Analysis of xanthine dehydrogenase mRNA levels in mutants affecting the expression of the *rosy* locus

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ABSTRACT

Xanthine dehydrogenase (XDH) mRNA levels were measured in a number of mutants and natural variants affecting XDH gene expression. Two variants, *ry*⁺⁴ and *ry*⁺¹⁰, contain cis-acting elements which map to a region flanking the 5' end of the XDH gene. *Ry*⁺⁴, which has 2-3 times more XDH protein than a wild type strain, has 3.2 times more XDH mRNA. *Ry*⁺¹⁰ has 50% of the wild type XDH level and 54% of the wild type XDH mRNA level. Three *rosy* mutants which map within the structural gene were also examined. Two of these had little if any XDH mRNA, but the third mutant had 1.3 times more XDH mRNA than wild type flies. Another mutant, *ry*², which contains no XDH protein and has a 9KB transposable element inserted into the XDH gene, has normal levels of XDH mRNA transcripts which are also the same size as those found in the wild type strain. Changes in XDH mRNA levels were measured during *Drosophila* development and found to parallel changes in the amount of XDH protein. In addition, there were no large changes in the size of XDH mRNA during development.

INTRODUCTION

The XDH gene is one of the best characterized genetic units in *Drosophila*. The coding sequences of this gene are located at 3-52, the *ry* locus (1,2). Xanthine dehydrogenase negative flies are readily selected as *rosy* mutants because of their visible dull red eye color. Moreover, they behave as conditional lethals when reared in purine-enriched media, permitting high resolution recombinational analyses which have been used to generate a detailed genetic map of the XDH gene (3). Further studies estimated the boundaries of the XDH coding element by mapping a large number of unambiguous structural mutants representing XDH-negative *rosy* eye color mutant sites, sites of electrophoretic variants, and leaky mutant sites (4).

A class of natural variants has been described which are promising candidates for regulatory mutants which affect the expression of the XDH gene. One of these, *ry*⁺⁴, was found to have a 2-3 fold increase in XDH enzyme activity compared to a standard wild type strain, *ry*⁺⁵. Another variant, *ry*⁺¹⁰, has about half the wild type activity (5). Fine structure mapping

experiments have revealed the presence of two separable sites, i409 and i1005, which are responsible for the variation seen in ry⁺⁴ and ry⁺¹⁰. These sites map close to, but outside, the genetic boundaries for the structural information of the XDH gene. Since the variants have increased and decreased amounts of XDH enzyme molecules, as defined by immunological criteria (6), it is probable that these cis-acting sites define flanking DNA sequences which serve in some manner to regulate the expression of the XDH gene.

In addition to these putative regulatory variants, there are a number of alleles located within the structural limits of the XDH which result in decreased XDH enzyme activity. These mutants have varying amounts of cross reacting material (CRM), ranging from undetectable to nearly wild type levels. Thus far, studies concerning both structural and putative control variants for XDH have compared enzyme activity levels with levels of CRM. The underlying assumption in these studies has been that CRM levels are reflective of XDH mRNA levels. In an extension of these studies we have used a cloned genomic fragment containing the entire XDH gene (7) to measure XDH mRNA levels in a group of rosy mutants by Northern blot analysis. We have analyzed X-ray and EMS induced mutants, as well as spontaneous mutants.

While there are no simple a priori expectations concerning the effects of these mutants on XDH mRNA accumulation, one might expect to identify several classes of mutants. These would include possible nonsense or missense mutants containing wild type levels of XDH mRNA but no CRM for XDH, deletion or insertion mutants in which the size of the XDH mRNA is altered, and mutants in which XDH mRNA accumulation is altered. This latter class would be particularly useful in a molecular analysis of XDH gene expression.

Another important aspect of regulation at the rosy locus concerns changes in the expression of XDH activity during Drosophila development. Previous studies have shown that XDH enzyme activity levels change at different times during development (4,8). We have extended these studies by measuring XDH mRNA levels at several times during Drosophila development to determine if changes in XDH enzyme activity are a reflection of XDH RNA levels, and therefore probably also a reflection of XDH gene transcription, or the result of translation of preexisting XDH mRNA. Although changes in enzyme activity during development generally reflect mRNA levels, there are enough exceptions to warrant measuring developmental changes in XDH mRNA accumulation prior to undertaking a detailed molecular analysis of XDH gene expression during development. This is particularly important in view of the recent demonstration that cloned genes inserted into the germline of Drosophila by

transposon mediated transformation are expressed in a developmentally correct manner (9,10,11).

MATERIALS AND METHODS

Drosophila strains

The mutants ry²⁶, ry⁶⁰³, ry², and ry⁶⁰ were a gift from V. Finnerty. The ry⁷⁷ⁱ strain was a gift from J. Bell and M. Green. The ry mutant strains, with the exception of ry⁷⁷ⁱ, are fully described in Lindsley and Grell (12) and Gelbart *et al.* (13). Ry⁷⁷ⁱ was generated from a P x M cross (M. Green, personal communication). The wild type isolalleles ry⁺⁴, ry⁺⁵, and ry⁺¹⁰ were a gift from V. Finnerty and are described in Chovnick *et al.* (4).

Stocks of mutant flies were routinely checked for contamination by wild type flies by examination for wild type eye color. Flies were maintained on a standard cornmeal-agar-sucrose medium at 25°C under a 12 hour light:12 hour dark period.

Staging of Organisms

Embryos, first and second instar larvae were collectively designated as early stage and collected by allowing adults to oviposit on grape agar plates for 2 hours and then harvesting the progeny 44 hours later. Third instar larvae were collected by transferring stock cultures to new media at an approximate density of 200 flies per bottle and allowing them to oviposit for 6 hours. Early third (3E) and late third (3L) larvae were collected 72 and 96 hours after the midpoint of the ovipositing period. Pupae were collected by setting up similar cultures and collecting organisms 12 hours after pupation and 72 hours after pupation, respectively. Newly eclosed adults (NEA) were collected 6-8 hours after emergence. After collection, flies from different stages were washed free of media, checked for contamination by flies from other stages, treated with diethylpyrocarbonate, weighed, frozen in liquid nitrogen, and stored at -80°C.

RNA Isolation

RNA was isolated by grinding frozen flies to a fine powder in a mortar and pestle, suspending the powder in 20 volumes of homogenization buffer (6M guanidine, 50mM potassium acetate, 10mM dithiothreitol), and homogenizing the suspension in a blender for 20 seconds. The solution was centrifuged at 20,000 rpm for 10 minutes and 0.6 volumes of ethanol added to the supernatant. After 2 hours at -20°C, RNA was pelleted at 5,000 rpm for 10 minutes and resuspended in 0.5 volumes homogenization buffer. RNA was reprecipitated as described above and dissolved in .1 volume of homogenization buffer. Twenty

volumes of 2M lithium chloride were added and the solution placed at 4°C overnight. RNA was again pelleted and suspended in 10mM Tris, 1mM EDTA. After a 30 minute incubation with 100µg/ml pronase the RNA was extracted once with phenol/chloroform and once with chloroform. Poly (A) RNA was prepared by chromatography on oligo dT cellulose, and generally constituted 1.8 - 2.0% of the total RNA.

Northern Blot Analysis

Poly(A) RNA was denatured and fractionated on denaturing formaldehyde gels as described by Goldberg (14), using bacterial and animal cell ribosomal RNAs as size markers. RNA was transferred to nitrocellulose paper according to Thomas (15). Baked filters were prehybridized for 4-6 hours in a prehybridization mix containing 50% formamide, 4XSET, 0.1% SDS, 0.1% sodium pyrophosphate, 2X Denhart's solution, and 10µg/ml denatured calf thymus DNA.

A probe was prepared by nick translating an 8.1 kb genomic DNA fragment containing the xanthine dehydrogenase gene (a generous gift from W. Bender). This probe was hybridized to the filter overnight and then washed in prehybridization mix for 1 hour, followed by four 1 hour washes in 1X SET, 0.1% SDS, 0.1% sodium pyrophosphate. The filter was dried and exposed for varying lengths of time to X-ray film in the presence of an intensifying screen.

Quantitation of Autoradiographs

The relative intensity on an autoradiograph produced by hybridization of the radioactive probe to mRNA was quantified by computerized densitometry. A TV camera was focused on the autoradiograph, and the image captured by a digisector board (The Micro Works, California) using an Apple II Plus computer. The relative intensity of each spot on the autoradiograph was then quantified with the aid of a program provided by C. Mariash. RNA isolated from newly eclosed *ry*⁺⁵ adults was included on each gel as a standard to which other signals were compared. To insure that film darkening was a linear response to the amount of radioactivity present on the filter, exposure and development times were carefully controlled. One of us (16) has previously shown that under these conditions film darkening is directly proportional to the amount of radioactivity present. Spradling and Mahowald (17) have demonstrated that quantifying the amount of radioactive probe bound to a filter is a reliable estimate of the concentration of molecules complementary to the probe on the filter.

Enzyme Assays and Immunophoresis

The fluorimetric assay for XDH was performed essentially as described by

Chovnick *et al.* (3). Rocket immunophoresis was done as described by Weeke (18), using anti-XDH antibody prepared by C. Warner.

RESULTS

XDH mRNA Levels in Putative Regulatory Mutants

In this study XDH mRNA levels from several ry mutant alleles were compared with levels of XDH mRNA in a standard wild type, ry⁺⁵ (4). This strain was chosen to represent a standard wild type because its XDH enzyme activity level is similar to that of the majority of other strains assayed. The first strains chosen for comparison were the regulatory variants ry⁺⁴ and ry⁺¹⁰. The ry⁺⁴ strain has 2-3 fold greater than normal enzyme activity, while ry⁺¹⁰ has about 50% of normal activity. The variation in activity has been shown to result from a cis-acting regulatory region which maps outside the genetically determined boundaries of the XDH structural gene. Furthermore, immunological criteria indicate that these variants affect the number of enzyme molecules produced (6,19).

Our stock of ry⁺⁴ was assayed for XDH enzyme activity and shown to have a 3.4 fold increase compared with the wild type strain, ry⁺⁵, while ry⁺¹⁰ had only half the enzyme activity of the wild type strain. We have confirmed by quantitative immunophoresis that this difference is reflected in protein levels. These results are shown in Table 1. To determine if ry⁺⁴ and ry⁺¹⁰ also contain altered numbers of XDH mRNA transcripts, we measured XDH mRNA levels in ry⁺⁴, ry⁺⁵, and ry⁺¹⁰, using Northern blot analysis. Poly(A) RNA from late third instar larvae was fractionated on a formaldehyde denaturing

Table 1. Cross reacting material for XDH found in strains used in this study. CRM is expressed as a percentage of that found in the standard wild type, ry⁺⁵.

<u>Strain</u>	<u>Type</u>	<u>CRM</u>
<u>ry</u> ⁺⁵	natural variant	100%
<u>ry</u> ⁺⁴	natural variant	240%
<u>ry</u> ⁺¹⁰	natural variant	50%
<u>ry</u> ²	spontaneous	0%
<u>ry</u> ²⁶	X-ray	0%
<u>ry</u> ⁴¹	X-ray	70%
<u>ry</u> ⁶⁰³	EMS	0%
<u>ry</u> ⁶⁰	X-ray	0%
<u>ry</u> ⁷⁷¹	P x M Cross	0%

Figure 1A. Accumulation of XDH mRNA in the regulatory variants ry^4 and ry^{+10} . RNA isolated from late third instar larvae was fractionated on a denaturing agarose gel, transferred to nitrocellulose, and probed with nick-translated DNA containing the XDH gene. Animal and bacterial ribosomal RNAs were used as size markers on each gel: 28S size RNA is denoted by the arrow. Each lane was loaded with 30 μ g of RNA except lane 4, which contained 50 μ g. Lane 1, ry^{+5} ; lane 2, ry^{+4} ; lane 3, ry^{+10} ; lane 4, ry^{+5} .

Figure 1B. Analysis of actin mRNA levels in ry^{+4} , ry^{+5} , and ry^{+10} . The filter used in Figure 1A was boiled to remove bound probe and rehybridized with labelled Drosophila actin DNA.

gel, transferred to nitrocellulose paper, and hybridized to a nick-translated 8.1KB SalI fragment containing the entire XDH gene. The results are shown in Figure 1A. In lane 2, ry⁺⁴ is shown to contain significantly more XDH mRNA than the wild type ry⁺⁵ (lane 1), and ry⁺¹⁰ contains less mRNA (lane 3). The amount of hybridization in each lane was quantified using computerized densitometry as described in Materials and Methods. Ry⁺⁴, which has 3.4 fold more enzyme activity than ry⁺⁵, has 3.2 fold more mRNA. Ry⁺¹⁰, with only half the enzyme activity of ry⁺⁵, has 54% of the wild type levels of XDH mRNA. Clearly these two variants, which map outside the structural region of the XDH gene, exert their effect at the level of RNA accumulation.

Although the same amount of RNA was loaded per well, as judged by OD₂₆₀ measurements, the possibility exists that slightly different amounts of poly(A) RNA may be present in each RNA preparation. This could result from differing contamination of the poly(A) RNA with ribosomal RNA in the various RNA preparations or from slight inaccuracies in the optical density measurements. These experiments were repeated several times and with different preparations of RNA to minimize this possibility, and we also used an internal standard to insure that each lane contained the same amount of poly(A) RNA. The filter used to generate the data shown in Figure 1A was boiled to remove radioactive material and rehybridized with labelled DNA containing Drosophila cytoplasmic-specific actin sequences (a gift from E. Fyrberg). There should be no difference in actin mRNA content in the various rosy mutants, and thus each lane should produce the same hybridization signal. The results from this experiment are shown in Figure 1B. When the intensity of hybridization was quantified by computerized densitometry, there was never more than a 10% difference between lanes, indicating that each lane does indeed have comparable amounts of poly(A) RNA.

XDH mRNA Levels in Intragenic Mutants

A number of ry mutants have been classified as intragenic lesions by recombinational analysis (19). All of the intragenic mutants we examined displayed the mutant ry eye color phenotype, and are considered null activity mutants, since less than 1% activity is normally sufficient to produce the wild-type eye color (5). We would expect to be able to place these mutants into at least three general classes: mutants affecting the accumulation of message, potential nonsense or missense mutants in which mRNA levels are normal but CRM is reduced, and deletion or insertion mutants which result in altered mRNA size. We have compared XDH mRNA levels with levels of CRM in several of these mutants.

Figure 2. Accumulation of XDH mRNA in two intragenic mutants, ry⁴¹ and ry⁶⁰³. RNA was isolated from newly eclosed adults and treated as described in Figure 1. Lane 1, ry⁺⁵ (15μg); lane 2, ry⁺⁴¹ (30μg); lane 3, ry⁶⁰¹ (30μg).

Mutants tentatively classified as affecting message accumulation are ry⁴¹, ry⁶⁰³, and ry²⁶. The ry⁴¹ mutant has 70% of wild type CRM, while ry⁶⁰³ and ry²⁶ have no detectable CRM (Table 1). Northern analysis of RNA isolated from newly eclosed adults of these strains is shown in Figure 2. In lane 3 Ry⁶⁰³ is shown to contain less than 5% of wild type XDH mRNA. This is consistent with its lack of CRM and enzyme activity and indicates that this mutant affects mRNA accumulation rather than translation. Ry⁴¹ (lane 2) gives somewhat surprising results. It appears to have slightly increased levels of transcripts when compared to the wild type (130% as quantified by computerized densitometry), yet has only 70% of CRM and no enzyme activity. Either this intragenic mutation causes increased transcription of the XDH gene or, more likely, acts to increase the stability of the message. The ry²⁶ allele, shown in Figure 3 (lane 3), is similar to ry⁶⁰³ in that it has no CRM and no enzyme activity. It too has decreased levels of XDH mRNA, but the effect of the mutation on mRNA accumulation is not as severe as ry⁶⁰³ (also shown in lane 5 of Figure 3 for comparison).

XDH mRNA Levels in Insertion and Deletion Mutants

Several XDH mutants have been classified as insertion or deletion mutants. One of these, ry², is a mutant of spontaneous origin and found to be the result of the insertion of a B104 transposable element into the structural portion of the XDH gene (W. Bender, personal communication). Ry² is CRM-less for XDH and yet RNA isolated from newly eclosed adults of this strain contains XDH mRNA of normal size and amount, as shown in Figure 3, lane 2. The insertion of a transposable element might not alter the transcription or

Figure 3. Accumulation of XDH mRNA in several intragenic mutants. RNA was isolated from newly eclosed adults and treated as described in Figure 1. Each lane contains 30 μ g RNA. Lane 1, ry⁺⁵; lane 2, ry²; lane 3, ry²⁶; lane 4, ry⁶⁰; lane 5, ry⁶⁰³; lane 6, ry⁷⁷¹.

stability of the mRNA, but one would expect to see a message of increased size.

The ry⁶⁰ allele is also CRM-less (Table 1) and has been shown by restriction mapping of the cloned gene to be a 1kb deletion (W. Bender). Analysis of ry⁶⁰ transcripts, shown in lane 4 of Figure 3, reveals an XDH mRNA approximately 1 KB smaller in size than wild type XDH mRNA. Analysis of RNA isolated from another mutant, ry⁷⁷¹, (lane 6 of Figure 3) also shows an XDH message of smaller than normal size. This mutant, which is CRM-less, was generated by a P x M cross and was never observed to revert (M. Green, personal communication). It is likely that this mutation arose by the excision of a transposable element in which some XDH structural sequences were also removed.

The reproducible presence of a small amount of wild type size message in both ry⁶⁰ and ry⁷⁷¹ is puzzling. The mutant stocks were carefully examined by eye and were not found to be contaminated with flies containing wild type eye color. Since the 8.1Kb SalI fragment used as a probe also contains sequences adjacent to the XDH gene, we investigated the possibility that sequences from a closely adjacent gene might be hybridizing to a message of similar size to XDH mRNA. The 8.1 KB fragment was digested with EcoRI and a

4.6KB internal fragment was electroeluted and used as a probe. This fragment contains sequences located entirely within the structural limits of the XDH gene. The results obtained with this probe were identical to those obtained with the 8.1KB probe (data not shown). Hybridization of the probe to genomic DNA under conditions of low stringency only results in the expected XDH band, eliminating the possibility that the probe is hybridizing to a mRNA of similar size as the XDH mRNA produced from another locus. At this time we have no convincing explanation for the presence of this wild type size message.

Figure 3 also shows the presence of low molecular weight RNAs which hybridize with the XDH probe. These RNAs are 4S or smaller in size, and their appearance is variable from one experiment to another. They are not the result of overall RNA degradation since they were not present if actin sequences were used as probe (data not shown). They may represent products of XDH processing.

XDH mRNA Accumulation during Drosophila Development

We have also compared levels of XDH mRNA with corresponding levels of XDH enzyme activity during Drosophila development. Since XDH enzyme activity levels show stage-specific changes during development it was of interest to determine whether these changes in enzyme activity levels are a reflection of mRNA accumulation or due to the activation of pre-existing RNA transcribed at an earlier time. Thus mRNA was isolated from several developmental stages as described in Materials and Methods and analyzed for XDH mRNA levels as described earlier. The results, shown in Figure 4, indicate that XDH mRNA is not detectable in embryos, first or second instar larvae. The message first appears in early third instar larvae and steadily increases in amount, reaching a peak in late third instar larvae. The level of XDH mRNA drops abruptly during pupation, and peaks again in newly eclosed adults. In Figure 5 relative levels of XDH mRNA and enzyme accumulation are compared with one another. There is close agreement between the developmental profiles of both mRNA accumulation and enzyme activity, indicating that translational control mechanisms do not play a significant role in the expression of the XDH gene during Drosophila development.

XDH mRNA accumulation during development was also measured in the regulatory mutants, ry⁺⁴ and ry⁺¹⁰. The overall developmental profile of XDH mRNA accumulation in these variants was also identical to the wild type pattern, with ry⁺⁴ exhibiting increased levels of mRNA at all stages, and ry⁺¹⁰ decreased levels (data not shown). This indicates that the mechanism respon-

Figure 4. XDH mRNA accumulation during development of ry^{+5} . RNA was isolated from various developmental stages as described in Materials and Methods, and treated as described in Figure 1. Lane 1, early stage RNA (embryo, first and second instar larvae); lane 2, early third instar larvae; lane 3, late third instar larvae; lane 4, early pupae; lane 5, late pupae; lane 6, newly eclosed adults.

Figure 5. Comparison of XDH enzyme activity with mRNA accumulation during development. The autoradiogram from Figure 4 was quantified by computerized densitometry and the highest value (late third instar larvae) set to 100. All other RNA values are relative to this. Similarly, enzyme activity was measured at each stage of development, the highest level set at 100, and other values put in relation to that value. Shaded bars represent enzyme activity; clear bars represent RNA accumulation.

sible for the increase and decrease of XDH mRNA in these variants is not stage specific, but operates throughout the life of the organism.

The results shown in Figure 4 also indicate there is no significant variation in the size of the XDH transcripts throughout Drosophila development, as has been reported for alcohol dehydrogenase (20), myosin heavy chain (21), and dopa decarboxylase (10). XDH mRNA from the intermediate pupal stage and from newly eclosed adults appears to be slightly larger than the transcripts from earlier stages, but the difference in size would be very slight, no more than 50-100 bases. Since the size of the XDH mRNA is very large (about 4500 bases), our Northern blotting procedure would not accurately measure very small size differences. However, this aspect bears further investigation, most likely by a detailed analysis of the 5' and 3' ends of the XDH gene.

DISCUSSION

We have shown that the 3.2 fold increase in XDH enzyme activity in the variant ry⁺⁴ and the 50% decrease in XDH activity in the variant ry⁺¹⁰ is a reflection of corresponding increases and decreases in the level of XDH mRNA. The electrophoretic gel patterns of heterozygotes of these variants indicate that the sites are cis-acting. Thus it is likely that these sites define a DNA sequence flanking the structural gene which affects the amount of RNA production. This is probably at the level of gene transcription, although differences in the stability of transcripts cannot be ruled out at this time. These regulatory variants offer a unique opportunity to examine mechanisms of XDH gene expression and are particularly interesting in view of a number of recent theories of gene regulation based on multiple cis-acting elements associated with each gene (22,23,24).

We also compared XDH mRNA levels with CRM in three mutants containing intragenic lesions. Two of these were induced by X-ray (ry⁴¹ and ry²⁶) and one by EMS (ry⁶⁰³). Ry²⁶ and ry⁶⁰³ had no detectable XDH CRM, and this is selected by greatly reduced levels of XDH mRNA in these mutants. On the other hand, ry⁴¹ had 70% of wild type CRM and 130% of wild type XDH mRNA. It is possible that these intragenic mutations act by affecting transcription of the XDH gene, but there is little precedence for this. More likely, alterations in XDH mRNA stability are responsible for the different levels of message observed in the mutants. At this time it is not clear what mechanism would act to cause an increase in mRNA stability in ry⁴¹ such that greater than wild type levels of XDH mRNA are present. The XDH mRNA produced in all three

mutants is the same size as XDH mRNA produced in wild type flies, indicating that all three mutants are the result of point mutations or very small deletions. EMS is known to cause predominately point mutations and approximately half of the wholly intragenic lesions induced by X-ray are also point mutations (25).

We also examined XDH mRNA levels in a known deletion mutant (ry⁶⁰), a known insertion mutant (ry²), and a mutant generated during a dysgenic P x M cross (ry⁷⁷¹). Ry⁶⁰ has been shown to contain a 1 KB deletion in the structural portion of the XDH gene (W. Bender, personal communication); and, as expected, XDH mRNA in the mutant was approximately 1 KB smaller than in wild type flies. Ry⁷⁷¹ XDH mRNA was also smaller than wild type XDH mRNA. Since this mutant was generated by a P x M dysgenic cross and never seen to revert (M. Green, personal communication) it is possible that the deletion could have arisen by the excision of a transposable element in which some XDH gene sequences were also removed.

A small amount of wild type XDH mRNA is also present in RNA isolated from ry⁶⁰ and ry⁷⁷¹ flies. We have no explanation for the presence of this RNA in the mutants, but we know it is not due to contamination of our stocks by wild type flies, the presence of sequences coding for another mRNA on the probe, or incomplete denaturation of mRNA during electrophoresis. In addition, mutant DNA was cut with Sal I and the size of the XDH gene determined by Southern blot analysis. The presence of an 8.1 KB fragment (the size of the wild type gene) was never detected, even after overexposure of the film to detect sequences making up only a small percentage of the XDH DNA present.

The CRM-less ry² mutant has been recently shown to contain a 9KB insertion of the transposable B104 element (W. Bender) in the structural portion of the XDH gene. Thus it was surprising that ry² had wild-type levels and size of XDH mRNA. Insertion of the element into an intron so that splice junctions are preserved could explain why the message is not larger than wild type, but is unlikely since the mutant has little CRM and therefore translation into normal immunoprecipitable protein is not occurring. It is also possible that the insertion element contains DNA sequences that resemble a consensus splicing junction near the 3' end of an XDH intervening sequence, generating a non-functional message of nearly normal size. Similar findings have been reported in a beta-thalassemic in which an aberrant splicing reaction resulting in the production of a nonfunctional mRNA was demonstrated to be the result of a single point mutation that creates an additional 3'

splice site (26). Another possibility is that an imperfect excision of the B104 element has occurred in our stocks, generating a non-functional message of nearly correct size. This alternative is presently being examined by in situ hybridization, using the B104 element as a probe.

The XDH gene is subject to complex developmental regulation, and in the past enzymatic developmental profiles were used as a first approximation of the level of gene activity. While this approach has proved correct in many cases, there are instances in which mRNA is synthesized at an early developmental stage and translated later. We have therefore compared XDH mRNA accumulation with CRM and enzyme activity at several times during Drosophila development. Our results indicate that the appearance of enzymatic activity is closely correlated with mRNA accumulation, which suggests that the developmental regulation of XDH gene expression is primarily at the level of RNA accumulation.

Recent evidence suggests that an additional step in gene regulation during development might be the differential transcription of multiple RNAs from a single gene. The single myosin heavy chain gene has been demonstrated to produce at least three different sized transcripts, which differ by the pattern of splicing at their 3' ends and their relative steady state levels during development (21). The alcohol dehydrogenase gene in Drosophila has been demonstrated to have different sized larval and adult transcripts. They differ in their 5' untranslated sequences, the larval mRNA being some 50 bases shorter than the major adult transcript (20). Our results indicate there is no major size difference in XDH transcripts during development, arguing against multiple RNAs of markedly different size being produced from the same XDH gene. However, we cannot rule out the possibility of small size changes, because the large size of the XDH mRNA (about 4500 bases), precludes the detection of changes of 50-100 bases. There does appear to be a slight but reproducible difference in the size of XDH mRNA isolated from late pupae and adults when compared to earlier stages, but this is better addressed by closer analysis of the 3' and 5' ends of the XDH gene and RNAs.

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