The promoter of the *Anopheles gambiae* trypsin 1 gene retains a rudimentary inducibility in transgenic *Drosophila melanogaster*.

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Abstract: The use of chimaeric antiparasitic constructs to control malaria transmission through its mosquito vectors requires, in addition to the availability of transgenic technology, the understanding of *Plasmodium-Anopheles* interactions at different levels. Moreover, should this strategy be based on the exploitation of the interactions that take place in the mosquito midgut, the primary site of interaction between the insect and the parasite, a detailed knowledge of gene physiology in this tissue is a *conditio sine qua non*. Here we demonstrate that a 261 bp long DNA segment upstream of a trypsin-encoding gene (*Antryp1*) of *An. gambiae* carries elements that respond to food uptake, up and down-regulating the expression of the reporter gene in transgenic *D. melanogaster*.

Introduction

The haematophagous behaviour of insects is of utmost importance for mankind since it is directly associated to a variety of serious diseases, the most important of which is malaria. *Plasmodia*, transmitted through man-biting and blood-sucking anopheline mosquitoes, are responsible for more than two million deaths annually, of which the large majority affect the populations of sub-Saharan Africa. Although malaria has now been vastly eradicated from temperate countries through a combination of vector control and improved health care, the situation in tropical areas of the world is still critical; it is assumed that more than 400 million people get sick every year. Since no malaria vaccines are available yet and *Plasmodia* are now, to a large degree, resistant to most classical antimalarial drugs, novel integrated vector control programs may offer a possibility to restrain the disease in tropical countries. In this sense, molecular biology could offer some breakthrough both by helping to understand the biology and genetics of anopheline mosquitoes as well as by designing insect control mechanisms based on modern strategies. These could encompass, among others, engineered insects that would no longer be able to carry the parasites, thus becoming dead-end hosts (Collins, 1994; Curtis, 1994). Since *Plasmodia* are ingested by mosquitoes in a blood meal, parasite transmission first requires their successful development in the insect midgut and the penetration of the midgut wall by the ookinetes. The possibilities to confer *Plasmodium*-resistance to anopheline mosquitoes, therefore, include the design of a strain that could express antiparasitic proteins in the mosquito midgut. This requires an in-depth study of potential gut-specific promoters. For *Anopheles gambiae*, the most important malaria vector (Coluzzi and Sabatini, 1967), such promoters became available by the isolation of its trypsin-encoding genes (Müller *et al.*, 1995). Seven genes are found clustered within 11 kb and these genes are differentiated by their temporal pattern of expression. For example, *Antryp1*'s expression is dramatically upregulated after a blood meal while *Antryp4* is shut off in response to the blood meal. Since germline transformation is not yet available for *An. gambiae* the promoters of these genes were difficult to study. To overcome this problem, we transformed *Drosophila melanogaster* with several constructs that allowed the study of *Antryp1* and *Antryp2* in terms of the sequences that are necessary

and sufficient to confer midgut specificity to the expression of a reporter gene (Skavdis *et al.*, 1996). These experiments defined the DNA containing the *cis*-acting elements responsible for the spatial specificity of these two blood-meal promoters in transgenic *D. melanogaster*, and study whether the DNA segments conferring midgut specificity also respond to the uptake of protein-supplemented food by the fruit flies.

Materials and Methods

Unless otherwise indicated, all molecular techniques were performed as described by Sambrook *et al.* (1989).

All lines in this study (see Figure 1) have been obtained by P element-induced germline transformation (Spradling, 1986) and have been described earlier (Skavdis *et al.*, 1996). Two independent lines each were used for the constructs *TylcXho, TylcHin* and *TylcBst* (labeled a and b in Figure 3 and Table 1), while only one line was examined for the remaining three constructs. Line "*Actin5C*" (*noc*^{Sco}/*CyO, P{w*$^{+mC}$ = *act-lacZ.B}CB1*) obtained from the Bloomington stock center (#582) was used as a control. It represents an enhancer trap line in which the P element promoter is under the control of the *Actin5C* enhancer, ubiquitously driving the expression of *lacZ*. All flies were kept at 25°C. For the experiments described, the flies were treated in one of the following ways:

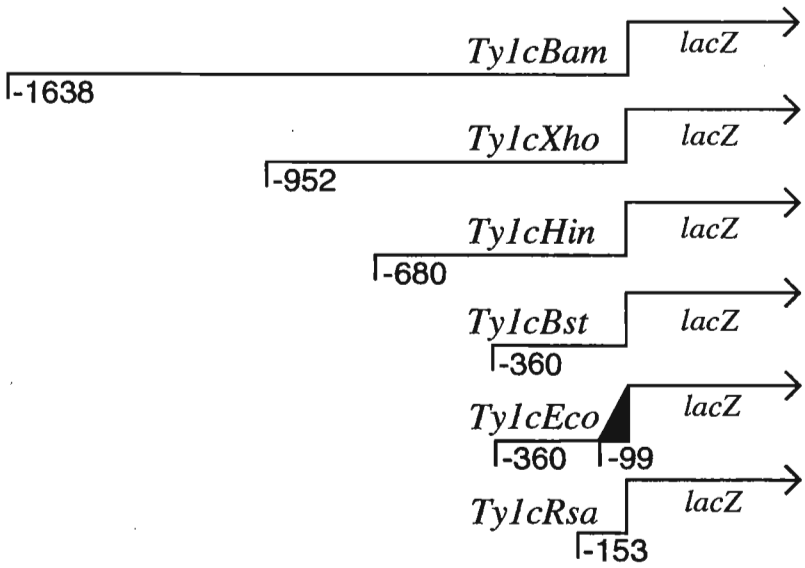

Figure 1. Schematic representation of the *An. gambiae Antryp1* promoter constructs used in this analysis. The length of the sequences derived from the *An. gambiae Antryp1* promoter, present in front of the *lacZ* gene, is indicated in base pairs. The black triangle denotes the *An. gambiae Antryp1* promoter segment that has been deleted in *TylcEco*.

(a) Late pupae were transferred to empty bottles prior to eclosion and adults were allowed to feed for 4 days on cotton balls that had been soaked in a 10% aqueous sucrose solution. Half of the flies were then kept for a further 48 hours under the same conditions while the other half were transferred to bottles containing standard cornmeal food supplemented by live yeast.

(b) Flies were allowed to hatch in bottles containing standard corn-meal food supplemented by live yeast. At least 4 days after eclosion, half the flies were transferred to new bottles and were given cotton balls that had been soaked in a 10% aqueous solution as a food source, while the remaining half were kept in bottles containing the standard yeast-supplemented food. The β-galactosidase assays were performed 48 hr later.

The quantitative CPRG (chlorophenol red β-D-galactopyranoside) assay for the determination of β-galactosidase activity (Ashburner, 1989) was performed as indicated by Skavdis *et al.* (1996). 100 flies were used for each assay that was performed after shifting to the new food condition at the times indicated.

Standard statistical tests were used in the analysis (Zar, 1996). In the cases of homoscedasticity in the distribution of the data, the one-tailed t-test was used for the comparison of means while in the contrary cases the Mann-Whitney-test was used. Barttlet's test was used to probe for differences in the effect of sex and diet. The effect of sex and diet on the activity of the promoter was examined by a two-way analysis of variance of the log-transformed data.

Results and Discussion

Our previous finding, that the small 261 bp long *An. gambiae* trypsin 1 promoter contained all elements necessary to drive the expression of the reporter gene in transgenic *D. melanogaster* in a seemingly correct way (Skavdis *et al.*, 1996) was not entirely unexpected, since it was already known that insect promoters could function over longer evolutionary distances (Mitsialis and Kafatos, 1985). In this particular case, though, the promoter is not only regulated in a spatial and grossly developmental way, but it also responds to a yet unidentified signaling mechanism that, triggered by the uptake of the bloodmeal, induces its expression. This mechanism is obviously hard to study in a transgenic fruit fly, since food uptake in this insect is continuous and digestion is, thus, completely different from mosquitoes (see Romoser, 1996). To test whether this small promoter element also contained the putative bloodmeal-responsive elements we devised further experiments using our transgenic lines. In a first series of experiments we studied whether ingestion of a bloodmeal would lead to an increase of the *lacZ* reporter gene's expression. Flies from the line *TylcBam* that carried ~ 1600 bp of *Anopheles* sequences in front of the *lacZ* gene (see Figure 1) were allowed to feed on cotton balls soaked in human blood for different lengths of time. The flies did indeed feed on the

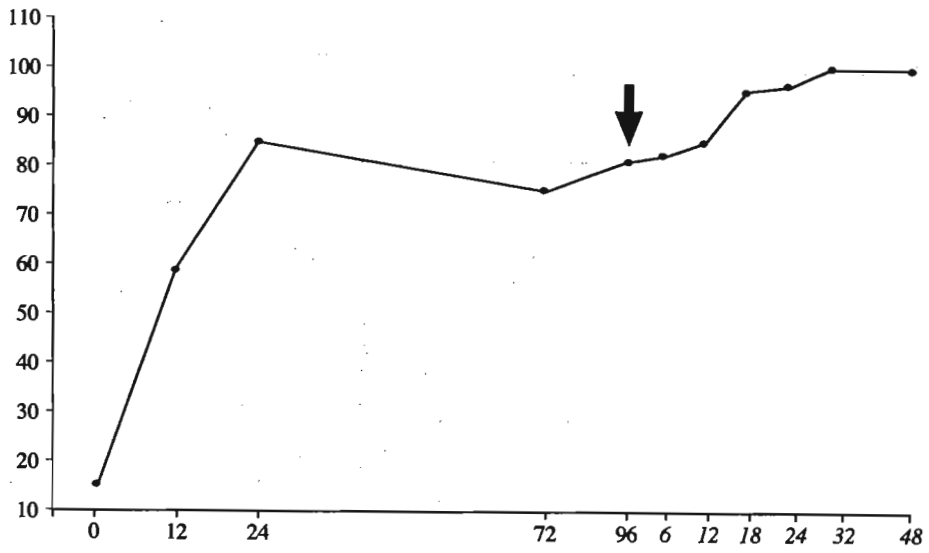

Figure 2. Time course of *Antrypl* promoter induction. After eclosion, flies carrying the Ty1cBam construct were fed 10% sucrose (see Materials and Methods) for four days and were then shifted to yeast-supplemented food (arrowhead). β-galactosidase activity was measured at the time of eclosion (0), throughout the first 4 days, and at the times indicated in hours after the shift (times after the shift indicated in italics). The abscissa shows the relative amount of β-galactosidase activity (B = 100%).

blood (especially if acetic acid was added to a final concentration of 0.2%), but they survived the treatment for several days only if the blood had been supplemented with 10% sucrose. In these cases, the activity of the promoter did not differ from the control experiments in which the transgenic *Drosophila* was fed the standard cornmeal food (results not shown). This was obviously not unanticipated. In An. gambiae, the induction of genes encoding digestive enzymes is complicated and does not follow a generalized path. Food protein composition plays a major role, as does the developmental age of the mosquito. The response of the trypsin gene is different from that of the gene encoding aminopeptidase (Lemos *et al.*, 1996), but substantial differences also exist between the expression pattern of the 7 trypsin genes studied in the malaria mosquito (Müller *et al.*, 1993). Given the basic differences of the food uptake and digestion as well as the fact that *D. melanogaster* under normal conditions never eats blood, we had to devise an alternative approach to study the problem.

Given the lack of inducibility of the trypsin promoter in its *Drosophila* environment through the uptake of blood, we tested to see whether the effect of high-protein food could have any effect. For this we tested the expression of the reporter gene using the quantitative CPRG assay in the line *TylcBam* (Figure 1). The times chosen were right after the eclosion (less than 30 min), 12 hr, 24 hr, 36 hr, and 48 hr. During this time the flies were fed only on a 10% sucrose solution, avoiding any protein uptake. After a total of four days they were then switched to standard cornmeal food, supplemented with live yeast. The results can be seen in Figure 2. Within the first 12 hr of imaginal life, and without the presence of any protein in the food, the *Antryp1* gene is expressed at levels that are about 5-6 times as high as that found for newly eclosed flies. The reporter gene's activity then remains at this level for 4 days. If the flies are then switched to standard cornmeal food, supplemented with live yeast, the β-galactosidase activity increases again and it reaches a new, higher plateau about 24 hours after the food change. The β-galactosidase activity now is about 20-30% higher than before the change of the diet. Can this change be attributed to the addition of protein in the food? It should be stressed that even if only sugar is present in the food right after eclosion, *Drosophila* has come "in contact" with protein since the alimentary canal already contains hydrolysed tissues that were used as energy sources for metamorphosis. Thus, if one can make a statement on induction of gene expression, this may have to refer to the initial burst of β-galactosidase activity, rather than to the significant but not so dramatic increase after the supplementation of the food with protein. Nevertheless, in both cases the results indicate that the *An. gambiae* segment in front of the reporter gene would contain signals that react, directly or indirectly, to the need of protease activity to digest the food.

Table 1. The β-galactosidase activity (in OD_{595}/mg protein) determined for the individual promoter construct lines. The activity was measured in flies raised first in rich food (F->) and the transferred to poor food (->S), or in flies first fed 10% sucrose (S->) and then transferred to rich food (->F). The ratios of the two values are also indicted (F/S) as are the individual probabilities.

Construct	F->	-> S	F/S	P	S ->	->F	F/S	P
Ty1cBam	77.8 ± 10.3	56.2 ± 8.1	1.38	< 0.0001	39.9 ± 7.6	71.0 ± 11.0	1.78	< 0.0001
Ty1cXho (a)	104.2 ± 7.2	76.9 ± 9.2	1.35	< 0.0001	88.8 ± 10.7	108.0 ± 4.9	1.22	< 0.0001
Ty1cXho (b)	110.1 ± 5.4	87.5 ± 11.9	1.26	< 0.0001	87.8 ± 10.3	106.1 ± 6.6	1.21	< 0.0001
Ty1cHin (a)	107.9 ± 7.0	93.3 ± 5.1	1.15	< 0.0001	68.7 ± 8.9	89.7 ± 8.0	1.30	< 0.0001
Ty1cHin (b)	102.8 ± 8.2	83.8 ± 6.4	1.23	< 0.0001	46.8 ± 9.2	90.4 ± 10.9	1.93	< 0.0001
Ty1cBst (a)	100.9 ± 15.3	80.3 ± 11.3	1.26	< 0.001	59.1 ± 16.4	90.7 ± 14.0	1.53	< 0.0001
Ty1cBst (b)	112.9 ± 6.3	89.9 ± 12.6	1.26	< 0.0001	56.5 ± 8.1	90.2 ± 10.9	1.60	< 0.0001
Ty1cEco	140.2 ± 12.8	120.5 ± 14.7	1.16	< 0.001	84.7 ± 5.1	123.7 ± 7.0	1.46	< 0.0001
*Ty1c*Rsa	0.3 ± 0.4	0.13 ± 0.3			0.3 ± 0.2	0.3 ± 0.4		
Actin5C	23.95 ± 2.6	23.02 ± 2.2	1.04	< 0.1	26.19 ± 3.2	24.01 ± 3.4	0.92	< 0.1

In a further series of experiments whose results are shown in Table 1, we checked the individual transgenic lines described by Skavdis *et al.* (1996) for their ability to modulate the expression of the *Antrypl* gene depending on the protein content of the food used. Two different experiments were used, namely the flies were either raised on sugar-only diet for two days and then protein-rich food was given for another two days before measuring the activity of the reporter gene, or the flies were first fed yeast-supplemented cornmeal food for two days before being switched to the protein-free diet. In addition to the transgenic lines carrying the different constructs of the *Antrypl* promoter we also checked, as a control, the β-galactosidase activity in a transgenic line, in which the reporter gene is regulated by the *Actin 5C* enhancer of *D. melanogaster*. This control was deemed necessary since the increase of β-galactosidase activity detected by the previous experiments was not considered to be of an extreme nature. Since our intention was to discriminate between a general effect of the protein-poor food and a possible induction, we specifically chose a promoter that was of a general nature, rather than one whose expression was somehow linked to the digestion. The results of the analysis are shown in Table 1.

The data indicate that, with the exception of the construct *Tylc Rsa* that was previously shown not to be able to drive the expression of the reporter gene, all constructs tested showed a protein-dependent response. Whether the flies were first fed on yeast and then "starved" or whether they were fed on sugar and then shifted to the rich medium, one consistently observed a higher expression level when protein was fed to the flies, the differences ranging between 15% and 93%. Interestingly, these extremes were observed with different lines carrying the same construct (*Tylc Hin*). The differences were more pronounced when the flies were shifted from low to high protein food with an average of 50% increase as opposed to 25% decrease when the flies were "downshifted." We stress the fact that no statistically significant differences were found in the control experiments where the *β-galactosidase* gene was under the control of the homologous *Actin 5C* enhancer. It should be mentioned here that β-galactosidase activity was measured in whole flies. Since the *Actin 5C* construct is expressed throughout the body, and not only in the midgut as is the case for the trypsin-constructs, a potential up or down regulation of the control in this tissue would have been undetected. Although this can not be excluded *a priori* (the numbers expected from the analysis of the *Actin 5C*-derived β-galactosidase activity in the gut would be too low to warrant statistically relevant results), the control experiment still demonstrates that the food-related modulation of the *Anopheles* promoter constructs does not represent a

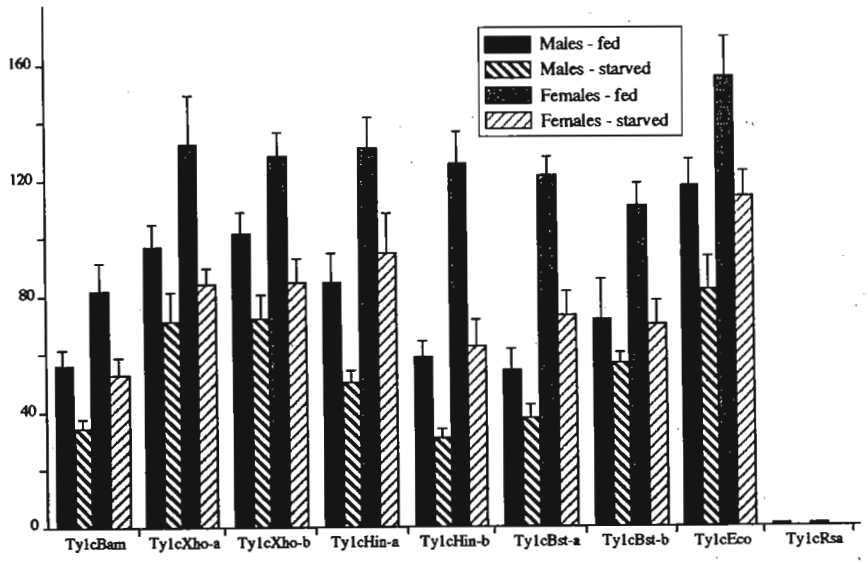

Figure 3. Sex-specific expression of the promoter constructs. The charts show the β-galactosidase activity (in OD_{595}/mg protein) for males and females, determined for the individual lines indicated before ("starved") and after ("fed") the switch to a diet containing live yeast.

general diet-related phenomenon. Finally, all promoter constructs retained about the same quantitative ability to drive the expression of the reporter gene. The presence of some individual variation, as already mentioned by Skavdis *et al.* (1996) was confirmed, and all constructs were seen to be expressed at similar levels; the possible exception to the rule could be the construct *Ty1cEco*, which again showed a somewhat higher expression rate.

In *An. gambiae* the *Antryp1* gene is inducibly expressed only in females while little amounts of trypsin are found in males. This is expected since its expression is only induced by the bloodmeal and males never bite, and only feed on nectar. Nevertheless, it has previously been reported that small amounts of *Antryp1* mRNA can be found in adult male mosquitoes, perhaps as a remnant of transcription during the pupal stage (Müller *et al.*, 1995). We therefore again tested all lines, this time separating the two sexes in the determination of the β-galactosidase activity. The results are shown in Figure 3. It becomes immediately apparent that the inducibility of the promoter constructs is retained in both sexes, although males express the reporter gene at a significantly lower rate. Our results are, on one hand, similar to the early, non-bloodmeal expression of the *Antryp1* gene in the malaria mosquito. On the other hand, inducibility is retained in males in contrast to the situation in *An. gambiae*. These results, taken together, indicate no sex-specific induction mechanisms are responsible for the differences observed in the two sexes in the mosquitoes. The fact that adult male malaria mosquitoes don't express the *Antryp1* gene could simply be due to the fact that they miss the putative initial trigger of the induction process provided by the uptake of a bloodmeal. Why, on the other hand, do the males have lower β-galactosidase activity than their sisters? This can not be answered with the experiments performed, but this behaviour can be observed consistently in all lines tested. A simple explanation could be that females eat more protein than males, due to the increased exigency of amino acids needed for oogenesis.

Our findings show that the relatively short DNA segment that contains the elements responsible for conferring spatial specificity to the expression of the An. gambiae *Antryp1* gene also contains the elements that respond, with a still unknown mechanism, to the food uptake. The fact that two species used in this analysis, and that have diverged since more than 180 million years, recognise the same elements is not unexpected. Indeed, functional recognition of specialised promoter elements is known to be the case among very distantly related insects such as moths and fruit fly (Mitsialis and Kafatos, 1985), but mutual recognition of this kind has also been described for organisms so far apart as vertebrates and invertebrates (Martinez *et al.*, 1991). This short DNA stretch has also been shown to contain several binding sites for nuclear factors isolated from either midgut or non-gut tissues (Shen and Jacobs-Lorena, 1998). Which one of them is responsible for which function remains to be elucidated.

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References: Ashburner, M., 1989, Drosophila: *A Laboratory Manual*. Cold Spring Harbor Press, Cold Spring Harbor, NY; Collins, F.H., 1994, Parassitol. Today 10: 370-371; Coluzzi, M., and A. Sabatini 1967, Parassitologia 9: 73-88; Curtis, C.F., 1994, Parassitology Today 10: 371-374; Lemos, F.J., A.J. Cornel, and M. Jacobs-Lorena 1996, Insect Biochem. Mol. Biol. 26: 651-658; Martinez, E., F. Givel, and W. Wahli 1991, EMBO J. 10: 263-268; Mitsialis, S.A., and F.C. Kafatos 1985, Nature 317: 453-456; Müller, H.M., J.M. Crampton, A. della Torre, R. Sinden, and A. Crisanti 1993, EMBO J. 14: 2891-2900; Müller, H.M., F. Catteruccia, J. Vizioli, A. della Torre, and A. Crisanti 1995, Exp. Parasit. 81: 371-385; Romoser, W.S., 1996, The vector alimentary system. In: *The Biology of Disease Vectors* (Beatty, B.J., and W.V. Marquardt, eds.). University Press of

Colorado, Niwot, CO, pp. 298-317; Sambrook, J., E.F. Fritsch, and T. Maniatis 1989, *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Press, Cold Spring Harbor, NY; Shen, Z., and M. Jacobs-Lorena 1998, Insect Biochem. Mol. Biol. 28: 1007-1012; Skavdis, G., I Siden-Kiamos, H.M. Müller, A. Crisanti, and C. Louis 1996, EMBO J. 15: 344-350; Spradling, A.S., 1986, P element-mediated transformation. In: Drosophila: *A Practical Approach*. (Roberts, D.B., ed.). IRL Press, Oxford, pp. 175-197; Zar, Z.H., 1996, *Biostatistical Analysis*. Prentice Hall International, Upper Saddle River, NJ.

Position-specific expression of β-galactosidase under heat shock conditions in the *hsp70-lacZ* insertion mutant of *Drosophila melanogaster*.

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Summary: Evidence suggests that P element vectors, which contain a bacterial β-galactosidase gene *lacZ*, often express β-galactosidase in a pattern that corresponds to the expression pattern of genes located near the insertion site. However, without having appropriate controls, it can be difficult to distinguish between a gene-specific expression pattern and the endogenous β-galactosidase expression. We have used a heat-shock inducible *lacZ* gene carried on a HBΔ-89 P element vector inserted in region 31B on the *Drosophila melanogaster* second chromosome to visualize the expression pattern of a nearby gene, *Dsur*, previously mapped to the same chromosomal region. The heat shock inducibility of this particular P element construct provides a unique opportunity for visualizing the tissue-specific expression of the *Dsur* gene at different stages of embryonic development.

Introduction

Extensive searches for cis-acting regulatory elements in the *Drosophila* genome have been carried out since the late 1980s. This approach was revolutionized with the introduction of a *Drosophila* transformation vector that carried a bacterial *lacZ* gene fused to a P-element promoter (O'Kane and Gehring, 1987). This method allowed the *in situ* visualization of gene expression in *Drosophila*. Upon the insertion into the genome, many vectors that carry the P-*lacZ* construct reveal position-specific expression patterns of β-galactosidase. It has been suggested that these patterns may reflect the expression of the genes neighbouring the insertion site (Bier *et al.*, 1989). On the other hand, extensive data show that when stained for β-galactosidase, some embryonic tissues reveal endogenous expression of *lacZ* (Bier *et al.*, 1989; O'Kane and Gehring, 1987). Nevertheless, some of what is perceived to be endogenous β-galactosidase expression in P-*lacZ* insertion mutants may, in fact, be true site-specific expression of P-*lacZ* (Bier *et al.*, 1989). The *hsp70-lacZ* construct (Lis *et al.*, 1983) provides a unique opportunity to differentiate between the position-specific expression of P-*lacZ* inserts and the endogenous β-galactosidase expression. In this model, the embryos carrying *hsp70-lacZ* can be heat shocked and stained for β-galactosidase, while non heat-shocked embryos from the same lay serve as a control.