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FUNCTIONAL ANALYSIS OF AMYLASE GENE PROMOTERS IN *DROSOPHILA MELANOGASTER*

By

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Thesis submitted to the
the School of Graduate Studies and Research
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ABSTRACT

The duplicated amylase genes of Drosophila melanogaster show a characteristic pattern of tissue-specific and developmental expression, in addition to regulation by dietary glucose. Germline and somatic transformation of Drosophila larvae has been used to monitor the expression of cloned amylase genes or derivative constructs, with the purpose of identifying regions important in gene expression.

We were able to demonstrate that amylase genes carrying short promoter sequences are still fully functional and show their characteristic pattern of glucose repression in transformed larvae. Amy (amylase) promoter sequences linked to the coding sequences of unrelated genes, the luciferase gene of the firefly, or the Adh (alcohol dehydrogenase) gene of D. melanogaster, mediate a pattern of tissue-specificity and glucose repression typical of amylase. A reciprocal gene construct, in which Amy coding sequences are controlled by upstream sequences of the Adh gene (Adh-Amy hybrid construct) confirmed that Amy coding sequences do not contribute to glucose repression or tissue-specificity.

Amylase promoter sequences were further analyzed in order to localize promoter elements mediating expression and glucose repression. A deletion analysis and site-directed mutagenesis of the Amy-1 proximal gene showed that 109 bp of upstream sequences

are sufficient for full expression, and that a sequence element essential for gene expression is present between -109 and -82; no single region was found to be responsible for glucose repression in these tests indicating the possibility that multiple elements mediate the glucose response.

The study of the distal amylase gene indicated that a short promoter sequence is also sufficient to control expression of this gene. A deletion analysis combined with DNA sequence comparisons revealed similarities between the proximal and distal promoters, although with some variation in the position of sequence elements important for gene expression. The DNA sequence comparison of the coding regions of a proximal and distal amylase genes from the same chromosome allowed us to uncover unexpectedly high levels of nucleotide similarity, suggesting the occurrence of concerted evolution.

RÉSUMÉ

Le système amylase de Drosophila melanogaster est caractérisé, d'un part, par la duplication du gène de structure et, d' autre part, par l' expression spécifique des tissus, et par la répression du glucose de la diète. La transformation de lignées germinales et la transformation somatique de larves de Drosophila ont etées employées pour examiner l' expression de gènes clonées de l' amylase et de constructions dérivés, afin d'identifier des régions qui sont éssentiels pour l' expression du gène.

Nous avons démontré que les gènes de l' amylase ayant des séquences du promoteur tres brèves étaient exprimées normalment et étaient réprimées par le glucose de la diète dans larves transformées de Drosophila. Séquences du promoteur du gène encodant l' amylase fusionné à séquences encodant la luciferase de luciole ou à la région transcrite de l' alcool déshydrogénase (Adh) de D. melanogaster contiennent les signaux assurant l' expression spécifique des tissus et la répression du glucose caractéristique de l' amylase. Un gène chimèrique réciproque (Adh-Amy) constitué de séquences du promoteur du gène Adh fusionné à la région codant l' amylase a confirmé que ce dernier ne contribue pas à la répression du glucose ou à l' expression spécifique des tissus.

Les séquences du promoteur du gène amylase ont étées analysées

ultériorment afin de localizer éléments assurant l' expression du gène et la répression du glucose. L' examen des déletions et des constructions mutagenizés du gène Amy-1 proximale ont révélé que 109 pb de la séquence du promoteur était suffisants pour l'expression complète du gène, et que un élement nécessaire pour l' expression du gène est situé parmi -109 et -82; aucune région en soi est résultée responsable de la répression du glucose dans cette analyse, qu' indique la possibilité que éléments multiples assurent la réponse au glucose.

L' étude du gène distal de l' amylase a dèmontrè que une séquence brève du promoteur est aussi suffisant pour controller l'expression caractéristique de ce gène. L' analyse des déletions, additionée a la comparison des séquences nucléoditiques a révelé des similaritées entre les promoteurs du gène proximal et celle du gène distal de l'amylase, bien que avec quelque variations dans la position des elements néccessaires pour l'expression du gène.

La comparison de la séquence de l' ADN des gènes amylase nous a permis la détection d' un haut degré de similaritée nucléotidique parmi les séquences encodant l'amylase du gène proximal et distal d' un meme chromosome, ce qui suggère l'occurence d' évolution concertée des ces séquences.

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ABBREVIATIONS

aa	amino acid
bp	base pair(s)
°C	degree Celsius
cDNA	complementary DNA
DNA	deoxyribonucleic acid
kb	kilobase pair(s)
µg	microgram
mg	milligram
µl	microliter
ml	millilitre
mRNA	messenger RNA
PCR	Polymerase Chain Reaction
PEG	polyethylene glycol
RNA	ribonucleic acid

1. LITERATURE REVIEW

1.1 Introduction

The major mechanism of regulation of gene expression in eukaryotes is through regulation of transcription. In this chapter, I will first outline the basic mechanism of transcriptional initiation. A lot of information has accumulated in the last ten years on this subject, revealing a strong evolutionary conservation of these mechanisms between lower and higher eukaryotes (Lewin, 1990b; Guarente and Bermingham-McDonogh, 1992; Mitchell and Tjian, 1989). Secondly, I will briefly describe examples of gene-specific transcriptional activation and repression, through which the expression of genes is regulated to give tissue-specific and developmental stage-specific expression, as well as coordinated responses to external and physiological stimuli (see Mitchell and Tjian, 1989 for an overview of these systems in mammalian cells). Specialization of gene expression can be also obtained at a higher organizational level, as in the case of usage of multiple promoters and gene duplication, and I will give some examples of these mechanisms.

One approach to the study of gene expression begins with the identification of promoter sequences that mediate that expression. In Drosophila, it has been possible to introduce genes into the genome of the fly through the P transposable elements. In Section

1.3, I will describe the transformation assays that have been used in Drosophila and that have facilitated the study of gene regulation in this higher eukaryote.

Finally, the amylase gene system of Drosophila, on which I did the experimental work, will be presented in Section 1.4. This gene-enzyme system is interesting because, in addition to being subjected to developmental regulation, it is affected in its expression by an external stimulus, glucose. Moreover, the amylase genes are duplicated, and this allows us to compare the evolution of regulatory and coding regions of these two genes.

1.2 Control of gene expression in eukaryotes

Control of gene expression in eukaryotes occurs mainly at the level of gene transcription (Lewin, 1990a). Two basic steps can be recognized in this process, summarized here and described in Sections 1.2.1 and 1.2.2.

The first step is the formation of an initiation complex in the promoter at the TATA box. This complex contains different general transcription factors and the RNA polymerase II protein; these factors associate in a ordered pathway and allow only low levels of gene expression. This complex is conserved throughout eukaryotes. The second step is the activation of this complex to achieve high levels of expression, through sequence specific DNA-binding proteins that have a double function of recognizing

specific promoters and also of interacting directly or indirectly (through other protein intermediaries) with the initiation complex and thus stimulate its expression. Moreover, repressor proteins can act in a way similar to activators, and repress transcription of a gene.

1.2.1 Basal transcriptional apparatus

Figure 1A illustrates a model of the formation of the initiation complex at eukaryotic promoters. The initial step consists in the binding of transcription factor TFIID to the TATA box, through the TATA-binding protein (TBP) component. This is followed by the binding of other general transcription factors (TF) in a preestablished order and the recruitment of RNA polymerase II (Sharp, 1991; Roeder, 1991; Gill and Tjian, 1992).

In the last ten years, isolation of proteins involved in transcription and cloning of the genes encoding them has facilitated the study of structure and function relationship, based on both in vivo and in vitro activity tests with nuclear extracts. Such studies have been carried out in both yeast and mammalian cells.

A high level of evolutionary similarity was found between factors of the initiation complex, indicating that the basic mechanism of transcriptional activation is highly conserved in eukaryotes (Guarente and Bermingham-McDonogh, 1992). For instance, RNA polymerase II proteins are similar in subunit structure and

Figure 1

Transcriptional activation in eukaryotes.

A) Formation of an initiation complex at eukaryotic promoters.

The ordered pathway of assembly of the general transcription factors at the TATA box is shown. The initial step of TFIID (D) binding to the DNA at the TATA box is followed by the binding of TFIIA (A), TFIIB (B), polymerase II (polII), and TFIIE (TFIIE/F) (E) respectively. The TATA-binding protein (TBP) of TFIID determines the linkage to the promoter (not shown).

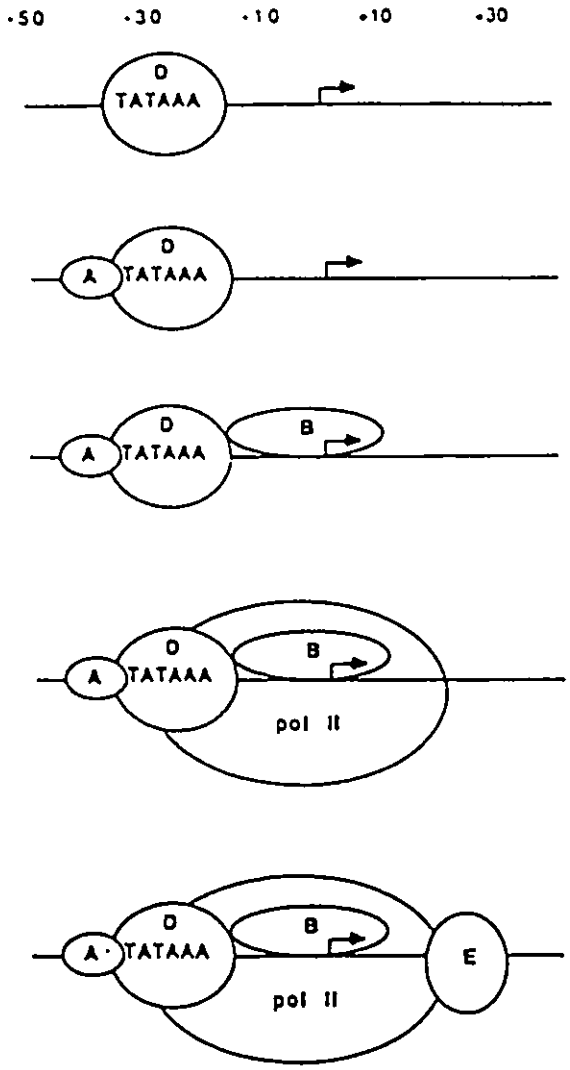
The initiation complex will work at a low level to give basal transcription, unless stimulated by activators (see B).

B) Current model of transcriptional activation.

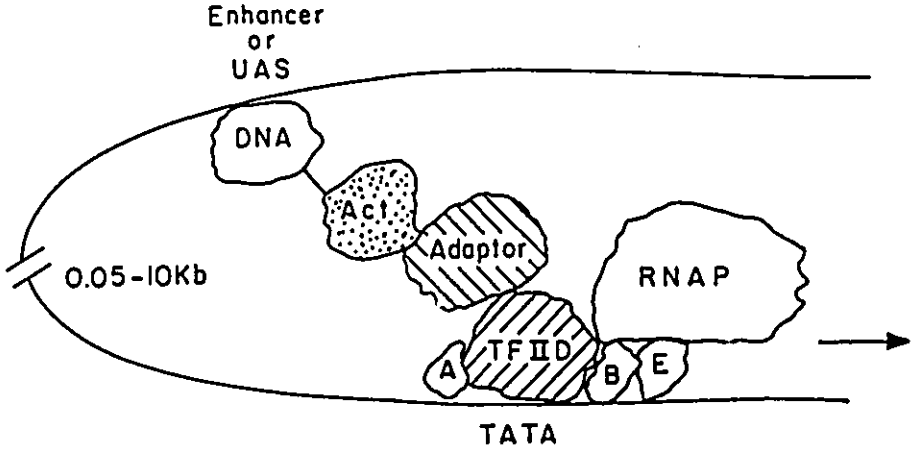
The initiation complex - with the general transcription factors and polymerase II - is stimulated by an activator protein (Act) with its DNA-binding domain (DNA) and activation domain (Act). Also shown is the adaptor (or co-activator) which links the activator to the initiation complex. This contact can be with TFIID, as shown, or with TFIIE, or other parts of the initiation complex. Repressors can change the ability of the activator or adaptor to link to the initiation complex (not shown).

From Guarente and Bermingham-McDonogh, 1992.

A.


-50
-30
-10
+10
+30
D
TATAAA
A
B
pol II
E

B.


Enhancer
or
UAS
DNA
Act
Adaptor
RNAP
0.05-10Kb
A
TFIID
B
E
TATA

function in eukaryotes and also prokaryotes (Young, 1991). TATA-binding factors (TFIIDs) of yeast and humans function as acidic activators in vitro and are functionally equivalent (Buratowsky et al., 1985; Kelleher et al., 1992).

At the structural level the TATA-binding component of TFIID, TBP, is characterized by a carboxyl terminal highly conserved core that is observed in yeast, Drosophila and humans (Peterson et al., 1991). On the other hand, there is relatively little conservation of the amino terminal region of TFIID (Hoffman et al., 1990, Lewin, 1990b, Guarente and Bermingham-McDonogh, 1992). Variation in length of the amino terminal tail is observed, from short in yeast and long in higher eukaryotes (Guarente and Bermingham-McDonogh, 1992). This region may be involved in the mediation of transcriptional activators and may have evolved to perform functions characteristic of each species (Lewin, 1990b). Thus, while the basic mechanism of transcription is maintained over evolution, variation in some of its parts may indicate adaptation to the needs of different organisms.

The other well characterized general transcription factors are TFIIA, TFIIB, TFIIE, and TFIIF. They assemble in a characteristic order and they recruit RNA polymerase II (Figure 1A) (Sharp, 1991; Roeder, 1991). Evolutionary conservation has also been observed for these factors (Peterson et al., 1991; Thompson et al., 1993).

In conclusion, while TFIID represents the protein-DNA interaction of the initiation complex, protein-protein interactions

have recently emerged as being both very refined and essential for the formation of the initiation complex. There are protein-protein interactions between the different TFs and between the TFs and polymerase II.

Recently some of these protein-protein interactions have been clarified. In yeast, Thompson et al. (1993) have demonstrated that the carboxyl terminal domain of RNA pol II interacts physically and functionally with the TATA binding protein (TBP) in the pre-initiation complex. Also, Peterson et al. (1991) have shown the existence of interactions the same carboxyl terminal domain of Pol II with a negative regulator, SIN1 in yeast, a chromatin component probably involved in general inhibition of transcription, thus illustrating the complexity of the system.

The carboxyl terminal domain of TFIID, as is the case for other regulatory proteins, is characterized by the presence of tandem copies of short amino acid sequences - heptapeptide in this case - repeated many times. The SSN6 and TUP1 general repressors of yeast, have repeats that consist of longer modules, with stretches of 34 and 43 amino acids repeated a few times (Keleher, 1992).

1.2.2 Regulation of transcriptional initiation

To obtain high levels of transcription, the initiation complex has to be stimulated by activators, Fig 1B, (Mitchell and Tjian, 1989; Lewin, 1990b; Roeder, 1991; Gill and Tjian, 1992; Guarente and Bermingham-McDonogh, 1992). These are gene-specific

transcription factors that usually include both a DNA binding domain and a transcriptional activation domain.

The DNA binding domain usually consists of 60-100 aa and recognizes a short sequence element in the promoter. This region is necessary but not sufficient for activation. Characteristic structures in these regions have been identified, such as the zinc-finger motif first identified in the polymerase III transcription factor TFIIIA, that is also present in the mammalian transcription factor Sp1 and in the steroid hormone receptors; or leucine zipper sequences required for DNA binding and dimerization (Mitchell and Tjian, 1989; Gill and Tjian, 1992).

The transcriptional activation domain is about 30-100 aa long and displays characteristic aa structures, such as acidic domains, glutamine rich domains and proline rich domains (Mitchell and Tjian, 1989).

The activating transcription factors recognize classes of promoters and enhance their expression by interacting with the initiation complex. They may increase the number of initiation complexes assembled, or increase activity of preassembled complexes (Gill and Tjian, 1992). This interaction between activators and the initiation complex (Figure 1B) can be direct, or occur through other proteins called adaptors or co-activators (Pugh and Tjian, 1990; Gill and Tjian, 1992). These adaptor proteins may serve as a bridge to connect the activator and the general factors or they may act by altering the conformation of the general transcription

factors. The activators or co-activators interact with some of the general transcription factors, and both TFIID and TFIIB (Sharp, 1991) have been shown as possible intermediaries, but this is still matter of study.

An activating transcription factor can also bind to a DNA element that is distant from the initiation of transcription, as is the case for the UAS (Upstream Activating Site) of yeast and the enhancers of mammalian promoters. Binding can be at distances of .5 to 10 kb from the promoter (Guarente and Bermingham-McDonogh, 1992). The function of different parts of transcriptional activators has been studied through hybrid/chimeric proteins that link a known binding domain to a heterologous activating domain or vice versa and through the study of their ability to mediate activation in vitro.

Repression of transcription, although a less common mechanism than activation in eukaryotes, can be achieved through repressors, cis-acting proteins, that bind to a specific element in the promoter inhibiting the formation of the initiation complex, possibly competing with TFIID or TFIIA. Alternatively, repressors may act through protein-protein interactions, for example, by inhibiting the action of activators.

The GAL system of yeast (reviewed in Johnston and Carlson, 1992) provides examples of both activation and repression mechanisms. The GAL1 gene is activated by the DNA binding GAL4 activator protein, that has been completely characterized in both

its DNA binding domain and activating domain (Ma and Ptashne, 1987). Repression of galactose expression is achieved through two different mechanisms: 1 - through a protein-protein interaction, via the GAL80 protein that inhibits the GAL4 activator by binding to a small region of the same and disrupting its ability to contact the initiation complex (Ma and Ptashne, 1987); 2 - through a DNA-binding repressor, possibly the MIG1 protein, that contacts the initiation apparatus through a recently identified intermediary repressing protein complex (Keleher et al., 1992). The intermediary complex, consisting of the SSN6 and TUP1 proteins, is being recognized as a general repressor complex of yeast. Its specificity is mediated by DNA binding proteins specific for different kinds of repression, such as MIG1 for glucose repression or alfa2-MCM1 for repression of cell type determination (Keleher et al., 1992; Johnston and Carlson, 1992). Thus a single general repressing mechanism is used in yeast to mediate different kinds of repression by using different DNA-binding proteins.

Conserved regulation of gene expression has also been reported between widely different organisms. A clear example is represented by the heat-shock response (Bienz, 1985; Sorger, 1991). This phenomenon is common to many organisms from yeast to Drosophila to mammals and it is singular in that the stimulus, a temperature increase, arrives quite directly to the nucleus. A common consensus sequence has been identified in all heat-shock gene promoters of the widely different species studied. A heat-shock factor that

binds to the heat-shock elements and induces transcription has been characterized in many different organisms; although presenting low aa identity it shows functional and organizational conservation (Sorger, 1991).

From these examples it appears that both positive and negative regulatory mechanisms are conserved and modified during evolution, and this conservation is especially true for responses to fundamental stimuli. This conservation is noted between different genes in the same organisms (repression in yeast), and among different organisms in response to a common stimulus (heat-shock response).

Control of gene expression can also be achieved through other mechanisms such as the duplication of promoters. An example of this type of control is provided by the Adh genes of Drosophila that contain a distal adult promoter and a proximal larval promoter. Both of these promoters control the expression of the same coding region but produce different transcripts (Heberlein et al., 1985). Gene duplication is often followed by divergence in promoter sequences which can result in the specialization of gene expression of the different members of the family. Often this variation in gene expression is also accompanied by an evolutionary divergence of coding sequences to obtain a "better" functioning protein for a specialized function, as observed for the globin genes (Li, 1983).

Another, very general mechanism of control of gene expression is represented by the inclusion of genes in the nucleosome, thus

rendering promoters less accessible for the formation of the pre-initiation complex (Roeder, 1991).

Finally, alternative splicing has also been recognized as a possible mechanism for differentiated gene expression (Sosnowski et al., 1989).

One can begin the study of transcriptional regulation at either end of the pathway that goes from the first environmental or physiological signal to the final step of activation or repression of transcription in the nucleus. In the case of hormones, the study often begins with the isolation of proteins that act as hormone receptors, and the isolation of the genes encoding them (see Talbot et al., 1993, for a study of genes encoding Drosophila ecdysone receptors). Once the receptors have been identified, such studies proceed with the identification of promoter sequences and elements to which the receptor binds.

The reverse approach is to study promoters of highly specialized genes, that may present expression in a limited number of cells or in a short developmental stage. Deletion analysis and mutagenesis of the promoter can be used to identify regions of importance for specific gene expression. DNA sequence comparison of promoters from similar genes and from the same gene in different species can lead to the identification of sequences necessary for a characteristic expression pattern. Subsequently, protein binding to these promoter elements can be identified. The final step is the identification of the complete signal transduction pathway.

In my thesis, I will report on the regulation of amylase genes of Drosophila. We studied glucose repression and tissue specificity of these genes at the level of promoter sequences through functional analysis of molecular constructs prepared with the aim of identifying sequences important for its expression. For this purpose, we used transformation assays, which have been developed in Drosophila since the discovery of P transposable elements.

1.3 Transformation in Drosophila

In 1982 Rubin and Spradling (Spradling and Rubin, 1982 and Rubin and Spradling, 1982) first published results of experiments that showed that P transposable elements of Drosophila could be introduced in the genome of the fly. They also showed that genes inserted between the P element ends would be introduced into the germline and would be stably inherited; these introduced genes are expressed in a normal manner. Plasmids carrying P elements were introduced into Drosophila through microinjection of young embryos and transformed lines were selected in the following generations. The technique was later used to introduce other known genes into the Drosophila genome, such as the Adh (Alcohol dehydrogenase) gene (Goldberg et al., 1983). In all cases correct expression of the inserted gene was observed.

In the last 5 years the transformation technique has been further utilized for the introduction of hybrid genes or in vitro

modified regulatory sequences to test the in vivo role of regulatory sequences. In this way the ability of promoter sequences to regulate expression of an easily-scored protein from a suitable reporter gene can be tested.

Recently, somatic transformation experiments have been carried out, where plasmids carrying the gene of interest are simply introduced into Drosophila embryos and the expression of the construct is monitored in the developing larvae or adults. The expression from the DNA sequences is in this case only transient.

1.3.1 Germline transformation

The germline transformation assay (reviewed in Spradling, 1986; Karess, 1985) is based on the introduction of plasmids carrying P elements with the sequences of interest, in addition to a marker of transformation, in a stock of Drosophila lacking the gene under study. The P element, mimicking its natural behaviour, can transpose from the plasmid and integrate into the chromosomal DNA of the germline.

Recently, P elements have been engineered in a very elegant way to obtain the introduction of foreign sequences. A typical P element is 2.9 kb long and contains at its ends inverted repeats that are 31 bp long (O'Hare and Rubin, 1983). For germline transformation, a mixture of plasmids carrying two kinds of engineered P elements is used. One has internal sequences, that normally encode transposase, substituted with the exogenous

sequences of interest, and the second plasmid, called a helper (Karess and Rubin, 1984) has defective ends, and is not able to integrate, but is still able to provide the transposase activity for the integration of the first plasmid.

Usually the P element that will integrate will carry, in addition to the sequences under study, a marker of transformation that allows recognition of transposition events in the fly through a change in phenotype. The rosy genes or the Adh gene have been used as such markers (Laurie-Ahlberg and Stam, 1987; Romano et al., 1988). Also a neomicyn-resistant gene has been introduced in a P element vector and used as a selection marker for transformation (Steller and Pirrotta, 1985).

Microinjection is carried out in young embryos at the preblastodermal stage (Wieschaus and Nuesslein-Volhard, 1986), between 1 - 2 hours from fertilization, when the embryo is at a syncytial stage and nuclei are not separated by cell membranes. The microinjection is carried out at the posterior end of the embryo where the germline cells develop, and in which the integration of the P element occurs, and which will transmit the inserted sequences to the following generation.

Suitable Drosophila strains are required for transformation, specifically those that have a cytoplasm of the M type which allows transposition of P elements. These strains must also lack the function under study, to allow restored activity to be scored.

Insertion of engineered P elements occurs at random in the

genome and single or multiple insertions may occur. The frequency of integration is high, since 1/10 of adults developing from injected embryos will produce transformants (Spradling, 1986).

As mentioned above, genes introduced into the Drosophila melanogaster genome are stably inherited and correctly expressed. More recently, hybrid genes have been introduced to study expression patterns. Mismer and Rubin (1989) have used germline transformation in conjunction with oligonucleotide-directed mutagenesis to show the existence of cis-acting elements in promoter of the ninaE opsin gene.

The chorion genes of D. melanogaster show a highly specialized temporal and tissue-specific expression pattern that has been successfully studied with transformation techniques. Romano et al. (1988) carried out germline transformation studies of various hybrid constructs carrying promoters of the chorion genes and Adh sequences as the reporter gene. They demonstrated that sequences necessary for developmental expression of the chorion genes are present in the 5' DNA flanking sequences and also elements for their temporal and developmental expression were identified (Mariani et al., 1988). Based on this information, DNA-binding proteins which recognize these elements have been isolated that may represent new transcription factors. Similarities of the DNA-binding and receptor binding domains of these factors with those of known transcription factors were noted, pointing again to the existence of families and modules that have evolved to adapt to

different functions in different organisms (Shea et al., 1990).

1.3.2 Somatic transformation assays

Recently the expression of genes carried by plasmids without P element sequences which were introduced through micro-injection into Drosophila melanogaster embryos has been monitored; these genes were expressed in both larvae and adults (Martin et al., 1986; Meyerowitz et al., 1987).

The occurrence of transient expression was first noted during germline transformation experiments (Rubin and Spradling, 1982) when the rosy marker was expressed in adults derived from injected embryos, before integration of DNA into the genome. The expression of the rosy gene occurred in a tissue- and stage- specific manner.

Martin et al. (1986) first used the somatic transformation assay systematically to study expression of the Adh gene. They found that ADH expression occurred in a developmentally correct way. They also monitored the fate of the injected plasmid as embryos proceeded through larval and adult development. The plasmid DNA is relatively stable and persists in diminishing quantities from embryo to adult, and even at the adult stage some expression is seen.

This system provides a convenient assay for in vivo testing of the expression of genes and of derivative constructs that have been modified in vitro. It is faster than germline transformation since expression is monitored within a few days from the injection in

developing larvae or adults. It can be used, like germline transformation, for the identification of sequences essential for gene expression.

Using this technique Shore and Guild (1987) were able to identify cis-acting elements involved in the regulation of the Sgs-5 gene of Drosophila. The SGS, Salivary Gland Secretory proteins are a major component of the glue, produced by the salivary glands. The genes expressing them show a pattern of highly characteristic tissue-specific and temporal expression that allows their characterization. These studies were able to localize a short region sufficient for correct expression; this region is adjacent to the site of transcriptional initiation.

In conclusion, embryo transformation experiments in Drosophila have permitted the identification of cis-regulatory elements in the case of genes with characteristic expression patterns. Also, studies of mutations of developmental processes of the Drosophila embryo have uncovered important mechanisms of regulation in this organism and similarities with mammalian regulatory proteins (Nuesslein-Volhard, 1991; for a review see St.Johnston and Nuesslein-Volhard, 1992).

1.4 The amylase system in Drosophila

The amylase genes have been studied in many different organisms from bacteria, to yeast, to insects and mammals. The

function of the amylase enzyme is a very fundamental one, being involved in the utilization of carbohydrates. Thus lots of information has been accumulated that allows comparison of the amylase protein and amylase gene regulation in different organisms.

The amylase (Amy) genes of Drosophila melanogaster are present in the genome in two closely linked copies. They are transcribed in opposite directions and separated by about 4 kbp of DNA that contains the 5' flanking regions of the two genes (Gemmill et al., 1985; Boer and Hickey, 1986; Benkel et al., 1987). Using classical methods, the two genes had been mapped on the second chromosome at position 77.8 (Doane, 1969); at a distance 1cM from the structural locus a trans-acting regulator of tissue-specific expression has been mapped, called map (see below). The gene duplication is found in all the 8 species of the D. melanogaster species subgroup (Payant et al., 1988).

The amylase genes of Drosophila encode alpha-amylase (alpha-1,4-glucan,glucanohydrase; EC 3.2.1.1), a monomer protein that hydrolyses the internal alpha-1,4-glucosidic bonds of starch molecules (Bernfeld, 1955).

The amylase enzyme accounts for up to 0.1% of the total soluble protein in Drosophila. The amylase protein of D. melanogaster as predicted from the DNA sequence resembles the mammalian amylase. The protein shows 55% aa identity with the mouse alpha-amylase, including short stretches of complete identity and conservation of regions previously recognized as being important

for amylase function (Boer and Hickey, 1986).

1.4.1 Electrophoretic and developmental expression

The amylase genes were first studied at the protein level through the analysis of activity and electrophoretic variation. With the cloning and sequencing of the amylase genes (Gemmill et al., 1985; Levy et al.,1985; Benkel et al.,1987; Boer and Hickey, 1986) the study has shifted to the molecular level, both for the coding sequences as well for the regulatory sequences. For reviews of amylase genes in Drosophila see Doane et al. (1983) and Hickey et al. (1989).

At least 6 electrophoretic variants, from AMY-1 to AMY-6 are known, with the AMY-1 being the most common one and also the one migrating fastest on acrylamide gels. (This is an unusual pattern; usually the most common variant is the one that migrates in an intermediate position). Due to the presence of duplicated genes, flies expressing two different variants are often found in isogenic lines. Electrophoretic variation has been examined in laboratory strains (Doane, 1969) and in world wide populations. These surveys show that the level of polymorphism at this locus can be very high (Hickey, 1979; Singh et al., 1982).

An $\underline{Amy}^{null}$ strain, which lacks amylase activity, has been found in a laboratory population and characterized at the molecular level (Hickey et al., 1988). In this stock, an inversion in the intergenic region results in the production of very low levels of

mRNA, that do not produce functional amylase protein.

The amylase genes are normally expressed in the midgut, in particular in the anterior and posterior midgut of the adult and in the posterior midgut of the larva. Variation in the tissue-specific expression of amylase has been observed. Expression of amylase activity varies over the length of the midgut within different regions of the adult fly (Abraham and Doane, 1978). With the use of crosses between isogenic strains of Drosophila displaying different electrophoretic variants and different patterns of midgut expression, a trans-acting regulator was identified, map (midgut activity pattern) which controls tissue-specific levels of amylase activity in different regions of the posterior midgut (for a review of tissue-specificity in adults see Doane et al., 1983).

Recently, Thompson et al. (1992) showed that the effects of map occur at the level of mRNA, suggesting that it may act as a positive trans-activator. The behaviour of the midgut pattern has been examined in genetic crosses, confirming the dominant effect of map on the expression of amylase activity in the posterior midgut (Klarenberg et al., 1986). The map locus has been mapped at 1cM from the amylase locus.

In the larva, amylase activity is mainly found in the posterior midgut and although variation in the length of the region that expresses this activity is found, this is strictly linked to the Amy locus, indicating that sequences in cis determine this behaviour (Klarenberg et al., 1986). Amylase is expressed at

highest level in third instar larvae; amylase activity drops following formation of the puparium; it reaches again constant higher levels in the adult (Doane, 1969). It is worth noting that the cells that form the adult and larval midgut are of different origin (Demerec, 1950). The anterior and posterior midguts of the larva are derived from two separate embryonic primordia near the germline. After histolysis during pupation, the adult midgut is formed from embryonic stem cells found along the basement membrane of the larval gut wall.

1.4.2 Glucose repression

Variation in amylase activity was reported in early studies of larvae and adults grown on different media varying in the carbohydrate components (for references see Benkel and Hickey, 1986a). Hickey (1981) noticed that the variation was not due to a increased activity of amylase protein but to a variation in the number of protein molecules. Subsequently, it was shown that the amylase activity is repressed by dietary glucose (Hickey and Benkel, 1982; Benkel and Hickey, 1986a) and that this repression was at the mRNA level, suggesting a transcriptional regulation (Benkel and Hickey, 1987).

It was also shown that the variation in amylase activity previously observed between strains is mainly due to the glucose-effect: all strains showed comparable amylase activity when raised in glucose-free medium, but a large variance in the degree of

reduction of the activity on glucose food was noted (Benkel and Hickey, 1986a). The differential glucose repressibility was shown to be genotype specific. Genetic polymorphism for the level of repressibility has been found in natural populations (Benkel and Hickey, 1986b), although most strains are highly repressible.

The glucose repression in Drosophila melanogaster is counteracted by addition of exogenous cAMP and sequences responsible for this effect have been recently localized in the promoter region close to the transcriptional start site (Magoulas et al., 1993a). cAMP regulation of glucose repression is well known in bacteria; in E. coli exogenous cAMP alleviates the repression mediated by glucose on the activity of catabolic enzymes (de Crombugghe et al., 1969; Pastan and Perlman, 1970). Thus the amylase system of Drosophila presents common aspects with the regulation in prokaryotes, making it an interesting system to be studied, while at the protein level it shows high similarities with the mammalian amylases.

1.4.3 Recent studies

Recent work in our laboratory (in which I have participated) has led to the identification of the promoter region mediating glucose repression of the amylase gene (Magoulas et al., 1993b). In particular, we showed that amylase genes carrying only a short sequence of 109 bp upstream the transcriptional start site were fully functional and that they mediate glucose-repression that is

sensitive to effect of exogenous cAMP (Magoulas et al., 1993a). It was also found that the glucose repression is an evolutionary conserved feature since in a distant species of Drosophila, D. virilis, the phenomenon is observed and it is mediated by short promoter sequences; the latter sequences are functional in transformed D. melanogaster larvae (Magoulas et al., 1993c).

Also, the evolutionary aspects of the two amylase coding sequences have been examined (Hickey et al., 1991). It was observed that the coding sequences of the two amylase genes are very similar within each of the two species examined, D. melanogaster and D. erecta, while they diverged as expected between species based on phylogenetic estimates. Promoter sequences also showed expected amounts of variation. It was proposed that gene conversion mechanisms are operating on the coding sequences, leading to their concerted evolution. To confirm this hypothesis, we have sequenced the entire amylase locus, including both the coding and intergenic regions (see Section 3.2).

In addition to glucose repression, the amylase genes of Drosophila present a characteristic developmental and a tissue-specific expression pattern, limited to the midgut. They are the only example of glucose repression in higher eukaryotes, and a rare example of regulation of expression by an external stimulus. This combination of tissue-specific and dietary-dependent regulation makes amylase an interesting gene-enzyme system to be studied. In this thesis I report results from experiments aimed at identifying

sequences important for both glucose repression and tissue-specificity. In addition, a functional analysis of the expression of various amylase genes was carried out, allowing a functional and structural comparison of the duplicated amylase genes.

2. MATERIALS AND METHODS

2.1 Fly stocks and culture media

The Oregon R and Canton S strains of Drosophila melanogaster were obtained from the Mid-America stock centre (Bowling Green); these are wildtype strains that produce normal levels of both amylase (AMY) and alcohol dehydrogenase (ADH) activity. They produce an AMY-1 and AMY-1/AMY-3 phenotype respectively for the amylase genes. An isogenic strain from a wildtype stock from Makokou, West-Africa, was also used; this presents the AMY-4/AMY-6 electrophoretic phenotype for amylase.

An Amy^{null} strain of D. melanogaster that does not produce any amylase enzyme (Hickey et al., 1988) was used as a recipient strain for somatic transformation experiments. An Adh^{null} pr cn Amy^{null} strain was also used for somatic transformation, which gave identical results as the Amy^{null} stock; this stock, though, showed lower viability than the Amy^{null} strain and thus its use was discontinued.

An Adh^{null} strain, Adh^{fn8} cn; ry^{506} (Laurie-Ahlberg and Stam, 1987) was used as recipient stock for germline transformation experiments. This strain has a defective Adh gene and does not produce wildtype mRNA. Rather, it produces low amounts of an aberrant transcript that is 850 bp larger than the normal Adh message and it produces no functional ADH enzyme (Benyajati et al.,

1982). This strain was used as a negative control for the ADH expression studies, as well as being the recipient stock for germline transformation experiments.
Homozygous lines of the transgenic stocks carrying intact Adh genes or chimeric Amy-Adh genes were used in this work (see below).

Drosophila larvae and adults were grown and maintained in two types of food, prepared according to Benkel and Hickey (1986a). Both food types contained killed yeast and agar, but one food contained added glucose (10%); this added glucose causes repression of the amylase promoter (Benkel and Hickey, 1987).

2.2 Somatic transformation assay

2.2.1 Somatic transformation procedure

Microinjection of plasmids (pIBI.24, 25 or pUC) carrying different Amy genes or derivative constructs was carried out in embryos of the Amy^{null} stock of Drosophila melanogaster. Expression of amylase activity was monitored in the transformed larvae developing from these embryos. The transient expression assay, first used for the Adh gene (Martin et al.,1986), turned out to be a very efficient way to assess the expression capability of intact and engineered Amy genes (see Results).

DNA used for microinjection was purified by a standard PEG/NaCl precipitation and dissolved in buffer containing 5mM KCl and 0.1 sodium phosphate (pH 6.8).

The protocol of Karess (1985) and Spradling (1986) was followed for the microinjection procedure with minor modifications.

Injected embryos were allowed to develop under mineral oil for 2 days at 18°C in a humid chamber. Young larvae were transferred to small petri dishes (35mm x 10 mm in size) containing food medium. Two types of food were used: 1) A glucose-free medium containing 5% killed brewer's yeast, 0.4% agar (w/v), 0.8% propionic acid (v/v), 0.1% potassium phosphate, in H_2O; 2) a glucose-rich medium containing the above mentioned ingredients plus 10% glucose. The larvae were reared at 25°C till the late third instar stage and then harvested and frozen at -20°C.

2.2.2 Amylase expression assay of transformed larvae

Amylase expression assays of transformed third instar larvae were carried out as follows. Five to ten larvae for each kind of food were homogenized in distilled water (15 microliters /larva). After centrifugation the supernatant was run on an electrophoretic gel (see amylase activity assay, below). One larval equivalent was loaded in each lane, two replicates for each homogenate. Activity of larvae grown on glucose-rich food was compared to activity of larvae grown on food lacking glucose; as a reference for comparison, a tenfold dilution of the latter was loaded on the same gel. A sample of non-injected larvae was also tested. As an internal control, constructs were co-injected with a second plasmid construct with known expression pattern, but producing a different

amylase variant. All gels contained a standard dilution series of wildtype Oregon R stock larvae grown on medium lacking glucose, for comparison between gels.

All constructs were examined in at least 2 - 3 independent somatic transformation experiments. DNA concentration was usually .1 microgram/microliter.

2.2.3 Luciferase activity assay of transformed larvae

Emission of light by the amylase promoter - luciferase coding construct was tested in transformed larvae at the third instar stage. Larvae were stored at - 70°C, prior to testing. The Luciferase Assay System kit from Promega was utilized and a protocol was developed, following recommendation from Promega for assays in cell cultures and plants tissue (Technical Bulletin 101. 1990).

Briefly, single larvae were homogenized in cell culture lysis reagent (1x), 18 microliter/larva, and the homogenate was kept at room temperature for 10 - 20 minutes, then it was briefly centrifuged to eliminate debris. 20 microliters of the homogenate were added to 100 microliters of a previously defrosted Assay Reagent, containing tne substrates luciferin, coenzyme A, and ATP. The reaction is based on the oxidation of beetle luciferin by the luciferase enzyme, with production of a photon. Production of light decays rapidly (half time of the reaction with the Promega assay system is five minutes). Measurements of light production were

carried out in a scintillation counter within one minute from the mixing and for the duration of three minutes; the average value per minute was recorded.

2.3 Germline transformation

2.3.1 Transformation procedure

Germline transformation by P element-mediated integration was carried out as described in Spradling and Rubin (1982). Each plasmid was coinjected with the pπ25.7wc helper plasmid (Karess and Rubin, 1984) in a 2 : 1 ratio (600 μg/ml to 300 μg/ml of helper plasmid). The recipient stock was an ADH^{null} stock, $\underline{Adh}^{fn6}\underline{cn;ry}^{506}$. A successful transformation was indicated by restored non-rosy phenotype in the eyes of adult flies.

DNA was purified by a standard PEG/NaCl precipitation step and dissolved in microinjection buffer (5mM KCl and 0.1 M sodium phosphate, pH 6.8). Embryos were injected at the posterior end before the formation of pole cells, covered with halocarbon oil and kept at 18°C for 48 hrs. First instar larvae were collected and cultured on Instant Drosophila medium (Carolina Biologicals). All first generation (G_0) adults were back-crossed singly to the recipient stock. G_1 adults, with restored non-rosy eye colour phenotype, which indicated germline integration of the transforming DNA, were backcrossed once more individually to the recipient stock flies; the G_2 progeny were brother - sister mated, in single

pairs, to establish independent transgenic lines on which selection for the eye colour marker phenotype continued for several generations until homozygous stocks were obtained.

2.3.2 Plasmid constructs for germline transformation

Two P element based vectors were used in this study to introduce Adh sequences into the Drosophila germline: (i) plasmid pΔ1wa2a, which carried an intact Adh gene and ry^+ sequences (which encode xanthine dehydrogenase, XDH, and act as a visible eye colour marker of transformation) inserted between the P element ends of the germline transformation vector pΔ1 (Laurie-Ahlberg and Stam, 1987), and: (ii) a similar plasmid vector in which the Adh gene was replaced by an Amy-Adh chimeric gene that has promoter sequences of the Amy gene and transcribed sequences of the Adh gene (see Figure 14, Benkel et al., 1992). These chimeric genes contain either 1580 bp or 428 bp of 5' flanking sequences of the proximal Amy gene of Drosophila melanogaster fused to the transcribed sequences of the Adh gene.

2.4 Amylase and alcohol dehydrogenase enzyme activity assays

2.4.1 Enzyme gel electrophoresis

Crude larval homogenates were centrifuged and the supernatants were electrophoresed. The resulting gels were stained for amylase activity or alcohol dehydrogenase activity.

For testing amylase activity, electrophoresis was combined with a starch/iodine staining method as described in Benkel and Hickey (1986a and 1987). After polyacrylamide electrophoresis amylase activity was observed as the result of the disappearance of substrate (starch). Gels were incubated in a 2% starch solution (0.1 M Tris-HCl buffer, pH 7.5, 0.02 M $CaCl_2$) for 1 hr. Gels were then washed and stained with iodine-potassium iodide solution (1:2:1 solution of I_2:KI:H_2O diluted 30 times). Within a few minutes white bands appear as the result of amylase activity and gels were photographed.

For ADH activity the method described in Benkel and Hickey (1987) was followed. Gels were incubated after electrophoresis in 100 ml of 0.1 M Tris-HCl, pH 8.5 with addition of 25 mg of MTT, 30 mg of NAD, 0.75 ml of isopropanol at room temperature. After 1 hr of incubation, 2 mg of phenazine methosulfate was added. Gels were photographed when bands were clearly visible.

2.4.2 Histochemical assays for ADH activity

ADH activity was tested *in situ* on different tissues of larvae transformed with the *Amy-Adh* chimeric construct and control larvae transformed with the intact *Adh* gene, and non-transformed larvae from the Adh^{null} recipient stock.

Transgenic adult flies and control flies were allowed to lay eggs in two different media without, or with, glucose (10% glucose) and developing late third instar larvae (feeding stage)

were dissected and stained for ADH activity following the methods of Martin et al. (1986) and Ashburner (1989) with some modifications. Larvae were rinsed twice in PBS (50 mM sodium phosphate, pH 7.2, 0.8% NaCl) and then dissected. Fixing was omitted. Different organs were incubated in staining solution (0.7 mL sodium phosphate buffer pH 7.5, 50 μL NAD (50 mg/mL, Sigma Type V), 250μL nitroblue tetrazolium (5 mg/mL), 25μL phenazine methosulfate (2 mg/ml), 50 μL 2-butanol) for 15 minutes in the dark. The reaction was stopped by rinsing with PBS twice and precipitation of dark blue stain recorded. Midguts and attached Malpighian tubules and hindguts were stretched on slides and photographed. For each line and culture medium between 10 and 20 larvae were examined. The ADH expression pattern (tissue specificity) was repeatable between replicate larvae for each line and food combination.

2.4.3 Enzymatic assays in adult flies

In order to quantify ADH activity in transformants carrying the Amy-Adh hybrid gene, young transgenic adult flies, from the transgenic homozygous lines, and control flies (1-2 days old) were transferred for 5-7 days to culture medium lacking glucose and then collected and stored at -80°C. Homogenates of twenty adults were prepared and assayed for alcohol dehydrogenase (ADH) and amylase (AMY) enzyme activity by specific staining following native polyacrylamide gel electrophoresis, as described by Benkel and

Hickey (1986a, 1987). Homogenates from the same fly collection were used to carry out RNA extraction to test mRNA levels of Amy and Adh transcribed sequences.

2.5 Recombinant gene constructs

2.5.1 Amylase gene constructs

Amylase genes were isolated from genomic clones from different stocks of D. melanogaster and subcloned in pUC or pIBI vectors (Benkel et al., 1987; Abukashawa Ph.D. thesis, 1990; Magoulas Ph.D. Thesis, 1992; Magoulas et al., 1993b).

The following amylase genes were examined in this study (see Figure 1.1, A and C, and amylase genes map in Figure 7):

- Amy-1 proximal genes from Oregon R and Canton S stocks : pOR 5.0, that is a 4.7 kbp EcoRI fragment containing the Amy-1 gene and 1600 bp of upstream sequences; pOR H/E, derived from the above, that has 477 bp of promoter sequences; pCS3.8 from Canton S stock that has 198 bp sequence upstream of the ATG (or 163 from transcription start site). These constructs derive from original cloned genes trimmed at promoter sequences utilizing naturally occurring restriction sites.
- Amy-3 distal from Canton-S, pCS5.5, that has AMY-3 phenotype;
- Amy-4 proximal gene and Amy-6 distal gene from the Makokou strain of West Africa; these have AMY-4 and AMY-6 phenotype respectively.

Figure 1.1

Scheme of amylase gene constructs analyzed in somatic transformation experiments.

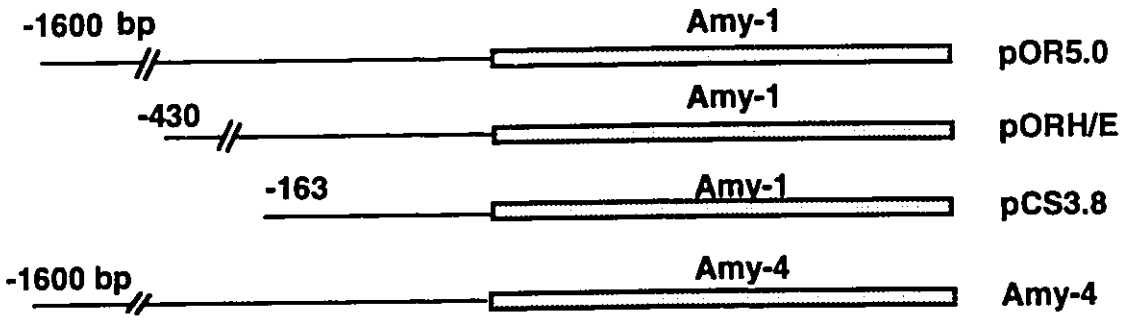
A set of constructs carrying cloned Amy proximal genes (A) or fine scale deletions of the same (B) were examined in somatic transformation assays to test their ability to express amylase. A set of constructs carrying cloned Amy distal genes (C) or derivative constructs (D) were examined with the same purpose.

Fine deletions constructs were prepared by C. Magoulas (see Magoulas et al., 1993b and C. Magoulas Ph.D. Thesis, 1992 and Materials and Methods). All constructs were subcloned in pUC or pIBI vectors.

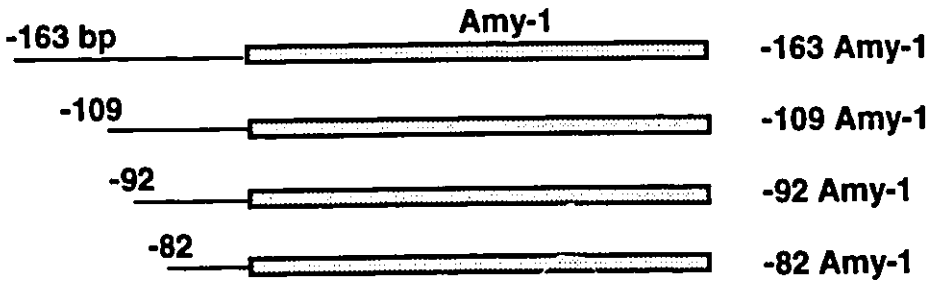
A fine line indicates upstream sequences till transcription initiation start. Grey boxes comprise leader sequences and coding regions. Downstream sequences are not shown and are about 1.5 kbp long. Sizes of constructs are not exactly to scale.

A set of -109 Amy-1 mutagenized constructs were also examined (see Figure 6).

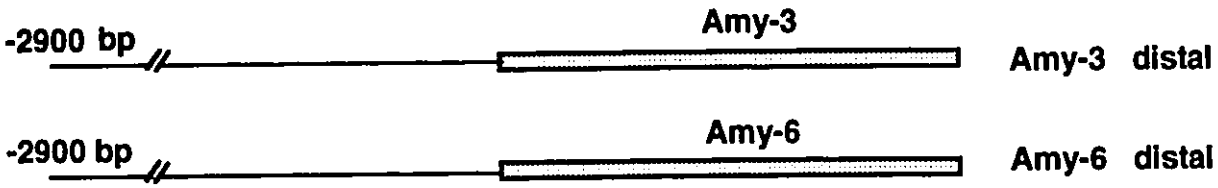
A. Proximal genes



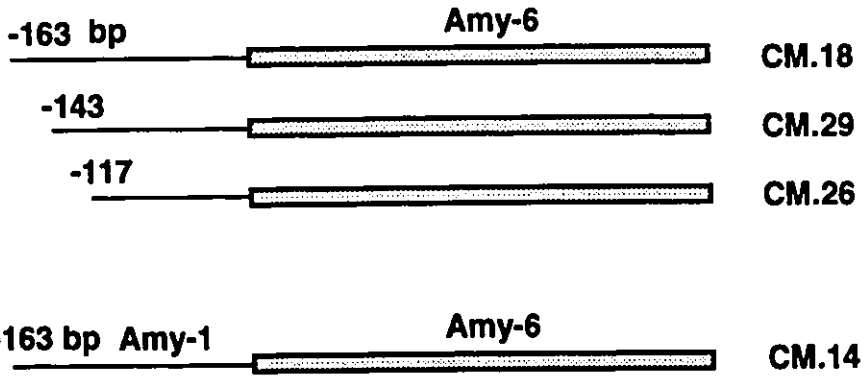
B. Fine scale deletions



C. Distal genes



D. Fine scale deletions



These genes were first isolated as EcoRI fragments of about 5 and 6 kbp respectively - see figure 7 in Section 3.2.1.

2.5.2 Amylase gene deletions

Amy-1 proximal genes carrying short promoter sequences (see Figure 1.1 B) were tested for functionality. Constructs - see C. Magoulas, Ph.D. Thesis, 1992; Magoulas et al., 1993b - had been prepared utilizing the CS/BclI* plasmid to which promoter sequences isolated with PCR technology were linked. CS/BclI* carries coding and downstream sequences of the Amy-1 gene - representing a "promoterless" gene - with a mutation to create a BclI site 8 bp past ATG. A reconstructed Amy-1 gene carrying the BclI restriction site has been tested and behaves as the original Amy-1 gene.

Also a series of mutagenized constructs based on the -109 Amy-1 construct were provided by C. Magoulas and examined in this study (see Section 3.1.2 and Figure 6).

Constructs carrying deleted Amy-6 distal genes, see Figure 1.1 D, were also examined in somatic transformation experiments. The Mk-d, Amy-6, distal gene from Makokou strain has 2.9 kbp of upstream sequences. Deletions of the Mk-d clone were prepared by C. Magoulas (Ph.D. thesis, 1992) by combined PCR and crossover linking technology.

First, short promoter sequences from the Mk-d clone were isolated with use of PCR technology, utilizing a forward and

reverse primer with sequences complementary to the ends of a short promoter fragment. The primers also had at their 5' ends, HindIII and ClaI sites respectively, so that the amplified fragment could be linked to a modified Mk-d clone, Mk-d*ClaI. This is a Mk-d gene that has a new ClaI site (resulting from the change of a C to G) 21 bp from transcription start, and at its 5' end is linked with a HindIII site to pIBI24. The ClaI site is utilized to link previously isolated short promoters. In this way, a series of Amy-6 gene constructs carrying a 163 bp, 143 bp and 117 bp long promoters were constructed (CM18, CM.29 and CM.26, respectively).

A similar process was used to create a hybrid gene carrying Mk-d promoter sequences linked to Amy-1 coding sequences (CM.14, Figure 1.1D). In this case the forward and reverse primers used to amplify short promoter sequences from the Mk-d clone carry a HindIII and BclI sites in order to link the amplified fragment to a suitable p24/CSBcl*I (Amy-1) clone. This clone carries coding and downstream sequences of the proximal Amy-1 gene and also has an introduced BclI site at 8 bp past ATG to allow construction of genes with different promoter sequences.

2.5.3 Hybrid genes with alcohol dehydrogenase (Adh) sequences

For the construction of an Adh-Amy hybrid gene, promoter sequences of alcohol dehydrogenase gene of D. melanogaster were amplified via Polymerase Chain Reaction technology (Saiki et al.

1985; Higuchi, 1989) and linked to the coding sequences of the Amy-1 proximal gene in pCS/BclI* plasmid (see Figure 19).

The pΔ1wa-f clone (provided by Laurie-Ahlberg, Laurie-Ahlberg and Stam, 1987) containing the Adh fast gene was used as template to amplify promoter sequences of the Adh proximal (larval) promoter. Appropriate primers (35-45 bp long) were custom-made and contained sequences identical to the 5' and complementary to the 3' endings of the promoter sequences, moreover they carried sequences containing the HindIII and BclI restriction sites at their 5' ends to allow linkage to the Amy-1 coding sequences in the pCS/BclI* plasmid. The following times and temperature were used for the amplification reaction: 30 cycles, at 95°C for 30 seconds (denaturation), 50°C for 30 seconds (primer annealing), 72°C for 1 minute (primer extension) per cycle.

A different approach was used to construct an Amy-Adh hybrid promoter (see Figure 21) that had Amy-1 sequences from bp -109 to the TATA box (78 bp of Amy sequences) and alcohol dehydrogenase sequences (Kreitman, 1983) from TATA box to the ATG (102 bp long), for a total length of 180 bp: two long primers, forward and reverse primer, of 113 bp each were prepared with sequences identical or complementary to the hybrid promoter that we wanted to construct and which also had 20 bp overlap in the central region. By running through one amplification cycle without template, we obtained a few long molecules with the desired

sequence. By adding to these molecules short upstream and downstream flanking primers, and amplifying for several cycles, we selected the long molecules and obtained the desired promoter sequences. These again were linked to the Amy-1 coding sequences of the pCS/BclI* plasmid.

2.5.4 Construct with luciferase coding sequences

A construct carrying promoter sequences of the Amy-1 proximal gene, from just downstream the HindIII site (-477) to the ATG fused to luciferase coding sequences was provided by B.Benkel.

The construct was micro-injected together with Mk-p, Amy-4 gene construct in Amy^{null} embryos and developing larvae tested for luciferase activity (see Section 2.2.3).

3. RESULTS

3.1 Studies of amylase gene expression via somatic transformation assays

In this first section of the results I will report on a series of experiments by which the expression pattern of the amylase genes of D. melanogaster are examined in transient expression assays via somatic transformation experiments. These studies were done in cooperation with Charalambos Magoulas, who engineered the different constructs to be examined (see Section 1 and 2 of Results, and Materials and Methods).

I will describe the first somatic transformation experiments with the amylase genes that show how this technique is suitable for expression tests in vivo and how expression and glucose repression of amylase genes are determined by cis-acting sequences present on the cloned sequences examined (Section 1.1). In section 1.2 an already published analysis of the promoter sequences of the Amy-1 gene will be summarized and in section 1.3 the analysis of an hybrid gene, Amy-luciferase is reported, that shows that amylase promoter sequences mediate characteristic amylase-specific expression patterns of the luciferase reporter gene.

3.1.1 Somatic transformation with amylase genes in *Drosophila melanogaster*

The somatic transformation assay (see Materials and Methods) is based on micro-injection of *D. melanogaster* embryos of an Amy^{null} stock with plasmids carrying intact *Amy* genes or derivative constructions (e.g. deletions). Transformed third instar larvae derived from the injected embryos were examined for amylase activity, because at this stage amylase activity is very high and also the glucose repression effect is greatest.

The transient expression assay was first applied to the *Adh* gene of *D. melanogaster* by Martin et al. (1986). It was shown that this gene was normally expressed when injected in Adh^{null} embryos.

At first, intact *Amy* genes were tested. These genes contain non-mutated amylase promoter and coding sequences. The *Amy-1* proximal genes from the Oregon R and Canton S stocks were examined. *Amy-1* clones which contained 1600, 430, 163 bp of promoter sequences were used (pOR5.0, pORH/E, pCS3.8 respectively). Also various concentrations of plasmid DNA carrying the amylase genes were tested: 1 mg/ml, 0.5 mg/ml and 0.1 mg/ml.

Expression of amylase activity by an intact *Amy-1* cloned gene is observed in each single transformed larva (Figure 2A). The expected amylase enzyme mobility is observed. The level of activity expressed is comparable to that of wildtype Oregon R larvae. This

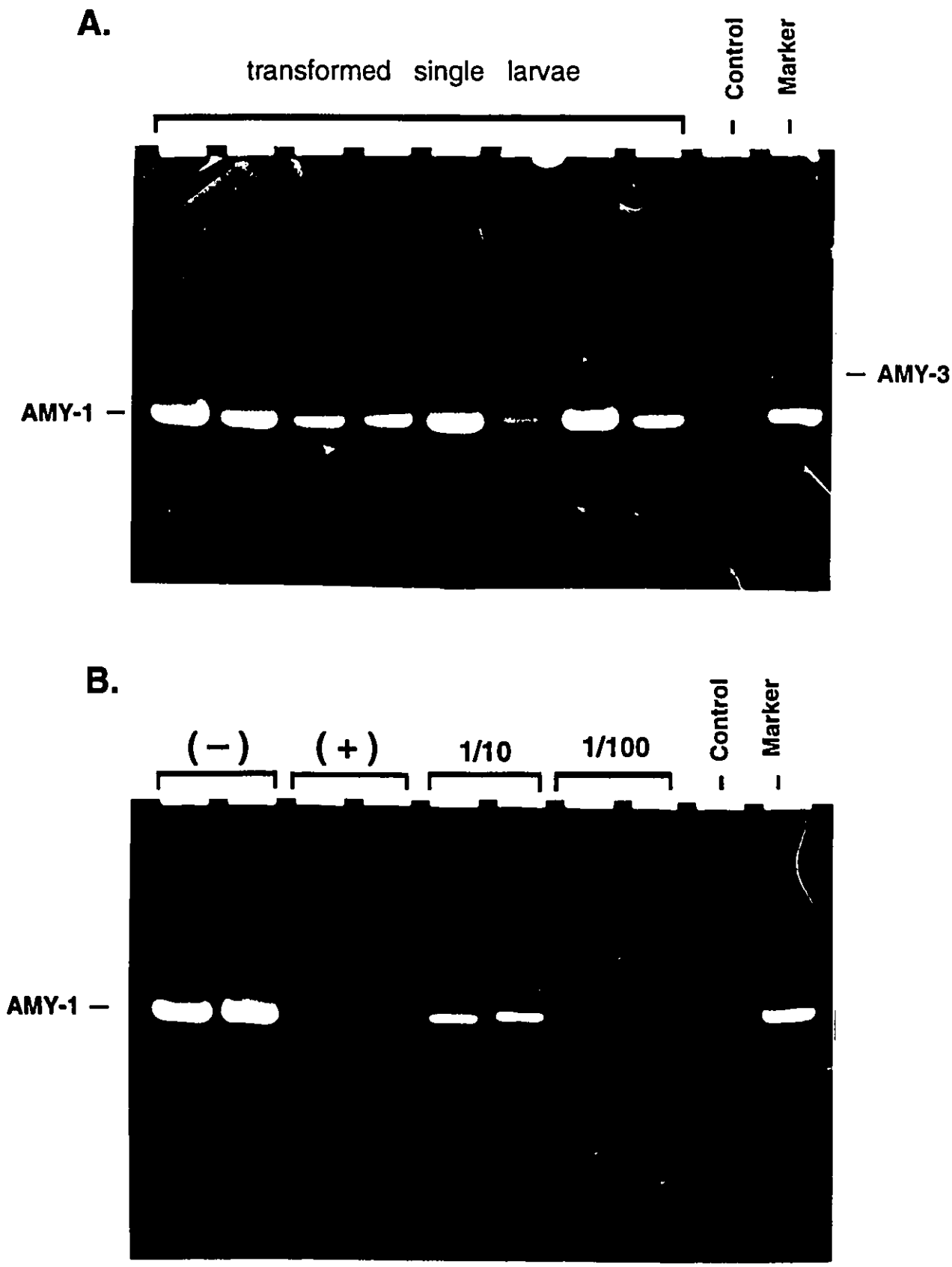
Figure 2

Transient expression assays with the Amy-1 gene of Drosophila melanogaster.

A. The Amy-1 gene is expressed in somatically transformed larvae: Single larvae transformed with an intact Amy-1 gene construct, (pOR5.0), and grown on food lacking glucose for full expression, were tested for amylase activity in electrophoretic gel. Larvae developed from embryos of the Amy^{null} stock which had been micro-injected with the construct. Control = sample (1 larva equivalent) from an homogenate of non-transformed Amy^{null} larvae, also grown on glucose-free medium; Marker is a sample from a Canton S fly homogenate, which produces an AMY-3/AMY-1 phenotype.

B. Wildtype expression and glucose repression by a short Amy-1 gene construct: Homogenates of larvae transformed with an Amy-1 construct (pCS3.8), carrying only 163 of promoter sequences, were prepared and assayed for amylase activity in electrophoretic gel (see Materials and Methods). One larval equivalent was loaded in each lane of the gel. Larvae were raised on two types of food; Food lacking (-) glucose for full expression of amylase activity and on glucose-rich food (10% glucose) to test glucose repression. A ten times (1/10) and a hundred times (1/100) dilutions of the larval homogenate from the glucose-free treatment are included.

Control as in A. Marker is a sample of Oregon R larval homogenate, which produces AMY-1 phenotype.


A.
transformed single larvae
Control
Marker
AMY-1
AMY-3
B.
(−)
(+)
1/10
1/100
Control
Marker
AMY-1

indicates normal expression of the Amy-1 gene. In this case the Amy-1 clone carried 1600 bp of upstream sequences.

Homogenates from multiple larvae were subsequently examined in an experiment testing a shorter version of the Amy-1 gene (pCS3.8) (Figure 2B). The expression of amylase activity in transformed larvae is again wildtype and also glucose repression of amylase activity is observed in larvae grown glucose-rich food. The level of glucose repression can be estimated by comparison with the dilution series of the non-glucose homogenate, and is about 100-fold as seen in the original stock (Benkel and Hickey, 1987).

This expression pattern was observed for all the above mentioned constructs and concentrations, indicating that the Amy-1 gene is regulated normally in transient expression assays. Also the short CS3.8 Amy-1 construct, with only 163 bp of promoter sequences is still completely functional and thus must contain all sequences necessary for normal expression and glucose repression.

Other amylase genes derived from various stocks that presented different mobility and or glucose repression behaviour were also tested. All genes tested retain the electrophoretic mobility in somatic transformation experiments and the characteristic glucose repression pattern shown in the stock of origin. Some genes show only a mild response to presence of glucose. For instance, the Amy-3 gene construct from Canton S shows normal expression levels and a low response to glucose repression, when micro-injected in Amy^{null} embryos, since activity on glucose-rich food is higher than

activity of 1/10 dilution of the glucose-free homogenate (Figure 3A).

Next we examined the behaviour of two different genes when co-injected as a mixture in *Amy*^{null} larvae. The *Amy-3* construct was co-injected with the *Amy-1* gene which has a different mobility and glucose repression response. Both constructs retained their characteristic expression pattern (Figure 3B). This indicates that *cis*-acting sequences on the cloned genes are responsible for the expression and glucose repression pattern, independently from environmental or experimental conditions.

Amylase expression was also examined in transformed adult flies, derived from embryos injected with *Amy-1* and *Amy-6* constructs, presenting different electrophoretic mobility and glucose repressibility. The amylase activity was tested in homogenates from several adult flies and larvae. The amylase activity resulted lower (about 1/10) in adults than in the larvae from the same experiment, but activity was clearly visible. The glucose repression pattern was similar to the one of the larvae. Probably in adults plasmids are partially degraded, as observed by Martin et al. (1986), accounting for the lower expression observed.

The results of our tests show that intact cloned amylase genes are expressed normally, and are glucose repressed in transformed larvae. *Cis*-acting sequences are thus present on the cloned sequences that determine expression of different electrophoretic variants, encoded by the structural gene, and also mediate

Figure 3

Test for expression and glucose response of the Amy-3 distal gene of Drosophila melanogaster.

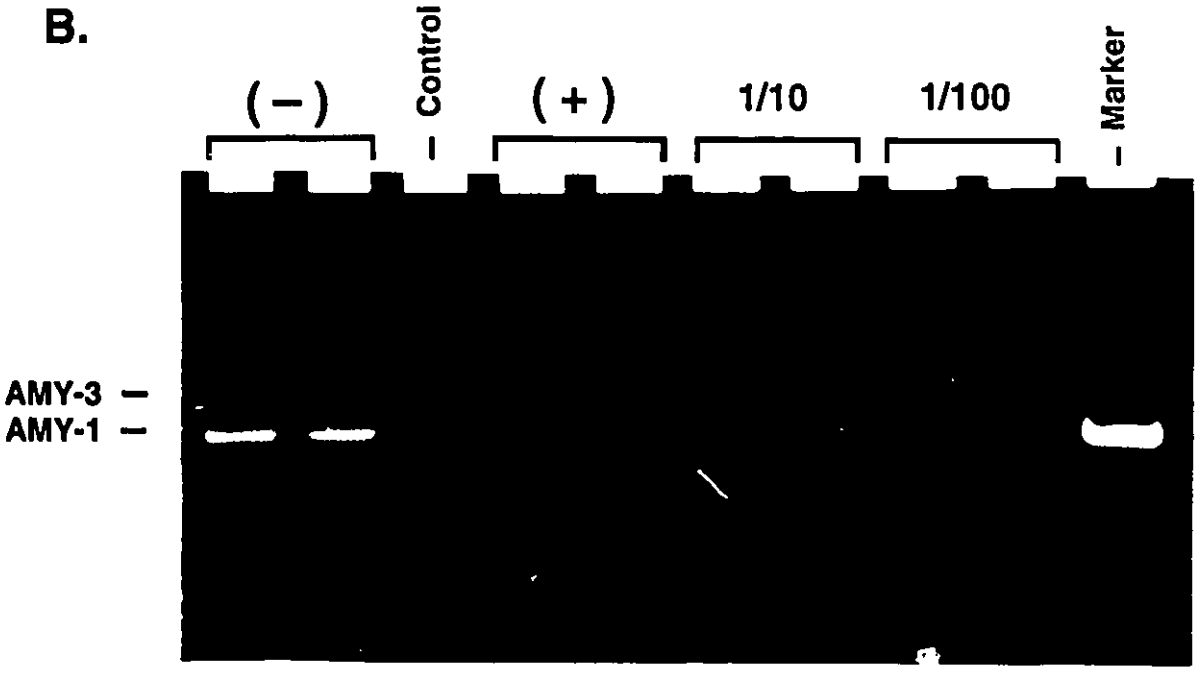
A plasmid carrying the Amy-3 distal gene from Canton S strain (pCS5.5) was injected in embryos of the Amy^{null} stock by itself (panel A) or together with the Amy-1 gene (pCS3.8) (panel B). Developing larvae were grown on food without (-) or with (+) added glucose to assess expression and glucose response. Homogenates of larvae grown on different foods were tested for amylase expression in electrophoretic gel stained for amylase activity. A ten times (1/10) and one hundred times (1/100) dilutions of the non-glucose treatment homogenate were included for comparison.

The control sample is from a homogenate of non-transformed larvae of the host stock. Marker is a sample from the Oregon R larval homogenate producing AMY-1 phenotype.

A.


(−)
(+)
1/10
1/100
Control
Marker
AMY-3
AMY-1

B.


(−)
Control
(+)
1/10
1/100
Marker
AMY-3
AMY-1

variation in the degree of sensitivity to glucose.

Next we proceeded in an analysis of newly engineered constructions of the Amy-1 gene with the aim of identifying regions on the amylase gene important for its expression.

3.1.2 Analysis of amylase promoter sequences using somatic transformation assays

The Amy-1 gene has been the subject of studies aimed at defining regions important for expression and glucose repression within the promoter sequence. Newly engineered Amy-1 constructs were examined for expression ability against a control Amy-6 gene, that produces a distinct electrophoretic variant and is only mildly repressed.

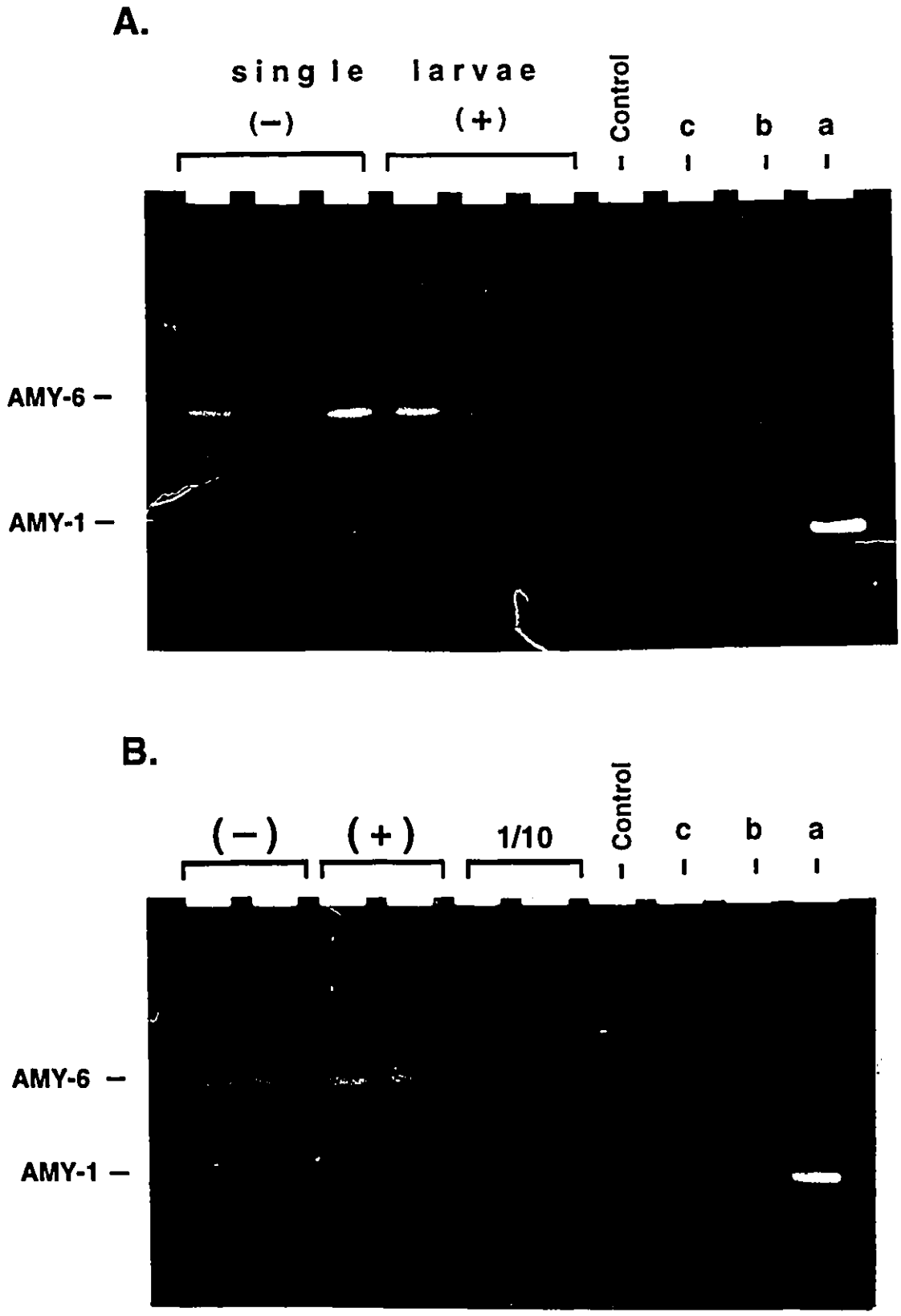
In Figure 4, the result of a typical experiment of somatic transformation using an intact Amy-1 gene is shown, as an example of the procedure used in these experiments. Somatic transformation of the Amy^{null} stock was carried out with a 1:1 mixture of the Amy-6 gene, as control, and the pCS3.8 Amy-1 gene. Clearly both genes are expressed at normal levels, but the activity shown by the two genes are different in larvae that are exposed to glucose: Amy-1 is highly repressed while Amy-6 is not affected by the glucose treatment. This result is apparent both in the single larvae gel (4A) and in the homogenates (4B), and the latter analysis was routinely used when assessing expression capacity of new constructs. The presence of AMY-6 activity in the glucose-rich homogenate serves as control. It is worth noting that the two genes have only 163 bp upstream of transcription start and still present wildtype expression pattern characteristic of the stock of origin.

Using this kind of analysis a series of deletions and

Figure 4

Expression and glucose repression of cloned Amy-1 and Amy-6 genes in somatically transformed larvae.

The pCS3.8 Amy-1 gene and the CM.18 Amy-6 gene each carrying 163 bp of promoter sequences were co-injected in embryos of the Amy^{null} stock and developing larvae were grown on medium without (-) or with (+) 10 % added glucose. Single third instar larvae (in panel A) or homogenates of multiple larvae (panel B) were examined for amylase expression in electrophoretic gels. Control = homogenate of not injected larvae grown on food lacking glucose; c, b, a = dilution series of wildtype Oregon R larvae grown on glu(-) food (1/100, 1/10, 1 larval equivalent). In B, homogenates were prepared from 8 larvae and replicates of 1 larval equivalent were loaded in each lane; a ten times (1/10) dilution sample of the glu(-) homogenate was run for quantification.


A.
single larvae
(−)
(+)
Control
c
b
a
AMY-6
AMY-1
B.
(−)
(+)
1/10
Control
c
b
a
AMY-6
AMY-1

mutagenized construct of the Amy-1 gene were tested. Here I will summarize the results of these investigations already published in Magoulas et al. (1993b).

The first study consisted of an analysis of constructs carrying fine scale deletions of the Amy-1 gene, in order to find the minimal promoter length sufficient for expression and glucose repression. Plasmids carrying further deletions of the 163 bp long Amy-1 gene were constructed using Polymerase Chain Reaction (PCR) and cross-over linker techniques (see Materials and Methods and Magoulas et al., 1993b). The constructs were tested against the Amy-6 gene in somatic transformation experiments.

A construct carrying 109 bp of promoter sequences till transcription start site shows full expression and glucose repression (data not shown). In Figure 5 the results obtained with a deletion carrying only 92 bp is reported. Expression of the Amy-1 gene is lowered to less than a tenth (Figure 5A), but glucose repression is still mediated by this construct, as can be clearly seen when higher concentrations of the construct are micro-injected (Figure 5B). Thus, sequences between -109 and -92 are important for gene expression. When only 82 bp of promoter sequences are left in the construct, expression of the amylase gene cannot be scored. With this study the minimal sequences of Amy-1 promoter necessary for wildtype expression are defined at 109, while sequences between -109 and -92 result to be relevant for gene expression.

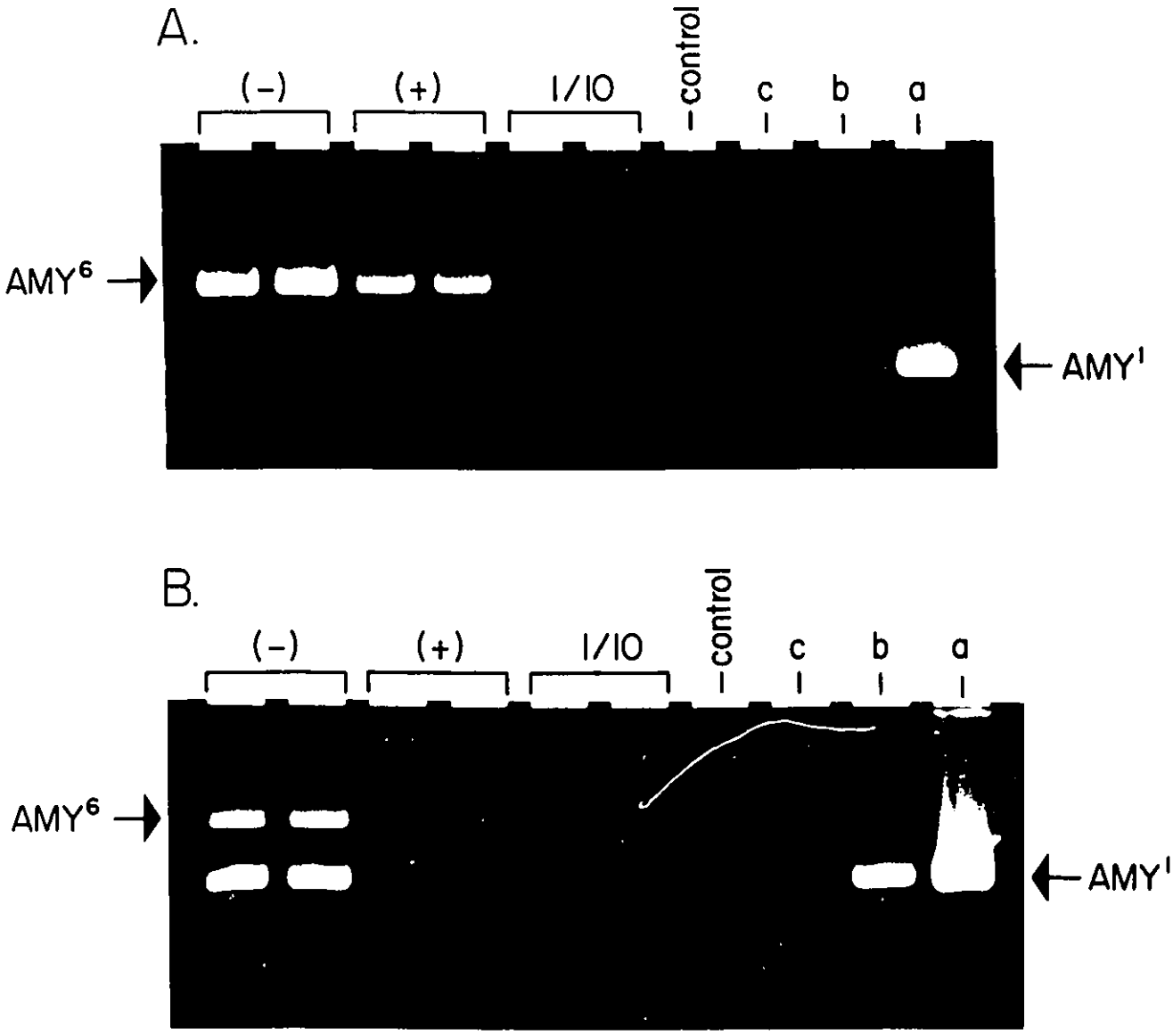
In a second study, the 109 bp promoter was subjected to site-

Figure 5

Expression and glucose repression of Amy-1 gene constructs with shorter promoter sequences.

In A and B the expression of the Amy-1 gene carrying only -92 bp of upstream sequences (provided by C. Magoulas, see Magoulas et al., 1993b and Materials and Methods) is tested in somatic transformation experiments.

The -92 bp construct was co-injected in Amy^{null} embryos together with the Amy-6 gene plasmid in a 1 :1 ratio (Panel A) and in a 20 : 1 ratio (.5 mg/ml of Amy-1 to .025 of Amy-6) (panel B) to increase amylase activity. Third instar larvae were grown on medium without (-) and with glucose (+). Replicates of samples of the different homogenates were run on electrophoretic gels and stained for amylase activity. Also a ten-fold dilution of the sugar-free grown larval homogenate was run for quantification; control = sample of homogenate of not injected larvae; c, b and a = 1/100, 1/10 and 1 larval equivalent dilution series of wildtype Oregon R larvae grown on glucose free medium.
(From Magoulas et al., 1993b).


A.
(-)
(+)
1/10
control
c
b
a
AMY6
AMY1
B.
(-)
(+)
1/10
control
c
b
a
AMY6
AMY1

directed mutagenesis analysis, in order to find, within this smaller region, elements responsible for repression and glucose repression. Within the promoter, between positions -92 and -17, small regions of 10 bp at a time were mutagenized and expression of the constructs was tested in somatic transformation assays. In Figure 6, the results of this analysis are shown: When the regions that include the TATA box or the CAAT-like box are disrupted, activity of the Amy-1 gene is reduced greatly, confirming the importance of these two promoter elements in gene expression. This result also indicates that the site-directed mutagenesis analysis is useful for the identification of elements that are essential for transcriptional activity. From Figure 6 it can also be seen that a region to the left of the CAAT box is important for full gene expression, confirming the results of the deletion analysis, where the -82 promoter did not produce visible amylase activity.

None of the constructs examined showed disruption of glucose response since all the mutagenized constructs showed glucose repression capability. This result raises the possibility that multiple elements mediate the glucose repression response, and thus the disruption of one element through mutagenesis does not bring about the absence of glucose repression. A similar result was obtained by Flick and Johnston (1990) in the study of glucose repression of the galactose gene GAL1 in yeast. They showed that a short region upstream the GAL1 gene is sufficient for glucose repression and a saturation mutagenesis analysis of this region did

Figure 6

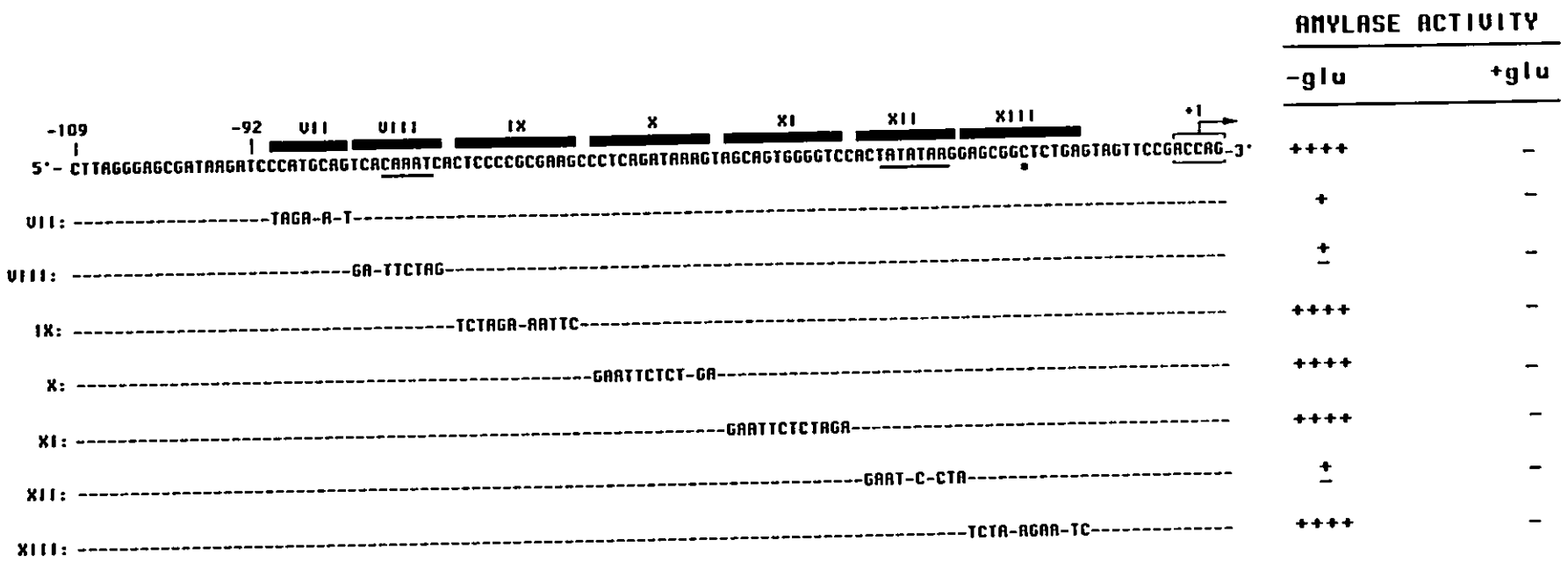
Site-directed mutagenesis of the amylase promoter.

A set of amylase constructs, VII to XIII was assayed for e::pression in somatically transformed larvae. The constructs were provided by C. Magoulas (Magoulas et al., 1993 b) and present each a different mutagenized region, stretching over the regulatory upstream sequences of the Amy-1 gene. The regions that were the targets of mutagenesis are shown by a black bar over the wildtype amylase sequence; the individual nucleotide substitutions for each amylase construct are shown. An asterisk marks position -17. (A promoter carrying sequences from -430 till -17 was shown to mediate correct expression and glucose repression of a reporter gene - see Section 3).

Each construct was co-injected with an amylase control construct, the Amy-6 gene, into Amy^{null} embryos.

The relative amylase activity in transformed larvae grown on a medium with(+) or without (-) glucose is presented as ++++ = 100%; + = less than 25%; +/- = less than 5%; - = no activity or less than 1%.

(from Magoulas et al., 1993b).


AMYLASE ACTIVITY
-glu
+glu
-109
-92
VII
VIII
IX
X
XI
XII
XIII
+1
5'- CTTAGGGAGCGATAAGATCCCATGCAGTCACAAATCACTCCCCGCGAAGCCCTCAGATAAAGTAGCAGTGGGGTCCACTATATAAGGAGCGGCTCTGAGTAGTTCCGACCAG-3'
++++
-
VII: ----------TAGA-A-T----------
+
-
VIII: ----------GA-TTCTAG----------
±
-
IX: ----------TCTAGA-AATTC----------
++++
-
X: ----------GAATTCTCT-GA----------
++++
-
XI: ----------GAATTCTCTAGA----------
++++
-
XII: ----------GAAT-C-CTA----------
±
-
XIII: ----------TCTA-AGAA-TC----------
++++
-

not localize a single regulatory element, suggesting the possibility of functionally redundant multiple elements. More recent direct evidence of these multiple elements has been reported (Flick and Johnston, 1992). Also elements that mediate the heat-shock response have been found in multiple copies in all promoters of the duplicated heat-shock genes of different species (Bienz, 1985; Sorger, 1991). These elements often present some variation in their position in respect to fixed elements like TATA motifs. The heat-shock response, like the glucose effect, is a basic response to environmental change.

In summary, the study of the Amy-1 promoter region has shown that a construct with only 109 bp from transcription start, instead of the 1600 originally present on the cloned gene, has sufficient information to mediate full expression of the gene. A region important for high levels of expression is between -92 and -82. Upstream deletions that extend to position -82 lack the ability to express amylase.

Mutagenesis studies of the Amy-1 gene with 109 bp of upstream sequences confirmed that a region important for gene expression is present upstream of -82. It also confirmed the importance of the TATA motif and CAAT-like motif in this promoter, as already known for many other eukaryotic gene promoters (Hoey et al., 1990; Nussinov, 1990). Finally it pointed out that multiple elements in the Amy-1 promoter may be mediating glucose repression.

3.1.3 Amylase promoter sequences regulate expression of an unrelated reporter gene, firefly luciferase

Using the somatic transformation assay, Amy promoter sequences (500 bp) were examined for their ability to regulate the expression of an unrelated gene, the luciferase gene from the firefly. The coding sequences of the luciferase gene were used as reporter of gene expression. This gene has been previously used as reporter gene in bacteria and in plants (Ow et al., 1986), both in transient expression assays and in transgenic plants, and proved to be a very sensitive reporter gene.

A construct was prepared (by B. Benkel, see Materials and Methods) that has 477 bp of 5' flanking sequences of the Amy-1 gene from Oregon R strain linked to luciferase coding sequences. The purpose was to determine if expression and glucose repression mediating sequences, present on the cloned Amy-1 genes previously examined, are in fact localized in the promoter region of the amylase gene.

Somatic transformation experiments were conducted following the usual protocol. Third instar larvae transformed with the construct and grown on food with and without glucose were tested for emission of light in a scintillation counter (see Materials and Methods) In Table 1 the values of luciferase activity in transformed larvae and in non-injected larvae are reported. While transformed larvae grown under full expression conditions (on food lacking

Table 1

Luciferase activity expressed by the Amy-luciferase construct is glucose repressible.

Food Treatment	N	Luciferase activity (average cpm ± S.E.)
Glu $^{-}$	10	5,400,000 ± 1,500,000
Glu $^{+}$	5	318 ± 101
Control	7	200 ± 82

Embryos of the Amy^{null} stock were micro-injected with the Amy-luciferase construct (1mg/ml) provided by B. Benkel (see Materials and Methods). Transformed larvae were grown on food without glucose (glu^{-}) and with glucose (glu^{+}) and tested individually for light emission in a scintillation counter (see Materials and Methods) as well as non-injected larvae (control).

Average count per minute (cpm) and S.E. is reported for each group of larvae.

N = number of larvae examined.

glucose) show very high levels of light emission, transformed larvae grown in glucose food, present values not significantly different from the one observed in the non-injected larvae. These latter samples represent the background level. Although the number of larvae examined is not great, a few conclusions can be drawn: Expression of the hybrid construct is efficient, (also 1/10 of larval equivalent showed readings around 10^6 - 10^5); in glucose grown larvae the expression returns to background levels, indicating that the Amy promoter sequences act as mediators of glucose repression. The difference in expression between light emission values in the two media is around 10^4, much higher than the one observed in amylase activity in intact amylase genes. This might be due to the high sensitivity of the luciferase assay.

In conclusion, the luciferase gene appears to be an efficient reporter gene in Drosophila. We were able to show that Amy promoter sequences mediate glucose repression of an unrelated coding sequence. Evidence confirming this observation will be reported in Section 3.3, where a hybrid gene with Amy promoter sequences linked to Adh coding sequences is examined in germline transformants.

3.2 Analysis of proximal and distal amylase genes of D. melanogaster

The proximal and distal amylase genes from the Makokou strain of D. melanogaster have been previously cloned (S. Abukashawa, Ph.D. thesis, 1990) and here I report results from expression tests carried out on these genes and derivatives, via somatic transformation assays. The Makokou strain is interesting since it presents two electrophoretic variants, AMY-4 and AMY-6, that allow phenotypic discrimination of the proximal and distal genes. Also, the two amylases of the Makokou strain are repressed differently when exposed to dietary glucose (Benkel and Hickey, 1986b). The whole amylase region of the Makokou chromosome was completely sequenced in our lab, thus allowing a comparison between the amylase genes structure and expression, both at the level of the structural genes and at the level of regulation of gene expression with an analysis of promoter sequences.

Data on the Amy-1 proximal gene (see Section 3.1) are used in a comparison, to test variation between alleles at the proximal locus (Amy-1 and Amy-4) versus variation between duplicated genes.

3.2.1 Study of proximal and distal amylase genes of the Makokou strain

The expression of proximal and distal amylase clones was tested in somatic transformation experiments. The two EcoRI to EcoRI clones with the proximal and distal genes, transcribed in opposite directions, are respectively about 5 and 6 kbp long and carry about 1.6 and 2.9 of upstream sequences (Figure 7A) (see Materials and Methods).

In somatic transformation experiments, when injected separately in embryos of the Amy^{null} stock, the cloned *Amy* proximal gene produced the AMY-4 phenotype while the *Amy* distal produced the AMY-6 variant, both typical of the stock of origin (Figure 7B). When injected together, they give the AMY-4/AMY-6 electrophoretic pattern characteristic of the Makokou stock. Thus we can assign to *Amy* proximal the AMY-4 phenotype and to *Amy* distal the AMY-6 phenotype.

The availability of DNA sequence for the region containing the two amylase genes from the Makokou strain, along with the DNA sequence of *Amy-1* proximal gene from the Oregon R strain (Hickey et al., 1987; Boer and Hickey, 1986) allows us to compare the characteristics of the amylase proteins (in the expression tests and in the stock of origin), with the one expected on the basis of the available DNA sequence of these genes.

Figure 7

Amylase genes from the Makokou chromosome: gene structure and expression.

Panel A

Map of an 11 kbp region from a Makokou strain of *D. melanogaster* including the amylase genes.

The map is derived from the DNA sequence of genomic clones. *Amy-p* = Amylase proximal; *Amy-d* = Amylase distal. Black blocks indicate amylase coding regions; shadowed block indicates coding region of serpin gene. Arrows show direction of transcription. B = *Bam*HI ,E = *Eco*RI, H = *Hind*III, S = *Sal*I.

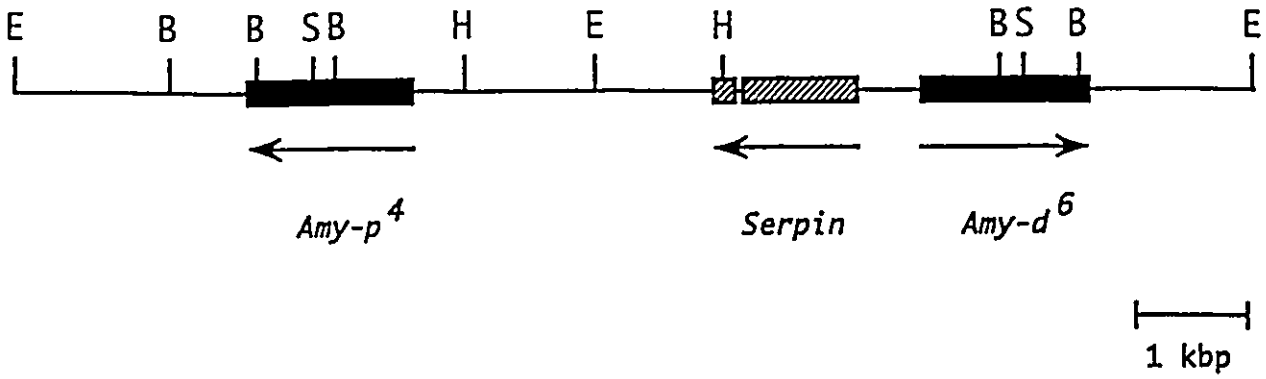
Panel B

Expression of cloned *Amy* proximal and *Amy* distal genes from Makokou.

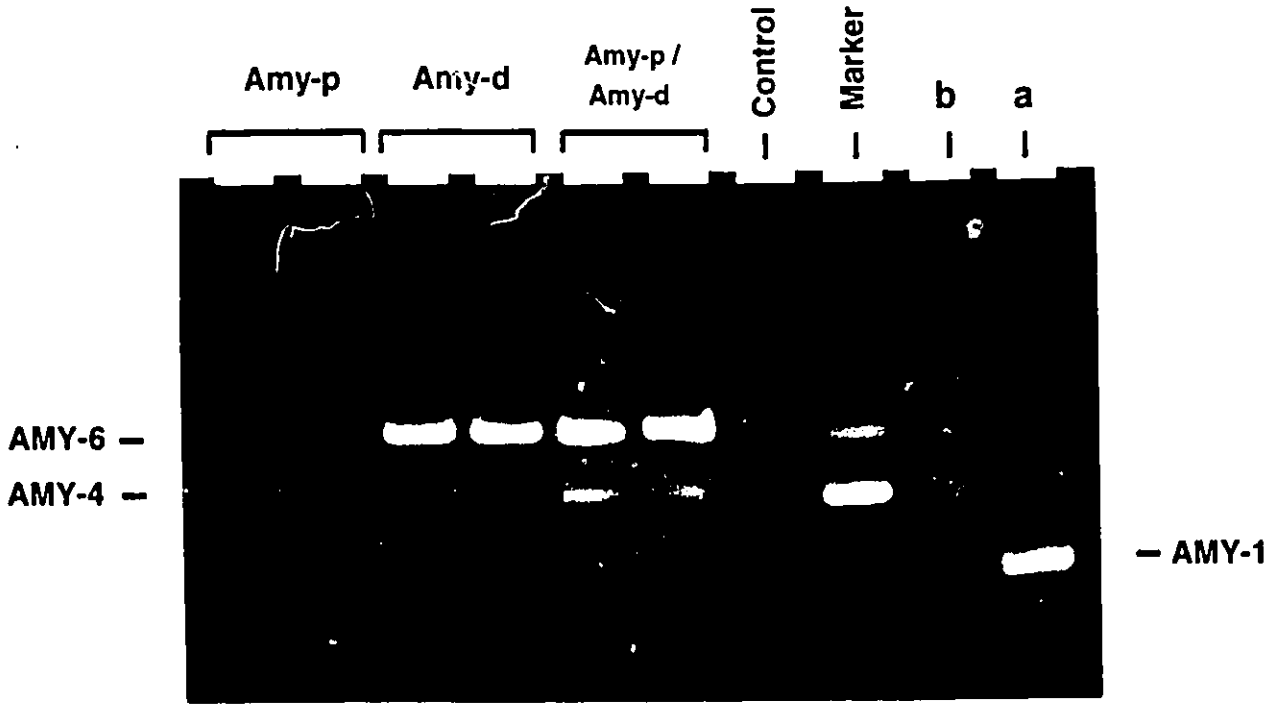
Embryos of the Amy^{null} stock were somatically transformed with the 5 and 6 Kbp long *Eco*RI-*Eco*RI fragments carrying the *Amy* proximal (*Amy-p*) and *Amy* distal (*Amy-d*) gene constructs or with a 1 : 1 mixture of the two (*Amy-p/Amy-d*). Third instar larvae derived from these embryos were raised in glucose-free medium for full expression and tested for amylase activity in electrophoretic gels. Control = homogenate of third instar larvae derived from non-injected embryos and raised in glucose-free medium; Marker = homogenate from Makokou stock larvae; b,a = samples (1/10, 1 larval equivalent) from homogenate of wildtype Oregon R larvae grown on glucose-free medium.

A.

Amylase Locus of *Drosophila melanogaster* (Makokou strain)



B.



For instance, the values of pI (isoelectric point), were calculated on the basis of the derived amino acid (aa) sequence for the Amy-4 and Amy-6 genes from Makokou and Amy-1 gene of Oregon R (Table 2). The calculated values of pI increase from AMY-1 to AMY-4 to AMY-6: Over the length of 495 amino acids, the number of aa with acidic side group (R) is the highest in AMY-1 and lowest in AMY-6, while, at the same time, the number of basic aa increases from AMY-1 to AMY-6. The calculated values of pI enable us to predict the migration pattern of the amylase proteins, based on the fact that a lower pI means that equilibrium between acidic and basic residues is reached at lower pH, indicating that a higher number of negatively charged side groups are present on that protein; thus, this will migrate faster towards a positive pole in electrophoretic gels. For the amylase variants, the calculated pI values are consistent with the actual migration pattern observed in electrophoretic gels (pH 8.9), where AMY-1 with lowest pI (5.39) is the fastest migrating and AMY-6, with pI of 5.95 is the slowest migrating protein. In conclusion, molecular studies are consistent with previous results obtained by protein analysis.

We can also compare the derived aa sequence and the nucleotide sequence of the coding regions of the three amylase genes. Only two amino acid substitutions, both causing changes in the net charge of the protein, are found between Amy-6 and Amy-4, distal and proximal genes from Makokou, and 4 substitutions are present between Amy-4 and Amy-1, three of which cause variation in the charge of the

Table 2

Values of isoelectric point (pI) calculated on the predicted amino acid sequence for three amylase variants.

Protein	Calculated pI	No. of acidic aa	(%)	No. of basic aa	(%)
AMY-1	5.39	52	(10.5)	40	(8.1)
AMY-4	5.72	50	(10.1)	41	(8.3)
AMY-6	5.95	49	(9.9)	42	(8.5)

Total length of amylase protein is 495 AA. The number and percentage of amino acids with acidic and basic sidegroups are shown.

protein.

At the nucleotide level, 18 substitutions are found between Amy-6 and Amy-4, that is equivalent to a 1.2 % nucleotide difference over the 1485 bp length; 16 of these are silent substitutions, all in the third position; of the remaining two that cause aa changes, 1 is in third position and 1 in second. Between Amy-4 and Amy-1, both proximal amylase genes, there are 23 nucleotide changes (1.6%), 4 of which are responsible for aa substitutions. It is interesting to note that the number of changes is very similar, actually lower, between the proximal and distal genes from Makokou than between the two allelic proximal genes. Greater variation might be expected between the duplicated coding regions, based on the sequence divergence between the two promoters. This similarity between the duplicated loci is true also when only silent substitutions are considered, which do not affect the protein and are thus not subject to selection pressure. There are 16 silent substitutions between Amy-4 and Amy-6 and 19 between the two proximal alleles Amy-4 and Amy-1. An explanation for such similarity between proximal and distal coding sequences could be found in the occurrence of gene conversion processes between proximal and distal genes on the same chromosome, as suggested by the observation of concerted evolution of the duplicated genes of D. melanogaster and D. erecta as reported in Hickey et al., 1991. The similarity is limited to the coding regions (see Section 2.4 where promoters are compared).

3.2.2 Regulation of expression of the amylase proximal and distal genes

The two cloned genes from Makokou strain, Amy-p (or Amy-4) and Amy-d (or Amy-6) where analyzed for their expression pattern and for the role of promoter sequences in the regulation of expression.

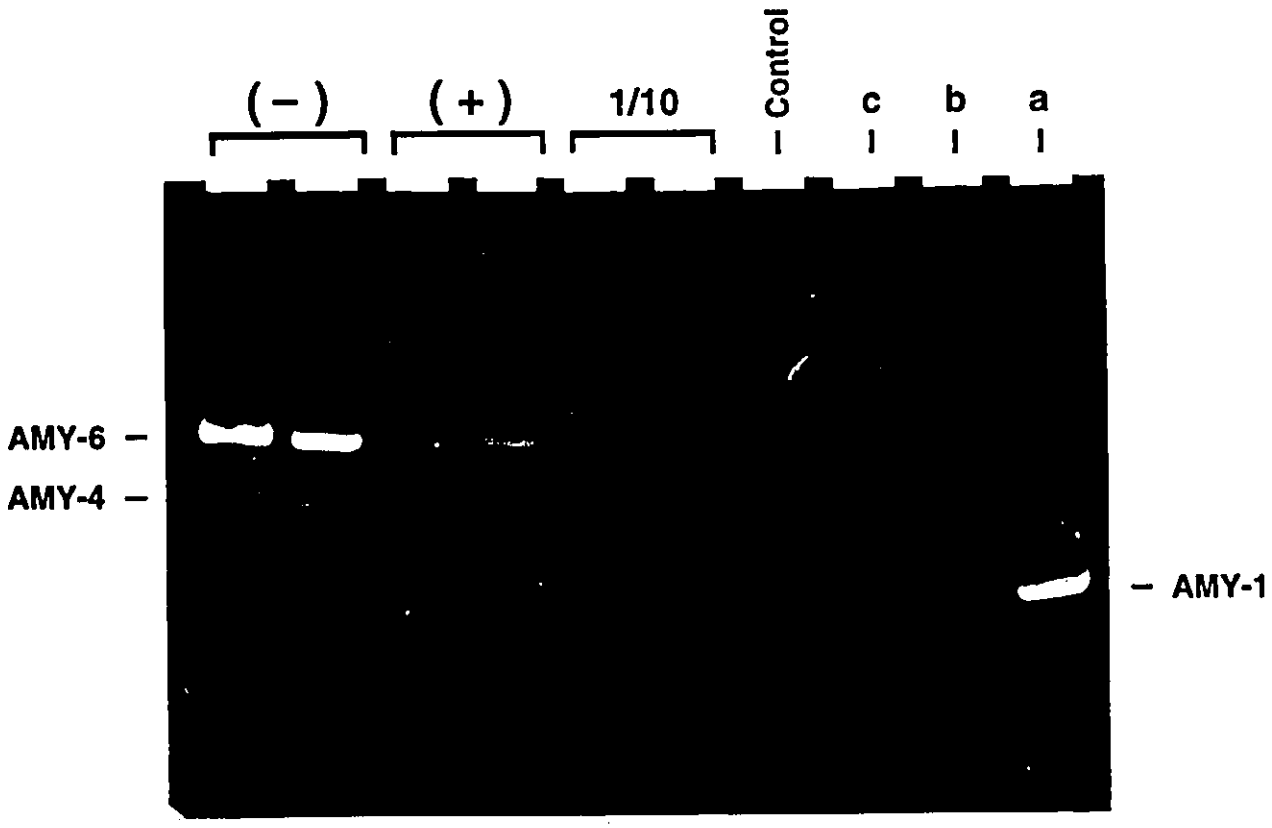
The Amy-4 and Amy-6 cloned genes present a different response to dietary glucose in somatic transformation experiments (Figure 8). While both constructs are expressed in glucose-free medium, in glucose-rich medium the Amy-4 construct shows very little activity (less than 1/10 of the non-glucose homogenate's activity), while the Amy-6 produces amylase activity almost as high as the one observed in the non-glucose expression food. This differential glucose response by the two genes was previously observed in the Makokou stock of origin (Benkel and Hickey, 1986b). From the somatic transformation experiments, it can be concluded that cis-sequences present on the two cloned genes are responsible for the high levels or low levels of glucose repression observed.

A deletion analysis of the proximal gene to identify promoter sequences important for expression and glucose repression phenomenon was previously done on the proximal amylase gene from Oregon R, Amy-1 (Section 3.1). The Amy-4 proximal gene gives results that are very similar to the Amy-1 proximal gene, not only for its expression and glucose repression, but also at DNA sequence level: In the upstream region there are only 4 polymorphic sites in the first 200 bp upstream from ATG, a region that is sufficient for

Figure 8

Different glucose response by Amy-4 and Amy-6, proximal and distal amylase genes from Makokou.

The Amy-4 and Amy-6 genes from Makokou show different glucose repression levels in somatic transformation experiments. Amy^{null} embryos were co-injected with the Amy-4 and Amy-6 gene constructs and derived larvae were grown on glucose free (-) or glucose-rich diets (+) to test for expression and glucose-response of the two constructs. Homogenates of the two types of transformed larvae were electrophoresed and assayed for amylase activity. A ten times dilution (1/10) of the larval homogenate from the glucose-free treatment is included. Control = sample from homogenate of non injected larvae; c,b,a = dilution series (1/100, 1/10, 1 larval equivalent) of homogenate from larvae of the wildtype Oregon R stock, raised on glucose-free diet.


(−)
(+)
1/10
Control
c
b
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AMY-6
AMY-4
AMY-1

normal expression. Since the Amy-1 and Amy-4 proximal genes give expression results that are so similar, the functional analysis carried out on Amy-1 will be considered characteristic of the proximal gene.

As for the distal gene, a deletion analysis, similar to the one carried out for the Amy-1 proximal gene, was performed on the Amy-6 distal gene. The purpose is to identify minimal promoter regions necessary for wildtype expression, and to compare the distal and proximal promoters' organization.

First, a deleted construct carrying only 163 bp of promoter sequences, instead of the 2.9 kbp of the original cloned Amy-6, has been examined in functional tests for expression ability. This Amy-6 gene carrying only 163 bp of promoter sequences, construct CM.18, (see Materials and Methods), is expressed normally and is not repressed by dietary glucose, when tested in somatic transformation experiments with the highly repressible Amy-4 gene as internal control (Figure 9). Its expression pattern is thus very similar to the one of the "long" Amy-6 gene from which it was derived, as can be seen in a direct comparison in Figure 9B. In conclusion, it seems that constructs of the distal gene, like the proximal gene, with only about 200 bp of promoter sequences are capable of normal gene expression.

It is interesting to note that from the DNA sequence analysis we find that upstream sequences of the Makokou distal gene encode a serpin protein (see Figure 7A). This gene is a trypsin inhibitor

Figure 9

Expression pattern of a "short" Amy-6 gene construct carrying 163 bp of 5'- flanking sequences.

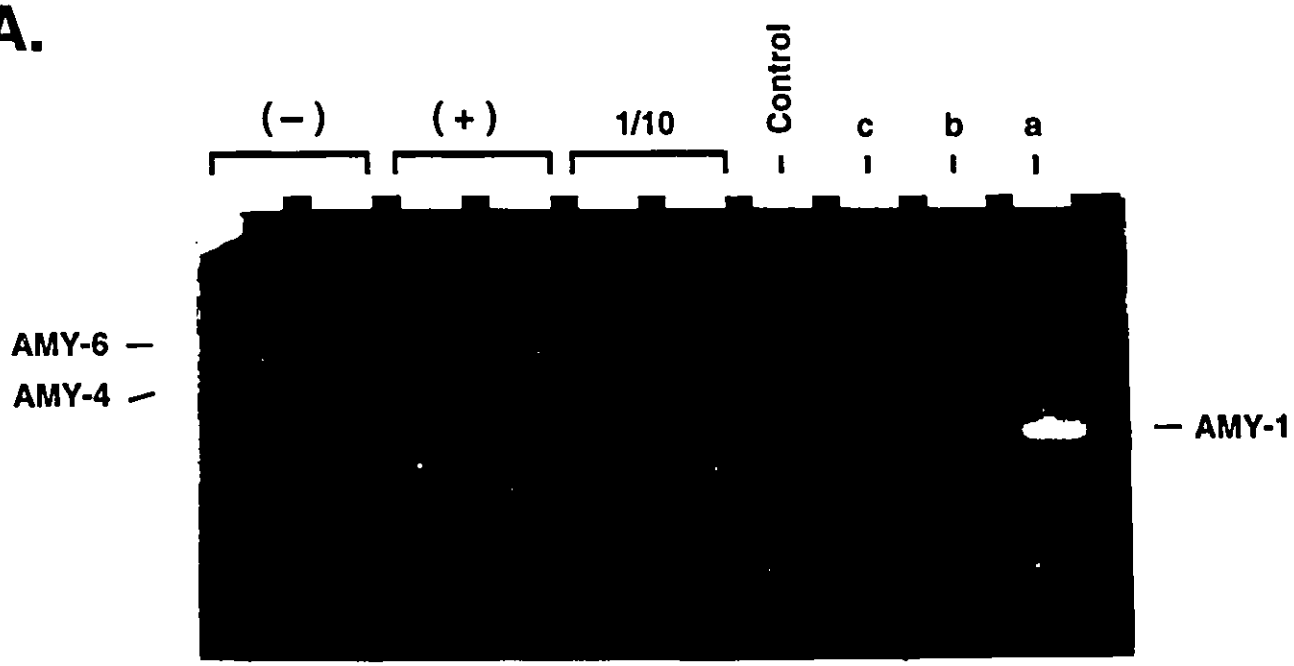
Panel A

Larvae somatically transformed with a 1:1 mixture of the -163 bp Amy-6 construct and Amy-4 construct, as control, are tested for amylase activity. Larvae were grown on medium without glucose (-) and with glucose (+). 1/10 = one tenth dilution of the glucose-free larval homogenate. Control = homogenate of Amy^{null} not injected larvae. c, b and a = dilution series (1/100, 1/10 and 1 larval equivalent) of wildtype Oregon R larval homogenate from larvae grown on glucose-free medium.

Panel B

Comparison of amylase activity and level of glucose response between "long" and "short" Amy-6 constructs carrying respectively 2.9 kbp and 163 bp of 5'-flanking sequences. Amylase activity of larval homogenates from two somatic transformation experiments in which the "long" and "short" Amy-6 construct were each injected in Amy^{null} embryos, with the Amy-4 control construct, in a 1:1 ratio, are examined on the same gel. (-) and (+) = homogenates from larvae grown on a glucose-free or glucose-rich diet. Controls = Homogenates from non-injected larvae.

A.


(−)
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Control
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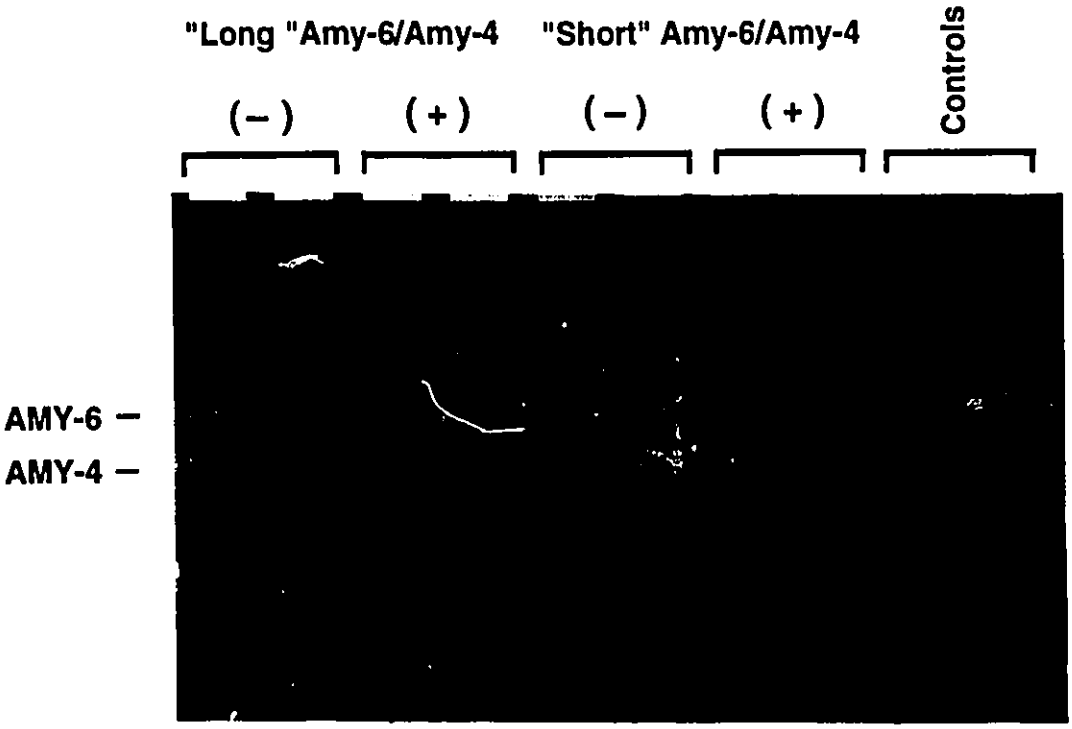
AMY-6
AMY-4
AMY-1

B.


"Long "Amy-6/Amy-4
"Short" Amy-6/Amy-4
(−)
(+)
(−)
(+)
Controls
AMY-6
AMY-4

and is transcribed in opposite direction from the amylase gene; thus, the about 500 bp that separate the serpin and amylase genes are occupied by their respective promoters; this is consistent with the fact that about 200 bp of promoter sequence probably contains all elements necessary for normal expression.

Recently Hawley et al. (1992) have reported that sequences upstream of -219 of the distal gene contain enhancer element(s). Our data indicate that regions upstream of -200 are not necessary for full expression, although the "short" Amy distal gene may be slightly less active than the "long" Amy gene. It is possible that small effects by enhancers on gene expression are magnified by experimental conditions.

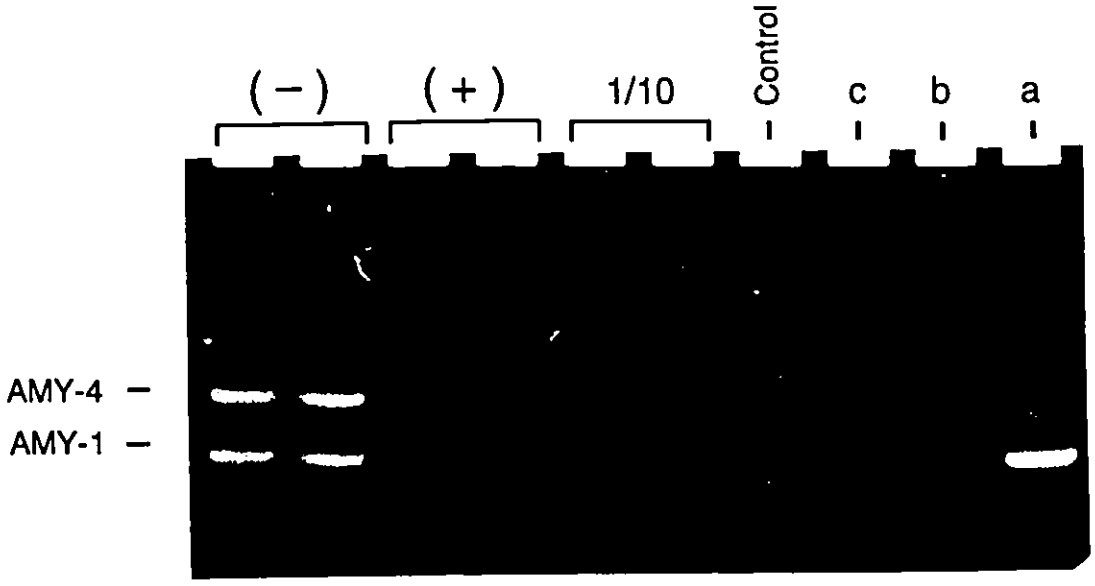
Also the tissue specific expression of the distal and proximal genes carrying only 163 bp of promoter sequences was tested by assaying amylase activity along the midgut of larvae transformed with respective Amy-1 and Amy-6 constructs (data not shown). The result was that the two genes are expressed basically in the same region, i.e. the posterior midgut.

To ascertain whether the Amy-6 promoter sequences are responsible for its expression pattern, a hybrid gene was constructed carrying the 163 bp long promoter of Amy-6 gene, linked to the coding and downstream sequences of the Amy-1 proximal gene, construct CM.14 (see Materials and Methods). A functional assay of this construct (see Figure 10), shows that it produces normal levels of the Amy-1 activity, but that the glucose response is the

Figure 10

Amylase expression by an hybrid construct (CM.14) carrying 163 bp of Amy-6 promoter sequences linked to Amy-1 coding and downstream sequences.

Amy^{null} embryos were injected with a mixture (1:1) of the hybrid construct (provided by C. Magoulas) and the Amy-4 gene, as control, producing Amy-1 and Amy-4 variants respectively. Larvae grown on two types of medium, glucose-free (-) and glucose-rich (+), to test expression and glucose repression of the construct were tested in electrophoretic gel stained for amylase activity. 1/10 = tenfold dilution of glucose-free treatment; Control = sample of homogenate of non-injected larvae; c,b,a = dilution series (1/100, 1/10, 1 larval equivalent) of wildtype Oregon R larvae,also grown on glucose-free medium.


(−)
(+)
1/10
Control
c
b
a
AMY-4
AMY-1

characteristic one of the distal gene, since activity in larvae exposed to glucose is still high. This experiment proves that differences in the amylase promoter sequences or in the organization of elements in this region, are responsible for the different glucose responses of the proximal and distal genes.

3.2.3 Fine scale deletion analysis of the Amy-6 promoter

A fine scale deletion analysis of the "short" Amy-6 gene was carried out with the purpose of defining regions of importance for expression within the 163 bp promoter region, similarly to what was done for the Amy-1 proximal gene. The constructs were prepared as described in Materials and Methods and tested for gene expression and glucose repression in transient expression assays. The Amy-6 derived constructs were co-injected in Amy^{null} embryos with the pCS3.8 Amy-1 construct. Third instar larvae grown on glucose-free and glucose-rich media were tested for amylase activity in electrophoretic gels. Results of the analysis are shown in Table 3.

The 143 bp construct shows reduction to about 10% of the activity from the 163 bp long construct, but still its glucose repression pattern remains unchanged (Figure 11, construct CM.29 = -143 bp promoter). A shorter promoter, 117 bp long, does not give detectable amylase activity. These results show that the region between -163 and -143 is important for high levels of expression, and elements mediating expression are present between -143 and 117, but deletions under -117 are not functional.

Table 3

Amylase activity shown by Amy-6 gene constructs carrying deletions of the promoter sequences.

PROMOTER	F O O D T Y P E	
	GLU -	GLU +
CM.18 165 bp	++++	+++
CM.29 143 bp	+	+
CM.26 117 bp	-	-

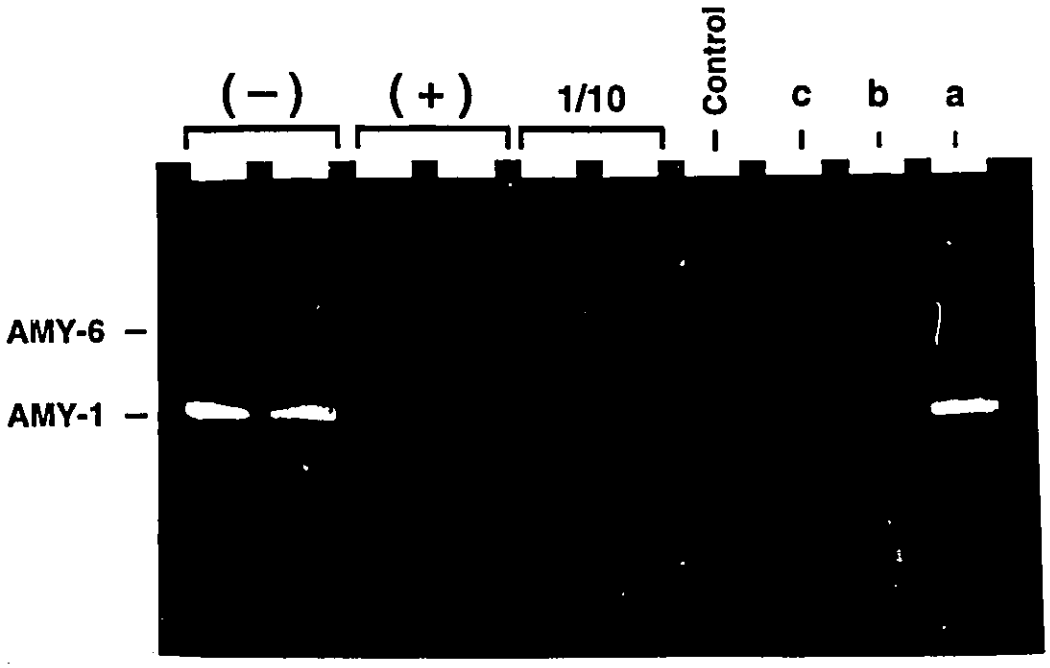
Estimates are based on amylase activity shown by third instar larvae somatically transformed with the indicated Amy-6 constructs provided by C. Magoulas (Magoulas, Ph.D. Thesis, 1992 and Materi. ls and Methods) and an Amy-1 construct, as internal control, which produces a distinguishable AMY variant.

Promoter = length of promoter sequences (in bp from transcription start point); GLU -, GLU + = larvae grown in glucose-free or glucose-rich food respectively. ++++ = 100% activity; + = 25% - 10% activity; - = no detectable activity.

Figure 11

Analysis of expression and glucose repression of a short Amy-6 construct carrying only 143 bp of promoter sequences (CM.29).

Electrophoretic gel showing amylase activity of third instar larvae transformed with equal amounts of CM. 29 construct, carrying a short Amy-6 gene, and the pCS3.8 Amy-1 construct as internal control. Amy^{null} embryos were injected with equal amounts of the CM.29 construct and the 3.8 Amy-1 construct. Larvae were raised in glucose-free (-) and glucose-rich (+) diet to test ability to express amylase and to respond to glucose. Homogenates of transformed larvae were tested together with a ten fold dilution of the glucose free treatment (1/10). Control = homogenate of Amy^{null} not injected larvae. c, b and a = dilution series (1/100, 1/10 and 1 larval equivalent) of wildtype Oregon R larval homogenate from larvae grown on glucose-free medium.


(−)
(+)
1/10
Control
c
b
a
AMY-6
AMY-1

3.2.4 Functional and sequence comparison of proximal and distal gene promoters

The results of the deletion analysis of the distal gene, Amy-6, can be compared to the one from the deletion analysis of the proximal amylase gene reported in Section 3.1. In the functional tests, both genes show normal regulation of expression when carrying only 163 bp of promoter sequences from transcription start (or circa 200 bp from ATG) (Figure 12). For both genes a reduced activity is observed when 5' flanking blocks are chopped away, till the expression ability is completely lost. However, in a small scale analysis, a 109 bp long Amy-1 promoter is expressed normally, while the Amy-6 promoter has to have at least 147 bp for some expression to occur. A comparison of the proximal and distal amylase promoters based on the functional assay is illustrated in graphic form in Figure 12A; here the similarity and differences in the promoter organization are evident.

The loss of activity occurs within a 30 bp region in a similar fashion in both promoters, but at a different position. If these regions, that are important for gene expression, are compared at the DNA level, an element showing high sequence similarity is found (Figure 12B). Over a stretch of 15 base pairs, 10 are identical. Moreover, this sequence is followed in both promoters by a CAAAT or CAAT element and also preceded by a GATAAG element (see below).

In order to clarify the differences in the organization and structure of the two promoters, a visual alignment of the 200 bp of

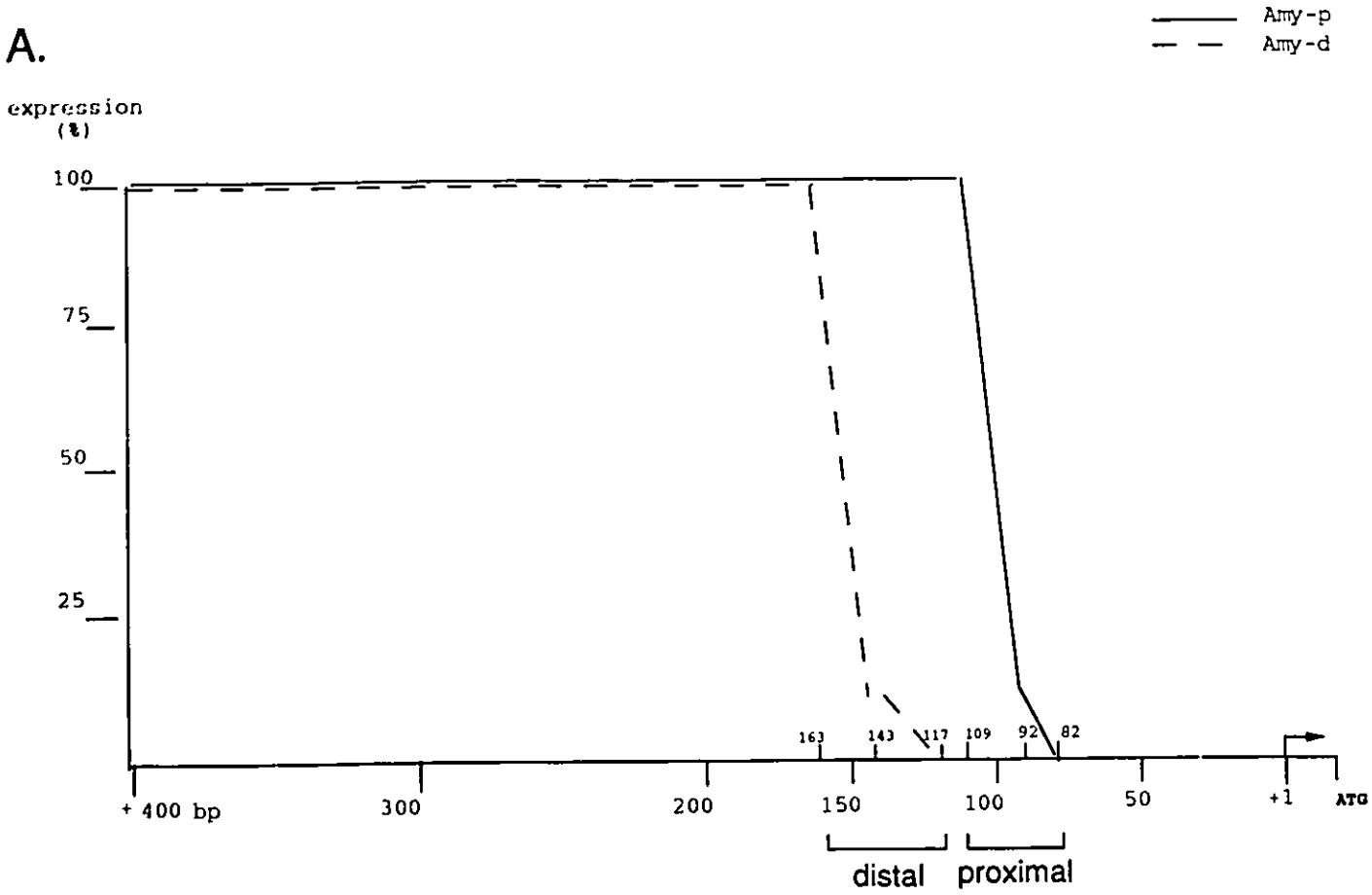
Figure 12

Comparison of loss of amylase expression shown by progressively deleted construct of the Amy-1 proximal gene and Amy-6 distal gene.

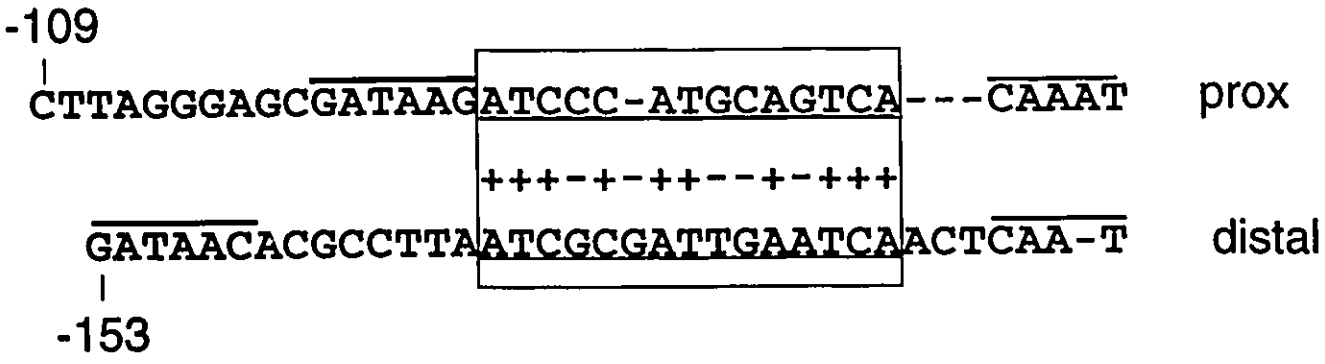
Panel A. Clones of the Amy-1 and Amy-6 gene carried initially 1.6 and 2.9 kbp of upstream sequences. Deleted constructs with 163 bp of promoter sequences still show normal level of expression for both Amy-1 and Amy-6 genes. Amy-1 proximal gene maintains high level of expression also when only 109 bp of promoter sequences are left; after that activity decreases quickly, within 30 bp, to almost null values when 82 bp long promoters are examined. For the Amy-6 gene normal levels of activity are present in a 143 bp long promoter construct, after that the activity decreases to null values at -117, in a 30 bp stretch, as for Amy-1.

Panel B. DNA sequence comparison of the regions important for gene expression in the two promoters.
Boxed is a 15 bp long sequence element that presents high sequence similarities. + and - = same or different nucleotide respectively. Other elements that are similar in the two promoters' regions are marked with a line.

A.


Amy-p
Amy-d
expression
(%)
100
75
50
25
163
143
117
109
92
82
+400 bp
300
200
150
100
50
+1
ATG
distal
proximal

B.


-109
CTTAGGGAGCGATAAGATCCC-ATGCAGTCA---CAAAT
prox
+++-+-++--+-+++
GATAACACGCCTTAATCGCGATTGAATCAACTCAA-T
distal
-153

promoters of proximal and distal genes (that is 167 from transcriptional start site) has been carried out (Figure 13) and correlated with the information obtained from the functional tests.

In the alignment of the two promoters, some important elements are easily identified and are present in similar positions relative to each other, and also are usually surrounded by region with high sequence similarity. These elements are the initiation of transcription, the TATA box, the CAAT box (actually a CAAAT box equivalent to the mammalian CAAT box). Also a GATAAG sequence is present on both promoters, in similar position (-98 and -86 respectively). The GATAAG sequence element was previously identified as a conserved element, possibly a tissue specific regulator, in a comparison of proximal amylase promoters of different Drosophila species (Magoulas et al., 1993c). Sequences similar to the GATAAG element (indicated as grey boxes in Figure 13) are present on the proximal promoter in two more copies; one of these is also present on the distal amylase promoter, at a similar position, while the other is missing. The GATAAG sequence elements are present in regions necessary to obtain high levels of expression, and may be functionally important for gene expression and/or tissue specificity.

Greatest similarity between promoters is noted from the TATA box downstream to ATG. Upstream of TATA, the CAAT motif is followed by a region of 14 bp that are almost identical; after that, some deleted sequences in the distal promoter are noted. Upstream of

Figure 13

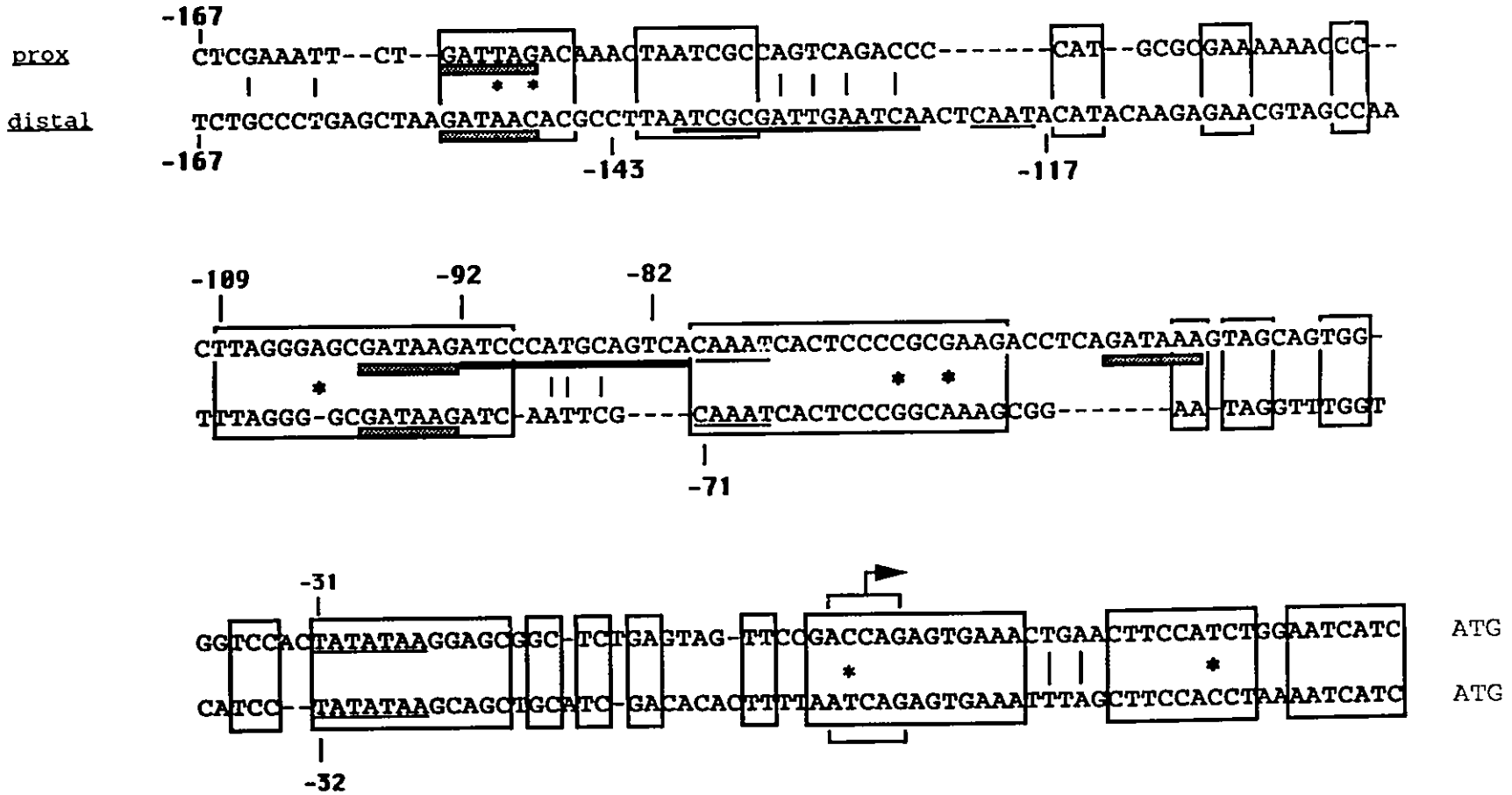
Sequence comparison of promoter regions of proximal and distal amylase genes from Makokou strain.

200 bp of promoter sequences of the Amy-4 proximal and Amy-6 distal genes, from -167 to transcription start point and to ATG, are visually aligned.

Sequences similar in the two promoters are boxed; * indicates single nucleotide differences within these boxes.

TATA and CAAAT (or CAAT) boxes are underlined. Arrow indicates transcription start site.

Bold line indicates element essential for expression in proximal and distal genes. GATAAG sequence elements or similar element are highlighted by grey boxes.


-167
prox
CTCGAAATT--CT--GATTAGACAAACTAATCGCCAGTCAGACCC-------CAT--GCGCGAAAAAACCC--
distal
TCTGCCCTGAGCTAAGATAACACGCCTTAATCGCGATTGAATCAACTCAATACATACAAGAGAACGTAGCCAA
-167
-143
-117
-189
-92
-82
CTTAGGGAGCGATAAGATCCCATGCAGTCACAAATCACTCCCCGCGAAGACCTCAGATAAAGTAGCAGTGG-
TTTAGGG-GCGATAAGATC-AATTCG----CAAATCACTCCCGGCAAAGCGG-------AA-TAGGTTTGGT
-71
-31
GGTCCACTATATAAGGAGCGGC-TCTGAGTAG-TTCCGACCAGAGTGAAACTGAACTTCCATCTGGAATCATC
ATG
CATCC--TATATAAGCAGCTGCATC-GACACACTTTTAATCAGAGTGAAATTTAGCTTCCACCTAAAATCATC
ATG
-32

CAAAT motif, a sequence from -92 till -80 in the proximal gene, and containing the element previously identified as essential for gene expression (in the deletion analysis and in mutagenesis tests, construct #7 of Section 3.1) is missing in the distal gene. That could explain why a -117 bp distal promoter is not functional while a -109 proximal promoter is.

As noted earlier, this element, between -92 and -80 in the proximal promoter, has great similarity with the region between -143 and -117 of the distal promoter (see Figure 12B), that in functional test also resulted essential for gene expression. It appears that this element is important for expression and is present in both promoters, but at different positions.

This is a similar result to the one obtained in the study, via both functional tests and sequence analysis, of the heat shock genes. For these genes also, multiple approaches allowed identification of important elements (Bienz, 1985 for a review) which are not necessarily always in the same position in the promoter. Also, these elements are repeated and the similarity is limited to groups of a few bp scattered along a 10 - 20 bp length.

Promoter sequences upstream of -200 do show lots of differences when the Makokou proximal and distal promoters are compared, whereas the two proximal promoter regions of Amy-1 and Amy-4 present quite similar sequences. Over a length of 1600 bp from ATG only 24 bp are different (1.5%). There are 16 bp mutations plus an 8 bp long deletion.

In conclusion, the sequence and functional comparison of promoters from proximal and distal genes shows a basic similarity between the two. Some differences are noted at a small scale analysis. These differences consist of substitutions and deletions that cause some variation in the functionality of the promoters. An element important for gene expression has been identified through functional and sequence comparison, and is present on both promoters at different positions.

3.3 Expression of an amylase-alcohol dehydrogenase chimeric gene in transgenic strains of D. melanogaster.

In Drosophila, P element mediated transformation can be used to introduce genes into the genome of the fly and it has been shown that the introduced gene often exhibits its normal wild-type expression pattern in the transformed flies (Rubin and Spradling 1982; Goldberg et al., 1983). Moreover, hybrid genes consisting of the upstream and transcribed parts of different genes can also be introduced into the Drosophila genome and tested for their expression patterns in order to identify which gene segment carries regulatory elements (e.g., Romano et al., 1988).

In order to identify regions responsible for glucose repression at the amylase locus in Drosophila, amylase-alcohol dehydrogenase (Amy-Adh) chimeric genes were constructed and introduced into recipient strains using P element-based vectors (Benkel et al., 1992). In these experiments, the expression of the Adh structural or "reporter" region is controlled by promoter sequences of the highly glucose-repressible gene of the Oregon R strain of D. melanogaster (Benkel et al., 1987). In contrast to amylase, the wildtype Adh promoter (gene) of Drosophila is not subject to glucose repression.

3.3.1 Germline transformation and expression of hybrid Amy-Adh gene constructs

The role of promoter sequences of the amylase gene in the glucose repression phenomenon was assayed by studying the expression pattern of hybrid genes carrying Amy promoter regions linked to Adh coding and downstream sequences.

Germline transformants were obtained that carry hybrid amylase-alcohol dehydrogenase gene constructs and their expression pattern examined. As a control, transformants carrying the intact Adh gene were tested.

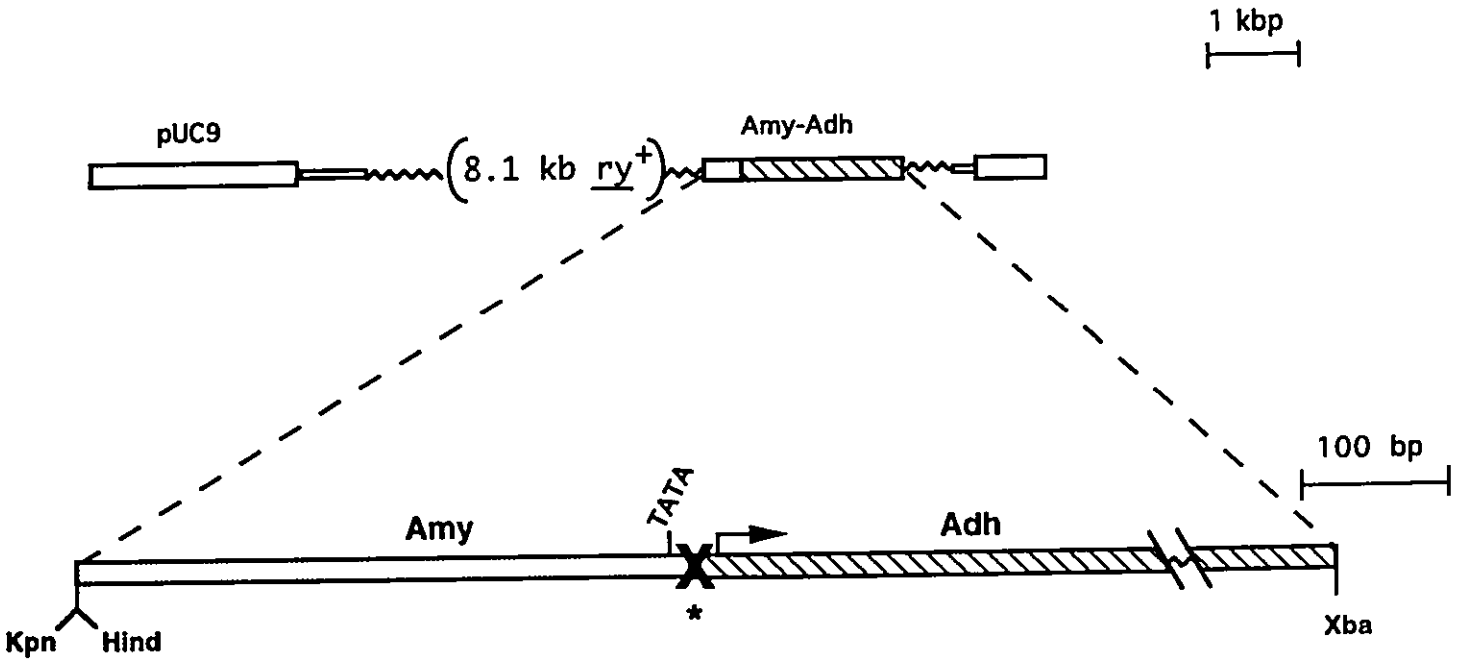
The hybrid construct used in the transformation experiments is outlined in Figure 14. Between the P element ends, the construct carries a hybrid gene consisting of promoter sequences of the Amy-1 gene (1600 bp or 430 bp) linked to coding and downstream sequences of the Adh gene. The junction between the amylase and Adh sequences is between the TATA box and the transcription start point; thus the transcribed region contains Adh sequences only.

The Adh gene was used as reporter gene to test expression ability of promoter amylase sequences since it is a well studied gene of D. melanogaster and is not subject to glucose repression phenomenon (Benkel and Hickey, 1987). The tissue specificity of the Adh gene is also well known (Ursprung et al.. 1970) as its gene structure and expression pattern (Kreitman, 1983; Benyajati et al., 1982).

Figure 14

P element plasmid used for germline transformation.

This construction is based on plasmid pΔlwa2a described by Laurie-Ahlberg and Stam (1987) and was provided by B. Benkel (Benkel et al.,1992). The chimeric genes have 428 bp, shown here, or 1600 bp of Amy upstream sequences linked to 1800 bp of Adh sequence that contains the Adh larval transcription start site and leader sequences, along with the Adh coding and downstream sequences. In the Figure, the asterisk marks the junction between the Amy and Adh sequences. ΛΛΛ = P element sequences; thin open blocks = white locus sequences. The ry^+ gene was used as a cotransformation marker; it encodes xanthine dehydrogenase (XDH) and its expression affects the eye color of the transformed flies.


1 kbp
pUC9
8.1 kb ry+
Amy-Adh
100 bp
TATA
Amy
Adh
Kpn
Hind
Xba
*

The plasmid used for transformation also carries the ry+ gene, that codes for the XDH (Xanthine dehydrogenase) enzyme; upon insertion in the fly genome these sequences restore the ry+ phenotype (wildtype eye colour) in the adult fly, acting as a marker of transformation.

Micro-injection of the chimeric construct to obtain germline transformant was carried out as described in the Materials and Methods, in embryos of the Adh^{null} stock, Adh^{fn8} cn; ry^{506}. Transformants were recognized by a change in phenotype following the insertion of the ry+ gene in the genome.

In a first series of experiments, three independent lines (#1-1, 5-3 and 10-2) carrying the -1600 Amy-Adh construct were selected and examined for Adh expression and glucose response in electrophoretic gels. As a control, one line transformed with the intact Adh gene was examined. All three lines presented ADH activity that was absent in the recipient Adh^{null} strain, although the activity was lower than in the controls. Moreover, larvae exposed to the glucose treatment did not show ADH activity (Figure 15A). The control line presented ADH activity regardless of the type of food. From these data we can conclude that the promoter region of the amylase gene is able to mediate glucose repression of the Adh gene.

In order to restrict the length of the promoter region responsible for expression and glucose response, a shorter Amy sequence, with 428 bp of promoter sequences was tested and a

Figure 15

Expression and glucose-repression of the Amy-Adh hybrid genes in transgenic larvae.

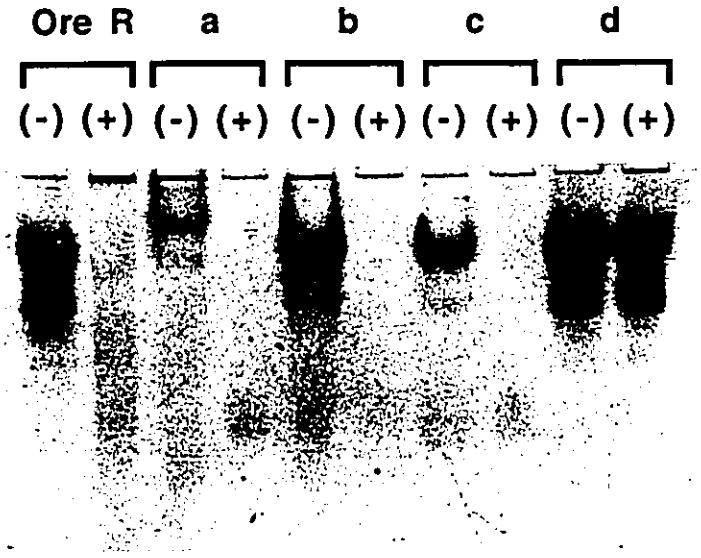
Results for independent transformant lines of D. melanogaster are shown. The flies were transformed with the Amy-Adh hybrid constructs shown in Figure 14; these constructs contain the amylase promoter region linked to the alcohol dehydrogenase transcribed region.

In panel A the transformants carry the -1600 bp long Amy promoter sequences.

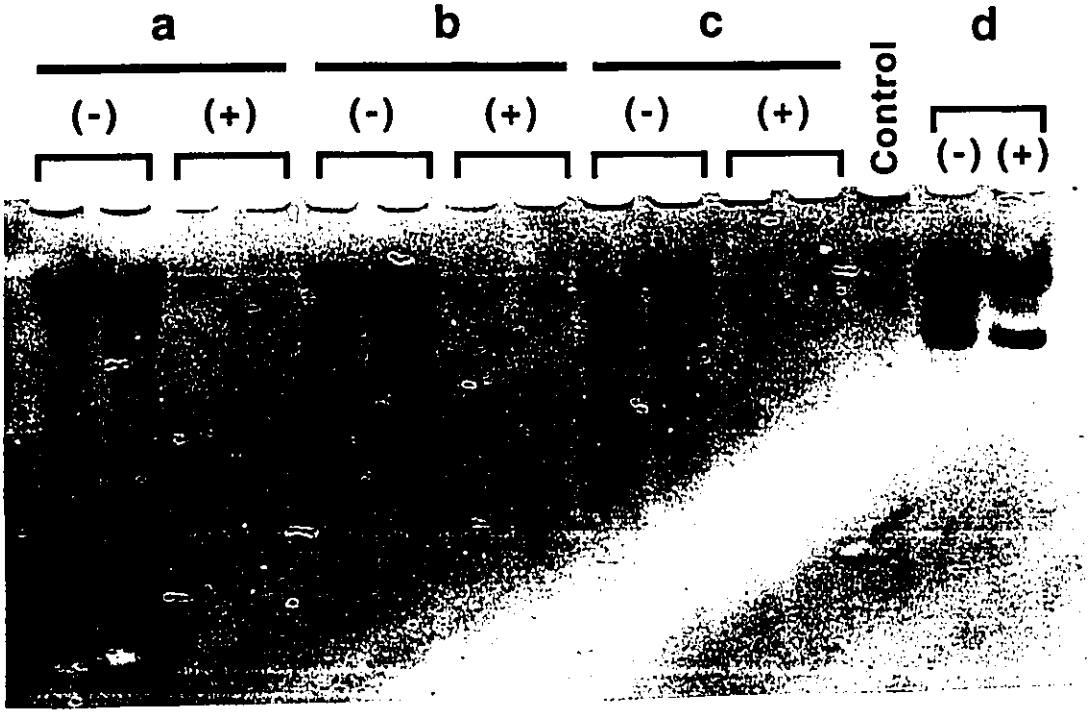
In panel B (from Magoulas et al., 1993a) the transformants carry the 430 bp long promoter region from Amy.

Larvae from these transgenic flies were grown in a medium with (+) or without (-) glucose and larval homogenates were assayed for ADH activity as described in Materials and Methods. The ADH activity in the larval homogenates from each of the transgenic lines is shown in the lanes under **a**,**b**, and **c**. In **d** the ADH activity of a control transgenenic line, which was transformed with the intact Adh gene, is shown. Ore R = homogenate (1/10 of a larval equivalent) from the wildtype Oregon R larvae also grown on a glucose-free medium. The control lane contains an homogenate of untransformed Adh^{null} larvae grown in glucose-free medium.

A.



B.



detailed analysis was carried out on the germline transformants.

Three independent transformant strains (strains: 2b, 4b and 13a) were examined. Each strain resulted from the transformation of the $\underline{Adh}^{null}$ recipient stock, with the plasmid shown in Figure 14, carrying the Amy-Adh chimeric gene with 430 bp of amylase promoter sequences. These strains were made homozygous for the Adh and rosy markers. One of the transformant strains, strain 2b, displayed a sex-linked segregation pattern of the ry^+ marker. As a control, a fourth transformant strain was used; in this strain, the transforming plasmid contained the Adh coding region linked to the native Adh promoter sequences. Consequently, one expects the normal, wildtype patterns of Adh activity in this strain.

The three lines and the control lines were tested for the number of copies of inserted genes (Grunder et al., 1993) via Southern blotting. All the lines carry one copy of the exogenous gene, except line 13a which carries two gene copies.

When tested for Adh expression, larvae transformed with the hybrid gene again showed expression and glucose repression of ADH activity (Figure 15B), indicating that less than 500 bp of promoter sequences mediate glucose repression. The larvae grown on glucose-free medium showed the ADH fast variant, as expected, and an activity lower than the Adh transformant. Larvae raised in glucose-rich medium did not present any ADH activity.

A detailed study of these lines and control line was carried out. This involved an analysis at the tissue-specificity level and

a quantification of expression levels of ADH in adults.

3.3.2 Tissue-specific expression of ADH activity in transformed larvae

The tissue-specific distribution of ADH activity expressed by the chimeric gene carrying Amy promoter sequences, was assessed by means of histochemical staining of intact organs of transgenic third instar larvae. Larvae were grown on diets with and without glucose in order to test for glucose repression of the chimeric gene. The strain transformed with the intact Adh gene and the recipient Adh^{null} strain were included as controls. The main purpose was to determine whether amylase promoter sequences would confer amylase tissue-specificity to an unrelated gene (Adh).

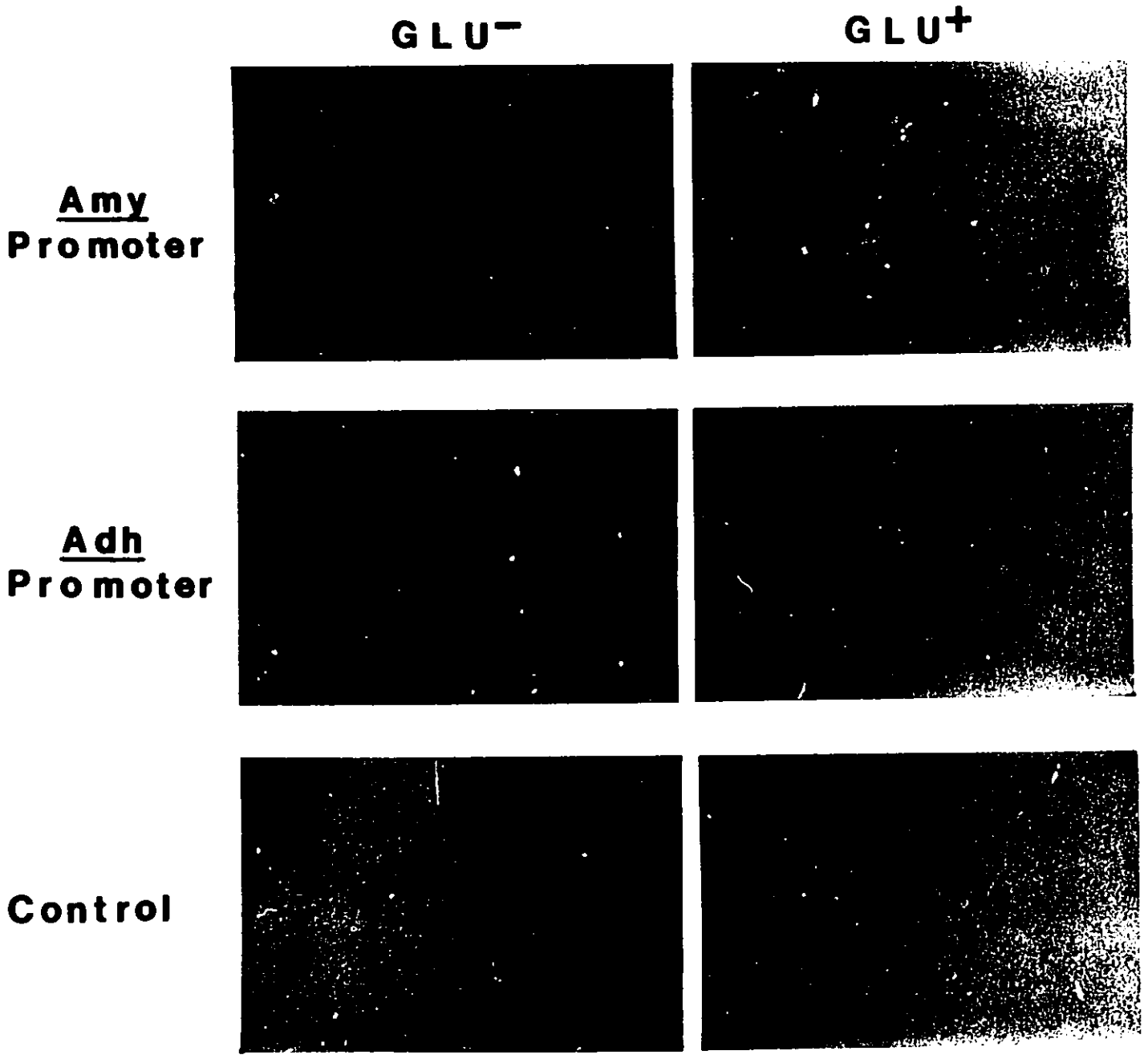
The results (see Figure 16) show a very different tissue specific distribution of ADH activity in the two kinds of transformants, and this difference is consistent with the expectation based on the two different promoters. Under non-repressing dietary conditions, the chimeric gene was expressed in the posterior midgut in the location where normally the amylase is expressed (shown by an arrow in Figure 16). This activity disappeared when the larvae were grown on the repressing food containing glucose. In contrast, transgenic larvae carrying an intact Adh gene showed a wildtype pattern of Adh activity; staining was observed in the gastric caeca, anterior and posterior mid-gut

Figure 16

Histochemical staining pattern of ADH activity in transgenic larvae.

Transgenic larvae transformed with the Amy-Adh construct (provided by B. Benkel, Benkel et al., 1992) or with the construct carrying an intact Adh gene (provided by Laurie-Ahlberg) were examined for ADH activity (see Figure 14 for constructs description).

ADH activity is shown as a dark blue precipitate in midgut preparations of transgenic and control genotypes. Larvae were grown on food without glucose (GLU $^-$), left column, or containing glucose (GLU $^+$), right column. The arrow indicates the region of ADH staining in the posterior midgut of transgenic larvae containing the chimeric Amy-Adh gene. The midgut extends anteriorly from the gastric caeca (1) to the attachment point of the Malpighian tubules (2). The three strains shown are labelled as follows: Amy promoter = transgenic strain 4b with the Amy-Adh chimeric gene; Adh promoter = transgenic strain with the intact Adh gene; Control = Adh^{null} stock, Adh^{fn8} cn; ry^{506}, used as transformation recipient.


GLU⁻
GLU⁺
Amy
Promoter
Adh
Promoter
Control

(but not in middle midgut or hindgut), in the Malpighian tubules, and fat bodies (not shown); this is similar to the pattern observed by Goldberg et al. (1983) in transformed larvae carrying the intact Adh gene or in wildtype larvae (Ursprung et al., 1970). Moreover, the staining pattern of the native Adh gene was not subject to glucose repression. The non-transformed recipient strain showed no detectable Adh expression regardless of dietary treatment. Figure 16 shows the staining patterns for transgenic strain 4b. The results for the expression patterns from the other two transgenic strains (2b and 13a) were indistinguishable from that of strain 4b. Overall, the results of this experiment showed that the Amy promoter sequences control both tissue-specificity and glucose repression of the Adh transcript in a manner that is typical of the amylase gene.

With this study we have shown that a less than 500 bp long amylase promoter region is sufficient to confer an amylase-specific pattern of expression on an unrelated transcript (Adh) in transgenic Drosophila larvae. This result is important since it limits the search of glucose repression mediating elements to the promoter region of the genes, while transcribed sequences of the amylase gene are not necessary for the glucose repression to occur. This also confirms that glucose repression operates at the level of transcriptional regulation, since the hybrid gene examined contained only Adh transcribed sequences.

This result complements other studies being done in this system.

For instance, when the luciferase gene from the firefly was used as reporter of Amy promoter sequences in somatic transformation experiments, the latter conferred glucose repressible expression upon this unrelated gene. Moreover, as seen in Section 1 of Results and in Magoulas et al. (1993b), we also used somatic transformation of larvae in a promoter deletion analysis to show that the glucose response mapped within the first 109 bp from transcription start point of the Amy-1 promoter region. Here, we have shown that cis-acting elements controlling both glucose repressibility and midgut specificity are both closely linked to the structural locus (within 500 bp).

Despite the variations in insertion site, as demonstrated by the linkage to the sex-chromosome in one case and to the autosomes in the other cases, and by the Southern blotting analysis (Grunder et al.,1993), there were not large differences in the expression patterns between the three strains transformed with the chimeric gene. This indicates that there were not significant position effects due to flanking sequences at the site of insertion on the level of gene expression. It may be that the ry^+ marker sequences in the plasmid that were inserted upstream of the chimeric gene acted as a buffer against the effects of flanking chromosomal DNA. The fact that one strain (13a) had an elevated level of gene expression is consistent with the finding of two copies of the gene in that strain.

3.3.3 Levels of Adh expression in transgenic strains

Although all of the transgenic lines produced Adh activity, the levels of expression of the hybrid gene appeared to be relatively low in both larvae and adults. We compared gene expression levels in adults of transgenic and control strains, both at the level of enzyme activity and at the mRNA level.

Samples from homogenates of adult flies were examined by means of gel electrophoresis for Adh enzyme activity; AMY enzyme activity was assayed as an internal control. The results (Figure 17) showed that the strain transformed with the wildtype Adh gene had ADH activity similar to wildtype flies (compare samples 1 and 3); the three strains transformed with the chimeric gene showed the presence of ADH activity, but at a reduced level relative to the control strains. Line 13a, which contains two copies of the chimeric gene, showed stronger activity than the two other transgenic strains, but still had less than 1/10 of the activity shown by the wild-type control. On the other hand, AMY activity in the same homogenates that were tested for ADH was, as expected, similar in all the transformed stocks, and the recipient stock, as well as in the wild type stock (Figure 17B). This uniformity of the AMY activity shows that the variation found for the ADH activity was real and not due to non-specific variations in enzyme activity. Since we assayed enzyme activity by comparing the intensities of electrophoretic bands, we can conclude that ADH expression in the transformant strains was less than 10% of

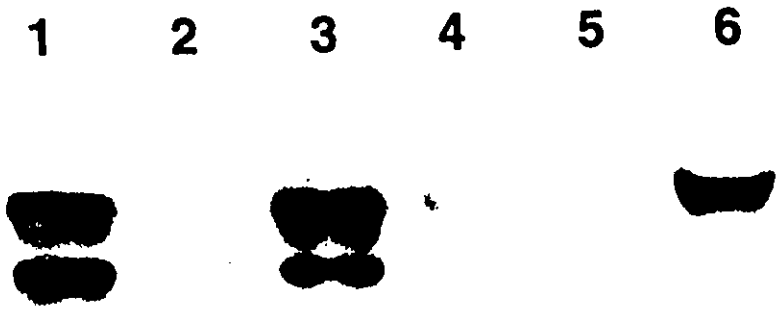
Figure 17

ADH and AMY enzyme activity in adult flies fron three transgenic strains.

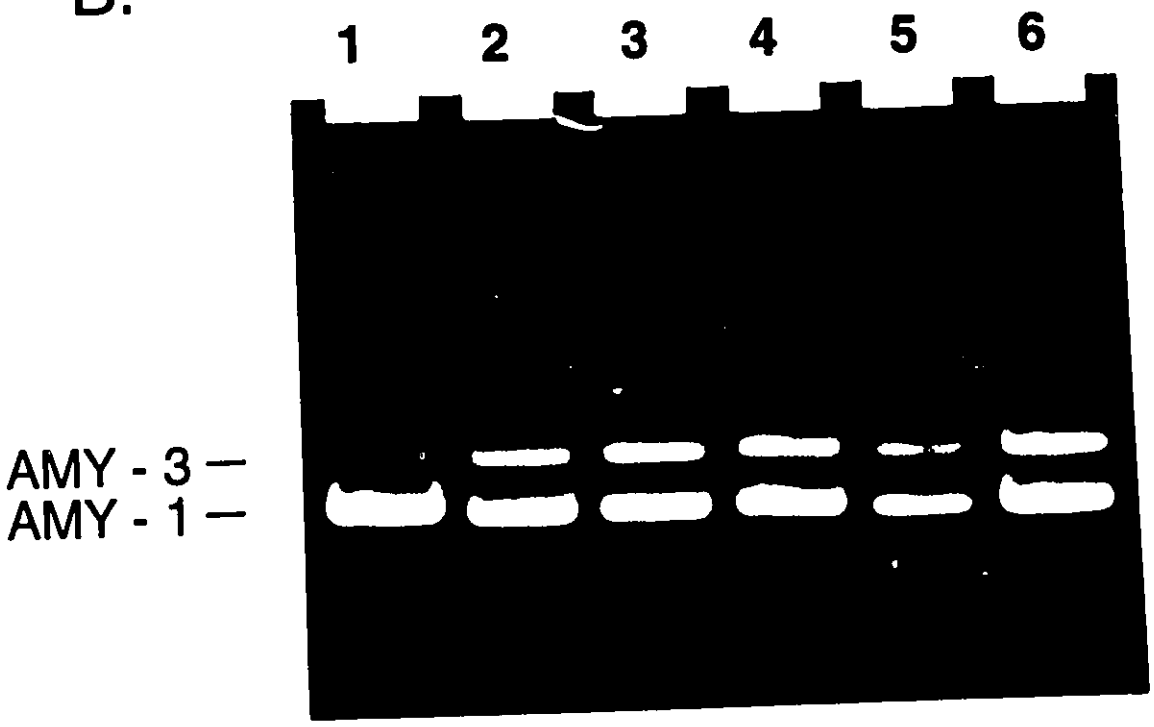
Flies from both control and transgenic strains were cultured on medium without glucose. Homogenates from 20 flies per strain (about 1 week old) were prepared and run on 5% acrylamide gels, which were stained for ADH activity (Panel A) and AMY activity (Panel B). In Panel A, 1 fly equivalent of homogenate was loaded for the Amy-Adh transgenic strains and Adh^{null} strain, and 1/10 of a fly equivalent was loaded for the positive controls (Oregon R and the Adh control transformant).

In Panel B, equal amounts of all homogenates (1 fly equivalent) were loaded in each lane. Lane 1, Oregon R; Lane 2, Adh^{fn6} cn; ry^{506}, i.e., Adh^{null} recipient stock; Lane 3, Adh control transformant; Lanes 4, 5 and 6, Amy-Adh transgenic strains 2b, 4b and 13a, respectively.

A.



B.



wildtype levels, but we cannot say exactly how much less.

In Grunder et al. (1993), Adh mRNA levels in adult flies were assayed by Northern blot hybridization. This was done to determine if the low ADH activity in the strains transformed with the chimeric gene was due to a pre-translational defect or if actually transcription of the Amy-Adh gene occurs at low levels. From those results, it seemed that the low ADH activity for Amy-Adh transformant strains shown in Figure 17A was paralleled by a reduction in mRNA. This result confirms that the reduced enzyme level in the transgenic strains is largely due to a reduction in message level.

Although the chimeric gene exhibited the amylase-specific pattern of gene expression, alcohol dehydrogenase activity levels in these transgenic strains were clearly less than that of Oregon R wildtype strain. We estimated that the enzyme levels in the transgenic lines was of the order of 10%, or less, of the wildtype levels. Since the longer Amy promoter (1600 bp long) also showed low expression, it is not the absence of enhancer or other upstream regulatory elements that is responsible for the low expression. The fact that transformants carrying the wildtype Adh gene show full wildtype levels of activity suggests that the reduced expression is due to the amylase promoter. Northern blot analysis of Adh mRNA levels (Grunder et al., 1993) showed that the reduction in enzyme activity was paralleled by a reduction in message levels. This reduced expression of the chimeric gene is reminiscent of the

result reported by Romano *et al.* (1988). They also used the *Adh* gene as a reporter, but in their experiments it was linked to a chorion gene promoter. The expression pattern of the chimeric gene was typical of the chorion promoter but the level of transcript was reduced eighteen fold relative to the endogenous promoter. In our experiments, it is worth noting that the histochemical staining for *Adh* activity (see Figure 16) shows that expression from the amylase promoter is spatially more restricted than expression from the *Adh* promoter. In other words, the reduction in total expression from the amylase promoter in the chimeric gene may simply reflect the fact that it is expressed in a more limited set of cells (i.e., midgut cells only) than is the *Adh* promoter.

3.4 Expression of recombinant gene constructs carrying promoter sequences of the Adh gene of Drosophila melanogaster

In this section we analyze the transient expression of a plasmid carrying promoter sequences of the Adh gene of D. melanogaster linked to the coding and down stream sequences of the highly glucose repressible Amy-1 gene. We have previously shown in the analysis of the Amy-Adh hybrid gene that promoter sequences of the amylase gene confer glucose-repressible expression and tissue-specificity to the Adh reporter gene. In the reciprocal construct examined here we checked the possibility that sequences within the amylase coding region might also contribute to glucose repression.

As outlined in Figure 18, we have switched the promoter and coding sequences of the Amy-1, amylase gene, and Adh, alcohol dehydrogenase gene (A and B in Figure 18). These two genes are both expressed in the midgut of the larva of D. melanogaster, but differ for two characteristics that are easily determined in our system, i.e., glucose sensitivity and tissue specific expression. The amylase gene is highly glucose repressible and is expressed only in the posterior midgut; the Adh gene is glucose insensitive and is expressed both in the anterior and posterior midgut and also in the fat bodies and Malpighian tubules. By completing the analysis of the expression pattern specified of the hybrid genes C and D in Figure 18 (construct C was examined in Section 3 of Results), we

Figure 18

Outline of genes and hybrid gene constructs examined.

A and B represent the Amy and Adh genes of Drosophila melanogaster. In wildtype strains and in transformants, these genes present a different expression pattern. In order to localize the sequences responsible for the expression pattern hybrid genes C and D were examined. The hybrid genes carry upstream sequences of one gene and coding sequences of the other. They are expected to produce protein specified by the coding region and present regulatory patterns specified by the upstream sequences. This has been demonstrated to be the case for construct C, Amy-Adh hybrid gene, whose expression was examined in transgenic larvae. As a confirmation of those findings the expression pattern of the reciprocal Adh-Amy construct (D) was examined.

	Upstream	Coding		Glucose repression	Tissue specificity
A			Amy	Yes	Amy
B			Adh	No	Adh
C			Amy-Adh	Yes	Amy
D			Adh-Amy	?	?

can verify whether promoter sequences determine the characteristic expression pattern on either one of the coding regions and whether the latter do not contribute to the regulation of gene expression. If this is the case we can also conclude that the major control of gene expression is at the transcriptional level.

A second purpose of this study is to find a glucose insensitive promoter (Adh promoter) suitable for further dissection of the amylase promoter. One would try to identify sequences that are important for glucose repression by testing the expression pattern determined by hybrid promoters containing part of the glucose repressible Amy promoter and part of the non glucose-repressible Adh promoter. A successful example of this type of approach is reported below.

3.4.1 Expression of an Adh-Amy hybrid construct

A hybrid gene construct carrying upstream sequences of the Adh (alcohol dehydrogenase) gene fused to Amy coding sequences was obtained and tested via somatic transformation assays for its ability to express the amylase protein. An expression pattern different from the Amylase gene was expected since the Adh gene is not glucose repressible and is expressed in different tissues than the Amy gene. The purpose of this study was to ascertain if in fact the Adh upstream sequences confer their expression pattern on the Amy coding sequences and if the Amy coding sequences of the repressible Amy gene are able by themselves to mediate glucose repression (and tissue specificity).

The Adh-Amy hybrid gene used in this study (Figure 19A) carries about 510 bp of the Adh larval promoter of the Adh wa-f clone fused with the coding and downstream sequences of the Amy-1 proximal gene from Oregon R strain, as described in Materials and Methods and in Magoulas et al. (1993a).

The transient expression of the Adh-Amy construct was studied in somatic transformation experiments carried out as usual (see Materials and Methods). The Adh-Amy construct was co-injected in embryos of the Amy^{null} stock together with the Amy-4 gene that encodes a different electrophoretic variant and was shown to be glucose repressible.

Somatic transformation experiments were performed first using

Figure 19

Non repressible expression of the Adh-Amy hybrid construct.

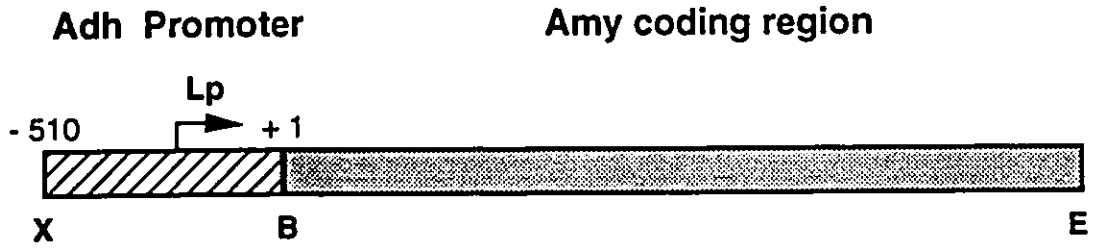
Panel A)

A hybrid Adh-Amy construct (provided by C. Magoulas, Magoulas et al., 1993c, and Materials and Methods) carries 510 bp of larval promoter sequences of the Adh gene (Kreitman, 1983) from plasmid Adh wa-f (provided by Laurie-Ahlberg, Laurie-Ahlberg and Stam, 1987) linked to Amy-1 coding sequences . L_p = transcription start site of larval Adh promoter. X=Xba, B=BamI, E=EcoRI.

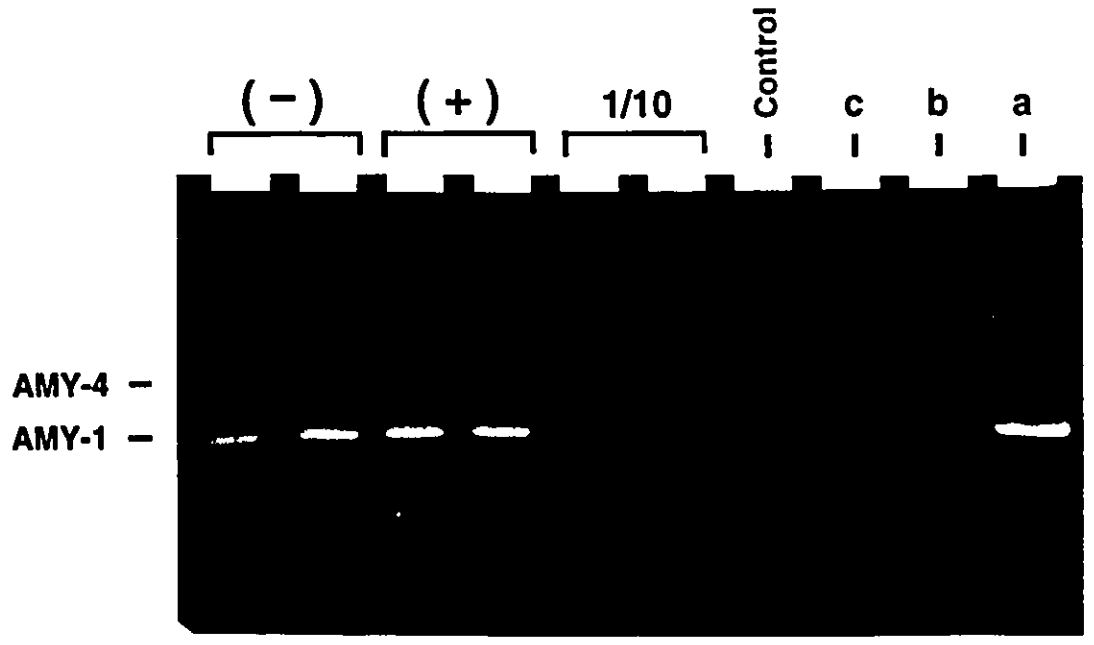
Panel B)

A mixture of plasmids carrying the Adh-Amy hybrid gene construct and plasmids carrying the cloned Amy-4 gene as internal control (.04 mg/ml and .2 mg/ml respectively) were micro-injected in Amy^{null} embryos. Samples of the homogenates of transformed larvae raised on glucose-free or glucose-rich media were run on a gel together with a dilution (1/10) of the glucose-free treatment, for comparison, and stained for amylase activity. The amylase activity of Adh-Amy construct (AMY-1) can be compared to the amylase activity of the control construct, (AMY-4), in glucose-free treatment and its repressibility can be monitored in glucose-rich treatment. Control = Samples of non-injected larvae. Lanes c, b, and a contain a dilution series of wildtype Oregon R larval homogenate (1/100, 1/10 and 1 larval equivalent).

A.


Adh Promoter
Amy coding region
Lp
- 510
+ 1
X
B
E

B.


(-)
(+)
1/10
Control
c
b
a
AMY-4
AMY-1

equal amounts of the hybrid Amy-Adh construct and Amy-4 construct. Subsequently, given that the AMY activity produced by the hybrid gene was higher than that of the control gene, the concentration of the hybrid gene was reduced to 1/5 of the Amy-4 gene construct, in order to obtain bands of equal intensity on the gel, which could be compared with the ones obtained from larvae grown in glucose-rich medium. The results of the two experiments were similar, and the one of the second experiment are shown.

As shown in Figure 19B, the hybrid construct is functional and not glucose repressible: the expression level of Adh-Amy in whole larvae is at wildtype level, comparable to that of the control Amy-4 construct and of the Oregon R wildtype flies, although the concentration of the hybrid construct is lower than that of the control Amy-4 gene. Moreover, while the control AMY-4 activity is not present in larvae grown on glucose rich food, the AMY-1 activity is present in these larvae, thus showing that Adh promoter confer glucose-insensitive expression on the Amy reporter gene.

In subsequent experiments we tested the tissue-specific pattern of the hybrid construct. The amylase activity expressed by the Amy-Adh hybrid construct was examined separately in the midguts and fat bodies of transformed larvae. These are the two tissues with highest Adh activity in wild type larvae (Ursprung et al, 1970). In the midguts, the Adh-Amy hybrid gene is expressed at high levels and is not glucose repressed, while the control construct is (Figure 20A). This confirms that the amylase activity

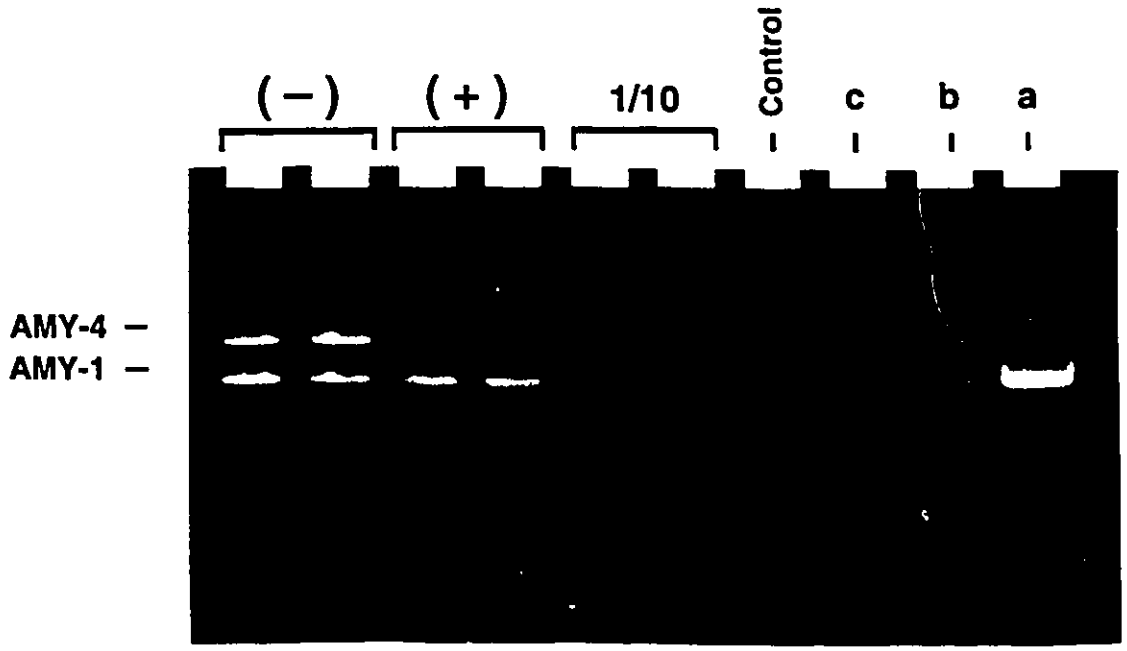
Figure 20

Expression and glucose response of the Adh-Amy hybrid construct in different tissues of the larva.

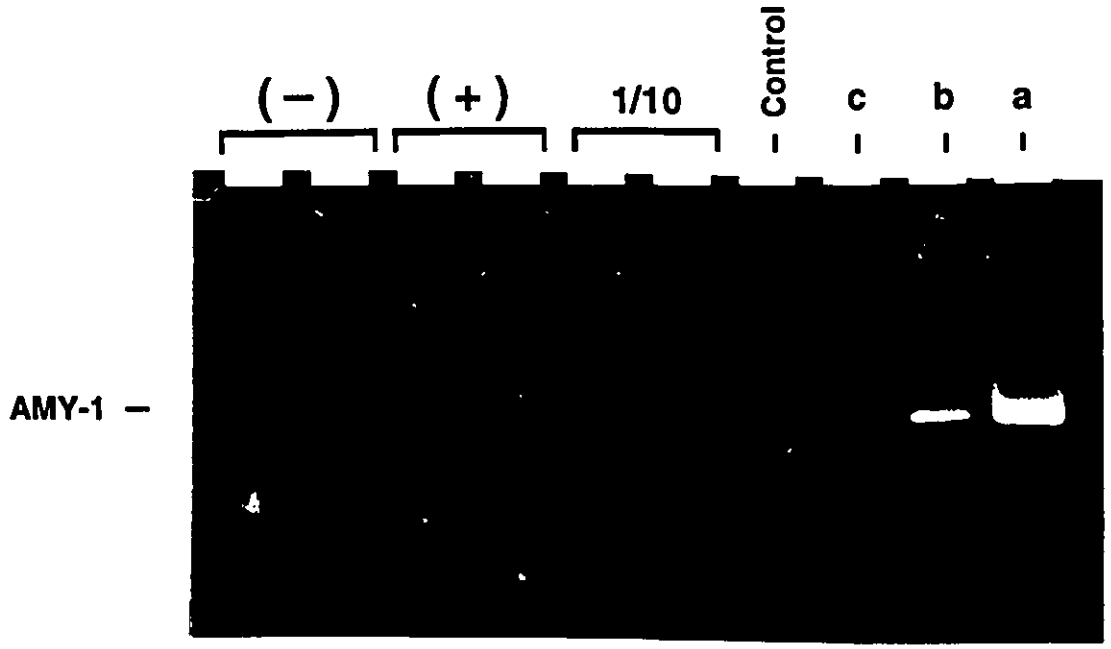
Third instar larvae, somatically transformed with a mixture of the Adh-Amy and Amy-4 constructs as described in Figure 19, were dissected and midguts and fat bodies examined separately for amylase activity. Homogenates of tissues from several larvae were prepared and run on electrophoretic gel stained for amylase activity. A = midguts; B = fat bodies.

(-) and (+) refer to samples from larvae grown on glucose-free and glucose-rich food respectively. 1/10 is a dilution of the glucose-free homogenate for comparison of activities with glucose-rich treatment. Control and c,b,a as in Figure 19.

A.



B.



driven by the Adh promoter is not glucose repressible, even in a tissue that is exposed to dietary glucose. In the fat bodies, where the Adh gene is normally expressed at high levels, only the AMY-1 activity is present indicating that the Adh promoter mediates expression of amylase in this tissue, whereas the AMY-4 band is not visible or present as a barely visible band (possibly result of tissue contamination) as expected (Figure 20B). The AMY-1 activity is lower in the fat bodies compared to the midguts; a possible explanation is that the fat bodies are quite a small tissue, and also it was not possible to collected all six of the fat bodies from each larva. Biochemical assays (Ursprung et al.,1970) had shown that ADH activity is very strong in both the midgut and fat bodies. It is also possible that the AMY enzyme is not fully functional in the fat bodies, a tissue where it is not usually present.

These data demonstrate that Adh promoter sequences confer the Adh tissue-specific pattern of expression on the Amy coding sequences. Earlier, we showed that Amy promoter determines tissue-specific pattern on Adh gene. Both the Amy and Adh genes thus carry tissue-specific signals in promoter sequences.

The localization of tissue-specific elements upstream of the transcription start site (and approximately within a hundred bp from it) has been determined for several other genes in D. melanogaster, such as the Ddc gene (Bray et al., 1988) where neural expression is mediated by an element at -60 from transcription

start; the Sgs-3 gene which produces the glue protein of the salivary glands (Meyerowitz, 1987; Martin et al., 1989); the ninaE opsin gene (Mismer and Rubin, 1989), and the chorion genes (Romano et al., 1988).

To find short sequences responsible for the tissue specificity is quite a difficult task, but as more examples of gene promoter organization are found a simpler representation of the "typical" gene can be drawn, in which relatively short promoters control the expression of a gene. This organization is much simpler than the one visualized until recently.

In conclusion, the study of the expression of the Adh-Amy hybrid gene construct that carries the Amy-1 coding sequences controlled by the Adh promoter sequences (500 bp) demonstrates that the latter promoter sequences determine the glucose insensitive expression of the amylase protein. Moreover, this expression occurs in tissues where usually Adh is expressed. They have also shown that the Amy coding and downstream sequences do not carry signals mediating glucose repression (or tissue-specificity). These results complement the previous observations that elements mediating glucose repression are present on the Amy promoter sequences.

The activity of the Adh-Amy hybrid gene construct (although we did not quantify this expression) was quite high with respect to the controls. This means that the hybrid gene is functional and the high expression levels compared to the amylase gene could be explained with the fact that the Adh promoters mediate expression

of a protein in a wider range of tissues than the amylase gene.

From these results it follows that we can use the Amy-1 coding sequences as reporter gene to test promoters for their ability to specify glucose repression.

3.4.2 Glucose repression of an hybrid promoter

We used promoter sequences from the Adh and Amy genes to construct a hybrid promoter to be tested for glucose repression ability in order to identify shorter regions in the Amy promoters that mediate glucose repression.

Figure 21 outlines the structure of the hybrid promoter that carries regions from the Amy and the Adh promoters, that respectively mediate or do not confer glucose repression pattern of expression of linked sequences. In particular, the 109 bp promoter sequences of the Amy-1 gene, that are sufficient for full expression, are substituted with sequences from the Adh promoter, in the region that goes from the TATA motif till ATG. The resulting hybrid promoter is 180 bp long and was linked to the Amy-1 reporter gene (see Materials and Methods). The construct was tested for its expression pattern in transient expression assays, in co-injection experiments with both the Amy-4 repressible amylase gene and the Amy-6 less sensitive to dietary glucose repression.

As shown in Figure 22, the construct is functional and confers glucose repressible expression on the amylase activity in third instar transformed larvae: While the Amy-6 control presents

Figure 21

Outline of an Amy/Adh hybrid promoter tested for the ability to regulate Amy coding sequences.

The structure of an Amy/Adh hybrid promoter is outlined: It carries sequences derived from the Amy-1, highly glucose repressible promoter, and from the Adh larval promoter (Kreitman, 1983), insensitive to glucose effect. The 180 bp long hybrid promoter was constructed using PCR technology and linked to Amy-1 coding sequences of the CS/BclI* plasmid (see Materials and Methods).

Amy sequences go from -109 (from transcription start site) till TATA box (78 bp) and are joined to Adh sequences that go from the TATA box till ATG (102 bp). 5 bp at the TATA box are the same in the two promoters (CTATA).

Continuous line = Amy sequences; dashed line = Adh sequences. Overlap means sequences are equal in the two promoters. The transcription start site is indicated by an angular arrow.

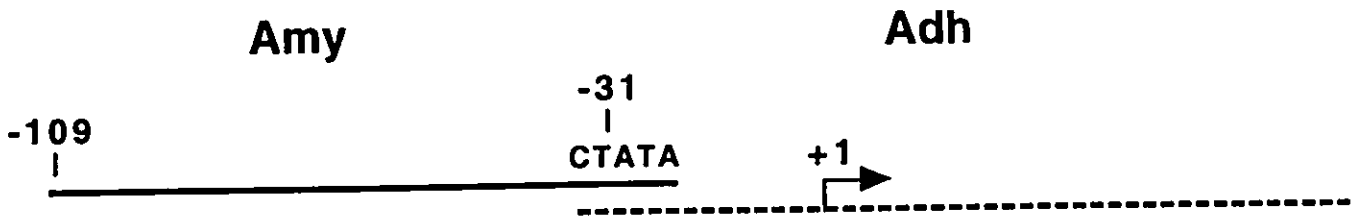
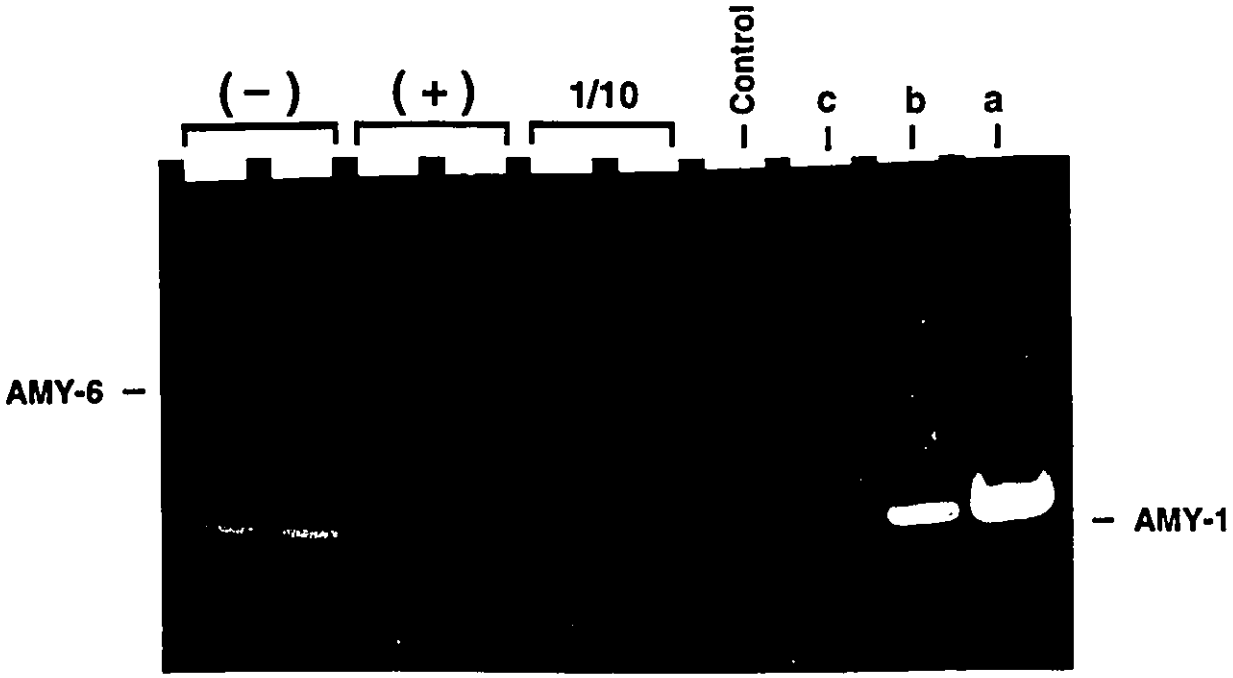

Amy
Adh
-31
-109
CTATA
+1

Figure 22

An hybrid Amy/Adh promoter mediates glucose repressible expression.

Transient expression of an Amy/Adh promoter (Figure 21) linked to the Amy-1 coding region is examined in somatically transformed larvae together with Amy-6 gene construct as internal control. Amy null embryos were injected with a mixture of the two constructs (250 and 50 mg/ml respectively) Transformed larvae were grown on glucose-free and glucose-rich medium and tested for expression and glucose repression in electrophoretic gel.
1/10 is a dilution of the glucose-free treatment. Control = samples of not-injected larvae; c,b,a are a dilution series of wildtype Oregon R larvae (1/100, 1/10 , 1 larval equivalent).


(−)
(+)
1/10
Control
c
b
a
AMY-6
AMY-1

amylase activity in both glucose-free and glucose rich food, the AMY-1 variant produced by the hybrid promoter is present in the glucose-free treatment and absent in the glucose treatment.

In conclusion, the sequences between 109 and -31 (where the TATA motif is located) contain elements that mediate glucose repression. With this experiment we have restricted the localization of glucose-repression signals in a 78 bp long sequence of the amylase promoter.

4. Discussion and Conclusions

4.1 Amylase expression and glucose repression

We used somatic and germline transformation assays as *in vivo* functional tests to study the expression of amylase genes of *Drosophila melanogaster*. The characters studied were glucose repression and tissue-specificity. Glucose repression of the amylase expression is an interesting character: like the heatshock response, it occurs in a wide range of organisms from bacteria, to yeast (Johnston and Carlson, 1992) and Drosophila (Hickey et al., 1989) and mammals. We do not know if tne underlying mechanism is the same and has a common evolutionary origin, or if more than one mechanism has evolved in different organisms.

In Drosophila, glucose repression is strongest in the larva where it determines a reduction of amylase activity to less than 1/100 both in wildtype strains (Benkel and Hickey, 1986b) and in transformants (Magoulas et al. 1993b and this thesis), making it an easily scorable character. Previous observations showed that glucose repression occurs at the mRNA level, probably involving a control of transcription (Benkel and Hickey, 1987).

In this study we have dissected the amylase proximal gene, *Amy-1*, that presents the strongest response to dietary glucose, into its major parts, promoter and coding sequences. We have examined the expression pattern of hybrid genes consisting of a combination of the *Amy* gene and other genes that do not show the

glucose repression phenomenon (see Figure 23). Upstream sequences of the amylase proximal gene contain sequences mediating expression and glucose repression since they were able to mediate a glucose repressible expression of linked coding sequences of the glucose insensitive alcohol dehydrogenase (Adh) gene or the unrelated luciferase gene from the firefly (Figure 23, constructs B and C). It has to be noted that in the Amy-Adh construct (B), the whole transcript was made out from Adh sequences. This demonstrates that glucose repression occurs at the level of transcriptional initiation.

Amylase coding sequences lack elements mediating glucose repression, complementing the previous observations. This was shown through a construct carrying the Amy coding and downstream regions, (Adh-Amy construct D), that loses the glucose repressibility, when driven by the glucose insensitive Adh promoter. A practical consequence of this observation is that we can use the Amy-1 coding sequences as reporter gene, knowing that it does not interfere with gene expression (tissue-specificity or glucose repression).

Finally, we demonstrated that a construct carrying only 78 bp of the amylase promoter, upstream the TATA motif, (E), contains elements mediating expression and strong glucose response. This latter observation was made possible based on results from the deletion analysis of Amy promoter sequences and a saturation mutagenesis test: from the deletion analysis we reduced from 1600 bp to 109 bp from transcription start site, promoter sequence

Figure 23

Outline of hybrid gene constructs examined in the thesis.

Different hybrid gene constructs derived from the Amylase-1 gene of Drosophila melanogaster, (A), were examined for expression ability in transformed larvae. The hybrid genes carried Amy-1 promoter sequences linked to coding regions of different genes (B and C), like the alcohol dehydrogenase gene of D. melanogaster and the luciferase gene of the firefly, or, they carried the Amy-1 coding region preceded by the promoter of the non-glucose repressible Adh gene (D). Also a Amy/Adh hybrid promoter linked to Amy coding sequences was examined (E).

	Promoter	Coding	Glucose repression ?
A.			Yes
B.			Yes
C.			Yes
D.			No
E.			Yes

sufficient to mediate full expression. Saturation mutagenesis analysis indicated that multiple functionally independent elements mediating glucose repression must be present on the amylase promoter, similarly to what was found for the glucose repression phenomenon in yeast (Flick and Johnston, 1990, 1992).

An element important for high level of gene expression was identified on the amylase promoter. This element is just downstream of -109 from transcription start, from -92 to -80 in the proximal amylase. This region is also conserved in amylase promoters from different species of Drosophila, as it results from sequence comparison studies (Magoulas et al. 1993a). We also identified this sequence in the promoter of the distal amylase gene. Also, a tissue-specific sequence element (GATAAG), that may mediate tissue-specificity and/or enhance gene expression is repeated in multiple copies both on the proximal and distal amylase promoters.

Sequence elements which mediate amylase glucose repression cannot be found solely on the basis of sequence comparison, though some interesting sequences have been noted by this method in the promoter of the amylase gene (Hickey et al, 1989, Hickey et al., 1987). Functional analysis of putative elements is necessary. From our results, these elements appear to be present in multiple copies. Much nucleotide variation is reported in the literature between repeated regulatory elements, which makes it difficult to identify the elements. These are usually made of a few (10-12) bp, and, in the case of heatshock elements, the basic motif can be

reduced to 5 bp, (nGAAn), three of which are constant; this motif can be repeated in inverted order (Sorger, 1991; Xiao et al., 1991). Also for the induction of specific genes in response to heavy metals, multiple elements have been identified (Stuart et al., 1985).

Two mechanisms can be envisaged, by which elements in the 78 bp long region of the amylase promoter can mediate glucose repression : 1) through a repressor protein that binds to these elements in the promoter and disrupts transcription; 2) through a protein-protein interaction that disrupts activation of transcription by an activator protein. Some observations point to the first possibility (see below).

Our results show similarities between the glucose repression of amylase in Drosophila and glucose repression in yeast, Saccharomyces cerevisiae. In yeast, glucose repression is well studied. It acts in different multigene families involved in carbon utilization (for a review see Johnston and Carlson, 1992), like the GAL genes involved in galactose utilization, SUC (sucrose) genes that encode the enzyme invertase, and the MAL (maltose) genes and others. The expression of these genes is highly regulated.

Recently the mechanism of glucose repression in yeast has been further clarified. Some of the cis-acting promoter elements responsible for glucose repression of the GAL gene were shown to be repeated and were isolated and tested for their functionality (Flick and Johnston, 1990,1992). A general system for glucose

repression has been hypothesized: It is thought that glucose repression acts via a repressor protein, MIG1, a zinc-finger protein, whose DNA binding ability has been demonstrated. MIG1 also binds to two proteins, SSN6 and TUP1, which contact the transcriptional machinery and repress transcription. This glucose repression mechanism is common also to the GAL4 gene, whose product activates transcription of GAL1, and to the MAL and SUC gene systems (Johnston and Carlson, 1992). Moreover, the SSN6 and TUP1 genes and encoded proteins, have been found through a mutational analysis and have been known to affect many regulatory processes, among which mating type determination. The current hypothesis (Keleher et al., 1992) is that SSN6 and TUP1 represent a general system of repression that affects not only glucose repression, via the MIG1 protein, but also determination of mating type trough a different DNA binding protein, alfa2,MCM1. SSN6 and TUP1 form a complex that does not bind directly to the DNA but is recruited to the promoter it suppresses by the different DNA binding proteins.

From this information it looks like repression of gene expression has one basic operating mechanism for different genes in yeast. Also other widespread regulatory phenomena have a common basis in widely different organisms like the heat-shock response (Bienz, 1985; Sorger, 1991). Thus, it is not unthinkable that the Drosophila glucose repression of amylase and the yeast repression nave a common evolutionary origin, especially since in both cases multiple cis-acting sequences have been identified just upstream

the TATA motif. These data also point to a classic repression mechanism as probably acting in Drosophila.

In the future, it will be important to find the sequence elements that mediate glucose repression and, through that information, isolate the repressor protein and eventually find the signal transduction mechanism in order to understand the glucose repression phenomenon and its evolution. In our lab, promoters carrying only short regions of the Amy-1 promoter are being constructed and will be tested and used for isolating a repressor protein.

4.2 Tissue-specificity

We studied the tissue-specific expression of the amylase gene in both germline and somatic transformed larvae.

Tissue-specific expression of amylase has been the subject of intensive studies. In the adult fly amylase is expressed in both the anterior and posterior midgut and this expression is regulated both by trans- and cis- acting factors (Doane et al., 1983), while in the larva only cis- acting regulation has been found (Klarenberg et al., 1986). The map trans-acting locus is located at 1 cM from the amylase structural locus. It determines a variation in the length of the posterior midgut showing amylase expression, while the anterior midgut expression is not affected. This variation also occurs at mRNA level (Thompson et al., 1992). In the larva, expression of amylase is mainly in the posterior midgut, and is

only regulated by cis-acting elements.

With respect to the tissue-specific expression of amylase genes in the larvae, we were able to demonstrate that this is mediated by cis-acting sequences close to the transcription start. The germline transformed lines carrying the Amy-Adh hybrid gene presented ADH activity with the tissue specific pattern typical of the amylase gene. These results also show that this regulation is at the trancriptional level. Preliminary data on somatic transformants, carrying constructs of the amylase genes, both proximal and distal, with only 163 bp from transcription startpoint show that they are expressed normally and this expression occurs in the posterior midgut only as in the strain of origin (data not shown, but mentioned in Results).

Our data show that also the tissue-specificity of the Adh gene is determined by a few hundred bp of 5' flanking DNA sequences. In Drosophila several genes, all presenting a very specialized expression pattern, have been examined in order to identify regions responsible for tissue-specific expression, usually by means of germline transformation of hybrid genes. In all cases, tissue-specificity is mediated within a couple of hundred of bp from transcription startpoint. Specific examples include the Ddc gene encoding Dopa decarboxylase (Scholnick et al., 1986; Bray et al., 1988), the Sgs-3 gene for the expression of the Drosophila salivary gland secretion protein (Martin et al., 1989), the chorion genes (Romano et al.,1988; Mariani et al., 1988; Shea et al.,

1990), the ninaE opsin gene (Mismer and Rubin, 1989).

These data show that tissue-specificity is usually determined by 5' flanking sequences. When more closely localized, it turns out that the signals for tissue-specificity are quite close to the transcription start point (within 100-200 bp from it). Thus a clearer concept of the gene is forming with the accumulation of data on the sequences regulating its expression. We can say that a few hundred bp of upstream sequences are sufficient for full expression of many genes in Drosophila, and that some of the major regulatory elements, like glucose repression and tissue specificity, are very near the transcription initiation point.

It appears that the concept of a typical gene can be formulated on the basis of molecular data. The control of gene regulation is basically determined by a relatively small upstream region (smaller than the coding sequences), although many exceptions and variations apply to this scheme. For example, enhancers of transcription have also been found very far from the transcription start, from .5 to 10 kbp (Guarente and Bermingham-McDonogh, 1992). Regulatory sequences often can act quite well on different coding regions (in hybrid genes), thus appearing functionally independent, although in the whole organism the right combination of upstream and coding sequences is needed.

To find, within a short region, elements responsible for tissue specificity is quite difficult because the disruption of tissue-specificity mediating elements could lead to a complete

disruption of gene expression, since these elements might be activators of the expression of the gene. In the amylase gene, a GATAAG sequence has been found in a region important for high levels of gene expression (Magoulas et al.,1993b and this thesis). This sequence is conserved in promoter sequences from different species of Drosophila (Magoulas et al.,1993a). In this report it has been noted that a GATAAG sequence is repeated in the amylase proximal and distal promoters. This observation reinforces the idea that this sequence may be a midgut-specific regulator of gene expression. GATAAG and similar sequences are also present in the promoters of three trypsin-like gene of D. melanogaster, at comparable distances from transcription start point (unpublished observation) and these genes, like the amylases, are expressed only in the midgut (Davis et al., 1985). Moreover they are present in an inverted order on the maltase gene promoters, also expressed in the midgut (Snyder and Davidson, 1983). The Adh gene, another gene that is expressed in many tissues, including the midgut, has one sequence similar to GATAAG (GATAAT). Thus GATAAG is both conserved and present in genes (expressed in the midgut) and occurs in multiple copies, presenting thus characteristics of elements mediating gene expression.

4.3 Amylase duplicated genes

The comparison of proximal and distal amylase genes has shown that the two promoters present similar expression patterns, but a

different degree of glucose repression. A DNA sequence comparison has shown that the proximal and distal promoters are quite similarly organized, since both are functionally fully expressed when carrying only 163 bp of promoter sequence. If we look at them in a more detailed way, some differences can be noted: short deletions and substitutions are present between the sequences of the two promoters; at the functional level, though, both promoters show a similar pattern of gradual loss of expression ability; this occurs in a short region about 30 bp long; in the distal promoter this region is localized about 40 bp upstream than in the proximal gene. These regions, identified as important for gene expression, although localized at different positions in the two promoters, contain similar DNA sequences. This indicates a functional conservation of the duplicated promoters.

It is interesting to note that mutations in promoter regions do not have drastic effects like mutations in the coding regions where the deletion of a single nucleotide disrupts the whole encoded protein; variation in promoter sequences may occur without major consequences since often the position of different elements may vary and the elements often are repeated. In fact, it is known that variation in the promoter of duplicated genes may lead to the evolution of differential developmental expression for the different genes and, when accompanied by mutations in the coding region, may lead to highly specific functions in the organisms (see the haemoglobin genes, Li, 1983).

The comparison of the amylase coding sequences shows that the proximal and distal genes from the same chromosome are more similar than two alleles at the proximal locus. This observation parallels observation conducted on the duplicated amylase genes from D. melanogaster and D. erecta, where it was also noted that coding sequences from the same species are very similar while mutations between species accumulated as expected (Hickey et al., 1991). Mechanisms of gene conversion were postulated to explain this concerted evolution of the amylase genes. Overall, the analysis of both promoter and coding sequences of the amylase genes in Drosophila has revealed interesting aspects in the evolutionary and functional aspects of gene organization at molecular level.

In conclusion, we have demonstrated that cloned amylase genes are expressed and regulated normally in in vivo expression assays involving both somatic and germline transformation experiments. We have shown that the promoter sequence, and not the coding region of the amylase gene, carries elements mediating glucose repression and tissue-specificity. Thus these genes are regulated at the transcriptional level. We also have restricted from the 1600 bp, originally present in the cloned amylase gene, to a 78 bp region, closely linked to the transcription start site, the location of signals mediating glucose repression.

A comparison of proximal and distal amylase genes at the functional level has shown that variation in glucose repression

levels depend on *cis*-acting sequences. Comparison of functional characteristics of DNA sequences of the promoters and coding sequences has revealed a basic similarity between the two promoters and confirmed concerted evolution of the coding sequences.

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