement (deletion, insertion, inversion, transposition) in a local, cis-acting, regulatory component of a cistron might well be progressive (cf. McClintock, 1956; Mottinger, 1973; Wallace, 1975). Perhaps Stadler’s emphasis on the negative aspects of chromosome derangement resulting from DNA ionizations was overly pessimistic. After all, only drastic phenotypes could be recognized as mutants.

(v) **Explaining the differences between the maize and Drosophila data**

It must be that the male-transmissible small X-ray-induced deletion-type mutants in maize are caused by a single spur. In addition, it is not realistic to suppose that essentially all single-hit events which result in a two-strand break are repaired with absolute efficiency. Given the above conditions, we envisage two reasonable explanations for the lack of intragenic deletions in plant male germ cells. (1) Two-strand breaks may be excised by exonucleases to a considerable extent (1–100 kilobases) before reunion may occur. The result is ‘chromosome suicide’ where two-strand breaks are generally filtered out through gametophytes. The degraded region would include lethal or semi-lethal adjacent genes or non-dispensable regulatory components used by the target structural gene. This chromosome suicide alternative may demand clustering of lethal cistrons on the chromosome in order to account for the numerous balanced chromosomal aberrations where the breakpoints themselves are not apparently associated with.
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gametophytic inviability. Evolutionary explanations for chromosome suicide would be interesting, but certainly academic at this point. (2) A second alternative would seem bizarre except for the distinguished history of transposable elements in maize. A two-strand break may attract an insertional sequence. The inserted DNA may then permit reunions. In addition, the insert need also adversely affect gametophytic viability. In terms of recombination and chromosomal cytology, this insert would be difficult to differentiate from a deletion. In short, this second alternative suggests that intragenic inserts may behave as expected of intergenic chromosomal rearrangements. A third explanation would include both of the above. It should also be clear that neither of these explanations should be called ‘repair’ unless a repaired chromosome is defined as practically anything with two telomeres on either side of a centromere. While we now feel that at least one of these alternatives must be true to account for the lack of intragenic radiation-induced lesions in maize, we nevertheless admit that ionizing radiations probably cause base-substitutions as well, but at far lower frequencies. Indeed, any source of energy should exacerbate the infidelities underlying spontaneous mutation, a process which, for Adh1-S, happens at a frequency below $2 \times 10^{-7}$ (Freeling, 1977). In addition, it may be that our explanations apply only to plant pollen or pollen mother cells and that data on ‘somatic’ cells will be canonical.

In the light of the above discussion, we are presently reevaluating our rationale (Introduction, paragraph 3) for using high-Z particles on male germ cells. For example, there is no gain from inducing multiple lesions along 200 nucleotide pairs in a cis-acting regulatory component if exonucleases were to cut out several kilobase-pairs on either side of each break.

(vi) On obtaining mutants in programmable components of the gene

As stated in the Introduction, the overall aim of these studies was to induce regulatory mutants near, but not in, the Adh1 structural gene. Definite proof of such mutants must await continued analyses. Mutant Adh1-S2008 appears to be allelic, entirely outside of the structural gene, and to act differentially in scutellar cells versus pollen. Our Class B (underproducers) and Class C (overproducers) also look promising. These mutants seem to involve the level of gene expression in the scutellum as influenced by breakpoints which are probably close to Adh1-S. Further, when these breakpoint-associated Adh1-S alleles are transmitted through backcrosses to Adh1-F testers, the allozyme ratios in backcross scutella vary from almost zero Adh1-S expression to an overabundance of Adh1-S expression relative to Adh1-F, as is the case for 1946. Mottinger (1973) reported on two unstable bronze-1 mutants induced by pre-meiotic X-ray treatment to the male germ cells. These mutants, designated bz-x3, bz-x4 and bz-x5, showed instability in the aleurone cells of the endosperm and also in leaf tissue. At bronze-1, the instability involves timing rather than level of gene action in development. Mottinger concluded that he had either added a component (like a transposable element) or altered regulatory components affecting bronze-1. The fact that our allozyme assay system detected ionizing-radiation-induced balance mutants (Classes B, C and D)
at high frequency (16/69 total mutants is 23%), immediately suggests that the 'programmable component' of Adhl is mutable. We trust that mutants in regulatory gene components will prove valuable in understanding development and progressive evolution.

Note added in proof

Data from the progeny of one subsequent round of crosses confirmed these initial results except that 1999 and 2008 also appear to be unstable. The NE1(H"°-underproducers are translocations cytologically; a stable derivative of 1951 is tightly linked but probably outside of the structural gene.

This work was supported by NIH grant GM 21734, University of California Biomedical Research Grant (DHEW) and California Agricultural Experiment Station project CA-2947-H. We thank Deverie Bongard and William Theiss for field and laboratory help, and Roy Sahara for nursery work. We also thank Jerry Howard and the crew at Bevatron, LBL; Laurie Craise and especially Tracy C. Yang for continued help and discussions.

5. REFERENCES


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