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MOLECULAR GENETIC ANALYSIS OF GLUCOSE REPRESSION
IN DROSOPHILA

By

Charalambos Magoulas

Thesis submitted to
the School of Graduate Studies and Research
in partial fulfilment of the requirements for the
Doctor of Philosophy degree in Biology

University of Ottawa



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ABSTRACT

The mechanisms of glucose-mediated repression have been extensively studied in both prokaryotic and lower eukaryotic organisms. The research described in this thesis focuses on glucose repression of gene expression in a higher eukaryotic organism, the fruit fly Drosophila melanogaster.

The results show that DNA sequences which are located upstream of the transcribed region of the amylase gene (Amy) control glucose repression in D. melanogaster larvae. Hybrid constructs, in which the upstream flanking sequences of the amylase gene were fused with the transcribed region of the alcohol dehydrogenase gene (Adh), were expressed in transgenic Adh^{null} larvae. The expression of ADH from the hybrid gene was found to be subject to glucose repression. The function of potential regulatory cis-acting elements within the glucose responsive upstream region of the amylase gene was examined by deletion analysis and site-directed mutagenesis, coupled with expression assays in somatically transformed Amy^{null} larvae. The upstream deletion analysis showed that essential elements, both for overall activity and glucose repression of the amylase gene, are located close to the transcription start site (within 109 base pairs). In vitro site-directed mutagenesis of upstream sequences revealed that the TATA motif, at position -28, and a novel 35 base pair element, at position -109 to -74 with respect to the transcription start site, were necessary for overall activity of the amylase promoter. None of the introduced mutations, which scanned the upstream regulatory

region of the amylase gene, resulted in the loss of glucose responsiveness. These results suggest that glucose repression, in Drosophila, is mediated by transcriptional mechanisms which involve multiple functionally redundant DNA elements.

Variation in the degree of glucose repression of the amylase genes was examined at a molecular level in D. melanogaster. Gene-specific elements located within a short upstream DNA region were shown to regulate the difference in response to dietary glucose between duplicated amylase genes. In addition, in order to assess the effect of interspecific variation of regulatory DNA sequences on glucose repression, the function of glucose repressible amylase promoters was examined in distantly related species. The D. virilis amylase gene was shown to be repressed by dietary glucose in somatically transformed D. melanogaster larvae. Conserved upstream DNA elements were identified and shown, by site-directed mutagenesis, to be significant for the activity of the amylase promoter. One of these upstream elements was shown to occur within the promoters of the D. melanogaster maltase-like genes. The expression of these latter genes was also shown to be repressed by dietary glucose.

Finally, cyclic adenosine-3',5'-monophosphate (cAMP) was shown to relieve glucose repression of amylase activity in D. melanogaster. The cAMP derepression effect occurred at the level of mRNA abundance and was mediated by sequences upstream of the amylase gene. In addition, the glucose repressed activity of amylase was relieved by cAMP in evolutionarily distant insect

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species, i.e. in diptera and in lepidoptera.

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1. LITERATURE REVIEW

1.1 Introduction.

Thirty years ago, Jacob and Monod (1961) proposed that genetic regulatory mechanisms mediate induction or repression of the synthesis of specific proteins, in response to environmental factors, in bacteria. Since then, these prokaryotic mechanisms, and other similar mechanisms in more complex organisms, have been studied at the molecular level. In higher eukaryotes, genetic regulatory mechanisms include those which mediate differential protein synthesis during development, in specific tissues, and in response to a variety of extracellular stimuli. From these studies, it has become evident that these mechanisms comprise similar molecular interactions, i.e. DNA-protein and protein-protein, which regulate the initiation of gene transcription in all phylogenetic systems. The challenge in molecular biology has been to elucidate the mechanics of such molecular interactions and to determine their significance in adaptive evolution.

Transcriptional repression of specific genetic systems by dietary glucose is an adaptive biological phenomenon which occurs in organisms of widely divergent phylogenetic origin (for a review see Hickey et al., 1989). Our understanding of the transcriptional mechanisms of glucose repression has been based largely on studies of microorganisms (reviewed in Adhya, 1989; Nicholson et al., 1987; Flick and Johnson, 1990). In the metazoa, the mechanisms which underlie transcriptional repression are not well known (see Renkawitz, 1990; Goodbourn, 1990). Characterization of such

transcriptional mechanisms, which control functions of common physiological importance to eukaryotic and prokaryotic organisms, will be essential to the understanding of macroevolution of transcriptional regulation. Similarly important will be the studies on intracellular transduction mechanisms which communicate the environmental cues for transcriptional regulation in phylogenetically divergent organisms.

I have divided this chapter into four distinct sections, taking into account the molecular and evolutionary significance of transcriptional repression and giving an emphasis to mechanisms which regulate functions of similar physiological significance in various kinds of heterotrophic organisms. Also, an emphasis is given to intracellular mediators, i.e. cAMP, which regulate transcription of well-studied prokaryotic and higher eukaryotic genetic systems. In the first section, I describe the regulatory molecular components and the mechanisms which are involved in glucose repression of transcriptional initiation in prokaryotes. In the second section, I review the molecular aspects of lower eukaryotic transcriptional regulation in systems similar to the glucose-repressible prokaryotic systems. In the third section, I will describe the transcriptional regulation of mammalian metabolic genes which have similar physiological significance as the glucose repressible microbial genetic systems. Finally, I will describe various transcriptional regulatory systems in D. melanogaster. I will end by describing the experimental system, the Drosophila amylase gene-enzyme system, used in the studies for this thesis.

1.2 Glucose repressible genetic systems in prokaryotes.

1.2.1 Regulatory transcriptional components.

Around the middle of this century, it was recognized that the activity of certain bacterial enzymes is influenced by the presence of specific chemical factors in the growth medium (Gale, 1943). Moreover, it was noted that the synthesis of catabolic enzymes is inhibited by extracellular glucose or other readily metabolizable carbohydrates (Monod, 1947). This "glucose effect" on the synthesis of specific enzymes was termed "catabolite repression" by Magasanic (1961) and was demonstrated to be regulated by genetic determinants (Jacob and Monod, 1961). Since then, the molecular basis of catabolite or glucose repression has been extensively studied in Escherichia (see review in Adhya, 1989). Generally, it is shown that the genetic systems or operons which respond to glucose repression consist of a set of structural genes which encode proteins involved in the metabolism of various carbohydrate sources. The expression of these genes is regulated at the level of transcription initiation by RNA polymerase. The molecular components which regulate the transcription of these operons include trans-acting protein factors, such as activator and repressor proteins, cis-acting DNA elements, and effector molecules. The significance of such transcriptional components has been characterized more extensively in the regulation of the lac and gal operons in E. coli than in any other glucose repressible system in prokaryotes (Adhya, 1989).

i) Negative regulatory components. The lacI gene has been genetically characterized as the trans-regulatory locus which represses expression of structural genes of the lac operon (reviewed in Miller, 1980). The product of this genetic locus has been isolated and characterized as a tetrameric protein of four 38 kDa subunits which, in the absence of the inducer isopropyl-1-thio- β -D-galactopyranoside (IPTG), binds with the cis-acting regulatory region of the lac operon (Gilbert and Muller-Hill, 1966). Extensive biochemical studies have been undertaken in order to characterize the structure-function relationship of the lac repressor (reviewed in Beyreuther, 1980). The amino-terminal of this protein contains a helix-turn-helix motif which is responsible for specific DNA binding (Lehming et al., 1987). The repressor also contains a core region which is required for the binding of the inducer (IPTG) (Manly et al., 1983).

The operator is defined as a regulatory cis-acting genetic locus of the lac operon in response to catabolite repression. This DNA region was genetically localized between the promoter and the structural gene of the lac operon and was first proposed to be the binding site of a repressor (Jacob and Monod, 1961). Later, Reznikoff et al. (1974) demonstrated that the purified repressor has a primary binding affinity with a site located within the operator (O1) and a lesser affinity with another site (O2) which is located 401 bp downstream of the O1 site and lies within the structural gene lacZ. In addition, another DNA site 97 bp upstream of the O1 site was shown to bind weakly to the

purified repressor (Fried and Grothers, 1981).

The sequence of the primary 27 bp operator site (O1), which was protected by the repressor from DNase I treatment, was determined by Gilbert and Maxam (1973). The operator sequence was found to have a two fold symmetry region. This operator structure, which is similar with the structure of the O2 operator, has been functionally characterized by oligonucleotide-directed mutagenesis experiments (Oehler et al. 1990). A significant part of the operator symmetrical structure is its interaction with the various residues of the turn-helix-turn motif of the lac repressor. These interactions have been characterized extensively in vitro (reviewed in Barkley and Bourgeois, 1980) and in vivo (Lehming et al, 1987). However, the rules which govern this protein-DNA recognition are still unknown.

Like the lac operon, repression of the activity of the gal operon is controlled in trans by the galR gene. This gene encodes a protein of 343 residues containing a region with high similarity to the lac repressor at the amino-terminus (Wilcken-Bergmann and Muller-Hill, 1982). The gal repressor is present in solution as dimer of 37 kDa subunits which binds with sequences found within the operator region of the gal operon. This repressor specific binding is inhibited by D-galactose, the in vivo inducer of this operon (Majumdar et al., 1987). Genetic and biochemical analysis shows that the gal repressor, in the absence of its inducer, interacts with two operator regions which are separated by 114 bp . The external operator (Oe) is upstream of the promoter

while the internal operator (O_i) is within a structural gene ($galE$) of the operon (Mujumdar and Adhya, 1987).

ii) cAMP as a mediator of glucose repression. Glucose in the growth medium of E. coli was shown to influence the intracellular level of cAMP (Makman and Sutherland, 1965). Furthermore, exogenous cAMP was found to relieve glucose repression of protein synthesis (Perlman and Pastan, 1968a,b; de Crombrughe et al., 1969). These early experiments suggested that cAMP may be a mediator of catabolite repression in bacteria. Similar studies, however, in bacteria other than enteric coliforms, such as in several gram-positive species of Bacillus, showed that the level of intracellular cAMP is not associated with the induction of glucose repressible enzymes (Botsford, 1981). Therefore, most of the known prokaryotic transcriptional effects due to an interaction of extracellular carbohydrate resources with intracellular mediators, i.e. cAMP, have been studied in E. coli.

At present, it is not known whether the intracellular cAMP concentration is the exclusive mediator of catabolite repression, or whether cAMP competes with a negative effect of protein synthesis caused by intracellular metabolite(s) in E. coli (reviews in Ullmann and Danchin, 1983; Danchin and Ullmann, 1985). It is clear, however, that extracellular carbohydrates can regulate intracellular cAMP concentrations (Saier, 1985; Peterkofsky, 1981). In turn, cAMP controls positively the synthesis of catabolic enzymes at the level of gene transcription (Busby, 1986).

The activation of transcription of the glucose responsive lac and gal operons by cAMP has been more extensively studied than any other prokaryotic cAMP regulated genetic system. Biochemical and genetic studies indicated that the transcription of these operons, including the transcription of other catabolic genes, is activated by a protein termed as the Catabolite Activator Protein (CAP) which is the intracellular receptor of cAMP (see reviews in Busby 1986, Ullmann and Danchin 1983, de Crombrughe et al. 1984). This trans activating factor, which is required for the expression of catabolite sensitive E. coli mutants, was determined to be the product of the crp genetic locus (Schwartz and Beckwith, 1970). The encoded protein from the crp gene consists of 210 amino acids (Aiba et al., 1982), and characterized as the cAMP receptor protein (CRP) which consists of a basic dimer of 22.5 kDa subunits (Zubay, 1980). Biochemical and biophysical studies showed that there are two binding domains within the structure of the CRP, one domain at the amino terminus which bind with cAMP and the other domain at carboxyl terminus which binds with DNA (de Crombrughe et al., 1984; Busby, 1986). The DNA binding domain contains an identical two α -helical structure (Steitz et al., 1982). Residues in the subunits of this helix-turn-helix motif bind in parallel with bases which appear in two successive major grooves of the lac operon promoter (Weber and Steitz, 1984).

CAP (catabolite activator protein), or CRP (cAMP receptor protein), was shown to bind with 18-24 bp located approximately 60 bp upstream of the transcription start of the lac

promoter. This CAP specific binding was characterized by genetic promoter-specific mutations (Dickson et al., 1977) and DNA protection assays (Simpson, 1980, Schmitz, 1981). Similarly, CAP was shown to bind a site approximately 35 bp from the transcription start of the gal operon (Busby et al, 1982). A sequence comparison of CAP specific sites, between various cAMP sensitive promoters, shows that there is a consensus sequence of over 22 bp which diverges in position and orientation with respect to the transcription start. This consensus, however, contains a highly conserved DNA core with the 5'-TGTGA-3' sequence (de Crombrugge et al., 1984; Busby, 1986). Point mutations within this motif were found to affect the CAP binding with the gal promoter (Busby and Dreyfus, 1983; Busby et al., 1982). Similarly, the genetic mutations which affect the activity of the lac promoter by cAMP have been localized within this element (Dickson et al., 1977).

1.2.2 Mechanisms of transcriptional repression

Initiation of transcription of each of the catabolite sensitive operons lac and gal is controlled by the Catabolite Activator Protein, CAP (see review in Busby, 1986). The activity of CAP depends on the intracellular level of cAMP which is regulated by the amount of extracellular glucose, as previously mentioned. The lac operon is transcribed from the P1 promoter, located in a region -35 bp from the transcription start, and by a weaker promoter which is located -22 bp relative to the P1 site. The cAMP-CAP complex has been shown to activate transcription from the lac

P1 and represses the lac P2 (Malan and McClure, 1984). It appears that CAP activates the lac P1 promoter by accelerating the initial binding of RNA polymerase (Malan et al., 1984). The function of the lac P2 promoter in vivo and the molecular basis of the dual promoter regulation is still unknown (Busby, 1986). Like the lac operon, the expression of the gal operon is controlled by two promoters (Musso et al., 1977; Adhya and Miller, 1979). In the gal promoter, CAP-cAMP causes transcriptional initiation to shift from one promoter (P2) to a second, downstream promoter (P1). Utilization of both promoters was first demonstrated in vitro (Musso et al., 1977) and subsequently was shown to occur in vivo (Aiba et al., 1981).

It is still unclear how binding of the CAP with its targeted site activates RNA polymerase. Two possible models, however, which underline the transcriptional activation of RNA polymerase by CAP have been favoured based on biophysical and on biochemical in vitro studies (reviewed in Busby, 1986). One model indicates that binding of CAP causes DNA conformational changes, such as DNA bending, which activate RNA polymerase. DNA bending upon binding of CAP with its target DNA has been determined based on hydrodynamic properties (Wu and Crothers, 1984; Kolb et al., 1983), crystallographic structure (Weber and Steitz, 1984) and visualization of binding complexes by electron microscopy (Gronenborn et al., 1984). An alternative model suggests that a direct interaction of CAP with RNA polymerase is necessary for initiation of transcription. This protein-protein interaction has

been predicted because CAP and RNA polymerase bind each other; moreover, because these two proteins bind co-operatively to the gal and lac promoters (Shanblatt et al., 1983; Spassky et al, 1984).

In vitro studies show that negative control of expression of catabolite sensitive operons is exerted by the repressor by at least two main mechanisms. One mechanism acts at the site of transcriptional initiation while the other provokes transcriptional termination (Adhya, 1989; Flashner and Gralla, 1988). In the past, repression of transcriptional initiation was perceived as distinct from transcriptional activation (Jacob and Monod, 1961; Magasanic, 1960). The repressor was assumed to inhibit initiation of transcription by hindering the binding of RNA polymerase to the promoter or halting the RNA polymerase at a certain transcriptional complex with the promoter (Straney and Crothers, 1987). Recently, it has been shown by in vitro studies that transcriptional regulation of the lac operon is mediated by the co-operative interactions of CAP and the lac repressor with the lactose promoter (Hudson and Fried, 1990). It has been suggested that these co-operative protein-protein interactions regulate the expression of the lac operon by two possible mechanisms (Hudson and Fried, 1990). One mechanism involves the direct interaction between CAP and repressor which together interact with RNA polymerase. This mode of action requires the formation of a DNA loop in order to bring together the regulatory proteins which are bound to distant recognition DNA sites. The other mechanism proposes that the co-operative protein interactions are mediated by

allosteric DNA conformations alone. These structural changes can be induced by the occupancy of a regulatory binding site and facilitate binding of the lac repressor to its multipartite DNA sites.

The in vivo activity of RNA polymerase is suggested to be inhibited also by a DNA looping mechanism which involves the interaction of the repressor with distant multipartite DNA sites (reviewed in Adhya, 1990). This model is supported by experiments which show that the co-operative occupancy of distant multipartite operators is necessary for in vivo transcriptional repression of the gal and lac operons (Mandal et al., 1990; Oehler et al., 1990; Flashner and Gralla, 1988). The feasibility of this model is also based on biophysical evidence in vitro, where the formation of a DNA loop by the binding of the repressor with two spaced operators was shown to be energetically possible (Kramer et al., 1987).

1.3 Glucose repressible genetic systems in lower eukaryotes

Saccharomyces cerevisiae is the most well-studied lower eukaryote with respect to the effect of extracellular carbohydrate sources on protein synthesis. Glucose can induce or repress the synthesis of certain enzymes which are involved in the metabolism of various carbon sources. In some genetic systems, this type of regulation is found to be at a transcriptional level (see review in Wills, 1991). The transcription of genes which are involved in the metabolism of sucrose, maltose, and galactose are shown to be repressed by glucose (Carlson, 1987). Similarly, glucose is shown to repress transcription of fructose diphosphatase (Sedivy and Fraenkel, 1985), ADH2 (Denis et al., 1981) and of genes encoding certain mitochondrial proteins (Szekely and Montgomery, 1984).

In S. cerevisiae, specific cis-acting elements and trans-acting factors are known to be important for activity of the glucose repressible promoters of the CYC1 (Lalonde et al., 1986; Pfeifer et al., 1987) and the MAL (Ni and Needleman, 1990; Hong and Marmur, 1987) genes. How these transcriptional components are inactivated by glucose remains to be found. However, glucose repression of two yeast genetic systems, GAL and ADH2, has been studied at a transcriptional level to some extent. In the following section, the transcriptional components of these systems are described.

1.3.1 The GAL system.

The expression of the GAL genes is co-ordinately induced by galactose and repressed by glucose (review in Oshima, 1991). The activation of these genes is dependent on cis-acting elements which are located upstream of the transcription start. A short DNA region containing a guanine-plus-cytosine -rich stretch, termed GAL upstream activating sequence or UASg, was localized approximately in the middle of the sequence which separates the divergently transcribed GAL1 and GAL10 genes. This DNA region was shown to be required for galactose-related induction of both genes (Johnston and Davis, 1984; ; Yocum et al., 1984). Four short sequences of partial dyad symmetry, two of which are required for expression of the GAL1 and GAL10 genes, were identified within this upstream activating DNA region. In addition, this upstream activating region was shown to mediate glucose repression of GAL1 gene expression (Flick and Johnson, 1990). Similarly, an 87 bp fragment between UASg and TATA box, termed GAL upstream repression sequence or URSg, was shown to be responsible for glucose repression, independently of the repression acting on the UASg.

Two upstream elements and a TATA box are necessary for galactose-controlled GAL7 transcription. It was shown that at least one of these elements is required for galactose induction and glucose repression (Tajima et al., 1986). These activating elements were found to have structural similarities, i.e. dyad symmetry, with upstream activating sequences of other genes of the GAL complex (Bajwa et al., 1988).

Two genetic loci, GAL4 and GAL80, are required for positive and negative regulation of the GAL genes (reviews in Johnston, 1987; Oshima, 1991). The product of the GAL4 gene was shown to bind with the upstream activating sequences of the GAL genes in vitro and in vivo (Bram et al., 1986; Selleck and Majors, 1987). Interaction of the UAS with the GAL4 product was shown to be required for transcription of the GAL7 gene in vivo (Tajima et al., 1986). The GAL4 gene has been sequenced and encodes a protein of 881 amino acids (Laughon and Gesteland, 1984). The N-terminal domain of this protein was shown to contain a cysteine-zinc finger structure which is important for DNA binding (Johnston and Dover, 1987). The C-terminal contains two transcription activating regions, region I comprises residues 148-196 and region II comprises residues 768-881, which bear a significant net negative charge (Ma and Ptashne, 1987a). The product of the GAL80 gene has been shown to inhibit the activity of the GAL genes by interacting with the activating region of the GAL4 trans-acting protein (Ma and Ptashne, 1987b).

It has been proposed that two possible mechanisms mediate glucose repression of expression of the GAL genes at the transcriptional level. One mechanism controls the activity of the GAL4 activator, while the other mechanism involves interactions at upstream repression sequences (see review in Johnston, 1987; Flick and Johnston, 1990). Activity of the GAL4 protein can be regulated at a level of protein abundance or by a dephosphorylation reaction which changes the physical state of this protein (Mylin et al.,

1989). In accordance with this mechanism, glucose specific inactivation of the GAL4 protein is shown to result in a loss of specific binding with UASg (Selleck and Majors, 1987; Lohr and Hopper, 1985). The other mechanism suggests that the URS (upstream repression sequence) of the GAL1 promoter, located between the UASg and the TATA motif, mediates glucose repression either by interacting with a repressor protein or by contributing to the necessary spacing for other protein-DNA interactions (Flick and Johnson, 1991).

1.3.2 The ADH2 gene.

Deletion analysis of the upstream of the ADH2 gene shows that a region from -216 to -257 bp from the transcription start site is required for induction but not for glucose repression of gene expression. Also, a 22 bp perfect dyad sequence, which was shown to be of significance for mediation of ADH2 expression by the ADR1 locus, was identified within this region (Beier et al., 1985). This perfect dyad element of the ADH2 upstream was further characterized by oligonucleotide-directed mutagenesis and shown to be significant for derepression and for response to the ADR1 locus (Shuster et al., 1986).

The product of the ADR1 locus, which is the best studied trans-activator of the ADH2 gene, encodes a protein of 151 kDa which is structurally similar with the transcription factor TFIIIA from Xenopus (Hartshorne et al., 1986). A zinc finger DNA binding motif of the ADR1 protein has been shown to be significant for

function. This domain binds with upstream ADH promoter sequences which contain the 22 bp activating palindrome (Eisen et al., 1988; Thukral et al., 1989; Blumberg, et al., 1988). Apart from the ADR1 positive trans-activator of the ADH2 activity, three negative regulatory loci, ADR4, CRE1 and CRE2 have also been identified (review in Wills, 1990). How these negative loci regulate ADH2 activity is still unknown.

The activity of the transcription factor ADR1, which regulates ADH2 expression, is itself regulated at a post-transcriptional level (Blumberg et al., 1988). Cherry et al. (1989) showed, by genetical and biochemical means, that the transcription of the ADH2 gene is regulated by a mechanism which involves the inactivation of the ADR1 by a phosphorylation reaction catalyzed by cyclic AMP-dependent protein kinase (cAPK). This mechanism is thought to control ADR1 function only partially, since the phosphorylated site of the ADR1 is not an absolute requirement for transcriptional function or for release of ADH2 expression from glucose repression (Bemis and Denis, 1988).

1.4 Transcriptional regulation in mammals

1.4.1 Transcriptional regulation of metabolic genes.

At present, examples of glucose-related transcriptional repression mechanisms in higher eukaryotes, analogous to the ones encountered in microorganisms, are not known. In this section, however, examples of positive and negative transcriptional regulation of mammalian metabolic genes will be described. The most studied metabolic regulatory genes are those which control carbohydrate and lipid metabolism in the mammalian liver (reviews in Goodridge, 1987, 1990). The transcription of glucokinase (Iynedjian et al., 1989) and L-type pyruvate genes (Vaulont et al., 1986), which are key regulatory genes of glycolysis, is shown to be reduced in starved, diabetic and glucagon treated (which acts via cAMP) animals. This transcription, however, is restored by carbohydrate rich diets and the administration of insulin. Also, the expression of key regulatory lipogenic genes, such as those which encode the malic enzyme and fatty acid synthetase, is regulated in the same way by similar carbohydrate and hormonal manipulations (Goodridge, 1987). The same treatments alter the mRNA level of the gluconeogenic cytoplasmic phosphoenol pyruvate carboxylokinase (PEPCK) gene in an opposite direction, in comparison to the regulation of the glycolytic and lipogenic genes (Cimbala et al., 1982).

The transcriptional mechanisms which regulate mammalian metabolic genes are not known. It is evident, however, that these

mechanisms are under a complex control in response to hormones and in specific tissues. Cis-acting elements, which mediate positive and negative effects of glucagon (or cAMP) and insulin respectively, have been identified within the 5'-flanking region of the PEPCK gene. The cAMP mediating effect was found to be conferred by a 47 bp upstream region which contains a 12 bp core sequence homologous to other cAMP responsive genes (Short et al., 1986). In similar experiments, a 15 bp insulin responsive sequence (IRS) was localized, between -416 bp and -402 bp from the transcription start site, with a capacity to bind with specific nuclear proteins (O'Brien et al., 1990). The 5'-flanking of the PEPCK gene has been shown to mediate not only hormonal responsiveness but also tissue specific expression in transgenic animals. Tissue specific cis-acting elements within this DNA region, however, remain to be identified (Goodridge, 1990).

Tissue specific regulation of metabolic genes has been characterized in the L-type kinase system. Specifically, upstream specific cis-acting elements were shown to be binding sites of the liver specific transcription factors HNF-1 (hepatocyte nuclear factor-1) and LF-A1 (liver factor-A1) (Ginot et al, 1989; Vaultont et al., 1989). A metabolic gene which is regulated under similar complex conditions is the pancreatic amylase gene, Amy-2.2. Expression of this gene is reduced in diabetic animals and is restored by administration of insulin. The 5'-flanking region of this amylase gene contains a cis-acting element which has an enhancer-like activity. This element is conserved in the upstream

of other pancreatic-specific genes and is shown to bind with a pancreatic nuclear protein PTF-1 (Howard et al., 1989). A 30 bp upstream region, which includes the PTF1-binding site, has been also shown to confer an insulin-dependent response of the amylase gene in transgenic mice. The mechanisms which regulate the tissue-specific and hormonal regulation of this gene, however, are still unknown (Keller et al., 1990).

In mammals, regulation of the expression of the gene encoding the low density lipo-protein (LDL) receptor is a rare example of transcriptional repression. The LDL receptor is involved in metabolism of cholesterol, where the end product of this process (sterol) represses transcription of the LDL gene. It has been shown that a 42 bp element within the 5'-flanking region of the human LDL gene confers repression by sterols. This element, containing three 16 bp imperfect repeats, is shown to be the site of nuclear proteins (Sudhof et al., 1987). Repeats 1 and 3 bind with a nuclear protein, Sp1 which stimulates the transcription of many other mammalian genes. Repeat 2, which contains a 10 bp designated as the sterol regulatory element -1 (SRE-1), was found to be the binding site of a different protein. This repeat-2-specific protein has been suggested to be the activator, with the co-operative interaction of the Sp1 factor, and the sterol specific repressor of LDL gene transcription (Sudhof et al., 1987). A gene encoding a cellular nuclear binding protein (CNBP) which interacts specifically with the repeat 2, and is up regulated by sterols, has been isolated. This protein contains several zinc finger DNA

binding motifs and is believed to be the previously proposed regulator of sterol mediated transcription. However, the mechanism of action of this transcription factor remains to be characterized (Rajavashisth et al., 1989).

1.4.2 cAMP transcriptional mechanisms.

Historically, cAMP was the first molecule discovered as a cellular mediator or second messenger of the extracellular effects of hormones and neurotransmitters. These hormonal effects were found to be on metabolic activity in mammalian tissues (Sutherland, 1972). The cAMP related post-translational mechanisms, which regulate activity of specific metabolic enzymes by causing structural modifications, have been studied in some detail (Cohen, 1985). Post-translational regulation by cAMP has been viewed as a short term regulatory mechanism, in comparison to the long term cAMP related mechanisms which act at the level of transcription. These transcriptional mechanisms, however, are not completely understood yet (review in Pilgis et al., 1988).

Phosphoenol pyruvate carboxylokinase (PEPCK) is the only metabolic gene of which response to cAMP has been related with specific cis-acting elements (O'Brien et al., 1990). The same cAMP responsive element has been also identified within the upstream sequences of a number of other cAMP induced genes with various biological functions (review in Roesler et al., 1988). This cAMP responsive element (CRE) is confined within a 8 bp palindrome and alone appears to not be sufficient for the cAMP effects (Silver et

al. 1987; Lewis et al., 1987). In addition, several other cis-acting elements have been found to be essential for the cAMP-regulated expression of the PEPCK (Quinn et al., 1988), the c-fos (Fisch et al., 1989) and the human proenkephalin genes (Comb et al., 1988). Such cis-acting elements appear to be the sites of distinct multiple factors (Hayman et al., 1988; Comb et al., 1988). These various transcription factors are suggested to be responsible for inducible and constitutive enhancer activities of the CRE in the various type of cells (Kanei-Ishii and Ishii, 1989). Also, neighbouring sequences of the cAMP responsive element appear to confer sites for specific protein binding. Mutations within the palindrome core of CRE of various genes result in a reduced binding activity of proteins from nuclear extracts of different tissues and species (Bokar et al., 1988; Fink et al., 1988). In addition, the contexts of the bases adjacent to CREs exert a profound influence on the transcriptional activities mediated by this element. (Deutch et al., 1988).

A purified 43 Kd protein, which binds with high affinity with a CRE of the somatostatin gene, has been isolated from rat brain and PC12 cells (Montminy and Bilezikjian, 1987). This factor, named CREB (cAMP responsive element binding protein), has been found to be necessary for transcriptional activation of the somatostatin gene (Andriani et al.; 1989). The gene encoding this rat CRE binding protein has been isolated and its deduced amino acid sequence is highly similar to the deduced amino acid sequence of a human placental CREB-cDNA analogue (Gonzalez et al., 1989;

Hoeffler et al., 1988). Several other CREB genes, however, have been cloned revealing that a large family of transcription factors bind with the same element (Habener, 1990). Recently, a mouse gene which is highly homologous to CREB has been isolated and encodes a protein which also can bind with the cAMP responsive element. This protein is termed CREM and has different tissue specific expression than CREB. Also, this protein, unlike CREB, uses alternative DNA binding domains and acts as a negative regulator of cAMP-induced transcription. The intracellular mechanisms which regulate the action of these positive and negative CRE-specific binding transcription factors are still unknown (Foulkes et al., 1991). It has been suggested, however, that activity of CRE-binding proteins is regulated by two hormonal responsive transduction mechanisms, a Ca^{++} /calmodulin-dependent protein kinase II and a cAMP-dependent protein kinase (kinase A) related pathways (Dash et al., 1991). The binding capacity of CREB has been shown to be induced by a phosphorylation reaction catalysed by cAMP-dependent protein kinase (Montminy and Bilezikjian, 1987; Yamamoto et al, 1988). It is proposed that protein kinase-A regulates the activity of CREB by causing structural changes which affect the interaction of CREB with the RNA polymerase transcriptional initiation complex (Lee et al., 1990; Gonzalez et al., 1991).

1.5 Transcriptional regulation in Drosophila

The fruit fly Drosophila melanogaster has been used as an organism of choice for studies which aim to characterize regulatory components of complex transcriptional mechanisms. Generally, in vitro manipulation of DNA sequences in combination with the P element transformation system have been used extensively to characterize elements which regulate expression of genes in response to environmental stimuli, during development, and/or in specific tissues (review in Maniatis et al., 1987). In addition, genetic and biochemical methods have been employed to identify a number of regulatory Drosophila trans-acting factors (review Biggin and Tjian, 1989).

In this section, transcriptional components of D. melanogaster, which regulate positively or negatively the expression of various genes, are described. An emphasis is given to the organization of regulatory cis-acting DNA elements within the 5'-flanking region of various genes. Finally, the type of mechanisms which regulate transcriptional repression in Drosophila will be mentioned.

1.5.1 Gene regulation in response to extracellular stimuli

Examples of potential mechanisms of transcriptional induction in response to extracellular stimuli are those underlying the regulation of the heat shock and metallothionein genes, respectively. Though evolutionarily conserved multiple specific DNA

elements have been identified within the promoters of these genes, only cis-acting elements which regulate the heat shock response have been studied in some detail (review in Sorger, 1991; Stuart et al., 1985). The *Drosophila* heat shock genes have multiple regulatory regions which contain a palindromic element, heat shock element (HSE). This consensus element is a binding site of the heat shock factor (HSF) (Wu et al., 1987). An inverted repeat of the nGAAn unit, which can be arranged in various orientations within the HSE, is the minimum structural requirement for HSF binding (Perisic et al., 1989). Each subunit of the multimeric complex of the HSF can bind with a single nGAAn unit of which increased number of copies can stimulate high levels of transcription (Xiao and Lis, 1988; Xiao et al., 1991). The *Drosophila* gene encoding HSF has been isolated and its product contains a short DNA binding domain similar to the putative DNA recognition helix of bacterial sigma factors (Clos et al., 1990). The overall structure of the *Drosophila* HSF, however, shares limited similarities with its counterparts from other organisms. At present, the type of mechanisms which activate transcription by an interaction between heat-shock related cis-acting elements and trans-acting factors in *Drosophila* and in other organisms are still unknown (Sorger, 1991).

Extracellular stimuli such as hormones and neurotransmitters can induce or repress the transcription of targeted genes. In *Drosophila*, a model system for the study of such hormonal interactions is that which describes the effects of the steroid hormone 20-hydroxyecdysone (20HE). This system, however,

has not been characterized in detail at a molecular level yet (Ashburner, 1991). So far, an ecdysone responsive element (EcRE), within the promoter of the hsp27 gene, is the only characterized Drosophila hormonal responsive element. This element exhibits a dyad symmetry which is partially homologous to certain mammalian hormone responsive elements (Riddihough and Pelham, 1987; Martinez et al., 1991). EcRE mediates both activation and repression of gene transcription. This dual effect has been proposed to be mediated by an interaction between EcRE and a receptor-hormone complex (Dobens et al., 1991).

1.5.2 Tissue-specific and developmental gene regulation

The two clusters of major chorion genes, which are expressed during the final stages of oogenesis in follicle cells, present a complex system which is under temporal and spatial control. Each cluster contains a set of genes which are expressed in different developmental periods. Each gene, however, is believed to be under a specific rather than a coordinate control (Romano et al., 1988). Cis-acting elements and trans-acting factors which are involved in the regulation of this D. melanogaster genetic system are generally unknown. The regulation of the s15 chorion gene, however, has been studied in some detail (Shea et al., 1990; Mariani et al., 1988; Romano et al., 1988). Regulatory elements were identified within a short proximal upstream region of the s15 gene. Specifically, a negative element and two positive elements were identified, within the distal and proximal part of this

regulatory region, respectively. One of these positive elements, i.e. TCACGT, occurs within the promoters of other chorion genes (Mariani, 1988; Romano et al., 1988). So far, only nuclear proteins which interact with the chorion conserved activator element have been identified. The deduced structure of two such proteins, CF1 and CF2, has been determined from their corresponding cDNA sequences. CF2 has a zinc-finger DNA binding motif while the CF1 is a member of the steroid hormone receptor family (Shea et al., 1990).

The family of the glue genes, which encode polypeptides in the salivary glands, is another genetic system under hormonal (i.e. ecdysterone), developmental and tissue specific control (review in Meyerowitz, 1987). The most characterized member of this family is the Sgs-3. This gene is regulated by two upstream DNA regions. A distal upstream region (around -600 bp from the transcription start) is necessary for high levels of expression and a proximal region (from -98 bp to -50 bp) mediates correct developmental and tissue specific expression. Furthermore, sequences within the proximal regulatory region were shown to have functional redundancy. A single point mutation within one of these sequences was found to be responsible for ecdysone-mediated repression at pupariation (Martin et al., 1989). The organization of the regulatory region of other glue genes such as the Sgs-4 gene appears to be different. For example, a distal region alone, which contains elements of functional redundancy, can regulate gene expression (Jongens et al, 1988).

Distal redundant upstream sequences were also found to be necessary for activation but not for tissue specific expression of the opsin gene (ninaE) (Mismer and Rubin, 1987). In addition, a proximal upstream region of this gene was characterized and shown to mediate tissue specific expression (Mismer and Rubin, 1989). Two elements within this region, A and B respectively, were found to be important for regulation of gene expression. Element B was found to be necessary for promoter tissue specific activity; while element A was found to mediate repression of RH1 gene in other tissues.

Similarly to the previous examples, distal upstream enhancers regulate developmental and tissue specific activity of the proximal and distal promoters of the Adh gene (Corbin and Maniatis, 1989). The proximal promoter is active through most of larval development while the distal promoter controls transcription of late third instar larvae and of adult flies. Specific expression of the proximal promoter is under dual cis-acting control. First, stage specific transcription of the proximal promoter is repressed by a transcriptional interference due to the active distal promoter in adults (Corbin and Maniatis, 1989). Secondly, two distal upstream enhancer sequences and DNA regions near the transcription start regulate the overall wild type tissue specific level of expression (Corbin et al., 1990). Recently, a 34 kd protein, which binds with specific sequences present in both proximal and distal promoters, has been characterized as a transcriptional activator which is termed Asf-1 (England et al.,

1990).

Finally, distal enhancers regulate expression of the Dopa decarboxylase gene (Ddc) in specific subsets of neurons (Johnson et al., 1989). The neural expression of this gene is also regulated by a cis-acting element located within a short DNA region close to the transcriptional start site (Scholnick et al., 1986; Bray et al., 1988). A 16 bp sequence (element I), located -60 bp relative to the transcription start, is required for Ddc expression in all neurons. This element alone, however, was found to not be sufficient for promoter activity (Bray et al., 1988). The gene encoding the protein Elf-1 or NTF-1 (Neurogenic element-binding transcription factor) which interacts with the element-I has been identified. In addition, this factor was shown to be significant for promoter activation of certain homeodomain genes. The importance of the NTF-1 for the regulation of genes which are under developmental and neuronal specific control remains to be elucidated (Bray et al., 1989; Dynlacht et al., 1989).

1.5.3 Mechanisms of transcriptional repression.

In Drosophila, the only well characterized examples of transcriptional repression are those which are mediated by homeodomain proteins. These proteins are transcription factors which regulate the expression of a variety of targeted genes in a temporal and spatial manner. These proteins have a DNA binding domain, i.e. the homeo-box, which has a highly conserved amino acid sequence between different genes and organisms. This domain

contains a helix-turn-helix motif very similar to the DNA binding motif of a number of prokaryotic repressors (Scott et al., 1989).

Homeodomain proteins can regulate transcriptional repression by a variety of mechanisms. For example, the engrailed protein blocks activity of transcriptional activators by binding with distantly located upstream DNA sites (Jaynes and O'Farrel 1991). The same protein can repress activity of other homeodomain proteins (i.e. fushi tarazu) by competing at common binding sites (Ohkuma et al., 1990). Also, repression can be mediated by a protein-protein interaction. This is the case in the inactivation of the CF1-a factor, which regulates dopa-decarboxylase gene expression, by the I-POU protein which contains a subfamily of the homeodomain (Treacy et al., 1991). In a different mechanism, some homeodomain transcription factors can autoregulate their own abundance (see a review in Serfling; 1989). Activity of the fushi tarazu (ftz) gene, which is expressed at the cellular blastoderm in a pattern of seven transverse stripes and later during neurogenesis, is partially regulated by this mechanism. The ftz gene contains two regulatory DNA elements; an enhancer element located few kb upstream from the transcription start and the zebra element which is located closer to transcription start. The distal enhancer element is shown to be activated by the ftz gene product (Hiromi and Gehring, 1987). In addition, the expression of the ftz gene is regulated by multiple activator and repressor DNA regions, located within the zebra element, which interact with the specific factors FTZ-F1 and FTZ-F2 respectively (Dearolf, 1989; Ueda et al.,

1990; Brown et al., 1991). These transcriptional repression mechanisms show, in comparison to the transcriptional repression mechanisms of prokaryotes, a diversity of molecular interactions which may reflect their role in higher eukaryotic morphogenesis.

1.5.4 Regulation of the amylase genetic system.

The Drosophila amylase gene-enzyme system is of special interest, among higher eukaryotic regulatory genetic systems, because it exhibits two forms of regulation (see for a recent review in Hickey et al., 1989). One type of regulation, developmental and tissue specific, is similar to the types of higher eukaryotic gene regulation described above. The other mode of regulation, however, is due to facultative responses, i.e. glucose repression of amylase activity, which is common only in the regulation of prokaryotic and lower eukaryotic genetic systems.

Amylase activity in D. melanogaster is detected mainly in adult and larval midgut. Within the midgut, distinct patterns of enzymatic activity are seen during various developmental stages of the fly (Doane et al, 1983; Doane, 1969). A trans-acting locus, which is located 2 to 3 map units distal to the amylase locus on chromosome 2, was shown by genetic analysis to control this midgut activity pattern (map) in young adults only (Doane et al., 1983). Similar genetic studies showed that the distribution of amylase activity within the anterior midgut of larva is controlled by a cis-acting element located very close, less than 0.1 cM, from the structural locus (Klarenberg et al., 1986).

A number of studies have shown that amylase activity is also modulated by dietary carbohydrate sources in D. melanogaster (review in Hickey and Benkel, 1987). Dietary glucose has been shown to repress activity of most naturally-occurring amylase variants (Benkel and Hickey, 1986a, 1986b). In this species, such amylase variants are encoded by two closely-linked amylase genes (Gemmill et al., 1986; Boer and Hickey, 1986; Benkel et al., 1987). The dietary regulation of amylase activity, including the developmental and tissue specific regulation, is shown to occur at the level of mRNA, suggesting possible transcriptional regulatory mechanisms (Benkel and Hickey, 1987; Hickey et al., 1989; Doane et al., 1983).

1.5.4 Thesis project.

In this study, glucose repression of the Drosophila amylase gene is analyzed at a molecular level. A series of experiments, which are presented in distinct sections in the Results chapter, were done. i) The regulation of glucose repression at the transcriptional level is addressed. In these experiments, the capacity of various 5'-flanking regions of the amylase gene to confer glucose repression in an heterologous gene, i.e Adh, is tested by germline transformation experiments. Then, the function of cis-acting elements, within the 5'-flanking regulatory region of the amylase gene, is examined by a combination of in vitro DNA site-directed mutagenesis and somatic transformation assays. In similar transgenic experiments, the relationship between genotype

specific regulatory DNA sequences and amylase allelic variation, in response to glucose repression, is examined. ii) In another section, the evolutionary conservation of intracellular mechanisms which mediate glucose repression is addressed. In these experiments, the effect of cAMP on glucose repressible expression of the amylase gene, in D. melanogaster, is examined. iii) Also, in an evolutionary context, the conservation of the amylase promoter is addressed. Specifically, the function of amylase promoters from distantly related species is tested in heterologous Drosophila transgenic systems. Then, the intraspecific variation in upstream DNA sequences of glucose repressible promoters, in Drosophila, is examined in order to identify functionally conserved cis-acting regulatory elements. iv) Finally, other glucose repressible genes, which have the same physiological function as amylase, were identified in D. melanogaster. Upstream sequences of such glucose repressible genes, i.e. maltase-like gene family, were analyzed in order to identify conserved DNA regulatory elements within the same species.

2. MATERIALS AND METHODS

2.1 Drosophila stocks.

Three wild type strains of D. melanogaster were used in this study: the two common laboratory stocks, Oregon R and Canton S and an isogenic strain collected from Makokou, West Africa. The amylase allozyme patterns produced by these strains have been characterized as AMY¹, AMY^{1,3} and AMY^{4,6}, respectively (Doane, 1969; Hickey, 1979, 1981). An Amy^{null} strain, which does not produce any amylase enzyme (Hickey et al., 1988) was used as the recipient strain for the embryo transformation experiments. An Adh^{null} strain (Laurie-Ahlberg and Stam, 1987) was used for germline transformation using the hybrid Amy/Adh gene construct. In addition, a wild type stock of D. virilis, which was obtained from Dr. Ron Blackman, Harvard Univ., was used.

2.2 Dietary media.

Food media were prepared according to Benkel and Hickey (1986). In all experiments two type of foods were used: i) a non-glucose medium which consisted of 5% killed brewer's yeast, 0.75% agar (w\v), and 0.8% propionic acid (v\v); and ii) a glucose medium which consisted of the above ingredients plus dextrose. The concentration of dextrose used depended on the experiment. All food ingredients were brought to boil, under continuous mixing. Then, they were cooled and 10 ml of food was placed in prechilled 8-dram shells.

2.3 Larval growth conditions.

Drosophila larvae were grown as described by Hickey (1981): 40-60 adult flies were placed in 8-dram shells containing test medium. Adults were allowed to lay eggs for 1-2 days and then were removed. Cultures were kept at 21°C or 25°C. Third instar larvae were harvested from their test medium by washing them with distilled water and they were kept at -20°C. A fraction of the larval population was used for protein assays and the rest, if required, for RNA extraction.

2.4 Protein assays.

10-40 third instar larvae or adults were homogenized in 0.25-1.00 ml of sterile water. The crude homogenate was micro-centrifuged for 2 min at room temperature and the aqueous fraction was assayed for protein content and alpha-amylase activity as described by Hickey (1981) and Benkel and Hickey (1986). In some experiments the activity of other metabolic enzymes was assayed. Specifically, the electrophoretic method of native polyacrylamide gels was used to assay the Drosophila homogenates for alcohol dehydrogenase (Benkel and Hickey, 1987) and 6-phosphogluconate dehydrogenase (Young, 1966) basically as described in the references.

Protein content was determined mainly by the method of Lowry et al. (1951) as following: A sample of 5-10 μ l of the soluble larva-homogenate was brought to 200 μ l with sterile water and it was placed in sterile tubes. Then, 1.0 ml of *reagent-C was added and the mixture was allowed to stand for 10 min. at room

temperature. Finally, 0.1 ml of 1N of Folling reagent was added, mixed immediately and the test tubes were placed in dark for 30 min. A spectrophotometric reading of the mixture, at 700 nm, was extrapolated on a standard curve, which shows absorbance at 700 nm versus μg of bovine serum albumin, and the protein content of the sample was quantified. A duplicate sample per homogenate was assayed. Also, the Bio-Rad protein assay was used, in some experiments, according to manufacture's instructions.

* All reagents used for protein assays are shown in Appendix-I.

Alpha-amylase activity was determined by the two following methods which are described by Bernfeld (1955):

i) The DNSA (dinitrosalicylic acid) method quantifies the production of reducing sugars when starch is used as substrate of the alpha-amylase. This method was basically performed as described by Hickey (1981) and by Benkel and Hickey (1986): 75-100 μg of soluble larval protein was brought to 0.1 ml with sterile water and placed in sterile test tube. Then, 0.9 ml of substrate solution was added and the reaction mixture was incubated for 30-60 min. at 37 °C in a shaking bath. The reaction was stopped by adding 2 ml of DNSA reagent and the mixture was immediately boiled for 10 min. Finally, the reaction mixture was cooled at room temperature and its absorbance was measured at 550 nm by a spectrophotometre. The absorbance reading minus the background reading was extrapolated on a standard curve, which shows absorbance at 550 nm versus μmoles of maltose, and it was expressed in UNITS. One UNIT = 10^{-4} μmoles of maltose produced \ minute \ μg of total larval soluble protein

at 37 °C. The amount of protein assayed and the incubation time was such as to yield an absorbance reading, at 550 nm, in the range of 0.0- 1.2. Also, the same amount of protein and incubation time was used for the various treatments within an experiment.

ii) The electrophoresis \ starch-iodine staining method, which quantifies the disappearance of alpha-amylase substrate (i.e. starch), was used as described by Hickey (1981) and by Benkel and Hickey (1986): 50-100 µg of soluble larval protein was brought to a loading volume of 20-50 µl with 10X loading buffer and sterile water. Then, the sample was applied in a polyacrylamide gel and electrophoresed at 200V (constant voltage) in a running buffer for 1-2 hours under cooling conditions. After electrophoresis, the gel was incubated with 200-300 ml of substrate solution for 45-90 min. at room temperature in a shaking bath. Then, the gel was washed with water and stained for 1-5 min. The stained gel was photographed and the negative film was scanned by an optical densitometre in order to determine the intensity of the bands formed due to alpha-amylase activity. Every gel contained a dilution series of a homogenate sample, high in alpha-amylase activity, in order to determine the relative enzymatic activity of the various treatments based on densitometre readings. The same amount of protein and loading volume of the various treatments was applied within an experiment.

The electrophoretic method of native acrylamide gels was also used to assay Drosophila homogenates for alcohol dehydrogenase (ADH) and 6-phosphogluconate dehydrogenase (6-PGD) activity. Gels

were stained for ADH and 6-PGD, for 1-3 hr's at room temperature, according to Benkel and Hickey (1987) and Young (1966) respectively. The contents of staining solutions are shown in Appendix-I.

2.5 Isolation of nucleic acids.

Total RNA extraction procedure was based on the Guanidine hydrochloride method of Cox (1969). DNA plasmid isolation was based on the protocols provided by Promega (In Protocols and Applications, 1989-90, p.73). Single stranded DNA was isolated for sequencing according to instructions of International Biotechnologies, Inc. (IBI). A detailed description of all the procedures used for nucleic acid isolation is shown in Appendix-II.

2.6 DNA sequencing.

Single stranded DNA from various pIBI plasmid clones was prepared according to the instructions of the International Biotechnologies, Inc. (IBI) (see Appendix-II) and sequenced by the chain termination method according to the instructions of the Sequenase kit of the United States Biochemical, Co. In addition, single stranded and double stranded DNA clones were sequenced by the DNA sequencing system of the Applied Biosystems Model 373A using the DyeDeoxy Terminator Taq polymerase sequencing kit.

2.7 Hybridization analysis of nucleic acids.

RNA dot blots were basically prepared as described by Benkel and

Hickey (1986) : 5 μ g of total RNA was mixed with 50 μ l of RNA loading buffer * (without glycerol) and it was heated at 65 °C for 5 min. Then, it was cooled on ice and brought to 5X SSC with 15 μ l of a 20X SSC stock solution. The final volume of the sample did not exceed 100 μ l. RNA samples were applied to a Biodyne membrane (Pall Ultrafine Filtration, Glen Cove, NY) which was placed in a Filtration Manifold device, SRC-96/0 (Schleicher and Schuell, Inc., Keene, N.H. 03431). The membrane was previously soaked in double distilled water and 10X SSC respectively. Low suction was applied on the device during RNA application on the Biodyne membrane. After RNA application, the membrane was removed, dried, irradiated with U.V. light for 5 min and finally was washed with 0.1x SSC.

* Reagents used for nucleic acids assays are shown in Appendix-II.

Northern analysis was performed according to Maniatis et al. (1982) : 10 μ g of total RNA (approx. 4 μ l) was mixed with 10-20 μ l of RNA loading buffer without glycerol. The sample was heated to 65 °C for 7 min., cooled on ice and 2 μ l of bromophenol blue solution was added. Finally, the sample was loaded on a formaldehyde agarose gel and it was electrophoresed at 50V (constant voltage) for 17hr's in formaldehyde running buffer. A standard sample, E. coli rRNA: 23S and 16S, was electrophoresed as well in order to have a visual inspection of the migratory pattern of RNA at the end of the run. The standard was visualized by staining the gel with EtBr. At the end of electrophoresis, the gel was washed with double distilled water and was equilibrated in transfer buffer (10X SSC) for 15-30 min. The transfer unit was set

as described by Maniatis et al., (1982) and the transfer of RNA, from the gel to the Biodyne membrane, was carried out over night. At the end of the transfer, the membrane was dried and U.V. irradiated for 5 min.

RNA-DNA hybridizations, for RNA dot blots and Northern analysis, were performed under high stringency conditions as described by Benkel and Hickey (1987). DNA-DNA hybridizations, for Southern analysis, were performed under low or high stringency conditions as described by Benkel et al. (1986). For the low stringency probing, hybridization was performed at 37 °C in a solution containing 5xSSC, 5xDenhardt's solution, 50 mM phosphate buffer pH 6.5, 0.1% SDS, 500 µg/ml of heterologous DNA and 50% formamide; blots were washed in 1xSSC, 0.1% SDS at 50 °C. For the high stringency probing, the blots were hybridized at 45 °C and washed with 0.1xSSC, 0.1% SDS at 65 °C.

DNA probes were prepared by the multiprime DNA labelling method according to Feinberg and Vogelstein (1983). The reaction mixture contained : 5-25 ng of DNA solution, multiprime buffer, 50-100 µCi of ³²P-dCTP and 1U of Klenow's DNA polymerase. The final volume of the reaction mixture was brought to 50 µl with double distilled water and let stand at room temperature overnight. DNA solution was heated to 90-100 °C for two minutes before labelling. If the incorporation of the radioactive nucleotide was less than 75% the mixture was spun through a Sephadex G-50 column as described by Maniatis et al. (1982).

Alternatively, DNA specific probes were made by using the

Polymerase Chain Reaction procedure. 10 pmole of specific DNA primers (20 mers) were used to amplify approximately 10 ng DNA template. The reaction mix was according to the instructions of the GeneAmp DNA amplification reagent kit of Perkin Elmer Cetus with the exception that the dNTP mix was deprived of dCTP which was substituted with the radioactive ^{32}P -dCTP. A typical amplification reaction consisted of 25 cycles performed at 92°C for 1 min , 55°C for 30 sec and 60°C for 2 min per cycle.

2.8 Engineering of DNA constructs.

2.8.1 Constructs for P element-mediated germline transformation.

The construction of the Amy/Adh hybrid genes is described in detail elsewhere (Benkel et al., in prep.). Briefly, the promoter region of the proximal amylase gene from the Oregon R strain (Benkel et al., 1987) was fused to the transcribed region of the Adh gene (derived from clone p Δ 1waf, Laurie-Ahlberg and Stam, 1987) using double-crossover linker methodology. The double-crossover linker promoted an in vivo intramolecular recombination event, in the host bacteria, which allowed the components of the two unrelated genes to be fused precisely while maintaining the fidelity of the sequence information. In effect, both the nucleotide sequence and the spacing between the promoter elements has been preserved in the hybrid genes. Two versions of the hybrid Amy/Adh gene were constructed, both with the fusion site downstream of the amylase TATA-box, and 17 nucleotides upstream of the Adh transcription start site. The shorter upstream version contained

amylase 5'-flanking sequences from -430 to -17 upstream of the transcription start site , while the longer version extended upstream to position -1595. The hybrid genes were incorporated into the P element germline transformation vector, p Δ 1wa2a (Laurie-Ahlberg and Stam, 1987) which co-transforms Adh and xanthine dehydrogenase (Xdh) genes. Digestion of p Δ 1wa2a with KpnI and XbaI separated the Xdh, pUC vector and P element sequences as a unit from the Adh gene, allowing the Adh sequences to be replaced by the hybrid Amy/Adh genes (see Figure 2). The cotransformant Xdh gene allows putative transgenic flies to be selected on the basis of a change in eye colour. Plasmid p Δ 1wa2a was used as the Adh/Adh control construct for the Adh expression tests.

8.2.2 Constructs for somatic transformation.

i) Wild type constructs of the proximal and distal amylase genes.

Two EcoRI fragments (approximately 5 kbp and 6 kbp long) of the lambda clone MK2, which was isolated from a genomic library of the Makokou strain of D. melanogaster, were shown to contain the proximal and distal amylase genes respectively (S. Abukashawa, PhD thesis, 1990). The EcoRI fragment which spans the proximal gene contains approximately 1.6 kbp upstream of the 5' end and 2.0 kbp downstream of the 3' end of the gene. The EcoRI fragment which contains the distal amylase gene contains approximately 2.0 kbp of the downstream flanking region and 2.5 kbp upstream of the 5' end of the gene. These EcoRI fragments were subcloned separately into a pIBI.24 vector and they were characterized by DNA sequencing. The

subclones were designated as p24.Mk/d and p24.Mk/p containing the distal and the proximal amylase genes which were shown to encode the AMY⁶ and AMY⁴ variants respectively.

ii) Constructs containing a series of upstream deletions of the proximal amylase gene.

Large-scale deletions were made by exploiting naturally-occurring restriction sites in the upstream region; fine-scale deletions were constructed by a combination of crossover linking and Polymerase Chain Reaction (PCR) techniques (see below for details).

The three constructs which contained large deletions of the upstream amylase sequences (AMY¹ variant), are shown schematically in Figure 4, panel A; they were made by using naturally occurring endonuclease sites as follow: Construct I consists of a 4.7 kbp Eco RI fragment, spanning the proximal amylase locus of the Oregon R type and is derived from the pOR5.0 clone (Benkel et al., 1987). This Eco RI fragment, which contains 1595 bp upstream of the transcription start of the amylase gene (Hickey et al., 1987), was subcloned into the pUC13 vector. For the making of construct II, the upstream of the pOR5.0 clone was deleted up to a HindIII site which is located 430 bp upstream of the transcription start. This deleted version is known as pOR5.0/H.E clone. Construct III is the p24/CS3.8 clone containing a 3.8 kb EcoRI fragment with only 163 bp upstream of the transcription start of the amylase gene subcloned into a pIBI.24

vector. This EcoRI fragment is derived from a genomic lambda clone, CS1:1 (Benkel et al., 1987), which contains the amylase proximal locus of the Canton S type.

A second set of three constructs, which contain short deletions in the amylase upstream region, were made by a combination of the crossover-linking method (Sung et al., 1989) and the Polymerase Chain Reaction (PCR) method (Higuchi, 1989), as shown schematically in Figure 4, panel B. First, a BclI cloning site was introduced close to the start codon of the amylase gene (clone p24/CS3.8) by the crossover linker insertion method described by Sung et al. (1989). A double stranded DNA crossover linker was produced by annealing the custom-made oligonucleotides K152 and K154:

	<u>BclI</u>
K152:	5'-CTAGAGGT GATCAAGAGCATAGT -3'
K154:	3'-TCCACTAGTT TCTCGTATCACACG -5'

The bold nucleotides correspond to the sequence of the coding region of the amylase proximal gene, located eight nucleotides downstream of the start codon (Boer and Hickey, 1986). The non-bold nucleotides AT, within the coding region, replace the wild type nucleotides GC in order to introduce the BclI cloning site (see Figure 5). The XbaI site was used to ligate the linker in the multiple cloning site of the p24/CS3.8 clone (construct III) at the 5' end of the EcoRI fragment which contains the amylase gene. The final crossover-linked construct, p24/CS*BclI, contained the amylase coding region, with an introduced BclI cloning site, but

lacked the entire upstream region. Subsequently, several deleted upstream regions of the proximal amylase gene were amplified, using the plasmid p24/CS3.8 as template, by the PCR method, basically as described by Higuchi (1989). The oligonucleotide primers K159, K186 and K289, shown below, were used to amplify the deleted 5' flanking sequences. The oligonucleotide K160 was used as the second primer for the amplification of all of the upstream regions priming in the opposite direction, i.e., from the start codon, going upstream.

-163

5'- TGCCAAAGCTTGAATTCTGATTAGACAAACTAATCGCCAGT- 3' (K159)

-109

5'- TGCCAAAGCTTAGGGAGCGATAAGATCCCATGCAGTC-3' (K186)

-92

5'- TGCCAAAGCTTCCCATGCAGTCACAAATCACTCCCCG-3' (K289)

+33

5'-GCTTGATCAGAAACATGATGATTCCAGATGGAAGTTCA-3' (K160)

The underlined letters in the primers K159, K186 and K289 indicate the introduced HindIII cloning site while in the primer K160 they indicate the introduced BclI cloning site. The bold letters of the primer K160 show the lower strand of the start codon. The numbers indicate the distance of the primer sequence with respect to the transcriptional start site. The amplified upstream sequences were purified by the MERmaid kit according to the instructions of the manufacturer (BIO 101, Inc). The purified fragments were digested by HindIII and BclI, and were repurified before ligation with the

crossover-linked plasmid p24/CS*BclI. The resulting constructs, containing the coding region ligated to the PCR-amplified upstream regions, amplified with the primers K159, K186 and K289, were named constructs IV, V and VI respectively. Finally, a construct having an upstream up to -83 bp from the transcription start, which is named as CM.30, was made by cloning the EcoRI insert of construct VII (which is described below) in the pIBI.24 plasmid vector.

The constructs, which are described in this section, are kept in the lab with storage codes which are shown in Appendix-III.

iii) Constructs containing a series of upstream deletions of the distal amylase gene.

These constructs were made by the PCR and crossoverlinking methods in a similar manner as described above. A ClaI cloning site was made 21 bp upstream of the transcription start site of the distal amylase gene (clone p24.Mk/d) by the crossoverlinking method using the following linker:

ClaI

K231: 5'- AGCTTGGGCATCGATACACTTTTAA - 3'
 K232: 3'- ACCCGTAGCTATGTGAAAATTAGTC - 5'

-21

The nucleotides of the linker which correspond to the sequence of the distal amylase gene, according to Boer and Hickey (1986) and the unpublished sequence of the Makokou distal gene, are bolded. Also, the introduced ClaI, by changing the nucleotide C to T, is overlined. The crossoverlinked product, Mk*ClaI, which contains the distal amylase gene with a ClaI site was constructed by ligating the linker in the HindIII site of the p24.Mk/d plasmid.

The plasmid p24.Mk/d was also used as a template for amplifying various upstream regions by the PCR method using the following primers:

K256:

-97

5' TGCCAAGCTTAGGGAGCGATAAGATCAATTCGCAAATCACTCACTCCCGCAAAGCCGGAATA3'

-117

K314: 5'-TGCCAAGCTTCATACAAGAGAACGTAGCAATTTAGGGGCGAT-3'

-143

K340: 5'-TGCCAAGCTTAATCGCGATTGAATCAACTCAATACATACAAG-3'

-163

K247: 5'-TGCCAAGCTTGAGCTAAGATAACACGCCTTAATCGCGATT-3' and

- 50

K279: 5'- GTGTATCGATGCGAGCTGCTTATATAGGATGACC- 3' (reverse).

The underlined sequence in the primers K256, K314, K340 and K247 indicate the introduced HindIII cloning sites while in the primer K279 indicates the ClaI cloning site. The numbers indicate the position of the nucleotide from the transcription start site (Boer and Hickey, 1986). The primer K256 was used to amplify the Makokou distal upstream for the construct **CM.19**; similarly, the primer K314 was used for the construct **CM.26**, K340 for the construct **CM.29** and K247 for the construct **CM.18**. The reverse primer K279 was used for all the amplified distal upstreams which were cloned as HindIII-ClaI fragments in the crossoverlinked construct of the distal amylase gene (Mk*ClaI).

iv) Site-directed mutagenesis of upstream amylase sequences.

Various upstream regions of the amylase proximal gene were mutagenized by a recombinant PCR method which is basically

K319:

-24 -11
 5'-AAGTCTAGAGAATTCGTAGTTCCGACCAGAGTGAAACTGAACTTCCAT-3'

Reverse primers

K313:

-60 -73
 5'- GGGAATTCTCTAGATGATTTGTGACTGCATGGGATCTTATCGCTC-3'

K323:

-47 -60
 5'-CTATCTAGAGAATTCGCTTCGCGGGGAGTGATTTGTGACTGCATGGGA-3'

K318:

-34 -47
 5'-GTGTCTAGAGAATTCACTTTATCTGAGGGCTTCGCGGGGAGTGATTTG-3'

K341:

-23 -34
 5'-CGCTCTAGAGAATTCGGACCCCACTGCTACTTTATCTGAGGGCTTCGC-3'

K320:

-11 -24
 5'-TACGAATTCTCTAGACTTATATAGTGGACCCCACTGCTACTTTATCTG-3'

The bolded nucleotides of these primers present the introduced substitutions which generate the EcoRI and XbaI, or vice versa, cloning sites which are underlined. The introduced 5'-flanking HindIII site is underlined in primers K321 and K337. Also, the primers K186 and K160, shown in a previous section, which introduce a HindIII and BclI flanking cloning sites, respectively, were used for these constructs.

A set of constructs, VII, VIII, IX, X, XI, XII and XIII, containing various mutagenised upstream sites were made by using these primers as follows: Construct VII was made by ligating the HindIII-BclI upstream fragment, which was amplified by the primers K321-K160, with the p24/CS*BclI construct. Similarly, the fragment amplified by the primers K337-K160 was cloned to make construct

K229 (Lower strand, reverse):

5'-GCCGCTCCTTATATAGGATGACCAAACCTATTCCGGCTTGGCCGGGAGTGATTTGTGACTGC
 -31 -72
 ATGGG-3'

K187 (Upper strand, forward):

5'-GAGTCGACTATATAAGCAGCTGCATCGACACACTTT-3'
 -32 -5
SalI

K188 (Lower strand, reverse):

5'-AGGTCGACCCCACTGCTACTTTATCTGAGGGCTTC-3'
 -32 -64
SalI

K251 (Lower strand, reverse):

5'-GCTCT**TGATC**AGAAACATGATGATTTTAGGTGGAAGCTAA-3'
BclI

The bolded letters indicate the introduced nucleotides in order to generate cloning sites which are underlined. Primer K251 contains a distal sequence, priming at the lower strand of the start codon, which corresponds to the proximal sequence of primer K160 (p.44).

The upstream of construct I-D contains only sequences of the distal amylase gene. This construct was made amplifying the distal upstream with the primers K186 (shown in p. 44) and K251. This fragment was cloned into the HindIII-BclI cloning sites of the p24/CS*BclI construct (described above) which contains the coding region of the AMY¹ variant of the proximal amylase gene. The HindIII and BclI cloning sites were introduced into the amplified upstream by the primers K186 and K160 respectively which have been previously presented. The hybrid upstream fragments containing fused sequences of the distal and proximal upstream, which were amplified for the making of the other constructs as described below, were similarly cloned in the p24/CS*BclI construct.

The hybrid upstream of the construct II-D was made by amplifying the proximal upstream with the primers K210 and K160. The primer K210 contains sequences of the distal upstream within the proximal upstream region which spans from upstream of the CAAAT box. The hybrid upstream of the construct III-D was made by amplifying the proximal upstream with a double-strand primer and the reverse primer K160. The double-strand primer was the amplified product of the primers K186 and K229 which have an 11 bp overlap at their 3' ends. The PCR cycle for the amplification of the double-strand primer was 92°C for 1 min, 25°C for 45 sec and 35°C for 45 sec. The primer K229 contains sequences of the distal upstream within the proximal region which spans between the TATATAA and the CAAAT motifs.

The upstream of construct IV-D was made by ligating two different amplified fragments. One fragment was amplified by the primers K186 and K188 using the upstream of the proximal amylase gene as template. The other fragment was amplified by the primers K187 and K251 using the upstream of the distal amylase gene as template. These two fragments were ligated by a SalI site which was introduced by the primers by changing the nucleotide C (at position -34 of the proximal) to G. In this ligated upstream, there are distal and proximal sequences which were located downstream and upstream the TATATAA box respectively.

Finally, a construct containing a distal upstream of 163 bp and the proximal coding region was made by a similar manner. Specifically, a HindIII-BclI fragment was amplified by primers K247

(shown in p. 46) and K251 using the distal gene as template. This upstream fragment was cloned with the proximal coding region (i.e. clone p24/CS*BclI). This hybrid construct, CM.14, is referred as construct *AMY¹ in the Results section.

vi) Constructs of the D. virilis amylase gene.

The wild type D. virilis amylase gene was constructed from three genomic fragments, (SalI-SalI: 2.5 kb; SalI-EcoRI: 0.25kb and EcoRI-EcoRI: 3.0 kb), which contained the entire coding region, 1.6 kb upstream and approx. 2 kb downstream of the amylase gene, into a plasmid vector (pIBI.24). These DNA fragments were derived from a phage clone isolated from a D. virilis genomic library (S. Abukashawa, PhD thesis, 1990).

A hybrid construct containing the D. virilis amylase promoter and the D. melanogaster amylase coding region, named Vir/Mel, was made by the PCR recombinant method as described above. A 337 bp of the upstream of the D. virilis amylase gene was amplified by the PCR method using the following primers:

-337 HindIII

K192 (Upper strand, forward): 5'-CATAAATTA**AAGCTT**GAGCTATGCGATA-3'

BclI +1

K193 (Lower strand, reverse): 5'-GCTCT**TGATC**AGAAACATGTTGCTGCT-3'

The bold nucleotides of the K199 indicate the lower strand of the start codon (ATG) of the amylase gene. The endonucleases HindIII and BclI, which are present in the D. virilis amylase sequence, were included in the primers as shown. The parameters for each of

the 25 cycles of the PCR amplification reaction were: 92°C for 1 min, 50°C for 30 sec and 55°C for 1 min and 30 sec. The amplified fragment was cloned into the HindIII-BclI cloning sites of the CS*BclI construct which contains the coding region of the D. melanogaster proximal amylase gene as described above.

2.9 Transgenic Drosophila assays

2.9.2 Germline transformation assay.

Germline transformation, by P element-mediated integration, was carried out essentially as described previously (Spradling, 1986). Embryos of the Adh^{fn6}cn:ry⁵⁰⁶ strain of D. melanogaster (provided by C. Laurie) were injected with a mixture of 100 µg/ml of the helper plasmid, pπ25.7wc, and 200 µg/ml of the test construct, in buffer containing 5 mM KCl and 0.1 M sodium phosphate (pH 6.8). Injected embryos were allowed to develop under halocarbon oil, and viable embryos were transferred to food vials for culture. Adult flies (G0) were collected and were singly mated to individuals of the recipient strain. Transformed progeny (G1) were selected on the basis of eye colour. These were again backcrossed to the recipient strain in single pair matings; this ensured that each transformant lines was derived from a single transformed genotype. Transgenic flies (G2 and subsequent generations) were brother-sister mated to produce homozygous transgenic lines. Finally, larvae, which were grown under experimental conditions, from the germline transformed flies were assayed for alcohol dehydrogenase activity as it is previously described by Benkel and

Hickey (1987).

2.9.2 Somatic transformation assay.

Amy^{null} embryos were somatically transformed by DNA plasmids, which contain the various amylase gene constructs, according to Martin et al. (1986) and Hawley et al. (1990). Microinjected embryos were incubated in mineral oil for two days at 18°C, followed a four day incubation, up to the third instar larva stage in experimental food media at 25°C. Two types of growth media were used, prepared according to Benkel and Hickey (1986), one enriched with 10% glucose and another control medium without glucose. Five to ten third instar transformed larvae were homogenized and the amylase activity was assayed as described above.

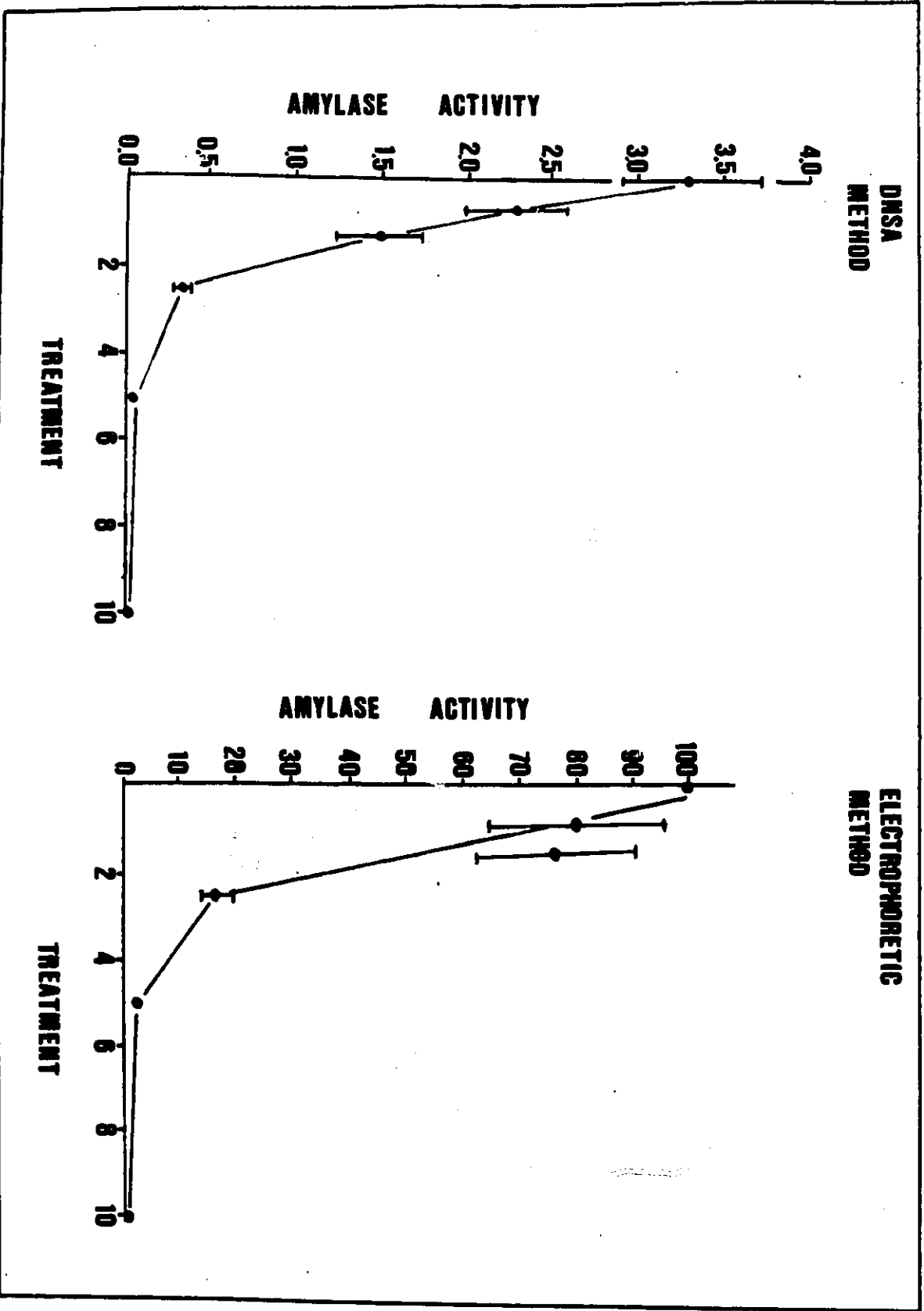
3. RESULTS

3.1 The effect of dietary glucose on amylase activity in *Drosophila melanogaster*.

Expression of the amylase gene has previously been shown to be repressed in *Drosophila* larvae which are cultured in a medium containing 10% glucose (Benkel and Hickey, 1986, 1987). The first experiment, described here, was aimed at understanding the relationship between glucose concentration in the food and the degree of repression of amylase activity. *D. melanogaster* (Oregon R strain) larvae were cultured in food media containing a range of glucose concentrations and then their amylase activity was determined. Two methods were used, the dinitrosalicylic (DNSA) method which quantifies the production of reducing sugars from starch and an electrophoretic native gel method which measures the disappearance of the enzymatic substrate. Both of these methods are described in detail in Materials and Methods (p. 35). As can be seen from Figure 1, amylase activity decreased exponentially as glucose concentration increased, and even 2% dietary glucose had a large effect on the degree of amylase expression. These results show that there is at least a hundred fold difference in amylase activity between larvae grown in 10% glucose and those larvae grown in a medium without glucose. In addition, absolute and relative values of amylase activity are found to be consistent with values from Benkel and Hickey (1986a, 1986b). Absolute values of the

Figure 1: Effect of various levels of dietary glucose on amylase activity in D. melanogaster larvae.

This figure shows the amylase activity in larvae treated with various levels of dietary glucose. The amount of glucose in food is shown as a percentage on the horizontal axis (labelled "treatment"). Amylase activity assayed by the DNSA method is expressed in UNITS. One UNIT = 10^{-5} μ moles of maltose produced/min/ μ g of total soluble protein at 37 °C. Relative amylase activity assayed by the electrophoretic method is expressed in percentage by taking the amylase activity of larvae treated with no-glucose as 100%. Values present the mean +/- S.E. . These values are derived by assaying three different larval populations per treatment.



amylase activity were determined by the DNSA method. The relative amylase activity of larvae grown in the various glucose media was estimated by comparison to the amylase activity of larvae grown under derepressed conditions, i.e., larvae grown in a medium without glucose.

3.2 Characterization of the 5' flanking region of the amylase gene in *Drosophila melanogaster*.

3.2.1 The 5' flanking region of the proximal amylase gene mediates glucose repression in germline transformant strains.

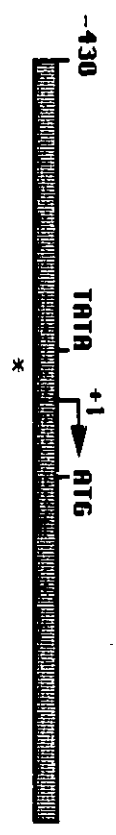
The next set of experiments were aimed at determining whether glucose repression of amylase gene expression is mediated by sequences located within the upstream region of the amylase gene. First, the capacity of the 5'-flanking region of the *Drosophila* amylase gene to confer glucose repression on another gene was examined by creating hybrid genes and testing their expression in transgenic flies. The 5' flanking region of the amylase (*Amy*¹) proximal gene, extending from position -430 to -17 bp upstream from the transcription start site (Boer and Hickey, 1986), was fused to the transcribed region of the alcohol dehydrogenase (*Adh*) gene as described in Materials and Methods, p. 40. This hybrid gene was cloned in a P element transformation vector, which is shown schematically in Figure 2, and was introduced into the germ line of *Adh*^{null} flies according to Laurie-Ahlberg and Stam (1987). The effect of dietary glucose on ADH enzyme activity was examined in larvae of three independent lines transformed with this *Amy/Adh* hybrid construct. The results of this experiment are shown in Figure 3.

ADH activity, as shown in Figure 3, is completely repressed in transgenic larvae grown in glucose in comparison to ADH activity in non-glucose fed larvae. In contrast, ADH activity

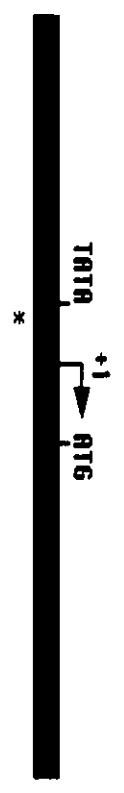
Figure 2: Construction of an Amy/Adh hybrid gene.

The 5'-flanking region of the amylase gene (Amy) was fused with the coding region of the alcohol dehydrogenase gene (Adh) as shown. The asterisk indicates the point of fusion located 17 bp upstream of the transcription start site of the amylase gene. The length of the fused amylase upstream is 430 bp from the transcription start site which is labelled "+1". The hybrid gene was then cloned into a P element germline transformation vector.

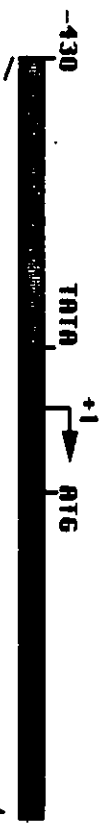
AMYLASE:



RDH:



**HYBRID:
(AMY/RDH)**



**TRANSFORMATION:
VECTOR**

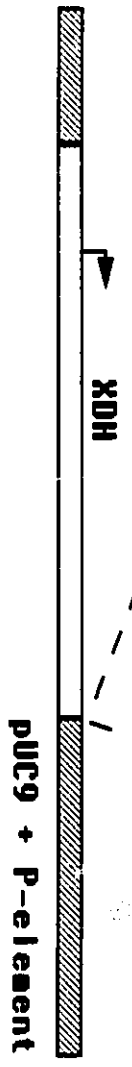
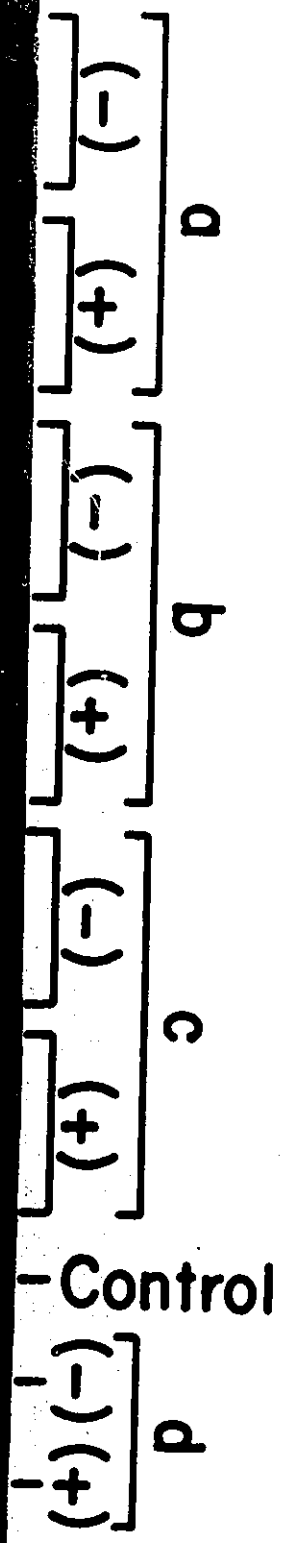
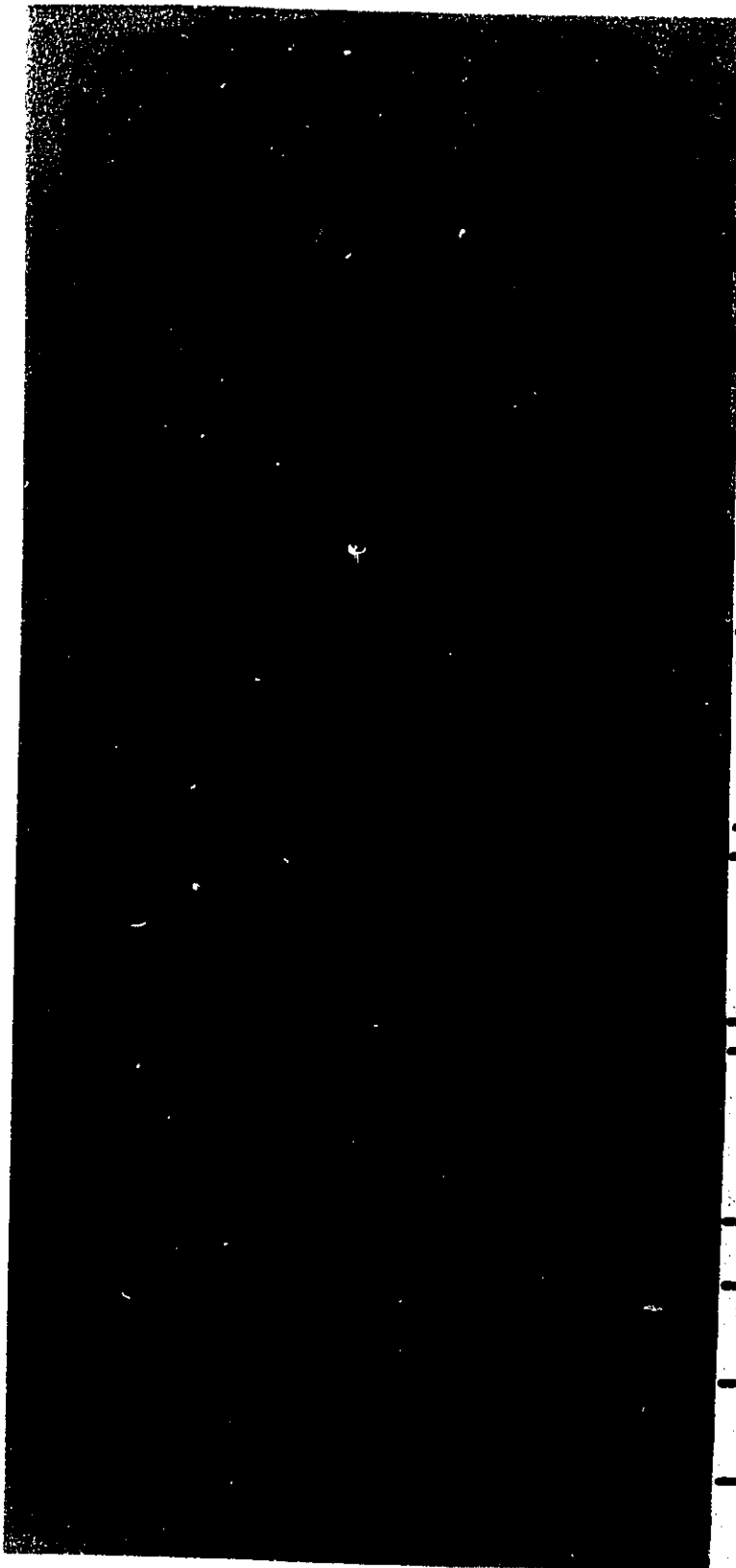


Figure 3: Glucose repression of the Amy/Adh hybrid gene in germline transformant larvae.

Results of three independent transformant lines of D. melanogaster are shown. Flies were transformed with the Amy/Adh hybrid construct shown in Figure 2. Larvae from these transgenic flies were grown in a medium with (+) or without (-) glucose and their homogenates were assayed for Adh activity. Lanes under a, b, and c show Adh activity in the larval homogenates from each of the three transgenic lines. Also, the effect of glucose on Adh activity of a control transgenic line which was transformed with the wild type Adh gene is shown in lanes under d. The control lane has a homogenate of untransformed Adh^{null} larvae grown in a medium free of glucose.



is not glucose repressible in control transgenic larvae that were transformed with a construct containing the native Adh upstream sequences (Figure 3, sample d).

In addition to the result shown in Figure 3, another set of transgenic lines were produced. These flies were transformed with a hybrid gene having a 1.6 kb of amylase upstream sequences linked to the Adh transcriptional unit. Results of these experiments (data not shown) were essentially identical to those presented in Figure 3, both for overall levels of Adh expression and for glucose repression. Thus, these germline transformation results taken together show that cis-acting elements located between positions -430 and -17 relative to the transcription start site are important for the control of amylase gene expression. Amylase constructs with further upstream deletions were tested by the somatic embryo transformation technique in order to localize the regulatory cis-acting elements (see below).

3.2.2 Deletion analysis of upstream proximal amylase sequences in somatic transformants.

In order to study in vivo expression of several amylase constructs, which have different deletion derivatives of the upstream region of the amylase gene, the somatic transformation technique was used. This technique has previously been described by Martin et al. (1986) and has already been shown to be successful for the amylase system (Hawley et al., 1990).

First, amylase constructs containing large-scale

deleted upstream sequences were made by using naturally-occurring restriction sites located at -1595 bp, -430 bp and -163 bp, respectively, from the transcription start site (see Figure 4, panel A). Then, a combination of polymerase chain reaction (PCR) and crossover linker techniques was used to construct more precise upstream deletions in the absence of naturally-occurring convenient cloning sites within the 5' flanking region (see Materials and Methods, p. 43). This latter approach has the advantage that any portion of the upstream region can be amplified and then fused to the coding region to create the desired deletion construct for embryo transformation. The fusion of the amplified upstream sequences was possible by constructing a convenient cloning site in the start of the amylase coding region by the crossoverlinking method as shown schematically in Figure 5. By this approach, a series of amylase constructs having small-scale deleted upstreams, extending up to -163 bp from the transcription start, were made by the PCR method (see Figure 4, panel B).

These deletion constructs were injected into D. melanogaster Amy^{null} embryos. Each batch of transformed larvae was divided into two sets which were grown to the third instar stage. One set of larvae were grown in a medium which lacked added glucose and the other set of larvae were grown in a medium containing 10% glucose. Amylase activity from each set of larvae was assayed by the electrophoretic native gel method as described in Materials and Methods (p. 36). Results for the three large upstream deletions which were based on naturally-occurring restriction sites

Figure 4: Constructs of the amylase gene containing a series of upstream deletions.

The three deletion derivatives of the amylase proximal gene, (Amy¹), containing upstream deletions up to naturally-occurring endonuclease sites, EcoRI (E) and HindIII (H), are shown in panel A. These constructs are designated as I, II and III and they were made as described in Materials and Methods, p. 42.

Panel B shows the scheme for the engineering of the amylase gene containing a series of fine-scale upstream deletions. The polymerase chain reaction and crossover-linking methods were used in the production of these latter constructs. The asterisk shows the site targeted by the crossover-linking method in order to engineer the promoterless amylase construct. Arrows represent the forward primers K159, K186 and K289, which contain at their 5'-end a HindIII cloning site, and the reverse primer K160 which introduces a 3'-end BclI (B) cloning site. Each of the forward primers was used in combination with the reverse primer K160 to amplify the upstream regions, which were ligated with the promoterless crossover-linked amylase construct, to produce constructs IV, V and VI respectively (see in Materials and Methods, p. 43). Construct IV is the engineered version of construct III. The numbers indicate the position of the various upstream deletions relative to the transcription start motif which has been characterized by Boer and Hickey (1986).

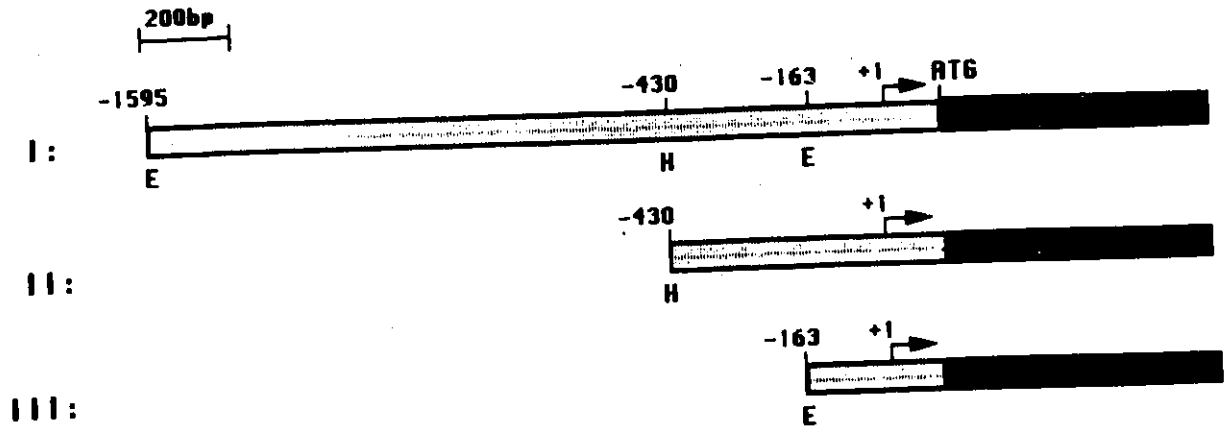
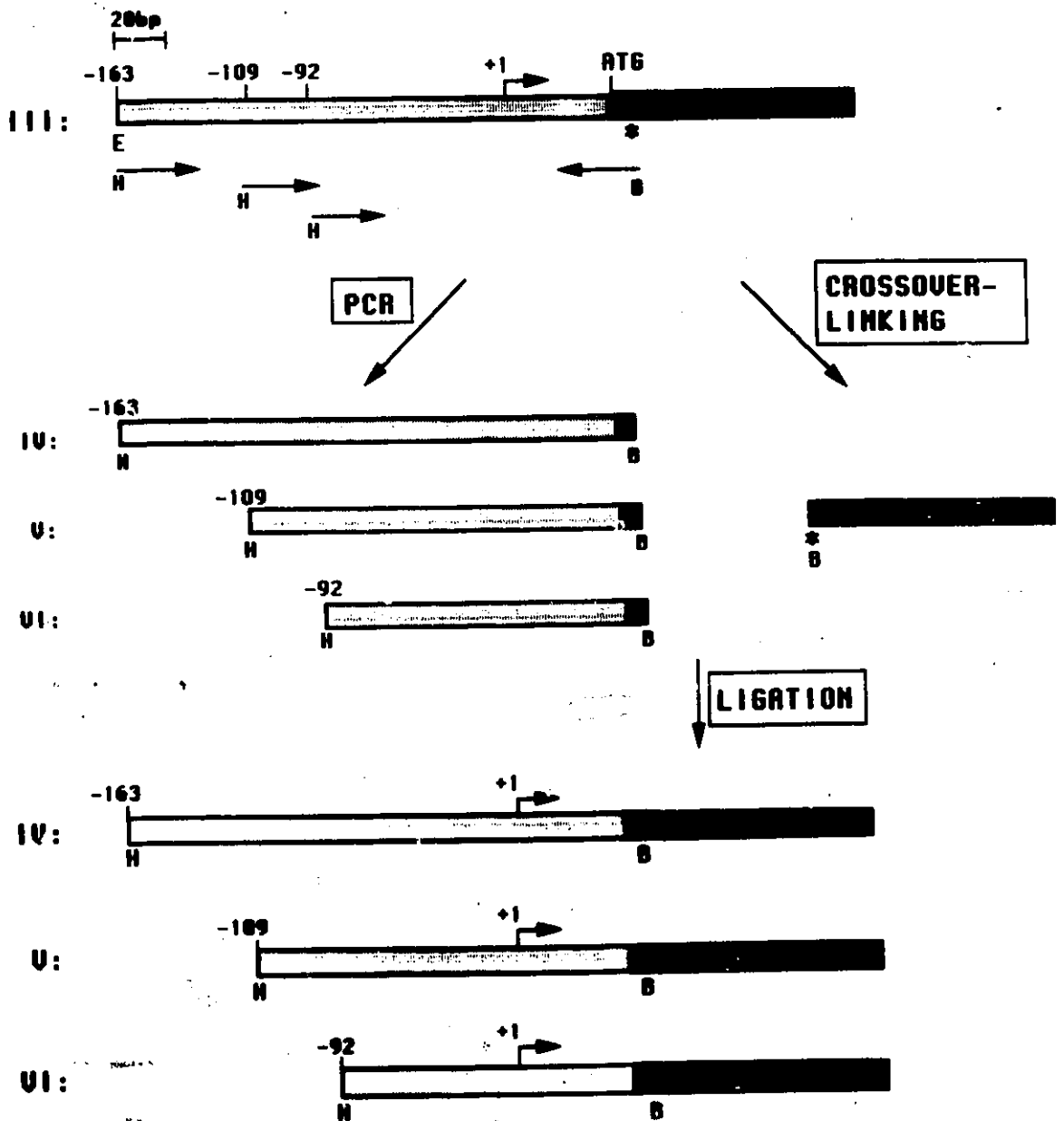
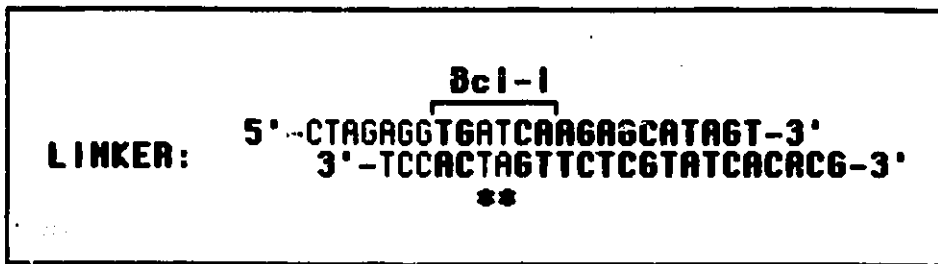
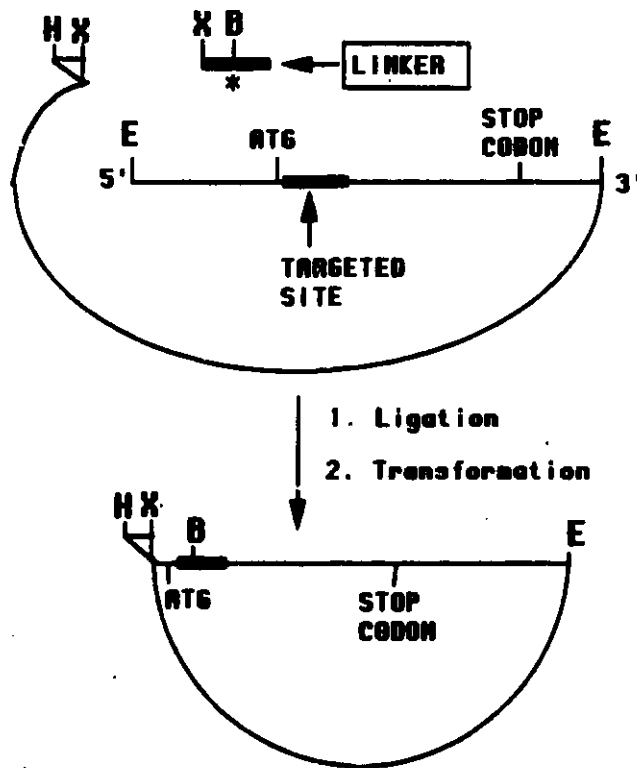
A.**B.**

Figure 5: Engineering of an amylase reporter gene by the crossover-linking method.

This figure shows schematically the steps used to generate a cloning site in the beginning of the amylase gene. A synthetic DNA linker was made with sequences targeting the beginning of the coding region. The bold nucleotides indicate the targeted amylase sequence. The linker contains the substitution of two wild-type nucleotides GC with AT, marked with asterisks, in order to introduce a BclI cloning site. The crossoverlinked product was used as a reporter gene for characterization of the 5'-flanking region of the amylase gene in the gene transformation experiments.



(Figure 4, Panel A) were identical. In all cases, amylase was expressed at normal wild-type levels and the activity was fully repressed by glucose (see Table 1). The results for the shortest of these constructs (construct III) is shown in Figure 6. A dilution series of a sample from a homogenate of de-repressed larvae is included to demonstrate that glucose feeding reduces amylase expression to about one per cent of the de-repressed level. Figure 6, panel B, shows an electrophoretic gel of the same larval homogenates stained for ADH activity; in this case there is no significant effect of dietary glucose on Adh expression. This result shows that 163 bp of upstream sequence is sufficient for gene-specific glucose repression of Drosophila amylase. Also, these results demonstrate that glucose represses expression of the cloned amylase gene in the transformed Amy^{null} larvae to the same degree as the endogenously-expressed AMY¹ variant in wild-type strains (Benkel and Hickey, 1987).

PCR-generated deletions were then used to determine the minimum length of upstream sequence necessary for full expression of the amylase gene. Deletions to within 109 bp from the transcription start (construct V, Figure 4 B) still gave high levels of expression; when a further 16 bp was deleted (construct VI) the activity was reduced significantly, this lower level of expression was still repressed by dietary glucose (Table 1).

Table 1: Effect of glucose on transient expression of amylase gene constructs having deleted upstreams regions.

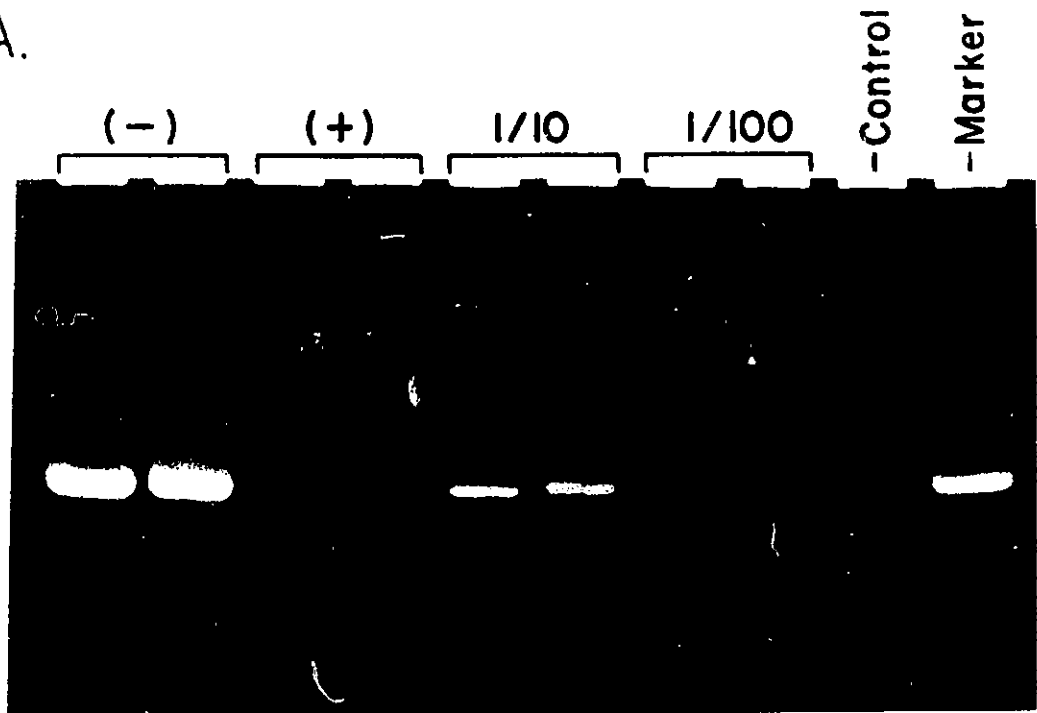
GENOTYPE	AMYLASE ACTIVITY	
	- glu	+ glu
Oregon R (wild type)	++++	-
<u>Amy</u> ^{null} (transformation host)	-	-
Construct I	++++	-
Construct II	++++	-
Construct III	++++	-
Construct IV	++++	-
Construct V	++++	-
Construct VI	+	-

These constructs represent a series of upstream deletions (shown in Figure 4). They were used to somatically transform D. melanogaster Amy^{null} embryos. Amylase activity, in the transformed third instar larvae which were grown in a medium with (+ glu) or without (- glu) glucose, was assayed as described in Materials and Methods. The relative amylase activity was estimated by a comparison to a dilution series of a wild type larval homogenate (external control) and by a comparison to the expressed activity of a wild-type co-injected construct producing a single AMY⁶ phenotype (internal control) as shown in Figure 7. (++++ = 100%; + = less than 25%; - = no-activity or less than 1%).

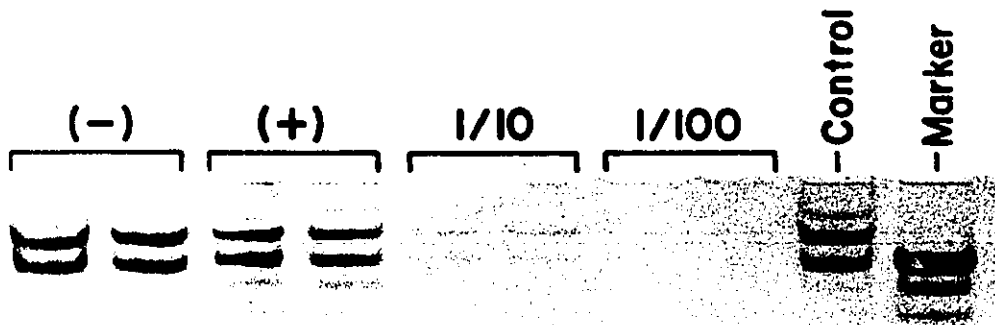
Figure 6: Glucose repression of the amylase gene in somatically transformed D. melanogaster Amy^{null} larvae.

Amy^{null} embryos were somatically transformed with the amylase construct III. This construct contains the amylase proximal gene, encoding the AMY¹ variant, with only 163 bp of its 5' flanking region from the transcription start site. Homogenates with equivalent protein content from transformed larvae grown on a glucose-free (-) and on a 10% glucose-enriched (+) diet were assayed for amylase activity (panel A) and ADH activity (panel B). A ten times (1/10) and a hundred times (1/100) dilution sample from the larval homogenates from the glucose free treatment is included. The control sample is from a homogenate of non-transformed Amy^{null} larvae which were also grown in a glucose free medium. The marker is a sample from a wild type (Oregon R) larval homogenate which produces the AMY¹ variant (Doane, 1969; Hickey, 1981).

A.



B.



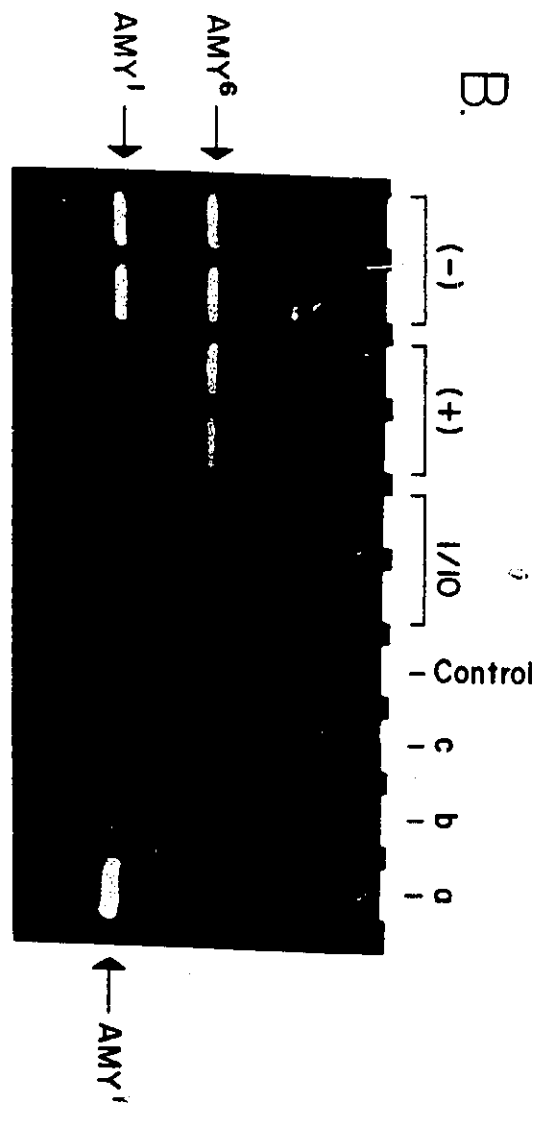
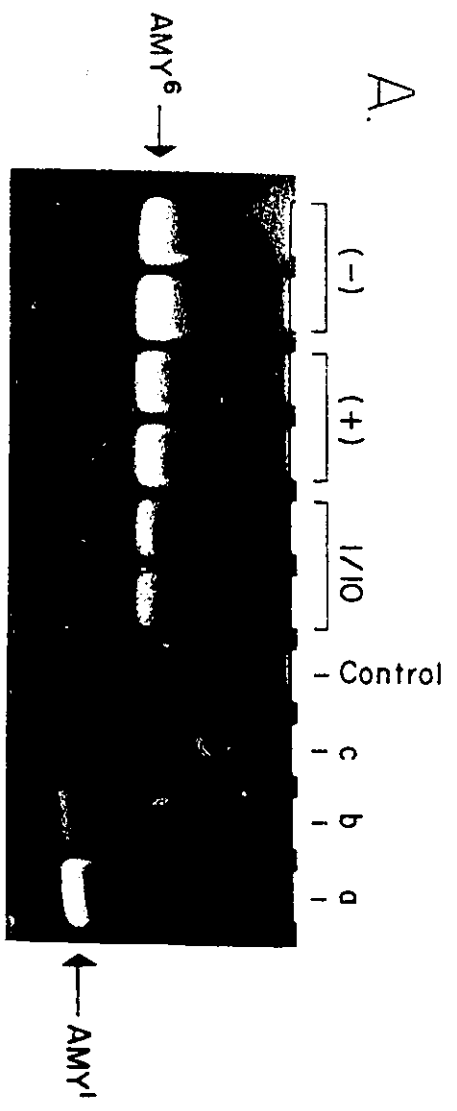
3.2.3 Allelic variation in glucose repression of the amylase gene.

Although most naturally-occurring amylase variants are highly repressible by dietary glucose, a number of D. melanogaster strains possess allelic variants at the amylase locus which show a relatively low response to dietary glucose, i.e., larvae from these strains show significant levels of amylase expression even in the presence of glucose (Benkel and Hickey, 1986b). One such strain is the Makokou strain used in this study. The electrophoretic amylase phenotype of this strain is AMY^{4,6} and the AMY⁶ band is the least responsive to glucose repression (Benkel and Hickey, 1986b). The duplicated amylase genes from this strain have been cloned (Abukashawa et al., unpublished). In addition, these proximal and distal genes have been confirmed to produce the AMY⁴ and AMY⁶ allozyme bands, respectively, in transformed embryos (results not shown). In a transformation experiment of the Amy⁶ gene, the somatically-transformed embryos were divided in two treatment groups, glucose-fed and non-glucose-fed, and were grown to third instar larvae. The amylase activity of these transformed larvae was assayed by the electrophoretic gel method as shown in Figure 7, panel A. It can be seen that glucose-fed transformed larvae produced significant amounts of amylase activity; this can be visualized most easily by comparing the glucose treatment (lanes labelled "+" in Figure 7) to the ten-fold dilution of the non-glucose treatment (lanes labelled "1/10" in Figure 7). In summary, the cloned Amy⁶ gene was expressed in the transformed Amy^{null} larvae

Figure 7: The effect of dietary glucose on the expression of cloned proximal and distal genes in transformed D. melanogaster Amy^{null} larvae.

The clone p24/Mk.d containing the distal amylase gene, Amy^δ, (see Materials and Methods for a description of this clone) was injected into Amy^{null} embryos. The effect of glucose on transient expression of this gene was assayed in third instar larvae grown in a medium with glucose (+) or without glucose (-) as shown in panel A. A ten times (1/10) dilution sample from the larval homogenates from the glucose free treatment is shown. There is also an one, ten and a hundred times serial dilution, which is shown in lanes a -, b - and c - respectively, from an equivalent protein sample of a wild type (Oregon R) larval homogenate which produces the AMY¹ variant. The control sample is from a homogenate of non-transformed Amy^{null} larvae which were grown in a glucose-free medium.

Panel B shows the results of a similar experiment where constructs CM.18 and IV, which contain the distal and proximal amylase genes respectively, are co-introduced into the Amy^{null} embryos. Both genes contain 163 bp of upstream sequences from the transcription start site. For a detailed description of the engineering of these distal and proximal amylase constructs see in Materials and Methods, pp. 45 and 42 respectively.



and showed the same reduced response to dietary glucose as did the Makokou strain from which the gene was derived. This indicates that the low response to dietary glucose is mediated by cis-acting elements contained within the cloned DNA.

In order to characterize this allelic variation in the degree of glucose repression further, the upstream region of the Amy⁶ clone was deleted to -163 bp from the transcriptional start site; this is the same point as the upstream deletion of the Amy¹ construct IV shown in Figure 4. Amy^{null} larvae were then transformed with a 50:50 mixture of these two constructs. As expected, transformed larvae expressed both allozymic bands in the absence of dietary glucose (Figure 7, panel B). When these transformed larvae were fed glucose, however, the AMY¹ band was greatly reduced while the AMY⁶ retained most of its activity. Since both constructs were co-injected into the same embryos, this result clearly demonstrates that the variation in glucose response is controlled by differences in the injected DNA sequences rather than any variation of genetic background or experimental conditions.

3.2.4 Effect of site-directed mutagenesis of upstream sequences of the proximal amylase gene.

In the previous experiments, it was shown that the expression of the amylase proximal gene and its response to dietary glucose are mediated by a 109 bp region flanking the transcription start site. In addition, it was shown that a further 16 bp deletion (up to -92 bp) reduced the amylase activity significantly but not

completely (see Table 1). In the following experiments, first the activity of a transformed amylase construct with a deleted upstream up to -83 bp is examined. Note, this is a further 26 bp deletion of the full active upstream which is flanking up to -109 bp from the transcription start site. Secondly, I aimed at defining which elements within the 109 bp upstream sequence are responsible for mediating overall activity and glucose repression of the amylase gene expression. For this purpose, the effect of dietary glucose on the expression of a set of amylase constructs with mutagenized sites, which span the regulatory region of the amylase gene, was examined.

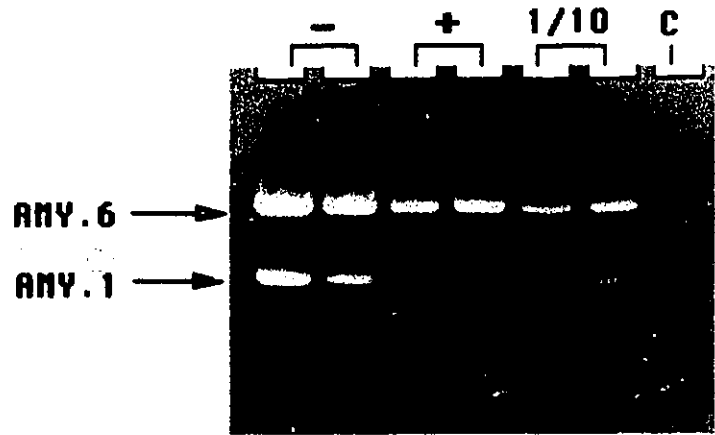
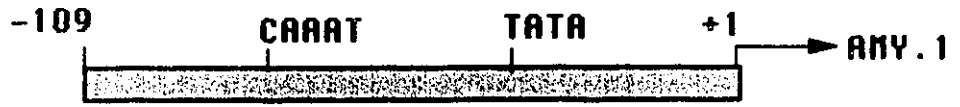
Figure 8 shows the results of the experiment which examines whether the short DNA region, between -109 to -83 bp, of the amylase promoter is necessary for overall amylase activity. Panel I shows that full expression of the amylase proximal gene (Amy¹) is mediated by 109 bp of upstream (control experiment). Lanes under - and + contain homogenates of transformed larvae grown on a non-glucose and on a glucose rich medium respectively. Again, a co-injected construct which produces the AMY⁶ variant was included (internal control), as was described in previous experiments. Panel II shows that amylase activity of a construct with an upstream deletion to -83 bp is greatly reduced in comparison to the internal control and the external control (a wild type larval homogenate). A deletion to -92 bp has been previously shown to cause a 4 to 5 fold reduction in amylase activity (see Table 1). In this experiment, a further 9 bp

Figure 8: Identification of an upstream activating region of the proximal amylase gene.

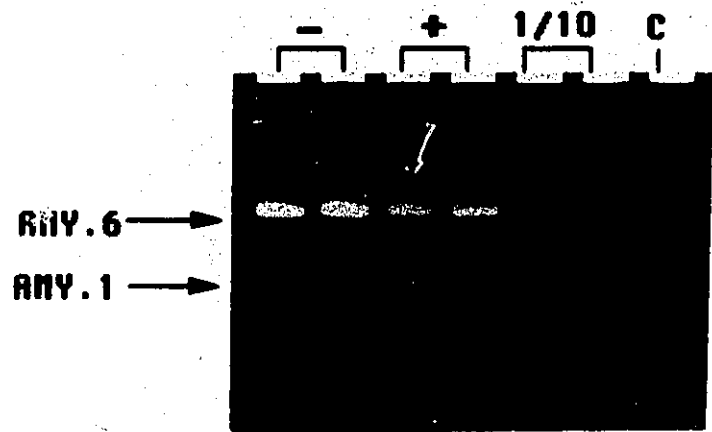
I. Expression of the amylase construct **V** in somatically transformed Amy^{null} larvae is shown. This construct contains the shortest upstream sequence (109 bp) required for full activity of the amylase proximal gene (see also Results of Table 1). Construct **V** was co-introduced with a control construct, i.e. the amylase gene Amy⁶. Amylase activity was assayed in transformed larvae grown in medium with glucose (+) or without glucose (-). The control lane contains a homogenate from Amy^{null} larvae grown in non-glucose medium. The 1/10 dilution is a sample from the homogenate from the transformed larvae grown in non-glucose food (-).

II. Expression of an amylase construct having a deleted upstream up to -83 bp (construct **CM.30**) is shown. The amylase activity is assayed in somatically transformed larvae which were treated the same way as described in Panel I.

I.



II.

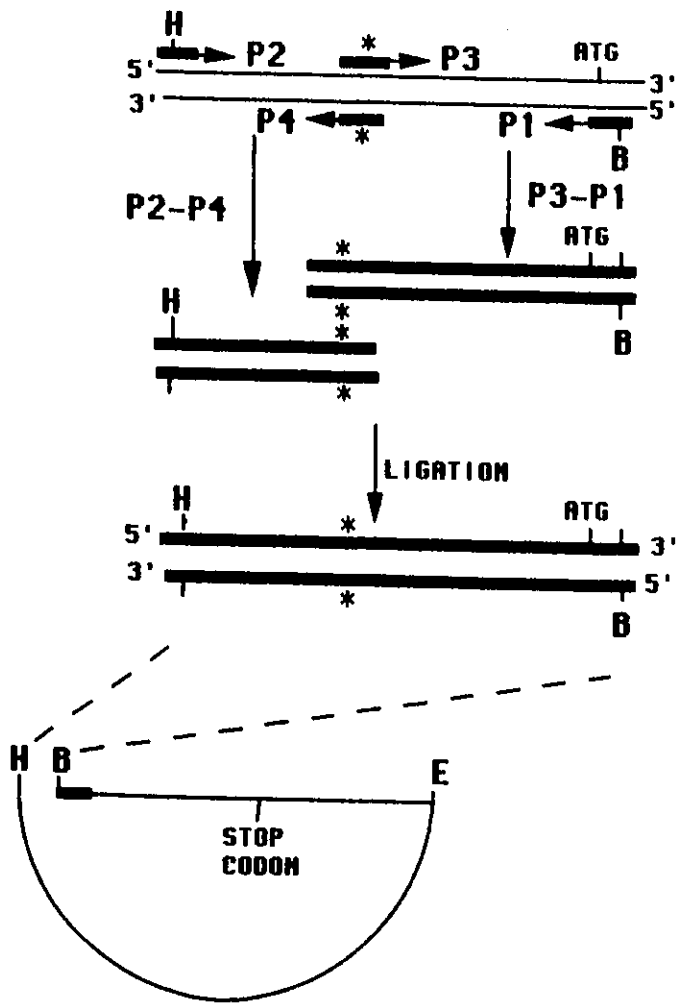


deletion, from position -92 to position -83, reduced amylase activity at least 50 fold.

Next, the effect of site-directed mutagenized sequences, within the 109 bp upstream region flanking the transcription start site of the proximal amylase gene, were characterized by the somatic transformation assay. In these experiments, a set of amylase constructs having various mutagenized upstream regions, 8 to 12 bp, were examined. These mutagenized regions were engineered by using a combination of PCR and crossover-linking methods, as shown schematically in Figure 9. A detailed experimental description of these constructs is given in Materials and Methods, p. 46. Figure 10 shows, in detail, the various upstream regions which were targeted for site-directed mutagenesis. Also, nucleotides which substituted wild type sequences are shown. Each of these constructs was co-injected with the control *Amy*⁶ gene (which is mentioned in previous similar experiments) into *Amy*^{null} embryos. Transformed embryos were grown in a medium with or without glucose up to third instar larvae. Amylase activity of these larvae was assayed by the electrophoretic method. Results of this amylase activity assay are shown in Figure 10. It is shown that any introduced changes of nucleotide content between the TATA and CAAAT motifs, as well as between the TATA motif and the transcription start site, did not affect overall activity or glucose responsiveness of the amylase promoter. In contrast, mutations which included the TATA or CAAAT sequences considerably reduced promoter activity. In both cases, this reduction of activity was

Figure 9: In vitro site-directed mutagenesis of upstream sequences by the Polymerase Chain Reaction method.

This figure shows schematically the use of a PCR method for introducing mutations into any desired upstream region. Four different primers (P1 to P4) can be used. Two external primers, P1 and P2, introduce cloning sites such as BclI and HindIII at the ends of the amplified upstreams. Overlapping internal primers, P3 and P4, introduce any desired changes, such as substitutions, deletions, and insertions. An example of substitution mutagenesis is given by using primers K339 and K341. Substitution mutations of targeted sequences are shown by asterisks. These primers were used for engineering of construct XII (see Methods and Materials, p. 46, for a detailed description). These primers contain sequences, shown in a shade, which substitute of TATA motif of the template. Note, this mutagenesis introduces an EcoRI cloning site. This cloning site is used to ligate the PCR amplified products by primers P1xP3 and P4xP2. The final mutagenized upstream was ligated as an HindIII-BclI fragment with the amylase reporter gene.



P3: 5'-TCC ********* GAGCGGCTCT...-3' →

TEMPLATE: 5'-...GGGGTCC ********* GAGCGGCTCT...-3'
 3'-...CCCCAGG ********* CTCGCCGAGA...-5'

← 3'-...CCCCAGG ********* CTGGC-5' **:P4**

Figure 10: Effect of site-directed mutagenesis of upstream sequences of the proximal amylase gene.

This figure shows the effect of various mutagenized regions, within upstream sequences, on amylase activity. Various DNA sites which scan the regulatory region of the amylase gene, from position -109 to -17, were targeted for mutagenesis. An asterisk marks position -17. These targeted regions are overlined by a black bar. The complete wild-type amylase sequence is shown (control construct). Also, the type of introduced nucleotide substitutions used for each amylase construct (VII to XIII) are shown. Each of these constructs was co-injected with an amylase control construct, the Amy⁶ gene, in Amy^{null} embryos. Relative amylase activity in transformed larvae, which were grown in a medium with (+) or without (-) glucose, was estimated by comparison to a dilution series of a wild type larval homogenate (external control) and by comparison to the expressed activity of an internal amylase control construct (++++ = 100%; + = less than 25%; +/- = less than 5%; - = no-activity or less than 1%).

AMYLASE ACTIVITY

-glu +glu

5'-CTAGCGAGCGATAGGATCCCAAGCAGTCGACATTCACCCCGAGAGCCCTCGATAGAGTAGCAGTGGGATCCACTATATAGGAGCCGCTCTGGTAGTCCGACCG-3'



U11: -----TAGA-A-I-----

U111: -----GA-TICTAG-----

IX: -----ICTAGG-AGATC-----

X: -----GATTCICT-GA-----

XI: -----GATTCICTAGG-----

XII: -----GAAT-C-CTA-----

XIII: -----TCTA-AGAA-TC-----

++++

+

+

++++

++++

++++

+

++++

estimated to be approximately 50 fold. Similarly, nucleotide changes within an element directly upstream of the CAAAT motif caused a large reduction in amylase activity. In all cases, full or highly reduced activity due to micro-injected constructs was completely repressed by dietary glucose.

3.2.5 Characterization of the 5'-flanking region of the distal amylase gene.

The distal amylase gene, Amy⁶, has previously been shown to have a low response to glucose repression, in comparison to the proximal amylase gene (see Figure 7). Other proximal and distal amylase genotypes, which are isolated from various Drosophila strains, have also been examined for their response to glucose repression by using the somatic transformation assay (results not shown). From these experiments, a common pattern of gene regulation is observed, i.e. expression of proximal genes is highly glucose repressible in contrast to expression of distal genes. These observations, which point that genotypic specific cis-acting elements regulate the degree of glucose repression, were addressed by the following experiments.

The significance of upstream sequences of the distal and proximal amylase genes, respectively, in the genotype specific variation in the degree of glucose repression was examined. In a control experiment, glucose regulation of the expression of proximal and distal amylase genes, containing 163 bp of wild type upstream sequences respectively, was examined in co-transformed

larvae as shown in Figure 11, panel A. Again, it can be seen that the expression of the proximal Amy¹ gene is highly glucose repressible in contrast to the expression of the distal Amy⁶ gene. In another similar experiment, glucose regulation of the expression of a hybrid amylase gene (*AMY¹), which contains a coding region of a proximal gene and an upstream region (163 bp from the transcription start) of a distal gene, was also examined in somatically transformed larvae. This *AMY¹ hybrid gene is described as construct CM.14 in Materials and Methods (p. 51). The result of this experiment is shown in Figure 11, panel B. It can be seen that the expression of AMY¹ from the hybrid gene has lost its high response to dietary glucose. This is clear when the degree of glucose repression of the hybrid AMY¹ expression is compared with the degree of glucose repression of the co-injected distal Amy⁶ gene expression. Therefore, the results of Figure 11 show that a substitution of a short upstream sequence of the proximal gene with the corresponding upstream sequence of the distal gene can change the degree of glucose response. This indicates that the variation in the degree of glucose repression of amylase genes is dependent on genotype specific upstream sequences.

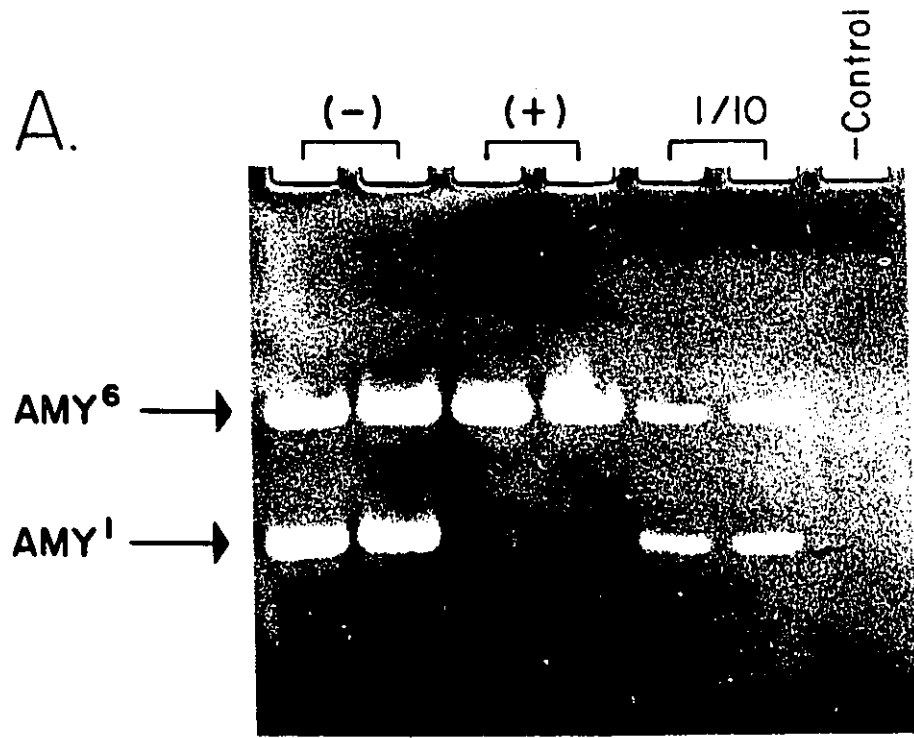
A sequence comparison analysis between the regulatory upstream sequence of the proximal and distal genes was examined in order to identify potential regulatory genotype specific cis-acting elements. A high degree of divergence, more than 30%, between these amylase sequences can be observed (Boer and Hickey, 1986). Based on this sequence comparison, a number of amylase constructs with

Figure 11: Characterization of upstream sequences of the distal amylase gene in response to glucose.

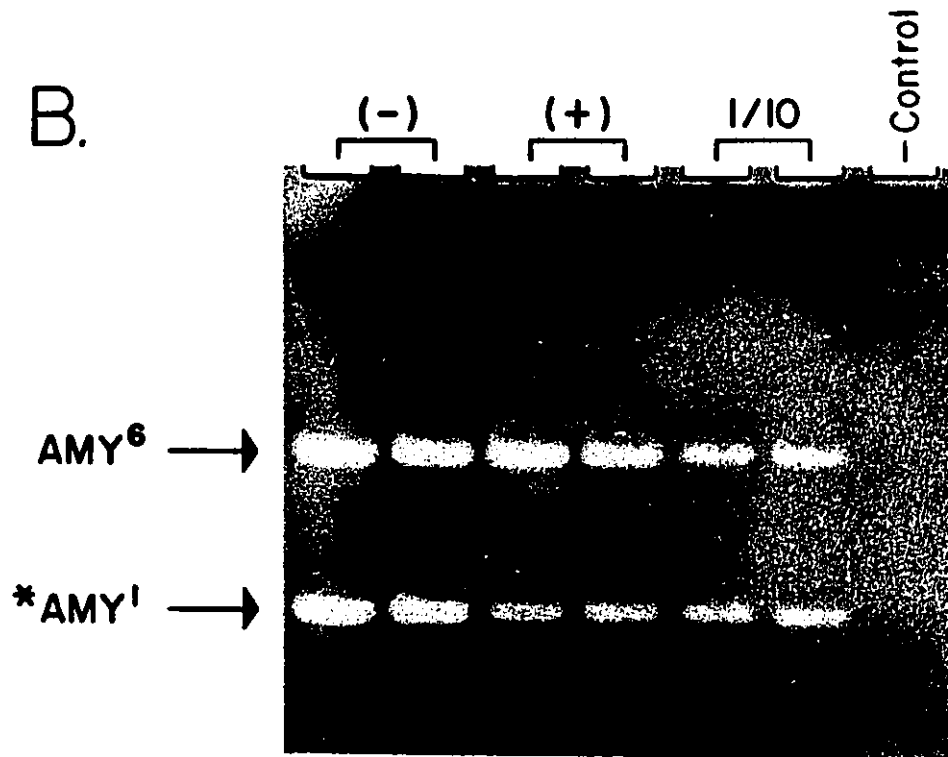
Panel A shows the effect of glucose on expression of two co-transformed constructs, IV and CM.18, which have the amylase proximal (Amy¹) and distal (Amy⁶) genotype, respectively. These amylase constructs contain only 163 bp of upstream sequence. The amylase activity is assayed in somatically transformed Amy^{null} larvae which were grown in a medium with (+) or without (-) glucose. An 1/10 dilution of the larva homogenate of the no-glucose (-) treatment is included. Control no-transformed Amy^{null} larvae were grown in a non-glucose medium.

Panel B shows the results of a similar experiment where the distal gene (construct CM.18) is co-injected with a hybrid construct, *AMY¹, into Amy^{null} embryos. This hybrid amylase construct contains the coding region of a proximal gene (Amy¹) and the upstream region of a distal gene (Amy⁶).

A.



B.



hybrid proximal/distal upstream sequences were made in order to characterize the observed genotype specific differences. In these constructs, short DNA regions of a proximal upstream region, which is enough to regulate amylase gene expression, were substituted with distal sequences located at a similar distance from the transcription start site. A detailed presentation of these fused proximal/distal upstreams is shown in Figure 12. These upstream hybrids were ligated with the coding region of the Amy¹ gene. The glucose effect on amylase activity, due to expression of these constructs, was examined in transformed larvae as described in previous similar experiments. The results of these experiments are also summarized in Figure 12. From these results it can be seen that a substitution of a proximal region, which flanks downstream of the TATA box, with distal sequences did not affect overall activity or glucose responsiveness of amylase. In contrast, overall amylase activity was reduced, in a magnitude of approximately ten times, by introducing distal sequences into any other location of the proximal upstream region. In all cases, none of the fused distal sequences changed the proximal genotype from high to low glucose responsiveness. These results, however, suggest that activating sequences of the distal promoter do not correspond to the same location (with respect to the transcription start) with activating sequences of the proximal promoter.

The next experiments of this section aim at identifying activating sequences of the distal amylase gene. For this purpose, a set of distal amylase constructs having deleted upstreams up to

Figure 12: Effect of dietary glucose on expression of amylase constructs having hybrid, proximal/ distal, promoter sequences.

Three constructs, II, III and IV, which have hybrid proximal/distal upstream sequences are shown. Construct I contains an unmodified 98 bp distal upstream sequence. This distal region corresponds to 109 bp of the proximal upstream sequence, which is also shown in the Figure, based on a sequence alignment by Boer and Hickey (1986). Dashes within the distal upstream sequence represent identity with nucleotides of the proximal sequence. Asterisks indicate nucleotides which are absent in distal sequences. The distal regions which substitute the proximal sequences are boxed. Each of these constructs, which produces the Amy¹ variant, were co-injected with the amylase Amy⁶ gene into Amy^{null} embryos. Amylase activity in transformed third instar larvae, which were grown in a medium with (+) or without (-) glucose, was determined as described in Figure 10.

Proximal 5'-TTAGGAGAGCGATAGATCCCAATCGAGATCCAAATCACTCCCGCCGAGAGCCCTCAGATG AAGTAGCAGATGAGGATCCACATAATAGAGAGCCGC TCTAGATAGATCCGAGCAG

-108
 -97
 -73
 -72
 -80
 -101
 -109
 -80
 -31
 -32
 -31
 -32
 -31
 -32

AMYLOSE ACTIVITY

-glu +glu

++++ -

+ -

+ -

+ -

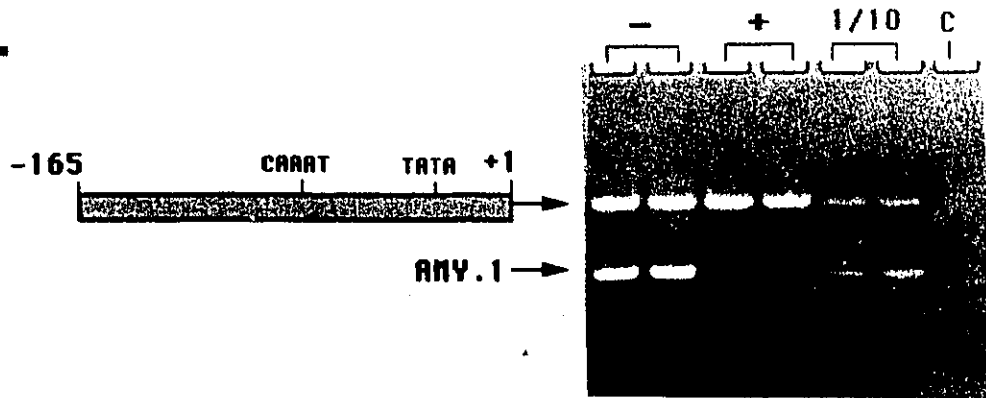
++++ -

the points -165, -143, -117 and -97 base pair from the transcription start site were engineered. In a control experiment, a construct of the distal (Amy⁶) gene which contains an upstream of -165 bp, is shown to have the same overall expression as a native wild type proximal gene in transformed Amy^{null} larvae (Figure 13, panel I.). Overall activity of the distal gene, however, is shown to be reduced considerably by a further upstream deletion up to -143 bp (Figure 13, panel II.). These results, taken together with previously presented results of the deletion analysis of proximal 5' flanking sequences, show that there are genotype specific upstream activating sequences. These sequences are located in different distances from the transcription start site as shown in Figure 14.

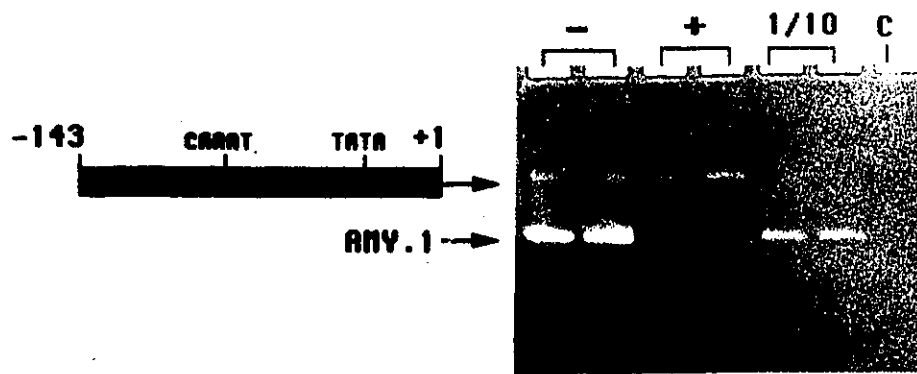
Figure 13: Deletion analysis of upstream sequences of the distal amylase gene.

This figure shows the transient expression of various amylase distal (Amy⁶) constructs which contain deleted upstreams in somatically transformed larvae. These constructs contain -165 bp, -143 bp and -117 bp of upstream sequences and are described as constructs **CM.18**, **CM.29** and **CM.26**, respectively, in Materials and Methods (p. 45). The proximal Amy¹ gene (internal control) was somatically co-transformed with each of these constructs in Amy^{null} embryos. Amylase activity of transformed third instar larvae, which were grown in a medium rich in glucose (+) or without glucose (-), was assayed. A sample of untransformed Amy^{null} larvae which were grown in a medium without glucose is also assayed (control lane). A 1/10 dilution sample of a larva homogenate from glucose (-) treatment is included.

I.



II.



III.

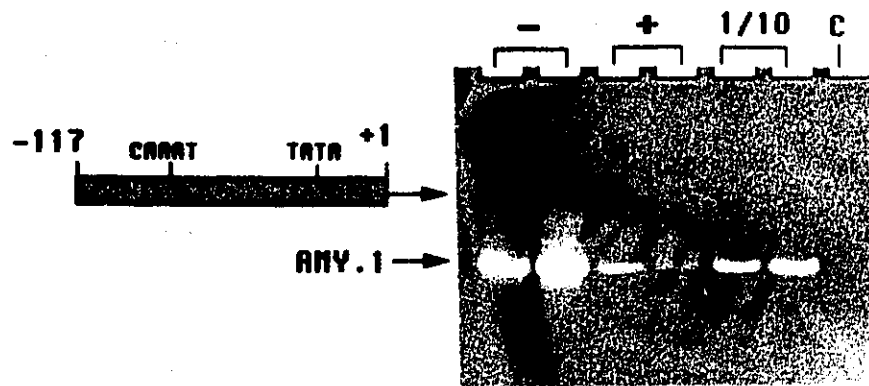


Figure 14: Upstream activation sequences of the distal amylase gene.

The deletion analysis results of upstream sequences of the distal amylase gene are summarized in Panel A. The numbers indicate the deletion points from the transcription start site. Panel B shows an alignment between distal and proximal upstream sequences. Asterisks indicate sequence identity between the two genes (Boer and Hickey, 1986). Regions which were identified to be important for promoter activity are shadowed. Arrows indicated the points which were used for specific upstream deletions.

3.3 Role of cAMP in glucose repression of Drosophila.

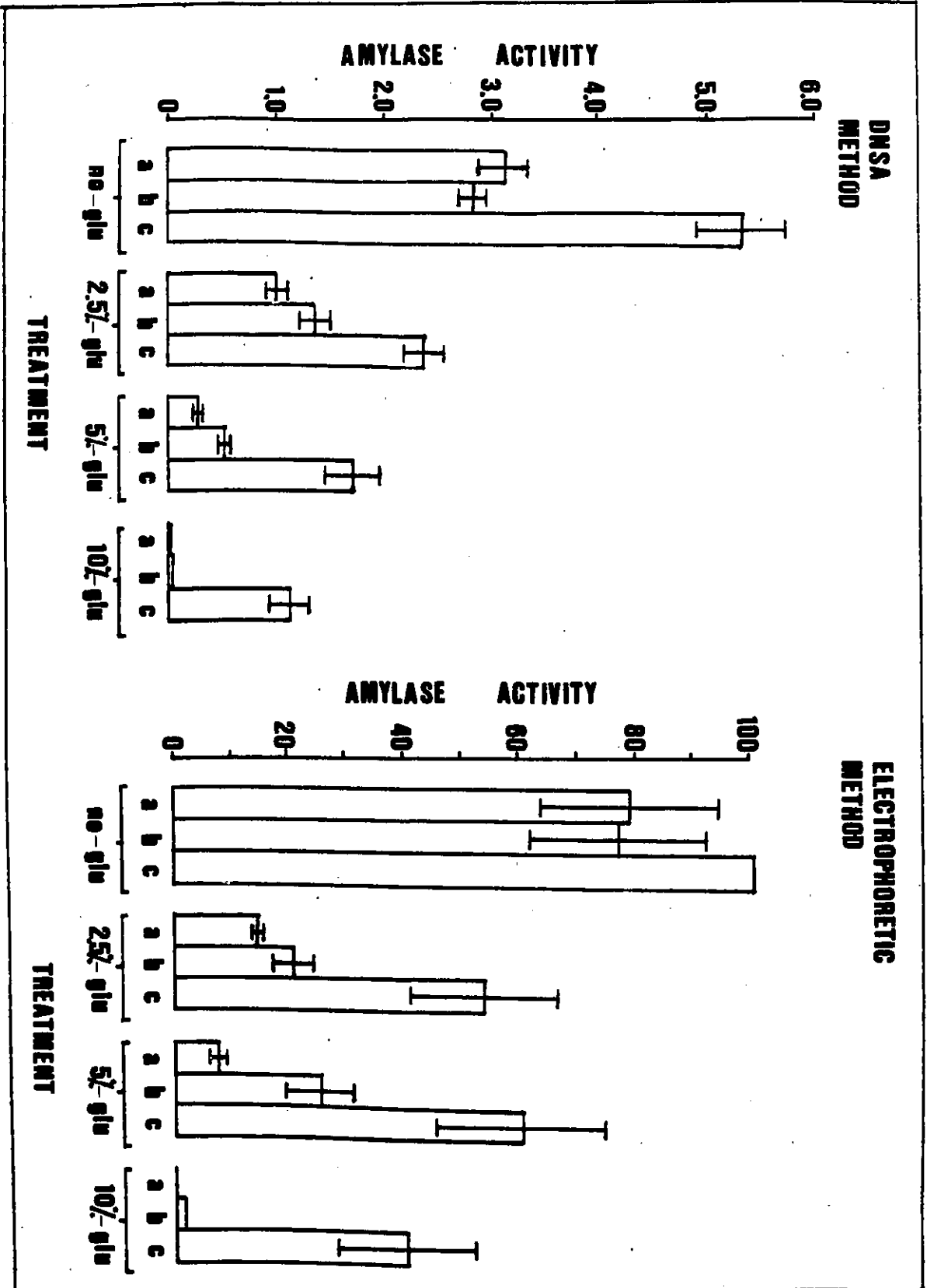
3.3.1 Effect of exogenous cAMP on glucose repressible amylase activity.

In experiments of this section, the effect of exogenous cAMP on glucose repression of the amylase activity in D. melanogaster larvae was investigated. Furthermore, whether this cAMP effect reflects a regulation at the DNA level was examined.

Preliminary results (Benkel and Hickey, 1986) indicated that repression of amylase activity by a diet with 10% glucose can be counteracted by exogenous cAMP in D. melanogaster larvae. In this experiment, the effect of exogenous cAMP on amylase activity of larvae grown in various levels of dietary glucose was examined. Results of this experiment are shown in Figure 15. In addition, the effect of exogenous AMP, which is an analogue of cAMP, was tested in order to define the specificity of the cAMP effect. These results show that glucose repression was relieved by cAMP regardless of the concentration of dietary glucose. The magnitude of cAMP effect on amylase activity was found to be the same by both methods used in this experiment, the DNSA and the electrophoretic gel methods which are described in Materials and Methods (p. 35). In addition, it is shown that the degree of relief, due to exogenous cAMP, is proportional to the degree of glucose repression. The magnitude of relief is maximum when cAMP is added to larvae which were grown in the most repressive conditions (10% glucose) and is estimated to be about 40

Figure 15: The interaction of exogenous cAMP and various levels of dietary glucose on amylase activity.

This figure shows amylase activity of larvae which were grown in media with various levels of glucose and which were treated with exogenous cAMP. In treatment-c, 150 μ moles of cAMP were added to the food, 24 hr's before larval harvesting. In treatment-b, an equal amount (150 μ moles) of AMP was added to the food. Finally, in control treatment-a, no exogenous substance (cAMP) was added. Amylase activity assayed by the DNSA method is expressed in UNITS. One UNIT = 10^{-5} of μ moles of maltose produced \ min\ μ g of total soluble protein, at 37 °C. Amylase activity determined by the electrophoretic gel method is expressed in percentage (%) of relative activity. Values present the mean +/- S.E. and derived from assaying three different larval populations.



fold. This estimation of the cAMP effect was confirmed, when a direct dilution series of a homogenate from larvae treated with cAMP and glucose was used in the electrophoretic gel assay. Amylase activity of this dilution was compared with the amylase activity of larvae treated with glucose alone.

The cAMP effect on glucose repressed amylase activity was found to be dependent on the dosage of exogenous cAMP, results shown in Figure 16. Also, in another study, the cAMP effect was found to be dependent on the duration of the treatment (results not shown). Specifically, days rather hours of cAMP treatment are required for a significantly measurable effect on amylase activity. In summary, these results suggest that glucose repression of amylase activity is due to a reduction of endogenous cAMP which can be compensated by exogenous cAMP. Also, the full compensation of endogenous cAMP is limited by the amount of exogenous cAMP applied and by the duration of cAMP treatment.

The specificity of exogenous cAMP on amylase activity was further examined by comparing the effect of two classes of cAMP analogues. One class comprises nucleotides which are adenosine phosphates while the other class of analogues are guanosine phosphates. The following considerations have been taken into account in order to compare the potency of these compounds: first, an equal molar amount of these compounds was applied and second, a time course of their effect was monitored. A summarized version of results of these experiments is shown in Figure 17. The Figure shows that the effect of exogenous cAMP, 5'-AMP, dibut.cAMP and

Figure 16: Effect of increasing levels of exogenous cAMP on amylase activity.

Amylase activity of third instar larvae grown on a diet containing 10% glucose and supplemented with various levels of exogenous cAMP is shown. The following amounts of cAMP were added: a - no added cAMP (control), b - 15 μ moles of cAMP, c - 60 μ moles of cAMP, d - 150 μ moles of cAMP. Also, 150 μ moles of AMP was added, treatment-e, as a control for specificity. cAMP or AMP was added to the medium 24 hr before larval harvesting. Amylase activity was determined by the DNSA method and expressed in units. One unit = 10^{-4} of μ moles of maltose produced \ min \ μ g of total soluble protein, at 37 °C. Values represent the mean +/- S.E. (n = 4) and by assaying two replicate samples in two separate experiments.

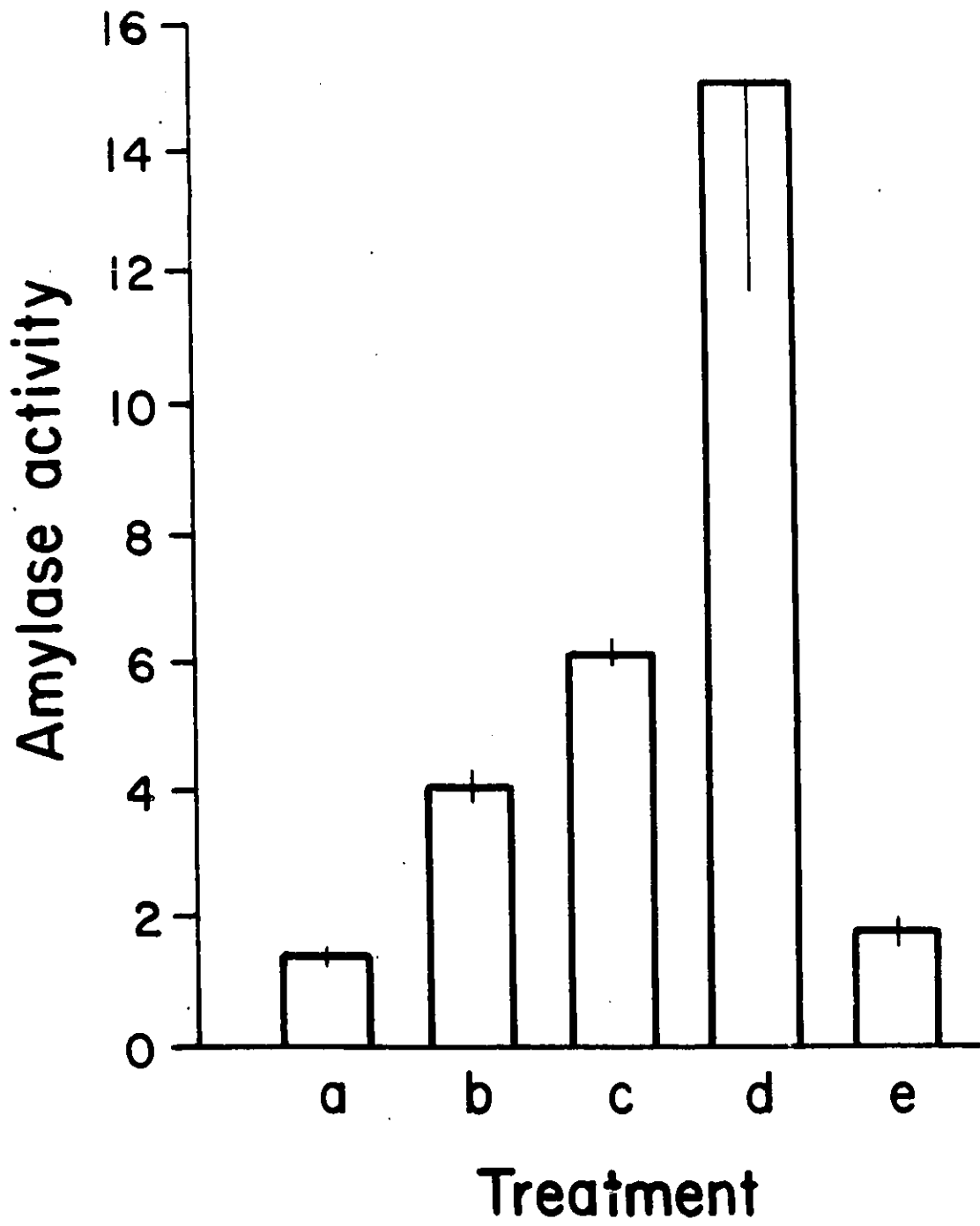
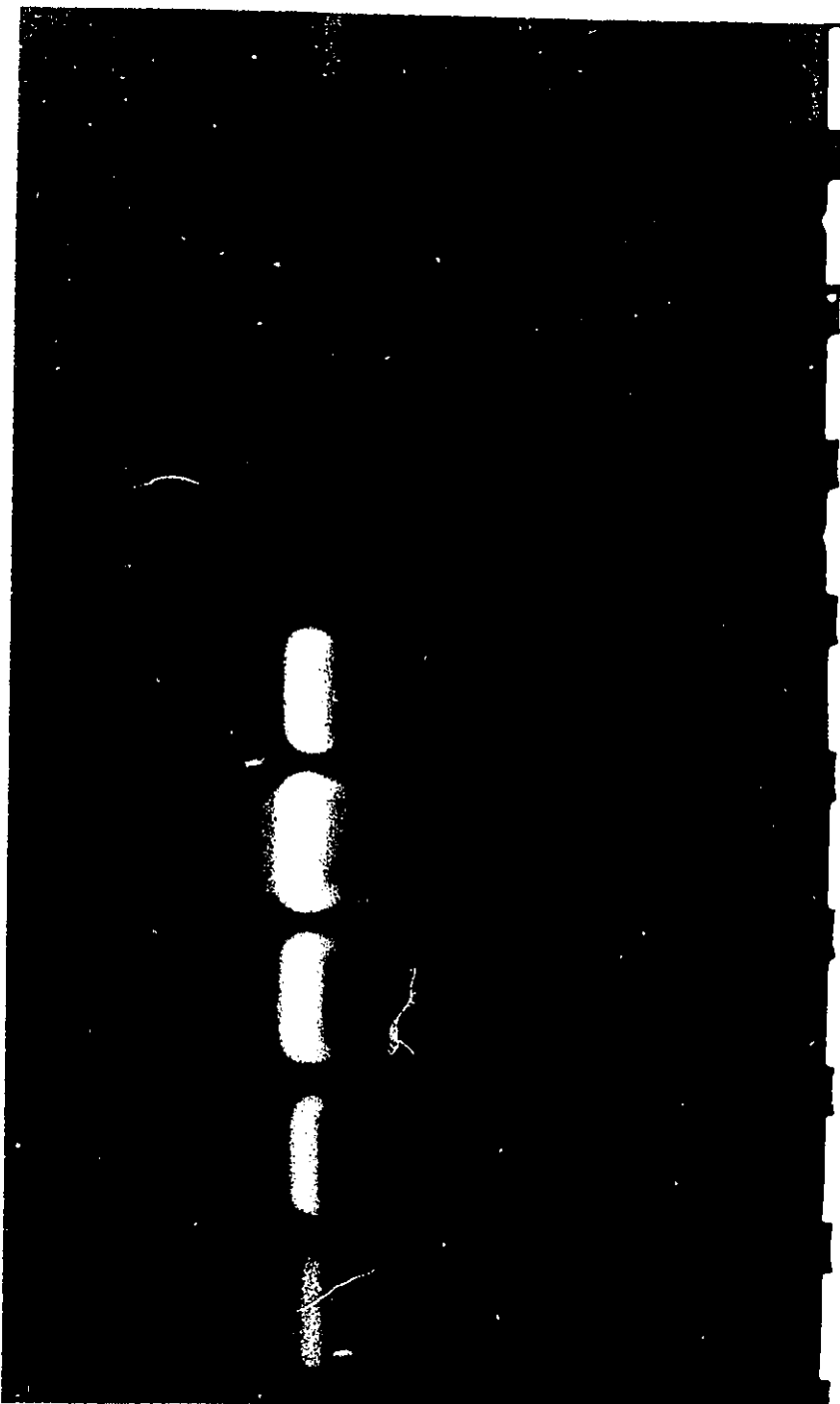


Figure 17: Effect of cAMP analogues on amylase activity.

This is a native electrophoretic gel stained for amylase activity. It contains homogenates of third instar larvae which had been grown on glucose-supplemented (10%) foods containing the following additives (30 μ moles of each): sample a - no additives, sample b - butyrate , sample c - dibutyryl cAMP, sample d - AMP, and sample e - cAMP. Each of these compounds was added to the medium 48 hr's before larval harvesting. Sample f, larvae grown on a glucose-free medium, serves as a control for non-repressed levels of expression. Various dilutions of the control sample are shown.

a | b | c | d | e | f | 1/4 | 1/16 | 1/64



butyrate over a period of 48hr's of treatment. cAMP is shown to be the most potent compound in the relief of glucose repression of amylase activity, compared with its analogues. This relatively potent cAMP effect, relative to its analogues, was seen regardless of the duration of treatment (results not shown). After 48hr's of treatment, cAMP was found to be around 10 times more potent than AMP and dibut.cAMP. However, 5'-AMP and dibut.cAMP had a small effect close to background.

In other similar experiments, the effect of cGMP, 5'-GMP, cAMP and 5'-AMP on glucose repressed amylase activity was examined (these results are not shown). Again, cAMP was found to be the most potent compound for relieving glucose repression of amylase activity. Also, cGMP and 5'-GMP were found to counteract glucose repression approximately four fold less than cAMP.

3.3.2 Counteraction of glucose repression by exogenous cAMP at the amylase mRNA level.

The results of the previous section showed that exogenous cAMP counteracts glucose repression of amylase activity suggesting that the expression of the amylase gene may also be responsive to cyclic AMP. In the experiments of this section, the counteraction of glucose repression by cAMP was examined at the amylase mRNA level. The effect of exogenous cAMP on amylase activity was first determined in larvae grown in a glucose rich medium as shown in Figure 18. The native gel stained for

Figure 18: The interaction of dietary glucose and exogenous cAMP on the activity of amylase.

This is an electrophoresis gel stained for amylase activity. It shows the enzymatic activity of third instar larvae treated as following: sample a - larvae grown on a glucose-free diet, sample b - larvae grown in a glucose-supplemented (10%) food medium and sample c - larvae grown in a glucose-supplemented medium to which cAMP was also added. In this case, 150 μ moles of cAMP was added 48 hr's before larval harvesting. Included in the gel is a dilution series of sample c; eight-fold and sixty four-fold dilutions.

a

l

b

l

c

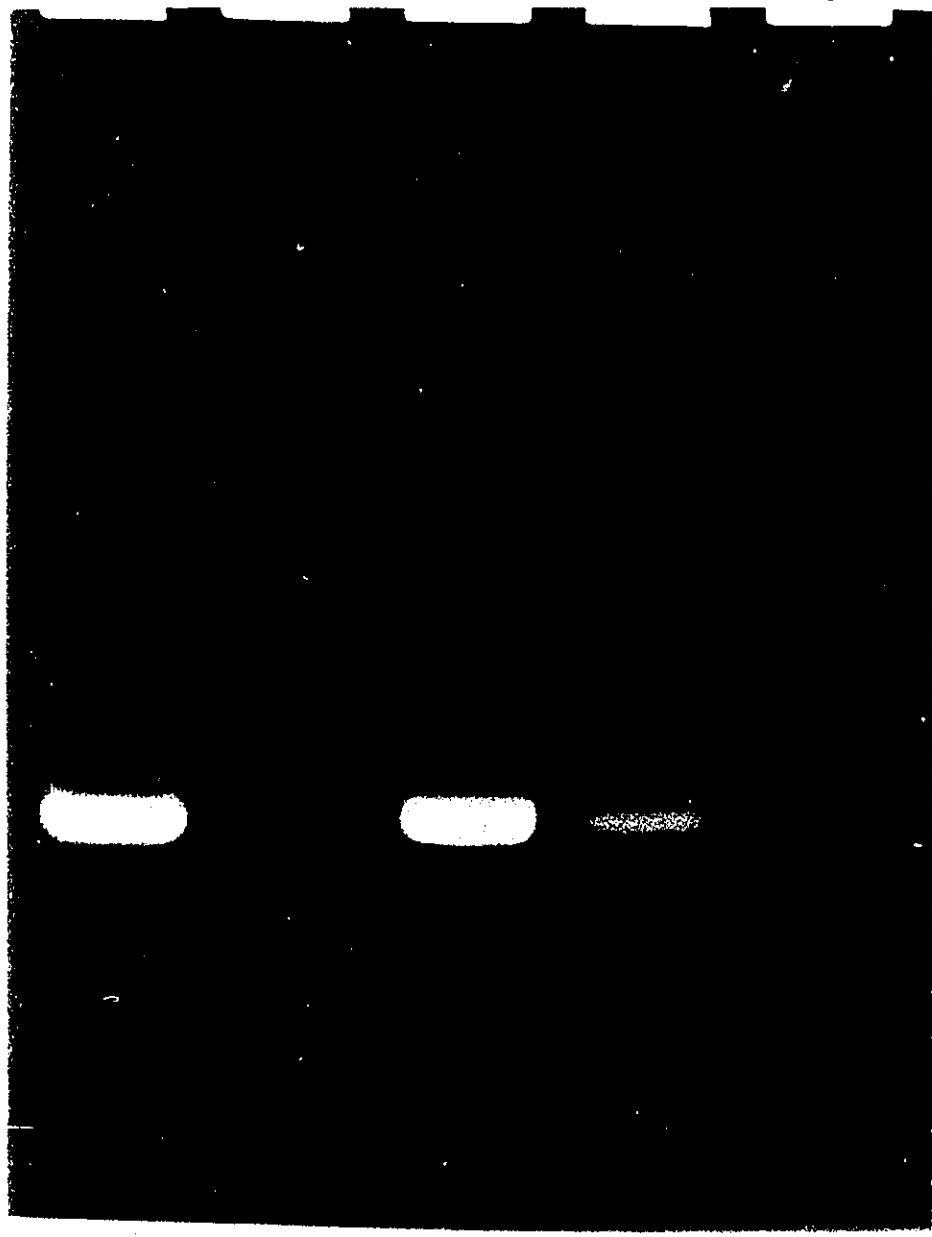
l

$\frac{1}{8}$

l

$\frac{1}{64}$

l



amylase activity shows a strong band of enzyme activity in lane a; this sample is from larvae grown in the absence of dietary glucose. The second lane, b, shows an equivalent sample from larvae grown in the presence of 10% glucose; note the virtual absence of any detectable amylase activity. The third lane, c, contains a homogenate from larvae which were grown in the presence of both glucose and cyclic AMP; in this case the activity is restored to a level comparable to that of larvae grown in the absence of glucose. Eight-fold and sixty four-fold dilutions of the cAMP-treated homogenate (sample c) are included to indicate the magnitude of these dietary-induced changes.

Then, RNA was extracted from the larval homogenates described in Figure 18 above. Northern blots and dot-blot of this RNA were probed with amylase-specific cDNA probes. As can be seen from Figure 19, the changes in amylase mRNA levels paralleled the changes in enzymatic activity (shown in Figure 18). Sample a is RNA from larvae grown in the absence of glucose, sample b is from the glucose treatment, and sample c is from the glucose plus cyclic AMP combined treatment. Again, glucose reduces the level of amylase mRNA and cyclic AMP restores it. This is seen both in the Northern-blot (Figure 19A) and in the dot-blot (Figure 19B). Few bands, other than the amylase transcript, are visible in the Northern-blot. These extra bands are larger than the standard E. coli rRNA, 23S, and their appearance is found to be variable from experiment to experiment. The RNA blots were also probed with an alcohol dehydrogenase probe (Adh is not glucose repressed) as an internal

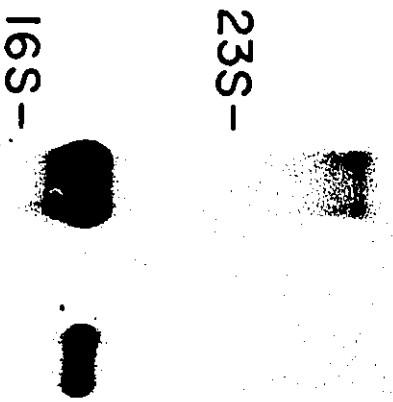
Figure 19: Dietary glucose and exogenous cAMP affect amylase mRNA levels.

RNA was extracted from third instar larvae which were treated as following: sample a - larvae grown on a glucose-free food medium; sample b - larvae grown on a glucose-supplemented (10%) medium and sample c - exogenous cAMP was added to the glucose-supplemented medium. The alpha amylase mRNA was detected by hybridization with an amylase-specific probe. Panel A, Northern blot analysis; Panel B, dot blot analysis; Panel C is a the same dot blot as shown in B, washed and re-hybridized with an Adh specific probe as control.

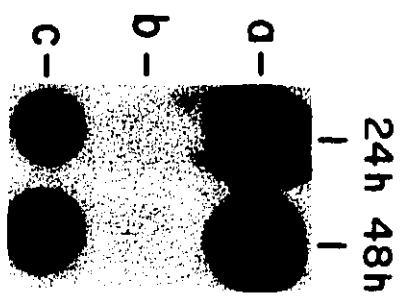
In the Northern analysis larvae were treated with 150 μ moles of cAMP for 48 hr before harvesting. Also, a ten-fold dilution of the cAMP treatment is shown. In the dot blot analyses, two larval homogenates are shown; these two sets of larvae were treated with cAMP for 24 and 48 hr's respectively.

A

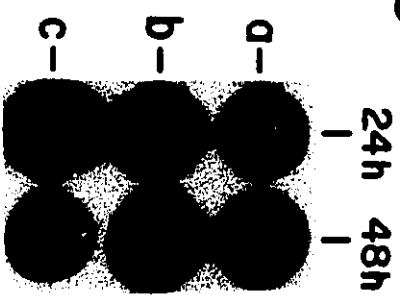
ORI-
| a | b | c | 1/0



B



C



control. As can be seen from Figure 19C, high levels of Adh message are produced regardless of the dietary treatment.

A quantitative summary of a larger data set on the effects of glucose and cyclic AMP, on both amylase activity and amylase mRNA levels, is presented in Table 2. In this case, amylase activity was measured both by a spectrophotometric method and by the densitometric scanning of electrophoretic gels. Amylase mRNA levels were assayed by the densitometric scanning of autoradiogrammes of Northern-blot hybridizations. Two data sets are shown, one for larvae that were harvested twenty four hours after treatment and the other for larvae that were harvested forty eight hours after treatment. The general conclusion to be drawn from these data is that, regardless of the assay technique used, glucose represses amylase expression and cyclic AMP enhances it. It is also noteworthy that the effects of cyclic AMP continue for at least forty eight hours after treatment.

3.3.3 The interaction of exogenous cAMP and dietary glucose on the expression of the amylase gene in transgenic larvae.

The results of the previous section show that exogenous cAMP counteracts glucose repression of the amylase activity at the mRNA level. The next step is to determine whether this effect is related with DNA regulatory sequences. The embryo somatic transformation assay, which is described in previous sections, was used in order to examine the response of the upstream amylase sequences to cAMP. In this transient expression experiment, the

Table 2: The interaction of dietary glucose and cAMP on the expression of the amylase gene.

	Enzymatic activity				mRNA level
	DNSA method		Electrophoretic method		Northern hybridization
	24 h	48 h	24 h	48 h	48 h
A:	36 ± 3	47 ± 3	100 ± 14	100 ± 3	100 ± 9
B:	1 ± 1	0 ± 0	1 ± 0	1 ± 0	1 ± 1
C:	13 ± 1	25 ± 0	41 ± 8	84 ± 16	51 ± 11

Sample A: larvae grown on a glucose-free diet; sample B: larvae grown on a glucose-enriched (10%) diet; sample C: larvae grown on a glucose-enriched diet which was also supplemented with cAMP. Two sets of data are shown for enzyme activity: one for larvae which were harvested 24 hr after treatment and the other where harvesting took place 48 hr post-treatment. For a definition of units of amylase activity scored by the DNSA method, see legend to Figure 16. In the electrophoretic assay method, gels were scanned by densitometer, and the values expressed as a percentage of the highest activity (which was given the arbitrary value of 100%). Values derived from the densitometric scanning of Northern blot autoradiogrammes were also expressed as percentages of the highest value.

amylase construct IV, which has a short upstream sequence with 109 bp from the transcription start, was injected into Amy^{null} embryos. This length of upstream sequence is the shortest required for full amylase expression (see Table 1). Then, activity of transformed embryos, which were grown in media containing no glucose (a), 10% glucose (b) or 10% glucose plus cAMP (c), was assayed by the electrophoretic method. The results of this experiment are shown in Figure 20, panel A. It can be seen that the transiently expressed amylase gene responds to the cAMP treatment in the same way as the wild-type endogenous gene which has been characterized in previous experiments. Also, endogenous ADH activity of the same larvae was assayed indicating that this response is specific to the transformed amylase gene (results not shown).

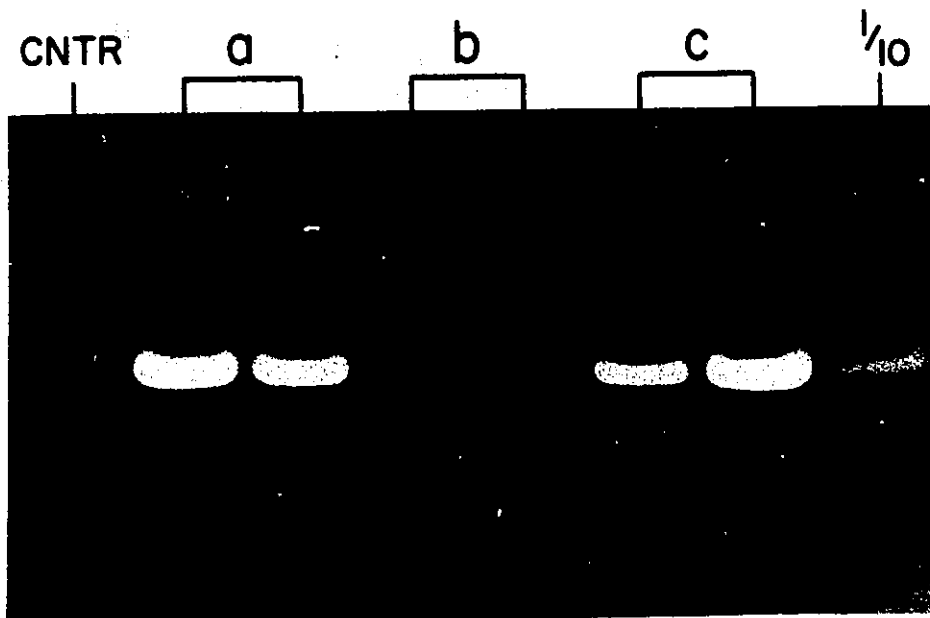
Upstream sequences of the transformed amylase construct IV which are fully responsive to cAMP, are shown in panel B of Figure 20. An element which shares similarities with the mammalian cAMP responsive element (CRE) was identified closely upstream of the CAAAT motif, as shown in the figure. This element has been functionally characterized, in previously described experiments, showing that is important for activity of the amylase promoter (see results of Figure 10).

Figure 20: The interaction of exogenous cAMP and dietary glucose on amylase gene expression in somatically transformed D. melanogaster larvae.

Panel A: This is a native electrophoretic gel showing the amylase activity in Amy^{null} larvae which were transformed somatically with an amylase construct (IV) of the proximal gene (Amy¹). This construct contains a 109 bp upstream from transcription start site. Transformed embryos were grown to third instar larvae under the following treatments; a: no-glucose in the diet; b: glucose-enriched (10%) diet and c: exogenous cAMP was added (48 hr's prior to larval harvesting) to the glucose-enriched diet. A duplicate sample of five larvae from each treatment were homogenized and 40 µg equivalent of total protein content was assayed for amylase activity. A sample of untransformed Amy^{null} larvae grown in a glucose-free diet was assayed as a control. Also, a lane containing 1/10 dilution of the cAMP treatment is included.

Panel B: This is the sequence of the promoter region of the amylase construct used in this transformation experiment. This DNA region is sufficient to confer the cAMP derepression. The TATA and CAAAT motifs, as well as, the ATG start codon of the amylase gene are underlined. The arrow indicates the transcription start site identified by Boer and Hickey (1986). The consensus of the mammalian cAMP responsive element (CRE) is compared with the putative Drosophila cis-acting element.

A.



B.

5'-TGACGTCA-3'
**

-145
5'-AAGCTTAGGGAGCGATAAGATCCCATGCAGTCACAAATCACTCCCCGCGAAG
HindIII

CCCTCAGATAAAGTAGCAGTGGGGTCCACTATATAAGGAGCGGCTCTGAGTAGT

┌→ +1
TCCGACCAGAGTGAAACTGAACTCCATCTGGAATCATCATGTTTCTGATCA-3'
BclI

3.4 Evolutionary aspects of glucose repression of amylase gene expression in insects.

3.4.1 Glucose repression of amylase activity in D. virilis.

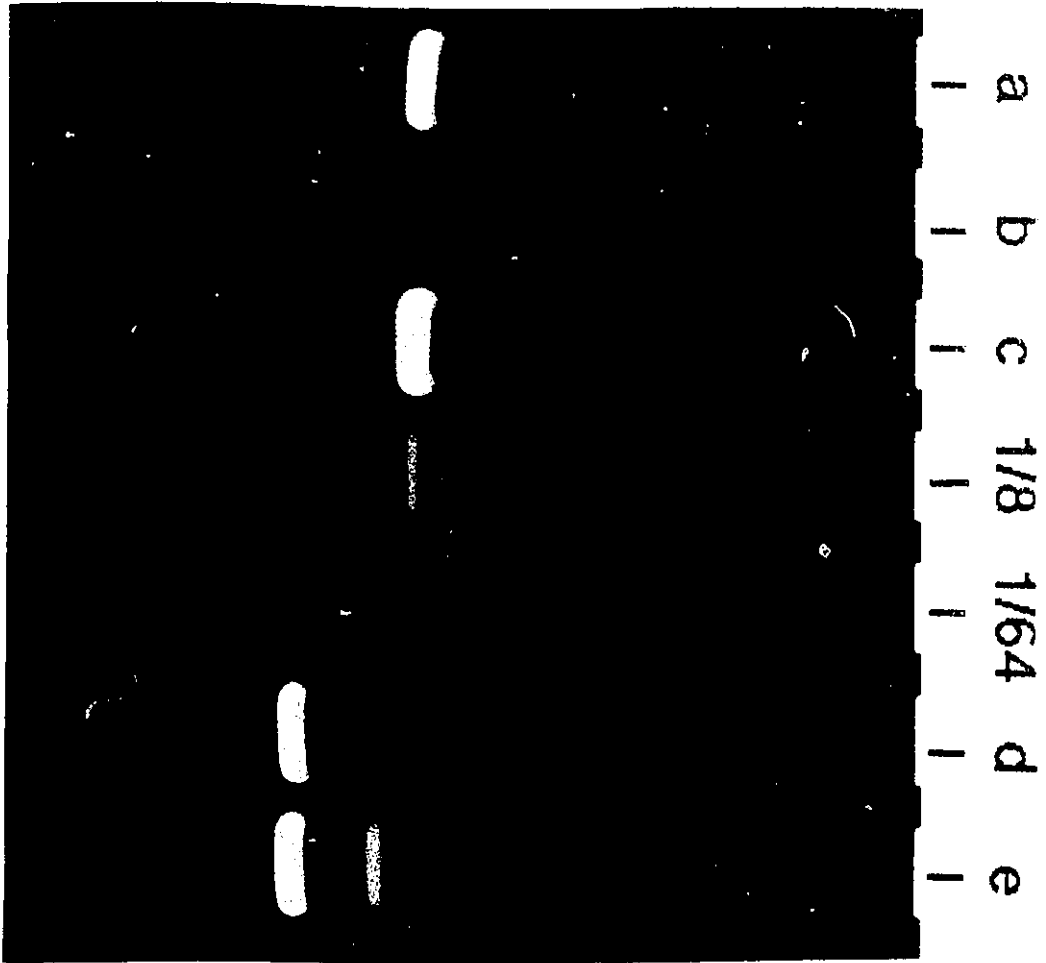
In this experiment, glucose repression of amylase activity in D. virilis, a species which is distantly related to D. melanogaster, was examined. As is shown in Figure 21, the amylase activity of homogenates from larvae which were grown in a glucose diet is at least one hundred fold repressed in comparison to the enzymatic activity of a control sample. It is also shown that exogenous cAMP (50 mg for a 24 hour treatment) counteracts this glucose repression. An electrophoretic method, which is described in previous experiments, was used to detect amylase activity. Samples of two D. melanogaster strains (Oregon R and Canton S) were included in the electrophoretic run in order to characterize the specific mobility of the D. virilis amylase. The effect of the glucose rich diet on D. virilis amylase activity was also found to occur at the level of mRNA abundance (results not shown).

3.4.2 Expression of the D. virilis amylase gene in D. melanogaster larvae.

Expression of the amylase gene is repressed by glucose in D. virilis (as shown in the previous section) and in D. melanogaster (Benkel and Hickey, 1987). Then, the conservation of this phenomenon was examined at the transcriptional level by using an embryo transformation assay.

Figure 21: The effect of dietary glucose on the activity of amylase in D. virilis.

This is an electrophoretic gel stained for amylase activity. It shows enzymatic activity of homogenates, with equal protein content, from D. virilis third instar larvae treated as follows: sample a - larvae grown on a glucose-free diet, sample b - larvae grown on a glucose-supplemented (10%) food medium and sample c - larvae grown on a glucose-supplemented medium to which 150 μ moles of cAMP was also added 48 hr's before larva-harvesting. In addition, an eight-fold and sixty four-fold dilution series of sample c is shown. For electrophoretic mobility markers, I included sample d and sample e which are homogenates from D. melanogaster flies of the Oregon R and Canton S strains respectively. The Oregon R strain produces the amylase electrophoretic variant AMY¹ while the Canton S strain produces the AMY¹ and AMY³ isozymes (Doane, 1969).



↑
↑
AMY-3
AMY-1

The D. virilis amylase gene has been isolated and characterized by DNA sequencing (Abukashawa, Ph.D. thesis, 1990). A construct was made containing -1600 bp of upstream sequence along with the coding region and 2000 bp downstream of the gene as described in Materials and Methods. This construct was microinjected into Amy^{null} embryos of D. melanogaster and transient activity of the amylase was assayed, in third instar larvae which had been grown on media with or without glucose, as shown in Figure 22, panel A. The results show that the transformed D. melanogaster larvae expressed the D. virilis amylase gene, as the D. virilis amylase product can be identified by its specific electrophoretic mobility. Furthermore, this expression was repressed by dietary glucose as in the wild type which has been characterized in the previous section (Figure 21). This experiment demonstrates that expression of the D. virilis amylase gene is appropriately regulated in D. melanogaster. The overall expression of the D. virilis gene was also quantified, in comparison to the endogenous D. melanogaster amylase expression, by using a serial dilution of the activity of the latter. The overall expression level of the transformed gene was found to be approximately one tenth that of the endogenous amylase gene.

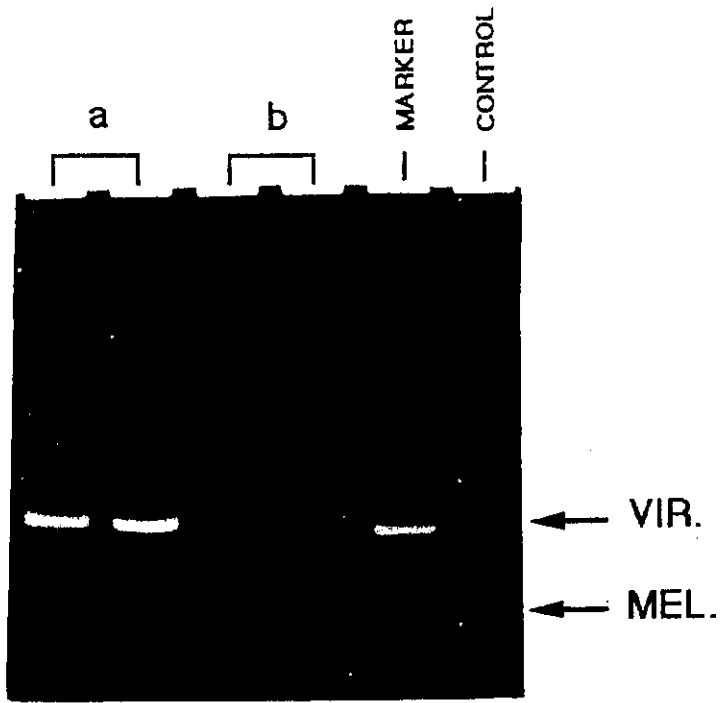
Finally, a hybrid gene construct was made in order to confirm the functional conservation of the D. virilis amylase promoter in D. melanogaster. This construct contains 330 bp upstream of the start codon of the D. virilis amylase gene coupled with the coding region of the D. melanogaster gene and is made as

Figure 22: Glucose repression of expression of the D. virilis amylase gene in transformed D. melanogaster larvae.

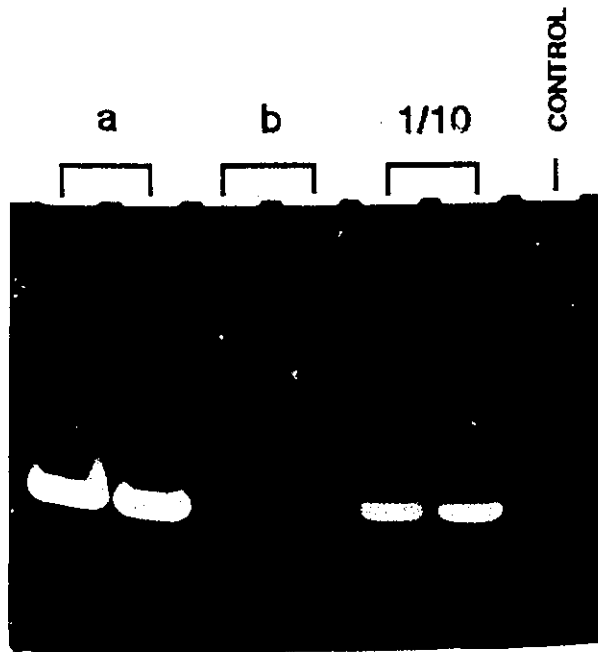
Panel A shows the effect of dietary glucose on the amylase activity of D. melanogaster Amy^{null} larvae which are somatically transformed with the D. virilis amylase gene. Transformed larvae were treated as follows: a-larvae grown on a glucose-free medium, b-larvae grown on a glucose-supplemented (10%) diet. In each lane of the electrophoresis gel, a homogenate of equal protein content from five to ten transformed larvae was loaded. In the control lane was run a sample of untransformed larvae grown on the glucose-free medium. The electrophoretic mobility marker is a mixture of a D. virilis wild-type homogenate and a D. melanogaster (Oregon R strain) homogenate.

Panel B shows the glucose repression of the expression of a D. virilis/D. melanogaster hybrid gene in D. melanogaster larvae. This hybrid amylase gene contains the promoter of the D. virilis amylase gene and the coding region of the D. melanogaster amylase gene. The transformed D. melanogaster Amy^{null} larvae were treated the same as described in panel A. This electrophoresis gel assay also shows the amylase activity of a ten time dilution sample from homogenates of transformed larvae which were grown on a glucose-free medium (treatment a).

A.



B.



described in Materials and Methods (p. 52). This hybrid amylase gene was microinjected into D. melanogaster Amy^{null} embryos. The transformed embryos were grown to third instar larvae under a glucose diet and a control, without glucose, diet. Amylase activity of third instar larvae was assayed by the electrophoretic method. Results of this assay are shown in Figure 22, panel B. It can be seen from this Figure that the short region of the D. virilis upstream was sufficient to give high overall expression of the D. melanogaster amylase gene. In addition, this expression was repressed by glucose.

3.4.3 DNA sequence comparison between the D. melanogaster and the D. virilis amylase genes.

Drosophila virilis has only one copy of the amylase gene (Abukashawa, Ph.D. thesis, 1990). The expression of this gene is glucose repressible in D. virilis wild-type strains and is also repressible when it is transiently expressed in D. melanogaster Amy^{null} embryos, as shown in the two previous sections. Therefore, the promoters of both genes, i.e. the D. virilis and the D. melanogaster amylase genes, must contain conserved cis-acting elements which confer glucose repression. Moreover, elements from both species are appropriately recognized by the transcriptional machinery of D. melanogaster.

Previously, a dot-matrix sequence comparison between the coding region and the upstream flanking region of the D. melanogaster and D. virilis amylase genes showed that there no

significant sequence similarities in the promoter region (Abukashawa, PhD thesis, 1989). In this comparison, a stringency high enough (80% homology per 20 nucleotides) to detect a high level of similarity in the coding region without any background was used. These stringency conditions were set by using the Microgenie sequence analysis programs of Queen and Korn (1984).

A more detailed dot-matrix sequence comparison between the promoters of the amylase genes of D. virilis and D. melanogaster was performed (results not shown). Only 330 bp upstream of the start codon of the amylase promoter was compared, since this region is sufficient for the constitutive and glucose-regulated expression of the amylase gene as shown in Figure 22, panel B. In this comparison, a stringency low enough (80% homology per 10 nucleotides) to detect any possible significant similarities was used. Only short regions of sequence similarity sporadically distributed between the two promoters were identified even under this low stringency.

3.4.4 Sequence alignment of the promoters of glucose repressible amylase genes of distantly and closely related Drosophila species.

Promoter sequences of the D. melanogaster and D. virilis amylase genes were aligned based on the results of the Dot-matrix comparison. First, the sequence of the conserved clusters between the promoters of the D. melanogaster and D. virilis were aligned by the computer. It was found that two of these regions of the D. virilis amylase gene promoter contain the TATAA and CAAAT motifs

respectively. These motifs have previously been identified in the D. melanogaster amylase gene promoter (Boer and Hickey, 1986) and in other promoters of various eukaryotic genes (Nussinov, 1990). Then, taking these sequence motifs as reference points, the remainder of the promoter sequences were aligned visually. This visual alignment was facilitated by comparing the sequences of other glucose-repressible amylase promoters from other less diverged Drosophila species such as D. erecta and D. pseudoobscura (see Figure 23).

3.4.5 Glucose repression of amylase activity in Lepidoptera.

The promoter of the amylase glucose repressible genes contains conserved clusters between distantly related species in Drosophila, as shown in Figure 23. A question worth pursuing, related to the evolution of functionally significant DNA sequences, is whether the function and structure of the amylase gene promoter is conserved between more distant species, i.e between diptera and lepidoptera. I examined the glucose repression of the amylase activity in larvae of the lepidopteran Ostrinia nubilalis. The results of this experiment, see Figure 24, shows that this insect has at least two amylases, with different electrophoretic mobilities, which are highly responsive to glucose repression. Furthermore, it is shown that exogenous cAMP counteracts glucose repression reflecting similarities with the regulation of the amylase gene in Drosophila and with the catabolite repressible genetic systems in prokaryotes.

Figure 23: Sequence alignment of the promoters of glucose repressible amylase genes of distantly and closely related Drosophila species.

A region of the proximal D. melanogaster amylase gene promoter, up to 141 bp from the start codon, which is sufficient for glucose repression is aligned with the sequences of the proximal amylase gene promoters of D. erecta (Hickey et al., 1991), D. pseudoobscura (Brown et al., 1990) and with the glucose repressible amylase promoter of D. virilis. Sequences which are homologous with the D. melanogaster sequence are shadowed. The transcription start site, characterized in D. melanogaster by Boer and Hickey (1986), is shown by an arrow. Conserved CAAAT and TATAA motifs are underlined. Conserved identified elements I, II and III have been functionally characterized in D. melanogaster, see Figure 10.

Figure 24: Glucose repression of amylase activity in
Lepidoptera.

This figure shows the effect of dietary glucose on amylase activity in Ostrinia nubilalis larvae. This electrophoretic gel assay contains samples of homogenates from larvae treated as follows: a- larvae growing on a medium without glucose; b- larvae growing on a medium enriched with glucose (10%) and c- exogenous cAMP was added to the glucose-rich medium.

A

B

C



3.5 Identification of other glucose repressible gene-enzyme systems in Drosophila.

3.5.1 Effect of dietary glucose on various metabolic enzymes.

Experiments which are described in this section were aimed at identifying gene-enzyme systems, other than the amylase gene-enzyme system, which are regulated by glucose in D. melanogaster. Genetic systems, which are under the same control, can be used in comparative studies in order to identify common transcriptional elements. First, the glucose regulation of a series of key regulatory metabolic enzymes, such as hexokinases, pyruvate-kinase, fructokinase and 6-phosphogluconate dehydrogenase (PGD) was examined (results not shown). These experiments showed that only the activity of 6-PGD is significantly repressed by glucose in Drosophila larvae and adults. Figure 25 shows a summary of the results from such experiments, the 6-PGD and amylase (control) activity is assayed in third instar larvae and in adults (males and females) which have been grown on a medium supplemented with or without glucose. These results show that the activity of 6-PGD is repressed by dietary glucose in larvae and in adults (Figure 25, Panel B). Similarly, in an experiment which controls the various experimental treatments, the amylase activity is repressed by dietary glucose in the same larvae and adults (Figure 25, Panel A). In addition, results of this Figure show that cAMP has a negative effect on the activity of 6-PGD in comparison to the cAMP effect on activity of the amylase. These results indicate that 6-PGD can be

Figure 25: Effect of dietary glucose and exogenous cAMP on activity of the phosphogluconate dehydrogenase.

Amylase (control) and 6-phosphogluconate dehydrogenase activity in homogenates of equal protein content, from Drosophila melanogaster adult and larval populations, is shown. Electrophoretic native gel assays of amylase and 6-PGD activity are shown in Panel A and Panel B respectively. Enzymatic activity of three different populations was scored by a densitometre and is expressed in percentage of relative activity of the highest activity (i.e. 100 %).

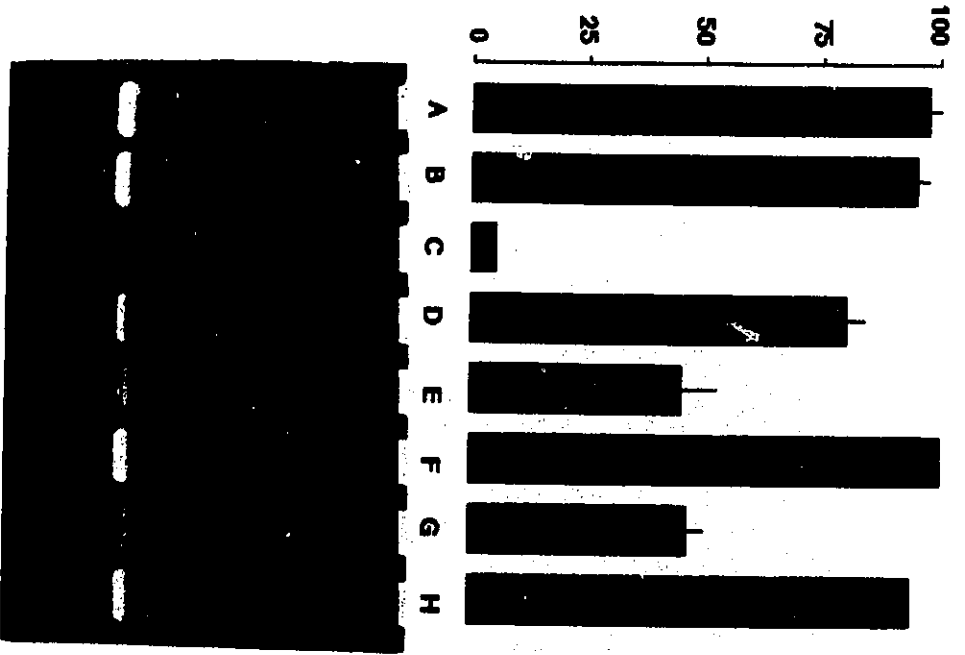
Larval populations were treated as follows:

- A - grown on a medium without glucose.
- B - cAMP was added on the glucose free medium.
- C - growth medium contained 10% glucose.
- D - cAMP was added on the glucose enriched medium.

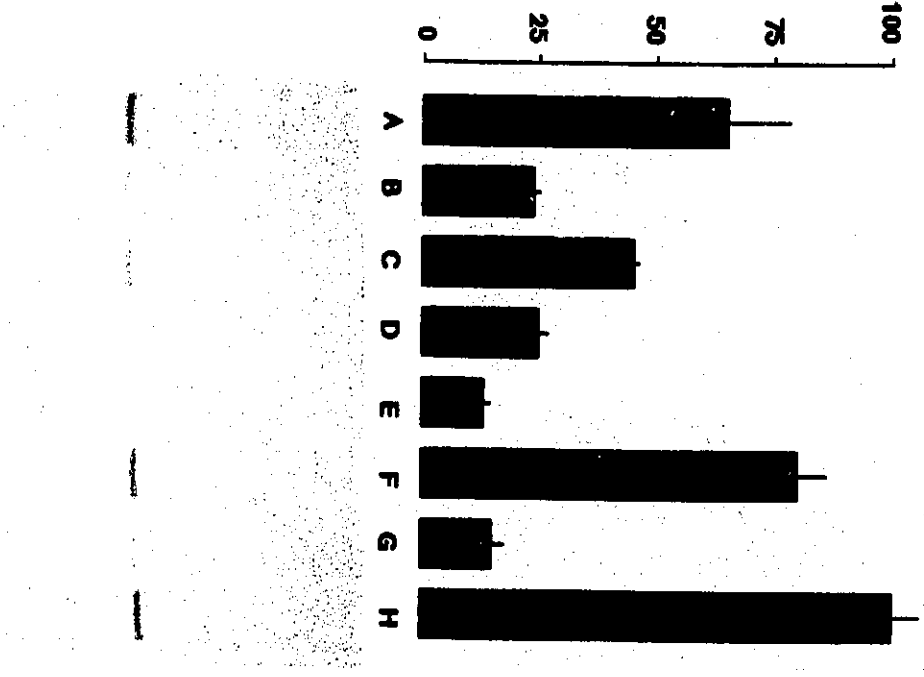
Adult populations were treated as following:

- E and G - glucose enriched medium of female and male populations respectively.
- F and H - no-glucose in growth medium of female and male populations respectively.

A.



B.



regarded as a putative candidate for comparative studies in order to identify glucose regulatory DNA elements. However, the present unavailability of the Drosophila 6-PGD gene makes such studies impossible at the DNA level.

3.5.2 Common cis-acting elements within promoters of glucose repressible genes.

The effect of glucose on expression of the maltase-like family of genes was examined. These genes have previously been isolated (Snyder and Davidson, 1983) and specific DNA probes were used in a Northern analysis (see Figure 26, panel A). RNA isolated from larvae grown on media with or without glucose, respectively, was analyzed. Results of this Northern analysis are shown in Panel B of Figure 26. It is shown that expression of the genes encoding the H and L maltase genes is glucose repressible. Glucose repression conditions were confirmed by probing with Drosophila amylase specific sequences. In addition, it is shown that the D maltase has a much less overall expression (under non-repressive conditions). Generally, all of the maltase genes were expressed less than the amylase gene. Therefore, autoradiogrammes of the maltase Northern analysis were exposed approximately four times longer (three days) in comparison to autoradiogram of the amylase Northern analysis.

This experiment shows that expression of maltase-like and amylase genes is repressed by glucose. Then, a comparative analysis between sequences of these genes was performed in order to

identify common DNA elements. Previously, a conserved sequence had been identified between the maltase-like genes (Snyder and Davidson, 1983). This conserved sequence, which is located 47 to 72 nucleotides upstream from the start codon of each maltase gene, is shown in Figure 27, panel A. A comparison between the maltase-like consensus and amylase upstream sequences shows that there is a similar element. However, this similar element is within the lower strand of the amylase gene, as shown in panel B. In addition, this conserved sequence is also present within the promoters of amylase genes of different Drosophila species and is designated as element-III in Figure 23.

Figure 26: Glucose repression of expression of the maltase gene family.

Panel A: The Drosophila genome contains three genes (H, D and L) which are members of the maltase gene family (Snyder and Davidson, 1983). The overlined regions I, II and III, spanning each of the respective genes, were used for probes in a Northern analysis shown below.

Panel B: This is a Northern analysis of the maltase-like genes in response to dietary glucose. Total RNA was extracted from third instar larvae grown in a medium with (+) or without (-) glucose. An assay of total RNA content is shown in the panel under the blots of the Northern analysis. RNA blots were hybridized with specific probes I, II and III (shown in panel A) which contain the coding regions of the H, D and L genes respectively. A control blot was hybridized with an amylase specific probe, A. All blots were hybridized with a probe which contains sequences of the Drosophila alcohol dehydrogenase (Adh) gene which is non-glucose repressible (internal control).

Figure 27: Identification of a conserved cis-acting element within various glucose repressible promoters in Drosophila.

Promoter sequences of the three D. melanogaster maltase-like genes contain a conserved DNA region as shown in Panel A. Results of this comparison analysis are taken from Snyder and Davidson (1983). Expression of these genes is repressed by glucose as shown in Figure 26. This conserved region contains a sequence core shown in a shadowed box, which is also found within a consensus sequence (shown as cluster-III in Figure 23) of glucose repressible promoters of the amylase gene of closely and distantly related Drosophila species, as shown in Panel B.

A.

MALTRASE -H: 5'-C-C-A-C--G-A-C-T-a-G-3'
MALTRASE -D: 5'-g-t-g-C--G-A-C-T-g-g-3'
MALTRASE -L: 5'-C-C-A-C--a-A-t-g-g-g-3'

B.

ANYLASE CONSENSUS: 5'-G-A-T-A-A-G-3'
ELEMENT-111 3'-C-T-A-T-T-C-5'

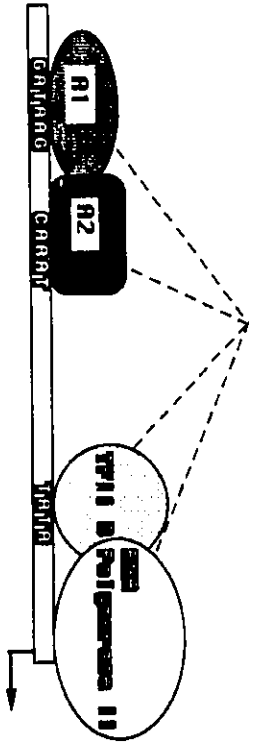
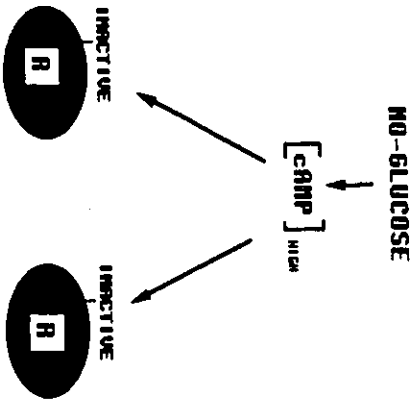
4. DISCUSSION

The results of this study demonstrate that the repression of Drosophila amylase activity by dietary glucose occurs at a transcriptional level. This conclusion is based on the observation that expression of the Amy/Adh hybrid construct, in which the entire transcribed region of the amylase gene was replaced by the Adh transcript, is still subject to glucose repression. The analysis of upstream deletions shows that functionally-important cis-acting elements are closely linked to the amylase structural gene. In fact, only a 109 bp region upstream of the transcriptional start site is sufficient for both high levels of in vivo expression and glucose repression. A detailed deletion analysis and site-directed mutagenesis of upstream sequences show that a 35 bp region, from -109 to -74 bp from the transcription start site, contains elements which are necessary for overall amylase activity. In addition, these experiments show that the TATA motif is significant for amylase expression. In vitro site-directed mutagenesis of various sites within the upstream regulatory region of the amylase gene, however, did not affect glucose responsiveness. Based on these results, repression of transcription initiation of the Drosophila amylase gene, in response to glucose, can be mediated by two possible mechanisms (see Figure 28). Both of these mechanisms take into account the redundancy of functional cis-acting elements. One mechanism involves a glucose related inactivation of activators, by protein-

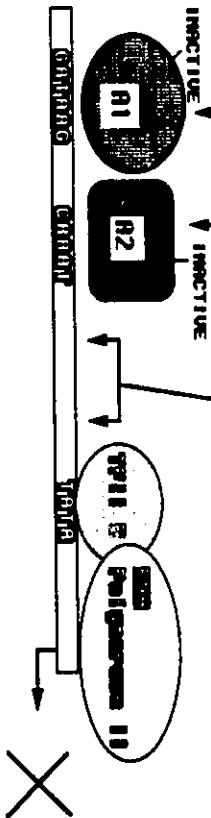
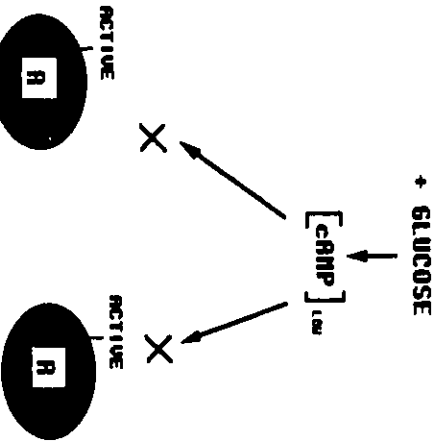
Figure 28: Models of glucose transcriptional repression
in *Drosophila*.

The panel A of this figure shows a schematic presentation of the minimum required DNA elements for transcription of the amylase gene. The TATA and CAAAT motifs were defined, by site-directed mutagenesis, to be necessary for amylase gene expression. The *Drosophila* general transcription factor TFIID has been shown to interact with the TATA motif and RNA polymerase II (Hoey et al., 1990). Panel B shows the glucose repression of amylase transcription initiation by two possible pathways designated as I. and II. respectively. Pathway I involves the concomitant inactivation of multiple activators, designated as A1 and A2, while pathway II shows the direct interaction of a repressor protein, designated as R, with multiple DNA elements.

A.



B.



protein interactions, which bind with multiple distinct upstream elements. The other mechanism indicates that a repressor protein may interact directly with multiple elements located within the DNA regulatory region of the amylase gene.

A number of questions can be raised from these results.

- 1) Do potential mechanisms which regulate transcription of the D. melanogaster amylase gene share any characteristics with any other known mechanisms of eukaryotic transcriptional repression ?
- 2) Are there any obvious structural similarities, i.e DNA cis-acting elements, which can reflect a functional conservation of the same phenomenon, i.e. glucose repression, between divergent species.
- 3) Can we determine the mode of evolution of this transcriptional mechanism by characterizing and comparing the DNA sequences which regulate glucose repression between related species?

The following sections present a discussion of the results of this thesis in a frame set by these questions.

4.1 Molecular aspects of transcriptional glucose repression in *Drosophila*.

In eukaryotes, transcriptional repression mechanisms can be mediated by a competition of proteins at common DNA binding sites, by protein-protein interactions and by distinct DNA binding of repressor proteins (see reviews in Renkawitz, 1990; Goodbourn, 1990; Levine and Manley, 1989). In some genetic systems, competition of transcription factors at common DNA binding sites can involve a CCAAT motif, which is commonly found within eukaryotic promoters (Nussinov, 1990). For example, activating factors which bind with this DNA motif, in vertebrates and in invertebrates, are displaced by other proteins under conditions which repress transcription (Superti-Furga et al., 1988; Barberis et al., 1987). A CAAAT element, present within the upstream of the amylase gene, shares a structural similarity with the CCAAT motif. In addition, this *Drosophila* element shares more similarities with a mammalian octameric motif, ATGCAAAT. This mammalian octamer is also the binding site of various proteins (Fletcher et al., 1987; Scheidereit et al., 1987) and is shown to mediate transcriptional repression in embryonal carcinoma cells (Lenardo et al., 1989). These structural similarities may reflect a common transcriptional mechanism. The results of the site-directed mutagenesis experiments, however, show that the *Drosophila* CAAAT element is not a repression controlling site; if any, this element is shown to be necessary for the activity of the amylase promoter.

Protein-protein interactions, between repressor and activator factors, may regulate transcriptional repression of the amylase gene. This type of interaction can prevent potential activator(s) from binding with upstream DNA sites (see Figure 28). A few examples are known where a protein-protein interaction prevents the binding of an activator protein. These examples include the inactivation of the yeast GAL4 activator by the GAL80 protein (Ma and Ptashne, 1987b), the interaction of the human transcription factor NF-kB with a cytoplasmic protein (Lenardo and Baltimore; 1989) and the regulation of Drosophila-development related transcription factors (Treacy et al., 1991). By a similar mechanism, the activator can be inactivated by the catalytic properties of another protein. This is the case of the inactivation of the yeast transcription factor ADR1 by a phosphorylation reaction which is suggested to be catalyzed by cyclic AMP-dependent protein kinase (Cherry et al., 1989). In other cases, however, a similar phosphorylation reaction appears to activate transcription. These cases include the mammalian transcription factor CREB (Yamamoto et al., 1988; Lee et al., 1990) and the yeast activator GAL4 (Long et al., 1991). It is possible, that a glucose mediated binding of activators with elements of the Drosophila amylase promoter is dependent on a cAMP -phosphorylation pathway. If glucose repression is regulated by this mechanism, however, then the potential activator(s) has to interact with multiple elements, since a single element was not found to be associated with both loss of activity and glucose responsiveness. It is also possible

that a single protein, the activity of which is regulated by glucose, mediates transcriptional repression by interacting simultaneously with multiple transcription factors. Recently, it has been shown that the eukaryotic transcriptional apparatus can be activated by such mediator proteins (Flanagan et al., 1991).

Eukaryotic transcriptional repression is also known to be mediated by a direct interaction of a repressor with distinct upstream DNA sites which are located distantly or closely to the structural gene. Distal regulatory cis-acting elements, which inhibit transcription, have been characterized in yeast genetic systems (Keleher et al., 1988; Hofmann et al., 1989) and in a number of higher eukaryotic genetic systems. Such elements, or silencers, appear to share structural similarities between some eukaryotic regulatory DNA sequences (Farrel et al., 1990). The high level of overall expression of a number of Drosophila genes is dependent on upstream distal elements, while the differential expression of these genes, i.e. developmental and tissue specific, is dependent on elements proximal to the transcription start (Martin et al., 1989; Mismar and Rubin, 1987). In other cases, Drosophila distal elements can mediate differential gene expression (Jongens et al., 1988; Corbin and Maniatis, 1989; Johnson et al., 1989, Ip et al., 1991). In this respect, the regulation of the Drosophila amylase gene is different. All sequences necessary for high amylase activity and glucose repression are located close to the transcriptional start. Therefore, it is possible that the activating sequences of the amylase gene, located between -109

and -74 bp from the transcription start site, mediate complex transcriptional processes, since this system is also under developmental and tissue specific control (Doane et al., 1983). The regulation of different genes, in temporal and spatial manner, by a short DNA region close to the transcription start has been identified in Drosophila genetic systems before. For example, a proximal short DNA region mediates developmental and/or tissue specific expression of the Sgs-3 gene (Martin et al., 1989), the opsin gene (ninaE) (Mismer and Rubin, 1987) and the dopa decarboxylase gene (Ddc) (Scholnick et al., 1986; Bray et al., 1988).

Negative elements proximal to the transcription start have not been characterized in eukaryotes (reviews in Renkawitz, 1990; Goodbourn, 1990, Levine and Manley, 1989). The transcription of the amylase gene may be repressed by a direct interaction of such elements with a repressor protein. In this case, the amylase promoter should have multiple redundant negative elements in order to explain the site-directed mutagenesis results in this thesis (see Figure 28). Specifically, if a single distinct element was responsible for glucose repression then the destruction of its binding capacity by in vitro mutagenesis should relieve, at least partially, glucose repression of amylase gene expression. Finally, if there is a redundancy in repressor-DNA interactions then the regulatory DNA region of the Drosophila amylase gene should contain multiple elements with structural similarities. A high sequence similarity between elements within the upstream of the amylase gene

is not obvious. However, there are short DNA stretches rich in GC content between the CAAAT and TATA motifs. Elements rich in GC content have previously been found to be present in higher eukaryotic promoters and to be involved with transcriptional repression (Rajavashisth et al., 1989).

4.2 Macroevolutionary aspects of glucose transcriptional repression.

The demonstration that the Drosophila amylase gene is regulated by glucose, at a transcriptional level, means that this metazoan system is similar to microbial glucose repression, in that a significant component of the regulation is at the level of transcriptional initiation. Therefore, negative elements in the Drosophila amylase promoter may share structural similarities with the known prokaryotic operator sequences. First, however, the generality of the operator structure within prokaryotes has to be addressed in order to have macroevolutionary comparisons with some significance. So far, a catabolite repression regulatory sequence, characterized in the promoter of the α -amylase gene in Bacillus subtilis, is the only element with operator-like properties in prokaryotes other than gram-negative bacteria. This sequence is shown to have a two fold symmetry which is conserved within the promoters of other catabolite-repressed genes in B. subtilis (Weickert and Chambliss, 1990). Furthermore, a consensus between the Bacillus amylase operator sequence and the Escherichia gal and lac operator sequence has been identified (Nicholson et al., 1987). This consensus appears to be the site of prokaryotic repressors which share an α -helix-turn- α -helix DNA binding motif (Henkin et al, 1991). From these comparisons, however, it is obvious that there is a reduced similarity between the Bacillus and Escherichia operator sequences (Nicholson et al., 1987). At present, the

evolutionary significance of these few similarities between operator elements can not be assessed. This is because the rules governing protein-DNA recognition are not known (Lehming et al., 1987); furthermore, the forces which mediate a high frequency of nucleotide substitutions within an element without affecting function in long laps of evolutionary time have not been determined (Shabalina et al., 1991).

The Drosophila glucose repressible amylase promoter does not have any obvious sequence similarities with the sequence of the prokaryotic operator consensus, i.e. T N T N A N . | . N T N A N A (Nicholson et al., 1987). In addition, upstream sequences of the Drosophila amylase gene do not share any similarity with regulatory sequences of the mammalian counterpart, i.e. pancreatic amylase A2.2 (Keller et al., 1990). In this latter case, the lack of identifying similar elements may reflect a divergence in the regulation of the same gene among higher eukaryotes. It should be noted, however, that a 15 bp element of a mammalian gene, i.e. phosphoenol pyruvate carboxylokinase (PEPCK), which mediates repression of transcription in response to insulin (O'Brien et al., 1990) shares certain similarities with the insulin responsive element of the mammalian pancreatic amylase gene. There are cases, however, where comparisons of regulatory sequences between divergent species can provide the identification of a conserved motif. For example, it has been shown that vertebrate and invertebrate hormone responsive elements have evolved from common repeated motifs. Furthermore, it has been suggested that the

spacing between these motifs determines the differences in the specificity of these elements (Martinez et al., 1991). Similarly, the spacing between the symmetric DNA motifs of certain eukaryotic activator sequences appears to be the only requirement which determines the recognition of phylogenetically divergent factors (Sellers et al., 1990).

Elements with a symmetrical structure have been commonly found within upstream activating sequences (UAS) of the GAL genes (Bajawa et al., 1988). Similarly, a symmetrical dyad sequence motif is necessary for transcriptional activation of the yeast ADH2 gene (Eisen et al., 1988; Thukral et al., 1989). The transcription factors GAL4 and ADR1 bind with the symmetrical activating sequences of the GAL1 and ADH2 genes, respectively. These factors share a common DNA binding structural motif, i.e. the zinc finger, (Johnston and Dover, 1987; Blumberg et al., 1988). The zinc finger DNA binding motif is also conserved in other eukaryotic transcription factors including Drosophila (Latchman, 1990; Schuh et al., 1986). The GAL4 factor, apart from its evolutionary conserved DNA binding motif, contains activating sequences which are functional in divergent heterologous systems (Ptashne and Gann, 1990).

It is difficult to speculate whether the potential Drosophila activating transcription factors of the amylase gene have similar structural characteristics with other eukaryotic activators. It is evident, that the amylase promoter does not contain any obvious dyad symmetrical motifs which are the binding

sites of the zinc finger motif of eukaryotic transcriptional activators. Activating upstream sequences of the Drosophila amylase gene, however, may have similar functional characteristics with the yeast GAL1 upstream activating sequences. That is, these sequences mediate both repression and activation of gene expression (Flick and Johnston, 1990).

4.2.1 Evolutionary aspects of cAMP transcriptional mechanisms.

A potential transcriptional activator of the Drosophila amylase gene may have a similar mode of action with the prokaryotic catabolite activator protein (or cAMP receptor protein). This speculation is based on a number of experiments, presented in this thesis, which show that cAMP mediates glucose repression in Drosophila. In E. coli, and in other microorganisms, the role of cAMP was established by testing the following experimental criteria: i) whether exogenous cAMP elicits the same effect, i.e. induction of glucose repressed enzymatic activity, as the predicted effect of endogenous cAMP, ii) whether exogenous cAMP effect is specific and iii) whether endogenous cAMP levels are controlled by extracellular glucose.

i) In E. coli, exogenous cAMP was shown to counteract glucose repression of activity of a number of catabolic enzymes (Perlman and Pastan 1968; DeCrombrughe et al. 1969). Results of similar studies, in the lower eukaryote of S. cerevisiae, appear to be controversial. Exogenous cAMP did not relieve the glucose repressed activity of galactokinase (Matsumoto et al., 1982),

while cAMP positively affected glucose repression of the synthesis of a number of mitochondrial enzymes (Mahler and Lin, 1978). Similar experiments in this thesis show clearly that exogenous cAMP relieves glucose repression of Drosophila amylase activity.

ii) Only exogenous cAMP, among its adenosine analogues, was found to counteract the glucose effect on activity of β -galactosidase in E. coli (DeCrombrugghe et al, 1969). However, Fang and Butow (1970) found that the cAMP effect, which is related to a relief of glucose repression in mitochondrial function of S. cerevisiae, was not specific in comparison to various cAMP analogues. Similar experiments, performed in this study, show that the cAMP effect is specific. Furthermore, it was found that cGMP has a similar effect (results not shown). This result may reflect the similarities exhibited in certain regions of the chemical structure of cGMP and cAMP or that these compounds regulate functions of the same physiological importance in fruit fly. In bacteria, however, the concentration of intracellular cGMP appears to be dependent on the availability to dietary glucose in a manner opposite to that of cAMP (Bernlohr et al., 1974). In vitro studies confirmed that cGMP opposes the action of cAMP with respect to the expression of glucose sensitive genes (review in Goldberg et al., 1973). cGMP was found to inhibit the binding of the CAP with the lac operon (Riggs et al., 1971). However, cGMP was found to antagonize the complex of catabolite activator protein (CAP) and cAMP, and the cAMP stimulation of β -galactosidase synthesis (Zubay et. al., 1970; Emmer et al, 1970). Also, Nisley et. al. (1972)

showed that cGMP does not oppose the binding of CAP with DNA, in various organisms, rather it competes with cAMP for binding with CAP. In contrast, glucose was shown to reduce the cGMP level concomitant to the cAMP level under aerobic or anaerobic conditions in S. cerevisiae (Eckstein, 1988). Therefore, the interaction of glucose and cGMP, with respect to enzymatic activity, appears to be the same in both yeast and Drosophila.

iii) Finally, the effect of dietary glucose on endogenous cAMP levels was examined. Previously, it was found that the intracellular level of cAMP is lower in E. coli cells grown in a medium containing glucose (Mackman and Sutherland, 1965). Furthermore, this correlation of endogenous cAMP and glucose was found to reflect repression of the synthesis of various catabolic enzymes (Epstein et al., 1975; Pastan and Perlman, 1970; Buttner, et al, 1973). Similar results were found in certain yeasts such as in Schizosaccharomyces pombe (Schlanderer and Dellweg, 1974), in S. carlsbergensis (Van Wijk and Konijn, 1971) and in S. fragilis (Sy and Richter, 1972). In contrast, Eraso and Gancedo (1984) did not find a relationship between low cAMP levels and catabolite repression of the activity of a number of enzymes in S. cerevisiae. A number of similar experiments were done in order to determine the significance of a dietary glucose and endogenous cAMP interaction in repression of amylase activity in D. melanogaster larvae. However, the results of these experiments (which are not presented) were not consistent. This inconsistency may have been due to cAMP degradation during assaying, an interference of the total larval

cAMP pool with the level of cAMP in cells which respond to glucose repression, and due to an insensitivity of the assay used.

In summary, the cAMP-related results presented in this thesis satisfy clearly two of three criteria mentioned above. Specifically, exogenous cAMP relieved glucose repression of the Drosophila amylase activity and this cAMP effect is specific in comparison to various cAMP analogues. Furthermore, it is shown that the cAMP effect occurs at the level of mRNA abundance and is mediated by sequences located close to the coding region of the amylase gene. These latter results indicate that expression of the Drosophila amylase gene is regulated by cAMP responsive cis-acting elements and/or at the stability of amylase mRNA abundance. Both of these cAMP effects have been documented to regulate higher eukaryotic gene expression (see review in Roesler et al., 1988; Hod and Hanson, 1988).

The molecular link between prokaryotic and eukaryotic cAMP transcriptional mechanisms is still unknown (Roesler et al., 1988). Several mammalian cAMP regulated genes have been found to contain upstream regions which are homologous with the consensus of the CAP (catabolite activator protein) DNA binding site of the E. coli catabolite sensitive genes (Tsakada et al., 1987). This similarity, at the DNA level, has been shown to confer the same functional cAMP induction between mammals and protozoa. For example, the E. coli CAP can bind with the mammalian CRE (cAMP responsive element) in order to induce transcription of a mammalian gene. Similarly, a mammalian transcription factor, i.e. ATF, can

bind with the E. coli CAP specific DNA sites (Lin and Green, 1989). In addition, the mammalian CRE has been found to activate transcription in yeast and to bind with a yeast factor which resembles that of the mammalian ATF (Jones and Jones, 1989).

The evolutionary relationship between various transcription factors which bind with CRE is still unclear. Between some mammalian CRE-binding (CREB) factors appears to be a high overall sequence similarity, while in other cases only a minimum structural conservation of the leucine zipper DNA binding motif is identified. The same structural motif has been also identified within CRE-binding proteins in plants (Katagiri et al., 1989; Tabata et al., 1989). The evolutionary link of cAMP related transcription factors between mammals and microorganisms, however, is controversial. It has been proposed that the regulatory subunit of the eukaryotic cAMP-dependent protein kinase (cAdPK) protein has the same function as the prokaryotic catabolite activator protein (CAP) (Nagamine and Reich, 1985). This speculation has been supported by the structural similarities between the cAMP binding sites of the regulatory subunit of cAdPK of eukaryotes and the cAMP binding sites of the CAP of E. coli (Weber, et al., 1987). In addition, the catalytic subunit of cAdPK is directly associated with transcriptional active chromatin (Sikorska et al., 1988), and binds with oligodeoxyribonucleotides which contain the CRE (Wu and Wang, 1989). Other experiments, however, show that the catalytic subunit of the cAdPK is the only component of the cAdPK holoenzyme which is responsible for stimulation, by activating CRE binding

factors, of gene expression in higher eukaryotes (Riabowol, 1988; Montminy and Bilezikjian, 1987; Grove, et al., 1987; Nigg, et al., 1988; Mellon et al., 1989). Also, the catalytic core of the cAdPK is shown to be conserved among different types of eukaryotic cells (Taylor , 1989; Toda et al., 1987). In yeast, however, this regulatory protein has been associated to have an opposite effect on transcription. Specifically , it is shown to inactivate transcriptional activators of environmentally regulated genes (Cherry, et al., 1989).

My results show that potential cAMP related cis-acting DNA elements can mediate transcription of the Drosophila amylase gene. Characterization of the regulatory proteins which interact with such elements will be of importance to trace the evolutionary relationship of fruit fly, mammalian and bacterial cAMP related transcription factors. In a series of experiments, which are not shown in this thesis, I tried to isolate the Drosophila homologue of the rat gene encoding CRE-binding protein. My experiments indicated that the gene encoding the rat CREB does not have any significant similarity with genomic Drosophila sequences based on species cross-hybridization experiments. The results of these experiments may reflect the high structural divergence of CREB related proteins, which has been already encountered, within and between certain species (Habener, 1990).

4.3 Evolutionary aspects of glucose transcriptional repression in *Drosophila*

In this thesis, some evolutionary aspects of the regulatory mechanism of glucose transcriptional repression were examined in *Drosophila*. First, whether the variation in the degree of glucose repression of different amylase genotypes is due to intraspecific changes in cis-acting regulatory sequences was examined in *D. melanogaster*. Also, the presence of evolutionarily conserved cis-acting elements between various glucose repressible genes and the amylase gene was examined in the same species. Secondly, whether the long range evolutionary changes in sequences of amylase promoters affect transcriptional function was assessed in distantly related *Drosophila* species.

4.3.1 Intraspecific aspects of cis-acting regulatory elements.

The expression of co-injected proximal (*Amy*¹) and distal (*Amy*⁶) amylase genes in the common *Amy*^{null} genetic background indicates that there is variation in some natural populations for the degree of glucose repression. It is shown that this variation is due to genotype specific upstream sequences. It is also shown, by a series of experiments where the function of fused proximal/distal promoter sequences was examined, that the absence of a repressor binding sequence within the upstream of the distal gene is not what accounts for the low response to glucose. It is found, however, that the sequence content and the location of DNA

elements, which are necessary for overall promoter activity, are different between the distal and the proximal amylase genes. An evolution of such novel activating cis-acting sequences points to two basic mechanisms which can explain the low glucose response of expression of the distal amylase gene. In one mechanism, the distal specific activating element recognizes cell-specific trans activator factors which are not sensitive to glucose repression. In the other possible scenario, both proximal and distal upstream sequences recognise the same cell specific factors but the further upstream location of the distal activating region requires a different structural rearrangement, i.e. DNA looping, for an interaction of the activator with the basic transcriptional apparatus, thus this conformational requirement prevents the activator from inactivation. Therefore, one mechanism correlates the evolution of a molecular interaction between cis-acting elements and trans-acting factors while the other mechanism involves a change in the structural organization of the same cis-acting elements and trans-acting factors within a species.

Common specific transcriptional components, which are required for regulation of a network of genes of similar biological functions within a species, are not well-known. It has been proposed, however, that a common DNA element can mediate the expression of a network of genes which are involved in energy metabolism in mammals (McKnight et al., 1989). In E. coli, such a proposed relationship has been demonstrated by the interaction of

the cAMP binding protein with conserved elements of various catabolic genes (Busby, 1986). I aimed at identifying conserved elements within promoters of various glucose repressible genetic systems, such as the amylase and the maltase genetic systems, in D. melanogaster. Expression of the maltase-like family of genes was shown to be repressed by glucose. The only conserved cluster between the maltase-like genes was found to contain a core element which is also conserved in the amylase gene promoter of closely and distantly related Drosophila species. This conserved element, designated element III, was shown to be significant in the expression of the amylase gene (Figures 8 and 10). Whether this conserved element interacts with a common trans-regulator, however, remains to be demonstrated.

4.3.2 Interspecific aspects of cis-acting regulatory elements.

In this work, I addressed the question of whether glucose repression of a specific gene-enzyme system, i.e. amylase, in D. melanogaster also occurs in distantly related species. Second, whether the conservation of glucose repression of amylase activity reflects a conservation at the transcriptional level.

Previously, it was shown that glucose represses expression of the amylase gene in D. melanogaster and in closely related species of the D. melanogaster species complex. (Payant et al., 1988). In this work, the effect of glucose on amylase activity of D. virilis, which is a distant relative of D. melanogaster, was examined. It is shown that glucose represses the activity of the D.

virilis amylase to the same extent as in the previously characterized D. melanogaster gene-enzyme system (Benkel and Hickey, 1986, 1987). Furthermore, it is shown that exogenous CAMP can alleviate this repression, as in the case of the well characterized prokaryotic glucose repressible genes (Danchin and Ullman, 1987). The evolutionary conservation of glucose repression on amylase activity, in the two distantly species of D. melanogaster and D. virilis, was examined at the level of transcription by using the embryo transformation assay. It is shown that the D. virilis amylase gene, when it is introduced into D. melanogaster amylase-null embryos, is transcribed and this transcription is repressed by glucose. This result demonstrates that transcription of the amylase gene, for expression and for repression by glucose, is functionally conserved. In these experiments, however, the overall activity of the transiently expressed heterologous genes in transformed larvae was found to be much less than the activity of the homologous expressed gene in D. melanogaster or in D. virilis (results not shown). Whether this discrepancy in activity is due to a partial function of the heterologous promoter was further examined. It was shown that the D. virilis promoter can fully express and glucose regulate the expression of the D. melanogaster gene when the hybrid gene is transformed into D. melanogaster amylase-null embryos (see Figure 22, Panel B). This indicates that the transcriptional interactions between promoter and transcriptional factors are highly conserved between these two species. In similar studies, the promoters of

genes with an evolutionarily conserved function have been found to regulate gene expression not only in different Drosophila species (Bray and Hirsh, 1986; Moses et al, 1990) but also in species which have an evolutionary distance of approximately 250 million years (Mitsialis et al., 1989; Peek et al., 1990). Recently, it was shown that within this range of evolutionary time a transcriptional mechanism can be conserved even when its biological function diverges (Bello and Couble, 1990).

I have shown that repression of amylase gene expression by glucose is controlled by a small region of the promoter, 141 bp proximal from the start codon or 109 bp from the transcription start, in D. melanogaster. Similarly, I have shown that only 330 bp of the 5' flanking region the D. virilis amylase gene is sufficient for glucose repression in D. melanogaster embryos. Therefore, it is possible that putative regulatory cis-acting elements can be identified by sequence comparison of the glucose repressible amylase promoters in Drosophila.

A DNA sequence comparison between the distantly related species of D. melanogaster and D. virilis indicates that the 5' flanking region of the amylase gene diverges much more than the coding region. The degree of divergence is so great that no significant sequence similarities can be detected by computer analysis even when the stringency of the comparison is reduced. In addition, sequences of glucose repressible amylase gene promoters were aligned from species which are evolutionarily closer to D. melanogaster than is D. virilis, in order to obtain a more

significant comparison. The Drosophila species used for this comparison have a wide range of phylogenetic relationships, (Throckmorton, 1975). For instance, D. virilis belongs to the subgenus of Drosophila which separated around 40 million years from the subgenus of Sophophora. The other three species, D. melanogaster, D. erecta and D. pseudoobscura are all members of the Sophospora subgenus; where D. pseudoobscura belongs to the obscura group separated from the melanogaster group around 30 million years. D. melanogaster and D. erecta are sibling species belonging to the melanogaster group and it is estimated that they separated around 15 million years ago. The estimated evolutionary divergences of these species are in agreement with the level of sequence divergence of the coding region of the alpha amylase gene. Specifically, it is shown that there is approximately 4% divergence between D. erecta and D. melanogaster (Hickey et al., 1991), 13% between D. pseudoobscura and D. melanogaster (Brown et al., 1990) and 20% between D. virilis and D. melanogaster (Abukashawa, Ph.D. thesis, 1990).

The sequence comparison of the Drosophila amylase gene promoters shows that there is relatively high sequence conservation within the 5' untranslated region of the transcript. This region is defined as untranslated transcript based on the previous characterization of the transcriptional start motif and the start codon of the amylase gene in D. melanogaster (Boer and Hickey, 1986). There are also conserved sequence clusters which contain the TATAA and CAAT motifs, shown as regions I and III in Figure 23.

These motifs are commonly found in the promoters of eukaryotic genes (Nussinov, 1990). Similar conserved promoter regions, which contain the TATAA motif and the untranslated transcript, have been also found by similar comparisons of the genes encoding the hs82 protein (Blackman and Meselson, 1986) and of the chorion genes s16 and s19 (Fenerjian et al., 1989) between distant Drosophila species. In other cases, for example the s36 chorion genes in D. melanogaster and D. virilis, there is high degree of divergence even in the region of the untranslated transcript (Konsolaki et al., 1990). From these comparative studies, however, a common pattern emerges, i.e., that the promoter diverges to a much higher degree than the coding region but that it retains very short regions of sequence conservation.

This sequence comparative analysis, taken together with the functional analysis of the upstream sequences of the D. melanogaster amylase gene, shows that conserved elements are significant for transcriptional activation. It is evident from these studies that the identification of the trans-acting regulatory proteins, which interact with such conserved cis-acting elements of the Drosophila amylase gene, is necessary in order to elucidate further the molecular and evolutionary aspects of glucose transcriptional repression.

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APPENDIX-I: REAGENTS FOR PROTEIN ASSAYS**Lowry-assay**

To make reagent C add 0.5 ml of reagent B.1 to 0.5 ml of reagent B.2, then add 49 ml of reagent A.

Reagent A: 5gr of Na_2CO_3 , 1gr of NaOH in 250 ml of dd. H_2O .

Reagent B.1: 0.25gr of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 25 ml of dd. H_2O .

Reagent B.2: 0.5gr of NaK.Tartrate in 25 ml of dist. H_2O .

Alpha-amylase assays

DNSA reagent: Add 1 gr of 3,5-dinitrosalicylic acid in 20 ml of 2N of NaOH and 50 ml of dd. H_2O . Then, add 30 gr of KNa.Tartrate (Rochelle salt) and make up to 100 ml with dd. H_2O .

Substrate solution: 2% starch in 0.1 M of Tris.HCl buffer, pH 7.3, and 5-10 mM of CaCl_2 were boiled for 5 min. The solution was cooled to experimental temperature before use.

5%-acrylamide gel: mix 20 ml of 10% acrylamide in 0.1 M of Tris.Borate, pH 9.0 / 10 ml of 0.2 M Tris.Borate, pH 9.0 / 10 ml of dd. H_2O / 0.4 ml of 10% Ammonium persulfate and lastly 50 μl of TEMED.

Electrophoresis running buffer: 0.1 M Tris.Borate, pH 9.0. Made as a 10x stock.

Staining solution: I₂ : KI : H_2O = 1(gr) : 2(gr) : 20(ml) .

10x-loading buffer: 1 M Tris.Borate, pH 9.0; 50% sucrose; a "pinch" of bromophenol blue.

6-Phosphogluconate dehydrogenase assay

Gel buffer: 21 mM Tris

20 mM Boric acid

1 mM EDTA

The pH was adjusted to 8.5

Running buffer: 0.21 M Tris

0.15 M Boric acid

5 mM EDTA

The pH is adjusted to 8.5

Staining solution : 1.0 mM 6-Phosphogluconate

5 mM MgCl₂

0.13 mM NADP

0.12 mM tetrazolium

0.13 mM phenazine methosulfate

0.05 M Tris pH 8.0

Alcohol dehydrogenase assay

Staining solution (for 100ml):

25 mg of 3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyl-2H-tetrazolium bromide

30 mg of NAD

2 mg of phenazine methosulfate

0.75 ml of isopropanol

0.1 M of Tris.HCl, pH 8.5

APPENDIX-II: PROCEDURES AND REAGENTS FOR NUCLEIC ACID ASSAYS**RNA isolation**

Total RNA was extracted from maximum of few hundred larvae or adults by the Guanidine Hydrochloride method of Cox (1969):

1. Larvae were homogenized in 8 vol. (500 μ l) of 8M Guanidine HCl, 0.01M EDTA, pH 7.0 *. Homogenate was filtered through an autoclaved nylon mesh (62 m) into a polypropylene sterile tube. The final volume of the filtrate was 1-2ml after rinsing. Alternatively, the homogenate was cleared by few minutes of micro-centrifugation.
2. 0.5 Volume of EtOH was added, mixed and stored in -20 °C for 1hr or in -70 °C for 15 min. Then the sample was centrifuged at 16000g for 10 min. at 4 °C. The pellet was resuspended in 2.0 ml of 4M Guanidine HCl, 0.01M EDTA, pH 7.0. Then, 0.5 volume of EtOH was added, mixed and stored in -20 °C for 1hr or -70 °C for 15 min and then, it was centrifuged (16,000g at 4 °C).
3. The above step was repeated 5 times.
4. Finally, the pellet was resuspended in 6 vol. (600 μ l) of sterile water, incubated at 56 °C for 10 min., spun at 16000g and 10 μ l of the soluble sample were assayed for RNA content and purity. One unit of absorbance at 260nm = 50 μ g of RNA \ ml. Pure preparation of RNA has a ratio of absorbance at 260nm \ absorbance at 280nm = 2.0.
5. The last step was repeated by resuspending the pellet with 8 vol. (800 μ l) of sterile water. The supernatant or the water

soluble phase of the purest and richest, in RNA content, fraction was divided in eppendorf tubes and it was precipitated with 0.1M of Ammonium Acetate and 2.2 volumes of EtOH. The final concentration of the RNA sample was brought to $2.5 \mu\text{g} \backslash \mu\text{l}$. The quantity and quality of the extracted RNA was checked by electrophoresis in a denaturing agarose gel stained by EtBr.

- * 38.2 gr of Guanidine.HCl (M.W.=95.53 gr/mole) is made up to 50 ml of sterile H₂O and 0.5 ml of 0.5 M of EDTA is added. The pH is adjusted to 7.0.

Reagents for RNA analysis (Northern and Dot-blot)

RNA loading buffer: 50%-formamide / 6%-formaldehyde / 1x MOPS-RNA buffer / 10% glycerol.

To make 10 ml of loading buffer mix : 5 ml of formamide / 0.6 ml of formaldehyde / 1 ml of 10x MOPS-RNA buffer / 1 ml of glycerol (which is omitted for dot-blot hybridizations) and 2.4 ml of H₂O.

10x MOPS-RNA buffer: 0.2 M of MOPS, pH 7.0 / 50 mM NaAct. / 10 mM EDTA.

To make 500 ml of 10x MOPS- RNA buffer mix: 3.4 gr of NaAct (M.W. 136.08) / 23.13 gr of MOPS (M.W. 231.3) / 10 ml of 0.5 M of EDTA. Adjust to pH 7 with acetic acid and autoclave.

Formaldehyde gel: To make 200 ml of 1.2% agarose gel is mixed: 2.4 gr of dissolved agarose in 148 ml of H₂O with 20 ml of 10xMOPS-RNA buffer and 32 ml of formaldehyde.

RNA running buffer: 80 ml of formaldehyde per liter of 1x MOPS-RNA

Hybridization solution: 5x Denhardt's / 5x SSC / 50 mM NaPhosphate, pH 6.5 / 0.1% SDS / 500 µg per ml non-homologous DNA / 50% formamide.

To make 100 ml of hybridization solution mix: 10 ml of 50x Denhardt's / 25 ml of 20x SSC / 5 ml of 1 M of NaPhosphate, pH 6.5 / 1 ml of 10% SDS / 50 ml of formamide and 9 ml of H₂O.

20x SSC: 3M NaCl, 0.3M NaCitrate, pH 7.

50x Denhardt's: 1% Ficoll (M.W. 400,000) / 1% Polyvinyl-pyrrolidone (M.W. 360,000) / 1% Bovine Serum Albumin.

DNA plasmid isolation (small scale).

1. Incubate bacteria in 1.5 ml of 2xYT medium overnight at 37 °C.
2. Pellet bacteria in an eppendorf tube by microcentrifuging for 5 min.
3. Resuspend pellet in 150 μ l of lysis buffer. Mix and incubate for 5-10 min in ice.
4. Add 300 μ l of 0.2 N of NaOH / 1% SDS. Mix and keep on ice for 10-15 min.
5. Add 225 μ l of KAct (pH 4.8) and mix (optional, keep in ice for 10 min).
6. Spin in a microcentrifuge for 10 min. Place supernatant in clean tubes and phenol/chloroform once, spin for 5 min and keep 500 μ l of the supernatant in a clean eppendorf tube.
7. This step is optional: Add supernatant in clean tube and add 2 vol. of EtOH. Let it stand at room temperature for 5 min. Spin in a microcentrifuge for 10 min. Resuspend pellet in 500 μ l of TE.
8. Add 20 μ g/ml of RNase. Incubate at 37 C for 1 hr. Phenol/chloroform and precipitate the supernatant.

DNA plasmid isolation for transgenic assays

1. Incubate bacteria in 150 ml of LB medium overnight at 37 °C.
2. Centrifuge bacterial culture at 5,000 g, 4 °C for 15 min.
3. Resuspend pellet in 6 ml of lysis buffer. Transfer in a polypropylene tube (40 ml) and incubate in ice water for 20 min.
4. Add 12 ml of freshly prepared 0.2N-NaOH/1%-SDS, mix gently without vortexing, incubate in ice water for 10 min.
5. Add 7.5 ml of 3M of NaAcetate, pH 4.6, mix gently without vortexing, incubate in ice water for 20 min.
6. Centrifuge at 10,000 g (15,000 rpm) for 30 min (or until the supernatant is clear). Divide supernatant into two corex tubes (appx. 12 ml each).
7. Add 50 μ l of 10mg/ml of RNAase A into the supernatant, mix and incubate at 37 °C for 1 hr.
8. Add equal volume of phenol:chloroform (1:1) in each tube, mix thoroughly by vortexing for 5 min, spin at 10,000 g for 10 min at room temperature.
9. Transfer upper phase in a clean tube and repeat step-8.
10. Place 8 ml of the supernatant into a clean corex tube, add 2 volumes (16 ml) of 100% EtOH, mix and incubate at -20 °C for 30 min.
11. Centrifuge, at 8,000 rpm at 4 °C for 30 min, resuspend the pellet from each tube with 500 μ l of dd water, add 100 μ l of 4 M of NaCl and 725 μ l of 13% PEG, mix and incubate in ice water for 1 hr.

11. Spin in a microcentrifuge at 4 °C for 15 min.
12. Discard supernatant and rinse pellet twice with 70% EtOH (spinning as in step-11 every time).
13. Dry pellet and resuspend with dd water to a desired final concentration.

Lysis buffer50 ml

25 mM Tris-Hcl, pH 7.5;	1.25 ml of 1 M.
10 mM EDTA;	1.0 ml of 0.5 M.
15 % Sucrose;	7.5 gr sucrose.
2 mg/ml lysozyme.	

50 ml of 0.2 N of NaOH / 1%-SDS

1 ml of 10 N of NaOH,
 5 ml of 10% SDS
 44 ml of dd water.

50 ml of 3M NaAct, pH 4.6

30 ml of 5 M of NaAct.
 5.75 ml of Acetic acid
 14.25 ml of dd water.

Preparation of single stranded DNA**I. DNA amplification**

1. Grow a colony in 2 ml of 2xYT + ampicillin (50 $\mu\text{g/ml}$) at the stationary phase.
2. Transfer 100 μl of the culture in a flask containing 20 ml of 2 x YT + ampicillin. Shake for 1/2 hr.
3. Add 20 μl of helper phage M13 K (appx. 10^6 pfu/ μl).
4. Add 15 μl kanamycin final concentration 50 $\mu\text{g/ml}$.
Shake overnight (appx. 14 hr's).

II. DNA purification

1. Spin o.n. culture at 6000 rpm, at 4 C, for 10 min in corex tubes.
2. Transfer supernatant in clean corex tubes and repeat step 1.
3. Transfer supernatant in clean corex tubes, add 0.25 x of 2.5 M NaCl / 20% PEG, mix and keep solution in ice for 1 hr.
4. Spin at 9000 rpm for 10 min.
5. Discard supernatant, dissolve pellet in 500 μl of water.
6. Phenol/chloroform twice.
7. Precipitate supernatant with 0.3 vol of NH_4Ac . and 3 vol. of EtOH. Keep at -80°C for 30 min.
8. Spin for 10 min. Wash the pellet with 70 % EtOH and redissolve in 20 μl of water.

APPENDIX-III: AMYLASE CONSTRUCTS FOR SOMATIC TRANSFORMATION

The following constructs have been described with Roman numbers in the text. The engineering of these constructs is described in detail in Materials and Methods. The constructs are kept in the lab with storage codes as shown:

<u>Construct</u>	<u>Storage code</u>	<u>*Construct</u>	<u>Storage code</u>
I	pOR5.0	I-D	CM.15
II	pOR5.0/H.E	II-D	CM.8
III	p24/CS3.8	III-D	CM.10
IV	CM.2	IV-D	CM.6
V	CM.4	*These constructs are presented as constructs I, II, III and IV in Figure 12.	
VI	CM.20		
VII	CM.24		
VIII	CM.27		
IX	CM.21		
X	CM.25		
XI	CM.22		
XII	CM.28		
XIII	CM.23		