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Genetics

Regulation of *Cat1* gene expression in the scutellum of maize during early sporophytic development

(differential gene expression/temporal regulatory gene/enzyme turnover)

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ABSTRACT **A regulatory element has been identified in maize that appears to exert an effect specifically on *Cat1* gene expression in the scutellum of maize during early sporophytic development. *Cat1* encodes CAT-1 catalase, one of two forms of catalase expressed in the scutellum during this developmental time period. Density-labeling experiments indicate that the regulatory element influences the overall levels of CAT-1 protein synthesis in the scutellum but has no effect on CAT-2 protein synthesis. Immunoprecipitation experiments of *in vitro* translation products suggest that this element has an effect on the level of translatable *Cat1* mRNA associated with the scutellar polysomes. The element exhibits additive inheritance and is tissue and time specific in its action. This element, therefore, meets all the criteria of a regulatory gene and has been designated *Car2*. The element acts to regulate the temporal expression of the *Cat1* structural locus in maize.**

It is firmly established that genes are differentially expressed both temporally and spatially during eukaryotic development (1). How the cell regulates the activity of its structural genes to bring about this differential gene expression is one of the central concerns of developmental genetics. In recent years, there has been a considerable advance toward understanding the regulatory processes occurring in higher eukaryotes. Cases of genetically controlled variation in enzyme level are particularly interesting, because they allow for the identification of genetic elements with a strict regulatory function. Several regulatory genes have been identified and characterized that influence the timing and/or level of expression of various structural genes (2–5). There are several instances of distant regulatory genes (relative to the structural gene being regulated) in a variety of eukaryotes, including mice (2), maize (4, 5), and *Drosophila* (6, 7). However, proximate regulatory genes are more commonly observed in these organisms. Cases have been identified in mice (2, 8–12), maize (3, 13), *Drosophila* (14–17), and rainbow trout (18). Proximate regulatory genes exhibit additive inheritance and, in those cases tested, exert an effect on the synthesis of the enzyme being regulated (2, 3, 8–16).

The catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) gene–enzyme system of *Zea mays* L. has been under investigation for several years (for a review see ref. 19). Maize catalase is a tetrameric enzyme encoded by three distinct structural genes, *Cat1*, *Cat2*, and *Cat3*, which have been mapped to the chromosome arms 5S, 1S, and 1L, respectively (20). The catalase of maize scutella is coded for by two loci, *Cat1* and *Cat2*, which are differentially expressed in this tissue during early seedling growth (19). The expression of the two structural genes is regulated by several factors (21, 22), one of which is differential rates of synthesis and degradation of the two gene products, CAT-1 and CAT-2 (21).

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The inbred maize line R6-67 exhibits significantly increased levels of total scutellar catalase activity after the fourth day of post-germinative development, as compared to the characteristic catalase activity developmental program of most "typical" maize inbred lines examined (e.g., W64A). Biochemical and genetic analyses have shown that an increase in synthesis of CAT-2 protein in R6-67 is controlled by a distinct temporal regulatory gene, designated *Car1*, which is located 37 map units from the *Cat2* structural gene (5). The *Car1* regulatory gene exhibits additive inheritance and is tissue (scutellum) specific, time specific (acts 4 days post-imbibition), and isozyme structural gene (*Cat2*) specific in its action. Being distally located, *Car1* likely acts to modulate the expression of *Cat2* via a diffusible regulatory molecule.

This report details the characterization of a second regulatory gene, designated *Car2*, which influences the developmental program of catalase expression in the maize scutellum. Unlike *Car1*, this element influences the expression of the *Cat1* locus by affecting the levels of CAT-1 protein synthesis. This regulatory element demonstrates additive inheritance and is tissue and time specific in its action.

MATERIALS AND METHODS

Plant Material. The highly inbred maize lines, W64A and Tx303, that were used in these studies are maintained in our laboratory. Seeds were surface-sterilized by soaking in a 1% hydrochlorite solution for 10 min. They were then soaked for 24 hr in distilled water and placed in plastic containers with moistened germination paper for subsequent germination and growth at a constant temperature of 23°C in the dark.

Sample Preparation. Isolated scutella of post-germinative seedlings or the isolated endosperm or scutellum of developing kernels were ground with a mortar and pestle in glycylglycine buffer (25 mM; pH 7.4). The homogenate was centrifuged in an Eppendorf microfuge for 3 min, and the supernatant was used for either starch gel electrophoresis, spectrophotometric assays, or rocket immunoelectrophoresis.

Starch Gel Electrophoresis. Starch gel electrophoresis using the Tris citrate (1.0 M; pH 7.0) buffer system was carried out as described (23). Catalase was stained by first incubating a gel slice in 0.01% H_2O_2 for 20 min, and then the slice was rinsed and stained with a solution consisting of 1% $FeCl_3$/1% $K_3Fe(CN)_6$.

Enzyme Assays and Protein Determination. Catalase (24), isocitrate lyase (25), malate synthase (25), malate dehydrogenase (26), alcohol dehydrogenase (27), and glutamate oxaloacetate transaminase (28) were assayed spectrophotometrically as described. Protein concentration was determined according to Lowry *et al.* (29) using bovine serum albumin as a standard.

Immunoelectrophoresis. The technique of rocket immunoelectrophoresis was carried out as described (30) with minor

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modifications. Fifty milliliters of 1% agarose (Bio-Rad; low molecular weight) in Tris·HCl buffer (50 mM; pH 8.6) was boiled and then cooled to 57°C. At this point, an appropriate amount of CAT-1 antibody, prepared as described (31), was added to the solution, which was then poured onto a 23 × 12.5 cm glass plate. After cooling, samples of identical protein content were added to 1-mm-diameter wells. Electrophoresis was conducted at 4°C with 60 V for 48 hr. The gel was then pressed and washed as described (30) except that the electrophoresis buffer was used and the whole procedure was carried out at 4°C to avoid enzyme inactivation. The gel was then stained for catalase activity as described above.

Density Labeling and Density Gradient Centrifugation. Seeds (60–100) of W64A, Tx303, or the F_1 hybrid W64A × Tx303 were surface-sterilized in 1% hypochlorite for 10 min and soaked in either a solution of 10 mM KNO_3 in H_2O or 10 mM $K^{15}NO_3$ in 70% ^2H_2O (Bio-Rad) for 24 hr. The seeds were then transferred to Petri dishes containing germination paper moistened with the same solution the seeds were soaked in. Growth was allowed to proceed at 23°C in the dark until a total labeling time of either 42 or 48 hr was achieved. Afterward, the scutella from both treatments were isolated, extracted [60 scutella per 2 ml of glycylglycine buffer (25 mM; pH 7.4)], and the extract was applied to starch gels. After electrophoresis, a thin slice of the gel was stained for catalase activity and was used as a template to isolate the CAT-1 and CAT-2 homotetrameric bands from the rest of the gel (21). The fluid was extracted from the gel by centrifugation and then concentrated. Density gradient centrifugation was done by mixing the extracts with 3.0 ml of the glycylglycine buffer and 2.26 g of cesium chloride in polyallomer centrifugation tubes. Fifty micrograms of β-D-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) was added to each tube as a marker. The mean density of each sample was then adjusted to 1.300 g/ml. The samples were layered with paraffin oil and then centrifuged (160,000 × *g*, Type 65 rotor, L5-65 Beckman ultracentrifuge) at 4°C for 40 hr. After centrifugation, the tubes were punctured and 3-drop fractions (75 μl) were collected. The refractive index of every other fraction was determined with a Bausch and Lomb Abbe-3L refractometer and converted to density units.

Immunoprecipitation of Translation Products. Polysomes were isolated from the scutella of 5-day post-imbibition seedlings of W64A and Tx303 as described (32) and translated *in vitro* in a rabbit reticulocyte lysate system (Bethesda Research Laboratories) using 1 OD of polysomes per 30-μl reaction mix. Antiserum against CAT-1 was prepared (31) and used to immunoprecipitate CAT-1 from the total translation products (33). The immunoprecipitates were then subjected to electrophoresis on one-dimensional $NaDodSO_4$/polyacrylamide gels and analyzed by fluorography.

RESULTS

Catalase Developmental Programs in the Scutellum of Inbred Lines W64A and Tx303. Two distinct catalase genes, *Cat1* and *Cat2*, are differentially expressed in the scutellum of maize during early sporophytic development (19). In a typical line (e.g., W64A) CAT-1 is expressed in the dry seed and early days post-imbibition. CAT-2 production becomes evident on days 2–3 post-imbibition. When both genes are expressed, a 5-banded zymogram pattern is observed (Fig. 1*A*) due to the random association of the CAT-1 and CAT-2 subunits and the tetrameric structure of maize catalase (34). As development proceeds, CAT-1 levels decrease and CAT-2 levels increase, resulting in a shift of catalase gene expression. By day 10 post-imbibition, only CAT-2 is expressed. This shift in expression has been shown to be due, in part, to differential turnover rates (21). A variant line, Tx303, exhibits a more rapid shift in catalase gene expression than is typically observed. By days 5–6 post-imbibition, only CAT-2 is expressed in the scutellum of this line (Fig. 1*A*). Mixing experiments of day-5 Tx303 scutellar extracts with day-2 W64A scutellar extracts were carried out and gave no evidence for CAT-1 specific inhibitors or inactivators in the Tx303 extract. Rocket immunoelectrophoresis was performed on scutellar extracts of the two lines to determine the developmental profile of CAT-2 protein, specifically. Both W64A and Tx303 exhibit similar levels of CAT-2 protein in the scutellum during early development (Fig. 1*B*).

The catalase specific activity level in the scutellum normally increases to a peak on days 4–5 post-imbibition and declines thereafter. This developmental program for catalase is primarily the result of CAT-2 production. There are no significant differences in the specific activity levels for scutellar catalase between W64A and Tx303 (Fig. 2*A*). Thus, the accumulated data suggest that the altered pattern of catalase expression in the scutellum of Tx303 is due to an alteration in *Cat1* gene expression.

Expression of Other Enzymes in the Scutellum of W64A and Tx303. Scutellar catalase is known to be associated with the glyoxysomes, an organelle involved in lipid metabolism during seedling growth (19, 22). To determine whether an alteration in the developmental expression of the glyoxysomes

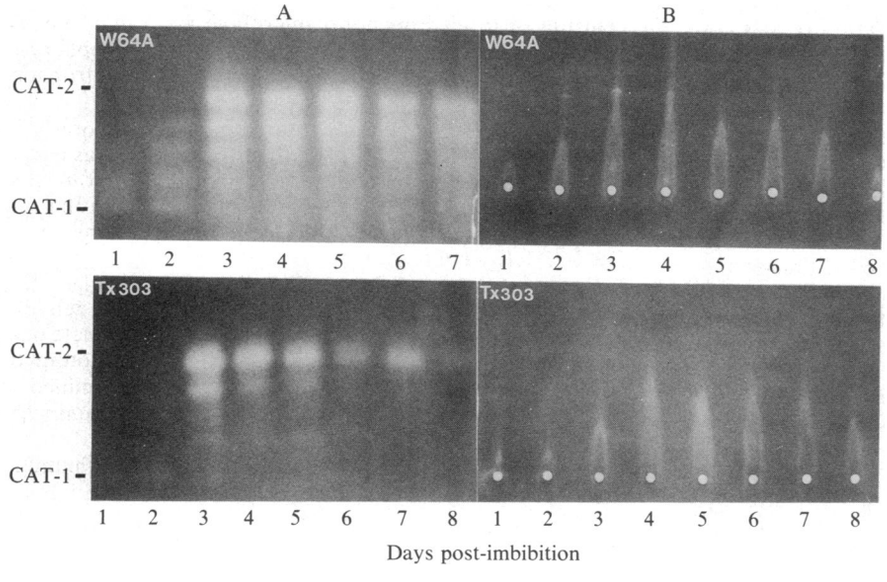

FIG. 1. (*A*) Zymogram analysis of catalase gene expression in the scutellum of W64A and Tx303 during early sporophytic growth. When both the *Cat1* and *Cat2* genes are expressed, a 5-banded isozyme pattern is generated, due to the tetrameric nature of catalase and intergenic complementation. As development progresses, there is a shift in catalase gene expression from CAT-1 to CAT-2. As shown, the shift in gene expression occurs more rapidly in Tx303 than in the more typical line W64A. (*B*) Rocket immunoelectrophoresis, using monospecific CAT-2 antibodies, of scutellar extracts from W64A and Tx303 during the first 8 days post-imbibition. The gels were stained for catalase activity. The height of each rocket is proportional to the amount of CAT-2 protein present in each sample. Identical amounts of total protein were added to each well. Migration is anodal.

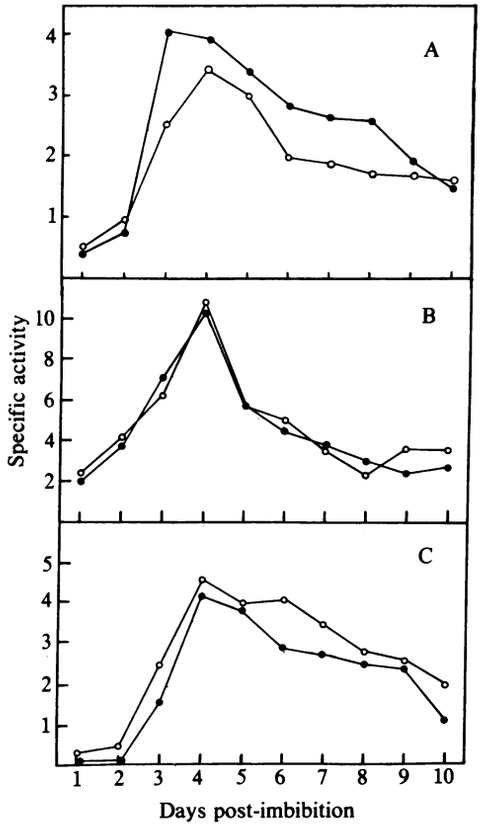

FIG. 2. Developmental programs in the scutellum of W64A (○) and Tx303 (●) for catalase (*A*), malate synthase (*B*), and isocitrate lyase (*C*) during the first 10 days post-imbibition. Catalase activity is primarily due to *Cat2* gene expression. Along with catalase, malate synthase and isocitrate lyase are component enzymes of the glyoxysome, an organelle involved in lipid metabolism in seeds. Each point represents the mean of three independent experiments, and the standard errors indicated no significant differences between the two lines.

leads to the altered pattern of catalase expression in Tx303, extracts were assayed for malate synthase and isocitrate lyase, two component enzymes of the glyoxysome. No differences in activity profiles of these enzymes were found between the two lines (Fig. 2 *B* and *C*). The developmental programs for other enzymes expressed in the scutellum were also compared and no differences were found for malate dehydrogenase, alcohol dehydrogenase, or glutamate oxaloacetate transaminase. Comparison of various growth parameters such as root growth and shoot growth also revealed no differences between the lines.

Expression of Catalase in Other Tissues of W64A and Tx303. CAT-1 is the only form of catalase expressed in the developing maize kernels. The developmental pattern of catalase specific activity in the endosperm and scutellum of developing kernels of W64A and Tx303 is shown (Fig. 3), and no differences exist between the two lines. Therefore, the altered pattern of *Cat1* gene expression is both tissue and time specific, being restricted to the scutellum during early sporophytic development.

Biochemical Characterization of Purified CAT-1 from W64A and Tx303. CAT-1 was purified from the two lines as described (31). Both lines carry the same CAT-1 allelic variant (*Cat1V*) and the purified proteins do not differ with respect to specific activity, antigenic cross-reactivity with CAT-1 antiserum, pH optimum, pH stability, thermostability, and sensitivity to various known catalase inhibitors (data not shown).

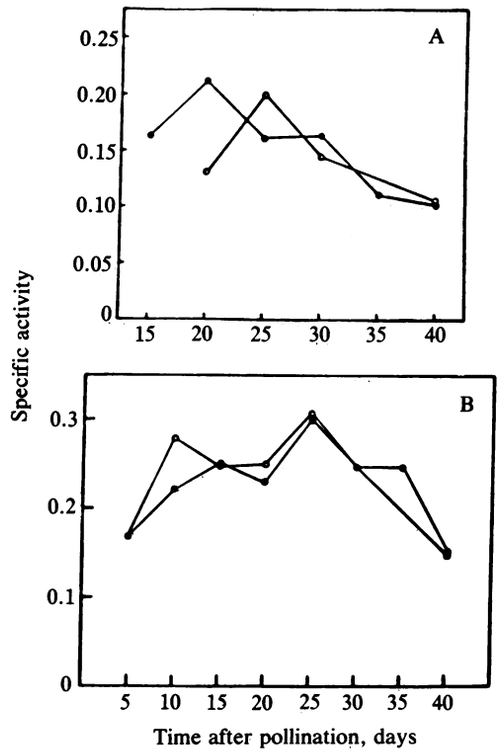

FIG. 3. Developmental profiles for catalase activity in the scutellum (*A*) or the endosperm (*B*) of developing kernels of W64A (○) and Tx303 (●) on various days after pollination. CAT-1 is the only form of catalase expressed in either of these tissues. Each point represents the mean of three independent experiments.

Density Labeling Experiments. Density labeling experiments were carried out to compare the levels of CAT-1 protein synthesis in the scutellum of the two lines. The results of

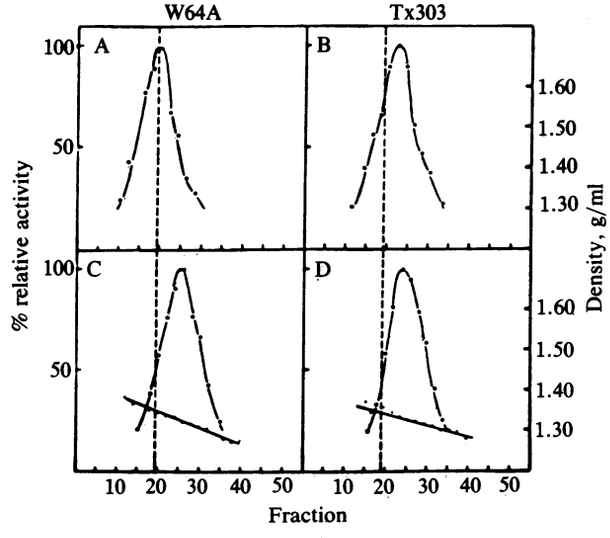

FIG. 4. Results from a representative density labeling experiment after isopycnic equilibrium centrifugation in CsCl. W64A or Tx303 seeds were grown in either 10 mM KNO_3 in H_2O (*C* and *D*) or 10 mM $K^{15}NO_3$ in 70% ^2H_2O (*A* and *B*) for 42 hr. The CAT-1 homotetramer was separated from the CAT-2 homotetramer on preparative starch gels and isolated. The CAT-1 extract was then centrifuged on density gradients. As shown in this figure, CAT-1 from W64A exhibits a density shift of 0.021 g/ml when grown in the labeling medium as compared to the unlabeled control. CAT-1 from Tx303 only exhibits a density shift of about one-half this value when grown in the labeling medium as compared to the unlabeled control. The dotted line represents the internal β-galactosidase marker.

Table 1. Density shift for catalase after isopycnic equilibrium centrifugation in CsCl

Line	Labeling time, hr	Density shift, g/ml CAT-1	Density shift, g/ml CAT-2
W64A	42	0.0215 ± 0.0015	0.0227 ± 0.0018
Tx303	42	0.0110 ± 0.0006	0.0247 ± 0.0017
W64A	48	0.0220 ± 0.0010	0.0240 ± 0.0030
Tx303	48	0.0115 ± 0.0005	0.0250 ± 0.0020
W64A × Tx303	42	0.0156 ± 0.0005	0.0224 ± 0.0015

a representative experiment with CAT-1 are shown (Fig. 4). The CAT-1 from W64A exhibits a density shift of ≈0.021 g/ml when grown in the labeling medium as compared to the unlabeled control. However, the CAT-1 from Tx303 exhibits a density shift of only ≈0.011 g/ml when grown in the labeling medium. The summary of a series of similar experiments for both CAT-1 and CAT-2 is presented in Table 1. CAT-1 from W64A consistently incorporated about twice as much label as did CAT-1 from Tx303, resulting in a greater density shift for W64A CAT-1. CAT-2 was always labeled to about the same extent in both lines. The labeling was carried out for both 42 and 48 hr to verify the fact that the populations of CAT-1 and CAT-2 molecules were fully labeled. Similar results were obtained using either incubation time.

Genetic Studies. The results of a genetic analysis of the rapid shift (as seen in Tx303) vs. the slow shift (as seen in W64A) of catalase gene expression (from CAT-1 to CAT-2) in the maize scutellum are summarized in Table 2. Zymogram analysis was conducted on day 5 post-imbibition scutella, because it is easiest to distinguish the rapid- vs. slow-shift phenotypes on this day (Fig. 1*A*). It is suggested by the zymogram data (Table 2) that the rapid-shift phenotype of Tx303 scutella may be controlled by a single gene exhibiting recessive inheritance. It is possible, however, that the rapid-shift phenotype may in fact be inherited in an additive manner, because it would have been difficult to identify an "intermediate" shift in gene expression (expected with additive inheritance) on the zymograms used for screening during this genetic analysis. To clarify whether the inheritance of this regulatory element exhibits either dominant/recessive or additive inheritance, F_1 seeds of W64A × Tx303 were density labeled and analyzed as before. The results summarized in Table 1 show that the density shift of CAT-1 in the F_1 hybrid is intermediate between the parental lines. Therefore, the regulatory element affecting CAT-1 synthesis demonstrates additive inheritance. The genetic element responsible for this trait has been designated *Car2* (*c*atalase *r*egulator-2).

Table 2. Genetic analysis of the "shift" phenotype of catalase expression in the scutellum of maize

Cross	Phenotype* Slow shift	Phenotype* Rapid shift	χ^2	p
R667†	35	0	—	—
Tx303	0	33	—	—
Tx303 × R667	26	0	—	—
R667 × Tx303	31	0	—	—
(Tx303 × R667) × Tx303	59	58	0.008	>0.90
(Tx303 × R667) × R667	109	0	0	>0.95
(R667 × Tx303) × Tx303	72	58	1.510	>0.20
(R667 × Tx303) × R667	103	0	0	>0.95
(Tx303 × R667) × (Tx303 × R667)	154	58	0.629	>0.30
(R667 × Tx303) × (R667 × Tx303)	129	52	1.360	>0.20

*The scutella from seeds 5 days post-imbibition were screened on starch gels.
†This line has a slow-shift phenotype as in W64A.

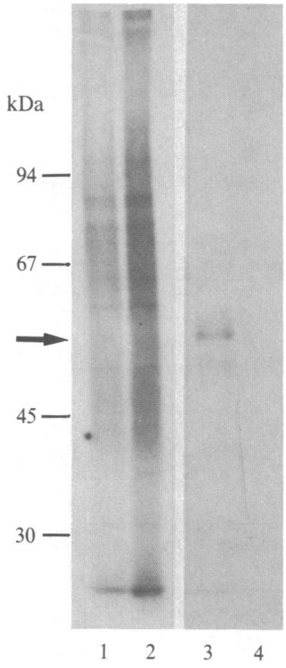

FIG. 5. Polyacrylamide gel electrophoresis of anti-CAT-1 immunoprecipitated *in vitro* translation products produced by polysomes isolated from 5-day post-imbibition scutella of W64A and Tx303. Lanes: 1, total translation products of W64A polysomes; 2, total translation products of Tx303 polysomes; 3, translation products of W64A polysomes immunoprecipitated with anti-CAT-1 antiserum; 4, translation products of Tx303 polysomes immunoprecipitated with anti-CAT-1 antiserum. Arrow indicates the purified CAT-1 marker with a subunit molecular size of 60 kDa.

Immunoprecipitation of Translation Products. In preliminary studies designed to identify the level of gene regulation at which *Car2* operates, polysomes were isolated from the scutella of 5-day-old seedlings of W64A and Tx303. *In vitro* translations were carried out using the rabbit reticulocyte lysate system. Both polysome preparations were equally active in the translation reaction (7.8×10^6 cpm incorporated per 1 OD of polysomes). CAT-1 was immunoprecipitated from the total translation products and subjected to electrophoresis on one-dimensional polyacrylamide gels, which were then analyzed by fluorography. The results (Fig. 5) indicate that the polysomes of Tx303 produce significantly less immunoprecipitable CAT-1 than the polysomes of W64A. This suggests that the overall levels of translatable CAT-1 mRNA associated with the polysomes of Tx303 are much lower than that observed in the polysomes of W64A. Therefore, it appears that *Car2* operates by affecting the amount of translatable *Cat1* mRNA produced by the *Cat1* structural gene in the scutellum after germination.

DISCUSSION

Understanding the genetic and epigenetic factors that regulate differential gene expression in higher eukaryotes is one of the major challenges facing experimental biologists today. The identification and characterization of regulatory genes that control different aspects of gene expression will be a valuable contribution toward this end. In recent years, it has become apparent that the structural genes of higher eukaryotes can be influenced by either proximally or distally located regulatory loci whose sole apparent function is to regulate the expression of specific structural genes (2–5). The distally located elements most likely act by producing diffusible regulatory molecules. They demonstrate dominant/recessive or additive inheritance and affect either the synthesis or degradation of the gene product under regulation (2–7). On the other hand, all proximate regulatory elements exhibit additive inheritance and, in all cases examined, influence synthesis levels; therefore, these elements likely act at the trancriptional or post-transcriptional level (2–5, 8–18).

A number of genetic systems have been found in higher

plants in which a regulatory locus appears to control the activity or level of expression of a specific structural gene product (3). This report describes the characterization of a genetic regulatory locus that influences the developmental program of catalase expression in the scutellum of maize. The characterization of this genetic element was made possible through the analysis of a variant line, Tx303, which demonstrates an altered pattern of catalase expression in the scutellum during early seedling development (Fig. 1A). Specifically, this element affects the overall levels of synthesis of CAT-1 protein, which is encoded by the *Cat1* gene, in the scutellum during early sporophytic growth. The variant in Tx303 appears to accelerate the decline in CAT-1 activity normally observed in this tissue. *Cat2*, a second catalase gene of maize expressed in the scutellum, is unaffected by this element as judged by specific activity levels, rocket immunoelectrophoresis, and density labeling experiments. This regulatory locus exhibits additive inheritance as shown by density labeling experiments and is tissue and time specific in its action. This element has been designated *Car2* (catalase *r*egulator 2).

It was not possible to determine whether *Car2* is located proximal or distal to the *Cat1* structural gene. This was because of the difficulty in scoring a "rapid shift" vs. a "slow shift" in catalase gene expression with allelic combinations other than CAT-1V and CAT-2Z, the allelic combination found in both W64A and Tx303. Therefore, linkage studies proved to be ambiguous.

Immunoprecipitation of CAT-1 from the total *in vitro* translation products produced by polysomes of W64A and Tx303 (5-day post-imbibition scutella) suggest that *Car2* influences the levels of translatable *Cat1* mRNA associated with the scutellar polysomes. It is possible that it accomplishes this by regulating the transcription rate of the *Cat1* gene. However, alternative possibilities such as *Car2* affecting *Cat1* mRNA processing or stability or affecting the ability of CAT-1 mRNA to associate with ribosomes also exist.

The catalase gene–enzyme system of maize represents an excellent model with which to analyze gene expression in a higher eukaryote, because the genes are highly regulated during the development of the corn plant (19). The developmental program for maize scutellar catalase activity results from the differential expression of two *Cat* genes, *Cat1* and *Cat2*. The expression of these two genes is regulated by several factors including an endogenous maize catalase inhibitor, hormones (abscisic acid), and differential turnover rates (19, 21, 22). Also, maize scutellar catalase activity is influenced by two temporal regulatory genes. The first, *Car1*, specifically affects *Cat2* gene expression (5) and the second *Car2*, affects expression of the *Cat1* gene, specifically. Both loci demonstrate additive inheritance. The *Car1* gene is located distant to the *Cat2* gene and presumably functions via a diffusible regulatory molecule. The location of *Car2*, relative to the *Cat1* gene, is presently unknown. Preliminary data suggest that *Car2* is located proximal to the *Cat1* gene and may represent either a closely linked regulatory site or a promoter region contiguous with the *Cat1* structural gene. However the possibility that *Car2* is unlinked or loosely linked to the *Cat1* locus has not been eliminated. Eventual sequencing of the 5′ upstream region of the *Cat1* genes from the two lines will help to clarify this matter. *Car1* is a *trans*-acting regulatory gene, whereas it is not yet possible to determine whether *Car2* is *cis*- or *trans*-acting. It is apparent that the final realization of the catalase developmental program in maize scutella is attained through extensive regulation (both genetic and epigenetic) of the expression of the *Cat1* and *Cat2* genes.

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