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Analysis of a *fushi tarazu* autoregulatory element: multiple sequence elements contribute to enhancer activity

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Regulatory sequences or factors involved in the regulation of target genes of *Drosophila* homeodomain proteins are largely unknown. Here, we identify sequence elements that are involved in the function of the *fushi tarazu* (ftz) autoregulatory element AE, a direct *in vivo* target of the homeodomain protein ftz. A systematic deletion analysis of AE in transgenic embryos defines multiple elements that are redundantly involved in enhancer activity. Sequences juxtaposed to ftz binding sites are not strictly required for enhancer function. Several sequence motifs are conserved in other developmentally regulated genes of *Drosophila melanogaster* and in the AE homologue of *Drosophila virilis*. The *D.virilis* AE is functional in *D.melanogaster*. The sequence motifs identified here are candidate elements contributing to the target specificity of the homeodomain protein ftz.

Key words: Drosophila/enhancer/*fushi tarazu*/homeodomain proteins/transcription

Introduction

Homeodomain proteins comprise a large family of DNA-binding transcription factors and developmental regulators (Affolter *et al.*, 1990b; Hayashi and Scott, 1990; Gehring, 1992). The homeodomain is the 60 amino acid DNA-binding domain of these proteins (Desplan *et al.*, 1988; Hoey and Levine, 1988; Müller *et al.*, 1988). Although highly related in homeodomain sequence and structure (Qian *et al.*, 1989; Scott *et al.*, 1989; Kissinger *et al.*, 1990; Otting *et al.*, 1990), the more than 30 different *Drosophila* homeodomain proteins play very distinct roles during development (Ingham, 1988; Nüsslein-Volhard, 1991; McGinnis and Krumlauf, 1992). Part of this functional specificity is due to the unique spatial and temporal expression patterns of homeodomain proteins (Struhl, 1985; Schneuwly *et al.*, 1987; Driever *et al.*, 1990). However, ectopic expression assays have shown that a given group of cells can enter very different developmental pathways depending on which homeodomain protein they express (Schneuwly *et al.*, 1987; Kuziora and McGinnis, 1988; Gibson *et al.*, 1990; Gonzales-Reyes and Morata, 1990; Mann and Hogness, 1990). Sequences in and immediately flanking the homeodomain are important for these specific regulatory effects (Kuziora and McGinnis, 1989; Gibson *et al.*, 1990; Mann and Hogness, 1990; Lin and McGinnis, 1992). The question emerges of how the related regulators specifically recognize and regulate their appropriate target genes *in vivo*.

Specificity could be determined by the sequence-specific DNA-binding activity of homeodomain proteins and/or the specific interaction with auxiliary protein factors. Studies on the yeast homeodomain protein $\alpha 2$ suggest that both mechanisms make important contributions to target recognition *in vivo*. In this system the general transcription factor MCM1 and $\alpha 2$ bind cooperatively to a 31 bp operator upstream of a-specific genes (Keleher *et al.*, 1988; Smith and Johnson, 1992). This operator consists of an MCM1 dimer binding site in the central portion and two flanking $\alpha 2$ binding sites, one on each side. The cooperative interaction of $\alpha 2$ with MCM1 at the operator increases the affinity for the $\alpha 2$ DNA-binding sites in the operator and thus allows $\alpha 2$ to specifically bind to these sites *in vivo*. In this system specific protein-protein (MCM1-$\alpha 2$) and DNA-protein ($\alpha 2$ binding site-$\alpha 2$) interactions seem to determine the functional specificity of $\alpha 2$.

In *Drosophila* the mechanisms providing the functional specificity of homeodomain proteins are not understood. Although some homeodomain proteins have different DNA-binding specificities *in vitro* and *in vivo* (Hanes and Brent, 1989, 1991; Treisman *et al.*, 1989; Percival-Smith *et al.*, 1990, 1992; Schier and Gehring, 1992), multiple homeodomain proteins have been found to bind similar or identical sites *in vitro*. For instance, the homeodomain proteins encoded by *fushi tarazu (ftz), engrailed (en), even-skipped (eve), zerknüllt, Antennapedia, Ultrabithorax* and *Deformed* all bind *in vitro* to DNA sites containing a CAATTA motif (Desplan *et al.*, 1988; Hoey and Levine, 1988; Müller *et al.*, 1988; Affolter *et al.*, 1990a; Ekker *et al.*, 1991; Florence *et al.*, 1991; Dessain *et al.*, 1992). Furthermore, reporter genes that contain an oligomerized version of the CAATTA-type binding site are regulated by several homeodomain proteins in transient transfection assays in tissue culture cells (Jaynes and O'Farrell, 1988; Han *et al.*, 1989). This promiscuity of *Drosophila* homeodomain proteins seems paradoxical in the light of the highly specific regulatory functions *in vivo*. It is not known whether very fine tuned DNA-binding specificity differences and/or specific protein-protein interactions are responsible for *in vivo* specificity. Factors or regulatory sequences contributing to the functional specificity of *Drosophila* homeodomain proteins have been largely elusive.

We have been studying the autoregulation of the homeobox gene *ftz* to learn more about the regulatory properties of homeodomain proteins *in vivo*. Genetic studies have shown that ftz enhances its striped expression pattern via two autoregulatory enhancer elements that lie far upstream of the *ftz* transcription start site (Hiromi *et al.*, 1985; Hiromi and Gehring, 1987; Pick *et al.*, 1990). We have recently demonstrated that ftz directly interacts with one of these elements *in vivo* (Schier and Gehring, 1992).

Having identified a direct regulatory target for ftz *in vivo*, the determinants contributing to the specific regulatory interaction of ftz with this element are amenable to analysis. Several observations indicate that the sequence-specific DNA-binding activity of ftz is an important, but not sufficient determinant of target recognition. A DNA-binding specificity mutant of *ftz (ftzQ50K)*, in which Gln50 in the ftz homeodomain is substituted by lysine, changes the regulatory efficiency of ftz *in vivo* (Schier and Gehring, 1992). Instead of efficiently recognizing ftz binding sites (e.g. CAATTA), the mutant *ftz* protein recognizes binding sites that contain the motif GGATTA. Accordingly, *ftzQ50K* confers only partial ftz activity to embryos mutant for the endogenous *ftz* gene (Schier and Gehring, 1993). These results exemplify the importance of DNA-binding specificity in the efficient interaction of ftz and its target sites. However, DNA-binding specificity *per se* does not account for all functional specificity *in vivo* (Schier and Gehring, 1993). When expressed at sufficiently high levels, *ftzQ50K* protein can confer wild type ftz activity to *ftz* mutant embryos. ftz binding sites with changed specificity also retain some wild type activity *in vivo* (Schier and Gehring, 1992, 1993). Furthermore, *ftzQ50K* does not activate natural or artificial target genes of the homeodomain protein bicoid, despite a similar DNA-binding specificity. The view that DNA-binding specificity interactions are not sufficient to explain the target specificity of homeodomain proteins is further supported by recent observations of Vincent *et al.* (1990). An artificial enhancer element, which contains oligomerized ftz *in vitro* binding sites of the CAATTA-type, has been found to direct reporter gene expression in glia cells of transgenic embryos. However, no ftz-dependent expression, e.g. in stripes, was observed. This indicates that ftz *in vitro* binding sites are, although necessary (Schier and Gehring, 1992), not sufficient to constitute a ftz-regulated *cis*-element. Therefore, ftz-regulated enhancers seem to contain important sequences other than binding sites for *ftz* protein.

In the present study we describe experiments designed to identify candidate sequences involved in the constitution of the ftz autoregulatory element AE, a direct *in vivo* target for *ftz* protein (Schier and Gehring, 1992). In a systematic deletion analysis we have defined multiple sequences that are redundantly involved in enhancer function. Several of these sequence motifs are conserved in the AE homologues of *Drosophila virilis* and *Drosophila hydei* and in other developmentally regulated *Drosophila* genes. We find that the AE homologue of *D.virilis* is functional in *Drosophila melanogaster*. Our results suggest that AE behaves as an autonomous enhancer element that requires the combinatorial interaction with both ftz protein and other transcription factors for full activity. The possible nature of these factors is discussed.

Results

All experiments reported here involve the ftz autoregulatory element AE, a ftz-dependent enhancer element including base pairs 2145−2574 in the upstream element (Hiromi *et al.*, 1985; Harrison and Travers, 1988; Pick *et al.*, 1990; Schier and Gehring, 1992). Previous studies have shown that AE directs the expression of seven ftz-like stripes from the minimal *hsp70* promoter in the P-element transformation vector HZ50PL. In this vector, the *Escherichia coli lacZ* gene is used as a reporter gene to monitor fusion gene expression (Hiromi and Gehring, 1987). ftz *in vitro* binding sites within AE are depicted as triangles in Figure 1. The sequences in the AE of *D.melanogaster* that are conserved at >80% in *D.hydei* (Maier *et al.*, 1990) and *D.virilis* (P.Baumgartner and W.J.Gehring, unpublished results) are indicated as black boxes (Figure 1).

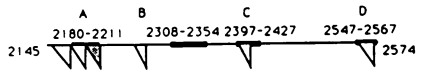
Deletion analysis of AE

To identify sequences that are required for the activity of AE, a systematic, high resolution deletion analysis was performed. PCR-mediated mutagenesis was used to generate 5′, 3′ and internal deletion derivatives of AE (Figure 1; Schier and Gehring, 1992). The deletion constructs cover every ~20 bp in AE, including quite precise deletions of conserved sequences. The resulting DNA fragments were cloned into the HZ50PL reporter gene vector and *lacZ* expression was assayed in several transgenic lines for each construct. As AE−lacZ fusion gene expression was most readily detectable at the germband elongation stage, *lacZ* expression was evaluated at this developmental stage.

The major detectable effect exerted by deleting different regions of AE was a reduction of fusion gene expression in all stripes. None of the deletion derivatives affected the expression of individual stripes, directed expression outside the normal domains of AE activity or led to an increase of β-galactosidase activity (Figure 1 and 2; data not shown). To monitor the quantitative changes of fusion gene expression, two assay systems were developed. In a semi-quantitative analysis, transgenic embryos were fixed and then stained for 48 h at 37°C using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Expression levels were then assigned a value of 0−8 according to the expression patterns shown in Figure 2. In independent experiments the staining intensity of a given transgenic line generally varied by ±0.38 arbitrary units. Staining intensities between different independent lines transgenic for a given construct varied by ±0.41 (Figure 1). We therefore considered a staining intensity difference of 0.8 or more as a significant difference in the expression levels of two constructs.

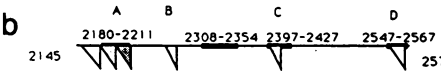
In a second, quantitative assay extracts from 4−6.5 hour-old embryos were prepared. β-galactosidase activity was measured using the fluorogenic substrate methyl-umbelliferyl-β-D-galactopyranoside (MUG; see Materials and methods). In independent experiments β-galactosidase activity of a given transgenic line generally varied by ± 15%; β-galactosidase activities between different independent lines transgenic for a given construct varied by ± 17% (Figure 1). Judging from standard deviations, the quantitative assay is more variable than the semi-quantitative assay (Figure 1). Furthermore, due to the rather high background activity (Figures 1 and 3; Materials and methods), the quantitative assay cannot distinguish between expression differences corresponding to semi-quantitative levels in the range between 0 and 3. At higher levels, however, the semi-quantitative and quantitative results are highly correlative (Figure 3). Figure 1 contains the semi-quantitative and quantitative results of the deletion analysis. In the following, we summarize the four major findings of this analysis. (i) The smallest AE deletion derivatives that are functional (semi-quantitative activity > 1) still contain overlapping sequences (constructs AE-7, AE-14, and AE-20). This

a



CONSTRUCT	SEMIQUANTITATION AVG	STD	LINES	QUANTITATION AVG	STD	LINES
AE-1	6.5	0.4	8	7.8	1.2	5
AE-2	6.4	0.2	6	7.4	1.3	4
AE-3	5.9	0.9	6	6.6	1.1	2
AE-4	5.7	0.4	7	6.9	0.3	2
AE-5	5.6	0.4	8	5.9	0.8	4
AE-6	5.0	0.3	5	6.3	1.5	2
AE-7	2.6	0.6	7	2.4	0.4	3
AE-8	0.5	0.3	6	2.8	0.1	2
AE-9	0.0	0.0	4	2.0	0.2	2
AE-10	0.0	0.0	3	N.D.		
AE-11	6.3	0.4	6	6.4	0.9	3
AE-12	6.2	0.5	8	6.8	1.7	5
AE-13	6.2	0.5	6	7.6	2.8	4
AE-14	2.6	0.9	5	3.7	1.2	2
AE-15	0.5	0.0	5	2.0	0.3	3
AE-16	0.0	0.0	4	2.7	0.3	2

b



CONSTRUCT	SEMIQUANTITATION AVG	STD	LINES	QUANTITATION AVG	STD	LINES
AE-17	5.3	0.5	6	4.8	0.5	4
AE-18	2.0	0.4	5	1.6	0.3	4
AE-19	4.9	0.5	6	4.0	0.2	3
AE-20	4.4	0.6	5	3.1	1.0	4
AE-21	0.2	0.2	6	2.1	0.5	3
AE-22	5.5	0.7	7	6.2	1.3	5
AE-23	4.9	0.3	6	4.8	1.3	4
AE-24	2.5	0.5	5	2.3	0.4	3
AE-25	4.2	0.3	14	3.5	0.3	3
AE-26	4.9	0.5	8	5.0	1.2	3
AE-27	3.2	0.2	6	3.1	0.3	4
AE-28	2.7	0.8	8	2.3	0.8	3
AE-29	3.0	0.0	4	3.2	0.6	3
AE-30	1.4	0.6	9	2.1	0.7	5
AE-31	2.6	0.3	6	2.8	0.4	3
AE-32	0.5	0.6	6	1.7	0.2	3
AE-33	0.1	0.4	7	2.5	0.1	3

Fig. 1. Structure and expression levels of AE derivatives. The structure, names and semi-quantitative and quantitative expression levels of the different AE derivatives are indicated. AE-1, 4, 11, 17, 22, 25, 30, 31, 32, 33, 10 have been described previously and were called AE, AEΔA, AEΔD, AEΔAD, AEΔB, AEΔC, AEΔCD, AEΔAC, AEΔACD, AEΔABCD (Schier and Gehring, 1992) and 5'F (Pick *et al.*, 1990) therein. Black boxes are sequences conserved at >80% in the AE homologue of *D.virilis* (P.Baumgartner and W.J.Gehring, unpublished results) and *D.hydei* (Maier *et al.*, 1990). Triangles indicate the location of ftz *in vitro* binding sites (Pick *et al.*, 1990; Schier and Gehring, 1992). **a.** 5' and 3' deletions. **b.** Internal deletions and combined deletions. AE derivatives were constructed as described in Materials and methods. Transgenic fly lines were generated and transgenic embryos were monitored for β-galactosidase activity as described in Materials and methods (the number of independent lines analysed for each construct is indicated). Staining intensity was semiquantitated according to the standards given in Figure 2 and quantified using embryonic extracts and the fluorogenic substrate MUG. Quantitative β-galactosidase activities are given as fluorescent product synthesis per min and 50 μg of extract. AVG, average; STD, standard deviation; N.D, not determined. Flies homozygous mutant for *rosy* have semi-quantitative and quantitative activites of 0.0 and 1.3 (STD = 0.1), respectively. A transgenic line carrying two copies of HZ50PL has semi-quantitative and quantitative activities of 0.0 and 2.1 (STD = 0.8), respectively.

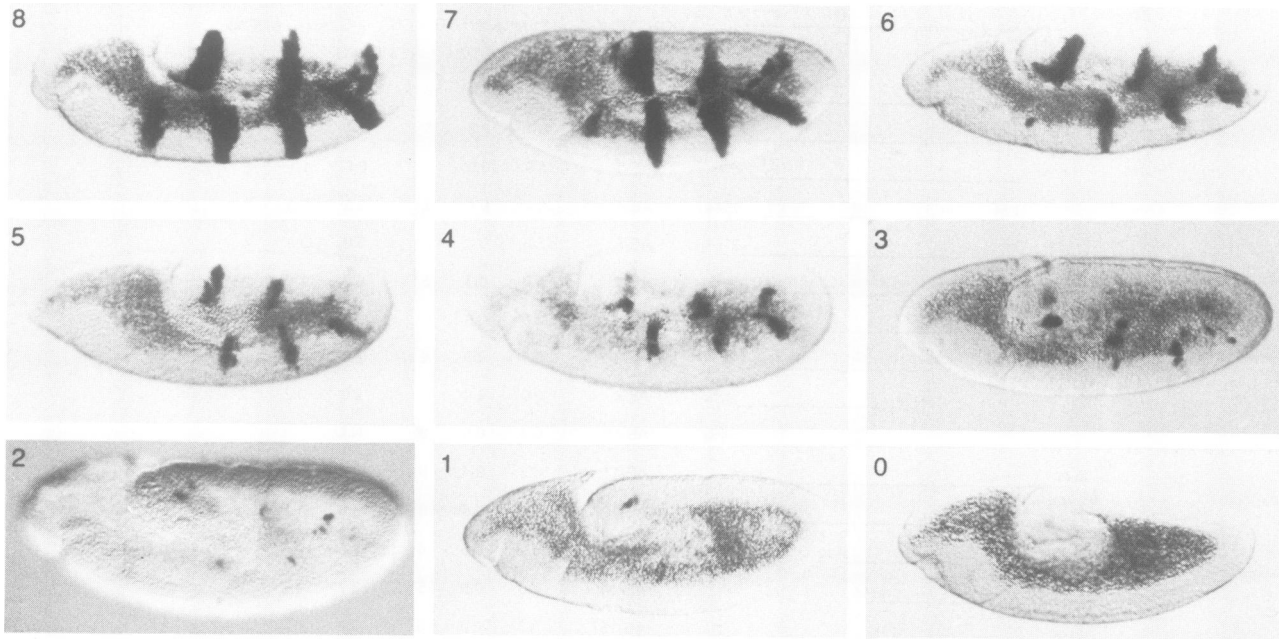


Fig. 2. Assignment of arbitrary expression level values. Photographs of different transgenic embryos were taken and ordered according to their apparent levels of expression. Nine values were assigned from 0 to 8. Expression in transgenic lines were semi-quantified by comparing staining intensities and expression patterns with the embryos depicted in this figure.

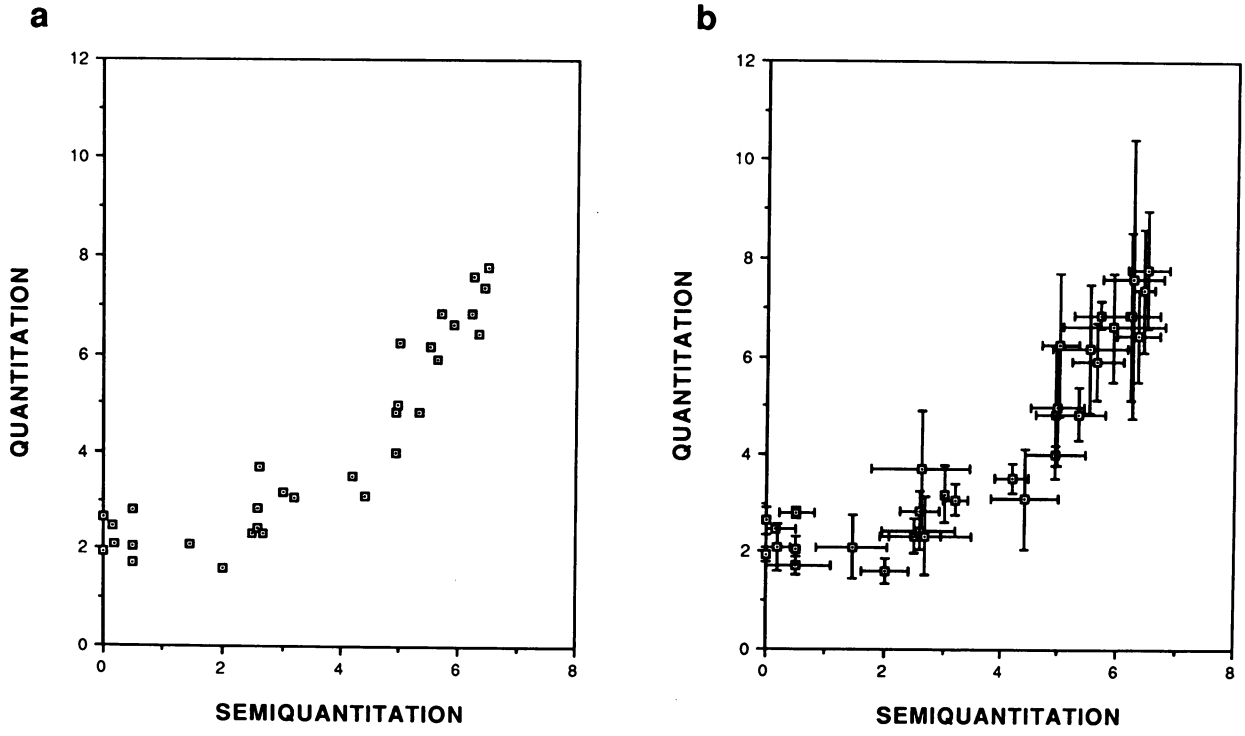


Fig. 3. Comparison of quantitative and semiquantitative assay. Results from Figure 1 are blotted in a coordinate system. *x*-axis and *y*-axis are semi-quantitative and quantitative levels, respectively. Standard deviations as indicated in Figure 1 are given as bars in panel b. Note that the background activity of the quantitative assay precludes a comparison with semi-quantitative levels below 3.

indicates that AE does not contain separable subelements that could act independently to direct expression in seven stripes. Thus, AE seems to function as a single autonomous unit in our assay. (ii) Up to 140 bp of any region in AE can be deleted without affecting its qualitative function, i.e. expression in seven stripes or dots (constructs AE-7, AE-14, AE-20, AE-29 in Figure 1). Accordingly, no single sequence element in AE is strictly required for stripe expression. These results point to an extreme redundancy of qualitative information present in AE. (iii) Sequences encompassing ftz *in vitro* binding sites are not sufficient for enhancer activity in some constructs. Deletion of ftz binding sites A1-3 and B reduces enhancer activity drastically but the remaining portion which contains two ftz binding sites still directs the

expression in seven stripes (construct AE-6). If the remaining ftz binding sites were sufficient for enhancer activity, deletions of non-ftz binding site sequences should not influence enhancer activity. However, deletion of a further 57 bp, which do not include any ftz *in vitro* binding sites, abolishes enhancer activity (AE-9). These results establish that sites (and factors) other than ftz binding sites (and ftz) are required for the activity of AE. (iv) Several sequence elements are important for the quantitative aspects of fusion gene expression. As transcription factors normally bind to sequences of a length of ~10 bp, a given deletion might also influence the functioning of DNA sequences juxtaposed to the deletion end point. The description below therefore reflects only the most parsimonious interpretation of the results.

AE region 2145−2224 contains one of the sequence elements conserved in *D.hydei* and *D.virilis* and three ftz binding sites. As described previously (Schier and Gehring, 1992), deletion of this region has a minor, but significant effect on enhancer activity (construct AE-4 versus AE-1, AE-31 versus AE-25 and AE-32 versus AE-30). 2224−2255 plays no or a very minor role in enhancer activity as judged by comparing constructs AE-5 with AE-4 and AE-20 with AE-19. 2255−2277 contains a ftz binding site and is weakly involved in enhancer activity as concluded from comparing AE-6 with AE-5, AE-22 with AE-1 and AE-33 with AE-32 (Schier and Gehring, 1992). 2277−2300 is important for enhancer activity as judged from AE-7 versus AE-6. 2300−2355 contains a long stretch of highly conserved sequences and clearly plays an important role as judged by comparing AE-24 with AE-1 and AE-9 with AE-7. Two important subelements can be identified in this region: 2300−2323 as judged by comparing AE-8 with AE-7. 2340−2355 as judged by comparing AE-24 with AE-23. Our deletion constructs do not allow us to clearly assess the role of 2323−2340. 2355−2396 is involved in enhancer activity as judged by comparing AE-15 with AE-14. 2396−2432 contains a stretch of sequences conserved at 100% in *D.hydei* and *D.virilis*. This region is clearly important for enhancer activity as judged by comparing AE-14 with AE-13. The region can be subdivided into two important elements: 2393−2418 contains a ftz binding site and is involved in enhancer activity (AE-25 versus AE-1; Schier and Gehring, 1992). The region 2417−2428 is also important for enhancer activity as judged by comparing AE-26 with AE-1. 2432−2542 does not seem to play a role in enhancer activity as concluded from comparing AE-13 with AE-12 and AE-19 with AE-17. 2542−2574 contains a ftz binding site. In the context of the complete AE this region seems to be dispensable (AE-11 versus AE1). However, this sequence clearly contributes redundantly to enhancer activity as judged by comparing AE-30 with AE-25 (Schier and Gehring, 1992).

We conclude from the deletion analysis that AE functions as an autonomous element that contains multiple redundantly combined sequences contributing to enhancer activity.

Sequence similarities in other Drosophila genes

We next wanted to determine whether the regions we have identified as important for enhancer activity in a functional assay are also present in other *cis*-regulatory regions, and whether factors have been identified that interact with these sites *in vitro*. P.Baumgartner and W.J.Gehring (unpublished) and Maier *et al.* (1990) have isolated the ftz homologues of *D.virilis* and *D.hydei*, respectively. These two species are very closely related, but seem to have diverged from *D.melanogaster* >60 million years ago (Beverly and Wilson, 1984). This phylogenetic distance implies that only functionally relevant DNA sequences have been conserved (Perler *et al.*, 1980). The AE sequences of *D.hydei* and *D.virilis* are virtually identical. As described previously (Maier *et al.*, 1990), four regions in AE show an overall homology of >80%. These sequences are embedded in regions of unrelated sequence. Strikingly, most of the regions found important in the deletion analysis are conserved at >80% in the AE homologue of *D.virilis*. A literature and computer search has revealed that several of these elements are also conserved in the *cis*-regulatory regions of the *ftz* zebra element, and of the developmental control genes *en*, *eve* and *Ubx*. Figure 4 shows the sequence comparison of these sites. A long stretch of the upstream element between base pairs 2290 and 2336 is highly conserved in four separate or partially overlapping boxes. The deletion analysis has revealed that this region is important for full AE activity. Below we describe the properties of these sequence motifs.

Sequences similar to region 2290−2299 are found in an autoregulatory element of eve (DENF2, Jiang *et al.*, 1991), in a control element of the *Ubx* gene (*pbx*, Zhang *et al.*, 1991) and twice in the *ftz* zebra element (Topol *et al.*, 1991). In the case of eve, this site has been implicated in transcriptional activation (Jiang *et al.*, 1991) and in the zebra element the two sites seem to be involved in general activation, interstripe repression and early zygotic repression (Dearolf *et al.*, 1989; Brown *et al.*, 1991; Topol *et al.*, 1991). The zinc finger protein tramtrack (tkk; Harrison and Travers, 1990; Brown *et al.*, 1991; Read and Manley, 1992) seems to bind *in vitro* to the DENF2 site in the eve gene and to the two sites in the zebra element (Harrison and Travers, 1990; Brown *et al.*, 1991; Jiang , 1991; Read and Manley, 1992). tkk protein is present in 0−2 h embryos and seems to be involved in the early repression of *eve* and *ftz* (Brown *et al.*, 1991; Read *et al.*, 1992).

Sequence similarities to the region 2296−2305 are found in an autoregulatory element of the *Ubx* gene (Saari and Bienz, 1987; Müller *et al.*, 1989). The role of this sequence in Ubx expression is not known.

Sequences similar to the region 2309−2319 are found in the ftz zebra element and the pbx region of the Ubx gene and also in AE itself, between positions 2420 and 2430. Both regions are also conserved in the AE homologue of *D.virilis*. A candidate factor recognizing these sites is FTZF1, a member of the steroid receptor super gene family (Lavorgna *et al.*, 1991) or a related factor. FTZF1 binds *in vitro* to the sites in the zebra element and its consensus binding site is very similar to the AE sequences analysed (Ueda *et al.*, 1990; Lavorgna *et al.*, 1991). The FTZF1 binding sites seem to be involved in both the activation and the interstripe repression mediated by the zebra element (Dearolf *et al.*, 1989; Ueda *et al.*, 1990; Topol *et al.*, 1991). FTZF1 RNA is detectable in a time window from 0 to 4 h (Lavorgna *et al.*, 1991). It might therefore overlap with *ftz* expression. However, neither the expression pattern nor a mutant phenotype have been reported for FTZF1, making its role in *ftz* regulation still speculative.

The region from 2305 to 2322 encompasses the putative FTZF1 binding site, but also shows a striking similarity to regulatory sequences in the first intron of *en* (Kassis *et al.*, 1986) and to a sequence element in the autoregulatory

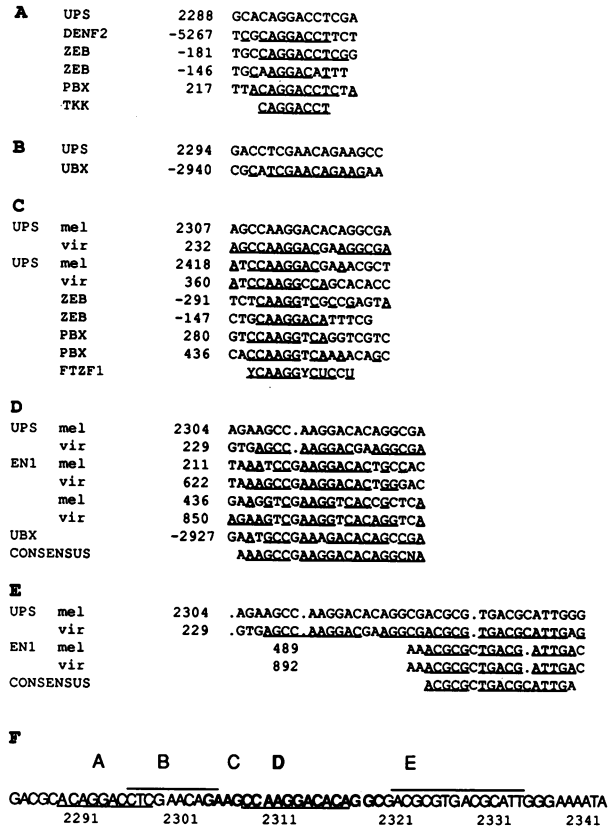


Fig. 4. Sequences in AE that are conserved in other *Drosophila* developmental control genes. A computer and literature search with the sequences 2285–2355 and 2417–2428 identified several similar sequences in the *cis*-regulatory regions of *ftz* (zebra element), *en*, *eve* and *Ubx*. A long stretch of the upstream element between base pairs 2290 and 2336 is highly conserved in four separate or partially overlapping boxes (F). Identical sequences are underlined. **A.** Sequences in the upstream element (UPS) from 2288 to 2301 are compared to the DENF2 site present in an autoregulatory element of the *eve* gene (Jiang *et al.*, 1991), two sequences in the *ftz* zebra element (*zeb*, Topol *et al.*, 1991) and the *pbx* region of the *Ubx* gene (Zhang *et al.*, 1991). **B.** Sequences in the upstream element (UPS) from 2294–2310 are compared to a sequence in a putative autoregulatory region of the *Ubx* gene (Saari and Bienz, 1987; Müller *et al.*, 1989). **C.** Sequences in the upstream element (UPS) from 2307 to 2324 and 2418 to 2435 are compared with the *ftz* zebra element (*zeb*, Topol *et al.*, 1991), the *pbx* region of the *Ubx* gene (Zhang *et al.*, 1991) and the *FTZF1* consensus binding site (Ueda *et al.*, 1990). **D.** Sequences in the upstream element from 2304 to 2324 are compared with sequences in the first intron of the *en* gene (EN1) in *D.melanogaster* and *D.virilis* (Kassis *et al.*, 1986) and to a sequence in an autoregulatory region of the *Ubx* gene (Saari and Bienz, 1987; Müller *et al.*, 1989). **E.** Sequences in the upstream element from 2304–2340 are compared with sequences in the first intron of the *en* gene in *D.melanogaster and D.virilis* (Kassis *et al.*, 1986). **F.** The different conserved boxes (A–E) in the upstream element region from 2285 to 2346 are highlighted.

element of *Ubx* (Saari and Bienz, 1987; Müller *et al.*, 1989). For a perfect match, one nucleotide has to be introduced into this region of AE. This sequence is also conserved in the *ftz* and *en* genes of *D.virilis* (Figure 1; Kassis *et al.*, 1986). The *en* intron directs *ftz*-dependent reporter gene expression in the *en* domain, but the role of this particular sequence is unknown (Kassis, 1990).

Sequences from 2323 to 2336 are similar to another region in the first *en* intron. These sequences are conserved in *D.virilis* (Kassis *et al.*, 1986). For a good match, two single nucleotides have to be introduced into the *en* sequence. The role of this particular region in *en* regulation is not known. The striking similaritiy of sequences in AE to other regulatory sequence elements suggests that these sites serve important functions not only in AE but also in other embryonically expressed genes.

The D.virilis homologue of ftz AE functions in D.melanogaster

The deletion analysis has identified several regions that are involved in the quantitative activity of AE. As pointed out, most of these regions are conserved at >80% in the AE homologue of *D.virilis*. To determine if these sites are sufficient for AE activity, we tested whether *D.virilis* AEvir can function in the context of *D.melanogaster*. AEvir was cloned into HZ50PL and transgenic fly lines were established by P-element-mediated germline transformation. Strikingly, AEvir directs the expression of seven *ftz*-like stripes in *D.melanogaster* embryos (Figure 5). The activity of both AEvir and AE is dependent on a functional *ftz* gene product (Schier and Gehring, 1992; data not shown). These results suggest that the evolutionary conserved regions are sufficient to constitute a *ftz*-regulated enhancer element in *D.melanogaster*.

Discussion

Sequences or factors involved in the regulation of homeodomain protein target genes in *Drosophila* have been largely elusive. The finding that the *ftz* autoregulatory element AE is a direct *in vivo* target for the homeodomain protein ftz has allowed us to study this problem (Schier and Gehring, 1992). In the present study, we have used three approaches to define sequence elements that are involved in the function of AE. First, the analysis of a large set of deletion derivatives of AE has identified several regions that are involved in the control of the activity of this element. Secondly, sequence comparisons indicate that several sequences within AE are also present in the control regions of other *Drosophila* genes. Finally, the *D.virilis* homologue of AE was found to function in *D.melanogaster*. Our results suggest that AE behaves as an autonomous enhancer element that requires the combinatorial interaction with both ftz and other transcription factors for full activity. A speculative model for protein–DNA interactions in the region of AE as deduced from our observations is shown in Figure 6. At least five factors other than ftz could interact with different regions of AE. Most but not all of the sequences identified in our functional assay are conserved in *D.virilis* (Maier *et al.*, 1990) and lie in DNAse I protected regions (Harrison and Travers, 1988).

What might be the nature of the factors interacting with AE? The major detectable effect exerted by deleting different regions of AE is a reduction of fusion gene expression. None of the deletion derivatives affects the expression of individual stripes, directs expression outside the normal domains of AE activity or leads to an increase of β-galactosidase activity. These observations suggest that the factors involved in AE activity are transcriptional activators that are expressed ubiquitously or whose activity is restricted to the domains which overlap with all the *ftz* stripes. We point out that the present study cannot address if repressors are involved in the spatial restriction of AE activity. Although none of the AE deletion constructs are expressed outside the normal

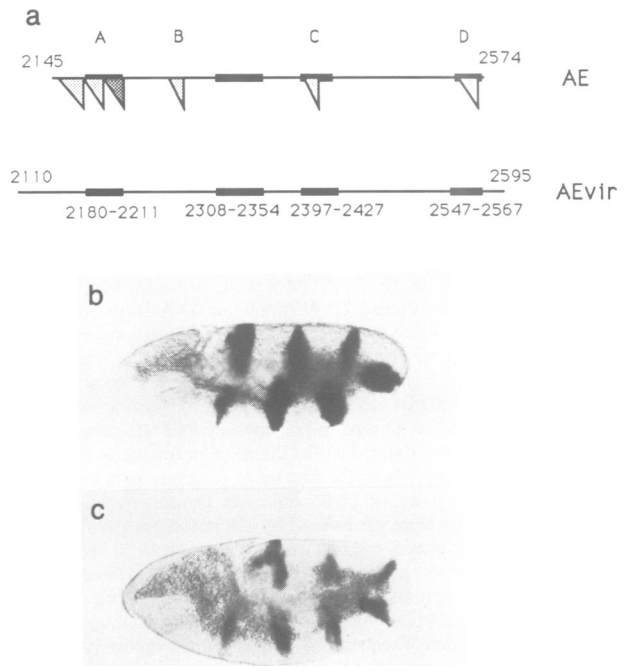


Fig. 5. Expression directed by the AE homologue of *D.virilis*. The region of the *D.virilis* upstream element corresponding to the sequences 2145−2595 of the *D.melanogaster* upstream element, was subcloned into the reporter gene vector HZ50PL. Transgenic fly lines were established and transgenic embryos were stained for β-galactosidase activity. **A.** Structure of the *D.melanogaster* AE and the *D.virilis* AE. Black bars highlight the four highly conserved regions. Triangles indicate where ftz binding sites are located. **B.** Expression directed by the *D.melanogaster* AE. **C.** Expression directed by the *D.virilis* AE.

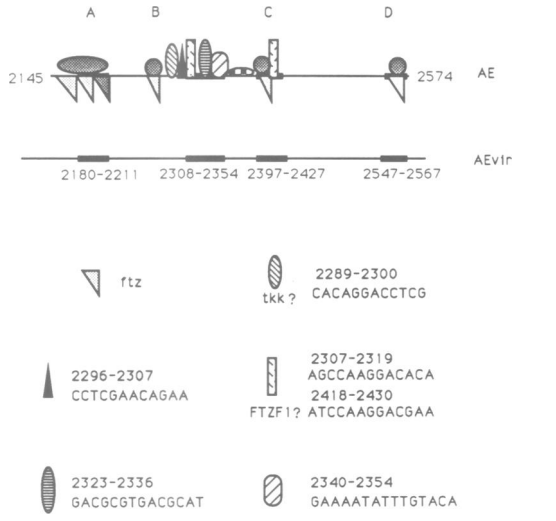


Fig. 6. Speculative model for DNA−protein interactions in AE. AE (2145−2574) is shown with the regions that are conserved in *D.virilis* (black bars) and the ftz *in vitro* binding sites (triangles). Binding sites for putative transcription factors were deduced from the deletion analysis and/or from the conservation in *D.virilis* and in other *Drosophila* genes. Two of the factors binding to AE might be FTZF1 and tkk. The deletion analysis indicates that region 2355−2396 is involved in AE activity (flat ovoid with large black dots). As for the case of ftz−AE interaction, site-directed mutagenesis, *in vitro* DNA-binding studies and the analyses of allele-specific second site suppressor mutants will be required to provide direct evidence for the proposed *in vivo* interactions. Circles over ftz *in vitro* binding sites indicate that apart from ftz, other transcription factors might also recognize these regions.

domains of ftz, this finding is simply explained by the ftz-dependent activity of AE. Due to the spatial restriction of ftz, sequences which might also contribute to the restricted expression of AE would not be identified in the present assay.

Three observations on the expression of AE provide further clues on the possible nature of the factors interacting with AE. (i) AE−*lacZ* fusion genes are not expressed in the developing nervous system although high levels of ftz are present there (Carroll and Scott, 1985; Hiromi and Gehring, 1987; Doe *et al.*, 1988). Apparently ftz is not sufficient to activate AE. (ii) Homeodomain proteins with very similar DNA-binding specificities as ftz (e.g. Antp or Ubx) do not detectably influence AE−*lacZ* expression (Hiromi and Gehring, 1987; Pick *et al.*, 1990; Schier and Gehring, 1992). (iii) *ftz* expression in the stripes and *ftz* autoregulation are restricted to early embryogenesis up to the gastrulation stage. How could the (unknown) factors interacting with AE contribute to this restriction of AE activity? If the factors that interact with ftz on AE were restricted in their temporal expression profile (or their activity) to the blastoderm and gastrula stages of embryogenesis, ftz could only activate AE during these very stages. Thereby, the temporally restricted availability of transcriptional activators might restrict the positive autoregulation of ftz to early embryogenesis and impede the activation by other homeodomain proteins like Antp or Ubx. A powerful mechanism to restrict the availability of these factors to early development would be their maternal origin. A short half-life would restrict their activity to the first few hours of embryogenesis when ftz autoregulation has to occur. In analogy to the MCM1/α2 system, these maternal factors might mark AE together with ftz binding sites, for recognition by ftz protein, making AE competent to respond to ftz.

We point out that apart from ftz, at least one other important activator or repressor must have a spatially restricted effect on AE activity. This suggestion is based on the effects of ectopically expressed *ftz* protein using a heat-inducible system (Struhl, 1985). The ubiquitous expression of *ftz* protein at the cellular blastoderm stage does not lead to the ubiquitous expression of the AE-1−*lacZ* fusion gene (A.F.Schier, unpublished). This result suggest that either a positive factor other than ftz or a repressor restrict the spatial activity of AE. Genetically defined candidate factors to exert this effect are the pair-rule proteins hairy, sloppy paired and odd-skipped, which are involved in *ftz* repression and runt, which is involved in *ftz* activation (Carroll and Scott, 1986; Hiromi and Gehring, 1987; K.Cadigan and W.J.Gehring, unpublished results).

The sequence similarities described here might suggest that two of the factors interacting with AE, at least *in vitro*, are FTZF1 and tkk, a member of the steroid receptor gene family and a zinc finger protein of the C_2H_2 class, respectively. The partial overlap of the consensus sequences of FTZF1 (CAAGGC/TCA/GC) and *tkk* (CAGGACCT) might be indicative of some overlap in target site recognition by these two proteins. Ueda *et al.* (1990) have suggested that FTZF1 acts as a transcriptional activator via the zebra element. The deletion analysis and sequence similarity might suggest a similar role in the regulation of AE. Tkk has been implicated in the activation of an autoregulatory element of

eve (Jiang *et al.*, 1991; Read and Manley, 1992; Read *et al.*, 1992) and the early zygotic repression of *eve* and *ftz* (Harrison and Travers, 1990; Brown *et al.*, 1991; Read *et al.*, 1992). The expression profiles of both FTHF1 and *tkk* are consistent with a maternal origin and a role in marking AE for recognition and activation by ftz. At this point, however, there is no direct evidence that these or related factors are indeed recognizing AE in the context of the developing embryo (see Bray and Kafatos, 1991). The *in vivo* role of FTZF1 and *tkk* will have to be tested in studies involving the introduction of point mutations, *in vitro* DNA-binding experiments, the isolation of mutations in these genes and the *in vivo* analysis of allele-specific second site suppressor mutants (Schier and Gehring, 1992).

The interaction of the yeast homeodomain protein α2 with both its binding sites and the juxtaposed MCM1 protein is a paradigm for the target recognition by homeodomain proteins (Keleher *et al.*, 1988; Smith and Johnson, 1992). Our results provide no direct evidence for the juxtaposition of ftz binding sites and binding sites for other transcriptional activators, with the exception of ftz binding site C. This might suggest that ftz is not binding to AE as a heterodimer with other factors and that the MCM1/α2 paradigm might not be directly applicable to the regulation by ftz. However, mechanistically similar strategies might still be used. For instance, DNA looping (Ptashne, 1988) would allow the cooperative interaction of ftz with other factors on AE. A further difference to the α2/MCM1 system is the observation that no single sequence element is absolutely required for the qualitative aspects of AE function. AE seems to consist of multiple elements that are redundantly combined, suggesting that the factors which interact with AE have overlapping functions. In this sense, AE seems to be much more complex than α2 operators. Apart from its contribution to full AE activity, the apparent redundancy in AE might serve at least three purposes. First, it might guarantee the fine tuning of *ftz* gene expression during development. Secondly, it might buffer *ftz* gene expression against variations in the concentration of cellular factors. Thirdly, redundancy might be an evolutionary strategy which allows the association and reshuffling of different sequence elements to create novel regulatory properties. A previous analysis of the *ftz* zebra element, a *cis*-regulatory region involved in the establishment of *ftz* expression, has also revealed extensive redundancy of *cis*-acting information (Dearolf *et al.*, 1989). Apparently, the principle building plan of viral enhancers—a combination of partially redundant sequence modules (Ondek *et al.*, 1988)—also applies to developmentally regulated enhancers like ftz AE and the *ftz* zebra element.

Finally, we point out the striking sequence similarity found between AE and the first intron of *en*. Both elements are regulatory targets to ftz (Kassis, 1990; Schier and Gehring, 1992), although a direct regulatory interaction has so far only been demonstrated for AE. This sequence conservation leads us to suggest that ftz regulated enhancer elements might have a common structure, consisting of ftz binding sites and some of the sequences identified in this study. This hypothesis predicts that *in vitro* designed enhancer elements containing a combination of these sequences direct the expression of seven ftz-dependent stripes in transgenic embryos. These studies, together with the isolation of factors interacting with ftz-regulated enhancers, will provide information on the mechanisms determining the functional specificity of the homeodomain protein ftz.

Materials and methods

DNA methods

AE (bp 2145–2574 of the upstream element; Harrison and Travers, 1988) and all subregions of AE were amplified using PCR (Saiki *et al.*, 1988). PCR reaction conditions for virtually all reactions were as follows: 2 μg of template, 50 pmol of each primer, 200 μM each dNTP, 10 mM Tris–HCl pH 8.3 at 25°C, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatine, 0.1% Tween 20 and 2.5 U AmpliTaq DNA Polymerase (Perkin Elmer Cetus) or Vent Polymerase (New England Biolabs) in a total volume of 50 μl. 10–13 cycles (1 min at 94°C; 1 min at 37°C; 1.5 min at 72°C) were run. To create 5′ and/or 3′ deletions, two appropriate primers were used. To create internal deletions, two inner oligonucleotide primers introducing deletions were used in two separate PCR reactions extending in opposite directions from the site of the deletion (reactions 1A and 1B). The gel-purified fragments of the first set of reactions were annealed and used for a second round of PCR with outer primers (reaction 2). The amplified fragments were gel-isolated by electroelution, purified over an Elutip column, precipitated, digested with *XbaI* and subcloned into the unique *XbaI* site of the reporter gene vector HZ50PL (Hiromi and Gehring, 1987). Orientations were first analysed with PCR, using an internal primer and a primer in the HZ50PL polylinker region. Orientation and sequences were then verified by dideoxy sequencing. Sequences of oligodeoxynucleotides are available upon request. The *D.virilis* homologue of AE (P.Baumgartner and W.J.Gehring, unpublished results) was subcloned into HZ50PL as follows (Sambrook *et al.*, 1989): A *SacI–PstI* fragment was isolated from the *D.virilis* upstream element region. This region corresponds to nucleotides 1–568 in the numbering system of P.Baumgartner and to the region from 2110–2595 in the upstream element, when aligning the conserved regions. Protruding ends were blunted and *XbaI* linkers were ligated to the free ends. After heat inactivation of T4 DNA ligase, *XbaI* was added. The fragment was gel-isolated and cloned into HZ50PL, giving rise to HZvir1. P-element-mediated transformation and establishment of balanced and homozygous transformant stocks was as described (Hiromi and Gehring, 1987).

β-Galactosidase activity detection

Semi-quantification. X-gal stainings for β-galactosidase activity in whole-mount embryos were performed essentially as described by Hiromi and Gehring (1987). Embryos were dechorionated in 3% chlorox, washed in 0.7% NaCl–0.03% Triton X-100 and tap water and fixed for 25–30 min on a rotating arm in heptane saturated with a glutaraldehyde–buffer solution (fixation solution: 0.05 M cacodylate buffer pH 7.3:50% glutaraldehyde: heptane = 1:1:4; after vigorous shaking the upper/heptane phase is used for fixation). Embryos were washed in heptane and deposited on a siliconized slide. When heptane had evaporated, the embryos were attached to a double sided sticky tape on a siliconized slide and covered with Ringer's medium. Embryos were popped out of the vitelline membrane using a sharp needle. Embryos were washed with Ringers into an Eppendorf tube, washed once in prewarmed (37°C) buffer B {10 nM sodium phosphate pH 7.2, 0.15 M NaCl, 1 mM $MgCl_2$, 3.1 mM $K_3[Fe(II)(CN)_6]$, 3.1 mM $K_3[Fe(II)(CN)_6]$} and stained in 0.5 ml buffer B containing 12.5 μl of 8% X-gal in dimethylsulfoxide (DMSO) on a rotating arm at 37°C for 48 h. Embryos were then washed with PBS containing 0.1% Triton X-100 and dehydrated in a series of 30, 50, 70 and 100% ethanol in PBS and mounted in 87% glycerol. Embryos were viewed under Nomarski optics or bright field and photographed with Kodak technical pan film TP 135-36 at a setting of 25 ASA. To assign arbitrary semiquantitative expression levels, at least 100 embryos per transgenic line were analysed and compared to the embryos shown in Figure 2.

Quantification. For quantification virtually all of the analysed lines were homozygous for a given insert. Embryos were collected from at least 200 female flies (3–5 days old) kept in bottles at 25°C. After a precollection for at least 2 h on grape juice plates, embryos were collected for 2.5 h and then kept at 25°C for a further 4 h. Embryos were dechorionated in 3% chlorox, washed in 0.7% NaCl–0.03% Triton X-100 and tap water, transferred to an Eppendorf tube containing 400 μl assay buffer (0.1 M potassium phosphate pH 8.0 and 1 mM $MgCl_2$) and either processed directly or frozen in liquid N_2 and stored at −25°C. Embryos were homogenized and after centrifugation for 15 min at 12 000 r.p.m. at 4°C, supernatant was transferred and centrifuged for 5 min at 12 000 r.p.m. Supernatant was transferred and protein concentration was determined (Bradford, 1976). β-galactosidase activity of lines transgenic for AE is

extremely low compared with regulatory elements previously used for quantification (e.g. Dearolf *et al.*, 1989). Activity of the *ftz* zebra element is at least 30 times higher than activity of AE. This precluded the use of the chromogenic substrates *o*-nitrophenyl-β-D-galactoside (ONPG) or chlorophenol-red-β-D-galactopyranoside (CPRG). Instead the fluorogenic substrate methylumbelliferyl-β-D-galactopyranoside (MUG) was used. Signal to noise ratio was found to be highly dependent on the pH of the reaction. For instance, at pH 6.0 no difference between background activity and AE-1 was detectable. Testing the signal to noise ratio at different pHs revealed that pH 8.0 is optimal for quantitative assays. Injection of MUG into *Drosophila* embryos results in a strong fluorescent signal in the yolk (A.F.Schier and F.Loosli, unpublished results). We conclude that background activity is most likely due to hydrolases in the yolk. For each reaction 50 μg total protein were used in a volume of 200 μl AB. 50 μl of substrate solution (10^{-3} M MUG in assay buffer were added. Reactions were performed at 37°C in microtiter plates (Dynatech). For each extract, reactions were incubated for 1, 2 and 3 h and stopped by addition of 50 μl 0.5 N NaOH. The fluorescent reaction product was measured on a Microfluor™ Reader (Dynatech) with λ_{ex} = 365 nm and λ_{em} = 450 nm. MUG turn over was calculated using a linear regression program. The correlation coefficient was routinely in the order of 0.99–1.0. β-galactosidase activities are given as fluorescent product synthesis per min and 50 μg of extract. For most of the transgenic lines two or three independent experiments were performed.

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