Redundant control of Ultrabithorax by zeste involves functional levels of zeste protein binding at the Ultrabithorax promoter

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SUMMARY

Many biological processes appear to be controlled by functionally redundant genes or pathways, but it has proven difficult to understand the nature of this redundancy. Here, we have analyzed a redundant regulatory interaction between the Drosophila transcription factor zeste and the homeotic gene Ultrabithorax. Mutations in zeste do not affect the cis-regulation of the endogenous Ultrabithorax gene; however, the expression of small Ultrabithorax promoter constructs is strongly dependent upon zeste. We show that this difference is due to redundant cis-regulatory elements in the Ultrabithorax gene, which presumably contain binding sites for factors that share the function of zeste. We also provide evidence suggesting that zeste and the gene encoding the GAGA factor have an overlapping function in regulating Ultrabithorax. Furthermore, we show that the zeste protein is bound at equal levels in vivo to a Ultrabithorax promoter construct, which zeste strongly activates, and to the identical promoter region in the endogenous Ultrabithorax gene, which zeste redundantly regulates. These results suggest that zeste is significantly active in the wild-type animal and not simply a factor that is induced as a back-up when other activators fail.

Key words: zeste, GAGA, Ultrabithorax, redundancy, transcription, Drosophila

INTRODUCTION

A variety of evidence indicates that many eukaryotic genes show no easily detectable phenotype when their expression is eliminated. For instance, molecular and genetic studies in Drosophila and Saccharomyces cerevisiae indicate that 60% to 70% of genes can be interrupted with no readily observable phenotypic consequence (Gausz et al., 1986; Oliver et al., 1992; Burns et al., 1994). Similarly, reverse genetic experiments show that the disruption of a gene that plays an important biochemical role or is prominently expressed in a particular tissue frequently leads to a surprisingly subtle or even no phenotype (e.g., O'Brien, 1973; Goldberg et al., 1989; Barad et al., 1991; Joyner et al., 1991; Soriano et al., 1991; Saga et al., 1992; Zhuang et al., 1992; Jones and McGinnis, 1993).

There are several conceivable reasons for these observations. It is formally possible that a gene could have literally no function. However, this is very unlikely since such situations are evolutionarily unstable. Another possibility is that a gene has an important and unique function, but that it may be necessary only in special circumstances, like the murine antiviral Mx gene (Haller et al., 1987; Pavlovic and Staeheli, 1991); alternatively, the gene’s function may be detected only by assays that are not standardly employed, such as the behavioral assays used to identify the per gene in Drosophila (Konopka and Benzer, 1971).

Another reason why some null mutations lead to no detectable phenotype is that the function of many genes are either partially or fully shared by other genes (reviewed in Tautz, 1992; Thomas, 1993). In this paper, we will refer to this as functional redundancy. Functional redundancy has been observed between homologous members of gene families, such that mutant phenotypes can only be detected when multiple members of the gene family are disrupted (Rykowski et al., 1981; Kataoka et al., 1984; Tatchell et al., 1984; Richardson et al., 1989; Higashijima et al., 1992; Rudnicki et al., 1993). Functional redundancy can also occur between genes in parallel genetic pathways (Ferguson and Horvitz, 1989; Hülskamp et al., 1992; Lamphier and Ptashne, 1992; Witke et al., 1992; Brown et al., 1995). In such instances, these functionally redundant genes are not necessarily homologous and appear to be members of interactive networks of genes with only partly overlapping activities. For example, strong mutant defects in Drosophila axonal pathfinding are observed only when two unrelated genes, fasciclin I and the Abelson proto-oncogene homolog, are mutated (Elkins et al., 1990).

Since the functions of redundant genes are only apparent in doubly or triply mutant organisms, one of the vexing questions concerning functional redundancy is what is normally occurring in the wild-type organism. For example, are all members of a redundant system fully active and in competition at all times in the wild-type organism, or are some only significantly active in situations of stress or in the artificial circumstances created in mutant organisms? In this paper, we have examined this question by analyzing the nature of the redundant regulatory interaction between the Drosophila genes zeste and Ultrabithorax (Ubx).
Flies with null mutations in *zeste* are almost wild type and have a subtle eye-color phenotype (Goldberg et al., 1989), and several lines of evidence suggest that *zeste* may be acting in a functionally redundant manner. *zeste* was initially identified as a component of a regulatory process termed transvection, in which regulatory elements of one homolog influence the expression of the other gene copy when the homologous chromosomes are paired (Lewis, 1954, 1985; Kaufman et al., 1973; reviewed in Wu and Goldberg, 1989). Although transvection occurs on several essential genes including *Ubx*, it is a dispensable process, which can take place even in the absence of *zeste* (Pattatucci and Kaufman, 1991; Martinez-Laborda et al., 1992; Hopmann et al., 1995). More recently, it has been shown that the *zeste* protein also activates the *Ubx* promoter in *cis*, both in vitro and in the embryo (Biggin et al., 1988; Laney and Biggin, 1992; Kennison, 1993). However, this role in *cis*-regulation is also not essential as *zeste* mutations do not affect the wild-type *Ubx* gene (Goldberg et al., 1989). Since the *zeste* protein is a transcription factor that binds to a variety of genes (Benson and Pirrotta, 1988; Biggin et al., 1988; Thummel, 1989; Pan et al., 1991), we previously suggested that *zeste* is a potent activator of many *Drosophila* genes, but that the in vivo function of *zeste* overlaps with other transcription factors. Here, we have sought to identify these other genes that redundantly share *zeste*’s function and examine the nature of this redundancy.

**MATERIALS AND METHODS**

**Fly strains**

The *z*, *z*1034 and *z*77h alleles were obtained from V. Pirrotta and the *bx*/pvd, *TBR(3)x3100* and *Df(3R)Ul* chromosomes were from the Bloomington Stock Center. These mutations are described in Lindsley and Zimm (1992). The *GAGA*Trl13c, *GAGA*TrlR85 and *GAGA*Trl062 mutations are described in Farkas et al. (1994) and were provided by K. Bhat and P. Schell. The *grh*832 and *grh*837 alleles were from S. Bray and are described in Bray and Kafatos (1991).

The 35UZ and 22UZ transgenes (Irvine et al., 1991) were provided by S. Jha and D. Hogness while the U*β-PBX* transformant line (called ABP in Müller and Bienz, 1991) was provided by M. Bienz. More transformants of this latter construct were obtained by crossing to a strain containing the stable transposase source Δ2-3 (99B) (Robertson et al., 1988).

**Analysis of expression patterns**

The analysis of transgene expression by β-galactosidase activity staining in *zeste* mutant backgrounds (*z*, *z*1034 and *z*77h) was performed as described (Laney and Biggin, 1992) except that transgenic chromosomes were crossed to *w*1118 for examination in a *z*+ background. Two different transformant lines of each construct have been examined in these three different *zeste* mutant strains with the same result.

Antibody staining with a monoclonal antibody against the *Ubx* proteins was performed as described previously (Laney and Biggin, 1992).

**zeste** interaction with *GAGA* and *Ubx*

To examine the interaction between *zeste, GAGA* and *Ubx*, virgin females of the genotype *z*+/z; *GAGA*/TM3*Sc* were crossed with *Sh*/*TM2*Ubx100 or *Dp(3;3)P5*, *Sh*/*Df(3R)Ul* males. Non-*Sh* progeny males (*z*/Y; *GAGA*/Ubx) were counted and examined for patches of wing tissue on the haltere and for notal transformants.

**RESULTS**

**zeste-dependent expression of *Ubx-lacZ* fusion genes**

At least five distant control regions regulate the pattern of *Ubx* expression (Fig. 1). When several of these elements are combined in transgenic *Ubx-lacZ* fusion constructs, they confer upon β-galactosidase an expression pattern closely resembling that of the endogenous *Ubx* gene (Müller and Bienz, 1991). Using these transgenes as an assay system, we have been studying the in vivo roles of three proteins (GAGA, NTF-1 and *zeste*) that activate *Ubx* transcription in vitro through sites in the proximal promoter (Fig. 1) (Biggin and Tjian, 1988; Biggin et al., 1988; Dynlacht et al., 1989).

Previously, we showed that expression from small *Ubx* promoter-*lacZ* reporter constructs containing *zeste* protein-binding sites (e.g., the Uβ construct, Fig. 1) is strongly dependent on a functional *zeste* gene. The behavior of this fusion gene contrasted with the endogenous *Ubx* gene whose embryonic expression pattern is unaffected in presumptive null *zeste* strains (Laney and Biggin, 1992). Since *zeste* shows no significant homology to any other genes in *D. melanogaster* (Chen et al., 1992) and because the *zeste* protein is the only factor to act on its binding site (Biggin et al., 1988; Laney and Biggin, 1992), *zeste* does not appear to be part of a homologous gene family whose members exhibit very similar activities. Therefore, we suggested that *Ubx* regulatory sequences present at the endogenous locus, but not included in the Uβ transgene, are bound by transcription factors unrelated to *zeste* which redundantly share *zeste*’s function. To test this hypoth-

**In vivo cross-linking and immunoprecipitation**

Two independent lines of each transgenic construct were analyzed. Embryos were collected from population cages as described (Walter et al., 1994), except that embryos were collected for 6 hours and allowed to age for 6 hours at 25°C. The embryos were irradiated and the chromatin was isolated, purified and restriction digested as described (Walter et al., 1994). Immuno precipitation with 1 μg of affinity-purified anti-*zeste* antibody or 3 μg of anti-IgG antibody and Southern blotting of co-precipitated DNA were performed as described by Walter et al. (1994). The blots were probed with a 1 kb *StuI-EcoRI* fragment from *Ubx* genomic clone 3102.

Affinity purification of anti-*zeste* antibodies was performed essentially as described in Bickel (1991). For the affinity column, a gel-purified *zeste*-β-galactosidase fusion protein (Mansukhani et al., 1988; plasmid provided by M. Goldberg) was cross-linked to CNBr-activated Sepharose (Pharmacia) at a concentration of 1 mg per ml of resin. A depletion resin was made from a crude soluble extract from bacteria containing pUR291 (Rüther and Müller-Hill, 1983) coupled to Actigel (Sterogene Bioseparations, Inc.) at approximately 10 mg protein per ml resin. Antibodies were purified from crude ‘7386’ serum (a gift of V. Pirrotta).
esis and to identify these redundant elements, we have examined the ability of *zeste* mutations to effect the expression of larger *Ubx-lacZ* constructs, which include additional regulatory regions (Fig. 1). In these experiments, we assumed that constructs containing DNA elements bound by factors that redundantly act with *zeste* would no longer be affected by loss-of-function *zeste* mutations.

To determine if transgenes containing more extensive *Ubx* promoter sequences were responsive to *zeste*, the expression of constructs containing 35.4 kb and 22.2 kb of upstream sequences was examined in wild-type and *zeste* mutant backgrounds (35UZ and 22UZ, Fig. 1) (Irvine et al., 1991). 35UZ is expressed in a pattern that is virtually identical to normal *Ubx* expression (Irvine et al., 1991, Fig. 2A). We find that this expression pattern is unchanged when two different lines of this transgene are crossed into three different *zeste* mutant strains (Fig. 2B and data not shown). Therefore, the large 35UZ construct, like the endogenous *Ubx* gene, is not affected by mutation in the *zeste* gene. In contrast, expression of both lines of the 22UZ transgene is dramatically reduced in *zeste* mutant embryos (compare Fig. 2D to 2C). In the lateral epidermis, staining is strongly diminished when the 22UZ construct is crossed into *zeste* mutant backgrounds, even though these *zeste* embryos are stained three times longer. Thus, the 13 kb of sequence between the endpoints of the 22UZ and 35UZ transgenes contains regulatory information that converts a *zeste*-responsive construct (22UZ) into an unresponsive construct (35UZ).

The 6 kb PBX element, which acts as an enhancer element in transgenic promoter constructs (Müller and Bienz, 1991; Qian et al., 1991), lies in the 13 kb interval between −22 kb and −35 kb (Fig. 1). We have therefore examined if addition of this regulatory element to a small, *zeste*-responsive construct would convert the transgene into a construct that no longer responds to mutation in *zeste*. The Uß fusion gene lacks the PBX element and is strongly expressed in the ectoderm of stage 13 wild-type embryos (Fig. 2G), but not in *zeste* mutant embryos (Fig. 2H) (Laney and Biggin, 1992). In contrast, expression of two different lines of the Uß+PBX construct 1991; Qian et al., 1991). The start site of transcription is denoted with a bent arrow and the scale is in kilobases (kb). Below is a summary of the characterized transcription factors binding to the *Ubx* proximal promoter. The scale is base pairs (bp). (G) GAGA; (N) NTF-1; (Z) zeste. (Bottom) Schematic representations of the *Ubx* DNA in the 22UZ, 35UZ, Uß and Uß+PBX fusion genes are shown (Irvine et al., 1991; Müller and Bienz, 1991; Laney and Biggin, 1992).
Mutations in the endogenous Ubx gene that remove the PBX element are not dependent on zeste

From the above results, it was possible that mutations that remove the PBX element from the endogenous Ubx locus might yield a chromosomal Ubx gene that responds to mutation in zeste. Therefore, we examined the expression of Ubx proteins in embryos doubly homozygous for zeste and mutations that delete the PBX element. Fig. 3A diagrams the two cis-regulatory mutations used. The bxd100 chromosome has a translocation breakpoint that removes Ubx DNA upstream of about −17 kb while the bx3 pbx1 recombinant chromosome has a 18 kb deletion in the pbx region as well as an insertion of two transposable elements between the BX regulatory element and the start site of transcription (Bender et al., 1985; Peifer and Bender, 1986).

Although both of these cis-regulatory mutations remove the PBX element, there are no detectable differences in the expression of Ubx proteins in zeste mutant (Fig. 3B) and zeste; bx3 pbx1 doubly mutant embryos (Fig. 3C). Similarly, embryos doubly homozygous for zeste and bxd100 did not show any detectable interaction (data not shown). These results suggest that, at the endogenous locus, PBX is not the only regulatory element that contains information that is redundant with that mediated by zeste.

zeste mutations increase the penetrance of the interaction between GAGA and Ubx

In addition to defining redundant cis-regulatory elements, we have examined whether other potential trans-regulators of Ubx functionally overlap with zeste. Like the zeste protein, the GAGA and NTF-1 proteins bind and activate the Ubx promoter in vitro (Biggin and Tjian, 1988; Dynlacht et al., 1989). Binding sites for the zeste, GAGA and NTF-1 proteins also activate the Ubx promoter in the embryo (Laney and Biggin, 1992). Furthermore, all three proteins are expressed in the same cells of the epidermis (Pirrotta et al., 1988; Bray et al., 1989; Dynlacht et al., 1989; Soeller et al., 1993). Because of these similarities, we looked for interactions between mutations in the genes encoding these factors and zeste.

Mutations in the gene encoding the GAGA factor (also called Trithorax-like (Trl)) were identified on the basis of their dominant interaction with Ubx alleles (Farkas et al., 1994). Flies doubly heterozygous for GAGA and Ubx mutations exhibit larger halteres than Ubx+/+ flies and, with incomplete penetrance and variable expressivity, show homeotic transformations of the haltere and postnotum (Farkas et al., 1994; Fig. 4A,B). When zeste mutations were crossed into this GAGA +/+ Ubx background, a similar range of phenotypes is observed: small areas of wing tissue on the haltere to strong transformations of the haltere into wing (Fig. 4C). However, the fraction of animals displaying the enhanced Ubx phenotype is increased 2- to 19-fold, depending on the GAGA allele used (Table 1). This increase in penetrance is observed with two different zeste alleles and with either of two Ubx mutations. Therefore, these results indicate that mutation in zeste increases the likelihood that limiting amounts of GAGA factor and Ubx products will lead to reduced expression of Ubx and to the homeotic transformation of haltere into wing.

Neither of the Ubx chromosomes used (TM2Ubx130 and Df(3R)Ubx109) support transvection effects. These chromosomes are multiply inverted or deficient for the Ubx locus and do not allow the pairing-dependent interaction between Ubx genes situated on homologous chromosomes. Thus, the effect...
of *zeste* is not due to the disruption of transvection and reflects the loss of *zeste* activity in regulating *Ubx* in cis. This is the first demonstration of a role for *zeste* in the cis-regulation of the endogenous *Ubx* gene.

We have also examined if *zeste* mutations enhance the phenotype of *GAGA* homozygous mutant animals. Homozygotes of a null allele of *GAGA* (*GAGA*¹¹¹) die during the third larval instar with no obvious homeotic or other specific defects (Farkas et al., 1994), possibly because many systems are simultaneously impaired. Similarly, double homozygotes of *zeste* and *GAGA* alleles show no obvious cuticular defects, particularly no *Ubx* loss-of-function phenotypes (data not shown). However, the weakly viable allele *GAGA*¹³ are even larger halteres that are variably transformed into wings and have transformation of the postnotum (arrowhead) (Farkas et al., 1994). (C) Similar phenotypes are observed in *zeste* mutants. *GAGA*¹³ homozygous flies, except for an increase in the penetrance of this interaction (see Table 1).

**Fig. 4.** *zeste* mutations increase the penetrance, but not the expressivity, of the interaction between *GAGA* and *Ubx*. (A) The halteres of flies heterozygous for the *Ubx*¹³ mutation (*Ubx*¹³/+ ) are enlarged relative to wild type, due to the haplo-insufficient character of *Ubx*. (B) Flies heterozygous for both *GAGA* and *Ubx* mutations (*GAGA*¹¹¹+ /+ + *Ubx*¹³) have even larger halteres that are variably transformed into wings and have transformation of the postnotum (arrowhead) (Farkas et al., 1994). (C) Similar phenotypes are observed in *zeste* mutants. *GAGA*¹³ homozygous flies, except for an increase in the penetrance of this interaction (see Table 1).

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*The number of animals of that particular genotype examined.
†The fraction of animals of the indicated genotype that exhibited an enhanced *Ubx* phenotype: haltere or notal transformations.
‡Of the remaining individuals of these genotypes, 20%-30% displayed flattened, paddle-like halteres that were easily distinguished from the bulb-like halteres of the control genotype.

mutations in *NTF-1*, like *GAGA*, would dominantly interact with *Ubx* alleles. We observed no such interaction (data not shown).

**The endogenous *Ubx* gene and a *zeste*-dependent *Ubx* transgene are bound by similar levels of *zeste* protein in the embryo**

In an attempt to understand how *zeste* is acting in this redundant regulatory situation, we have utilized in vivo UV cross-linking to determine the relative levels of *zeste* protein occupancy on identical *Ubx* promoter sequences in targets dependent upon *zeste* gene activity or targets not affected by mutations in *zeste*. With this method, covalent bonds between protein and DNA are induced in the embryo with UV light; these protein-DNA complexes are then immunoprecipitated and the co-precipitating DNA is finally analyzed by Southern blot hybridization. The technique gives a high resolution view of the in vivo distribution and level of proteins on DNA and has been used successfully to examine the DNA binding of several different proteins, including *zeste* (Gilmour and Lis, 1986; Gilmour et al., 1986; Champlin et al., 1991; Walter et al., 1994; O’Brien et al., 1995).

Embryos carrying the *Ubx* transgene were subjected to UV cross-linking and the purified chromatin was restriction...
discriminating with an anti-zeste antibody (Fig. 5B, lanes 6 and 7), the signal observed on the Uβ proximal promoter is due to the presence of the cluster of high-affinity zeste protein-binding sites, since the signal from the Uβ Δ-200/-31 construct, which lacks these sites, is reduced 20- to 25-fold (compare lane 6 of Fig. 5C with 5B). Thus, the proximal promoter of the endogenous Ubx gene is bound by levels of zeste protein that function to strongly activate the Uβ transgene in the embryo, suggesting that the zeste protein acts on the Ubx gene in wild-type embryos. Furthermore, the in vivo cross-linking studies provide another piece of evidence that these small Ubx-lacZ fusion genes are good model systems for studying the function of individual transcription factors in the regulation of Ubx.

**DISCUSSION**

The nature of a redundant regulatory interaction

In this study, we have used in vivo UV cross-linking to determine the level of zeste protein binding on identical Ubx promoter sequences in two different regulatory contexts. The results indicate that the zeste protein binds at the same level to the Ubx proximal promoter regardless of whether zeste is potentially activating or redundantly regulating Ubx transcription.

One model for a functionally redundant regulatory situation is that a redundant regulator is utilized only in emergency circumstances: when other transcription factors fail to regulate, a redundant factor is induced as a back-up system to regulate its target gene. The data presented here suggest that this is not the case for zeste, since zeste protein is normally bound at functional levels to the endogenous Ubx proximal promoter. Although we cannot determine the extent to which zeste protein bound at the proximal promoter is contributing to the activation of Ubx in normal embryos, it seems likely that it is playing a significant role in activation. If this is the case, then the fact that zeste acts redundantly suggests that the zeste protein must be in competition with other bound activators to promote the assembly of an activated transcription complex.

Apparently it is not the case that all redundant regulators act in the same way as zeste. For example, a different scenario is likely to exist for the redundant myogenic transcription factors MyoD and Myf-5 (Braun et al., 1992; Rudnicki et al., 1992, 1993). Singly mutant MyoD mice are viable and show no visible phenotype. However, the level of Myf-5 expression is significantly increased in these animals and probably compensates for the loss of MyoD activity (Rudnicki et al., 1992). Since these two transcription factors bind with the same heterodimerization partners to identical regulatory sequences (Braun et al., 1989; Braun and Arnold, 1991; Weintraub et al., 1991), they must directly compete to occupy the promoters of their downstream target genes. Therefore, both MyoD and Myf-5 cannot be fully active in binding to their regulatory targets in wild-type animals and Myf-5 could be viewed, at least in part, as an inducible backup of MyoD. To date, few examples of functionally redundant situations exist where there is any evidence for what is actually occurring in the wild-type organism. Further experiments examining other redundant systems will be necessary to fully appreciate the nature of these prevalent regulatory interactions and to understand how redundant regulators act.
Redundant regulation of Ubx

Ubx enhancer elements containing regulatory information redundant with that mediated by zeste have been identified by comparing the activity of different Ubx-lacZ fusion constructs in wild-type and zeste mutant embryos. The results suggest that the PBX distant enhancer element is bound by transcription factors whose function overlaps with zeste. Furthermore, the PBX element cannot be the only element at the Ubx locus that contains regulatory information that is redundant with that mediated by zeste, since embryos doubly homozygous for zeste and mutations that delete the PBX element express the Ubx gene normally. Earlier experiments also suggest that there is considerable redundancy in Ubx cis-regulatory elements (Kennison, 1993). For example, separate transgenic promoter constructs containing either the upstream or downstream regions of Ubx both give similar patterns of expression, which closely resemble that of the wild-type gene (Simon et al., 1990; Irvine et al., 1991). It appears that part of this redundancy is due to the presence of multiple binding sites for the same regulators occurring in several different regions of Ubx (Müller and Bienz, 1992; Qian et al., 1993). However, our experiments suggest that the redundancy within the Ubx locus is also due to different transcription factors with partially overlapping functions binding to different regulatory regions. Furthermore, our results define zeste as one of these redundant regulators.

Since there appears to be extensive redundancy among the regulatory elements of Ubx, we suggest that this redundancy is particularly important for the proper regulation of Ubx and other master regulatory genes in the wild-type organism. As with the other homeotic genes, the precise spatial and temporal control of Ubx activity is essential for normal development since the loss of expression and the ectopic expression of Ubx both result in dramatic developmental defects (Lewis, 1978; Mann and Hogness, 1990). The Ubx gene comprises >100 kb of genomic sequence and has rather extensive regulatory regions (Bender et al., 1983). These large regions may be necessary because they supply redundant regulatory information which ensures the highly faithful and reproducible control of Ubx expression and therefore safeguards against any developmental miscues.

In this paper, we have also provided evidence suggesting that the GAGA factor is one of the trans-regulators whose function overlaps with zeste, since mutations in zeste increase the penetrance of the genetic interaction between GAGA and Ubx. A number of observations, however, suggest that these two genes do not have identical functions and have only partly overlapping activities. Protein-binding sites for GAGA or Ubx overlap, while these other unique functions of GAGA and zeste presumably provide the necessary selective pressure so that these genes are maintained during evolution.

The zeste protein has been shown to bind to several hundred gene loci in Drosophila polytene chromosomes (Pirrotta et al., 1988). With the exception of white and Ubx, there is little direct evidence that zeste regulates these genes. However, in light of our experiments with Ubx, we suggest that zeste is involved in the expression of many of these genes and that zeste acts redundantly with a number of unrelated transcription factors.

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