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MOLECULAR CLONING OF
DROSOPHILA MELANOGASTER AMYLASE
SEQUENCES AND THE REGULATION
OF AMYLASE GENE EXPRESSION.

by

Bernhard F. Benkel

A thesis
presented to the University of Ottawa
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in the Department of Biology

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ABSTRACT

I have shown that amylase enzyme activity in Drosophila melanogaster is repressed by dietary glucose. This repression of activity occurs in a strain-specific and developmental stage-specific manner. In larvae of sensitive strains, such as Oregon-R, dietary glucose can repress amylase activity by over one hundred-fold. Glucose repression of amylase activity has implications for the interpretation of previous studies on amylase in Drosophila, including experiments designed to demonstrate fitness differences between amylase genotypes.

It has previously been demonstrated that amylase repression involves a reduction in the abundance of stable amylase enzyme. Here I used in ovo translation studies to demonstrate that the regulation of amylase activity in response to dietary carbohydrate affects the level of translational activity for amylase enzyme in RNA samples. In order to measure amylase mRNA levels directly, cloned D. melanogaster amylase sequences were isolated and characterized. Amylase copy DNAs were identified using two different approaches: (i) cross-species hybridization using a mouse amylase cDNA probe, and (ii) probing with a differential probe generated from Drosophila RNA. Amylase cDNA sequences were used, in turn, to isolate amylase genomic clones. The D. melanogaster amylase mRNA was sized at 1650 b.

Cloned amylase sequences were used to measure changes in amylase mRNA levels, in response to dietary carbohydrate, in a

cross-section of strains. It was found that the regulation of amylase activity, in response to glucose, takes place at a pretranslational, and probably transcriptional, level. The strain-specific as well as dietary-induced differences in enzyme activity are reflections of differences in levels of stable amylase messenger RNA. Dietary regulation of amylase activity in D. melanogaster is complex and, based upon evidence at the biochemical level, appears to involve the interaction of cis-acting elements and trans-acting regulatory factors.

In larvae of sensitive strains, amylase mRNA can account for as little as 0.01% of the poly A⁺ RNA under conditions of dietary glucose repression, or greater than 1% of poly A⁺ RNA, under depressing dietary conditions.

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1. INTRODUCTION

The current state of understanding of the genetic factors that regulate amylase gene expression in *Drosophila* resembles that for most other eukaryotic gene regulatory systems in that it is far from complete. A thorough knowledge of amylase gene regulatory systems in this organism will be rewarding for a number of reasons. First, because of the wealth of genetic information available on *Drosophila melanogaster*, this is an obvious organism of choice for experiments aimed at an understanding of gene regulation in multicellular higher eukaryotes. Secondly, the wide variety of regulatory phenomena which have already been described for this gene-enzyme system make it exceptional among eukaryotic gene-enzyme systems (see Laurie-Ahlberg, 1985, for a recent review). Thirdly, since amylase is involved in the facultative utilization of secondary carbohydrate resources, the study of amylase gene regulation may help us to understand some aspects of gene regulation among higher eukaryotes, in the context of the well characterized prokaryotic gene regulatory systems.

Finally, many studies on *Drosophila* amylase have focused on the biochemical adaptation of the organism to its environment and on the general biological significance of genetic variation in natural populations. These questions continue to motivate current research along with the aim of eventually understanding some evolutionary processes,

especially the evolution of gene regulatory mechanisms, at the molecular level.

1.1. Structural Gene Products

Drosophila melanogaster produces a single major amylase protein. It is a monomeric enzyme with a molecular weight of approximately 54,500 daltons (Doane et al., 1975). The monomeric nature of the enzyme can easily be visualized on electrophoretic gels stained for amylase activity when one uses homogenates of individuals heterozygous for electrophoretic variants (Hickey, 1981; Doane et al., 1983). Enzyme substrate specificities, activators and inhibitors resemble those that generally characterize animal alpha-amylases (Doane 1969a) and the enzyme can be readily purified from crude homogenates by precipitation as a glycogen-amylase complex in ethanol (Doane et al., 1975; Hickey, 1981). The amino acid composition of Drosophila amylases is similar to that of the mammalian enzymes although no immunological cross-reaction could be detected between the fly and human amylases (Doane et al., 1975).

The existence of electrophoretic variants of the amylase enzyme has greatly facilitated the genetic mapping of the amylase structural gene locus. The mapping studies of several workers (Kikkawa, 1964; Bahn, 1967; Doane 1969a) place the Amy structural gene at approximately position 77 on the genetic map of chromosome 2. This map position is consistent with cytogenetic results which locate the amylase gene in section 54

of the right arm of chromosome 2 (Doane, 1969a; Bahn, 1971). Recently, Gemmill et al. (1985) have shown that cloned amylase gene sequences also hybridize specifically to the same chromosomal region.

Many strains of Drosophila melanogaster are characterized by a double-banded electrophoretic pattern for amylase (Bahn, 1967; Doane, 1969b; Hickey, 1979a and 1979b; Singh et al., 1982). This pattern is heritable and the duplicated pattern segregates as a single genetic unit. Furthermore, the pattern observed is qualitatively similar in adults and larvae of any given strain. The most logical explanation for this phenomenon is the existence of closely-linked duplicate copies of the amylase structural gene. Based on the occurrence of rare recombinants between these loci, Bahn (1967) estimated that the distance between the duplicated genes is 0.008 cM. Heterozygotes between single-banded and double-banded genotypes give a triple-banded pattern, whereas heterozygotes between two distinct double-banded genotypes will yield four electrophoretic bands in the heterozygotes. Levy et al. (1985) have found evidence for a duplication of the amylase coding sequences in the DNA of a double-banded amylase genotype and, based upon more recent evidence (Gemmill et al., 1986), it appears that all strains of D. melanogaster possess duplicated gene copies.

It is not known whether, in single-banded strains, both copies of the structural gene strains are active and code for

electrophoretically indistinguishable gene products, or if one of the gene copies is silent. Several closely-related species within the melanogaster group show evidence for the structural gene duplication (Dainou et al., 1987) although the more distantly related species of *Drosophila* appear, based upon evidence from banding-pattern studies, to have only a single copy of the amylase gene.

Natural populations of *D. melanogaster* are usually polymorphic for amylase electrophoretic variants and the degree of this polymorphism shows a distinct geographical pattern (Hickey, 1979b; Singh et al., 1982). Early population studies by McCune (1969) and Langley et al. (1974) showed that a single-banded, Amy¹, variant was the most frequent in North American and Japanese populations. This finding contrasted with a report by Puyk and DeJong (1972) who scored a total of sixteen different genotypes among a sample of one hundred individuals from West Africa. This difference between African and other populations was confirmed by Hickey (1979b) and Singh et al. (1982). While the Amy¹ variant is the most frequent in North American, European, Asian and Australian populations, this is not the case for West African populations where a total of 23 different amylase phenotypes were found with the most common phenotype accounting for only 17% of the total frequency distribution (Singh et al., 1982).

The high degree of amylase structural gene variation which is seen in African populations of *D. melanogaster* also

seems to be present in African populations of other closely-related species (Dainou et al., 1987). This richness of electrophoretic variation which shows a distinct geographic localization can be explained by postulating that the African region represents the area of origin of D. melanogaster and that other populations represent relatively recent samplings from this diverse gene pool. An alternative explanation is that environmental conditions in Central and West Africa favor amylase variants which are very rare or absent elsewhere.

1.2. Natural Selection of Amylase Variants

The biological significance of allozyme variation within natural populations has been a major source of controversy in population genetics since the first quantitative estimates of such variation were made (Lewontin and Hubby, 1966). A variety of theoretical and experimental strategies have been used in an effort to discriminate between "neutralist" and "selectionist" explanations for the maintenance of this variation. Experimental approaches to the problem have been hampered by the fact that little is known about the physiological role of many of these enzyme proteins. In particular, it has been difficult to isolate an environmental challenge that could be deemed, with some certainty, to be relevant to the gene locus in question.

One possible approach is to relate changes in selection coefficients of different alleles at a specific locus to

environmental variables. The experiments of DeJong et al. (1972) and DeJong and Scharloo (1976) indicate that electrophoretic variants of the amylase enzyme in Drosophila melanogaster affect the fitness of individuals and that this effect is dependent on the carbohydrate source in the food medium. A controlled selection experiment by Hickey (1977) showed a predictable response of gene frequencies in experimental populations grown on different food media. The amylase variant which has the higher enzyme activity is at a selective disadvantage when the dietary carbohydrate source is in the form of simple sugars. However, the relative selective value of this variant is enhanced when the dietary sugars are replaced by starch. A possible complication in the interpretation of these results is the question of linkage disequilibrium with variants at other loci in the genetic background (Doane, 1980b). Therefore in a subsequent study, the effects of linked loci was eliminated by using several independently derived high-activity and low-activity amylase variants (Hickey, 1979a). In these latter experiments, the predicted divergence in gene frequency between high and low-activity variants was seen for some, but not all, allozyme pairs. More recent experiments (Yamazaki and Matsuo, 1984; Matsuo and Yamazaki, 1984) again show evidence for selection on amylase variants in response to dietary carbohydrate supplements.

The general conclusion to be drawn from all of these

experiments is that, on the one hand, there seems to be a clear relationship between the relative fitness of some amylase genotypes and the nature of the dietary carbohydrate source while, on the other hand, the magnitude of the fitness differences are rarely as large as one might have predicted from the in vitro enzyme activities of the amylase variants. The results reported by Haj-Ahmad and Hickey (1982) and the results on glucose repression of amylases reported in this study help to clarify the interpretation of the selection experiments. In particular, the problem with the selection experiments is that genotypes which may show large differences in enzyme activity on "standard" *Drosophila* medium (which usually contains significant amounts of simple sugars) may be much closer as regards activity when grown on experimental diets containing little sugar. Thus, the phenotypic difference between genotypes, upon which the prediction of selective advantage is based, tends to disappear under the experimental conditions used. This phenomenon is what has been recently referred to as "inducibility" variation (Yamazaki and Matsuo, 1984) although it might more properly be called "repressibility" variation (Hickey and Benkel 1982; this study).

1.3. Genetic Control of Amylase Activity Levels

Amylase gene expression in *Drosophila* is subject to a complex system of control factors. Biochemical analyses support

the existence of both cis and trans-acting components in activity control system. Amylase activity in D. melanogaster is subject to developmental and tissue-specific control (Doane, 1969a) including regulation by temporal, midgut-specific factors (Abraham and Doane, 1978). Paigen (1979) has reviewed the differences between "systemic regulators" which result in uniform changes in all tissues and "temporal regulators" which are more localized in terms of their tissue distribution and developmental stage specificity. Strain-specific variation in amylase activity has been documented extensively (e.g. Doane, 1969b; Hickey, 1979b). In addition, a number of studies have shown that amylase activity is modulated by dietary carbohydrate source (Abe, 1958; Hickey 1977; Hoorn and Scharloo, 1978; Hickey and Benkel, 1982; Echo and Doane, 1984; Yamazaki and Matsuo, 1984). Both interstrain variation in activity and the repression of amylase activity by dietary sugars have been shown to result from differences in amounts of active amylase enzyme (Hickey, 1981; Hickey and Benkel, 1982). The results of this present study clarify the relationship between strain-specific activity differences and diet-induced changes in activity. Furthermore, using cloned amylase sequences as molecular probes, this study shows that glucose repression takes place at the pretranslational level.

1.3.1. Genotypic Effects on Total Amylase Activity: Large differences in amylase activity between inbred strains of

Drosophila melanogaster have been well documented (Doane, 1969a; De Jong et al., 1972; Hickey, 1977; Hickey, 1979a; Hickey, 1981; Hoorn and Scharloo, 1980; Doane et al., 1983). These differences between strains are normally within a two to five-fold range in adult flies and the activity differences have been shown to be genetically linked to the amylase structural gene locus (Hoorn and Scharloo, 1978; Hickey, 1979a; Doane, 1980a). However, no fine-structure mapping has been done specifically to localize these putative systemic regulators relative to the structural locus. There is a general trend in all the studies mentioned above to observe a positive correlation between relatively high levels of amylase activity and the presence of double-banded electrophoretic patterns in homozygotes. This correlation has not been extensively studied and a number of exceptions certainly exist.

The question of whether these differences in amylase activity between genotypes are due to variations in the amount of enzyme produced or due to differences in the catalytic efficiencies of the various amylase structural gene products was addressed by Hickey (1981). Amylase enzyme was purified from high-activity and low-activity strains and the specific activities of the purified enzymes was compared. There was no significant difference in the activity of the purified amylases despite the several-fold differences in enzyme activity between the crude homogenates. This result was confirmed in an independent study by Treat-Clemons and Doane (1982).

Purified amylases were also used to raise amylase-specific antibodies. Immunological quantification of the level of enzyme present in crude larval homogenates, using the technique of rocket immunoelectrophoresis (Hickey, 1981), showed differences in levels of cross-reacting material among strains. Furthermore, these differences correlated with the enzyme activity differences in crude homogenates. These results, taken together, indicate that the major portion of this between-genotype variation in amylase activity reflects differences in enzyme quantity rather than differences in catalytic efficiency between enzyme variants.

The results of this present study show that: (i) there are genotype-specific differences in amylase activity between different D. melanogaster strains, however, these differences are relatively small when flies or larvae are grown on medium which derepresses amylase activity, and (ii) any interstrain differences in activity, irrespective of food type, are due to variations at a pretranslational, and probably transcriptional, level of regulation of amylase gene expression.

1.3.2. Tissue and Developmental Specificity of Enzyme Activity:

The major site of amylase activity in *Drosophila* (contrary to mammalian amylases) is not in the salivary glands but rather the larval and adult midgut (Kikkawa, 1964; Doane et al., 1983; this study). Abraham and Doane (1978) showed that there are genetically-controlled variations in the distribution of

amylase activity within the midguts of young adult flies. They defined three distinct distribution patterns in the posterior midgut and showed that the more extensive map A pattern behaves as a trans-acting dominant allele when made heterozygous with a chromosome carrying the genes for the more restrictive map C pattern. A recombinational analysis showed that the gene controlling the map (midgut activity pattern) patterns was located 2 to 3 map units distal to the amylase structural locus on chromosome 2. This result has recently been confirmed by Klarenberg et al. (1986).

It has been proposed that the map locus produces a diffusible substance which might interact with a cis-acting element associated with the structural gene (Doane et al., 1983). It should be noted that the map variants affect the distribution of enzyme activity in young adults only; as the flies age, all genotypes acquire the more extensive distribution-pattern (Abraham and Doane, 1978; Doane et al., 1983).

Subsequent work (Doane, 1980a; Klarenberg et al., 1986) provides evidence for an independent genetic control of the activity distribution in the anterior adult midgut which, in turn, appears to be unrelated to activity patterns in the larval midgut. It has been shown that the activity patterns in the posterior adult midgut reflect patterns of amylase protein and translatable mRNA distribution (Doane et al., 1983). Klarenberg et al. (1986) found that the distribution of

activity in the anterior midgut of *Drosophila* larvae is controlled by a closely-linked cis-acting element, less than 0.1 cm from the structural gene locus. The same study provides evidence that the midgut activity patterns of both larvae and adults are affected by dietary glucose. Specifically, the amount of amylase produced by any given region of the midgut, and the extent of the area along the length of the midgut producing amylase, are both reduced in the presence of dietary glucose. In general, the more posterior regions of the midgut appear to be more sensitive to glucose repression.

The patterns of amylase expression in the midgut of another *Drosophila* species, *D. pseudoobscura* have also been characterized and analyzed genetically (Powell and Lichtenfels, 1979) but no gene of major effect could be identified in this case. It was concluded that the genetic control of the enzyme patterns is polygenic in this species.

1.4. Glucose Repression of Amylase Activity

The ability to exploit environmental resources is of prime importance to all forms of life. In general, environmental resources can be either simple or complex in nature. The utilization of complex resources requires the production of catabolic enzymes; a specific set of polypeptides for any specific complex substrate. Moreover, the composition of the environment changes over time and space. Organisms respond to these changes by adapting their activities to

changes in environmental parameters.

Modulation of enzyme activity by one such environmental parameter, carbon source, has been studied extensively in prokaryotes. In Eschericia coli, the simple sugar glucose represses enzyme activities required for the catabolism of other more complex carbon sources by affecting intracellular levels of cAMP. In the absence of glucose, and in the presence of a complex carbon source, E. coli adapts by producing the relevant set of catabolic enzyme activities. Bacterial adaptation to a specific carbon source involves dramatically increased transcription of the gene(s) coding for the polypeptide(s) involved in metabolizing the carbohydrate in question. This "glucose effect" has also been described in Saccharomyces cerevisiae.

In yeast, as in bacteria, the adaptive response involves regulation at the level of transcription. Higher eukaryotes, such as Drosophila, face the same problem as E. coli and S. cerevisiae in exploiting environmental resources. For instance, D. melanogaster grows well on a medium containing the complex carbohydrate starch, and adapts to the addition of simple sugars to the medium by dramatically reducing the enzyme activity necessary to degrade starch, i.e. amylase activity.

Superficially, therefore, it would appear that E. coli, S. cerevisiae and D. melanogaster have solved a common problem in a similar manner. For the Drosophila system, however, the level at which the regulation of amylase activity in response

to dietary sugar takes place has yet to be established. In fact, testing for regulation at a pretranslational level occupies a major portion of this thesis.

It has been known for some time that amylase activity in D. melanogaster varies with carbohydrate source (Abe, 1958; Doane, 1969a; Hosbach et al., 1972). Only recently, however, has it been clearly demonstrated that dietary sugar represses amylase activity (Hickey and Benkel, 1982; Echo and Doane, 1984). In addition, most studies on dietary regulation of amylase activity in Drosophila have been done using young adults. One of the conclusions of this present study is that the effects of dietary glucose on amylase activity are much more visible in larvae than in adults of any given strain. In fact larvae of sensitive strains, such as Oregon-R, show mere trace levels of activity on medium containing 10% glucose. Not all strains are as sensitive to glucose as Oregon-R, however, and any given strain displays its own particular level of repression when grown on glucose-containing food.

Previously, when flies grown on glucose medium were compared to non-glucose treated flies, the difference in amylase activity was found to reflect a difference in the abundance of stable amylase enzyme molecules (Hickey and Benkel, 1982). In this study, the effort to identify the level at which dietary regulation of amylase takes place is advanced using: (i) an in vivo translation assay to measure translational activity of amylase messenger RNA and; (ii)

molecular probes to compare stable levels of amylase mRNA under both repressing and derepressing dietary conditions.

Both the in ovo translation and the molecular probing studies show that glucose regulation of amylase activity takes place at a pretranslational, and probably transcriptional, level. Regulation at the transcriptional level implies the existence of DNA elements, associated with the amylase structural sequences, which mediate glucose repression. Here I present the isolation of D. melanogaster genomic amylase sequences, including the region between the inverted gene copies; that is, the region presumed to contain the hypothetical regulatory cis-acting DNA elements.

Finally, the relative abundance of the amylase messenger RNA in relation to other well characterized mRNAs is determined. It is shown that under derepressing dietary conditions, amylase mRNA is more abundant than the alcohol dehydrogenase mRNA (Adh is estimated at about 1% of poly A⁺). In contrast, in sensitive strains grown under repressing conditions, amylase mRNA is relatively rare.

2. MATERIALS AND METHODS

2.1. Fly Stocks

Five strains of Drosophila melanogaster were used in this study. The **Oregon-R**, **Canton-S**, **Belinga** and **Makokou** strains were chosen because they span the amylase activity range found in nature. The fifth strain, an amylase null variant, was isolated as a spontaneously occurring mutant from a Brownsville, Texas population (Hickey and Haus, unpublished; Haj-Ahmad and Hickey, 1982). It shows no amylase activity and no detectable material which cross-reacts with amylase antibody.

Oregon-R (Amy¹) is a common "wild type" strain which has been described as a low-activity variant. It shows a single fast-migrating amylase band (AMY-1) on native electrophoretic gels (Doane, 1969b; Hickey, 1981). **Canton-S** (Amy^{1,3}) is another common laboratory strain which has an intermediate level of amylase activity. It shows a double-banded electrophoretic phenotype (AMY-1, AMY-3). **Belinga** (Amy⁶) is derived from an African population and has been described as a high amylase activity variant (Hickey, 1981). It displays a single slow-migrating amylase electrophoretic band (AMY-6). **Makokou** (Amy^{4,6}) is another African strain which is also a high amylase activity variant. However, it shows a double-banded (AMY-4, AMY-6) pattern on electrophoretic gels (Hickey, 1979).

2.2. Drosophila Culture Conditions

All strains were maintained on Carolina Biological Supply Instant Drosophila medium at 22°C prior to the experiments. The developmental profile for amylase activity shows two high points, the first during the late third instar of larval development and the second at about one week into the adult stage (Doane, 1969a). For this reason, the effect of dietary glucose on amylase activity was tested at these two stages in the life cycle.

To determine the sensitivity of larvae to dietary glucose, mature adults were transferred to 8-dram shell vials containing 10 ml of test medium for egg laying. After several days the adults were removed and the larvae allowed to grow to the late 3rd instar stage. Larvae were harvested by washing from the low-agar foods with distilled water. Adults, aged on the instant fly food for 3-5 days, were transferred to test foods for 2 days of exposure. Thus adults were 5-7 days old at the time of harvest. Flies and larvae were frozen at -20°C for short periods and kept at -70°C for long term storage.

2.2.1. Test Diets:

(i) "no-added-sugar" medium [(-) Gluc]

consisted of: 5% killed brewers yeast (w/v)

0.75% agar (w/v) and

0.8% propionic acid (v/v) in
distilled water

(ii) "glucose" medium [(+) Gluc]

consisted of: 10% dextrose (w/v)
5% killed brewers yeast (w/v)
0.75% agar (w/v) and
0.8% propionic acid (v/v) as mold
inhibitor, in distilled water.

These two diets are identical except for the sugar component; "glucose" medium contains 10% glucose whereas "no-added-sugar" medium lacks added carbohydrate.

2.3. Enzyme Activity Measurements

Mass extracts of flies or larvae grown on the test media were prepared by dounce homogenization. The water soluble fractions were assayed for protein content using the Lowry assay (Lowry et al., 1951). Results of the Lowry assay were checked by visual inspection of electrophoretic gels stained with Coomassie Blue.

2.3.1. Alpha-amylase Activity: Amylase enzyme activity was determined using two independent assay procedures as outlined below.

2.3.1.1. The DNSA (dinitrosalicylic acid) assay of Bernfeld (1955) measures the increase in reducing sugars resulting from the activity of amylase on starch. This assay was performed, and the result monitored, as described by Hickey and Benkel

(1982). Briefly, crude homogenates were added to a 2% solution of boiled starch (Connaught, hydrolyzed electrophoresis grade) with calcium ions, buffered to pH 7.4, and incubated at 37°C in a shaking water bath. After 30 minutes, the reactions were terminated by the addition of DNSA reagent and the mixtures boiled for 10 minutes. The mixtures were cooled to room temperature and assayed spectrophotometrically at 550 nm. Readings were converted to units of maltose released by reference to a maltose standard curve. Enzyme activity was expressed as nanomoles of maltose released per minute of incubation time per microgram of protein.

2.3.1.2. Amylase enzyme activity was also compared using the I-KI stained gel method (Hickey, 1981; Benkel and Hickey, 1986), which measures the disappearance of starch as a result of amylase activity. Briefly, crude homogenates were electrophoresed on native 5% polyacrylamide gels (Protean I or II by Biorad) buffered with 0.1 M Tris borate pH 8.9. Electrophoresis was carried out at 200-300 V for 2-3 hours. Following electrophoresis, gels were incubated in a 3% starch solution (Connaught, hydrolyzed electrophoresis grade) in 0.1 M Tris HCl pH 7.4, 20 mM CaCl_2 for 1 hour at room temperature and stained with Iodine-Potassium Iodide reagent. In order to visualize faint activity bands, gels were incubated in 0.1 M Tris HCl pH 7.4, 20 mM CaCl_2 without the starch at 37°C, following incubation in the starch solution, but before

staining with the I-KI reagent. All comparisons were made within a single gel which included a dilution series as an internal control. Band intensities were measured by scanning photographic negatives (4" x 5") of gels with an optical densitometer.

Neither of the assay systems described above is accurate over the broad range of amylase activities measured for the different homogenates generated in this study, at any single total protein concentration. In order to compare homogenates with large differences in specific activity, the higher-activity homogenate was diluted until the activity, measured spectrophotometrically or by the densitometer assay, was comparable to that of the low-activity sample. Consequently, the relative activities of the original homogenates were calculated taking into account the dilution factors. A homogenate of the amylase null strain was used to measure the background activity for both assays.

2.3.2. Alcohol Dehydrogenase Enzyme Activity: ADH activity was visualized in gels using a modification of the method of Prakash et al. (1969). Crude homogenates were electrophoresed as described above. Following electrophoresis, gels were incubated in 0.1 M Tris-HCl pH 8.5, containing 250 mg/L MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), 300 mg/L NAD (nicotinamide-adenine dinucleotide), and 7.5 ml/L of isopropanol at 37°C. After 1 hour of incubation, 20 mg/L of

PMS (phenazine methosulfate) were added and the gels were stained until bands appeared.

2.4. Midgut Isolation

Third instar larvae were placed in droplets of distilled water on a microscope slide. Midguts were dissected, under low power magnification, using glass microneedles to pull the larvae apart and to cut the midgut away from foreguts and hindguts. Midguts were stored at -20°C and prepared for electrophoresis as described above for whole larvae.

2.5. Oocyte Injections

2.5.1. The Microinjection System: Injection was accomplished using a system consisting of a glass microneedle (tip O.D. 20-30 μm) mounted on a plexiglass support which was itself mounted on a micromanipulator. The microneedle was attached to a 2 ml glass syringe by way of a loop of fine bore stainless steel tubing and a glass capillary (5 μl) calibration loop. The entire hydraulic system was filled with colored paraffin oil except for the calibration loop region where the colored oil was interspersed with small droplets of clear water. Injection volumes were calibrated by following the movement of oil/water interfaces as they moved past the graduations on a photographic reduction of a ruler, mounted beside the calibration loop.

2.5.2. Injection: Large Xenopus laevis females were purchased from "Xenopus One" (Ann Arbor, Michigan, USA). Frogs were anesthetized by cooling in ice-water. Ovaries were removed and oocytes scraped from the ovary wall with a platinum wire. Only large (1 mm diameter or greater) stage V or VI (Dumont, 1972) oocytes were used for injection experiments. Routinely, 10-20 oocytes were lined up against one edge of a glass microscope slide in a glass petri dish in preparation for injection. Then 1-3 μ l of RNA solution was sucked into the tip of a microneedle and each oocyte pierced and injected with approximately 50 nl. Modified Barth's medium (10 mM HEPES pH 7.0, 88 mM NaCl, 1.0 mM KCl, 2.5 mM NaHCO_3 , 0.82 mM M_2SO_4 , 0.33 mM $\text{Ca}(\text{NO}_3)_2$, 0.41 mM CaCl_2 , 10 μ g/ml tetracycline and 10 μ g/ml streptomycin) was used throughout the injection procedure. RNA, at 5-10 mg/ml in sterile water, was injected into the vegetal hemispheres of Xenopus oocytes according to Gurdon (1977).

Following injection, oocytes were incubated individually in microtitre plates (Terasaki, Falcon 3034) containing sterile modified Barth's medium for 3 days at 20°C in the dark. Homogenates were prepared from 20-30 oocytes, centrifuged for 3 minutes in a microfuge, and the supernatants brought to 0.1 M with Tris Borate pH 8.9 and 5% with sucrose. Then 5 oocyte equivalents were loaded on a 5% native polyacrylamide gel and electrophoresed. Conditions for electrophoresis and gel staining (for amylase activity) are described in the section entitled: "Enzyme Activity Measurements".

2.6. RNA Analysis

RNA was extracted from flies and larvae using the guanidine hydrochloride method of Cox (1968). Briefly, samples were homogenized in 8 M guanidine hydrochloride and the RNA precipitated from the mixture with ethanol. Following 4 washes with 4 M guanidine hydrochloride, the RNA was dissolved from the pellet with sterile RNase-free water. RNA was stored in water at -20°C for short periods and under ethanol at -20°C for long-term storage. Poly A⁺ RNA was prepared by one round of selection on messenger affinity paper (Hybond-mAP, Amersham) according to Wreschner and Herzberg (1984).

2.6.1. Northern Blots: RNA was separated on gels containing formaldehyde (Maniatis et al., 1982) and blotted to Biodyne (Pall Ultrafine Filtration Corp., Glen Cove, NY) or Hybond-N (Amersham Canada Ltd., Oakville, Ont.) membranes. Gels were blotted, hybridized and washed as described in the Pall Biodyne Manual with the following modifications: (1) for high stringency probing, hybridizations were carried out at 45°C in buffer containing 5x SSC, 5x Denhardt's solution, 50 mM phosphate buffer pH 6.9, 0.5% SDS and 50% formamide - blots were washed in 0.1x SSC, 0.1% SDS at 60°C ; (2) for low stringency probing using the mouse amylase cDNA (described below), hybridizations were carried out at 38°C in buffer as described above with the addition of 50 ug/ml poly C and 50 ug/ml poly G - blots were washed in 1x SSC, 0.1% SDS at 52°C .

Blots were probed for amylase mRNA with the PvuII restriction fragment of pOR-M3 (this study), and for Adh messenger with p30-8/7/8 (pEMBL8(-) containing a 2.8 kb HindIII-EcoRI fragment of the SAC1 genomic Adh clone of Goldberg (1980), with the two coding region introns removed, Craig Newton, unpublished). Probes were nick translated to high specific activity according to Rigby et al. (1977) or oligo-labelled using synthetic hexamer primers (Feinberg and Vogelstein, 1983).

2.6.2. RNA Dot Blots: Before application to Biodyne membranes using a Schleicher and Schuell Minifold device, RNA samples were heated to 65°C in a buffer containing 50% formamide and 6% formaldehyde, then quick-cooled on ice and brought to 5x SSC. Samples were applied to prewetted filters with low suction, and fixed to Biodyne by U.V. irradiation (Church and Gilbert, 1984). Conditions for hybridization are described above; high stringency. Quantification of signals was performed by scanning autoradiograms with an integrating densitometer. Appropriate dilutions of the stronger signal were included on each autoradiogram to allow for comparison of differences in signal levels between treatments. Amylase signal intensities were normalized with respect to Adh signals for the same samples.

2.7. Recombinant DNA Technology

Procedures such as; propagation of bacterial strains, restriction enzyme digestions, agarose gel electrophoresis, etc. were performed essentially as described by Maniatis et al. (1982). Manipulations involving recombinant DNA molecules were carried out in accordance with MRC Canada guidelines.

2.8. DNA Analysis

Plasmid DNA was prepared by the boiling method of Holmes and Quigley (1981) or the alkaline lysis method of Birnboim and Doly (1979). Large scale preparations were purified by centrifugation to equilibrium in cesium chloride gradients. Lambda DNA was prepared according to Silhavy et al. (1984). Briefly, phage were pelleted from medium scale (250 ml) lysates using PEG precipitation and purified, from host nucleic acid and protein, by glycerol step-gradient centrifugation and enzyme digestion. Lambda DNA was liberated from phage particles by treatment with SDS and proteinase K and purified by phenol extraction, followed by ethanol precipitation.

Drosophila genomic DNA was isolated using a modification of the procedure described by Davis and Davidson (1984). Briefly, flies or larvae were dounce homogenized and a nuclear pellet produced by low speed centrifugation. Nuclei were lysed by treatment with proteinase K and sarkosyl. Nuclear DNA was purified by digestion with DNase-free RNase and proteinase K followed by phenol extraction and ethanol precipitation.

DNA, digested with restriction enzymes, was separated on agarose gels as described by Maniatis et al. (1982) and blotted to Biodyne or Hybond-N membranes. Southern blots were performed as described in the Pall Biodyne Manual; capillary method (Southern, 1975). Blots were probed with nick translated or oligo-labelled probes using the high stringency conditions described under "RNA analysis".

Restriction fragments were isolated from low melting temperature gels (Seakem) or electroeluted (IBI model UEA) from DNA-grade agarose and transferred to pUC vectors (Vieira and Messing, 1982) or multipurpose vectors (Dente et al., 1983) for fine-structure restriction analysis.

2.9. Lambda Bank Screening

Plaque lifts of lambda banks containing cDNAs from RNA of the Drosophila melanogaster Oregon-R "wild type" strain (Poole et al. 1985), a lambda bank containing cDNAs generated from the Canton-S "wild type" strain (V. Pirrotta, unpublished), a Canton-S genomic bank in lambda Charon-4 (Maniatis et al., 1978) and, an Oregon-R genomic bank in EMBL 4 (Bialojan et al., 1984), were prepared according to Benton and Davis (1977), using Biodyne membranes. Filter lifts of the Oregon-R cDNA banks were probed with the 1650 bp PstI restriction fragment of pMSal04 (mouse salivary amylase cDNA, Hagenbuechle et al., 1980), under the low stringency conditions described above for the Northern analysis. These filters were also probed with a

differential cDNA probe enriched for glucose-repressible *Drosophila* sequences (stringent conditions). Canton-S amylase cDNAs as well as Oregon-R and Canton-S genomic amylase sequence-containing clones were identified using the 650 bp PvuII fragment of OR-M3 (Oregon-R amylase cDNA) as probe (stringent conditions). Plaques giving positive signals were purified and lambda DNA prepared according to Silhavy et al. (1984).

2.10. Preparation of Differential Probe

Labelled cDNA was prepared, essentially as described by Shaw et al. (1984), from 100 ug of total RNA from larvae of the Oregon-R strain, grown on "no-sugar-added" medium. After hydrolysis of the RNA templates, the cDNA was annealed, for 16 hours, to a 200x excess of total RNA from Oregon-R larvae, grown on "glucose" medium. Poly A+ RNA was estimated at 3% of total RNA and the efficiency of reverse transcription (AMV Reverse Transcriptase, Life Sciences) was measured at about 30% - 1st strand cDNA synthesized as a proportion of template added. The resulting mixture of cDNA:RNA hybrids and single stranded cDNAs was used as a probe, without further treatment, according to the high stringency hybridization procedure described above.

2.11. Relative Message Abundance Estimates

Plasmids were linearized by restriction enzyme digestion

and spotted onto Biodyne membranes with a Schleicher and Schuell minifold device under the conditions described in the Pall Biodyne Manual. Plasmids used were: (1) pPA-1, which is pUC9 containing 2.5 kbp of genomic Adh sequences, P element ends and some 17-C (*Drosophila* genomic) sequence (Goldberg et al., 1983); (2) pOR-M3, which is an 830 bp amylase cDNA insert in pUC13 (this study); and (3) Carnegie 20, which is the Carnegie 2 vector containing 7.2 kbp of the rosy gene and P element ends (Rubin and Spradling, 1984). All target sequences were from D. melanogaster and were present at large excess. Filters were probed with labelled 1st strand cDNA (Shaw et al., 1984) from Oregon-R larvae grown on both "glucose" and "no-sugar-added" media. Hybridizations were carried out as described above using stringent conditions. The 17-C sequence and the P element ends contained in pPA-1 and Carnegie 20 do not give measureable signals in either dot blots or Northern blots under the conditions used.

2.12. Primer Extension: Cold Primer, Hot Extended

A synthetic oligonucleotide [5'-AGCGATGTCGTCCTCCACTTCC-3'] complementary to the amylase mRNA at positions 110 to 129 (see Boer and Hickey, 1986, Figure 2), was used as primer. For primer extension, a 20X molar excess of cold primer was annealed to 10 ug of poly A+ RNA from "sugar-free" treated Oregon-R larvae by heating the RNA:primer mixture to 65°C for 3 minutes followed by slow cooling, at room temperature, in the

block of a heat block apparatus. Label (^{32}P dATP*) and AMV Reverse Transcriptase (Life Sciences) were added and the reaction mixture incubated at 42°C . Initial reaction conditions were optimized for the incorporation of label (low cold dATP concentration). Following 20 min of incubation, a dNTP chase solution was added to bring the final concentration of all 4 dNTPs to greater than 0.5 mM and the mixture incubated at 42°C for an additional 20 minutes. Finally, the products were treated with NaOH, the RNA hydrolysis products and unincorporated nucleotides removed by spun column chromatography and precipitation in the presence of ammonium acetate. The resulting material was separated on a sequencing gel for analysis.

3. RESULTS

3.1.¹ The Effect of Dietary Glucose on The Level of Amylase Enzyme Activity

In this first set of experiments, the effect of 10% dietary glucose was determined, on both late third instar larvae and one week old adults, for a total of five strains of D. melanogaster. As indicated in the section on fly stocks in MATERIALS AND METHODS, these five strains were chosen because: (i) they span the amylase activity range found in nature from none at all (Amylase null strain) or very low (Oregon-R), to very high (Belinga) and; (ii) all five strains are easily distinguishable based on their amylase isozyme banding patterns on native polyacrylamide gels.

For the strain-response comparison, a total of ten separate experiments were performed; tests were performed on two developmental stages for each of the five strains. Within each experiment, three replicates of each assay were carried out using the DNSA reagent and several further replicates of all assays were performed involving the quantification of starch-iodine stained electrophoretic gels.

¹ Results presented in section 3.1. also appear in: BENKEL, B.F. and D.A. HICKEY (1986) The Interaction of Genetic and Environmental Factors in the Control of Amylase Gene Expression in Drosophila melanogaster. Genetics 114: 943-954.

3.1.1. Glucose Repression in Larvae: The results for larvae are summarized in Figure 1 and Tables 1 and 2. The results in the Tables, including the gel data, are based on several replicates of the type of assay displayed in Figure 1. However, Figure 1 serves to give an effective visual impression of the main conclusions to be drawn from the set of experiments on larvae. Larvae of the Oregon-R strain (Figure 1A) show a greater than one hundred-fold reduction in amylase activity when grown in food containing ten percent glucose. The reduction in activity in the Canton-S strain (Figure 1B) is greater than ten-fold, but less than one hundred-fold. The difference between glucose-fed and sugar-free treated larvae of the Belinga strain (Figure 1C) is close to sixteen-fold and the degree of repression is less than 10-fold in the Makokou strain (Figure 1D). Hickey and Benkel (1982) reported differential repression of the two isozymes in the Makokou strain (Amy^{4,6}). The present results confirm our original observation: the AMY-4 isozyme band is more intense than the AMY-6 band when the larvae are grown in the absence of glucose. This difference disappears and is, in fact reversed on the sugar-containing medium. This means that while the AMY-4 isozyme is repressed more than sixteen-fold by 10% dietary glucose, the AMY-6 band is repressed by only four-fold.

The more complete results, based on both DNSA assays and densitometric scanning of the electrophoretic gels, are summarized in Table 1 and Table 2, respectively. The DNSA

FIGURE 1: Quantification of Repression of Enzyme Activity in Larvae.

Each panel represents a negative print of a separate gel on larvae from one of the four strains, as follows: A, Oregon-R; B, Canton-S; C, Belinga; and D, Makokou. In each panel, lane pair 1 represents two replicate samples of homogenate from glucose-treated larvae; lane pair 2 represents an equivalent amount of homomogenate (as assayed by the Lowry method) from non-glucose-treated larvae. The remaining two lane pairs on each gel represent dilutions of the non-glucose-treated homogenates. The dilutions are tenfold and 100-fold in the case of Oregon-R and Canton-S. Dilutions of fourfold and 16-fold were chosen for the Belinga and Makokou strains.

Samples were prepared and gels processed as described in the "Amylase Enzyme Activity Measurements" section of MATERIALS AND METHODS.

A 1 2 3 4 C 1 2 3 4

AMY⁶-  - -

AMY¹-  - -

B 1 2 3 4 D 1 2 3 4

AMY⁶-  - -
AMY⁴-  - -

AMY³-  - -
AMY¹-  - -

TABLE 1: Amylase Activity in Larval and Adult Homogenates of Four *Drosophila* Strains Assayed by the DNSA Method.

Strain	Developmental stage			
	Larvae		Adults	
	Glucose(10%)	No glucose	Glucose(10%)	No glucose
Oregon-R	0.03±0.01	3.77±0.08	1.39±0.25	4.16±0.16
Canton-S	0.14±0.02	5.12±0.13	4.24±0.19	5.28±0.25
Belinga	0.98±0.03	13.37±0.30	5.06±0.12	7.38±0.40
Makokou	1.60±0.17	7.73±0.15	4.78±0.18	7.34±0.18

Larvae and adults were maintained under repressing (10% glucose) or nonrepressing (no-added-glucose) conditions, as described in MATERIALS AND METHODS. All homogenates were equilibrated for total protein content before the assays. Amylase activity is given in nanomoles of maltose/microgram of protein/minute at 37°C. All values are the means of three replicate assays; standard errors are indicated.

TABLE 2: Relative Amylase Activities of Larval and Adult Homogenates of Four Drosophila Strains.

Strain	Developmental stage			
	Larvae		Adults	
	Glucose(10%)	No glucose	Glucose(10%)	No glucose
Oregon-R	0.5%	100%	24.5%	100%
Canton-S	2.6%	100%	73.5%	100%
Belinga	7.3%	100%	60.5%	100%
Makokou	12.1%	100%	67.7%	100%

The values shown were derived from scanning starch-iodine-stained electrophoretic gels (e.g., see Figure 1) rounded off to nearest 0.5%. Comparisons, in this case, are valid only within a single strain and developmental stage; these correspond to comparisons of patterns within gels, rather than between gels. In each case, the derepressed treatment is assigned a value of 100%.

assays (Table 1) indicate that the difference between glucose-treated and non-glucose-treated Oregon-R larvae is approximately 125-fold. Moreover, from the absolute activity readings one can see that this large difference is due to the very low activities in the sugar-treated larvae rather than elevated activity levels in the sugar-free treatment. The difference between the two treatments for Canton-S larvae, 36-fold is also very large but significantly less than the difference between the two sets of Oregon-R larvae. The reduced effect in the Canton-S strain can be accounted for by the fact that, in this case, the glucose-treated larvae have several-fold higher activities than glucose-fed Oregon-R larvae.

Larvae of the Belinga and Makokou strains have higher enzyme activities on both food treatments. However, the activities are especially high on glucose food relative to the Oregon-R and Canton-S strains. For instance, there is less than a three-fold difference in activity between the Belinga and Canton-S larvae on the no-added-sugar treatment. On the glucose medium, the difference between these two strains is more than seven-fold. Likewise comparing the values in Table 1 for Oregon-R and Makokou larvae, one can see that the difference between the strains is approximately two-fold in the absence of dietary glucose but this interstrain difference rises to over fifty-fold in the presence of dietary glucose. Generally, the results in Table 1 show that larvae of all four strains have significant levels of amylase activity in the absence of

added dietary glucose and that the differences between strains are rather modest, being within a four-fold range. When glucose is added to the diet, the amylase activity in all larvae is reduced, but in a strain-specific manner. Some strains, such as Oregon-R, retain only traces of activity while other strains, such as Makokou, retain a significant fraction of their amylase activity in the presence of dietary glucose.

This differential repression of amylase activity in larvae of the four strains can also be illustrated by the densitometric quantification of electrophoretic gels. The results are shown in Table 2. The within-strain, between-treatment comparisons using gel assays (Table 2) are similar to results of the same comparisons using the DNSA assay (Table 1). By comparing the two sets of results, we see that the treatment differences based on the DNSA assays may be overly conservative. For all strains, the gel assay yields larger differences between the treatments; this may be due to the fact that the gel assay is more specific and, consequently, the trace amounts of amylase activity in some of the glucose-treated larvae are not affected by background non-specific activities as in the case of the DNSA assays. However, the same general pattern of differential, genotype-specific, glucose repression emerges from both sets of results. For instance, using either type of assay, we see a very large effect of dietary glucose on amylase levels in Oregon-R larvae, a less striking effect in Canton-S larvae, and a much more modest

effect in larvae of the Makokou and Belinga strains.

The results for both the dietary and genotypic effects on larval activities are summarized visually in Figure 2. From the Figure, one can easily see the dramatic repression of activity in Oregon-R larvae (lane pair 1), the large difference in Canton-S (lane pair 2), and the more modest effects in the Belinga and Makokou strains (lane pairs 3 and 4). Using the ten-fold and one hundred-fold dilutions of the Makokou homogenate (lane pair 5) as a guide, one can see that the activity differences between Oregon-R and Makokou larvae in the absence of glucose (lanes 1B and 4B) is less than ten-fold, whereas the difference between the glucose-treated Oregon-R larvae and Makokou larvae not treated with glucose (lanes 1A and 4B) is greater than a hundred-fold. Finally, one should note that the amylase-null homogenates (lane pair 6 in Fig. 2) show no amylase activity regardless of the dietary treatment.

In summary, as is obvious from Figure 2, the between-diet, within-strain, effects in larval activity can be much larger than the between-strain differences.

3.1.2. Glucose Repression in Adults: The results for the parallel set of experiments on adult flies (one week old) are shown in Tables 1 and 2 and in Figure 3. The dietary and genotypic effects which were observed in larvae were also seen in the adult flies but the magnitude of the differences were much reduced. From the DNSA results in Table 1, it can be seen

FIGURE 2: Glucose Repression of Enzyme Activity in Larvae.

Samples, all of which represent larval homogenates, were equilibrated for protein content before loading on the gel. Samples are as follows: lane pair 1, Oregon-R; lane pair 2, Canton-S; lane pair 3, Belinga; lane pairs 4 and 5, Makokou; and lane pair 6, Amylase null. Within each lane pair (except pair 5), sample A is from the glucose-treatment and sample B is from larvae fed a glucose-free diet. Lane pair 5 contains tenfold and 100-fold dilutions of the material in lane 4B.

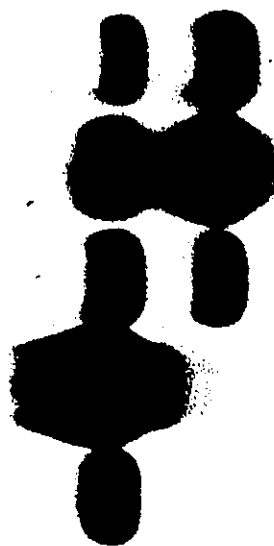
1 2 3 4 5 6
 A B A B A B A B A B A B

AMY⁶-

AMY⁴-

AMY³-

AMY¹-



that adults of all four strains are subject to glucose repression of amylase activity and that flies of the Oregon-R strain are the most sensitive to this repression. This is the same general result as for the larvae. However, the degree of repression for Oregon-R adults is only three-fold (as compared to over one hundred-fold in larvae of the same strain) and the level of repression is even less for adults of the other three strains. By comparing absolute activity values, one can see that adult and larval activities are comparable on the glucose-free diet. In the presence of glucose, however, adults of all four strains have much higher specific activities than the corresponding larval homogenates.

The essential difference between the two developmental stages is that the adults, while subject to glucose repression, are much less affected than are the larvae. This observation is confirmed by the quantification of electrophoretic gels as shown in Table 2. From both Tables 1 and 2, it can be seen that the differences between the dietary treatments are less than two-fold for all strains except Oregon-R where the differences between glucose-fed and non-glucose-treated adults are in the three to four-fold range. The results for the adult activities are summarized in Figure 3. It is clear from this Figure that although genotypic and dietary-induced differences do exist among the adult homogenates, these differences are much less dramatic than the large differences in larval activities as illustrated in Figures 1 and 2.

FIGURE 3: Glucose Repression of Enzyme Activity in Adults.

In each lane pair, sample A is a homogenate from glucose-treated flies; sample B is from non-glucose-treated flies. Samples, which were equilibrated for total protein content, are as follows: lane pair 1, Oregon-R; lane pair 2, Canton-S; lane pair 3, Belinga; lane triplet 4, Makokou; lane pair Amylase null. Lane 4C contains a tenfold dilution of the homogenate in lane 4B.

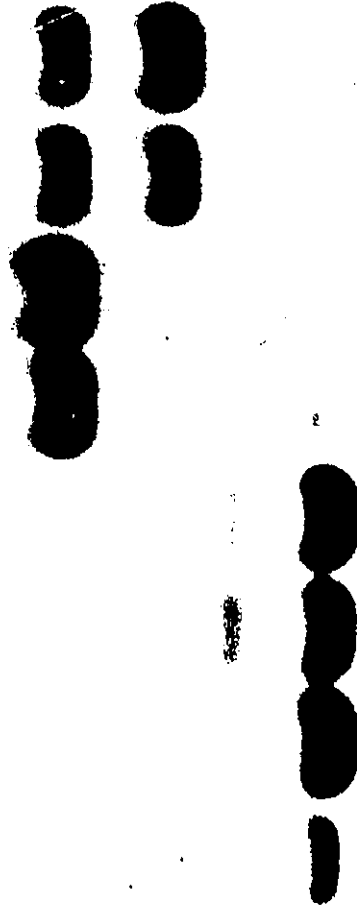
1 2 3 4 5
 A B A B A B A B A B

AMY⁶-

AMY⁴-

AMY³-

AMY¹-



3.1.3. Midgut and Whole Larval Homogenate Comparisons

The experiments described above were performed using crude extracts of whole larvae or adults. The major site of amylase enzyme production in both larvae and adults is the midgut and, in particular, the anterior and posterior midgut regions. There are, however, secondary sites of amylase activity and, presumably, amylase enzyme production such as salivary glands and fat body (Doane, 1969). One question, therefore, is whether there are tissue-specific differences in sensitivity to dietary glucose? In other words, how well do the assays using whole organisms reflect the response of the major site of activity, the midgut, to dietary repression?

The situation in larvae of sensitive strains, such as Oregon-R, is unambiguous. Since only trace levels of activity are present on glucose food, all tissues which can produce amylase in these larvae must be extensively repressed. The situation for less sensitive strains such as Makokou, however, is not as obvious. Here tests performed on whole larval extracts alone cannot distinguish between balanced, systemic repression and different, tissue-specific levels of repression.

Figure 4 shows a comparison of amylase enzyme activities measured in whole larvae (lanes 1 and 3) and dissected midguts (lanes 2 and 4) on both 10% glucose and no-added-sugar media. The Figure clearly shows that, regardless of food type, the midgut is the major site of amylase activity in Makokou larvae. Thus differences in sensitivity to glucose between different

Upper Panel:

FIGURE 4: Amylase Activity in Whole Larvae Versus Dissected Midguts: A Comparison.

A negative print of an amylase activity gel prepared as described in MATERIALS AND METHODS. Lane 1, whole organism homogenate from larvae of the Makokou strain grown on 10% glucose medium; lane 2, dissected midgut from larvae grown on 10% glucose medium, lane 3, whole larvae grown on no-sugar-added medium; lane 4, dissected midgut from larvae grown on no-added-sugar food. Each lane contains approximately 2 larval equivalents or 2 midgut equivalents of soluble protein.

Lower Panel:

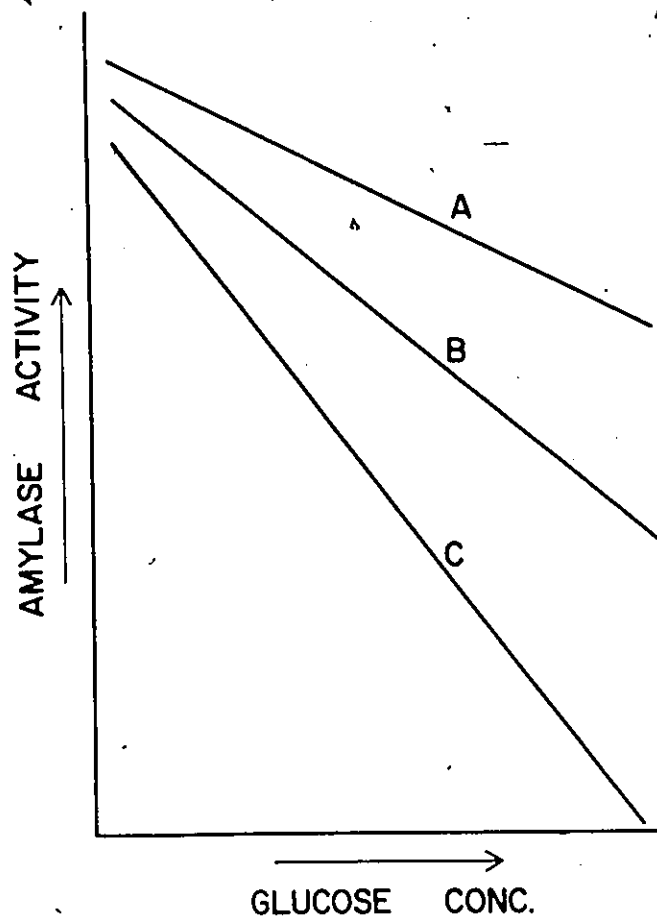
FIGURE 5: Relationship Between Dietary Glucose Concentration and Amylase Activity.

Line A, hypothetical response curve of low sensitivity strain such as Mokokou; line B, response curve of medium sensitivity strain such as Belinga; line C, hypothetical response curve of high sensitivity strain such as Oregon-R.

— — — —

 -AMY⁶
-AMY⁴

1 2 3 4



tissues which produce amylase must be relatively small. Furthermore, the results on activity modulation in extracts of whole organisms reflect the changes in activity which are taking place in the midgut of these organisms in response to dietary carbohydrate.

In summary, dietary glucose represses amylase activity in both larvae and adults of D. melanogaster. The repression of amylase activity is more dramatic in larvae of any given strain than in adults of the same strain. Most of the amylase activity is produced by the midgut in both larvae and adults, and the results on repression of activity in whole organism extracts reflect changes in activity in midguts. The pattern of response to dietary sugars displayed by the strains examined in this study is illustrated in Figure 5. The Figure shows that in the absence of added dietary carbohydrate, larvae or adults of all strains tested have relatively high levels of amylase activity. Different strains differ in their sensitivities to sugars. Thus, as the level of sugar in the medium is increased, significant differences begin to appear in amylase activities between strains. In the most extreme case observed in this study, Oregon-R larvae on 10% glucose, amylase activity is barely detectable.

3.2.¹ The Effect of Dietary Glucose on The Level of Translational Activity for Amylase Enzyme

From the previous section it is clear that amylase enzyme activity is repressed by dietary glucose and that this repression of activity is strain and developmental stage-specific. It has previously been demonstrated that the repression of amylase activity involves a concomitant, parallel change in the amount of amylase protein (Hickey and Benkel, 1982).

The next problem addressed in the current study was the level at which the regulation of amylase activity takes place. Initially, in order to determine whether control of amylase enzyme abundance is post or pre-translational, total RNA was extracted from Oregon-R larvae grown under both dietary conditions and injected into *Xenopus* oocytes. The oocytes show no endogenous amylase activity (Figure 6, lane 5) and provide a convenient, prepackaged translation system which is ideal for detecting low abundance messages (Gurdon et al., 1971). This system has been used previously to monitor *D. melanogaster* RNA-directed amylase production (Doane et al., 1983; Benkel and Hickey, 1984).

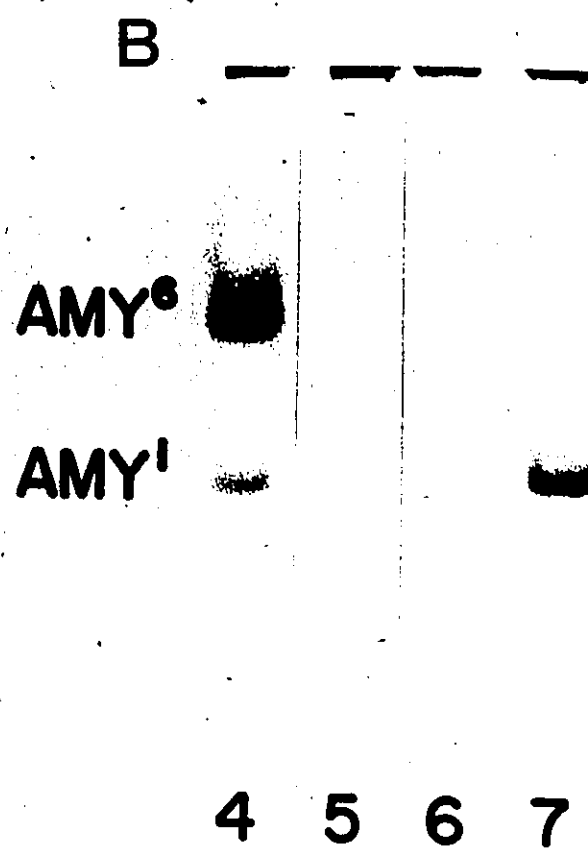
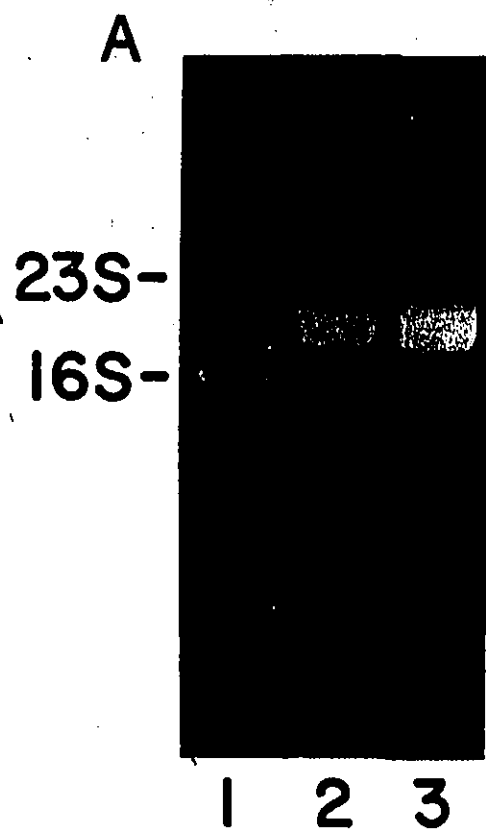
Total RNA from Oregon-R larvae grown on 10% glucose food did not direct the synthesis of any detectable *D. melanogaster*

¹ Results presented in section 3.2. also appear in: BENKEL, B.F. and D.A. HICKEY (1986). Glucose Repression of Amylase Gene Expression in *Drosophila melanogaster*. Genetics 114: 137-144.

FIGURE 6: Pretranslational Regulation of Amylase Activity in Oregon-R Larvae.

A, RNA integrity: lane 1, bacterial 23S and 16S rRNA standards; lane 2, total RNA from Oregon-R larvae grown on glucose medium; lane 3, total RNA from Oregon-R larvae grown on no-sugar-added medium. RNA was separated on a native agarose gel according to Sparks (1985).

B, Translational activity of Oregon-R RNAs: lane 4, amylase standards (mixed homogenate of Oregon-R, Amy¹, and Belinga, Amy⁶); lane 5, oocytes injected with Barth's medium; lane 6, oocytes injected with total RNA from Oregon-R larvae grown on glucose medium; lane 7, oocytes injected with total RNA from Oregon-R larvae grown on no-sugar-added medium.



amylase when injected into *Xenopus* oocytes (Figure 6B, lane 6). In contrast, a strong amylase band resulted after the injection of RNA from larvae fed the no-sugar-added food (Figure 6B, lane 7). This activity co-migrated with the Oregon-R, AMY-1 electrophoretic band (Figure 6B, lane 4). The amylase standard in Figure 6B, lane 4 consisted of a 50:50 mixture of Oregon-R (Amy¹) and Belinga (Amy⁶) larval homogenates. The difference in translational activity was not due to degradation of the RNA from the glucose treated larvae (see Figure 6A, lanes 2 and 3), nor was it due to batch to batch variation in the translational efficiency of oocytes (tested by co-injecting oocytes with RNA from an Amy⁶ strain which programs the synthesis of an electrophoretically distinguishable AMY-6 band as an internal control, data not shown). Therefore, the observed difference was due to the specific translational activities of the RNA samples.

The in ovo oocyte translation test cannot, however, tell us whether the *Drosophila* amylase messenger RNA is more abundant on "no-sugar-added" medium, and conversely rare on "glucose" medium since it does not measure mRNA levels directly. Thus, the oocyte translation results are consistent with a model which would, for instance, involve the modification of the amylase messenger RNA such that under conditions of glucose repression the normal level of amylase mRNA would be present, but in a translationally inactivated form.

In order to distinguish between these 2 possibilities, one must measure amylase mRNA levels directly, on the 2 test diets, using molecular probes specific for Drosophila amylase sequences. The isolation of D. melanogaster amylase sequences, both cDNA and genomic, is described in the next section.

3.3.¹ Molecular Cloning of Drosophila melanogaster Amylase Complementary DNA and Genomic Sequences

The initial isolation of amylase cDNAs from the Oregon-R "wild type" strain of Drosophila melanogaster was achieved by (i) exploiting the conservation of nucleotide sequence for the amylase structural region, between Drosophila and mouse (first employed for the cloning of amylase genomic sequences for the Canton-S strain of D. melanogaster by Gemmill et al., 1985); and (ii) using a differential probe enriched for D. melanogaster glucose-repressible sequences.) An amylase cDNA restriction fragment was subsequently used to isolate amylase cDNAs from the Canton-S "wild type" strain, as well as genomic sequence-containing lambda clones for both strains.

¹ Results presented in section 3.3. also appear in: BENKEL, B.F., ABUKASHAWA, S., BOER, P.H. and D.A. HICKEY (1987) Molecular Cloning of DNA complementary to Drosophila melanogaster alpha-amylase mRNA. Genome 29: 510-515.

3.3.1! Isolation of Amylase Clones: Under conditions of reduced stringency, the mouse amylase probe hybridized to a single band on Northern blots of D. melanogaster poly A⁺ RNA (Figure 7, lane 1). The size of this band was estimated at 1650 b. To isolate D. melanogaster amylase cDNAs, the mouse probe was hybridized to plaque lifts of Oregon-R cDNA banks in lambda gt10 under the same conditions of reduced stringency used for the Northern analysis.

In one experiment, of an estimated 20,000 plaques, eight positives (see Figure 8) were picked and rescreened with the mouse probe to yield six purified isolates. The six cDNA inserts differed in size ranging from about 700 bp - 1500 bp in length. They showed homology to each other under stringent hybridization and wash conditions (data not shown), and all six hybridized to the same band as the mouse amylase probe on Northern blots of *Drosophila* RNA (see Figure 7, lane 2).

In a second experiment, the differential 1st strand cDNA probe (see MATERIALS AND METHODS) was hybridized to a filter lift of an estimated 30,000 plaques (Figure 9A). A second lift from the same plate was probed with one of the fly cDNAs isolated using the mouse amylase cDNA (Figure 9B).

The mouse homology-derived probe established that the plate contained approximately 20 amylase cDNA clones. A similar pattern resulted from screening with the differential probe (Figure 9A) where, of eighteen strong signals, the eight strongest positives corresponded to amylase cDNAs. A total of

Upper Left Panel:

FIGURE 7: Northern Analysis: Identification of D. melanogaster Amylase mRNA.

Poly A⁺ RNA from 3rd instar larvae of the Oregon-R "wild type" strain of D. melanogaster was separated and blotted as described in MATERIALS AND METHODS. Lane 1, probed with the mouse amylase cDNA probe at reduced stringency; lane 2, probed with the PvuII fragment of OR-M3 under stringent conditions. Sizes refer to the positions of the 23S rRNA (2900 b) and 16S rRNA (1540 b) of E. coli as well as the Adh mRNA (1100 b) of D. melanogaster.

Upper Right Panel:

FIGURE 8: Identification of D. melanogaster Amylase Copy DNAs Using A Mouse Amylase Probe.

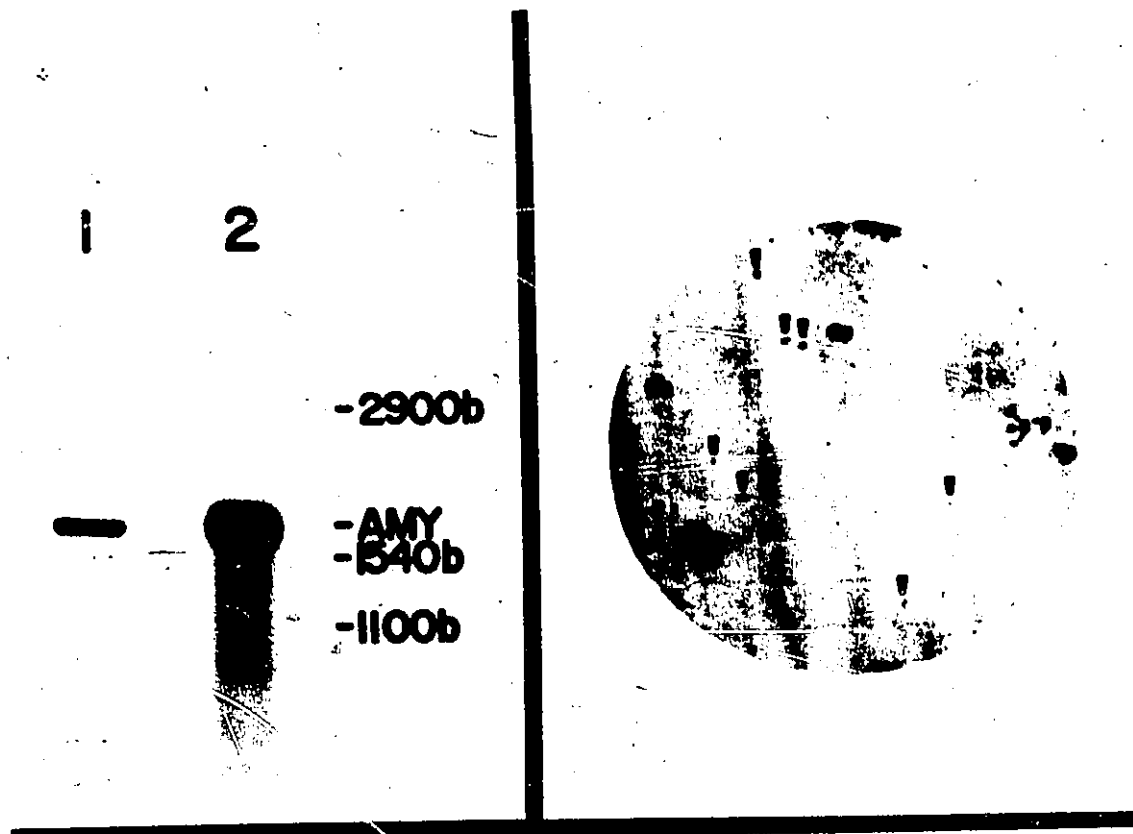
A plaque lift of a plate containing an estimated 20,000 recombinant lambda gtl0 plaques was probed with the gel purified insert of the plasmid pMSal04 (mouse salivary amylase cDNA, Hagenbuechle et al., 1980). Low intensity positives marked "v" indicate Drosophila amylase sequence-containing clones. Signals marked "p" represent plasmid sequences cloned into bank at a frequency of about 0.05%. Hybridization was carried out at reduced stringency as described in MATERIALS AND METHODS.

Lower Panel:

FIGURE 9: Differentially Expressed Sequences.

A, Plaque lift of lambda gt10 bank from larvae probed with the differential probe (described in MATERIALS AND METHODS) using stringent hybridization conditions. Plaques marked "a" are amylase cDNA-containing clones. Plaques marked "-" represent other putative glucose-repressed sequences.

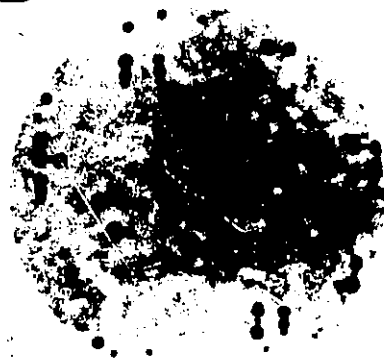
B, Duplicate plaque lift probed with pOR-M3, a mouse amylase homology-derived D. melanogaster amylase cDNA clone (in pUC13). Plaques marked with arrows "↑" represent cloned pUC9 inserts. All others are cloned amylase cDNAs; plaques marked "a" correspond to strong signals with the differential probe.



A



B



ten amylase cDNAs gave positive signals with both the differential and the mouse amylase homology-derived probes. On the other hand, ten weakly positive plaques identified with the differential probe did not light up with the mouse amylase cDNA-derived probe, therefore, these may represent other glucose-repressible sequences.

The Oregon-R cDNA clones, isolated as described above, were found to contain an internal PvuII restriction fragment of 650 bp (see Figure 11). This fragment was recovered from low-melting-temperature agarose gels and used as a hybridization probe, under stringent conditions, to isolate Canton-S amylase cDNAs and amylase genomic sequences from both the Oregon-R and Canton-S "wild type" strains of D. melanogaster (for a description of the banks screened see MATERIALS AND METHODS).

One such screening, the isolation of genomic clones from a Canton-S genomic bank in the vector lamdba EMBL 4, is shown in Figure 10. Part A of the Figure shows the first screening of 20-30,000 plaques at high density. Part B of Figure 10 shows the plaque purification of positives from the 1st screening by means of a second screening at low density.

3.3.2. Characterization of Amylase Clones: Three D. melanogaster amylase cDNA-containing clones were chosen for further analysis. The OR-M3 (Oregon-R) insert of 830 bp, the OR-M7 (Oregon-R) insert of 1500 bp, the longest amylase cDNA

FIGURE 10: Isolation of Genomic Amylase Clones.

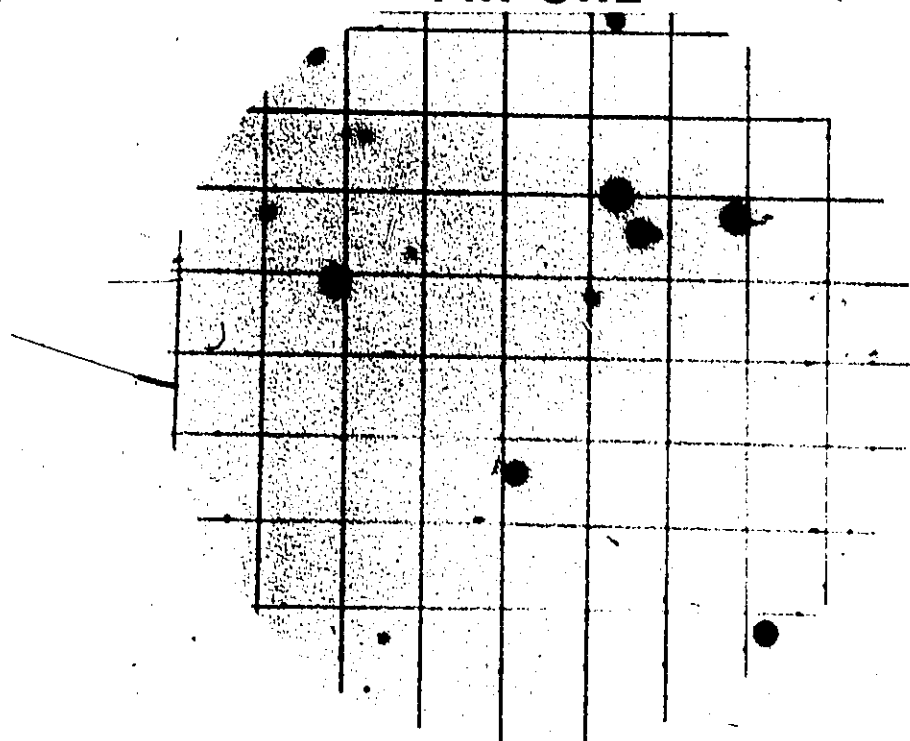
A, shows a plaque lift of a plate containing an estimated 30,000 recombinant clones. The library used here was the Oregon-R genomic bank in lambda.EMBL 4 (Biajolan et al., 1984).

B, shows the plaque purification of positives from the first probing (shown in part A) by means of a rescreening at low plaque density.

For both part A and part B, the probe consisted of the PvuII fragment of OR-M3 (Oregon-R amylase cDNA, 3'-coding region). Hybridization was carried out at high stringency as described in MATERIALS AND METHODS.

A

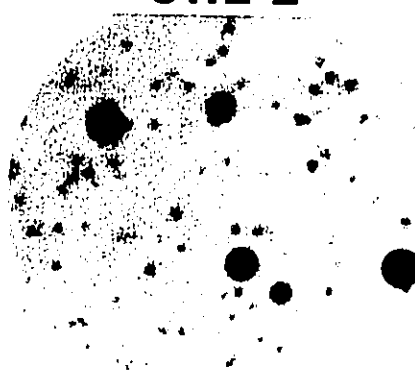
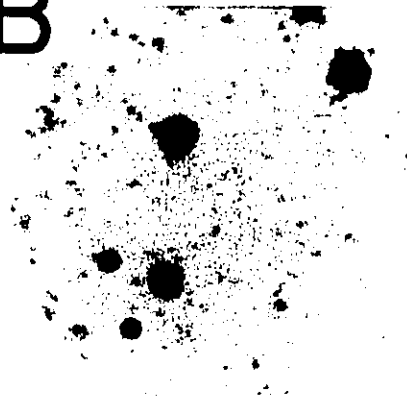
PIR-OR2



B

OR2:1

OR2:2



insert isolated in this study, and the CS-P4 (Canton-S) insert of 1400 bp were transferred to pUC13 for restriction analysis. Using these clones, and the genomic clones described below, the entire DNA sequence for the *Drosophila* amylase coding region has been determined, analyzed and compared to the mouse amylase sequence (Boer and Hickey, 1986). The authors find 57% homology, at the nucleotide level, over 1481 bp stretching from the "AUG" to the stop codon of the Oregon-R cDNA sequence and the equivalent murine pancreatic amylase coding region (Hagenbuechle et al, 1980). More importantly, the predicted protein product of the mature *Drosophila* amylase enzyme shows over 55% amino acid homology with the mouse pancreatic enzyme. This significant homology with the mouse sequence serves both to establish the clones described here as D. melanogaster amylase sequences and to explain why the mouse molecular amylase probe can be used successfully to isolate amylase clones from *Drosophila*.

Although the nucleotide sequences of the Oregon-R and the Canton-S amylase messengers differ by about 1% (Boer and Hickey, 1986), the restriction maps of pOR-M7 and pCS-P4 are identical, for the enzymes shown, over the extent of overlap of the two clones (Figure 11). Both cDNAs show a triad of sites; 5'-BamHI-0200 bp-SalI-500 bp-BamHI-3'. This pattern has also been found in regions of a D. melanogaster genomic amylase clone from the Canton-S strain, isolated by Gemmill et al. (1985), and in genomic amylase clones isolated in this study

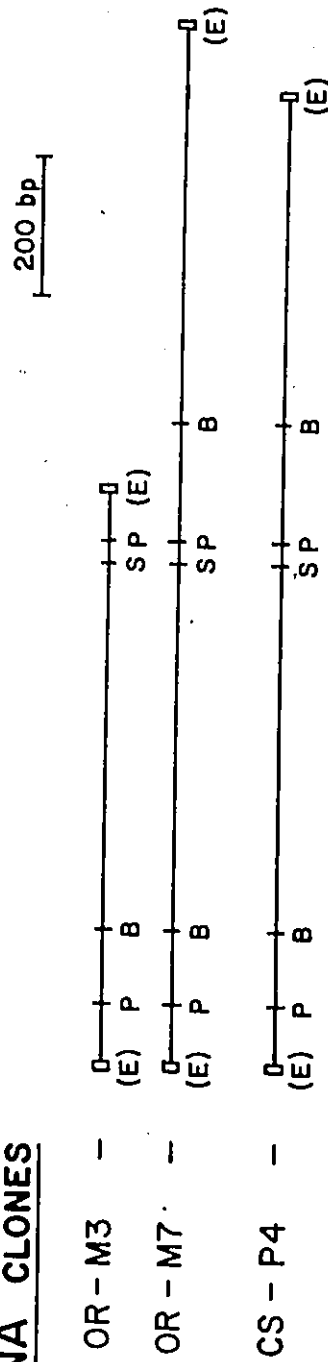
FIGURE 11: Restriction Maps of Amylase cDNAs and Genomic Clones.

A, Amylase cDNAs from Oregon-R (OR-M3, OR-M7) and Canton-S (CS-P4), poly-A tails on the left. Open boxes represent EcoRI linkers used in construction of banks.

B, Amylase genomic clones from Oregon-R bank (lambda OR2:1, lambda OR2:2) and Canton-S bank (lambda CS1:1). Open arrows indicate the locations of transcribed regions and the direction of transcription.. Note the duplication of the structural locus. Insert indicates the location of sequences corresponding to Oregon-R cDNAs. Open circles indicate EcoRI site polymorphisms between the two strains.

Restriction enzymes used were: B=BamHI; E=EcoRI; H=HindIII and S=SalI; P=PvuII used for cDNA analysis only.

A. CDNA CLONES

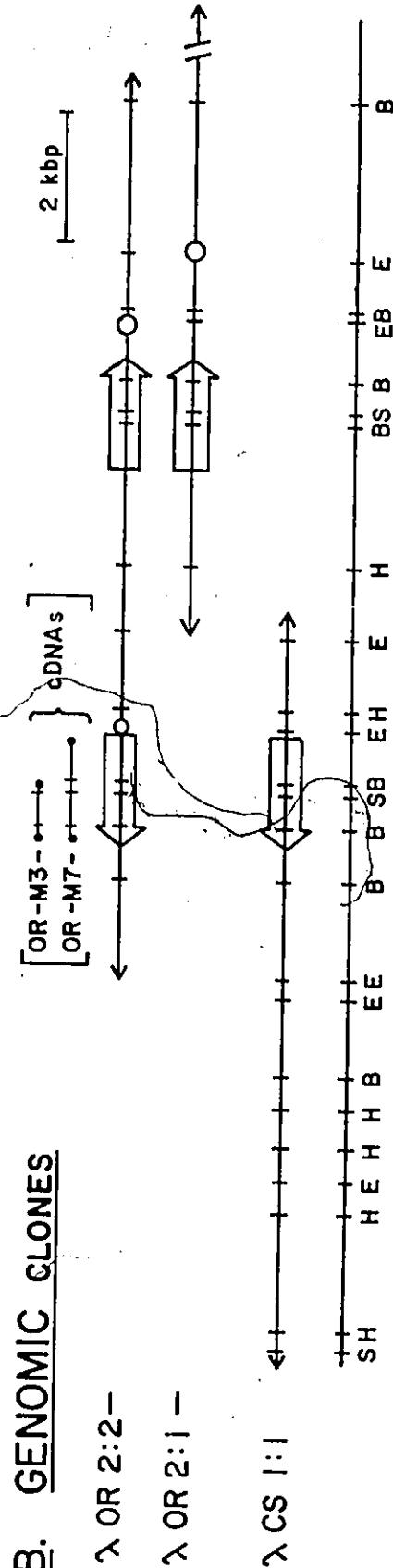


B. GENOMIC CLONES

λ OR 2:2-

λ OR 2:1-

λ CS 1:1



(see below).

Restriction maps of two clones isolated from an Oregon-R genomic library and one from a Canton-S genomic bank are shown in Figure 11. Overall, the maps agree with that of lambda Dm65, a Canton-S genomic clone isolated from the Maniatis Drosophila library (Maniatis et al., 1978) by Gemmill et al., (1985). The BamHI - SalI - BamHI site triad, found in the cDNA clones (see above) is clearly duplicated and inverted in the amylase structural region (Levy et al., 1985). Furthermore, the distance between the two coding regions is conserved in most strains studied to date (Gemmill et al., 1986).

There are, however, some differences between the Oregon-R and Canton-S amylase regions. For instance, the EcoRI site just upstream of the transcribed region of the locus on the left in Figure 11 (identified as the proximal locus by Gemmill et al., 1986) is present in Canton-S only. The presence of this site in Canton-S and its absence from the Oregon-R sequence is evident from Southern blots of genomic DNA digested with EcoRI (see Figure 12). This restriction polymorphism can also be inferred from a comparison of the DNA sequences of the two strains (Boer and Hickey, 1986). In addition, the size of the EcoRI fragment containing the transcribed unit on the right hand side in Figure 11 (the distal locus) varies between strains. Among strains which express amylase, this fragment may be approximately 6.0 kbp in length, as in Canton-S (Amy^{1,3}), or approximately 5.4 kbp, as the Copenhagen (Amy^{2,3}) strain, or

FIGURE 12: Southern Analysis of Genomic DNA.

Southern blot of genomic DNA from Oregon-R (lanes 2 and 4) and Canton-S (lanes 1 and 3) "wild type" strains of D. melanogaster, probed with an amylase coding region-specific probe (Oregon-R amylase cDNA - OR-M7 insert). Lanes 1 and 2, DNA digested with EcoRI. Lanes 3 and 4, DNA digested with both EcoRI and SalI. Oregon-R shows two EcoRI fragments in the 5.2 to 5.4 kbp range. In contrast Canton-S shows bands of 3.8 and 6.0 kbp. The EcoRI/SalI patterns are similar for the two strains. Differences in patterns are due to EcoRI site polymorphism in both "proximal" and "distal" restriction fragments (see Figure 11 Part B and text). The 1.5 kbp SalI/EcoRI fragment from the downstream portion of the distal locus in Oregon-R is replaced by a 2.2 kbp fragment in Canton-S.

Size estimates are based on positions of lambda digest fragments on same blot as well as fine-structure mapping and sequence information where available.

1 2 3 4

-6.0

-5.2

-3.8

-2.7

-2.5

-2.2

-1.5

-1.0

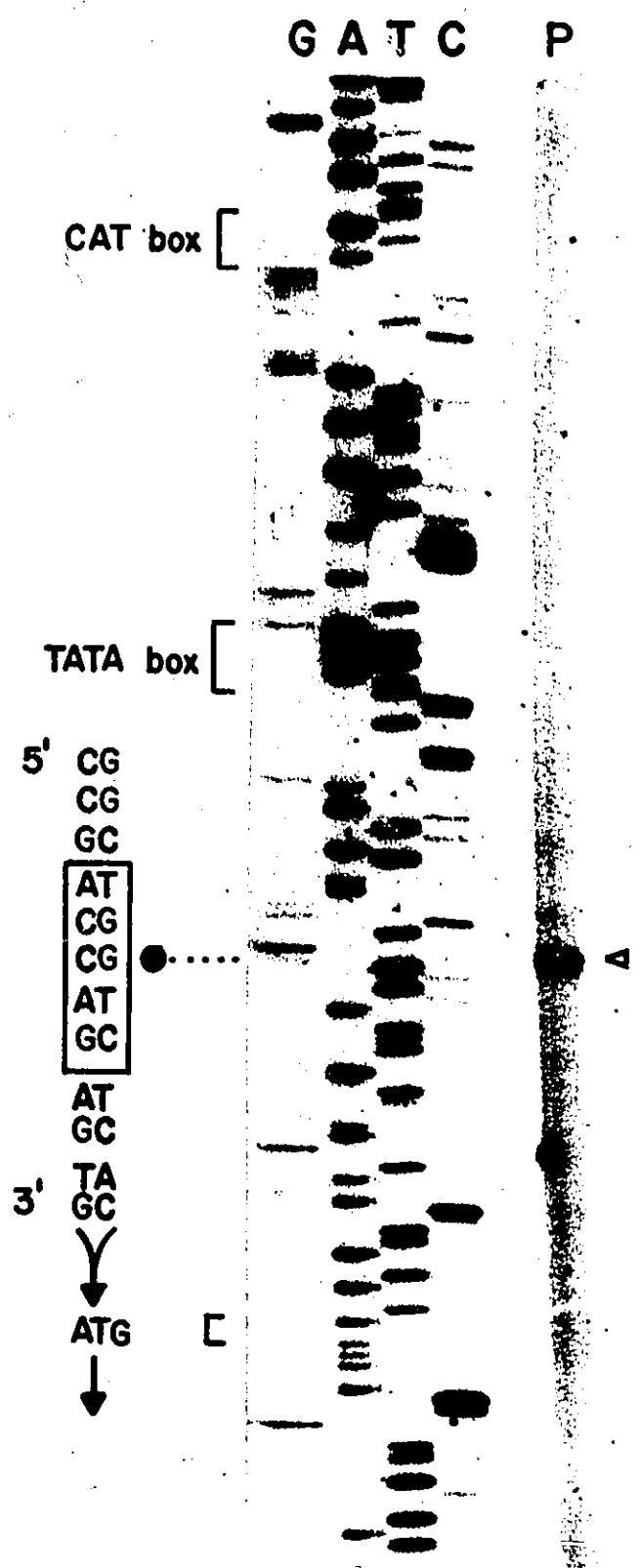
much greater than 6.0 kbp, as in the Copenhagen (Amy²) strain (Gemmill et al., 1986).

I have isolated clones containing 5.4 kbp EcoRI fragments (typical of Oregon-R, see Figure 12, lane 2), as well as clones with 6.0 kbp EcoRI fragments (typical of Canton-S, see Figure 12, lane 1) from the Oregon-R genomic bank. These fragments differ in size, by about 700 bp, in the SalI to EcoRI fragment; i.e. the downstream portion of the locus (see Figure 12, lanes 3 and 4). Thus it appears that the fly stock used for the construction of the Oregon-R genomic bank was segregating for the 5.4 kbp and the 6.0 kbp distal EcoRI fragments.

3.3.3. Expression Motifs: Figure 13 shows the mapping of the 5' end of the amylase transcript with respect to the upstream genomic sequence, in 3rd instar larvae of the Oregon-R strain. Transcription begins at the second C in the sequence ACCAG (Figure 13, arrowhead). This sequence resembles the ATCAG/TTC/T motif which is found to mark the start of transcription for a number of *Drosophila* genes (Hultmark et al., 1986). The typical eukaryotic RNA polymerase II promoter often contains a TATA-box and a CAAT-box, located about 30 bp and about 75 bp upstream of the transcription start site respectively. The start site identified by primer extension is downstream of both the motifs mentioned above with appropriate spacing (see Figure 13).

FIGURE 13: Identification of the Transcription Start Site for the Amylase mRNA.

Lane P shows the result of extending the synthetic primer (see MATERIALS AND METHODS) to the end of the amylase messenger RNA (marked with an arrowhead). Lanes G, A, T and C show the result of a sequence analysis of a subcloned genomic DNA fragment, which spans the promoter region. The CAAT, TATA, and ACCAG (transcription start site) promoter elements as well as the translation start codon (ATG) are indicated on the left side of the Figure.



3.4.¹ The Effect of Dietary Glucose on the Abundance of Amylase Messenger RNA

The effect of diet on amylase mRNA levels was investigated using 2 approaches. One approach used was to extract RNA from larvae or adults grown on the test foods and to perform either a dot blot or a Northern analysis. In this case, a large excess of radioactive, amylase-specific probe was used to drive the hybridization reaction, thus the resulting signal was proportional to the amount of filter-bound amylase mRNA. When combined with appropriate dilutions of the sample producing the stronger signal, this method is ideal for the quantification of differences in messenger levels due to changes in dietary sugar concentrations.

In the second approach the filter-bound, target sequences used were cloned DNA inserts in plasmid vectors. The probe used was 1st strand copyDNA reverse transcribed from RNA. In the case the resulting signal was roughly proportional to the amount of the particular mRNA in the reverse transcribed RNA population. This method is not useful for the quantification of

¹ The results pertaining to Oregon-R larvae presented in section 3.4. also appear in: BENKEL, B.F. and D.A. HICKEY (1987) A Drosophila Gene is Subject to Glucose Repression. Proc. Natl. Acad. Sci. USA 84: 1337-1339.

the differences in amylase mRNA on the two test diets, but it allows the abundance of amylase mRNA to be compared directly to that of other RNA species.

3.4.1. Glucose Repression of Amylase mRNA Abundance: Glucose repression of amylase expression was demonstrated by growing larvae of the Oregon-R "wild type" strain on the two different test media as described above. Third instar larvae were assayed for amylase enzyme activity by specific staining of native electrophoretic gels (Figure 14), run as described above. Duplicate gels were stained for alcohol dehydrogenase (ADH) activity, as an internal control. As can be seen from Figure 14A, there is a large difference in amylase activity between glucose-fed and non-glucose-fed larvae: both sets of larvae, however, show equivalent levels of ADH activity. In order to test whether dietary glucose affected the level of amylase mRNA, total RNA was extracted from larvae grown under both dietary conditions. The results of Northern blots probed with amylase-specific cDNA probes are shown in Figure 14B. It is clear that the glucose effect is also seen at the level of amylase mRNA abundance. Again, Adh was used as an internal control by simultaneously probing the filters with an Adh specific probe (Figure 14B). The difference in amylase mRNA levels between the treatments was quantified using dot blot analysis (Figure 14C). Several autoradiographs, of the type shown in Figure 14C, were scanned with an optical densitometer

FIGURE 14: The Effect of Dietary Glucose on Larval Amylase Activity and Amylase mRNA Abundance in Oregon-R.

Alcohol dehydrogenase (Adh) enzyme levels and mRNA levels were monitored as internal controls.

A, The effect of glucose on enzyme activity. Lane 1, larvae were grown on food containing 10% dextrose; lane 2, larvae grown on food lacking the dextrose component. Each lane represents approximately two larval equivalents. A duplicate gel stained for alcohol dehydrogenase (ADH) activity is shown as an insert.

B, Glucose affects the level of amylase mRNA. Each lane contains 10 ug of total RNA. Lane 3, RNA from larvae grown on food containing 10% glucose; lane 4, RNA from food with no glucose added. The positions of the amylase (1650 bp) and Adh (1100 bp) messengers are indicated.

C, Ten percent dietary glucose reduces amylase mRNA to less than 1% of its sugar-free level. The first dot from the left, (+) Gluc, represents 10 ug of total RNA from larvae grown on food containing 10% glucose. The second dot, (-) Gluc, represents 10 ug of total RNA from larvae grown on food with no added glucose. Each successive dot represents a 1:4 dilution of the previous dot, the last dot representing apparently 39 ng of "sugar free" RNA. The lower portion shows the strip, washed and reprobed with an Adh-specific probe.

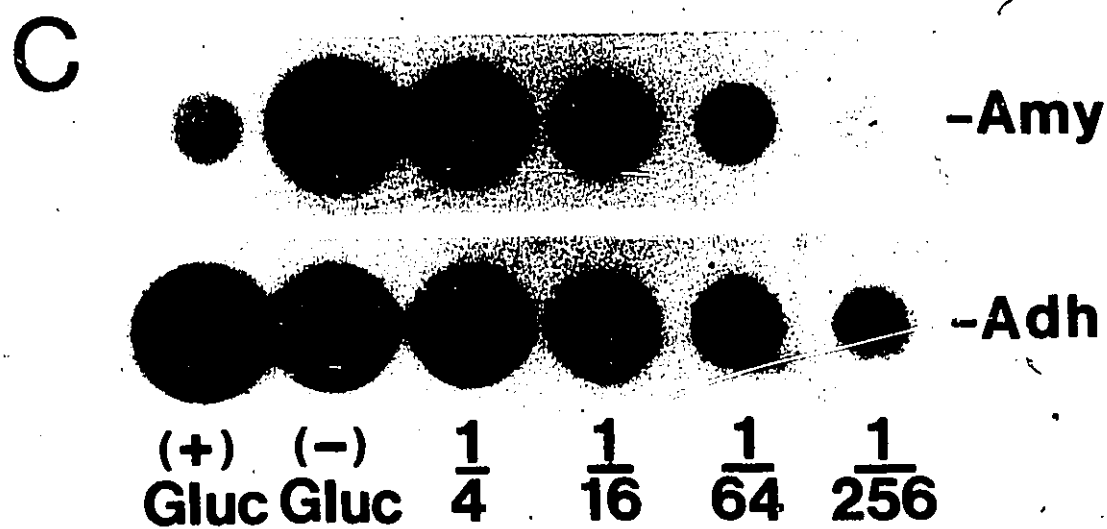


TABLE 3: Relative Abundance of Amylase mRNA in Glucose-Fed and Non-Glucose-Fed Larvae of the Oregon-R Strain.

Experiment	Dietary treatment		Amylase probe
	Sugar-free, %	Glucose, %	
1	100	2.3	A
2	100	1.6	A
3	100	0.5	A*
4	100	0.3	B

Dot blot hybridizations of the type described in Fig. 1C, including serial dilutions, were performed with several RNA batches from independent experiments. Alcohol dehydrogenase (Adh) probes were used to control for variations in total amount of RNA. Autoradiographs were scanned with an optical densitometer; amylase signal values were normalized relative to the Adh signal, and the glucose-treatment value was expressed as a percentage of the glucose-free value. Probe "A" consisted of the entire pOR-M3 plasmid containing 864 bp of D. melanogaster cDNA (including an A₁₇ tail) cloned in pUC13. Probe "B" was a gel-purified 650 bp fragment of the pOR-M3 plasmid containing only amylase coding sequence.

* Experiment 3 was corrected for nonspecific background hybridization by inclusion of a bacterial RNA dot on the filters.

and the results are presented in Table 4. From the Table, it can be seen that in all cases the amylase levels in glucose-fed larvae were less than 3% of that in the non-glucose-fed controls. Moreover, by using a variety of amylase-specific probes I could show that a large fraction of the low (+) Glucose signal was due to background hybridization. By either correcting for the non-specific background (Table 4, row 3) or by using gel-purified amylase coding region-specific fragments as probes (Table 4, row 4) it can be shown that the glucose-fed larvae have less than one percent of the control mRNA levels. These results for the effect of dietary glucose on amylase mRNA levels compare closely with the results on the glucose repression of enzyme activity/abundance and mRNA translational activity.

Quantification of differences in amylase mRNA levels can also be achieved by Northern Blot analysis, provided appropriate dilutions of the stronger signal-producing sample are included. Figure 15 shows the results of probing a Northern blot containing total RNA from Oregon-R larvae, grown on both 10% glucose and no-sugar-added foods, for amylase and Adh sequences (as internal control) simultaneously. Note that the difference in amylase mRNA abundance between the 2 food treatments is large; close to 100-fold. The difference, however, does not appear to be quite as large as estimated in the RNA dot blot analyses described above. This is probably due to the loading of different amounts of RNA in the 10% glucose

Upper panel:

FIGURE 15: Amylase mRNA Level Analysis in Oregon-R Larvae.

Northern blot prepared as described in MATERIALS AND METHODS. Lane pair 1, total RNA from 3rd instar larvae grown on 10% glucose food; lane pair 2, total RNA from larvae grown on no-sugar-added food; lane pair 3, a ten-fold dilution of the material in lane pair 2; lane pair 4, a 100-fold dilution of the material in lane pair 2. The blot was probed with a mixture of the PvuII fragment of OR-M3 (amylase cDNA) and plasmid pAdh (Adh genomic sequence) nick translated to high specific activity. The positions of the amylase and Adh messages are indicated.

Lower panel:

FIGURE 16: Quantification of Glucose Repression at the Level of Amylase mRNA in Larvae.

Dot blot analysis for larvae of three strains of D. melanogaster. Upper strip shows extent of repression in Oregon-R. Middle strip measures the effect of 10% dietary glucose in larvae of the Canton-S strain. Lower strip shows a similar analysis of amylase levels in Belinga larvae. Numbers refer to dilution factors of dots in relation to non-glucose treatment (-). Gluc dots. Total RNA was applied to Biodyne filters and probed as described in MATERIALS AND METHODS. The PvuII fragment of OR-M3 was used as probe under stringent hybridization conditions.

Amy-

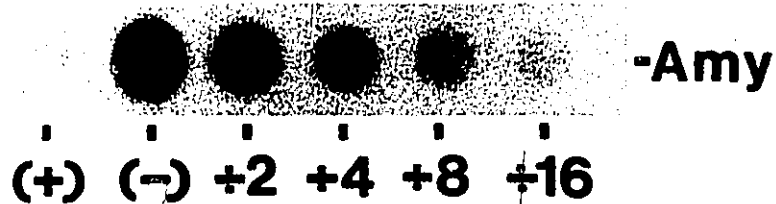
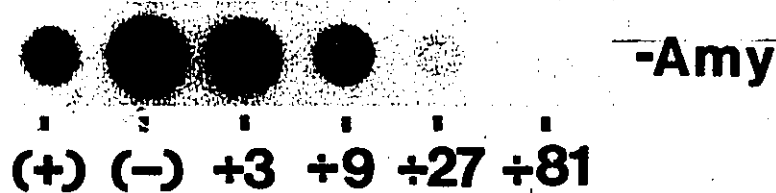
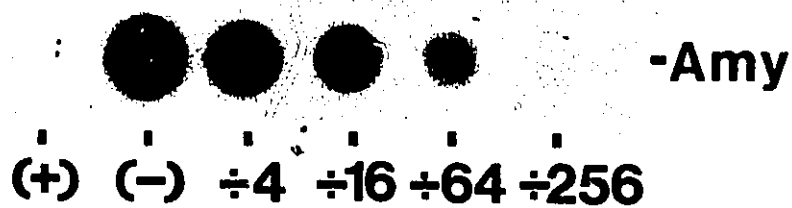
Adh-

1

2

3

4



and no-glucose-added lanes. In the figure, however, the Adh signal for the no-sugar-added sample (lane pair 2) is obscured by a heavy tail from the amylase band. This tail is presumed to be due partly to natural turnover of the amylase mRNA and partly to degradation of the RNA during isolation. Both the RNA blot analysis and Northern analysis, however, demonstrate that dietary glucose represses amylase mRNA levels in Oregon-R larvae dramatically.

The effect of dietary glucose on the abundance of amylase mRNA in both larvae and adults of the four amylase-producing strains of D. melanogaster examined in this study is shown in Figures 16 and 18, and summarized in Table 4. Figure 16 shows a dietary treatment analysis (amylase mRNA only) for larvae of each of the Oregon-R, Canton-S and Belinga strains. The extreme effect of 10% dietary glucose on amylase mRNA abundance in Oregon-R larvae (about 200x) is clearly evident. The difference in amylase messenger RNA level between the glucose and no-glucose-added treatments in the Canton-S strain (about 20x in Figure 16) is still dramatic but visibly smaller than the difference in Oregon-R. In Belinga larvae, using the serial dilutions as a guide, it is clear that 10% glucose represses amylase mRNA by greater than 10-fold.

Note that the values for glucose repression presented above, although uncorrected for RNA quantity differences, are similar to the values determined for repression at the level of amylase enzyme activity. A visible comparison of enzyme

activity and mRNA levels for larvae of three amylase-producing strains, as well as the amylase null strain, is presented in Figure 18. At the mRNA abundance level, as at the enzyme activity level, the three amylase-producing strains show relatively small differences on no-sugar-added medium (see the B lanes in each lane pair). The addition of 10% glucose to the medium represses amylase mRNA abundance in all three strains. This repression, however, occurs in a strain-specific fashion (see the A lanes in each lane pair).

The more complete results based upon replicates (as indicated in Table) analyzed by densitometer scans of autoradiograms for all strains and developmental stages tested are presented in Table 4. From the Table it is clear that the pattern outlined above for mRNA response in larvae can be extended to include adults as well. Thus, the overall pattern which emerges is that both larvae and adults of all strains of D. melanogaster show the glucose repression response. Changes in dietary glucose concentration affect the abundance of amylase messenger RNA. This, in turn, is the major factor in the regulation of amylase enzyme activity. The extent of glucose repression, however, is developmental-stage and strain-specific. Some strains, such as Makokou, are relatively insensitive to glucose, while in others, such as Oregon-R amylase expression is dramatically repressed by dietary glucose. One exception to the pattern described above is the amylase null strain. This strain shows no amylase enzyme

TABLE 4: Relative Abundance of Amylase mRNA in Glucose-Fed and Non-Glucose-Fed Larvae and Adults of D. melanogaster.

Strain	Developmental stage			
	Larvae		Adults	
	Glucose(10%)	No glucose	Glucose(10%)	No glucose
Oregon-R	0.5 ³	100	23.0 ²	100
Canton-S	5.5 ³	100	57.0 ²	100
Belinga	13.0 ¹	100	ND	ND
Makokou	28.5 ¹	100	75.0 ¹	100
Amy <u>null</u>	89.0 ²	100	ND	ND

¹ - based on a single test

² - average of two replicates

³ - average of three or more replicates

Autoradiograms of the kind shown in Figures 16 and 18 were scanned with an integrating densitometer. Differences between treatments were interpreted using dilution series as shown in the Figures, and values for amylase signal intensities were normalized for Adh internal control signal intensities before conversion to the nearest 0.5 percent of sugar-free values. Comparisons are valid only within a single strain and developmental stage.

activity, however, RNA dot blot analyses show the presence of low but detectable levels of amylase-specific sequences (see Figure 17). The null strain is examined more closely in the next section.

3.4.2. Amylase mRNA Abundance in Amylase Null Larvae: The amylase null strain produces no measurable amylase enzyme activity in either the adult or the larval stage, irrespective of the carbohydrate components of the food medium (Figure 18, lane pair 4). In contrast, dot blot analysis of total RNA from 3rd instar larvae of the null strain reveals an amylase signal level roughly equivalent to that in repressed Oregon-R larvae (see Figure 17; Figure 18, lane pair 4). Northern blot analysis shows that the amylase signal in dot blots is indeed proportional to an mRNA band at the amylase messenger position. In repressed Oregon-R larvae, which produce a roughly equivalent level of amylase mRNA, amylase enzyme activity is low but detectable (see Figure 1A, lane pair 1). Therefore, it appears that the amylase messenger RNA produced by amylase null larvae does not code for active amylase enzyme. Apparently, therefore, amylase null larvae produce a low level of defective amylase mRNA, and this level of amylase messenger is not effected by dietary carbohydrate components.

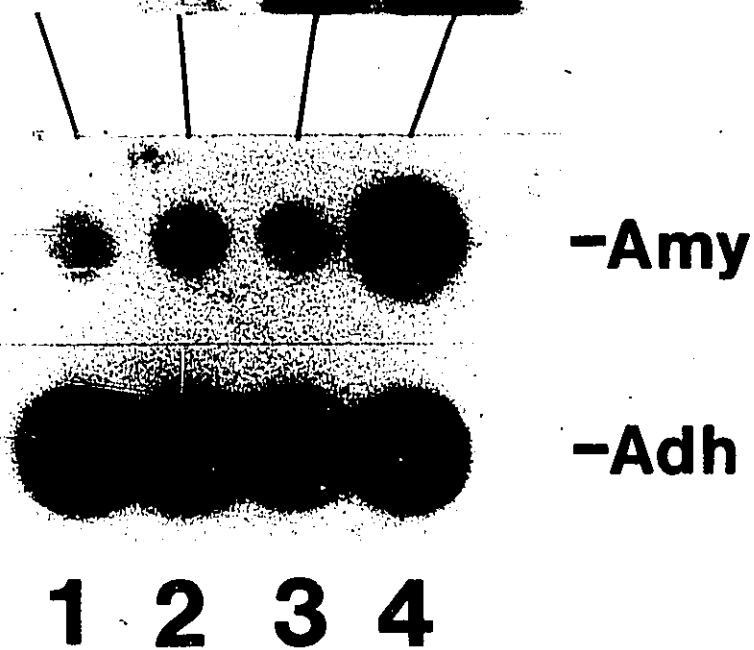
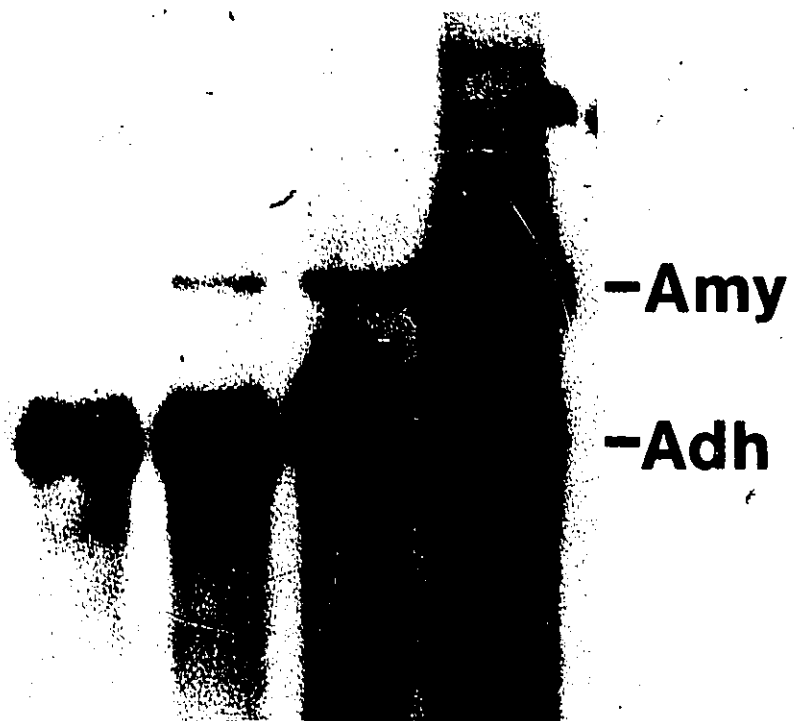
FIGURE 17: Analysis of Amylase mRNA Levels in the Amylase null Strain.

Top: A Northern blot probed for amylase (OR-M3, PvuII fragment) and Adh (pAdh) messenger RNAs.

Bottom: An RNA dot blot analysis for amylase and Adh mRNA levels. Upper strip probed with the amylase-specific probe, and lower strip probed with the Adh sequence-specific probe, under high stringency hybridization and wash conditions.

Lane 1, total RNA from amylase null strain larvae grown on 10% glucose medium; lane 2, total RNA from amylase null larvae grown on no-sugar-added medium; lane 3, RNA from Oregon-R larvae grown on 10% glucose food; lane 4, RNA from Oregon-R larvae grown on no-sugar-added food.

For both Northern and dot blot analysis, the Adh hybridization serves to measure the amount of RNA in dots and bands.



3.5. Amylase mRNA Abundance Estimates

As shown above, the abundance of the amylase messenger RNA in the Oregon-R "wild type" strain is sharply repressed by dietary glucose. Here, I was interested in estimating the relative abundance of the amylase messenger, in comparison to other mRNAs of well characterized gene-enzyme systems, under the two test dietary regimes. Figure 19 shows that the amylase mRNA is rare, and comparable to the level of Xanthine dehydrogenase (rosy) mRNA on repressing (10% glucose) food. In contrast, on derepressing (no-added-glucose) food, the amylase messenger RNA is more abundant than the Alcohol dehydrogenase (Adh) mRNA. Information on the absolute abundance of either mRNA in *Drosophila* is not available, however, the abundance of XDH (Seybold, 1974) and ADH (Goldberg, 1980) enzymes in total protein of *D. melanogaster* has been estimated at about 0.01% and 1% respectively. Thus the abundance of the amylase messenger RNA corresponds to a protein which is more abundant than ADH on "sugar-free" food and as rare as XDH on "glucose" food. Assuming that protein levels reflect mRNA levels, the abundance of the amylase messenger, in Oregon-R larvae, can be estimated to vary from 0.01% of poly A⁺ RNA to greater than 1% of poly A⁺ depending on the dietary regime.

Upper Panel:

FIGURE 18: Glucose Repression in Larvae: Comparison of Repression at the Enzyme Activity and mRNA Levels for Four Strains.

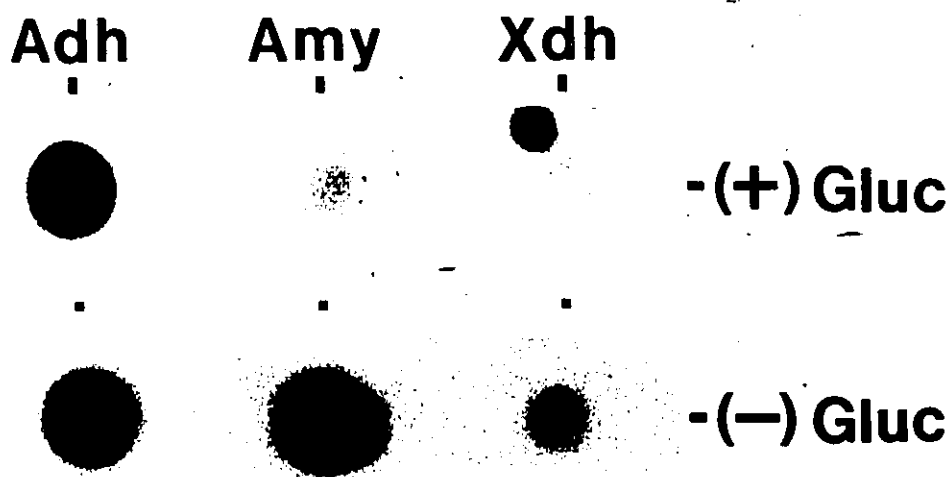
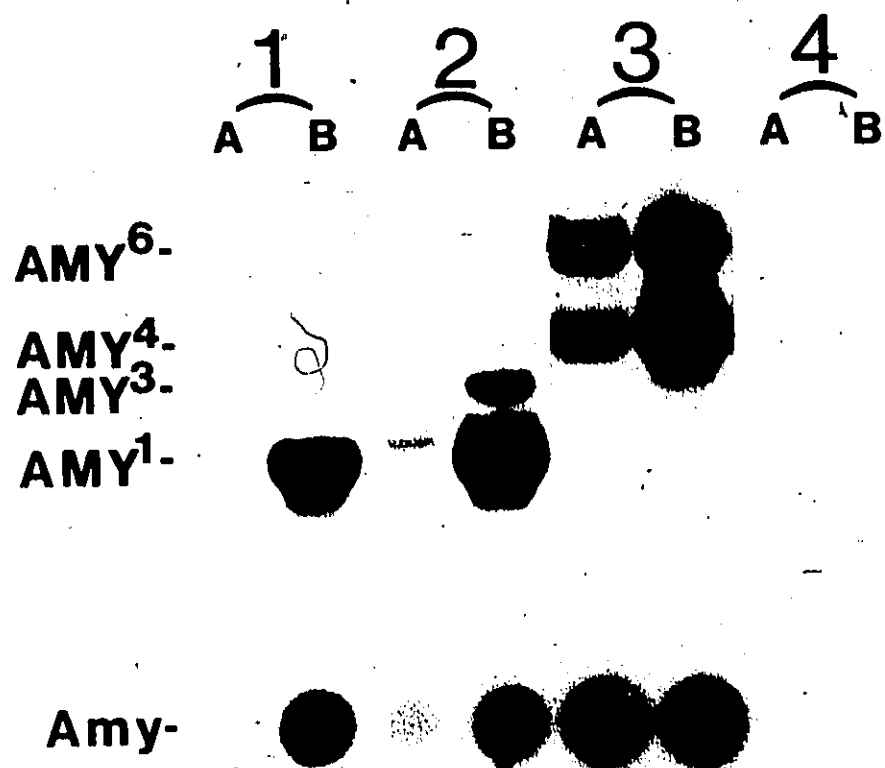
Top: Negative print of gel assay for enzyme activity (similar to Figure 2). Lane pair 1, Oregon-R; lane pair 2, Canton-S; lane pair 3, Makokou; lane pair 4, Amylase null. Within each lane pair, sample A is from glucose treated larvae and sample B is from larvae fed a no-glucose-added diet.

Bottom: RNA dot blot analysis of RNA extracted from larvae treated as described for the top part of the Figure. Filter probed for amylase sequences with the PvuII fragment of OR-M3.

Lower Panel:

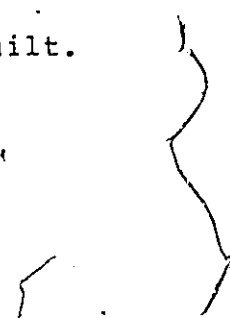
FIGURE 19: Relative Abundance of Amylase mRNA.

Plasmids pPA-1, pOR-M3 and Carnegie 20 were fixed to Biodyne as described in MATERIALS AND METHODS. Strips were probed with 1st strand cDNA generated from RNA extracted from Oregon-R larvae grown on: (i) 10% glucose medium (+) Gluc and; (ii) no-added-glucose medium (-) Gluc. Blots were exposed to Kodak X-Omat K film at -70°C with intensifying screens for 48h.



4. DISCUSSION

The results presented here serve a number of purposes. Firstly, the results from experiments at the enzyme activity level serve to consolidate much of the previous work on amylase activities in D. melanogaster. For instance, in the past the genotype-specific differences and the dietary-induced differences in amylase activities were considered separate phenomena. In this study I have shown that both the dietary and strain-specific effects are, at least in part, two aspects of the same phenomenon; namely, genotype-specific differential repression of amylase enzyme activity. Secondly, the interaction of genetic and environmental factors in the control of amylase expression has implications for the interpretation of many studies on amylase activity in *Drosophila*. This interaction is particularly important to those studies which attempt to link amylase allozyme variants with fitness differences in nature. Thirdly, the isolation and characterization of amylase cDNA and genomic sequences provides the raw material for further studies designed to elucidate amylase regulation in detail at the molecular level. Finally, measurement of changes in amylase messenger RNA levels for a cross-section of D. melanogaster strains provides the molecular groundwork upon which further work on glucose repression can be built.



4.1. Glucose Repression of Amylase Activity

This study clearly demonstrates that amylase activity in D. melanogaster is repressed by dietary glucose. Previous studies have shown that sugar-containing food represses amylase activity in young adult flies by 2 to 5-fold. These results are confirmed here and, in addition, it is shown that the response of flies, to the same level of glucose in the diet, occurs in a strain-specific fashion. In general, strains which have been characterized as "high" activity variants are relatively insensitive to glucose, whereas so-called "low" activity strains are more sensitive to dietary sugars.

The results for glucose repression in third instar larvae, however, are much more dramatic. In fact, for larvae of a sensitive strain, Oregon-R, repression by 10% glucose results in a reduction in amylase activity by over 100-fold. It should be noted, however, that even the dramatic reduction measured here for Oregon-R larvae is probably an underestimate for the following reasons. Firstly, traces of carbohydrate may have been introduced into the no-sugar-added food with the brewers yeast or agar components. In addition, the glucose levels in the 10% glucose medium were reduced by the growth of the test larvae. Despite these factors, which would tend to obscure the dietary effect, dramatic changes in amylase activity were nonetheless measured.

The reason for the difference in response to dietary glucose between adults and larvae (e.g. 4-fold in adults

compared to 100-fold in larvae of Oregon-R) is unclear. It is possible that, since larvae grow and feed much more continuously than adults, they are more sensitive to components of the food medium. Or, alternatively, amylase activity is normally required for adult survival in nature, whereas it is only essential for larvae in the absence of dietary sugars.

Regardless of the biological significance of the adult/larval differences, the fact remains that all D. melanogaster strains tested which produce amylase respond to dietary glucose, and that this response is strain and developmental stage-specific. It is now clear that, in the absence of dietary sugar, amylase activities measured in larvae and adults of all amylase-producing strains is relatively high. In other words, interstrain differences in amylase activity are minimal on no-sugar-added food. Because different strains show different sensitivities to dietary sugars, however, interstrain differences in amylase activity can be large when flies or larvae of different strains are grown on sugar-containing foods.

This result is critical to the interpretation of previous studies which sought to rank strains on the basis of their amylase activities. In such studies, flies were usually grown on "standard" *Drosophila* medium which invariably contains moderate levels of sugars; consequently, the observed interstrain differences reflect, to a large extent, different sensitivities to dietary sugar, rather than fixed differences

in activities between strains.

Other studies have attempted to relate variations in amylase expression to fitness differences between different amylase genotypes. These experiments were based on the expectation that high amylase activity variants should enjoy an advantage over low activity variants on foods containing starch as the sole carbohydrate source. In general, based upon selection experiments performed in the past, it appears that amylase expression levels do affect fitness, but the results have not been as consistent as expected.

The results on strain-specific repression of amylase activity outlined above point to an inherent difficulty in the selection experiments; namely, that the environmental challenge that is normally used in these experiments (dietary carbohydrate) can itself bring about a major response in terms of the levels of amylase expression. For instance, strains which are scored as having low activity on "standard" food may no longer show low activity when transferred to the experimental diets. Furthermore, the use of starch food complicates the issue. Haj-Ahmad and Hickey (1982) have clearly demonstrated that amylase enzyme is excreted from the adult and larval guts in an active form. Furthermore, *Drosophila* amylases remain active in the food medium, resulting in a breakdown of starch to simple sugars, leading to a deterioration of the environmental challenge during the course of the experiment.

In summary, it may well be that amylase activity levels

are selected in nature, depending on the available carbohydrate resource. It is not, however, as easy as was once imagined to demonstrate these selection effects in the laboratory due to a number of hidden complexities in the amylase gene-enzyme system.

4.2. Molecular Cloning of Drosophila Amylase Sequences

In this study I used both cross-species hybridization (with a mouse amylase cDNA as probe) and a glucose-repressible-sequence enriched probe (generated from Drosophila RNA) to identify cloned D. melanogaster amylase cDNAs. The differential probe resulted in a higher signal-to-noise ratio for amylase clones than the cross-species probe, but it was not exclusively amylase sequence-specific. Some of the other positives identified using the differential probe represent other putative glucose-repressible sequences. This result is consistent with studies on maltase and sucrase enzyme activities in D. melanogaster which demonstrated repression of these activities by as much as 5-fold in third instar larvae of some strains (Chow, Benkel and Hickey, unpublished).

The success of the cross-species hybridization using the mouse amylase probe depended upon conservation of nucleotide sequence between mouse and fly amylase genes. Since the divergence time between mouse and fly is estimated at about 600 million years, and since amylase is often considered a non-essential enzyme, at least in Drosophila, one might have

expected the fly and mouse amylase sequences to have diverged substantially. This expectation is reinforced by the lack of antigenic cross-reactivity between purified fly amylase and mammalian amylase-specific antibodies (Doane et al., 1975). Surprisingly, however, the mouse and fly amylase nucleotide sequences are an average of 55% similar over the entire coding region. This puts amylase into a class of intermediately conserved sequences; more highly conserved than e.g. rhodopsin (about 22% conservation between bovine and fly proteins, Zuker et al., 1985) and less conserved than e.g. cytochrome c (about 70% conservation between mammal and fly, Dickerson, 1971; Dayhoff, 1978).

The D. melanogaster amylase messenger RNA was sized at 1650 b, similar in length to the murine pancreatic amylase mRNA which encodes a mature protein of 55,000 daltons (Schibler et al., 1980). The molecular weight of the mature *Drosophila* enzyme has been estimated at 54,500 daltons (Doane et al., 1975) which is within the coding capacity of a messenger of 1650 b.

Restriction analysis of amylase cDNA and genomic clones reveals a general pattern of conservation of restriction enzyme sites within the transcribed regions of the duplicated, divergently-transcribed (see Boer and Hickey, 1986) structural gene copies. In contrast, sites upstream and downstream of the coding regions differ between the two "wild type" strains examined in this study.

4.3. Glucose Repression of Amylase mRNA

A substantial portion of this study was devoted to identifying the level at which glucose repression takes place, and in particular, assaying for dietary glucose-induced repression of the abundance of amylase messenger RNA. Initially, in the absence of amylase molecular probes from D. melanogaster, the Xenopus oocyte translation system was employed to measure the "translational activity" of RNA samples extracted from both repressed and derepressed Drosophila larvae.

From the results of the in ovo translation experiments it was clear that larvae which are grown on no-sugar-added medium contain RNA which has a much higher "translational activity" for amylase enzyme than the RNA from larvae of the same strain grown on 10% glucose food. This result rules out an important role for posttranslational regulation of amylase expression in response to dietary glucose. It does not, however, rule out the possibility that larvae grown on glucose contain abundant, translationally inactive amylase messenger RNAs.

The isolation of molecular amylase sequences from D. melanogaster made possible the measurement of amylase mRNA levels directly. The results of Northern and RNA dot blot analyses using molecular probes rule out the "inactivated mRNA" possibility mentioned above. Instead, the results show that glucose treatment reduces the abundance of stable amylase mRNA,

and that the reduction in messenger RNA is similar in extent to the reduction measured at the enzyme activity level. Furthermore, interstrain comparisons of both amylase activity and mRNA levels demonstrate that the genotype-specific differences in activities are also due to differences between strains in amylase mRNA abundance.

One exception to the pattern described above is the amylase null strain. Neither adults nor larvae of the null strain produce any measurable amylase enzyme activity. Third instar larvae of the null strain do, however, produce some amylase mRNA. It appears, therefore, that null strain larvae suffer from compounded defects affecting amylase expression. Firstly, the level of amylase gene expression is constitutively low (repressed level) and secondly, the small amount of amylase messenger RNA which is produced does not code for an active amylase enzyme.

In summary, in the absence of dietary glucose all D. melanogaster strains (except the null strain) contain relatively high levels of functional amylase mRNA. Dietary glucose acts to reduce the abundance of amylase messenger RNA in a strain and development stage-specific manner. Changes in mRNA levels are primarily responsible for parallel changes measured at the enzyme activity level in response to dietary conditions. Glucose regulation of amylase gene expression, therefore, takes place at a pretranslational, and probably transcriptional, level. It should be noted, however, that

direct measurement of the transcriptional activity at the amylase region was not performed as part of this study. Thus it is not possible, at this time, to estimate the contributions of changes in transcriptional rate as opposed to changes in mRNA stability (as in e.g. the yeast maltase system, Federoff et al., 1983) to the net effect measured in Northernblots and RNA dot blots.

4.4. What's Next?

There are a number of approaches which could be employed to assess the importance of changes in transcriptional rates in the dietary regulation of amylase expression in D. melanogaster. Perhaps the most informative approach involves the transformation of a suitable Drosophila host strain with recombinant P element vectors. P element vectors mediate the mobilization and stable integration of cloned genetic material into the Drosophila genome (Rubin and Spradling, 1982). The ability to introduce foreign genetic information, or to reintroduce cloned Drosophila sequences, into the Drosophila germline represents a powerful tool for the study of gene expression in fruit flies. For example, genetic deficiencies in recipient (null) strain embryos can be cured by the injection, into such embryos, of "wild type", functional genes. Alternatively, "wild type" genes can be modified and reintroduced into suitable recipient strains in order to determine the effect of the modification on gene expression.

A variation of the approach described above would allow us to assess the relative importance of transcription rate changes on the regulation of amylase gene expression by dietary glucose. Specifically, transcriptional regulation requires the presence of regulatory elements which are separable from the transcribed sequences and could be used to bring an unrelated coding region under the influence of glucose repression. In contrast, posttranscriptional regulation of amylase expression would require a message-specific "tagging" effect which would mediate changes in primary transcript stability. The test would involve the construction of hybrid genes built from parts of the amylase and Adh genes.

For a construct in which the putative regulatory region of the D. melanogaster amylase gene is fused to the coding region of the D. melanogaster Adh gene, one would expect the following result. Regulation of amylase expression exclusively at the transcriptional level would bring Adh expression from the chimeric gene under the influence of dietary repression. Thus Adh messenger RNA levels and ADH activity would become glucose repressible; Adh expression does not normally respond to dietary sugars. In contrast, if amylase expression is regulated exclusively at a posttranscriptional level, then the change in mRNA abundance in response to dietary glucose would be an intrinsic property of the amylase messenger. Therefore, the fusion of amylase regulatory signals to Adh transcribed sequences would not result in an acquired repressive effect on

Adh expression.

The approach described above has in essence been applied to the study of the expression of a number of Drosophila melanogaster genes. In general, transcriptional control, mediated by DNA elements upstream of the structural sequences, has usually been identified as the primary regulatory component (Posakony et al., 1985, Cohen and Meselson, 1985). There are exceptions, however, where a combination of transcriptional and translational effects contribute to the overall regulatory pattern (e.g. Hultmark et al., 1986). In addition, there are examples of coding region associated regulation, as in the case of the D. melanogaster alcohol dehydrogenase system (Laurie-Ahlberg and Stam, 1987).

At the present time, the mechanism by which glucose repression of amylase activity takes place in Drosophila remains unclear. Combining the results of this study with recent results by Klarenberg et al. (1986), however, allow us to build a rough working model of the amylase gene-enzyme system and to predict how some of the components of the system might interact. Briefly, it appears that each amylase structural gene is associated with its own, at least partially distinct, set of glucose repression-mediating elements. This expectation is based upon the observation that glucose repression can affect the expression of the proximal and distal loci differentially (e.g. AMY^4 and AMY^6 in Makokou larvae). These glucose-repression elements are probably short, conserved

stretches of DNA upstream of the structural genes, which are recognized and bound by trans-acting regulatory proteins involved in catabolite repression in *Drosophila*.

This system resembles the situation in yeast where negative control elements (see Brent, 1985) from a number of gene-enzyme systems have been characterized (Federoff et al., 1983; Sarokin and Carlson, 1985; Struhl, 1985; Bram et al., 1986). In general, negative control in yeast is mediated by short DNA elements located upstream of the transcription initiation site. Such elements can be located, as much as several hundred base pairs upstream of the initiation site and function by counteracting the positive effects on transcription mediated by UAS and TATA elements (Struhl, 1987).

In the absence of the negative element binding protein, transcription is promoted by the interaction of regulatory proteins (which specifically bind to the UAS and TATA elements, Gining̃er et al., 1985) and the RNA polymerase complex. Binding of the negative regulatory protein presumably disrupts the normal protein-to-protein interactions necessary for transcription initiation.

Very few transcriptionally repressible gene systems in higher eukaryotes have so far been studied in any detail. One notable exception is the low density lipoprotein (LDL) receptor gene in hamster cells. The LDL gene contains elements for both promotion and inhibition of transcription within the 500 base pairs upstream of the initiation site (Osborne et al., 1985).

b



One such element, a 42 bp element which is involved in sterol-mediated gene repression has been characterized (Suedhof et al., 1987). It is of interest to note that the hamster element, although only 42 bps long, confers both transcriptional activation and sterol-mediated repression to heterologous promoters. Thus higher eukaryotic regulatory elements may not be as easy to delineate as elements of similar function in yeast, or higher eukaryotic elements may be multifunctional. Furthermore, the gene activity repression mechanisms described above for lower eukaryotes (yeast) and higher eukaryotes (hamster) differ sharply from analogous systems in prokaryotes. In prokaryotes, control of gene activity depends on a short sequence upstream of the coding region. This sequence contains a binding site for RNA polymerase as well as closely spaced sites for regulatory proteins. Repressor proteins bind to repressor sites on DNA and thereby physically block the binding and movement of RNA polymerase (Brent, 1985).

Although the molecular mechanisms by which it functions to modulate gene expression remain to be elucidated, biochemical and genetic studies have already identified one trans-acting regulatory locus in the D. melanogaster amylase system (Abraham and Doane, 1978). In our simplistic model, the map locus serves a function similar to that of the ADRI locus, and the cis-acting DNA elements described above are equivalent to the UAS-elements of the ADH2 gene system in yeast (Beier and Young, 1982). The map locus functions only in the posterior

midgut and primarily in young adult flies. Thus the *Drosophila* system appears to involve other, yet uncharacterized, trans-acting factors.

As more information becomes available, it will be interesting to compare the data gained from studying the regulation of amylase gene expression in *Drosophila* with the molecular aspects of the "glucose effect" in prokaryotes, in lower eukaryotes and in other higher eukaryotes (e.g. Lin and Lee, 1984). At present it is too early to tell whether glucose repression in *Drosophila* involves a system similar to that in yeast, or whether *Drosophila* uses a regulatory system different from both prokaryotes and lower eukaryotes.

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