THE GENETIC CONTROL OF ALCOHOL DEHYDROGENASE IN MAIZE: GENE DUPLICATION AND REPRESSION*

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In an earlier paper¹ on the genetic control of alcohol dehydrogenase (ADH-EC 1.1.1.1) in maize, reference was made to the occurrence of a minor component in addition to the major ADH enzyme specified by the *Adh* gene. The concentration of this minor component can be significantly increased by growing the material under anaerobic conditions. A number of alleles of the *Adh* gene which specify isozymes differing in their migration rates have been identified. Zymograms of plant material which carry the different *Adh* alleles show a very striking correlation in the migration rates of the major and minor ADH isozymes. The most anodal minor band is always associated with the most anodal of the major bands, and the most cathodal major band is always found in genotypes which form the most cathodal minor band. A somewhat similar situation has been described for ADH in *Drosophila*.² This paper deals with the relationship between the major and minor ADH isozymes. Evidence will be presented for the existence of duplicate ADH genes. One of these genes which normally occurs in a highly repressed state specifies an enzymatically inactive polypeptide.

Methods and Materials.—The experimental techniques employed in this study are identical to those described in an earlier paper.¹ Starch gel electrophoresis was carried out at pH 8.5 in a borate buffer system. Zymograms were developed for ADH bands following a modification of the method of Dewey and Conklin.³ Immature kernels harvested 16 days after pollination were used as the source of the endosperm enzyme. Two treatments were employed for analyzing the scutellar enzyme: (1) mature kernels were soaked in water for 24 hr at 30°C, or (2) kernels were soaked for 24 hr, allowed to germinate for 24 hr on moistened filter paper in Petri dishes, then reimmersed in water for another 48 hr, all at 30°C. The former will be referred to as soaked scutellum and the latter as germinated-soaked scutellum. The scutellum was excised from the kernels, and the plumule and radical were removed. The scutellum was then squashed on the strip of filter paper which was inserted in the gels for electrophoresis. The alleles employed in this study were *Adh^S*, *Adh^F*, *Adh^{C(1)}*, *Adh^{C(m)}*, and the duplication gene *Adh^{FC}*.¹

Results.—Immature endosperm and soaked scutella from plants homozygous for the *Adh^S*, *Adh^F*, or *Adh^{C(1)}* alleles show a single intense ADH band which migrates to the anode at pH 8.5. *Adh^{C(m)}* homozygotes show a faint band at the same position in the gel as that occupied by the enzyme formed in the *Adh^{C(1)}* homozygote. Previous studies have shown that the enzyme specified by the *Adh^{C(m)}* allele is considerably less active than the enzyme formed by the other three alleles. The maize enzyme exists in the form of a dimer, and in heterozygotes involving any two of the alleles, a hybrid enzyme or allodimer is formed in addition to the two autodimers so that three enzyme bands appear in the zymogram. Plants homozygous for the *Adh^{FC}* duplication form three isozymes: *C^(m)C^(m)*, *C^(m)F*, and *FF*. *Adh^{FC}/Adh^S* heterozygotes show five rather than six bands since the

FIG. 1.—Relative intensities of the Set I and Set II ADH isozymes in the scutella of germinating kernels. (a) Right half—kernels soaked for 24 hr; (b) right half—kernels soaked 24 hr, then transferred onto moistened filter paper for 24 hr; left halves of gels (a) and (b)—kernels soaked 24 hr, on moistened filter paper for 24 hr, then reimmersed in water for an additional 48 hr. All treatments made at 30°C. Scutella are of the genotype Adh^F/Adh^F . The arrows indicate the positions of the Set II bands. The origin is at O.

migration rates of CS and FF isozymes are identical: $C^{(m)}C^{(m)}$, $C^{(m)}F$, $FF = C^{(m)}S$, FS , SS .¹ In the soaked scutellum, extremely faint bands can sometimes be detected in a more forward, faster-migrating position if the gels are developed for a prolonged period. The intensity of these faint bands can be increased relative to the more prominent ADH bands if the kernels are germinated and soaked (Fig. 1). The intensity of the forward bands is variable from line to line, but they do not attain the intensity of the more slowly migrating ADH bands.

For clarity in presentation, the prominent ADH bands specified by the various alleles will be referred to as the Set I ADH isozymes, and the faster-migrating minor bands which appear in the germinated-soaked material will be referred to as the Set II isozymes. As is the case with the Set I ADH isozymes, the number and positions of the Set II bands depend on the *Adh* genotype (Fig. 2). In the Adh^S , Adh^F , $Adh^{C(m)}$, and $Adh^{C(t)}$

homozygotes, only a single Set II band is observed, while in the heterozygotes two bands appear. Two Set II bands are also seen in zymograms of material homozygous for the Adh^{FC} duplication. In Adh^{FC}/Adh^S heterozygotes three Set II bands are observed. There is a complete correlation between the position of the Set I and Set II isozyme bands. Adh^S homozygotes form the slowest-migrating Set I and Set II bands. $Adh^{C(t)}$ and $Adh^{C(m)}$ homozygotes specify the fastest-migrating Set I and Set II isozymes. The Set II isozymes formed in $Adh^{C(m)}$ and $Adh^{C(t)}$ materials have identical migration rates. The positions of the Set I and Set II bands formed in the Adh^F homozygotes are intermediate. In contrast to the Set I condition, just two Set II bands are formed in the nonduplicate heterozygotes. Only the Set II bands formed in each parental homozygote are observed in the heterozygote. For example, an Adh^S/Adh^F heterozygote forms the same Set II isozymes as occur in Adh^S and Adh^F homozygotes. The Set II bands observed in the Adh^{FC} homozygotes are identical in migration rate to the single bands formed in Adh^F and $Adh^{C(m)}$ homozygotes. To date, no exceptions have been found to the correlation between the Set I and Set II isozymes in analysis of segregating populations from heterozygous plants and tests of diverse lines of maize carrying the Adh^S and Adh^F alleles.

The relative intensities of the two Set II isozyme bands in *Adh* heterozygotes can be quite variable from line to line.

In some germinated-soaked material in which the Set II bands are especially intense, another very faint band can be detected after prolonged development of

FIG. 2.—Zymograms showing the correlation in the positions of the Set I and Set II isozyme bands from scutella of different *Adh*₁ genotypes. (a) *Adh*₁^S/*Adh*₁^S, (b) *Adh*₁^F/*Adh*₁^F, (c) *Adh*₁^{C(v)}/*Adh*₁^{C(v)}, (d) *Adh*₁^S/*Adh*₁^F, (e) *Adh*₁^F/*Adh*₁^{C(v)}, (f) *Adh*₁^S/*Adh*₁^{C(v)}, (g) *Adh*₁^S/*Adh*₁^{C(v)}, (h) *Adh*₁^S/*Adh*₁^{C(m)}, (i) *Adh*₁^F/*Adh*₁^{C(m)}, (j) *Adh*₁^{FC}/*Adh*₁^{FC}. Bands below the arrows are the Set I isozymes, and bands above the arrows are the Set II isozymes. O indicates the origin.

FIG. 3.—Zymograms showing the position of the XX dimer (arrow) specified by the *Adh*₂ gene (see text). *Adh*₁^S/*Adh*₁^S scutellum was run on the left half of the gel, and *Adh*₁^S/*Adh*₁^{C(v)} on the right half. O indicates the origin.

the zymograms. This band (Fig. 3) migrates even faster than the Set II isozymes and occupies a constant position in the gel in all *Adh* genotypes.

The relative migration rates of the Set I and Set II isozyme bands are independent of the starch concentration. No significant difference is observed in the relative migration rates of the two isozymes in gels made up of 10 per cent and 15 per cent starch (Table 1).

Discussion.—The complete correlation in the inheritance of the Set I and Set II ADH isozymes strongly suggests that both sets are under the control of the same gene. The single-gene hypothesis is preferable over two very closely linked genes since the latter scheme would require concomitant mutations in each of two genes to give rise to the various allelic forms. *Adh*^F is the most common allele. If we assume that it is the ancestral form and all the others arose as the result of mutation of *Adh*^F, the changes to *Adh*^{C(m)}, *Adh*^{C(v)}, and *Adh*^S would require mutations in both of the genes specifying the Set I and the Set II enzymes, respectively. Furthermore, one of the linked genes never mutated without the other mutating in a like manner, resulting in a similar charge difference in both enzymes. The probability of this occurring in three different cases is so low that we are forced to the conclusion that the correlated charge difference between the Set I and Set II allelic isozymes results from the same mutational event.

We are thus faced with the problem of how a single gene can control synthesis of two different but related enzymes. The explanation which most readily comes to mind is that the Set I and Set II enzymes are composed of identical polypeptides but differ in degree of polymerization, i.e., the Set I enzyme is a dimer while the Set II represents the monomeric form, and the faster migration rate of the Set II isozyme is due to the molecular sieving property of the starch gel. However, this possibility can be eliminated on two counts. First, the migration rates of the Set

TABLE 1
COMPARISON OF MIGRATION RATES OF *Adh^F* SET I AND SET II
ISOZYMES IN GELS DIFFERING IN STARCH CONCENTRATION

	Average Migration (mm) for 3-Hr Run	
	Starch Conc. 10%	Starch Conc. 15%
Set II	48.5	32.8
Set I	34.3	23.7
Set I/Set II	0.71	0.72

I and Set II enzymes are equally affected by changing starch concentration of the gel, indicating that the enzymes do not differ significantly in size.⁴ Second, if a size factor were responsible for the difference in position of the Set I and Set II enzyme in the gel, the relative migration rates of these two should remain constant regardless of the allelic type analyzed. If the S monomer migrates twice as fast as the SS dimer because it is smaller, one would also expect the C monomer to migrate twice as fast as the CC dimer. However, this relationship does not hold (Fig. 2). The *Adh^S* Set II isozyme migrates 2.2 times as fast as the *Adh^S* Set I isozyme, while the *Adh^{C(u)}* Set II isozyme migrates only 1.3 times as fast as its respective Set I isozyme.

A second possible explanation for these results is suggested by the LDH system.⁵ An LDH polypeptide can polymerize either with homologous polypeptides specified by the same gene (types 1 and 5) or with nonhomologous polypeptides specified by a nonallelic gene (types 2, 3, and 4). Let us assume that all maize lines tested are homozygous for a gene *x* which controls the synthesis of polypeptide X. Gene *x* is not allelic to *Adh*. The hypothetical X protein has no ADH activity but is similar enough in structure to the ADH enzyme to allow for cross dimerization. The Set I isozymes are homologous dimers composed of two ADH polypeptides. It is postulated that the Set II isozymes represent heterologous dimers composed of one ADH and one X polypeptide. Thus, only the Set I enzyme will be formed when the *x* gene is not active. In tissue where both the *x* and the *Adh* genes are functional, both Set I and Set II enzymes will be formed. If the X polypeptide is more negatively charged than ADH, the Set II (X-ADH) dimers will migrate faster to the anode than the Set I (ADH-ADH) dimers. This hypothesis assumes that the ADH polypeptide retains its activity when associated in a Set II dimer with an X polypeptide. In homozygotes, as for example *Adh^S/Adh^S*, only two isozymes will be seen, a Set I SS and a Set II SX. In heterozygotes, as *Adh^S/Adh^F*, five isozyme bands with ADH activity will appear: the three Set I isozymes (SS, SF, FF), and the two Set II isozymes (SX, FX).

The appearance of the very faint, fastest-migrating band offers support for the proposed hypothesis. We suggest that this band represents the XX dimer which has retained a fraction of its ADH activity. It occupies the expected forward position in the gel, is seen only in material which gives intense Set II bands, and occurs in a constant position independent of the *Adh* genotype. As a matter of fact, this band was specifically looked for after its existence was predicted by the proposed hypothesis.

In view of this evidence for duplicate loci, gene *x* is redesignated *Adh₂*.

The conclusion drawn from the studies reported in this paper is that maize contains two nonallelic genes which specify two ADH enzymes. One of the enzymes

is active and the other much less so. The gene which specifies the enzyme with reduced activity is in a repressed state at least in those tissues where the other gene is functional. The inactive gene can be derepressed by maintaining the kernels under anaerobic conditions, completely immersed in water, after germination has been initiated. The two genes are probably of common origin since they show structural similarity in that dimerization can occur between polypeptides specified by the two genes as well as between homologous polypeptides specified by the same gene. Gene duplication must have occurred at least twice. In one strain the *Adh* gene is present in triplicate; two of the genes, *Adh*₁^F and *Adh*₁^{C(m)}, are closely linked and comprise the *Adh*^{FC} duplication¹ and the third gene is *Adh*₂. In the case of the *Adh*^{FC} duplication, both genes are active.

Gene duplication plays an important role in the evolution of genes with new functions.^{6, 7} In an organism possessing only one copy per genome of a gene concerned with a vital function, multistep mutation of this gene to a new form could not readily occur since in the process of mutating, the gene would likely suffer an impairment of its function leading to reduced survival of the organism. In those instances where gene duplication had occurred, the existing function could be carried out by one gene while the duplicate would be free to mutate to a new or improved function. However, although the selection pressure to eliminate the mutating gene from the population would definitely be reduced, it would still operate to some extent even in the latter case. The presence in the genome of a duplicate gene which specifies a nonfunctional or less efficient enzyme which may still compete for substrate would very likely be disadvantageous to the organism. On the basis of evidence presented in this paper, we propose that gene repression plays an essential role in the process of gene evolution. The selective pressure against the mutating gene would be eliminated if this gene were maintained in a repressed state during the course of change from the old to the new function. Although supporting evidence is lacking, it is possible that the two *ADH* duplications represent different stages in the process of gene evolution; *Adh*₁^{FC} being a recent duplication in which one component (*Adh*^{C(m)}) has not as yet become repressed, whereas the *Adh*₂ duplication is in a more advanced state of gene evolution.

The human hemoglobin system presents another example of gene duplication associated with gene repression. β and δ are duplicate genes.⁷ They are linked and specify closely related polypeptides which differ in only ten out of the total of 146 amino acids.⁸⁻¹⁰ In association with the α polypeptide the β chain forms the A₁ hemoglobin, and the δ chain forms A₂ hemoglobin. The δ gene is in a highly repressed state as A₂ accounts for only 2½ per cent of the total hemoglobin in the adult.

Electrophoretic analysis of isozyme and protein heterogeneity is an important and necessary part of such studies, and without it, evidence for the duplicate conditions would not have been readily detected. Thus, duplicate gene situations where one of the genes is repressed may be very common and widespread in the plant and animal kingdoms but are undetectable by most techniques of genetic analysis.

According to the proposed scheme, as long as the duplicate gene has not mutated completely to the form where it can carry out a new function, selection will tend to maintain it in the repressed state. Once it has mutated to the new desirable function, selective pressure will stabilize the gene in the derepressed state. The

new function may either complement the old function or supplant it. In the former case both genes would remain functional while in the latter, one would expect that the old gene would be repressed. McClintock¹¹ has shown that a mechanism for gene repression and derepression, which is nonspecific in that it can affect any locus, is operative in maize. It is conceivable that the *Ac-Ds* system plays such a role in gene evolution. Indeed it has been shown that *Ds* induces repression at the gene level so that not even a CRM is produced.¹²

Summary.—Evidence is presented for duplicate alcohol dehydrogenase genes in maize. One of these genes which specifies an enzyme with much reduced activity is normally repressed but can be derepressed in the scutellum. A possible role for gene repression in the evolution of genes with new function is discussed.

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