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The *Drosophila daughterless* gene autoregulates and is controlled by both positive and negative *cis* regulation

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SUMMARY

As the only class I helix-loop-helix transcription factor in *Drosophila*, Daughterless (Da) has generally been regarded as a ubiquitously expressed binding partner for other developmentally regulated bHLH transcription factors. From analysis of a novel tissue-specific allele, da^{lyh}, we show that *da* expression is not constitutive, but is dynamically regulated. This transcriptional regulation includes somatic ovary-specific activation, autoregulation and negative regulation. Unexpectedly, the diverse functions of *da* may require that expression levels be tightly controlled in a cell and/or tissue-specific manner. Our analysis of da^{lyh} identifies it as the first springer insertion that functions as an insulating element, with its disruptive activity mediated by the product of a fourth chromosome gene, *Suppressor of lyh [Su(lyh)]*.

Key words: bHLH, Autoregulation, Oogenesis, Insulation, *daughterless*, *Drosophila*

INTRODUCTION

Complex patterns of gene expression during development result from the combinatorial action of transcriptional regulators that vary in their specificities for their target genes. Multiple sequence-specific activators and their associated architectural proteins form enhanceosomes, which recruit the transcriptional initiation machinery to specific genes (Lee and Young, 2000; Wolberger, 1999). As some enhanceosome components are present in all cells, differential regulation of specific genes must be provided by temporal or spatial restriction of functional activator proteins.

The helix-loop-helix (HLH) family of transcription factors includes over 240 different proteins that are present throughout eukaryotes, including both ubiquitous and temporally/spatially restricted transcription factors (Massari and Murre, 2000). HLH proteins dimerize via amphipathic helices and interact directly with the major groove of DNA via a basic domain. These proteins fall into seven specific classes based on dimerization capabilities, tissue distribution and DNA target specificity (Murre et al., 1994). Class I HLHs, also known as E proteins, can form either heterodimers or homodimers, are widely expressed, and have DNA binding specificity for the E box (Ephrussi et al., 1985). The more numerous class II HLHs heterodimerize with class I HLHs and show tissue-restricted expression patterns and target sequence specificity that varies with different heterodimer partners and their conformation (Kophengnavong et al., 2000). In vertebrates, class I HLHs are essential for commitment to the B lymphoid lineage (Bain et al., 1994; Zhuang et al., 1994), T cell development (Barndt et al., 2000), regulation of V(D)J recombination (Romanow et al., 2000), muscle differentiation (Lassar et al., 1991) and expression of differentiated cell products such as insulin (Sharma et al., 1997). This partial list of vertebrate class I HLH functions does not include cases of transcriptional regulation for which a class II HLH is known, but its requisite class I partner has yet to be identified.

In *Drosophila*, there is only one class I HLH protein, encoded by the *daughterless* (*da*) gene, and this distinction not only reflects its apparent ubiquitous expression, but also accounts for the large number of developmental processes in which it functions (Cronmiller and Cummings, 1993; Massari and Murre, 2000; Moore et al., 2000). In the embryo, Da protein is required for the early transcriptional activation of *Sex lethal* (*Sxl*) during sex determination (Cronmiller and Salz, 1994; Keyes et al., 1992), for differentiation of the mesoderm (Gonzalez-Crespo and Levine, 1993), and for the establishment of the proneural field that gives rise to the central and the peripheral nervous systems (Caudy et al., 1988a). In larvae, *da* is again required for establishment of the neural field for adult sensory organ precursers (Modolell, 1997), for progression of the morphogenetic furrow of the developing eye (Brown et al., 1996) and for the differentiation of the salivary gland (King-Jones et al., 1999). In adults, *da* is required for ovarian follicle formation (Cummings and Cronmiller, 1994). Countless additional developmental functions have been implied, based on tissue culture experiments, identification of genes encoding tissue-restricted class II HLHs, and conservation of developmental processes requiring E proteins in other organisms.

It is generally believed that Da accomplishes its numerous discrete developmental roles through collaboration with

regulated HLH binding partners. During sex determination, Da associates with Sis-b/Sc (*sisterless-b/scute*), which is present in the early embryo at high enough levels to activate the *Sxl* early promoter only in females (Deshpande et al., 1995; Yang et al., 2001). Da associates with HLH proteins from the neural-specific *Achaete-Scute Complex* in establishment of the proneural field (Cabrera and Alonso, 1991). For the formation of multiple dendritic neurons in the peripheral nervous system, Da heterodimerizes with Amos, which is present in patches of ectodermal cells and soon thereafter is restricted to sensory organ precursors (Huang et al., 2000).

The Da protein, however, is not simply a generic unregulated binding partner for other developmentally regulated HLH proteins. Through analysis of a unique female sterile allele, we have discovered precise transcriptional regulation of *da*. And, at least in the ovary, either reducing or increasing the amount of Da causes distinct mutant phenotypes. Thus, among cells that contain both Da and its relevant Class II binding partner, variable Da levels may restrict the formation of functional transcriptional complexes, indicating regulatory specificity dictated by *da*.

MATERIALS AND METHODS

Drosophila stocks and genetics

The *fs(2)lyh* mutant was identified by L. Yue during an enhancer trap screen in the laboratory of A. Spradling (Spradling, 1993). da^{2} and da^{s22} have been previously described (Cronmiller and Cline, 1987; Cummings and Cronmiller, 1994); da^{7} and the chromosomal aberrations, *Tp(2;Y)cb50*, *Dp(2;Y)B231*, *Df(2L)J27* and *C(1)A* are described in Lindsley and Zimm (Lindsley and Zimm, 1992); the *Tp(2;Y)cb50* and *Dp(2;Y)B231* duplications on the *Y* chromosome include da^{+}. *Dp(2;2)*da^{+}*18* and *Dp(2;2)*da^{+}*20* are described in Cronmiller and Cline (Cronmiller and Cline, 1986). The stl^{a16} allele, provided by R. Nagoshi, was originally isolated as *fs(2)A16* (Bakken, 1973) and identified subsequently as a null allele of *stl* (N. Jones, MSc Thesis, University of Virginia, 1999). Transformant *w;* $P(w^{+}$, *hsp70-*$da^{+})$ flies were provided by A. Singson and J. Posakony (Cummings and Cronmiller, 1994). Transformant *w;* $P(w^{+}$, p_{da}*–gal4=da.G32)* flies were provided by E. Knust (Wodarz et al., 1995).

Suppressors of the da^{lyh} female sterility were isolated after standard ethyl methanesulfonate mutagenesis of males (Lewis and Bacher, 1968). Three independent dominant suppressors, including $Su(lyh)^{26H6}$, were recovered and subsequently mapped to the fourth chromosome.

Transvection experiment

Ovaries from flies of the genotype *C(1)A/Tp(2;Y)cb50;* da^{lyh} stl^{a16}*/Df(2L)J27* were examined. Standard polytene salivary gland squashes were carried out to examine chromosomes of male larvae carrying *Dp(2;Y)B231*; no pairing was seen between the *Y* duplication of the *da* region and the second chromosome.

Staining

Ovaries were fixed and DAPI stained as previously described (Cummings and Cronmiller, 1994). Whole-mount ovary immunohistochemistry was performed as previously described (Cronmiller and Cummings, 1993) with monoclonal mouse anti-Hts (1B1) (Zaccai and Lipshitz, 1996) (1:10 dilution) and polyclonal rabbit anti-Vasa (Styhler et al., 1998) (1:1000 dilution) using FITC- or TRITC-conjugated secondary antibodies (Jackson Immunoresearch).

Molecular analyses

Standard molecular techniques were used except where otherwise noted (Sambrook et al., 1989).

PCR

To amplify and identify the transposon insertion in da^{lyh}, PCR was performed using DyNAzyme EXT (Finnzymes) with primers 0A (5′-GGCTCAACGTCAACACTCGCTGCAAC-3′) and P1B (5′-CGTACATAAGGCTGTATACGCACGG-3′). The PCR product was cloned into the pGEM T-easy vector (Promega).

DNA sequence analysis

Sequence from both ends of the da^{lyh} springer insertion was obtained (Accession Numbers AF418012 and AF418013). A full-length springer contig of 7509 bp was constructed from unordered sequenced fragments from the Berkeley Drosophila Genome Project (BAC clones D849, D848, D841 and D823) using NCBI Blast, MacVector and AssemblyLIGN (Oxford Molecular Group). The restriction pattern roughly resembles that of the originally defined 8.8 kb springer element (Karlik and Fyrberg, 1985). Three full-length springer elements are included in the completed genome project sequence (GenBank Accession Numbers AE003580, AE003776 and AE003433) (Adams et al., 2000). Transcription factor binding sites within the *da* region were analyzed with MatInspector and the TransFac database (Quandt et al., 1995; Wingender et al., 2000).

Northern blots

PolyA^{+} mRNA was loaded (5 μg per lane). Hybridization was carried out using UltraHyb (Ambion) at 42ºC. ^{32}P-labeled probes were prepared by random priming of *da* cDNA MN6, *da* genomic fragment 5 and neighboring fragment 6 (to detect *Mdh1*) (Cronmiller et al., 1988), and EST LP12271 (to detect *rp49*). Band intensities were quantified using PhosphorImager Scanner and ImageQuant software (Molecular Dynamics).

Real-time RT-PCR

Four- to 6-day old female flies, carrying two copies of the p_{hsp70}-da^{+} transgene and one copy of the p_{da}-*gal4*=da.G32 transgene, were treated with heat shock in a 37ºC water bath or kept at room temperature for 3 hours. RNA was extracted immediately after treatment from 20 flies under each condition in duplicate using TRIzol reagent (Life Technologies) as per the manufacturer's protocol, followed by treatment with RQ1-DNase (Promega) and repurification with TRIzol reagent. Amplification reactions were prepared in triplicate using the Access RT-PCR System (Promega), with SYBR Green detection in a Cepheid SmartCycler (Morrison et al., 1998). Conditions used were 48ºC for 45 minutes; 94ºC for 2 minutes; 40 cycles of 94ºC for 10 seconds, 53ºC for 15 seconds and 68ºC for 20 seconds. Primer pairs: galRT1a (5′-TAACCGTCCACCCTCTCGTAACTC-3′) and galRT1b (5′-AAAAGGCGTGACTGAGCGATGC-3′); or mdh1a (5′-TACCATTGGCGGTCACCTTG-3′) and mdh1b (5′-TCATTATTTGGGGCAACCACTC-3′). Melting curves were analyzed for purity of product.

Statistical analysis

Conservatively, as RT-PCR reactions within RNA preps were not independent, the means of each RNA prep for each transcript were compared; no significant difference was seen between RNA preps for *Mdh1* under either treatment or for *gal4* with heatshock, although a significant change was seen between RNA preps for *gal4* without heat shock ($P<0.05$). To increase the power of statistical analysis each observation was then treated as an independent data point. A *t* test was used on *gal4* or *Mdh1* transcripts to test for differences between heat shock treatment (yes, no) using SAS v8.0 (SAS Institute, Cary, NC).

RESULTS

Identification of *fs(2)lyh* as an ovary-specific null allele of *da*

There are at least two well-described requirements for *da* function in the ovary: in the germline for progeny sex determination (Cronmiller and Cline, 1987) and in the somatic ovary for follicle formation (Cummings and Cronmiller, 1994). The functional unit of the ovary is the ovariole, and within the germarium of each ovariole, two distinct stem cell populations give rise to either germline or soma (Fig. 1A). A germline stem cell divides asymmetrically to produce a cystoblast, which undergoes four rounds of mitotic divisions with incomplete cytokinesis to produce an oocyte with its 15 interconnected nurse cells. It is in these germline cells that *da* mRNA is produced and eventually concentrated into the oocyte for the maternal sex determination function; *da* germline mRNA does not appear to be translated during oogenesis (Cummings and Cronmiller, 1994). As the cyst moves posteriorly through the germarium, the somatic stem cells give rise to somatic cells that (1) envelop the cyst to form a follicle and (2) form stalks that separate adjacent follicles. Da protein is found within these somatic cells, where it is required for proper encapsulation and separation of follicles (Cummings and Cronmiller, 1994).

The female sterile mutant, *fs(2)lyh*, which arose spontaneously in an enhancer trap screen for genes expressed during oogenesis (Spradling, 1993), is an allele of *da* that specifically disrupts function of the gene within the somatic ovary. In complementation tests with *da* alleles and genetic interaction tests with *Sxl*, *sis-a* and *sis-b*, *fs(2)lyh* showed no maternal effects on sex determination (data not shown). Similarly, *fs(2)lyh* was fully viable and showed no visible phenotypes in combination with null *da* alleles and deletions, demonstrating that no other *da* functions are disrupted. However, *fs(2)lyh* failed to complement *da* alleles for follicle formation: *fs(2)lyh*/*fs(2)lyh* and *fs(2)lyh*/*da*$^{-}$ ovaries had multiple indistinguishable follicular defects, such as missing stalks, multicyst follicles and late stage necrosis (Fig. 1C and data not shown, compare with wild type in Fig. 1B). In both cases, the phenotype worsened with age. Based on the mutant ovary morphology, *fs(2)lyh* behaved like a null allele: there was no discernible difference in mutant phenotypes from flies of equal ages homozygous for *fs(2)lyh* or transheterozygous for either a *da* null allele or a deletion, even in the youngest flies that exhibited the least extreme phenotype (Fig. 1D,E). Henceforth, *fs(2)lyh* will be referred to as *da*^{lyh}.

Da protein expression in *da*^{lyh} mutants is also disrupted specifically in the ovary. Staining of wild-type ovaries showed clear nuclear Da protein, while *da*^{lyh} mutant ovaries showed no nuclear localized protein within the ovariole (Fig. 2A). Western blots of ovary extracts detected Da protein but at reduced levels (data not shown). The protein seen in ovary extract probably corresponded to other Da-containing tissues included in the extract: the epithelial sheath that surrounds each ovariole and its associated muscles, the tracheae that infiltrate the ovary and the oviduct.

The molecular lesion in *da*^{lyh} is caused by the insertion of a springer element. Southern blot analysis of the *da* region indicated the insertion of approximately 8 kb of DNA. PCR amplification, cloning and sequencing of the inserted DNA identified the insertion as a gypsy-like springer retrotransposon. Our partial sequence data together with that of the Berkeley *Drosophila* Genome Project, which includes several full-length copies of the springer element, confirmed the strong similarity between springer and gypsy: homologous ORFs are 36% to 65% identical (Fig. 2C) (Adams et al., 2000). The *da*^{lyh} springer inserted 113 bp into the *da* intron, producing a characteristic TATA target site duplication (Davis et al., 1998; Karlik and Fyrberg, 1985; Kidd and Young, 1986; Voelker et al., 1990).

da^{lyh} alters levels of *da* transcription

The mechanism of *da* gene disruption by the springer element is different than other analyzed springer alleles. Molecularly characterized springer-induced mutations (*Tm2*^{3}, *N*^{fa3}, *f*^{36a}, *Mhc*^{2}, *Mhc*^{3}) each result from insertion within exons or near alternatively used exons to produce aberrant transcripts usually with premature transcriptional termination within the springer

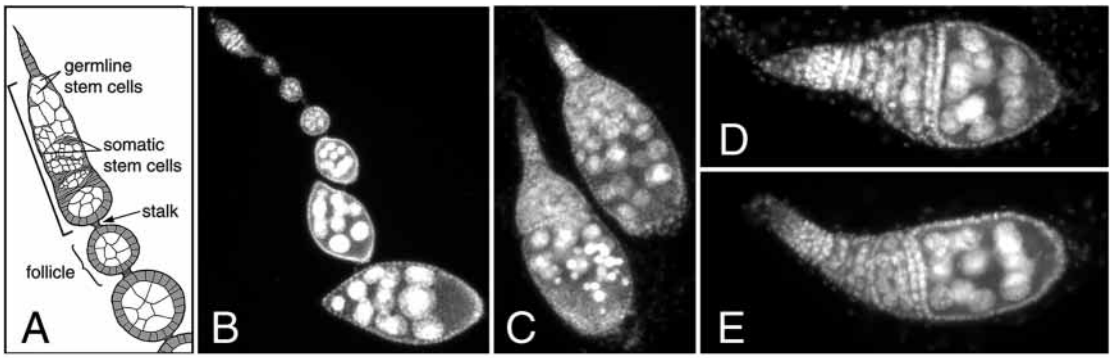

Fig. 1. In the somatic ovary, *da*^{lyh} is a null allele. (A) Diagram of the anterior *Drosophila* ovariole. The long bracket indicates the germarium, where stem cell divisions occur and follicle formation takes place. The somatic cells are shaded; the positions of the germline and somatic stem cells are indicated. (B-E) Ovarioles stained with the nuclear dye DAPI. The wild-type ovariole (B) illustrates normal ovarian morphology. By contrast, homozygous (*da*^{lyh}/*da*^{lyh}, C) and hemizygous (*da*^{lyh}/*da*$^{-}$: not shown) mutant ovaries from mature females exhibit gross disruptions in oogenesis, including the formation of multicyst follicles, failure of follicle individualization, and degeneration of late-stage cysts. The identification of *da*^{lyh} as an ovarian null allele is evident from a comparison of *da*^{lyh}/*da*^{lyh} and *da*^{lyh}/*da*^{2} ovaries (*da*^{2} is a lethal null allele.). Even in ovaries from newly eclosed mutant females, where the phenotype is least severe, *da*^{lyh}/*da*^{2} (D) and *da*^{lyh}/*da*^{lyh} (E) mutant ovaries are indistinguishable. In each panel anterior is upwards or towards the left; the magnification of D,E is approximately twice that of B,C.

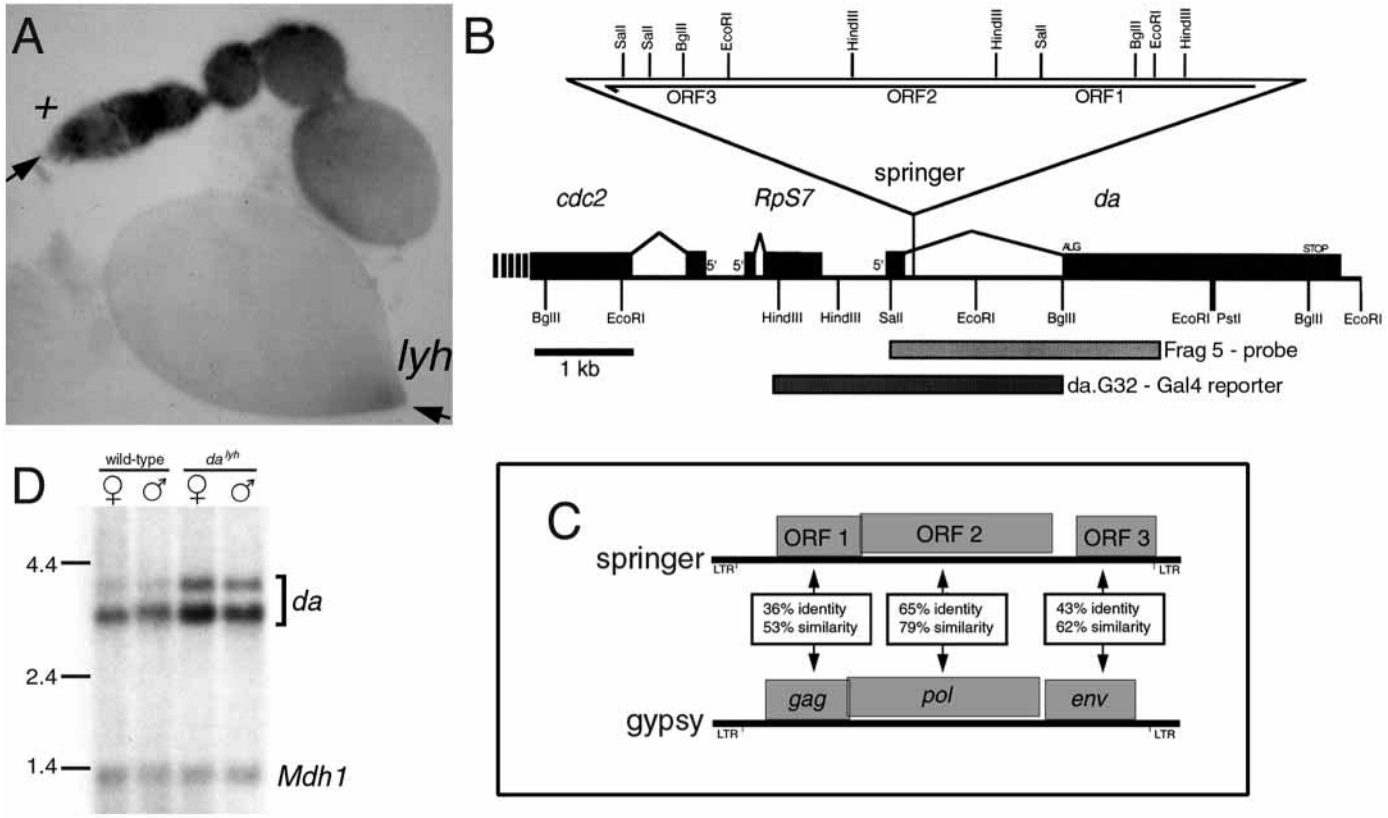

Fig. 2. Insertion of a springer retrotransposon is responsible for the da^{lyh} mutation. (A) A wild-type ovariole (upper) and a da^{lyh} mutant ovariole (lower) stained for Da protein. Arrows indicate the anterior end of each ovariole. Da protein is apparent in nuclei of wild-type somatic cells of the ovariole; no nuclear-localized protein is seen in da^{lyh} mutant ovarioles. (B) Graphical representation of the *da* genomic region. The da^{lyh} chromosome has a 7.5 kb springer element inserted within the single intron. Below the map are the extents of the Frag 5 DNA probe (Cronmiller et al., 1988), used for the northern blot shown in D, and the genomic fragment used in construction of the da.G32 reporter (Wodarz et al., 1995), used in the real-time RT-PCR analysis shown in Fig. 5. (C) Comparison of the conceptual translation products of the springer and gypsy retrotransposons. A complete springer nucleotide sequence was assembled from the ends of the da^{lyh} insertion and the *Drosophila* genome project sequences. Orientation of the springer is opposite of that in B. (D) Transcriptional analysis of da^{lyh}. Poly(A)$^+$ RNA from wild-type and mutant da^{lyh} adults was probed with the *da* fragment 5 (B) to detect *da* RNA and the 3′ adjacent fragment 6 (Cronmiller et al., 1988) to detect *Mdh1* RNA as a loading control. Both *da* transcripts and the single *Mdh1* transcript are indicated. RNA sizes are in kilobases.

LTR (Davis et al., 1998; Hoover et al., 1993; Ishimaru and Saigo, 1993; Karlik and Fyrberg, 1985; Kidd and Young, 1986). We tested whether da^{lyh} produced any novel transcripts by probing Northern blots with a full-length *da* cDNA probe or a genomic fragment that spanned the intron and included part of both exons (Fig. 2B). Both probes exclusively detected the two known *da* transcripts (Caudy et al., 1988b; Cronmiller et al., 1988), but the da^{lyh} mutation increased the *da* transcript levels. Normalized to the mRNA of the nearby *Mdh1* gene, which encodes malate dehydrogenase, *da* transcript levels were 1.5- and 1.9-fold higher (males and females, respectively) in da^{lyh} than in wild-type flies (Fig. 2D). Comparable increases were estimated when transcripts were compared with an *rp49* control (data not shown).

Taken together, the RNA and protein analyses suggest that the da^{lyh} springer insertion acts as a transcriptional insulator. The absence of Da protein in da^{lyh} mutant ovaries must result from blocked *da* expression in somatic cells: in this mutant, *da* is expressed everywhere except in the somatic ovary. Such a loss of *da* mRNA would not be apparent in whole fly mRNA; even in ovaries specifically, loss of somatic mRNA would be concealed by strong germline expression of *da*, whether assayed by northern blot or in situ hybridization (Cummings and Cronmiller, 1994). Consistent with this interpretation, we sequenced the entire *da*-coding region of da^{lyh} and found no additional changes (data not shown); thus, post-transcriptional loss of *da* product was ruled out. Additionally, the springer element also appears to insulate a negative regulatory element that results in an overall increase in *da* transcription, hence the elevated mRNA levels evident on the northern blots.

Genetic evidence for *da* autoregulation

Surprisingly, in genetic interaction tests da^{lyh} does *not* behave like a genetic null: ordinarily, the *da* somatic ovary function is particularly sensitive to gene dose, such that a *da* loss-of-function allele exhibits second site non-complementation with mutations in other genes involved in follicle morphogenesis (Cummings and Cronmiller, 1994; Grammont et al., 1997). By contrast, da^{lyh} did not. A specific example of this paradoxical genetic behavior was the interaction we observed between *da* and *stall* (*stl*), another gene required for follicle formation (Schupbach and Wieschaus, 1991). A null allele of *da*

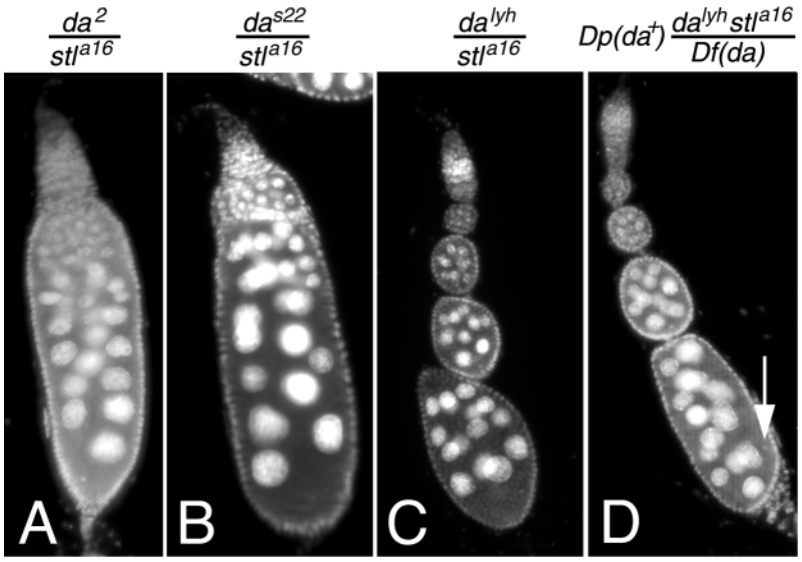

Fig. 3. Unlike *da* null alleles, da^{lyh} completely complements stl^{a16}. DAPI stained ovarioles dissected from various *da* mutant genotypes. (A) Ovaries of flies doubly heterozygous for stl^{a16} and the null allele da^{2} contain severe morphological defects in all ovarioles. (B) Similar defects are present in ovarioles that are doubly heterozygous for stl^{a16} and the hypomorph da^{s22}. (C) By contrast, da^{lyh} completely complements stl^{a16}. (D) In 15% of ovarioles from flies with an ectopic copy of da^{+}, mild defects include mislocalization of the oocyte nucleus (arrow). The remaining 85% of the ovarioles are completely normal, indicating that the wild-type allele of *da* does not have to be on the homologous chromosome to provide full function. In each panel, the anterior end of the ovariole is at the top.

completely failed to complement the null allele, stl^{a16}; ovaries of doubly heterozygous females had no normal ovarioles (Fig. 3A). Even the hypomorphic da^{s22} allele failed to complement stl^{a16}, with 65% of the ovarioles having defects (Fig. 3B). However, da^{lyh} fully complemented stl^{a16}; no defects were seen (Fig. 3C). Furthermore, we tested 46 chromosomal deletions with which the null allele, da^{2}, had exhibited dominant interactions to produce mutant ovary phenotypes (J. E. S. and C. C., unpublished); all produced completely normal ovaries in combination with da^{lyh} (data not shown). Thus, the da^{+} chromosome of the heterozygous da^{lyh} genotype appeared to induce wild-type function from its mutant da^{lyh} homolog, which we showed did not produce Da protein in the somatic ovary.

The apparent functional activation of da^{lyh} by da^{+} could be explained either by autoregulation or by transvection. In *Drosophila*, transvection is a common phenomenon where *cis*-acting enhancer elements on one homolog can act in *trans* to influence transcription of the paired homolog (Wu and Morris, 1999). If transvection activates da^{lyh}, then moving the wild-type allele away from a paired configuration should result in failure to activate da^{lyh}. To construct such a genetic scenario, we generated a genotype in which one chromosome was doubly mutant for da^{lyh} and stl^{a16}, its homolog carried a deletion of the *da* region, and an ectopic copy of da^{+} was carried on a translocation to the Y chromosome (see Materials and Methods). We examined the ovaries from these females to determine whether, in the absence of a paired wild-type homolog, da^{lyh} would still complement stl^{a16}. We found that 85% of the ovarioles were completely normal, and the remaining 15% had only mild defects (Fig. 3D). Thus, transvection was ruled out because pairing between da^{lyh} and its wild-type homolog was not required for activation of this allele in the somatic ovary. The results are consistent with autoregulation as an explanation.

Further genetic evidence in support of transcriptional autoregulation is the discovery that ectopic da^{+} can convert the da^{lyh} homozygous loss-of-function phenotype to a gain-of-function phenotype. We could observe only a subtle gain-of-function phenotype associated with an increased dose of da^{+} when we generated flies that had three copies of the gene, the extra copy being provided by a chromosomal duplication (either in tandem or by transposition). Although the ovaries from these flies had properly formed follicles, we occasionally observed interfollicular stalks that were distinctly longer than normal (Fig. 4B). In terms of the nature of this phenotype, it was not surprising that excess da^{+} could lead to these longer stalks, because reduced da^{+} resulted in loss of stalks. When we added an ectopic copy of da^{+} to the homozygous da^{lyh} genotype, we saw dramatic gain-of-function phenotypes that were similar to, but also more extreme than, those observed in the 3X-da^{+} ovaries. For example, in addition to long interfollicular stalks, we found ovarioles with shrunken germaria that occasionally were attached directly to mid-to-late stage follicles (Fig. 4C-E). Although the expressivity of these phenotypes varied, they were unlike any phenotypes that resulted from *da* loss of function. The conversion of da^{lyh} from a loss-of-function to a gain-of-function phenotype was not dependent upon the specific translocation, as two copies of a heat-inducible da^{+} transgene completely rescued the loss-of-function phenotype at 25°C and produced the gain-of-function phenotype when adults were placed at 32°C (Fig. 4F). These *da* overexpression phenotypes were also not dependent upon the da^{lyh} allele, as we could phenocopy such overexpression defects with the heat-inducible da^{+} transgenes in an otherwise wild-type background using 30 minute 37°C pulses every 6 hours (Fig. 4G). Thus, the da^{lyh}-associated overexpression phenotype resulted when wild-type Da protein transactivated the mutant da^{lyh} alleles, enabling them to produce their own wild-type *da* product. Furthermore, once transcriptionally activated, each da^{lyh} allele produced a greater amount of that product, consistent not only with the more extreme overexpression phenotype of the allele, but also with its overall increased mRNA levels.

Molecular evidence for *da* autoregulation

We validated the genetic evidence for *da* autoregulation by demonstrating molecularly that Da protein could transactivate the *da* promoter in vivo. We used a reporter transgene to detect activation in response to induced Da protein. This reporter

consisted of a genomic fragment that included the *da* promoter (molecular extents indicated in Fig. 2B), fused to the yeast *gal4* gene (Wodarz et al., 1995). Expression of this transgene produced a chimeric transcript that included all but the last 12 nucleotides of the *da* 5′UTR fused to the *gal4*-coding region. Using primers specific for the *gal4*-coding region and real-time RT-PCR, we assayed expression levels of this reporter transcript with or without induced Da protein, as regulated by heat-inducible da^+ transgenes. As the threshold cycle number (C_t) provides the most accurate comparison of transcript levels from RT-PCR (Higuchi et al., 1993), we plotted the second derivative of SYBR green fluorescence where the C_t is easily seen as a positive peak. For *gal4* transcripts without heat shock induction of Da, the C_t was 23.01±0.25; with heat shock induction of Da, the C_t decreased to 21.53±0.52 (Fig. 5). Control *Mdh1* transcript levels did not change after heat shock;

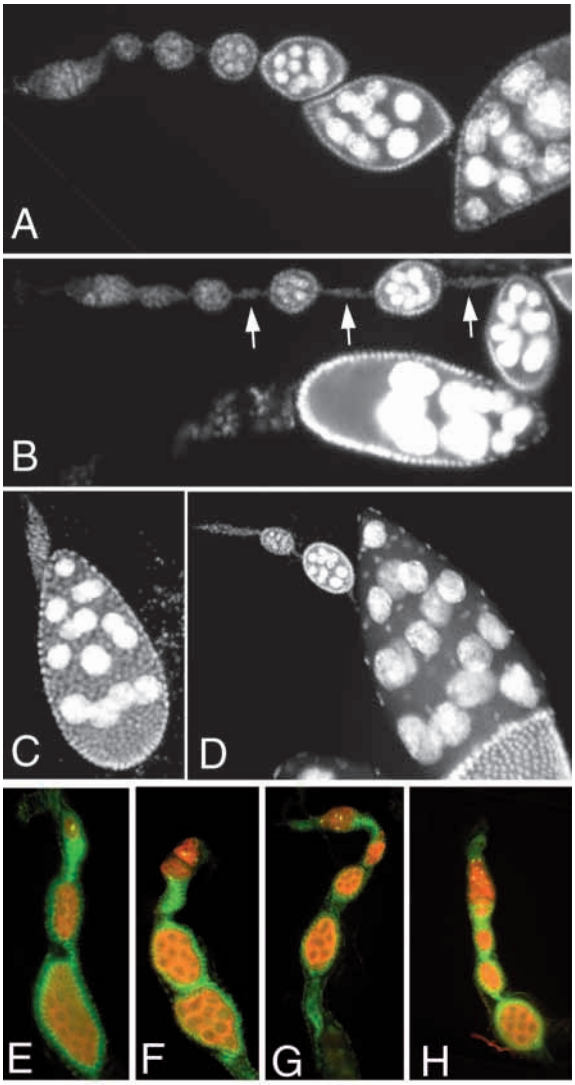

Fig. 4. Increased doses of da^+ produce a gain-of-function phenotype. (A-D) Ovarioles stained with the nuclear dye DAPI; (E-H) ovarioles double stained for Vasa (germline, red) and Hts (adducin, present in somatic cytoskeleton and germline spectrosome and fusome, green). (A) Wild-type ovariole. (B) Ovarioles from flies with three copies of da^+ contain abnormally long interfollicular stalks (arrows). The genotype of the ovariole shown here included a tandem duplication of da^+ ($da^+/Dp(2;2)da^+$); similar morphology was observed when the extra da^+ copy was provided by a transposition, carried on the *Y* chromosome (not shown). (C-E) Ovaries of homozygous da^{lyh} flies, carrying a duplication of da^+ on the *Y* chromosome, exhibit a more extreme phenotype. In these ovarioles, smaller germaria are attached directly to maturing follicles. (E) The staining for Vasa and Hts highlights the gap in follicle stages that is observed between germline in the germarium and the closest individual follicle. (F) These defects are also seen in flies homozgyous for da^{lyh} with two copies of a heat-inducible da^+ transgene at 32°C. (G) Likewise, the defects are phenocopied by 37°C pulses of the heat-inducible da^+ transgenes in a wild-type background. (H) These defects are not seen in wild-type flies exposed to the same heat shock regimen. In each panel anterior is at the top (C,E-H) or towards the left (A,B,D).

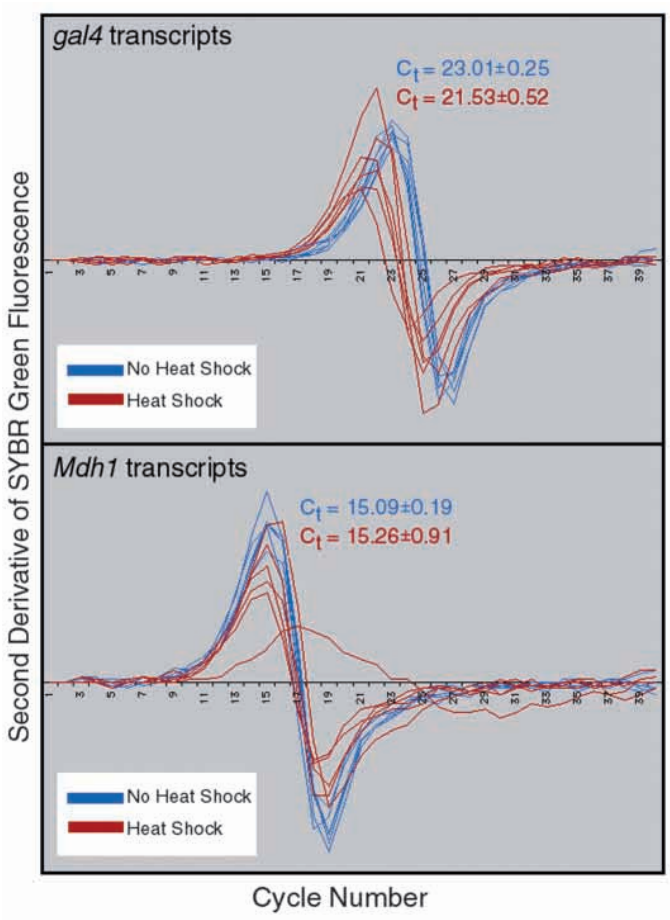

Fig. 5. An in vivo transcriptional reporter indicates Da can transactivate the *da* promoter. Flies were generated that carried a heat shock inducible da^+ transgene and a reporter transgene (da.G32), consisting of the *da* promoter fused to the *gal4*-coding region. Real-time RT-PCR was used to determine relative levels of *gal4* transcript in adult females maintained at 25ºC or after heat shock treatment. Both graphs plot the second derivative of SYBR green fluorescence for each replicate (blue, without heat shock; red, with heat shock). The most accurate measure of transcript level is the threshold cycle number (C_t), which is identifiable for each plot as the cycle corresponding to the second derivative peak. The mean threshold cycle numbers are indicated. The *gal4* graph shows a significant decrease of 1.48 cycles with heat shock induction of Da protein, indicating at least a two- to threefold increase in RNA. The *Mdh1* graph shows no significant change in the threshold cycle number with heat shock: control levels of RNA are unaffected by the treatment.

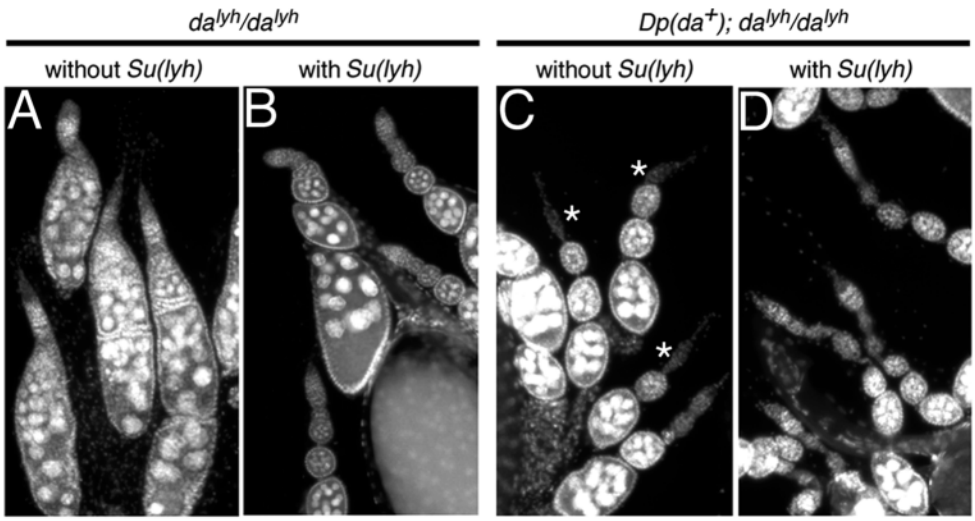

Fig. 6. *Su(lyh)* dominantly suppresses da^{lyh} phenotypes, both loss- and gain-of-function. (A-D) DAPI-stained ovaries. The da^{lyh} loss-of-function phenotype (A) is completely suppressed by a single copy of *Su(lyh)* (B) (genotype, da^{lyh}/da^{lyh}; $Su(lyh)^{26H6}/Su(lyh)^{+}$). The da^{lyh}-associated gain-of-function phenotype (C) (genotype, $Tp(2;Y)da^{+}$; da^{lyh}/da^{lyh}) is also dominantly suppressed by $Su(lyh)^{26H6}$ (D). Note that the reduced germaria (asterisks) that characterize the da^{+} overexpression phenotype are not present in the ovaries that carry the suppressor genotype.

without heat shock the C_t was 15.09±0.19, while with heat shock the C_t was 15.26±0.91. The change in reporter transcript level was highly significant ($P<0.0001$); there was no significant change in *Mdh1* transcript ($P>0.05$). As a decrease of one cycle number theoretically corresponds to a twofold increase in RNA levels under optimal PCR conditions, our decrease of 1.48 cycle numbers probably represented at least a 2.8-fold increase in *gal4* reporter transcript levels after heat shock induction of Da. To verify that the increase in *gal4* reporter transcript levels was dependent upon induced Da protein and not due to the heat shock treatment, the experiment was repeated in flies carrying the reporter transgene with no heat-inducible da^{+} transgenes. For *gal4* transcripts without heat shock treatment, the C_t was 21.74±0.55; with heat shock treatment, the C_t increased to 23.40±0.76. This significant ($P<0.05$) increase in C_t reflected a decrease in reporter transcript levels with heat shock treatment when Da protein was not induced. We were unable to make comparable reporter transgene measurements in a da^{lyh} mutant background because activation of the heat-inducible da^{+} transgenes under the same conditions resulted in lethality of this genotype. Thus, the genetic transactivation of da^{lyh} that we observed reflected a normal activity of Da protein on the wild-type *da* promoter, and in da^{lyh} this autoregulation was not insulated by the springer element of this mutant allele.

Isolation of *Su(lyh)*

That the da^{lyh} phenotypes resulted from insulating properties of the springer insertion of this mutant was strengthened by our identification of dominant suppressor mutations in another gene. We isolated four alleles of *Suppressor of lyh* (*Su(lyh)*) in a screen for rescue of da^{lyh} sterility. The strongest allele rescued da^{lyh} female sterility completely, with only 15% of the ovarioles still showing mild defects (Fig. 6A,B). In a homozygous *Su(lyh)* background, da^{lyh} ovaries were completely normal (data not shown). By contrast, *Su(lyh)* had no effect on the mutant ovary phenotype associated with other *da* alleles: it did not suppress da^{7}/da^{s22} ovary defects (data not shown). The specificity of *Su(lyh)* for da^{lyh} suggested that the *Su(lyh)* gene product was required for the insulating properties of the springer insertion that resulted in the da^{lyh} loss-of-function phenotype. The same *Su(lyh)* gene activity could account for the da^{lyh}-associated overexpression phenotype, as the *Su(lyh)* mutant also acted as a dominant suppressor of that phenotype (Fig. 6C,D). We were unable to detect any phenotype associated with the *Su(lyh)* mutations in an otherwise wild-type background; thus, the wild-type function of this gene is unclear.

DISCUSSION

da is under complex transcriptional control

Despite the expectation that a ubiquitously expressed binding partner for other developmentally regulated proteins would have a simple constitutive promoter, *da* is under precise transcriptional control. From the genetic and molecular analysis of a unique *da* allele, we have presented evidence for transcriptional autoregulation, as well as for the involvement of both positive and negative *cis*-acting regulatory sites. We suggest a model to describe the control of wild-type *da* expression in the somatic ovary. Our model also accounts for all aspects of the da^{lyh} misregulation.

In wild-type flies, several distinct transcriptional controls provide for a tightly regulated level of *da* expression within the somatic ovary (Fig. 7A). Initiation of *da* transcription requires an enhancer within the single intron of the gene. After activation of *da* transcription, the Da protein itself functions to maintain *da* expression. Indeed, since multiple canonical E-boxes (Ephrussi et al., 1985) are present in the *da* promoter region, this autoregulatory function may result from a direct interaction of Da protein with its own regulatory sequences. Finally, negative *cis*-acting sequences downregulate *da* transcription, thus preventing autoregulatory *da* expression from escalating to produce deleteriously high levels of Da protein. Such deleterious levels are achieved by overexpression of heat shock inducible da^{+} transgenes.

The insertion of the springer retrotransposon in the da^{lyh} mutant impacts the *cis*-acting transcriptional regulation of *da*, both positive and negative, without disrupting autoregulation (Fig. 7B). By insulating the *da* promoter from the intronic enhancer sequences, the springer insertion effectively blocks activation of *da* transcription in the somatic ovary. Thus, the da^{lyh} homozygote exhibits a *da* null phenotype in the ovary. By

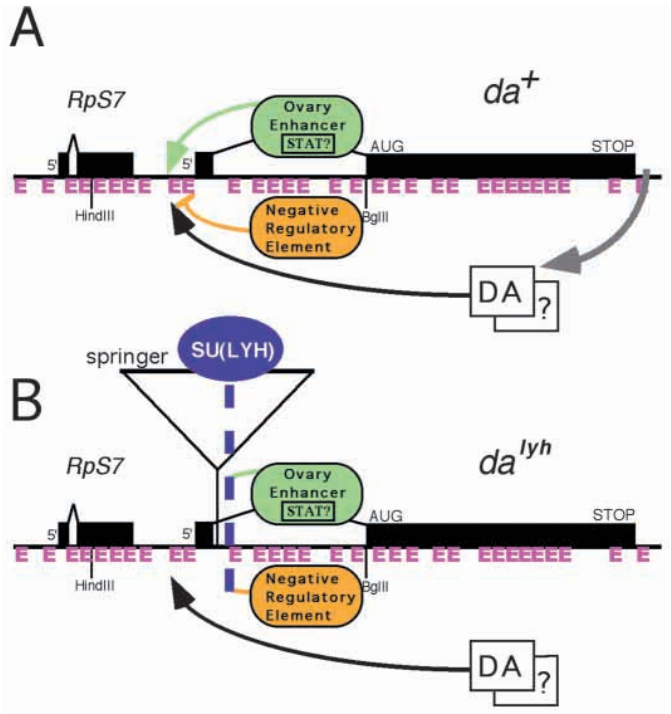

Fig. 7. A model for *da* transcriptional regulation. (A) Wild-type transcriptional regulation of *da* depends on both positive and negative control. An ovary-specific enhancer that is required for initiation of transcription in the somatic ovary is located within the 1.5 kb *da* intron. The location of this enhancer must be downstream of the da^{lyh} insertion site (indicated in B) and also within the sequence included in the da.G32 promoter reporter transgene (extents indicated by the *Hin*dIII and *Bgl*II sites). Within this region (500 bp into the intron), a STAT-binding site is one enhancer candidate. At least in this tissue, the initial expression of *da* leads to the production of Da protein, which subsequently acts to maintain the transcription of *da*. The binding partner of Da for this autoregulation is not known; however, numerous target E-box-binding sites (E) are dispersed throughout the genomic sequence. To keep *da* transcript levels from becoming too high, there is also a negative regulatory element, which must be located downstream of the da^{lyh} insertion site. (B) In the da^{lyh} allele, a springer retrotransposon acts as a transcriptional insulator that is capable of blocking the effects of both the positive and negative regulatory elements without disrupting Da autoregulation. In most tissues, the function of this allele appears to be normal, indicating that there is generally no problem with its transcriptional activation. In the somatic ovary, however, transcriptional activation fails because the promoter is insulated from the downstream ovary enhancer. But, da^{lyh} can be transactivated by Da protein from another allele. The springer element also insulates the negative regulatory element, so that the amount of *da* transcript produced from the da^{lyh} allele is higher than that from a wild-type allele. The insulating effects of springer depend on the *Su(lyh)* gene product. By analogy to Su(Hw)-mediated gypsy insulation, Su(lyh)-mediated insulation may result from direct binding of the Su(lyh) protein to springer sequences.

contrast, the mutant allele of a da^{lyh} heterozygote is functional: Da protein derived from expression of the wild-type allele transactivates the mutant allele, thus bypassing the need for a functional enhancer. In this way, transactivation of da^{lyh} by da^{+} accounts for the wild-type behavior of da^{lyh} heterozygotes in genetic interaction tests. The springer retrotransposon also insulates the *da* promoter from the negative *cis*-acting sequences that must lie downstream of the insertion site. In da^{lyh} homozygotes, in addition to the loss of *da* transcription in the somatic ovary, there is an overall increase in *da* transcript levels. Furthermore, in da^{lyh} homozygotes carrying an extra copy of da^{+}, the wild-type allele transactivates the mutant alleles, resulting in excessive *da* transcription and an associated gain-of-function phenotype in the ovary.

Based on the failure of transcription of da^{lyh} in the somatic ovary and from an analysis of a *da* promoter fusion transgene, we have identified a STAT (*Drosophila* Stat92E)-binding site as a candidate for the *cis*-acting enhancer. STAT (signal transducers and activators of transcription) proteins are activated by tyrosine kinases in response to cytokine or growth factor signals and play essential developmental roles in growth and differentiation, and they are constitutively activated in many cancers (Bromberg, 2001; Horvath, 2000). The Stat92E binding site in *da* (TTCATGGAA) is the only predicted transcription factor-binding site found exclusively downstream of the springer insertion and within the extents of the da.G32 reporter. The somatic ovary enhancer must be included in the da.G32 reporter (Fig. 2B), as this transgene is expressed in the ovary, even in da^{lyh} mutants (data not shown). Moreover, a temperature sensitive loss-of-function allele of *Stat92E* shows a *da*-like mutant ovary phenotype (K. Baksa and C. Dearolf, personal communication). We propose that Stat92E is essential for the initiation of *da* transcription within the somatic ovary.

Da protein appears to be necessary for maintenance of its own transcription, and the simplest molecular model for *da* autoregulation is direct transcriptional activation. Although Da homodimers can bind DNA in vitro (Murre et al., 1989) and the mammalian homolog, E47, does function as a homodimer in B cell development (Shen and Kadesch, 1995), there are no examples of Da protein homodimerizing to activate transcription in vivo. More likely, Da acts on its own promoter by collaboration with another bHLH-binding partner. We have identified several possible candidate binding partners, based on ovary phenotypes in genetic interaction tests (E. Basler and C. C., unpublished). For example, one candidate is *achaete* (*ac*), which transcriptionally autoregulates during the development of sensory bristles in wing imaginal discs (Van Doren et al., 1992). For this process, Ac protein heterodimerizes with Da; perhaps they collaborate again in the somatic ovary with the *da* gene as their target.

We have identified two *cis*-acting elements involved in *da* transcriptional regulation in the ovary, but there are likely to be more *cis*-regulatory elements whose use may differ between or within other tissues. Although nearly ubiquitous throughout development, Da protein is present at significantly different levels in various tissues, or even within individual tissues. For example, the CNS includes cells with levels of Da that range from very low to very high (Cronmiller and Cummings, 1993), and in eye discs, dynamic changes in Da protein levels correlate with the progression of the morphogenetic furrow (Brown et al., 1996). If Da protein levels directly reflect *da* transcript levels, these observations suggest that precise regulation of *da* is crucial for developmental processes, and the regulatory sites identified are probably not sufficient to account for the scope of regulation necessary. The da.G32 reporter, which shows a mottled expression pattern that is not attributable to position effect variegation (data not shown),

indicates that this transgene is missing crucial binding sites for regulatory factors. Additionally, this construct is unable to rescue embryonic lethality when driving a Gal4-dependent da^+ transgene (Giebel et al., 1997). However, a 15 kb genomic da^+ transgene that includes the da.G32 regulatory region and an additional 12 kb downstream rescues *da* mutant flies to adulthood (H. Vaessin, personal communication); this construct may contain all of the necessary *da* regulatory sequences. Thus, *da* expression, like that of many other protein-coding genes, is dependent upon a balance of multiple positive and negative regulators (Lee and Young, 2000).

da^{lyh} identifies springer as an insulator element

The da^{lyh} allele is the first springer-induced mutation in which this retrotransposon is documented to disrupt gene function by acting as an insulator; this discovery emphasizes the similarity between springer and the extensively-characterized gypsy retrotransposon. Like gypsy (Corces and Geyer, 1991), springer can disrupt gene function in two ways: either by altering the normal transcripts of a gene or by acting as an insulator. All other springer-induced alleles whose expression has been characterized to date produce aberrant transcripts (Davis et al., 1998; Hoover et al., 1993; Ishimaru and Saigo, 1993; Karlik and Fyrberg, 1985; Kidd and Young, 1986). This newly discovered similarity between springer and gypsy prompted us to look for Su(Hw)-binding sites in springer, as Su(Hw) mediates gypsy insulation (Roseman et al., 1993). Springer has no Su(Hw)-binding sites, so it must be bound by a different insulating protein.

Su(lyh) may encode the springer insulator protein. *Su(lyh)* dominantly suppresses the da^{lyh} insulation of both the *cis*-acting enhancer and the *cis*-acting negative regulator. The dominant nature of this suppression may be unique to the *da* locus, as autoregulation will amplify even the small amount of *da* gene product that results when the insulation is only weakly suppressed. However, *su(Hw)* can act as a dominant suppressor of some gypsy-induced alleles. (Hoover et al., 1992). Suppression of the da^{lyh} mutant phenotype is completely penetrant when the insulator protein is eliminated altogether, as in the case of *Su(lyh)* homozygotes. We expect that the Su(lyh) protein will function like Su(Hw) protein by binding specific sites within springer.

In addition to Su(lyh), other previously described insulator proteins may be involved in springer insulation. For one, the *zeste-white 5* (*zw5*) gene product binds to specific sequences within the *scs* chromatin domain boundary of the 87A7 heat shock locus (Gaszner et al., 1999), and there are two of these binding sites in springer. For another, BEAF binds to a clustered CGATA array within several chromatin boundaries (Cuvier et al., 1998), including the boundary of *scs*, and there are 12 unclustered CGATA sequences in springer. Finally, proteins of the polycomb and trithorax groups appear to collaborate with Su(Hw) in gypsy insulation (Gerasimova and Corces, 1998), and there is a polycomb response element (Mihaly et al., 1998) in springer. Future work will clarify what role, if any, these factors play in springer-associated insulation.

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