GENETIC BASIS OF THE MAJOR MALATE DEHYDROGENASE ISOZYMES IN MAIZE

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ABSTRACT

The mitochondrial MDH isozymes in the scutellum of the mature maize (Zea mays L.) kernel are encoded by three independently inherited nuclear genes. Mdhl is located on chromosome 8, close to the breakpoint (8L.35) of a waxy-marked reciprocal translocation between chromosomes 8 and 9. Mdh2 is located in the distal region of the long arm of chromosome 6. Mdh3 is on the long arm of chromosome 3, approximately 2.6 map units from sh2. A modifier of the mitochondrial MDH isozymes (Mmm) maps approximately 27.5 units proximal to Adh1 in the central portion of the long arm of chromosome 1. Independently assorting duplicate genes code for the soluble MDH isozymes. Mdh4 is located in the same region of chromosome 1 as Mmm, approximately 29 map units proximal to Adh1. Mdh5 maps approximately 20 units distal to a2 in the short arm of chromosome 5.—Intergenic and interallelic heterodimer formation occurs among gene products that occupy the same subcellular compartment. MDH isozymes were purified and analyzed by native-SDS two-dimensional polyacrylamide gel electrophoresis. The proposed mitochondrial MDH intergenic heterodimer bands were found to be composed of two subunits, which differ in their migrations on SDS gels; whereas, genetically defined homodimers contained only one type of subunit.—This evidence is discussed in terms of two genetic models proposed for the maize mitochondrial MDH isozymes.

ISOZYMES of malate dehydrogenase (L-malate NAD oxidoreductase; E.C. 1.1.1.37; MDH) are localized in particular cell compartments and participate in a multiplicity of metabolic processes, including the mitochondrial TCA cycle and malate shuttles between the compartments (reviewed by Ting et al. 1975).

The scutellum of the mature maize kernel contains cytosolic or soluble (s-MDH) and mitochondrial (m-MDH) malate dehydrogenase isozymes, as well as minor glyoxysomal (g-MDH) forms (Longo and Scandalios 1969). The m-MDH isozymes are active as dimers, encoded in the nucleus (Longo and Scandalios 1969) and translated on cytoplasmic polyribosomes (Yang and Scandalios 1975).

Recent studies indicate that multiple genes code for the m-MDH and the s-MDH subunits (Yang, Sorenson and Scandalios 1977; Goodman et al. 1978, 1980; Newton 1979a). Genetic analyses conducted in our laboratory and by M. Goodman at North Carolina State University have led to the following
hypothesis regarding the genetic basis of the major MDH isozymes active in
the scutellum of the mature kernel and in seedling tissues: (1) three independ-
ently inherited nuclear genes (Mdhl, Mdh2 and Mdh3) code for the mito-
chondrial MDH isozymes; (2) duplicate, unlinked loci (Mdh4 and Mdh5)
specify the major zone of soluble MDH activity; and (3) gene products that
occupy the same subcellular compartment from intergenic and interallelic het-
erodimers, but no heterodimers are formed between the s-MDH and m-MDH
subunits.

The gene Mmm, which assorts independently of the structural genes specify-
ing the mitochondrial MDH isozymes, modifies the m-MDH isozymes—possibly
effecting a post-translational modification of the m-MDH polypeptides (NEWTON
1979b). When a mutant allele mmm is homozygous, all the mMDH isozymes
migrate more rapidly to the anodal pole in starch gel electrophoresis. The soluble
enzymes are not altered.

The MDH structural gene model outlined above is based on analyses of the
segregation of variant MDH isozymes in testcrosses. Existence of the proposed
independently inherited multiple loci can be substantiated by gene mapping
demonstrating that they occupy different chromosomal sites. Although the genetic
evidence for the formation of intergenic heterodimers has been presented (GOOD-
MAN et al. 1980), a demonstration that they are composed of two different
subunits would provide biochemical substantiation.

In this paper, the chromosomal locations of the five postulated MDH struc-
tural genes and of the m-MDH modifier locus, Mmm, are reported. In addition,
we have studied the subunit composition of several proposed mitochondrial MDH
intergenic heterodimers, using a two-dimensional native-SDS polyacrylamide
gel electrophoresis (PAGE) system. Our results provide genetic and biochemical
confirmation of the proposed model.

MATERIALS AND METHODS

To localize MDH genes, we used electrophoretic and null variants. Genes were tentatively
assigned to chromosome arms after crosses with B-A translocation stocks, and their locations
were confirmed by determining genetic linkage to appropriate markers.

Stocks: Most of the B-A translocation stocks and many of the exotic lines and testers used in
this study originated from the Maize Genetics Cooperation stock center. J. BECKETT provided
the TB-3Lc, TB-3Ld and TB-6Lb stocks, while J. BIRCHLER constructed the compound TB-1La-
3L translocations. M. GOODMAN supplied many of the MDH variant lines identified in his
laboratory.

Use of B-A translocations: Behavior of the supernumerary B chromosomes in maize and the
use of reciprocal translocations between the B chromosomes and the normal A complement to
locate genes to chromosome arm have recently been reviewed (CARLSON 1978; BECKETT 1978).
Due to a high frequency of nondisjunction of the chromosome with the B centromere at the
second pollen mitosis, differences in the chromosomal content of the two sperm nuclei are gen-
erated. Since one of these fertilizes the egg nucleus to give rise to diploid embryonic and scutellar
tissues and the other sperm nucleus fertilizes the two polar nuclei to initiate the triploid endo-
sperm, the following major classes of progeny result when a hyperploid heterozygous B-A
translocation plant is used as a male parent: (1) Hyperploid scutellum with hypoploid endo-
sperm. Due to the preferential fertilization of the egg nucleus by hyperploid sperm this is
usually the most frequent class. (2) Hypoploid scutellum with hyperploid endosperm. Only the maternal variants of genes included in the A chromatin translocated to the B centromere are expressed in the scutella of these kernels. (3) Euploid scutellum and endosperm resulting from normal disjunction at the second microspore division. However, markedly different results are obtained when the B-A translocation plants are used as the female parent in crosses since there is no nondisjunction during the development of the embryo sac. Only euploid and hyperploid kernels are produced, and there is concordance between the scutellar and endosperm phenotypes of each kernel. Thus, when locating genes by the B-A translocation method, it is useful to make reciprocal crosses and to look for differences in the spectrum of progeny phenotypes in the two F₁ ears.

**TB crosses:** Hyperploid A A'B'B A'A ('"TB") plants were used in reciprocal crosses with MDH variant lines, as their use facilitates recovery of hypoploid progeny in the resultant ears. In most cases, the constitution of the parental hyperploids was ascertained by the expression of recessive markers in the correspondingly hypoploid endosperm. To confirm the presence of the B-A translocations, each TB-A plant was also crossed as a male in crosses to appropriate tester lines. To date, crosses with 23 different B-A translocation stocks covering portions of 15 of the 20 maize chromosome arms have been made. These chromosome arms are 1S, 1L, 2L, 3L, 4S, 4L, 5S, 5L, 6L, 7L, 8L, 9S, 9L, 10S and 10L.

**Starch gel electrophoresis:** Slivers of scutella were excised from kernels that had been softened by soaking in water overnight. (The embryonic axis was not disturbed so that the seeds could subsequently be germinated.) These were squashed through Miracloth onto Whatman #3 filter squares and inserted into 12% starch gels made with 0.015 M Tris-citrate buffer, pH 7 (23°C). The bridge buffer was 0.15 M Tris-citrate. Gels were subjected to electrophoresis for 5.5 to 6 hr at 4°C. Gel slices were stained in an MDH activity solution (e.g., SCANDALIOS 1969). In linkage studies, gel slices were also stained for ADH activity (SCHWARTZ and ENDO 1966).

**Purification of MDH isozymes:** Scutella, roots and shoots from 5-day etiolated seedlings were homogenized in 0.005 M sodium phosphate buffer (pH 7.5) containing 10⁻³ M 2-mercaptoethanol. The crude extract was subjected to a 50–70% ammonium sulfate fractionation. The pellet was resuspended in and dialysed against 0.005 M Tris-HCl (pH 8), loaded on a DEAE-Sephacel column and eluted with a gradient of 0.005–0.5 M Tris-HCl (pH 8). Fractions containing only one MDH isozyme, identified by starch gel electrophoresis, were dialyzed against 0.02 M sodium phosphate buffer (pH 7.5) and loaded on a Blue Sepharose CL-6B (Pharmacia) column; MDH was specifically eluted with 10 mM NADH. Testing on starch gels and on native polyacrylamide gels demonstrated that particular MDH isozymes had been purified. No contaminating protein bands were visible under our electrophoresis conditions.

Total s-MDH and m-MDH isozymes were also isolated by the same method, but omitting the ion exchange chromatography, which served mainly to separate isozymes. With this less extensive purification, minor contaminating proteins are observed, but these migrate well away from the MDH bands in two-dimensional PAGE.

**Polyacrylamide gel electrophoresis:** Native-SDS two-dimensional polyacrylamide gel electrophoresis (PAGE) was performed according to the procedure of FERL, DLOUHY and SCHWARTZ (1979). The second dimension (Laemmli-SDS) gel was 10% acrylamide.

**Nomenclature:** For cross-referencing purposes, band designations on zymograms are abbreviated from the published numbering system of YANG, SORENSON and SCANDALIOS (1977). s-MDH¹ is shortened to s-1, etc. Allele designations of the MDH structural genes are set off by a hyphen, e.g., MdhL-A1. To avoid confusion, we have followed the allelic nomenclature employed by GOODMAN et al. (1980).

**RESULTS**

The maize MDH isozyme banding pattern resolved by electrophoresis on tris-citrate starch gels is complex and many naturally occurring variant patterns exist. A pattern observed in a number of lines is illustrated in Figure 1. In this
Figure 1.—An MDH pattern observed in several lines after electrophoresis on a tris-citrate starch gel. Bands are numbered on the left side of the gel, and the subunit constitution is given on the right. The subunit specified by a particular gene is indicated by gene number. No allele designations are included. The s-2 band is apparently a modified form of s-1, as its position is correlated with the position of the major soluble MDH band (see Figure 5). * denotes proposed m-MDH intergenic heterodimers; s = soluble; m = mitochondrial.

case, the broad, heavily-staining s-1 band includes the products of three different genes. Two of these (Mdhl and Mdh5) encode cytosolic enzymes, and the third (Mdh3) specifies a weakly active mitochondrial MDH isozyme. The evidence for these co-migrations has been previously presented (Newton 1979a), and Figure 2B illustrates that a weak band (MDH3:MDH3) persists at the s-1 posi-

Figure 2.—Segregation of soluble MDH variants and evidence for MDH3-C16 homodimers at the s-1 position. (A) and (C) Scutella homozygous for Mdh4-D8 (a slow-migrating s-MDH variant) and containing an Mdh5-E12 standard allele. An MDH 5-4 intergenic heterodimer is formed. (B) A sib kernel homozygous for Mdh4-D8 and Mdh5 null. The weak band at the s-1 position is the mitochondrial isozyme MDH3-C16. (Note the absence of a heterodimer band between MDH3-C16 and MDH4.)
tion when the soluble MDH isozymes have been removed by the use of variants.

**Location of Mdh3:** The homodimer specified by the standard *Mdh3* allele, *Mdh3-C16*, migrates to the s-1 position and is masked by the soluble MDH enzymes. A faster migrating variant, designated *Mdh3-C18*, was found in the H25 inbred line by Goodman et al. (1980). *Mdh3-C16* homozygotes (e.g., Figure 1) show bands at the m-1 and m-2 positions, the proposed heterodimers of MDH3-C16-MDH1-Δ6 and MDH3-C16-MDH2-Δ3, respectively. *Mdh3-C18* homozygotes show a weak isozyme band (the variant homodimer) anodal to the s-1 position and no bands at the m-1 and m-2 intergenic heterodimer positions. It is proposed that m-MDH intergenic heterodimers do form between the MDH3 variant and the MDH1 and MDH2 isozymes, but that they migrate more rapidly and are obscured by the intense s-1 and s-2 bands. In fact, when the s-MDH isozymes are removed from the s-1 and s-2 positions by the use of variants (or by the use of an ascorbic acid-sucrose homogenizing solution, see Goodman et al. 1980), the two proposed heterodimer bands are clearly visible. In all cases, *Mdh3-C16/Mdh3-C18* heterozygotes can be distinguished from *Mdh3-C18* homozygotes or hemizygotes by the presence of the MDH3 interallelic heterodimer, MDH3-C16-MDH3-C18, migrating between s-1 and the variant homodimer (Figure 3).

In order to locate *Mdh3*, the H25 line was crossed with the various B-A translocation stocks. All of the TB lines used carry the standard *Mdh3-C16* allele. The hemizygous *Mdh3-C18* variant pattern with neither the MDH3 interallelic heterodimer band nor the m-1 or m-2 intergenic heterodimer bands should appear only in kernels from crosses where the B-A translocation involves the chromosomal region that includes the site of the *Mdh3* gene.

When plants of the H25 line were pollinated by TB-3La, TB-3Lc, TB-3Ld and TB-1La-3L5267 stocks, some scutella of ensuing kernels had only the maternal

![Figure 3](image)

**Figure 3.**—Zymogram illustrating that the positions of intergenic heterodimer bands, m-1 and m-2, are correlated with the segregation of the *Mdh3* alleles. An *Mdh3-C16/Mdh3-C18* heterozygote was backcrossed to the *Mdh3-C18* homozygous line. (A) and (C) are homozygous for *Mdh3-C18*; (B) is an *Mdh3-C16/Mdh3-C18* heterozygote. In (A) and (C) the intergenic heterodimers are masked by the s-MDH isozymes.
The Mdh3-C18 variant as determined by electrophoretic analyses. These were not found in any of the other TB crosses.

Compound B-A translocations, such as TB-1La-3L5267, carry portions of two different A chromosomes attached to the B centromere (see Rahka and Robertson 1975). The TB-1La-3L5267 translocation includes both the proximal portion (0.2–0.72) of the long arm of chromosome 1 and the distal region (0.73–end) of the long arm of chromosome 3 (Birchler 1980). Scutellar extracts of 39 kernels from the H25 × TB-1La-3L5267 cross were tested. Fifteen were small kernels (hypoploid endosperm, hyperploid scutella, see Birchler 1980) that were all heterozygous, having both Mdh3 alleles. However, the scutella of 11 of the 24 tested large kernels carried only the maternal Mdh3-C18 allele.

Since TB-1La-3L5267 uncovers genes located in the distal 25% of 3L, linkage between Mdh3 and the shrunken-2 (sh2) locus, a good endosperm marker in this chromosomal region, was examined. The H25 line (Mdh3-C18) was crossed with a sh2 tester carrying Mdh3-C16 and the F1 plants were self-pollinated. The shrunken F1 kernels were tested for MDH phenotypes. The data presented in Table 1 indicate approximately 2.6% recombination between Mdh3 and sh2.

**Location of Mdh1:** Efforts to locate Mdh1 using B-A translocation stocks were unsuccessful. Since it seemed likely that Mdh1 lies in a region of chromatin that is not included in any of the B-A translocations, we utilized another common cytogenetic method for locating genes in maize: waxy-marked reciprocal translocations between chromosome 9 and the other chromosome arms (see Anderson 1956). An artificial linkage is established through the use of these translocations, such that genes that do not normally show linkage with waxy (wx, 9-59), i.e., are not on chromosome 9, may be brought into close proximity to this endosperm marker.

No linkage between Mdh1 and wx is observed in the nontranslocated material. Of the waxy-marked translocations tested, only T8–9 (6673) obtained from the Maize Genetics Stock Center with breakpoints at 8L.35; 9S.31 and T8–9d (8L.09; 9L.16) gave positive linkage between Mdh1 and wx. The T8–9 (6673) translocation stock carrying the wx allele, is homozygous for the Mdh1-A6 allele and gives a MDH isozyme pattern similar to that seen in Figure 1. The Ky21 line

<table>
<thead>
<tr>
<th>Cross:</th>
<th>Mdh3-C16 sh2</th>
<th>Mdh3-C16 sh2</th>
<th>Mdh3-C18 Sh2</th>
<th>Mdh3-C18 Sh2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mdh3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16/C16</td>
<td>Parental</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16/C18</td>
<td>Recombinant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18/C18</td>
<td>Double Recombinant</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Recombination = 2.6%

F2 kernels of the shrunken phenotype (sh2/sh2) were scored for MDH3.
is homozygous for an allele of \textit{Mdhl}, \textit{Mdhl-Ai}, which specifies an easily distinguishable, slow-migrating MDH1 isozyme. The F\textsubscript{1} plants were self-pollinated, and the homozygous kernels were tested electrophoretically for MDH isozyme composition.

If \textit{wx} assorts independently of \textit{Mdhl}, 25\% of the F\textsubscript{2} waxy kernels should have been homozygous for the \textit{Mdhl-A6} allele. However, 48 of 54 (89\%) of the tested kernels were homozygous for \textit{Mdhl-A6}. This indicates less than 6\% recombination between \textit{Mdhl} and the 8\textit{L} breakpoint. All of the tested starchy (\textit{Wx}) kernels carried the \textit{Mdhl-A1} allele. If the genes segregated independently, 25\% of these kernels (8 of 33) should have been homozygous for \textit{Mdhl-A6}.

\textit{Location of Mdh2:} It was previously determined that \textit{Mdh2} is on chromosome 6 by studies with trisomics (Goodman \textit{et al.} 1980) and with TB-6\textit{Lc} (Newton 1979a). TB-6\textit{Lc} uncovers all the known markers in the long arm of chromosome 6 (see Beckett 1978). The TB-6\textit{Lb} translocation uncovers only genes located in the distal region of the long arm of chromosome 6 (Beckett 1978); its 6\textit{L} breakpoint lies between \textit{sm} (6-58) and \textit{py}(6-68), more than 40 map units distal to the position of \textit{Y} (6-17). Studies (similar to those described for \textit{Mdh3}) using TB-6\textit{Lb} in crosses with \textit{Mdh2} null lines indicated that \textit{Mdh2} is also carried by this translocation. This locates it in the distal region of the long arm of chromosome 6 and explains why no evidence for linkage between \textit{Y}, a proximally located endosperm marker, and \textit{Mdh2} was obtained.

\textit{Location of the mitochondrial MDH modifier (Mmm):} In scutella homozygous for the mutant m-MDH modifier gene, \textit{mmm}, all the m-MDH bands migrate slightly faster than normal to the anodal pole on starch gel electrophoretograms (Figure 4). We discovered this mutant MDH pattern in the Black Mexican Sweet Corn line (Newton 1979b), and Goodman \textit{et al.} (1980) reported it in four other lines. All of these modified phenotypes appear to be specified by

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure4.png}
\caption{Zymogram demonstrating the altered migrations of the m-MDH isozymes due to the action of the \textit{Mmm} gene. (A) Normal migration of m-MDH isozymes. (B) MDH isozyme pattern in a scutellum homozygous for \textit{mmm}. These patterns were segregating on a selfed ear of an \textit{Mmm/mmm} heterozygote. The s-MDH bands are unaffected.}
\end{figure}
alleles of the same locus since the mutant pattern is exhibited in the F₁ progeny of crosses between these lines.

The mutant \textit{mmm} allele is inherited as a simple Mendelian recessive. When homozygous \textit{mmm/mm} plants (Figure 4B) are crossed with \textit{Mmm} homozygotes (Figure 4A), all the F₁ kernels (\textit{Mmm/mmm}) have normally migrating mitochondrial MDH isozymes. When these F₁ plants were self-pollinated, a 3:1 (58 normal:20 mutant) F₂ progeny ratio resulted. In a backcross to the mutant modifier line, a 1:1 ratio (123 normal:120 mutant) was observed; whereas, only normally migrating mitochondrial MDH isozymes were observed in progeny kernels of the backcross of the F₁ heterozygote to the \textit{Mmm/MM} parent.

When the different TB stocks were crossed as the male parent to \textit{mmm/mm} plants, the modified m-MDH migration pattern characteristic of the female parent was uncovered only with those B-A translocations that produce hemizygosity for a region of the long arm of chromosome 1. The TB-1La translocation carries the distal 80% of the long arm of chromosome 1. The alcohol dehydrogenase-1 (ADH1) isozymes serve as markers for the dosage of \textit{1L}. BIRCHLER (1980) constructed a line carrying an \textit{Adh1-S} allele on the B-A translocation and an \textit{Adh1-C} allele on the normal chromosome. Pollen from hyperploid (\textit{Adh1-C/S/S}) plants was applied to \textit{mmm} silks (\textit{Adh1-F/F}). Hypoploid scutella, which carry only the maternal \textit{Adh1-F} allele, all showed the mutant MDH phenotype. Euploid (ADH1-F/S) and hyperploid (ADH1-F/S/S) scutella exhibited normal m-MDH migration. In the reciprocal cross, only normally migrating m-MDH isozymes were observed.

When Black Mexican Sweet Corn is used as the female parent in these crosses, the sugary-1 (\textit{sul}) gene provides an internal marker for self-contamination. None of the kernels scored for MDH isozyme patterns in the B-1 translocation crosses were sugary.

The \textit{Mmm} gene is linked to \textit{Adh1}. Black Mexican was crossed with a line homozygous for \textit{Mmm} and \textit{Adh1-S}. F₁ plants were backcrossed to the Black Mexican line, and two ears were scored for MDH and ADH. According to the data in Table 2, \textit{Mmm} lies approximately 27.5 recombination units from \textit{Adh1}. Results from the crosses with compound B-A translocation stocks demonstrated that \textit{Mmm} is localized proximal to \textit{Adh1}. \textit{Adh1} has been mapped to within 1.5 units of \textit{lw} (SCHWARTZ 1971) and located cytologically to between 0.80 and 0.90 of the long

\textbf{TABLE 2}

\textit{Linkage between Adh1 and Mmm}

<table>
<thead>
<tr>
<th>Cross: \textit{Mmm Adh1-S} \times \textit{mmm Adh1-F}</th>
<th>Class</th>
<th>\textit{ADH}</th>
<th>\textit{Ear 1}</th>
<th>\textit{Ear 2}</th>
<th>Totals</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental FF</td>
<td></td>
<td>42</td>
<td>45</td>
<td>87</td>
<td>72.5</td>
<td></td>
</tr>
<tr>
<td>Recombinant FS</td>
<td></td>
<td>16</td>
<td>17</td>
<td>33</td>
<td>27.5</td>
<td></td>
</tr>
</tbody>
</table>

Kernels with the recessive (\textit{mmm/mm}) MDH phenotype were scored for Adh1 recombinants.
Location of Mdh4: Two genetically independent loci, Mdh4 and Mdh5, specify the soluble MDH isozymes. The evidence for this has been presented elsewhere (Goodman et al. 1980; Newton 1979a). Basically, it was observed that the s-MDH variants within each group segregate as alleles from one another and assort independently from variants in the other group. The standard alleles of the s-MDH genes (Mdh4-D12 and Mdh5-E12) specify isozymes migrating to the s-1 position (Figure 1). In order to locate the s-MDH genes, lines carrying variants for both genes were used. The lines Tx303 and Tx325 (Figure 5A, C) are homozygous for an Mdh5 null and a faster migrating electrophoretic variant of Mdh4, Mdh4-D14.5 (Goodman et al. 1980). Tx303 was crossed with the B-A translocation stocks. Since the TB-A lines usually carry the standard alleles of both s-MDH genes, dosage differences in these crosses were expected to be discernible only by changes in the relative staining intensities of the s-MDH bands, not by the presence or absence of a particular band. Despite this difficulty, crosses with TB-1La suggested that one, but not both, of the s-MDH genes is located on the long arm of chromosome 1.

The demonstration of linkage between Adh1 and Mdh4 provides conclusive evidence that Mdh4 is located on the long arm of chromosome 1. Tx303, which is homozygous for Adh1-F and Mdh4-D14.5, was crossed with a line homozygous for Adh1-S and Mdh4-D12. The F1 heterozygote was backcrossed to the Adh1-S, Adh1-S,

**Figure 5.**—Soluble MDH variants. (A) and (C) MDH isozymes present in the Tx325 line. It is homozygous for Mdh4-D14.5, a faster migrating variant, and null for Mdh5. Note that the D14.5 variant has a trailing band at the approximate s-1 position, no band at the s-2 position, and the m-MDH isozymes are similar to those in Figure 1. (B) A variant s-MDH phenotype found in the Papago Flour corn line. It is homozygous for Mdh4-D12 (the standard, s-1 migrating, allele) and the Mdh5-PF ("Papago Fast") allele. An intergenic MDH 4-5 heterodimer is formed. In this particular scutellum, Mdh1 and Mdh2 are specifying isozymes of the same electrophoretic mobility at the m-3 position.
Mdh4–D12 line. Extracts of imbibed scutella from progeny kernels were subjected to electrophoresis and gel slices were stained for both ADH and MDH. The Mdh4–D12 homozygotes have a single major s-MDH band at the s-1 position and are readily distinguishable from the Mdh4–D12/Mdh4–D14.5 heterozygotes that have two additional, more anodally migrating bands: the MDH4–D14.5 homodimer and the 14.5–12 heterodimer. The backcross data are shown in Table 3. There is approximately 29% recombination between Adh1 and Mdh4.

Location of Mdh5: When Tx303 plants (homozygous for Mdh5 null and Mdh4–D14.5) were pollinated by the compound B-A translocation, TB–1La–5S8041, which carries both a 1L segment (0.20–0.80) and most of the short arm of chromosome 5 (0.1–end), some of the resultant F1 kernels had MDH patterns in which the paternal (standard) alleles of both Mdh4 and Mdh5 were missing (Figure 6C). Since the mitochondrial MDH band ratios are unlike those seen in the Tx303 parent, self-pollination can be ruled out. This indicated that both Mdh4 and Mdh5 are carried by TB–1La–5S8041. Since the Mdh5 gene is not carried by the TB–1La translocation and does not show linkage to Adh1, we tested for linkage between Mdh5 and an aleurone color gene, a2, located in the short arm of chromosome 5.

A colorless a2 tester line obtained from the Maize Genetics Co-op Stock Center carried the standard Mdh5–E12 allele. This lines was crossed with plants homozygous for a variant Mdh5 allele, Mdh5–PF ("Papago Fast"), which codes for a faster migrating isozyme (Figure 5B). The F1 kernels were all colored, indicating that the Mdh5–PF plants were also homozygous for the A2 allele. The F1 plants were backcrossed to the a2 tester line. Backcross data listed in Table 4 indicated approximately 20% recombination between Mdh5 and the a2 locus. Since the 5S breakpoint of TB–1La–5S8041, which uncovers Mdh5, is located approximately 6 map units proximal to a2 (see Beckett 1978) and there is 20% recombination

| TABLE 3 | Linkage between Mdh4 and Adh1 |
|-----------------|-----------------|-----------------|
| Genotype | No. | Class | % |
| Mdh4–D14.5 Adh1–F | 65 | Parental | 69.1 |
| Mdh4–D12 Adh1–S | | | |
| Mdh4–D12 Adh1–S | 53 | | |
| Mdh4–D12 Adh1–S | 23 | Recombinant | 28.9 |
| Mdh4–D12 Adh1–S | | | |
| Mdh4–D12 Adh1–S | 25 | | |
Figure 6.—The compound B-A translocation, TB-1La-5S8041, carries both Mdhl and Mdh5. Scutellar extracts were examined from F1 kernels of the cross: Tx303 (Mdh5 null, Mdh4-D14.5) × TB-1La-5S8041 (Mdh5-E12, Mdh4-D12). The designation “12” in the pattern includes the paternally derived MDH4-D12 and MDH5-D12 polypeptides, which cannot be distinguished on the basis of electrophoretic mobility. (A) A large kernel: an apparent euploid. (B) A small kernel carrying a hyperploid scutellum. This pattern was observed with all tested small kernels. (C) A presumptive hypoploid scutellum. The Tx303 s-MDH pattern is observed. The paternal contribution is absent for both Mdh4 and Mdh5. The arrow refers to the s-MDH trailing band—a modified form of the MDH4-D14.5 isozyme.

observed between a2 and Mdh5, we suggest that Mdh5 is located distal to a2 in the short arm of chromosome 5.

PAGE analysis of purified MDH and intergenic heterodimers: According to the model, polypeptides specified by different MDH genes, but which occupy the same subcellular compartment, associate to form heterodimers. The m-1 and m-2 bands in the MDH isozyme pattern shown in Figure 1 have been identified as heterodimers by genetic analyses, making use of Mdhl, Mdh2 and Mdh3 variants (GOODMAN et al. 1980). The m-1 band in Figure 1 is proposed to be an MDH1-MDH3 heterodimer and the m-2 band an MDH2-MDH3 heterodimer.

This interpretation of the genetic evidence is supported by two-dimensional PAGE analysis of purified MDH isozymes. The native-SDS two-dimensional

<table>
<thead>
<tr>
<th>Cross:</th>
<th>Mdh5-PF A2 Mdh5-E12 a2 × Mdh5-E12 a2</th>
<th>Mdh5</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class</td>
<td>Mdh5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parental</td>
<td>E12/E12</td>
<td>114</td>
<td>80.3</td>
<td></td>
</tr>
<tr>
<td>Recombinant</td>
<td>E12/PF</td>
<td>28</td>
<td>19.7</td>
<td></td>
</tr>
</tbody>
</table>

Colorless (a2/a2) kernels resulting from this cross were tested for MDH isozyme patterns.
system of Ferl, Dlouhy and Schwartz (1979) was used. In the native first dimension, the proteins are separated mainly on the basis of charge, preserving multimeric interactions and enzymatic activities. The second dimensional electrophoresis in the presence of SDS disrupts subunit interactions and separates monomers on the basis of size and SDS binding. If a certain band represents a heterodimer, two types of subunits should be present. If these monomers differ in molecular weight or SDS binding properties, they may be resolved in the SDS dimension and appear as a vertical double spot.

When total soluble and mitochondrial MDH isozymes were isolated by affinity chromatography with Blue Sepharose from the line whose starch gel pattern is represented in Figure 1, and electrophoresed in the two-dimensional PAGE system, a complex pattern resulted. In the regions of the m-1, m-2 and m-4 bands, double spots were seen. However, the multiple MDH bands were not well resolved. To overcome this, lines containing relatively uncomplicated MDH isozyme patterns with well separated m-MDH bands were chosen for further analysis of intergenic heterodimer formation.

The Mangelsdorf tester line obtained from the Maize Cooperation Stock Center contains the m-2 and m-5 bands, but lacks the m-1 and m-3 bands, a situation that was also observed with the inbred Line 59 of Yang, Sorenson and Scandalios (1977). In fact, when the scutellar MDH patterns from these two lines are directly compared by starch gel electrophoresis, they are indistinguishable. Genetic analyses conducted with these two lines suggest that the absence of the m-1 and m-3 bands in both lines may have the same basis. We conducted crosses with the Mangelsdorf line similar to those reported for Line 59 by Yang, Sorenson and Scandalios (1977). Similar F1 and backcross ratios for MDH patterns were observed. When the soluble and mitochondrial MDH isozymes were isolated from the Mangelsdorf line and run in the two-dimensional PAGE system, the m-2 band is clearly represented by two spots, one of which has the same mobility in the SDS dimension as the m-5 spot (Figure 7). Yang, Sorenson and Scandalios (1977) hypothesized that the m-2 band is a homodimer. Our analysis suggests that the m-2 band is a heterodimer and that it shares a subunit with the m-5 band.

A similar analysis was conducted on MDH isolated from kernels that contain m-1 and m-3 bands, but lack m-2 and m-5 bands. In Figure 8, the m-1 band is represented by a double spot in the second dimension, indicating that it is composed of two different types of subunits. In this particular case, the m-3 band contains only one type of subunit. Yang, Sorenson and Scandalios (1977) proposed that bands at the m-1 position are homodimers. However, in the case presented here, the m-1 band clearly represents a heterodimer.

The inbred Ky21 line has been analyzed genetically by Goodman et al. (1980). It is null for Mdh2 and variant for Mdh1. Bands are present at the m-3 and m-7 positions, but lacking at m-1, m-2, m-4 and m-5 (Figure 9A). The genetic analysis indicated that the band at the m-3 position in this particular line represents a heterodimer between subunits encoded by Mdh3-C16 and the slower migrating subunits specified by the variant Mdh1-A1 allele. When the total s-MDH and m-MDH isozymes from this line were isolated and subjected to the two-
Figure 7.—PAGE analysis of MDH purified from the Mangelsdorf line. (A) Native first dimension (Coomassie Blue protein stain). (B) Native first dimension stained for MDH activity. (No s-2 band is seen when MDH is purified by affinity chromatography.) (C) Two dimensional gel, resulting from electrophoresis of the native dimension into the SDS second dimension (Coomassie Blue protein stain).

dimensional PAGE (Figure 9C), a double spot was seen at the m-3 position. One of the proteins in this doublet had the same second dimension mobility as the single spot observed at the m-7 position.

Individual m-MDH isozyme bands can be separated from the other MDH isozymes by ion-exchange chromatography. Fractions containing a single band may be pooled and the individual isozyme purified by elution off a Blue Sepharose column. When the Ky21 m-3 band was purified in this manner and electrophoresed in the two-dimensional system, only a single protein band was present following the first-dimension (native) electrophoresis. This band showed MDH activity and migrated to the m-3 position. In the SDS second dimension, a double spot was resolved (Figure 9B). Clearly, the m-3 band in this genotype is a hetero-

Figure 8.—2-D PAGE of MDH purified from kernels containing m-1 and m-3 bands. The positions of the MDH activity bands in the first dimension are indicated. The m-1 band is composed of 2 subunits that differ in their migrations in the SDS dimension. The m-3 band in this case is an apparent homodimer.
McMillan, Rouphakias and Scandalios (1979) recently claimed that m-3 bands of the type present in the Ky21 line, which they call “MDH3a” isozymes, represent homodimers encoded by a gene that they propose to exist closely linked to the locus we designate as Mdh1. Furthermore, they propose that “MDH3a” subunits are defective in the ability to form the heterodimers that they expect to see at the m-5 position on the basis of the Yang, Sorensen and Scandalios (1977) model. Their explanation is not compatible with the demonstration that the Ky21 m-3 band is composed of two different subunits. Since the “MDH3a” isozymes are themselves heterodimers between MDH1-A1 and MDH3-C16 subunits, it is not surprising that a hybrid band at a position intermediate to the m-3 and m-7 (MDH1-A1 homodimer) bands is not observed!

**DISCUSSION**

Our mapping studies place Mdh3, one of three loci involved in the synthesis of the mitochondrial MDH isozymes, in the distal region of the long arm of chromosome 3, approximately 2.6 recombination units from sh2. Mdh2 is included in TB-6Lb, indicating that it is located distally in the long arm of chromosome 6. Mdh1 shows linkage to wx only in the waxy-marked reciprocal translocations, T8–9 (6673) and T8–9d, indicating that it is located in chromosome 8. A modifier of the mitochondrial MDH isozymes (Mmm) maps approximately 27.5 units proximal to Adh1, in the central portion of the long arm of chromosome 1. One of the duplicate genes coding for the soluble MDH isozymes, Mdh4, is located in
the same region of chromosome 1, 29 units proximal to Adh1. Close linkage between Mdh4 and Mmm has been reported previously (GOODMAN et al. 1980). The other s-MDH gene, Mdh5, is in the short arm of chromosome 5, 20 map units distal to a2. Thus, all genes specifying the major MDH isozymes active in the mature scutellum have now been localized to particular chromosomal regions.

That the mitochondrial MDH subunits specified by the different structural genes form intergenic heterodimers was first suggested by the correlated variation and segregation of certain "intermediate" bands with variants for both types of apparent homodimers (see GOODMAN et al. 1980). In this paper, we have analyzed by two-dimensional PAGE several zymogram bands, each of which by genetic analysis, appeared to represent an intergenic heterodimer between two different m-MDH isozymes. In each case these bands were shown actually to be composed of two distinct polypeptides.

The evidence presented in this paper distinguishes between two genetic models proposed for the maize mitochondrial MDH isozymes. The two models are in partial agreement. Both postulate that the m-5 and m-3 bands in the MDH banding pattern shown in Figure 1 are homodimers coded for by separate genes and that the m-4 band represents an intergenic heterodimer. They differ in their explanations for certain "intermediate" bands, such as m-1 and m-2 in Figure 1. According to YANG, SORENSON and SCANDALIOS (1977), the m-1 band represents a homodimer specified by a gene closely linked to that encoding the m-3 subunit, and the m-2 band represents a homodimer specified by a gene closely linked to that encoding the m-5 band subunit. According to the model presented in this paper, these "intermediate" bands represent intergenic heterodimers formed between subunits encoded by independently inherited genes. The "linked gene" hypothesis predicts correlated segregation of certain bands; e.g., m-2 with m-5, m-1 with m-3. However, Figure 3 illustrates that the positions of the m-1 and m-2 bands can be varied even when the m-3 and m-5 bands are constant. In this case, variation for m-1 and m-2 is absolutely correlated with segregation for the MDH3 fast-migrating electrophoretic variant (see GOODMAN et al. 1980 for additional examples). Such a result is consistent with the hypothesis that the m-1 and m-2 bands in Figures 1 and 3 represent intergenic heterodimers.

The "linked gene" hypothesis of YANG, SORENSON and SCANDALIOS (1977) predicts that the m-1 and m-2 bands would be composed of a single type of subunit, since they are supposed to be homodimers. However, in every case we have examined, bands migrating to the m-1 and m-2 positions are each composed of two different subunits. This finding is compatible with genetic evidence indicating that such bands represent intergenic heterodimers (GOODMAN et al. 1980) and is incompatible with the YANG, SORENSON and SCANDALIOS (1977) model. Based on that model, McMILLAN, Roupakias and SCANDALIOS (1979) recently proposed that the m-3 band present in the Ky21 MDH isozyme pattern (Figure 9) is a homodimer. However, genetic analysis indicated that this particular m-3 band represents an intergenic heterodimer (GOODMAN et al. 1980) and our two-dimensional PAGE analysis of the purified isozyme clearly demonstrates that it is composed of two distinguishable subunits. It should be emphasized that isozymes that
migrate to the same position (e.g., m-3) on zymograms do not always have a similar genetic basis. In every such case, careful genetic analyses should be performed; i.e., test crosses to variants for each MDH gene followed by analyses of F2 progeny ratios.

The Mdh3 locus we describe does not exist according to the model of Yang, Sorensen and Scandalios (1977), although co-dominant variants for it have been described and its chromosomal location has now been ascertained. Our localization of Mdh3 to chromosome 3 involved two separate methodologies: B-A translocations and linkage with a known marker. It assorts independently of Mdh2 and Mdhl. Mdh2 was located by cytogenetic criteria in the long arm of chromosome 6 (Goodman et al. 1978; Newton 1979a). Our analysis involving TB-6Lb allows placement of Mdh2 in the distal region of the long arm of chromosome 6. Linkages between distally located markers and Mdh2 recently reported by McMillan, Roupaikas and Scandalios (1979) are in agreement with our findings, although those authors claim to have mapped two closely linked MDH genes, rather than just Mdh2. Mdhl was located in chromosome 8 using waxy-marked reciprocal translocations between chromosomes 8 and 9. The demonstration of separate chromosomal locations for these three genes provides additional strong support for the model of mitochondrial MDH isozymes presented in this paper; i.e., that three independently inherited genes encode the m-MDH isozymes active in the mature scutellum. It is perhaps of interest to note that two of the three genes specifying m-MDH isozymes are located in regions of chromatin where few genes have previously been described: distal 6L and chromosome 8.

The isozymes designated as mitochondrial MDHs have certain properties that distinguish them from the soluble MDH isozymes: they are localized in the mitochondria, retain activity when tissues are homogenized in a sucrose-ascorbic acid solution (Goodman et al. 1980), their electrophoretic mobilities are influenced by a modifier locus, Mmm, and they all precipitate with antibody prepared against purified MDH2 homodimers (Newton, unpublished). The soluble MDH isozymes, however, are inactivated by the sucrose-ascorbic acid solution, do not respond to the mitochondrial MDH modifier and do not cross-react with antibody prepared against mitochondrial MDH isozymes (see Ting et al. 1975; Newton, unpublished).

The genetic basis of the major soluble MDH isozymes has also been elucidated. They are specified by duplicate genes, Mdh4 and Mdh5, which assort independently (Goodman et al. 1978, 1980; Newton 1979a). Our mapping of Mdh4 and Mdh5 to separate chromosomes (1 and 5, respectively) supports the conclusions drawn from testcross analyses.

Although Yang and Scandalios (1974) showed that the weaker s-MDH trailing band (s-2 in Figure 1) is not interconvertible with the major s-MDH form (s-1) by several methods, it is not genetically independent of the major form. In patterns variant for s-MDH isozymes, we always see a faint, slower migrating band trailing the major s-MDH isozyme (see Figures 5 and 6) and we suggest that it represents a modified form of MDH4.

Several isozyme systems in maize are specified by two or more unlinked loci
including the alcohol dehydrogenases (Freeling and Schwartz 1973), catalases (Roupakias, McMillin and Scandalios 1979), glutamic oxaloacetate transaminases (Scandalios, Sorenson and Ott 1975), phosphoglucomutases (Stuber and Goodman 1979) and certain esterases (MacDonald and Brewbaker 1974). In some of these cases, functional or regulatory differences have been demonstrated. With MDH, however, it is not yet apparent why duplicate s-MDH and multiple m-MDH loci exist. Goodman et al. (1980) have constructed homozygous plants null for two of the three nuclear genes encoding the m-MDH isozymes, so that one of the multiple m-MDH genes is sufficient for survival of the plants under normal growing conditions. Nulls for one of the s-MDH genes, Mdh5, also survive quite well. Since null and electrophoretic variants have been described for each MDH locus, the MDH isozymes of maize provide an interesting system for studying the structural and regulatory evolution of duplicate genes in a eukaryote that is well-characterized genetically.

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Note added in proof: MDH from line 59 has now also been analyzed by the native-SDS 2-D PAGE. Its 2-D gel pattern is very similar to the one shown in Figure 7.

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