**Molecular analysis of *cis*-regulatory sequences of the
 α -amylase gene in *D. melanogaster*: a short
5'-flanking region of *Amy* distal gene is
required for full expression of
Amy proximal gene**

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ABSTRACT

The structural locus for α -amylase (AMY) in *Drosophila melanogaster* is duplicated and divergently transcribed. These two genes are designated as *Amy-p* and *Amy-d*, respectively. We searched for the *cis*-acting regulatory elements for full expression of the duplicated *Amy-p* and *Amy-d* loci, by injecting plasmid constructs containing sequences from the *Amy* locus into preblastoderm embryos of an AMY-null strain and measuring exogenous AMY activity produced in transformed host larvae (i.e., the transient expression assay). Relative activities of endogenous amylase isozymes, AMY-1 and AMY-3, in extracts of AMY^{1,3} larvae of a Canton-S are almost the same. However, three independently isolated *Amy-p*¹ constructs with only the 5' upstream regions of *Amy-p*¹ expressed a very low AMY-1 activity. Two other *Amy-p*¹ constructs with the 5' upstream region of *Amy-d*³ in addition to that of *Amy-p*¹ produced a high activity. Thus, the 5' upstream region of *Amy-d*³ is necessary for full expression of *Amy-p*¹. In order to locate *cis*-regulatory elements within the 5' region of *Amy-d*³, a series of hybrid constructs including this region were tested to locate them. Our results clearly show that the *cis*-acting regulatory sequences required for full expression of *Amy-p*¹ are located between the base pairs at -304 and -372 upstream of *Amy-d*³ gene. In other words, only a short region located upstream of *Amy-d*³ was found to be necessary and sufficient for the full expression of *Amy-p*¹ in addition to its promoter. This region seems also necessary for the expression of *Amy-d*³.

1. INTRODUCTION

Drosophila α -amylase genes (*Amy*) are typically polymorphic with a large array of variants for electrophoretic mobility and thermostability in nature (Kikkawa, 1964; Prakash and Lewontin, 1968; Doane, 1969; Prakash, 1977; Hickey, 1979; Norman and Prakash, 1980; Singh et al., 1982; Yamazaki et al., 1984; Langley et

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al., 1988; Payant et al., 1988). There are significant differences for the amylase activity among inbred strains (Doane, 1969; DeJong et al., 1972; Hoorn and Scharloo, 1980; Hickey, 1981; Yamazaki et al., 1984) and some of the factors governing enzyme activity variations were genetically linked to the structural gene locus (Hoorn and Scharloo, 1978; Hickey, 1979; Doane, 1980). Factors controlling the expression of amylase which are not linked to the structural gene locus were also found (Matsuo and Yamazaki, 1986). The strain-specific differences in activity are controlled by regulatory factors which probably affect the levels of transcription at the *Amy* locus (Yamazaki, 1985; Benkel and Hickey, 1985, 1986). Thus, the *Drosophila* amylase is an excellent model system for the study of gene regulation in higher eukaryotes.

The structural locus for α -amylase in *Drosophila melanogaster* is at 54A on the polytene map for chromosome 2 (Gemmill et al., 1985). The gene is duplicated and divergently transcribed. The transcription units are typically spaced about 4 kb apart (Levy et al., 1985; Boer and Hickey, 1986; Gemmill et al., 1986; Benkel et al., 1987; Langley et al., 1988). Gemmill et al. (1985) isolated a recombinant clone, λ Dm65, which contains sequences that span the amylase locus from a Canton-S strain. Each of the amylase genes in λ Dm65 produces an active amylase when injected into *Xenopus* oocytes (Levy et al., 1985) or assayed in somatic transformants of *D. melanogaster* (Hawley et al., 1990).

It is known that amylase expression is controlled by a variety of *cis*- and *trans*-acting genetic mechanisms (Yamazaki and Matsuo, 1984; Hickey and Benkel, 1987). Here we present an analysis of the sequence necessary for full expression of amylase proximal gene in *D. melanogaster* using a transient expression assay. The about 4 kb region between the duplicated amylase genes was modified and a series of eleven constructs were obtained.

We have used the transient expression assay because there are certain advantages; for example, the rapidity with which potential control elements can be targeted (Martin et al., 1986), and the insensitivity to position effects resulting from germ line transformation (Spradling and Rubin, 1982; Kirkpatrick and Martin, 1992). When cloned genes were injected into preblastoderm embryos of the host, it can be somatically expressed within the same generation (Rubin and Spradling, 1982; Stellar and Pirota, 1984; Priess et al., 1985; Martin et al., 1986; Sofer and Martin, 1987; Hofmann et al., 1991). Somatic transformation in *D. melanogaster* appears to be associated with correct temporal and spatial expression of exogenous genes (Martin et al., 1986; Roark et al., 1990; Hawley et al., 1990, 1992).

Sequences tested for exogenous amylase activity with the transient expression assay were derived from the genomic clone λ Dm65 (Gemmill et al., 1985). The amylase genes in λ Dm65, *Amy-p*¹ and *Amy-d*³ [synonyms, *AmyA*¹ and *AmyB*³ (Gemmill et al., 1986)], encode amylase isozymes AMY-1 and AMY-3, respectively (Levy et al., 1985). Activities of AMY-1 and AMY-3 of a Canton-S wild type

strain, were almost the same. However, when we tested a construct which includes the *Amy-p*¹ structural gene and about 4 kb of its 5' flanking sequence, transformed host larvae expressed a very low level of AMY-1 activity relative to AMY-3 activity produced by another construct which includes *Amy-d*³ structural gene and about 500 bp of 5' flanking sequence. The result was compared with the endogenous patterns of AMY-1 and -3 expressions in larvae of the Canton-S strain. In this paper, we try to determine and describe the *cis*-acting regulatory elements that are necessary for full expression of AMY-1 activity. Our deletion analysis indicates that sequences required for full expression of *Amy-p*¹ are located between the base pairs at -304 and -372 upstream of the *Amy-d*³ gene. These sequences are probably required for the full expression the *Amy-d*³ genes.

2. MATERIALS AND METHODS

Plasmid constructions

All *Amy* fragments originated from λ Dm65. They were cloned into the pBluescript (pBT) phagemid vector (Stratagene) in the opposite orientation to the *LacZ* structural gene in pBT. The test constructs are shown in Fig. 1 and Fig. 2.

pE3.8, pE/H4.3, pX/Xb8.6 and pX/S10.2 were subcloned from λ Dm65 into the pBT vectors. The pE3.8 consists of the 3.8 kb *EcoRI* fragment containing the coding region of the *Amy-p*¹ gene plus 196 bp of 5' flanking sequence and about 2 kb of 3' flanking sequence. The pE/H4.3 contains an additional ~400 bp of 5' flanking sequence to that of pE3.8 (sequence data, Boar and Hickey, 1986; Hickey et al., 1987). The pX/Xb8.6 carries the 8.6 kb *XhoI/XbaI* fragment which includes upstream sequences extending from the *EcoRI* site upstream of the *Amy-p*¹ coding region to the *XbaI* site upstream of the *Amy-d*³ coding region and about 2.8 kb of 3' flanking sequence. The pX/S10.2 contains the same insert as pX/Xb8.6 plus upstream sequences extending to the *SalI* site of the *Amy-d*³ coding region. pX/S10.2-H/Xb was made by cutting pX/S10.2 with *HindIII* and *XbaI* and ligating the fragments together. The construct consists of the 5.1 kb *XhoI/HindIII* fragment which includes the *Amy-p*¹ with about 700 bp of 5' flanking sequence and 2.8 kb of 3' flanking sequence, and the 1.5 kb *XbaI/SalI* fragment which includes about 1.1 kb of *Amy-d*³ coding region and 463 bp of 5' flanking sequence. pE3.8, pE/H4.3, pX/Xb8.6, pX/S10.2 and pX/S10.2-H/Xb are called the *Amy-p*³ constructs.

Six hybrid constructs (pE3.8+dA, dB, dC, dD, dE and dF series) shown schematically in Fig. 2, were made by a combination of the polymerase chain reaction (PCR) method (Higuchi, 1989) and the crossover-linking method (Sung et al., 1989). The upstream regions of the *Amy-d*³ were amplified by PCR using two custom-made oligonucleotide primers for each constructs shown below:

dA;

Fig. 1. Outline of plasmid constructs used in somatic transformation experiments. *Amy* sequences were subcloned from λ Dm65. pXb/E3.1 is the *Amy-d*³ construct. The remaining constructs contain *Amy-p*¹.

- a forward primer: 5'-TCTAGAGGTGATACATGAG-3' and
- a reverse primer: 3'-ATCGTTAAATCCCCGCTATT-5'
- dB;
- a forward primer: 5'-TCTAGAGGTGATACATGAG-3' and
- a reverse primer: 3'-CAGAACTACAAATGAATACA-5'
- dC;
- a forward primer: 5'-ATTATAACAATATAAATTAC-3' and
- a reverse primer: 3'-CAGAACTACAAATGAATACA-5'
- dE;

Fig. 2. Outline of plasmid constructs used in somatic transformation experiments. The fragments amplified by PCR were ligated with the pE3.8 construct, to produce hybrid constructs. The symbols indicate that; ■: repeat motifs with the enhancer of *Adh* gene (Corbin and Maniatis, 1990), ●: a hexamer homology in chorion promoter (Mitsialis et al., 1987), ○: 5'-CCAAT-3' motif (Kim and Lee, 1989), □: the enhancer core homology (Falb and Maniatis, 1992), Ⓟ: a putative AP1 binding site (Bohmann et al., 1987; Rauscher et al., 1988), Δ: a sequence homology of glucose repressible gene in yeast (Boer and Hickey, 1986).

a forward primer: 5'-GTCAAATGGCCAATAAATC-3' and

a reverse primer: 3'-ACATAAGTAAACATCAAGAC-5'

dF;

a forward primer: 5'-ATTATAACAATATAAATTAC-3' and

a reverse primer: 3'-AGATCACCGCACGTT-5'

The plasmid pXb/E3.1 (described above) was used as a template. These primers were synthesized based on the sequences in the upstream region of the *Amy-d*³ from λ Dm65 (Hawley et al., 1992). The full length of the dD fragment, 44 bp, was made by synthetic oligonucleotides.

dD;

a sense strand:

5'-GTCAAGTGGCCAATAAATCTCCAATATCAACCAATACTTGAGGA-3'

The amplified fragments were then cloned into *EcoRV* endonuclease site of the plasmid pE3.8 (described above). All of these constructs were then verified by DNA sequencing.

pXb/E3.1 was cloned from λ Dm65. The pXb/E3.1 consists of the 3.1 kb *XbaI*/*EcoRI* fragment containing the coding region of the *Amy-d*³ gene plus 463 bp of 5' flanking sequence and about 1 kb of 3' flanking sequence. This construct is called the *Amy-d*³ construct.

The TN256 strain has *Amy-p*³ and *Amy-d*⁶. The plasmid p256d contained 5kb H/E fragment, which includes *Amy-d*⁶, about 1.5 kb of 5' flanking sequence and roughly 1 kb of 3' flanking sequence. This construct is called the reference construct.

Transient expression assay

Plasmids containing various amylase constructs were microinjected into *D. melanogaster* embryos of the *c,Amy*^{null} strain (Hawley, 1989) according to the method of Rubin and Spradling (1982), as modified by Martin et al. (1986). Eggs of the *c,Amy*^{null} host strain were collected, dechorionated and microinjected into the posterior end at the preblastoderm stage. Equal amounts of test plasmids and the reference plasmids (250 mg of each/ml) were dissolved in an injection buffer (5 mM KCl, 0.1 mM sodium phosphate, pH 6.8) and coinjected. Injected embryos were developed under halocarbon oil in a moist chamber at 25°C. Upon hatching, larvae were transferred to cornmeal medium (11% cornmeal, 2.8% molasses, 1.4% sugar, 2% brewers yeast, 0.6% agar, and 0.4% buffered propionic acid) and allowed to develop until they reached a length of 3–4 mm in the late third instar.

Electrophoretic analysis

Larvae were homogenized, two for each measurement, in 30 μ l buffer (0.1 M Tris Borate, 5 mM MgCl₂, 10% sucrose). Crude extracts were run at 300 V for 3 hr in 7% polyacrylamide gels. The electrode buffer was 0.1 M Tris Borate (pH

8.9). The separation gels were then incubated in 2% hydrolyzed starch solution for 2 to 4 hr, stained with 3% iodine solution ($I_2:KI:H_2O=1:2:20$) and quantified by scanning densitometry using whole band analysis (Doane, 1967).

3. RESULTS

Reference construct: p256d

In transient expression assays, we coinjected equal amounts of test plasmids and the reference plasmids in order to provide an internal control activity against a test plasmid activity. In this way, the relative activity of AMY-1 to that of AMY-6 can be assessed. The p256d was selected as the reference plasmid because when we tested p256d in transient expression assays (Choi and Okuyama, unpublished), it consistently expressed AMY-6 activity and its electrophoretic mobility was slower than that of the *Amy-p*¹ gene product.

*Transient expression assays of deletion constructs of Amy-p*¹

To determine the *cis*-regulatory sequences which are essential for full expression of the *Amy* proximal gene, we analyzed the effects of deletions and rearrangements on the expression of *Amy* constructs in somatically transformed hosts.

Transformed host larvae injected with pE3.8 expressed undetectable levels of AMY-1 activity relative to that of the AMY-6 activity produced by p256d in transient expression assays (Table 1A). AMY-1 activity was too low to be detected by densitometer under the given assay conditions. From the above results, it appears that additional flanking sequences are required for full expression of *Amy-p*¹.

The pE/H4.3 (Fig. 1) which contains the same construct as pE3.8 plus an additional about 400 bp of 5' flanking sequence, was then tested. The expression levels of transformed host larvae were similar to that of pE3.8 construct (Table 1A).

The pX/Xb8.6 (Fig. 1) includes the *Amy-p*¹ coding region with about 4.3 kb of upstream region and roughly 2.8 kb of downstream region. Forty six transformed host larvae also expressed very low levels of AMY-1 activity (Fig. 3A, Table 1A).

pX/S10.2 (Fig. 1) contained the same insert as pX/Xb8.6 plus additional upstream sequences extending to about 1.1 kb of the *Amy-d*³ coding region. The transformed larvae produced higher AMY-1 activity relative to those of the former three constructs (Fig. 3A, Table 1A). This indicates that some parts of the upstream sequence including the 1.5 kb *XbaI/SalI* fragment of the *Amy* distal gene is necessary for full expression of *Amy-p*¹.

To examine the effect of a part of the flanking region of *Amy-p*¹ in pX/Xb8.6, we tested the plasmid pX/S10.2-H/Xb (Fig. 1) which is deleted in the 3.6 kb *HindIII/XbaI* fragment from pX/S10.2. In transient expression assays with

pX/S10.2-H/Xb, AMY-1 activity was expressed at high level similar to that of pX/S10.2 (Fig. 3A, Table 1A). The result suggests that elements necessary for full expression of *Amy-p*¹ are within the 1.5 kb *XbaI/SalI* fragment of the *Amy* distal gene.

Table 1. Transient expression assay: Analysis of the *amylase* constructs

Construct	Host larvae transformed	Quantitation of amylase activities (AMY-1/AMY-6)	N ^a	Group ^b
(A) <i>Amy-p</i> ¹ constructs				
pXb/E3.1	12	0.93 ± 0.41 ^c	6	—
pE3.8	10	n.s.	5	—
pE/H4.3	8	n.s.	4	—
pX/Xb8.6	30	n.s.	15	—
pX/S10.2	20	0.66 ± 0.19	10	A
pX/S10.2-H/Xb	14	0.83 ± 0.23	7	A
(B) Hybrid constructs				
pE3.8+dA (−470 to −115) ^d	16	2.76 ± 0.62	8	B
pE3.8+dB (−470 to −234)	8	2.71 ± 0.21	4	B
pE3.8+dB (−372 to −234)	14	2.22 ± 0.42	7	B
pE3.8+dD (−304 to −261)	20	0.39 ± 0.19	10	—
pE3.8+dE (−304 to −234)	12	0.33 ± 0.13	6	—
pE3.8+dF (−372 to −304)	8	2.82 ± 0.36	4	B

The amylase constructs listed here correspond to the deletions diagrammed in Fig. 1. Exogenous amylase activity for each construct in transient assays is given as the relative activity (activity of test construct divided by that of the reference construct, i.e., AMY-1/AMY-6), ± standard deviation.

^a N is the number of samples quantified by densitometer.

^b Grouping was made by the Gabriel multiple-range test performed on the six constructs expressed at high level of the AMY-1 activity.

^c The mean relative activity of pXb/E3.1 construct was estimated by AMY-3/AMY-6.

^d The numbers in parenthesis indicate the end points of the PCR fragments.

Transient expression assays of hybrid constructs

To locate more precisely the unidentified *cis*-regulatory elements for full expression of *Amy-p*¹, a series of hybrid constructs were made. The fragments were amplified by PCR with upstream sequences of *Amy-d*³ and fused to the upstream of the plasmid pE3.8 which is in the same orientation as the endogenous genes (Fig. 2). The whole length of dD fragment was synthesized. The 196 bp upstream sequence of pE3.8 contains a CAAT-like motif (−122, nucleotide sequence of the *Drosophila* α-amylase gene numbered from the initiation codon) and the TATA (−64) box consensus sequences which are located at the expected positions for correct initiation of transcription of eukaryotic gene.

Fig. 3. Transient expression assays of the deletion and the hybrid constructs. Host were coinjected with equal amounts of reference plasmids p256d (AMY-6) and test construct (AMY-1). The AMY-1 and AMY-6 electrophoretic phenotypes appear in all sample lanes. The test constructs are; (A) deletion constructs: pXb/E3.1 (distal construct), pX/Xb8.6. Very weak AMY-1 activity was expressed by transformants of this construct compared to those of pX/S10.2 and pX/S10.2-H/Xb. (B) hybrid constructs: pE3.8+dA, pE3.8+dE and pE3.8+dF. ^aSomatically transformed host larvae expressed AMY-6 (reference plasmid) and AMY-3 (test construct).

Unfortunately, our transient assay does not distinguish between low and no expression. Thus, we constructed the plasmid pDm E3.8 which contains the same 3.8 kb *Eco*RI fragment as pE3.8. The plasmid was cloned into a P element transformation vector (data not shown). The pDm E3.8 was introduced into the germ line of *ry*⁵⁰⁶ flies by P element-mediated transformation. G₀ transformants

were crossed with *Amy-null* flies for three to five generations until the background of transformants was replaced with that of the *Amy-null*. In adults flies of four independent germ line transformant lines, the AMY-1 activity was one-tenth of that of the Canton-S strain which includes duplicated *Amy* genes, *Amy-p*¹ and *Amy-d*³. The result indicates that the 3.8 kb *Eco*RI fragment is sufficient for AMY-1 expression, even though its activity is very low. Thus, to test the effect of hybrid constructs, we ligated PCR fragments to the plasmid pE3.8.

pE3.8+dA (Fig. 2) consists of the dA fragment of the 5' flanking sequence of *Amy-d*³ and the E3.8 fragment of *Amy-p*¹. The dA fragment contains 355 bp amplified fragment from -115 to -470 of *Amy-d*³, which lacks the TATA box, CAAT-like motifs and the presumptive transcription start site (Fig. 4).

When pE3.8+dA was tested, the AMY-1 activity was three-fold greater than the AMY-6 reference activity (Fig. 2B, Table 1B). This indicates that *cis*-regulatory elements for full expression of *Amy-p*¹ is located between the base pairs at -115 and -470 of *Amy-d*³. Interestingly, the expression level of pE3.8+dA is higher than those of the pX/S10.2 and pX/S10.2-H/Xb (see DISCUSSION).

To further localize the regulatory element, we tested pE3.8+dB (Fig. 3). The amplified fragment dB contained 5' flanking sequences between the base pairs at -234 and -470 of *Amy-d*³ (Fig. 4). The dB fragment was linked to the upstream of pE3.8. The transformed larvae expressed a high level of AMY-1 activity similar to that of pE3.8+dA (Table 1B).

Then, we tested pE3.8+dC (Fig. 3) which includes 138 bp of 5' flanking sequence of *Amy-d*³ (from -234 to -372) fused to pE3.8. When pE3.8+dC was tested, the AMY-1 activity was about two-fold greater than the AMY-6 reference activity (Table 1B). The result was similar to those for the former two constructs (pE3.8+dA and pE3.8+dB). It suggests that the regulatory element for full expression of *Amy-p*¹ is located between the base pairs at -234 and -372 of *Amy-d*³ (Fig. 4).

In order to determine the location of the regulatory elements more precisely, we constructed the plasmids pE3.8+dD, pE3.8+dE and pE3.8+dF (Fig. 2, see also Fig. 4). The dD fragment contains 44 bp amplified fragment from -261 to -304 of *Amy-d*³, which is characterized by the presence of three 5'-CCAAT-3' motifs. The dE fragment contains 74 bp amplified fragment from -234 to -304 of *Amy-d*³. It consists of a posterior part of the dC fragment and includes the enhancer core homology [TGTGGAAA(TTT)G] plus three 5'-CCAAT-3' motifs. The dF fragment contains 70 bp amplified fragment from -304 to -372 of *Amy-d*³, which consists of anterior part of the dC fragment. These three amplified fragments were fused to the upstream of pE3.8 and tested in transient expression assays.

When pE3.8+dD and pE3.8+dE were tested, the AMY-1 activity of the hybrid constructs was one-fifth of that of pE3.8+dC on the average (Table 1B).

Fig. 4. Base sequences of 5' flanking region of the *Amy-d³* gene from λ Dm65. The TATA box and CAAT-like motifs are boxed by shaded areas. Some other consensus motifs are boxed (the symbols is the same as in Fig. 2). The nucleotide sequence are numbered from the initiation codon. Arrows mark the end points of seven PCR fragments.

On the other hand, a dramatic increase in AMY-1 activity relative to those resulting from pE3.8+dD or pE3.8+dE was observed in pE3.8+dF larvae (Fig. 3B, Table 1B). This AMY-1 activity level is similar to those of pE3.8+dA, dB and dC. Thus, we can localize the regulatory elements essential for full expression of *Amy-p¹* to the region from -304 to -372 of *Amy-d³*.

Statistical analysis of densitometric data

The six constructs that showed high AMY-1 activity in transient expression assays (pX/S10.2, pX/S10.2-H/Xb, pE3.8+dA, pE3.8+dB, pE3.8+dC and pE3.8+dF) were divided into two groups using the multiple-range test of Gabriel (1978); Group A (pX/S10.2 and pX/S10.2-H/X), Group B (pE3.8+dA, pE3.8+dB, pE3.8+dC and pE3.8+dF) (Table 1). The hybrid constructs pE3.8+dA, pE3.8+dB, pE3.8+dC and pE3.8+dF (Group B) expressed about four-fold higher activity of AMY-1 than those of pX/S10.2 and pX/S10.2-H/Xb

(Group A) and about 2-fold higher activity than the AMY-3 activity of the distal construct pXb/E 3.1.

4. DISCUSSION

Transient expression assay

Two copies of the α -Amylase gene in *Drosophila melanogaster* are divergently transcribed. A recombinant clone, λ Dm65, contains sequences that span the *Amy* locus from a Canton-S strain (Gemmill et al., 1985; Levy et al., 1985). Both *Amy* genes in λ Dm65 produce active amylase when injected into *Xenopus* oocytes (Levy et al., 1985) or assayed in somatic transformants of *D. melanogaster* (Hawley et al., 1990, 1992; Magoulas et al., 1993). Endogenous activities of *Amy-p*¹ and *Amy-d*³ in a Canton-S strain were almost at the same levels. However, when the duplicated genes from the *Amy* locus were inserted into separate plasmids, three independently isolated *Amy-p*¹ constructs (pE3.8, pE/H4.2 and pX/Xb8.6) expressed AMY-1 activity at very low levels even though an *Amy-d*³ construct (pXb/E3.1) expressed AMY-3 activity at a high level. Hawley et al. (1992) reported results some of which contradict those obtained for pE3.8, pE/H4.2 and pXb/E3.1 in our study. When they tested pBTSK-Xba/X8.6, although two of the four transformed larvae showed a low activity pattern as in our case, the remaining two displayed patterns similar to the endogenous *Amy*^{1,3} patterns with high activity of AMY-1. The difference between the two constructs, pX/Xb8.6 and pBTSK-Xba/X8.6, seems to be only the orientation of the insert in the vector. All *Amy* genes in our constructs were in the opposite direction to the *LacZ* structural gene in the vector whereas those of Hawley et al. (1992) are in the same direction as the *LacZ* gene. We hypothesized that if *Amy* genes are in the same orientation as the *LacZ* gene in the vector, the expression of the former genes is influenced by the promoter of the latter gene and sometimes becomes high. To test this, we constructed the same plasmid, pBTSK-Xba/X8.6. When we tested pBTSK-Xba/X8.6 in transient expression assay, four of the eight transformed larvae showed high activity. Thus, it is likely that the high AMY-1 activities observed by Hawley et al. (1992) were caused by the phagemid vector itself.

The results on pX/S10.2 and pX/S10.2-H/Xb in transient expression assays suggest that unidentified *cis*-acting regulatory elements for full expression of *Amy-p*¹ is located within the 1.5 kb fragment containing the anterior half of *Amy-d*³ coding region and 463 bp of 5' flanking sequence.

To determine the minimum length of upstream sequence necessary for full expression of *Amy-p*¹, we tested a series of hybrid constructs. The largest hybrid construct, dA, amplified between the base pairs at -115 and -470 of *Amy-d*³, lacks the TATA box, CAAT-like motifs and the presumptive transcription start site, but contains a putative AP1 binding site (TGAATCA) (Bohmann

et al., 1987; Rauscher et al., 1988) between the base pairs at -157 and -163, a sequence homologous to the elements of glucose repressible genes in yeast (Boer and Hickey, 1986) between the base pairs at -196 and -211, the enhancer core homology [TGTGGAAA(TTT)G] (Falb and Maniatis, 1992) between the base pairs at -257 and -265 and three 5'-CCAAT-3' motifs (Kim and Lee, 1989) between the base pairs at -269 and -294. pE3.8+dB lacking the putative AP1 binding site and the potential glucose repression element still fully expressed AMY-1 activity. The hybrid construct pE3.8+dC containing 5' flanking sequence from -234 to -372 produced AMY-1 activity at high levels. When we tested pE3.8+dD and pE3.8+dE which contain the posterior part of the dC fragment, the expression levels of *Amy-p¹* decreased to about one-sixth of those of the above three hybrid constructs, even though pE3.8+dD and pE3.8+dE contain three 5'-CCAAT-3' motifs and those motifs plus the enhancer core homology, respectively. However, when we tested pE3.8+dF which include anterior part of the dC fragment, the transformed host larvae expressed AMY-1 at high levels similar to that of pE3.8+dC. The results from a series of hybrid constructs suggest that regulatory elements for full expression of *Amy-p¹* is located between the base pairs at -304 to -372 of *Amy-d³*. The dF fragment contains a sequence similar to repeated motifs within the enhancer of *Adh* gene in *D. melanogaster* (ATAT-AAATTA) (Corbin and Maniatis, 1990) between the base pairs at -354 and -363 and a hexamer homology sequence which is essential for expression in both orientation in moth chorion promoter (TCACGT) (Mitsialis et al., 1987) between the base pairs at -325 and -320. These elements are candidates for the regulatory elements located in this region.

The cis-regulatory element may function bidirectionally for the duplicated Amy genes

Some shared regulatory elements were reported in the yolk protein genes *yp-1/yp-2* (Garabedian et al., 1985, 1986; Logan and Wensink, 1990) and the glue protein genes *Sgs-7/Sgs-8* (Hofmann et al., 1991) in *D. melanogaster* and the paired 18 and 401 chorion genes in the silkworm (Fenerjian and Kafatos, 1994). Hawley et al. (1992) reported that a sequence required for high levels of *Amy-d³* expression was located between the base pairs at -253 and -366 of *Amy-d³* in *D. melanogaster*. From these results, we suggest that the cis-regulatory element located between the base pairs at -304 and -372 of *Amy-d³* functions bidirectionally for divergently transcribed *Amy* proximal and distal genes.

Possibility of negative elements

Our statistical analysis identified two groups, Group A and B, among the constructs which expressed AMY-1 at high levels. Group B constructs (pE3.8+dA, pE3.8+dB, pE3.8+dC and pE3.8+dF) expressed about four-fold higher AMY-1 activity than Group A constructs (pX/S10.2 and pX/S10.2-H/Xb).

Since both Group A constructs include all fragments contained in Group B constructs, one possible explanation for this observation is that one or several negative regulatory elements are located between the base pairs at -196 (*EcoRI* site) and -480 (*HindIII* site) upstream of *Amy-p*¹. Boer and Hickey (1986) reported that repeated sequence motifs implicated for glucose repression of iso-1-cytochrome c and *Adh* II in yeast upstream regions are located between the base pairs at -199 and -217 upstream of the *Amy* proximal gene. It is likely that positive and negative regulatory elements cooperate for correct expression of both of *Amy* genes. To determine the negative regulatory element, however, further investigations are necessary. An alternative explanation is that the presence of *Amy-d*³ structure gene interferes with the formation of the transcripts of *Amy-p*¹. This may imply that specific transcriptional factors binding this regulatory element are shared by the divergently transcribed two *Amy* genes. To understand this mechanism, it is necessary to determine other *cis*-regulatory elements and to isolate the protein factors which mediate the interaction between the regulatory elements and the promoter.

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REFERENCES

- Benkel, B. and Hickey, D. A. (1985). Glucose repression of amylase expression in *Drosophila melanogaster*. *Genetics* **110**, (suppl.), s25.
- Benkel, B. and Hickey, D. A. (1986a). Glucose repression of Amylase gene expression in *Drosophila melanogaster*. *Genetics* **114**, 137–144.
- Benkel, B. and Hickey, D. A. (1986b). The interaction of genetic and environmental factors in the control of Amylase gene expression in *Drosophila melanogaster*. *Genetics* **114**, 943–954.
- Benkel, B., Abukashawa, S., Boer, P. H. and Hickey, D. A. (1987). Molecular cloning of DNA complementary to *Drosophila melanogaster* alpha-amylase mRNA. *Genome* **29**, 510–515.
- Boer, P. H. and Hickey, D. A. (1986). The alpha-Amylase gene in *Drosophila melanogaster*: Nucleotide sequence, gene structure and expression motifs. *Nucleic Acids Res.* **14**, 8399–8411.
- Bohmann, D., Bos, T. J., Admon, A., Nishimura, T., Vogt, P. K. and Tjian, R. (1987). Human proto-oncogene *c-jun* encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. *Science* **238**, 1386–1392.
- Corbin, V. and Maniatis, T. (1990). Identification of *cis*-regulatory elements required for larval expression of the *Drosophila melanogaster* alcohol dehydrogenase gene. *Genetics* **124**, 637–646.
- DeJong, G., Hoorn, A. J. W., Thorig, G. E. W. and Scharloo, W. (1972). Frequencies of amylase variants in *Drosophila melanogaster*. *Nature* **238**, 452–453.
- Doane, W. W. (1967). Quantitation of amylases in *Drosophila* separated by acrylamide gel electrophoresis. *J. Exp. Zool.* **264**, 363–378.
- Doane, W. W. (1969). Amylase variants in *Drosophila melanogaster*: Linkage studies and characterization of enzyme extracts. *J. Exp. Zool.* **171**, 321–342.
- Doane, W. W. (1980). Selection for amylase allozymes in *Drosophila melanogaster*: some questions.

- Evolution* **34**, 868–874.
- Falb, D. and Maniatis, T. (1992). A conserved regulatory unit implicated in tissue-specific gene expression in *Drosophila* and man. *Genes Dev.* **6**, 454–465.
- Fenerjian, M. G. and Kafatos, F. C. (1994). Developmental specificity of a bidirectional moth chorion promoter in transgenic *Drosophila*. *Dev. Biol.* **161**, 37–47.
- Gabriel, K. R. (1978). A simple method of multiple comparisons of mean. *J. Am. Stat. Assoc.* **73**, 724–729.
- Garabedian, M. J., Hung, M. and Wensink, P. C. (1985). Independent control elements that determine yolk protein gene expression in alternative *Drosophila* tissues. *Proc. Natl. Acad. Sci. USA* **82**, 1396–1400.
- Garabedian, M. J., Shepherd, B. M. and Wensink, P. C. (1986). A tissue-specific enhancer from the *Drosophila* yolk protein 1 gene. *Cell* **45**, 859–867.
- Gemmill, R. M., Levy, J. N. and Doane, W. W. (1985). Molecular cloning of α -amylase genes from *Drosophila melanogaster*. I. Cloning isolation by use of a mouse probe. *Genetics* **110**, 299–312.
- Gemmill, R. M., Schwartz, P. E. and Doane, W. W. (1986). Structural organization of the *Amy* locus in seven strains of *Drosophila melanogaster*. *Nucleic Acids Res.* **14**, 5337–5352.
- Hawley, S. A. (1989). *Analysis of alpha-Amylase Gene Regulation in Drosophila by Somatic Transcription Assay*. Ph. D. Thesis, Arizona State University, Tempe.
- Hawley, S. A., Doane, W. W. and Norman, R. A. (1992). Molecular analysis of cis-regulatory sequences at the α -amylase locus in *Drosophila melanogaster*. *Biochem. Genet.* **30**, 257–277.
- Hawley, S. A., Norman, R. A., Brown, C. J., Doane, W. W., Anderson, W. W. and Hickey, D. A. (1990). Amylase gene expression in intraspecific and interspecific somatic transformants of *Drosophila*. *Genome* **33**, 501–508.
- Hickey, D. A. (1979). The geographical pattern of an enzyme polymorphism in *D. melanogaster*. *Genetica* **51**, 1–4.
- Hickey, D. A. (1981). Regulation of amylase activity in *Drosophila melanogaster*: variation in the number of enzyme molecules produced by different amylase genotypes. *Biochem. Genet.* **19**, 783–796.
- Hickey, D. A. and Benkel, B. F. (1987). Regulation of Amylase gene expression: *Drosophila* amylases as a model experimental system. *CRC Crit. Rev. Biotech.* **5**, 229–241.
- Hickey, D. A., Genest, Y. and Benkel, B. F. (1987). Nucleotide sequence upstream of a glucose-repressible *Drosophila* gene. *Nucleic Acids Res.* **15**, 7184.
- Higuchi, R. (1989). Using PCR to engineer DNA. In: *PCR Technology* (ed.: H. A. Erlich), pp. 61–70. Stockton Press, New York.
- Hofmann, A., Garfinkel, M. D. and Meyerowitz, E. M. (1991). cis-Acting sequences required for expression of the divergently transcribed *Drosophila melanogaster Sgs-7* and *Sgs-8* glue protein genes. *Mol. Cell. Biol.* **11**, 2971–2979.
- Hoorn, A. J. W. and Scharloo, W. (1978). Functional significance of amylase polymorphism in *Drosophila melanogaster*. V. The effect of food components on amylase and α -glucosidase activity. *Genetica* **49**, 181–187.
- Hoorn, A. J. W. and Scharloo, W. (1980). Functional significance of amylase polymorphism in *Drosophila melanogaster*. III. Ontogeny of amylase and some α -glucosidases. *Biochem. Genet.* **18**, 51–63.
- Kikkawa, H. (1964). An electrophoretic study on amylase in *Drosophila melanogaster*. *Jpn. J. Genet.* **39**, 401–411.
- Kim, Y. K. and Lee, A. S. (1989). Cooperative interactions between the *GRP78* enhancer and promoter elements in hamster fibroblasts. *Gene* **77**, 123–131.
- Kirkpatrick, R. B. and Martin, P. F. (1992). Tissue-specific position effects on alcohol dehydrogenase expression in *Drosophila melanogaster*. *Mol. Gen. Genet.* **232**, 135–144.

- Langley, C. H., Shrimpton, A. E., Yamazaki, T., Miyashita, N., Matsuo, Y. and Aquadro, C. F. (1988). Naturally occurring variation in the restriction map of the *Amy* region of *Drosophila melanogaster*. *Genetics* **119**, 619–629.
- Levy, J. N., Gemmill, R. M. and Doane, W. W. (1985). Molecular cloning of alpha-amylase genes from *Drosophila melanogaster*. II. Clone organization and verification. *Genetics* **110**, 313–324.
- Logan, S. K. and Wensink, P. C. (1990). Ovarian follicle cell enhancers from the *Drosophila* yolk protein genes: Different segments of one enhancer have different cell-type specificities that interact to give normal expression. *Genes Dev.* **4**, 613–623.
- Magoulas, C., Bally-Cuif, L., Loverre-Chyurlia, A., Benkel, B. and Hickey, D. (1993). A short 5'-flanking region mediates glucose repression of amylase gene expression in *Drosophila melanogaster*. *Genetics* **134**, 507–515.
- Martin, P. F., Martin, A., Osmani, A. and Sofer, W. (1986). A transient expression assay for tissue-specific gene expression of alcohol dehydrogenases in *Drosophila*. *Dev. Biol.* **117**, 574–580.
- Matsuo, Y. and Yamazaki, T. (1986). Genetic analysis of natural populations of *Drosophila melanogaster* in Japan. VI. Differential regulation of duplicated amylase loci and degree of dominance of amylase activity in different environments. *Jpn. J. Genet.* **61**, 543–558.
- Michael, R. S. and Hans K. U. (1979). Tables of the studentized maximum modulus distribution and an application to multiple comparisons among means. *Technometrics* **21**, 87–93.
- Mitsialis, S. A., Spoerel, N., Leviten, M. and Kafatos, F. C. (1987). A short 5'-flanking DNA region is sufficient for developmentally correct expression of moth chorion genes in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **84**, 7987–7991.
- Payant, V., Abukashawa, S., Sasseville, M., Benkel, B. F., Hickey, D. A. and David, J. (1988). Evolutionary conservation of the chromosomal configuration and regulation of amylase genes among eight species of the *Drosophila melanogaster* species subgroup. *Mol. Biol. Evol.* **5**, 560–567.
- Prakash, A. and Lewontin, R. C. (1968). A molecular approach to the study of genetic heterozygosity in natural populations. III. Direct evidence of coadaptation in gene arrangements in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **59**, 398–405.
- Priess, A., Rosenberg, U. B., Kienlin, A., Seifert, E. and Jackle, H. (1985). Molecular genetics of *Kru'ppel*, a gene required for segmentation of the *Drosophila* embryo. *Nature* **313**, 27–32.
- Rauscher, F. J., Sambucetti, T. C., Distel, R. J. and Spiegelman, B. M. (1988). Common DNA binding site for fos protein complexes and transcription factor AP-1. *Cell* **52**, 471–480.
- Roark, M., Raghavan, K. V., Todo, T., Mayeda, C. A. and Meyerowitz, E. M. (1990). Cooperative enhancement at the *Drosophila Sgs-3* locus. *Dev. Biol.* **139**, 121–133.
- Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348–353.
- Singh, R. S., Hickey, D. A. and David, J. (1982). Genetic differentiation between geographically distant populations of *Drosophila melanogaster*. *Genetics* **101**, 235–256.
- Sofer, W. and Martin, P. F. (1987). Analysis of alcohol dehydrogenase gene expression on *Drosophila*. *Annu. Rev. Genet.* **21**, 203–225.
- Spradling, A. C. and Rubin, G. M. (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. *Science* **218**, 341–347.
- Stellar, H. and Pirota, V. (1984). Regulated expression of genes injected into early *Drosophila* embryos. *EMBO J.* **3**, 165–173.
- Sung, W. L., Benkel, B. F., Zahab, D. M. and Quin, X. (1989). Nucleotide sequence insertion directed by single-stranded crossover linker. *Methods Mol. Cell. Biol.* **1**, 8–13.
- Yamazaki, T. (1984). Measurement of fitness and its components in six laboratory strains of *Drosophila melanogaster*. *Genetics* **108**, 201–211.
- Yamazaki, T. (1985). Regulation and adaptive significance of inducibility of amylase in *Drosophila*

- melanogaster*. In: *Population Genetics and Molecular Evolution* (eds.: T. Ohta and K. Aoki), pp. 147–161. Springer-Verlag, Berlin.
- Yamazaki, T. and Matsuo, Y. (1984). Genetic analysis of natural populations of *Drosophila melanogaster* in Japan. III. Genetic variability of inducing factors of amylase and fitness. *Genetics* **108**, 223–235.
- Yamazaki, T., Matsuo, Y., Inoue, Y. and Matsuo, Y. (1984). Genetic analysis of natural populations of *Drosophila melanogaster* in Japan. I. Protein polymorphisms, lethal gene, sterility gene, inversion polymorphism and linkage disequilibrium. *Jpn. J. Genet.* **59**, 33–49.