# ROLE OF REPRESSORS IN FINE REGULATION OF DEVELOPMENT:

# *SXL* AND ITS NEW REPRESSORS *HEY* AND *MYC*

A Thesis

by

ELENA KOZHINA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2009

Major Subject: Genetics

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Approved by:

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December 2009

Major Subject: Genetics

# ABSTRACT

Role of Repressors in Fine Regulation of Development: *Sxl* and Its New Repressors *Hey* and *Myc*. (December 2009)

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Chair of Advisory Committee: Dr. James Erickson

In *Drosophila,* XX embryos express *Sxl* from the early promoter, *SxlPe*, and become females. At the same time, XY embryos with only one X chromosome become males.

I investigated the role of repression in the establishment of the strict regulation of *SxlPe*. I found that the co-repressor *Groucho,* is responsible for amplification of the two-fold difference in X-encoded activator genes into an all-or-nothing difference in *Sxl* expression. Three new basic helix-loop-helix repressors of *Sxl* were identified: *Hey*, Cwo and the prooncogene *Myc*, all of which are maternally supplied. I have shown that *Myc* specific repression is important as early as cycle 10, which is 2 cycles earlier than the onset of normal *Sxl* expression.

## ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Jim Erickson, for his help in experiment design and discussion of results, and my committee members, Dr. Deborah Bell-Pedersen, Dr. Ginger E. Carney, Dr. Keith Maggert and Dr. Rene Garcia, for their guidance and support throughout the course of this research.

Thanks to current and previous members of the Lab, Alejandra Gonzales, Sharvani Mahadevaraju, Guruswamy Mahesh, and Frank Avila, for discussion of my results. I would like to thank Dr. Boris Novikov and Dr. Hardin Lab for help with protein purification and Dr. Uwe Ohler for the non-canonical model of *SxlPe* and *SxlPm* promoter.

Thanks also go to my friends, colleagues, department faculty and staff for being open and friendly.

Finally, thanks to my husband, Serge Spraiter, for his encouragement, patience and love.

# TABLE OF CONTENTS

Page

# LIST OF FIGURES

Page

# LIST OF TABLES

# 1. INTRODUCTION*

## Role of *Sxl* repressors in sex determination in *Drosophila*

Dose-sensitive promoters respond to small differences in regulatory protein concentrations to produce large differences in gene expression. In some instances differential concentrations of activators alone appear to set promoters into their appropriate expression states, but the general rule is that the enhancers controlling switch-like promoters integrate concentration-dependent inputs from both activators and inhibitors to establish precise boundaries of expression (see (Mannervik et al., 1999), (Barolo and Posakony, 2002), (Clyde et al., 2003) and (Ochoa-Espinosa et al., 2005). In the developing nervous systems of flies and vertebrates, for example, antagonistic interactions between negatively and positively-acting proteins of the basic-helix–loop–helix, bHLH, family define the sharp boundaries of gene expression required for specification of neural precursor cells (reviewed in (Massari and Murre, 2000). Similar antagonistic interactions between bHLH proteins and their associated cofactors are hypothesized to play important roles in the specification of the alternative male and female fates in *Drosophila*.

Chromosomal sex determination in *Drosophila* is a textbook example of how two-fold changes in transcriptional regulatory protein concentrations can elicit different developmental outcomes (reviewed by (Cline and Meyer, 1996) and (Ashburner et al.,

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This thesis follows the style of Developmental Biology.

*Reprinted from Developmental Biology 323(2), Lu H, Kozhina E, Mahadevaraju S, Yang D, Avila FW, Erickson JW, Maternal Groucho and bHLH repressors amplify the dose-sensitive X chromosome signal in *Drosophila* sex determination, 248-260, Copyright © 2008, with permission from Elsevier Inc.

2005). In the fly, the collective dose of four X chromosome-linked signal element genes, XSEs, conveys X chromosome dose to the master regulatory gene *Sex-lethal*, *Sxl* (Cline, 1993) and (Erickson and Quintero, 2007)). In XX embryos the double XSE dose directs the transient activation of the *Sxl* establishment promoter, *SxlPe*, initiating a positive autoregulatory splicing loop that operates on pre-mRNAs produced from the constitutive promoter, *SxlPm*, thereby maintaining *Sxl* in the on (female) state for the remainder of its life (Cline, 1984; Bell et al., 1991; Keyes et al., 1992; Nagengast et al., 2003). In XY embryos, the single dose of XSEs leaves *SxlPe* inactive, precluding functional splicing of *SxlPm*-derived transcripts and thereby directing the male fate.

Three of the four XSE genes encode transcription factors that directly regulate *SxlPe*. The two strongest XSEs, *scute* and *sisA,* encode bHLH and bZIP activators, while *runt* encodes the founding member of the RUNX class of DNA binding proteins (Cline, 1988; Cline and Meyer, 1996; Ashburner et al., 2005). Although the dose-sensitive XSE proteins are of central importance in the X-counting process, their direct action at *SxlPe* requires additional protein factors. Maternally-supplied *daughterless* protein, for example, interacts with Scute to form the DNA binding bHLH heterodimer, Sc/Da, while maternally supplied STAT, and presumably, Bicoid stability factor, bind directly to *SxlPe* to facilitate expression (Yang et al., 2001; Bosch et al., 2006; Avila and Erickson, 2007; De Renzis et al., 2007). How these and other factors work to effectively amplify the two-fold difference in XSE dose into an all-or-nothing response at *SxlPe* is unknown. Cooperative or combinatorial interactions among the XSE and maternal activators in protein assembly, DNA binding, or via interactions with the general

transcription machinery, have been offered as possible explanations of how male and female XSE concentrations might be reliably distinguished at *SxlPe* (Cline, 1993; Erickson and Cline, 1993; Yang et al., 2001). Other models, however, focus on the means by which negative regulators might amplify the difference in XSE protein concentrations to generate a reliable sex-determining signal (see Parkhurst et al., 1990; Schutt and Nothiger, 2000; Gilbert, 2006).

Three negative regulators of *SxlPe* have been identified: the maternally supplied *extramachrochetae (emc)* and *groucho (gro)* products and the zygotically expressed product of the autosomal gene *deadpan (dpn)* (Younger-Shepherd et al., 1992; Paroush et al., 1994; Barbash and Cline, 1995). Emc is an HLH protein that lacks a basic DNA-binding domain and exerts its inhibitory effects by forming heterodimers with bHLH activators, such as Scute and Da, thereby preventing them from binding to DNA (Massari and Murre, 2000; Campuzano, 2001). While *emc* apparently plays a minor role in sex determination (Younger-Shepherd et al., 1992), loss of maternal *gro* has been reported to cause male embryos to express female levels of *Sxl* protein, suggesting that Gro-mediated repression of *SxlPe* may be essential for distinguishing X chromosome dose (Paroush et al., 1994). Gro is the archetypal example of the widely-distributed Gro/TLE family of transcriptional corepressors, that are recruited to DNA by virtue of their interactions with several different groups of sequence-specific DNA binding proteins; including bHLH repressors such as Dpn (reviewed in (Fisher and Caudy, 1998a; Chen and Courey, 2000; Buscarlet and Stifani, 2007; Fischer and Gessler, 2007).

The *dpn* gene was identified as an autosomal sex signal element, or ASE, because it functions as a zygotically expressed negative regulator of *Sxl* (Younger-Shepherd et al., 1992; Barbash and Cline, 1995). Present in equal amounts in XX and XY embryos the *dpn* product is needed to properly assess the male XSE dose as evidenced by the finding that loss of *dpn* function causes some XY cells to activate *SxlPe* and adopt the inappropriate female fate (Younger-Shepherd et al., 1992; Barbash and Cline, 1995). Dpn is a member of the Hairy-Enhancer of split, HES, family of bHLH repressors (reviewed in Fisher and Caudy, 1998a; Massari and Murre, 2000; Iso et al., 2003; Fischer and Gessler, 2007; Kageyama et al., 2007). HES proteins and the closely related HEY family (HES with YRPW) bind to the "E-box" CACGTG and the related sequence CACGCG, the later being the optimal sequence for Hairy and Dpn (Ohsako et al., 1994; Van Doren et al., 1994). HES factors also bind with reduced affinity to the "N-box" CACRAG suggesting that there is a range of allowable in vivo target sites.

HES proteins repress transcription by several different mechanisms. Best understood is the recruitment of the corepressor Gro to DNA via the C-terminal peptide sequence, WRPW, present in all HES family members (Paroush et al., 1994; Fisher et al., 1996; Fisher and Caudy, 1998a). Some HES proteins recruit other corepressors such as CtBP and Sir2 to DNA and there is evidence that mutual antagonism between different corepressors can influence HES protein function (Poortinga et al., 1998; Zhang and Levine, 1999; Bianchi-Frias et al., 2004). Repression may also be mediated directly by competition with activators for DNA binding or by sequestering bHLH activators

into inactive heterodimers (Fisher and Caudy, 1998a; Fischer and Gessler, 2007; Kageyama et al., 2007). Most of these schemes have been invoked to explain how Dpn might function during sex determination (Paroush et al., 1994; Dawson et al., 1995; Jimenez et al., 1997), but none have been examined in detail.

Although Dpn is the only known DNA-binding repressor of *SxlPe*, loss of *dpn* function has a relatively mild effect, causing low-level ectopic activation of *SxlPe* in a subset of male nuclei (Barbash and Cline, 1995). Given the efficiency of HES/Gro-mediated repression in other contexts (Barolo and Levine, 1997; Zhang and Levine, 1999; Courey and Jia, 2001) and the presence of two canonical CACGCG Dpn-binding sequences at *SxlPe* (Hoshijima et al., 1995; Winston et al., 1999), it is not clear why Dpn has such a modest effect on sex determination. One possibility is that Dpn function could be modulated, perhaps by chemical modification (Karandikar et al., 2005), or by competition with other DNA binding proteins (Yang et al., 2001; Louis et al., 2003). A second possibility is that additional repressors negatively regulate *SxlPe*: an explanation consistent with the report that loss of maternal *gro* function leads to high-levels of *Sxl* protein in XY embryos (Paroush et al., 1994).

To better understand the role of transcriptional repression in primary sex determination we characterized the cis-acting promoter elements recognized by Dpn, and analyzed the effects of maternal *gro* on *SxlPe*. Our studies revealed that *SxlPe* contains three functional Dpn DNA-binding sites, including one with the non-canonical sequence CACACT. Mutations in the Dpn-binding sites had stronger and earlier effects on *SxlPe* than did a null *dpn* mutation, suggesting that additional bHLH repressors

regulate *SxlPe*. We found that the *Hey* locus encodes one such maternal-effect repressor of *SxlPe,* but that the *E(spl)m3* gene, which had previously been proposed to regulate *Sxl* (Dawson et al., 1995; Poortinga et al., 1998), does not. The *gro* product influences *SxlPe* earlier and more strongly than does *dpn,* suggesting that the initial concentrations of XSE proteins needed to activate *SxlPe* in XX embryos are defined by Gro-mediated repression and then modulated upward to compensate for rising XSE levels in XY embryos. We propose a model for *SxlPe* regulation in which the XSE signal is amplified by a positive feedback mechanism that inhibits Gro-mediated repression in XX, but not XY, embryos.

# 2. MATERIALS AND METHODS*

## Immunohistochemistry and in situ hybridization

Cultivation, transfection, and assay of Schneider L2 cells were according to (Han et al., 1989). One µg of DNA was used per plate and included: 0.1 µg of *firefly luciferase* Dpn-binding site reporter, 0.1 µg *actin5Cp-dpn*-VP16 expression construct, 0.1 µg of SV40 -*Renilla* luciferase reporter to control for transfection efficiency (pRL-SV40 Promega), and carrier DNA. Luciferase activity was determined using a Dual-Luciferase assay kit (Promega) and a Berthold Lumat LB9501 luminometer.

Embryos were prepared for immunocytochemistry according to (Patel, 1994). Anti-Sxl mouse antibody was used as described (Erickson and Quintero, 2007). All embryos were stained with DAPI to visualize DNA and mounted in 70% glycerol. In situ hybridization was done using standard procedures including NBT/BCIP staining (Lehmann and Tautz, 1994). Briefly, digoxygenin-labeled RNA probes complementary to *Sxl* exon E1, or *lacZ* sequences were prepared using in vitro transcription of plasmid or PCR-derived templates (Avila and Erickson, 2007; Erickson and Quintero, 2007). *Sxl* exon E1 probes detect both *SxlPe*-derived mRNA and *Pe*-derived nascent transcripts, the later visible as dots of staining within nuclei (Shermoen and O'Farrell., 1991; Erickson and Cline, 1993; Barbash and Cline, 1995; Erickson and Cline, 1998; Erickson and Quintero, 2007). For X-linked genes, or transgenes, the number of nuclear dots

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corresponds to the number of X chromosomes. Embryo cell cycles were determined by nuclei density (Foe et al., 1993). Nuclei change in appearance through the cell cycle and we used this to closely stage embryos in cycles 11-13 (Edgar et al., 1994). Times within cycle 14 were estimated by nuclear shape and length, and by the extent of membrane furrow invagination (Foe et al., 1993; Grosshans et al., 2003). In wild-type females *SxlPe* expression begins during cycle 12. In typical embryo collections, only one quarter of cycle 12 embryos (one half of XX embryos) express *Sxl* and many of those express in a mosaic pattern with individual nuclei exhibiting one, two, or no nuclear dots, reflecting stochastic activation of the promoter during cycle 12 (Erickson and Cline, 1998; Erickson and Quintero, 2007). For $hey^{mat-}$ we observed that 10/21 cycle 12 embryos exhibited *Sxl* staining from both X chromosomes in most, or all, nuclei. The number of *Sxl*-expressing cycle 12 $hey^{mat-}$ embryos was not significantly different from wild-type (expected 5-6 with expression in some nuclei), but was consistent with our qualitative assessments of elevated staining levels in $hey^{mat-}$ XX embryos, and is thus suggestive of a repressive effect of maternal *Hey* on *SxlPe* activation in females.

**Fly culture and genetics**

Flies were grown on standard medium in uncrowded conditions at 25°C. Mutations and chromosomes are described: http://flybase.bio.indiana.edu. Null alleles used: $\Delta dpn^{2}$ (*Df(2R)dpn-2*) (Barbash and Cline, 1995), $gro^{E48}$ (Jennings et al., 2006), $Df(3R)E(spl)^{P11}$, $E(spl)^{-}$, $HLHm\gamma^{-}$, $HLHm\beta^{-}$, $HLHm3^{-}$, $HLHm5^{-}$, $HLHm7^{-}$, $HLHm8^{-}$ (Nagel et al., 2004), and $Df(2L)Exel6042, Side^{-}$. The *P(Bac)* insertion allele $hey^{f06656}$ is homozygous lethal but may retain partial function. *Hey* is located at position 44A2 on

chromosome 2R. The *FRT42B Hey$^{f06656}$* chromosome was made by selecting *P{FRT(w$^{hs}$)}G13 L$^{+}$* recombinant progeny of + *PBac{w$^{+mC}$}Hey$^{f06656}$ +/ P{FRT(w$^{hs}$)}G13 + L* females and screening for rare flies with slightly darker eye color than the *P{FRT(w$^{hs}$)}G13* parent. Darkest eyed flies were confirmed to carry *P{FRT(w$^{hs}$)}G13 PBac{w$^{+mC}$}Hey$^{f06656}$*. Germline clones (Chou and Perrimon, 1996) were generated following heat treatment of female larvae of the following genotypes: *P{hsFLP}1, y$^{1}$ w$^{1118}$/ w$^{1118}$ ; P{neoFRT}82B ry$^{506}$ gro$^{E48}$/P{neoFRT}82B P{ovoD1-18}3R* and *P{hsFLP}1, y$^{1}$ w$^{1118}$/ w$^{1118}$ ; P{neoFRT}82B Df(3R)E(spl)$^{P11}$/P{neoFRT}82B P{ovoD1-18}3R* and *P{hsFLP}12, y$^{1}$ w/y w$^{1118}$; P{FRT(w$^{hs}$)}G13 hey/P{FRT(w$^{hs}$)}G13 P{ovoD1-18}2R*. Females bearing recombinant germlines were crossed to *w$^{1118}$*/Y males and their *gro$^{mat-}$* or *hey$^{mat-}$* progeny analyzed. The *hb-hairy-en* transgene (Jimenez et al., 1997) was generously provided by G. Jimenez (IBMB-CSIC-PCB, Barcelona), *dpn* alleles were from T. Cline (University of California, Berkeley), *E(spl)P11* was a gift of A. Preiss (University of Hohenheim), *FRT82B gro$^{E48}$* was provided by P. Simpson (University of Cambridge). Other fly stocks, including those used for FLP/FRT recombination, were provided by the Bloomington *Drosophila* stock center: #13509 y1; P{SUPor-P}BgbKG03779 ry506/TM3, Sb1 Ser1; #11298 P{P-Sal}dmP0/C(1)DX, y1 f1; bw1; st1.

# 3. MATERNAL GROUCHO AND BHLH REPRESSORS AMPLIFY THE DOSE-SENSITIVE X CHROMOSOME SIGNAL IN *DROSOPHILA* SEX DETERMINATION*

My contribution to this section was to show that corepressor *Gro* is a potent negative regulator of *SxlPe*. I also identified that *Gro* has a stronger and earlier effect on *SxlPe* then does *dpn*, and I identified maternal Hey as a negative regulator of *SxlPe*.

**Dpn binds canonical and non-canonical sites at *SxlPe***

To identify Dpn-binding sites at *SxlPe* we expressed and a full length Dpn-maltose-binding protein fusion and used the pure MBP-Dpn to DNase I footprint the 1.4 kb region of *SxlPe* sufficient to confer high-level female-specific expression (Estes et al., 1995). We found three protected regions in the proximal 400 bp of *SxlPe* (Fig. 1A). One region was centered on the two canonical Dpn-binding sites located at -110 and -121 bp (Hoshijima et al., 1995; Winston et al., 1999). The other protected regions were centered at -160 and -330 bp where no sequences match identified HES protein-binding sites (Fig. 2) suggesting that Dpn, like the bHLH activator Sc/Da (Yang et al., 2001), binds non-canonical sites at *SxlPe*.

To identify the non-canonical sequences mediating Dpn binding, we carried out

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of a series of gel-mobility shift assays using a purified 6X His-tagged Dpn bHLH domain fusion protein. Oligonucleotides containing the previously characterized tandem sites 1 and 2 produced two gel-shifted complexes corresponding to dimeric and tetrameric

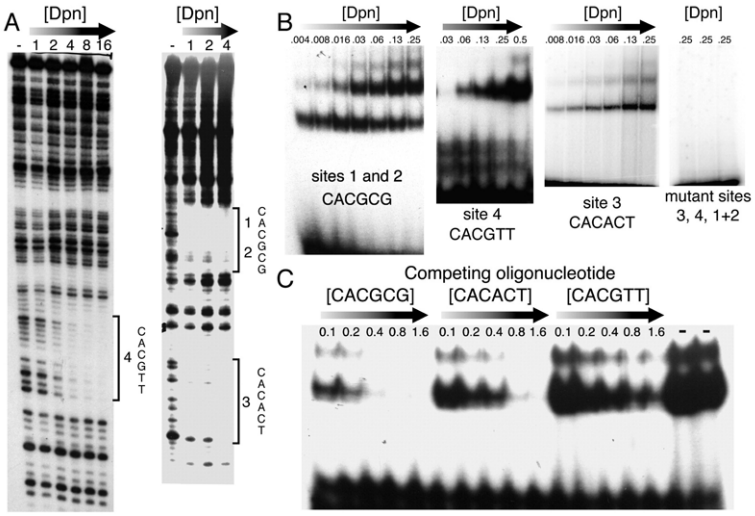



Fig. 1. Binding of Dpn to canonical and non-canonical DNA sequences at *SxlPe*. A) DNase I footprinting with the indicated units of full-length MBP-Dpn fusion protein. One unit equaled 0.3 pmole (15 nM) MBP-Dpn. Left panel, protection of Dpn-binding site 4, right panel, protection of Dpn-binding sites 1, 2, and 3. Six bp core sequences are indicated. Probes extended from -204 to -373 (left) and -229 to +72 (right). Protection of Dpn-binding site 3 is also visible in Fig. 6 of Hoshijima et al., (1995). B) Electrophoretic mobility-shift assays (EMSA). Indicated units of GST-Dpn bHLH fusion protein were incubated with $^{32}$P-labeled oligonucleotides and the complexes resolved on polyacrylamide gels. One unit equaled 0.3 pmole (15 nM) GST-Dpn bHLH protein. Core sequences for the Dpn-sites are shown. C) Binding site competition in EMSA. Complexes were formed between GST-Dpn bHLH protein (0.02 units) and a $^{32}$P-labeled site (1+2) probe and challenged with 10- to 160-fold molar excesses of oligonucleotides (1+2), 3, or 4 as competitors.

sequences eliminated Dpn binding (Fig. 1B). Consistent with the quantitative Dpn:DNA complexes (Winston et al., 1999) and mutations in the site 1 and 2 core study of (Winston et al., 1999), we found no evidence for cooperative binding to tandem sites 1 and 2 by the Dpn bHLH domain. To determine the sequences of Dpn-binding sites 3 and 4, we examined a series of overlapping oligonucleotides for their ability to bind the Dpn bHLH domain. We found that Dpn bound to oligonucleotides 3 and 3C containing the sequence CACACT but not to the similar fragment 3Cm carrying the single base change CAC<u>c</u>CT (Fig. 1B). Similarly, we found that Dpn bound to oligos 4 and 4C but not to 4L or 4R suggesting that the central CACGTT sequence is the core sequence for Dpn site 4. Consistent with this inference, mutations that changed the sequence to CA<u>a</u>G<u>c</u>T prevented Dpn binding in the gel-shift assay (Fig. 1C). The distal portion of *SxlPe* has a second CACGTT sequence at -1006. We found that the Dpn bHLH protein bound an oligonucleotide containing this distal site further supporting our conclusion that CACGTT is a Dpn-binding site. The distal site 5 was likely missed in our footprinting assays because it was too close to the ends of the probes.

The three different core sequences exhibited a range of Dpn-binding affinities in the DNase I protection experiments. Consensus sites 1 and 2 were always protected at lower Dpn concentrations than was site 3. Dpn-binding site 3 in turn, was protected by lower Dpn concentrations than was site 4 suggesting that the overall binding affinities are sites 1, 2 > 3 > 4. To further test the relative binding affinities of the Dpn sites, we performed DNA binding competition experiments. We found that Dpn could be competed off the tandem consensus sites 1 and 2 by oligonucleotides containing single

sites 1, 3, or 4, but not by a mutant site 1 sequence (Fig. 1C and unpublished data). Based on the footprinting and gel-shift data we estimate that Dpn-binding sites 1 and 2 are bound with approximately four-fold greater affinity than is site 3, which in turn is bound two to five times more tightly than site 4 (Fig. 1 and unpublished data). Binding to the non-canonical site 3 and site 4 sequences is not specific to Dpn, because the related protein Side (CG10446) bound the same sequences with similar relative affinities (unpublished data).

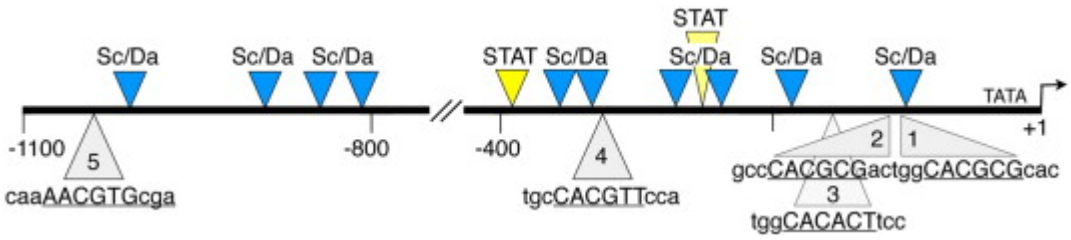



Fig. 2. Location of protein-binding sites at *SxlPe*. Diagram represents sequences from -1.1 kb to +1 relative to start of transcription. Triange apices denote positions of identified protein-binding sites. Ten binding sites for the activator Sc/Da (Yang et al. 2001) and two binding sites for the activator STAT (Avila and Erickson 2007) are shown above the line. The five HES-class repressor-binding sites are numbered and shown below the line. Core HES-binding sequences are capitalized. Sequences from + 42 to - 392 are sufficient for sex-specific expression of *SxlPe* but sequences to -1.4 kb are needed for near wild-type expression (Estes et al. 1995).

**In vitro defined Dpn sites bind HES proteins in vivo**

We employed three assays to determine whether the Dpn-binding sites we identified in vitro can be recognized by Dpn or related HES proteins in vivo. First, we asked whether Dpn could bind artificial promoters carrying multimers of the predicted Dpn-binding sites in cultured cells. Next, we asked if ectopic *hairy* protein could bind

the predicted sites in embryos, and finally, we asked whether the predicted Dpn-binding sites mediated repression of *SxlPe-lacZ* reporters in otherwise normal embryos.

To analyze Dpn binding in Schneider L2 cells, we created an activator form of Dpn containing the Dpn bHLH domain fused to the VP16 activation domain (Jimenez et al., 1999) and assayed for the ability of Dpn-VP16 to activate transcription from promoters carrying four tandem copies of the predicted Dpn-binding sites (Fig. 3A). When Dpn-VP16 was expressed from the *Actin5C* promoter it stimulated transcription

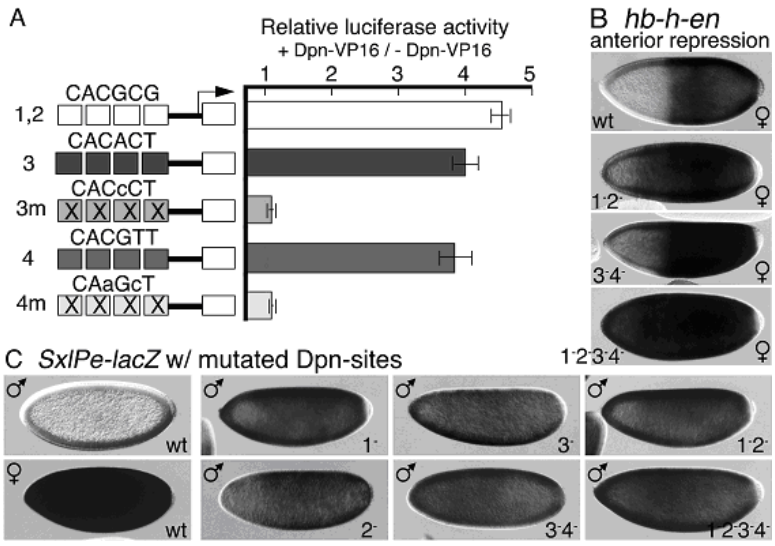



Fig. 3. Canonical and non-canonical DNA sequences mediate HES protein-binding at *SxlPe*. (A) Dpn-VP16 activates transcription in SL-2 cells via predicted Dpn-binding site 1, 2, 3, and 4 sequences. Four copies of Dpn-binding sequences were joined to a -95bp *SxlPe*-luciferase reporter and co-transfected with an *actin5C* promoter-Dpn-VP16 expression vector. Data are expressed as luciferase activity with actin-Dpn-VP16 relative to the *actin5C* promoter control (+/- one standard deviation). (B) Repression of *SxlPe-lacZ* by anteriorly-expressed *hairy-engrailed (hb-h-en)*. In situ hybridizations detect *SxlPe-lacZ* mRNA in embryos carrying wild-type (wt) or mutant ($1^{-}2^{-}$, $3^{-}4^{-}$, $1^{-}2^{-}3^{-}4^{-}$) Dpn-binding sites. Female embryos shown, Dpn-site mutant transgenes responded similarly to *hb-h-en* in males. (C) Ectopic expression of *SxlPe-lacZ* transgenes carrying Dpn-binding site mutations. In situ hybridizations to detect *lacZ* mRNA. All embryos carry two copies of the indicated *SxlPe-lacZ* transgenes inserted on an autosome.

from a luciferase reporter plasmid carrying four copies of the canonical CACGCG core sequence upstream of the otherwise inactive minimal *SxlPe* promoter. Plasmids carrying four copies of the site 3 CACACT or site 4 CACGTT core sequences supported levels of Dpn-VP16 activated transcription nearly equivalent to those seen with consensus sites. Point mutations in sites 3 and 4 blocked activation, confirming that

Table 1. Summary of expression of 1.4 kb *SxlPe-lacZ* transgene lines.
Abreviations: wt, wild-type 1.4 kb *SxlPe-lacZ*; $1^-$, $2^-$, $3^-$, $4^-$, Dpn-binding site mutations in 1.4 kb *SxlPe-lacZ*. Genotypes are normal + or deficient for maternal $gro^{mat-}$ or zygotic $\Delta dpn^2$. XY male, XX female chromosome complement.

| ***SxlPe-lacZ*** | **Embryo genotype** | **Onset *lacZ* expression (# transgene lines)** | **Comments** |
|---|---|---|---|
| wt | +/+ | XY cycle 13 (3)<br>XX cycle 11 (3) | XY: occasional nuclei express cycles 13-14. No detectable mRNA. XX: some nuclei express cycle 11. Trace mRNA cycle 13, strong mRNA cycle 14. |
| wt | $gro^{mat-}$ | XY (1)<br>XX (1) | XY: many nuclei express cycle 12. Strong mRNA cycles 13-14. XX: some nuclei express cycle 10, all in cycle 11. Strong mRNA cycles 13, 14. |
| wt | $\Delta dpn^2$ | XY cycle 13 (1)<br>XX cycle 11 (1) | XY: many nuclei express cycles 13, 14. Moderate mRNA accumulation cycle 13-14. XX: like +/+ genotype. |
| $1^-$ | +/+ | XY cycle 13 (3)<br>XX cycle 11 (3) | XY: most nuclei express cycles 13-14. Relatively uniform mRNA cycle 14. XX: nearly all nuclei express cycle 11. mRNA visible cycle 13, strong in 14. |
| $2^-$ | +/+ | XY cycle 12 (3)<br>XX cycle 10 (3) | XY: some nuclei express cycle 12, many cycle 13. Moderate mRNA cycle 13, strong mRNA cycle 14, lower than $1^-$. XX: Some nuclei express cycle 10, many to all, cycle 11. Strong mRNA cycles 13-14, lower than $1^-$. |
| $3^-$ | +/+ | XY cycles 12 (2) 13 (2)<br>XX cycle 11 (4) | XY, XX: like wt *SxlPe-lacZ* in $\Delta dpn^2$ genotype. |
| $4^-$ | +/+ | XY cycle 13 (2)<br>XX cycle 11 (2) | XY, XX: indistinguishable from wt transgenes in +/+ genotype. |
| $1^-2^-$ | +/+ | XY cycle 12 (2)<br>XX cycles 10 (2) | XY, XX: nuclei like $2^-$. mRNA like $1^-$ but elevated. |
| $3^-4^-$ | +/+ | XY cycle 12 (3)<br>XX cycle 11 (3) | XY, XX: indistinguishable from $3^-$ transgenes. |
| $1^-2^-3^-4^-$ | +/+ | XY cycle 12 (3)<br>XX cycle 10 (3) | XY: many nuclei express cycle 12. Strong mRNA cycles 13-14. XX: some nuclei express cycle 10, almost all by cycle 11. Strong mRNA cycles 13-14. |

these non-canonical sequences can mediate Dpn-binding in cultured cells.

To determine if the Dpn-binding sites can mediate HES protein-binding and transcriptional repression in embryos, we created a series of transgenic 1.4 *SxlPe-lacZ* reporters carrying mutations in the predicted Dpn-binding sites and assayed their effects in vivo. We first asked if the reporters could mediate repression by an ectopically expressed version of Hairy that carries the Gro-interacting repression domain from the *engrailed* protein (Jimenez et al., 1999). In this assay, first employed on endogenous *Sxl* (Parkhurst et al., 1990), zygotic expression of Hairy-Engrailed from the anteriorly-expressed *hunchback* promoter causes anterior-specific repression of target genes carrying HES protein-binding sites. We found that Hairy-En repressed *SxlPe-lacZ* even when both canonical Dpn-binding sites 1 and 2 were mutated ($1^{-}2^{-}$) although the degree of repression was less than seen with wild-type *SxlPe-lacZ* fusions (Fig. 3B). These findings indicate that Dpn-binding sites 1 and 2 bind Hairy-En, but also suggest that other, non-canonical, sequences can mediate Hairy DNA-binding in vivo. Those non-canonical sites appear to be at least one of Dpn-binding sites 3 and 4, because the ($3^{-}4^{-}$) *SxlPe-lacZ* transgenes were also less effectively repressed by Hairy-En than was wild-type *SxlPe-lacZ*, and because mutations in all four Dpn-binding sites ($1^{-}2^{-}3^{-}4^{-}$) eliminated nearly all Hairy-En mediated repression (Fig. 3B).

As a third test of the functions of the predicted Dpn-binding sites, we asked whether mutations affecting individual or multiple sites increased expression from *SxlPe-lacZ* transgenes, as would be expected if the sites normally mediate repression by Dpn or other HES proteins. We focused on male embryos because they do not express

detectable cytoplasmic *lacZ* mRNA from wild-type *SxlPe-lacZ* transgenes (Estes et al., 1995; Bosch et al., 2006; Avila and Erickson, 2007). We found that mutations affecting Dpn-binding sites 1, 2, or 3, led to ectopic *SxlPe-lacZ* expression in male embryos (Fig. 3C), confirming that these three sites mediate repressor-binding at *SxlPe*. A Dpn-binding site 4 mutation, in contrast, did not cause ectopic *SxlPe-lacZ* expression in males (Table 1), suggesting that the weakest in vitro Dpn-binding sites may not mediate repression in vivo. In the following sections we explore the function of Dpn-binding sites 1, 2, and 3 in relation to the actions of Dpn and other HES proteins, as well as those of the corepressor, Gro, in the sex-specific regulation of *SxlPe*.

**The corepressor Gro is a potent negative regulator of SxlPe**

Maternally supplied Gro interacts with several different types of DNA-binding proteins, including Hairy and Dpn, to repress transcription in the early embryo (Jimenez et al., 1997; Fisher and Caudy, 1998a; Chen and Courey, 2000; Buscarlet and Stifani, 2007). Paroush et al., (1994) identified *gro* as a negative regulator of *Sxl*, reporting that loss of maternal *gro* function caused strong ectopic activation of *Sxl* in males that rendered male and female embryos indistinguishable with respect to *Sxl* protein levels.

Because equality of *Sxl* expression between the sexes would have important implicationsfor the mechanism of X chromosome counting, as well as for maintenance expression of this X-linked regulator of dosage compensation, we examined the effects of maternal *gro* on both *SxlPe* activity and on *Sxl* protein levels. Staining with anti-Sxl antibody confirmed that XY embryos derived from mothers with $gro^{E48}$ germline clones

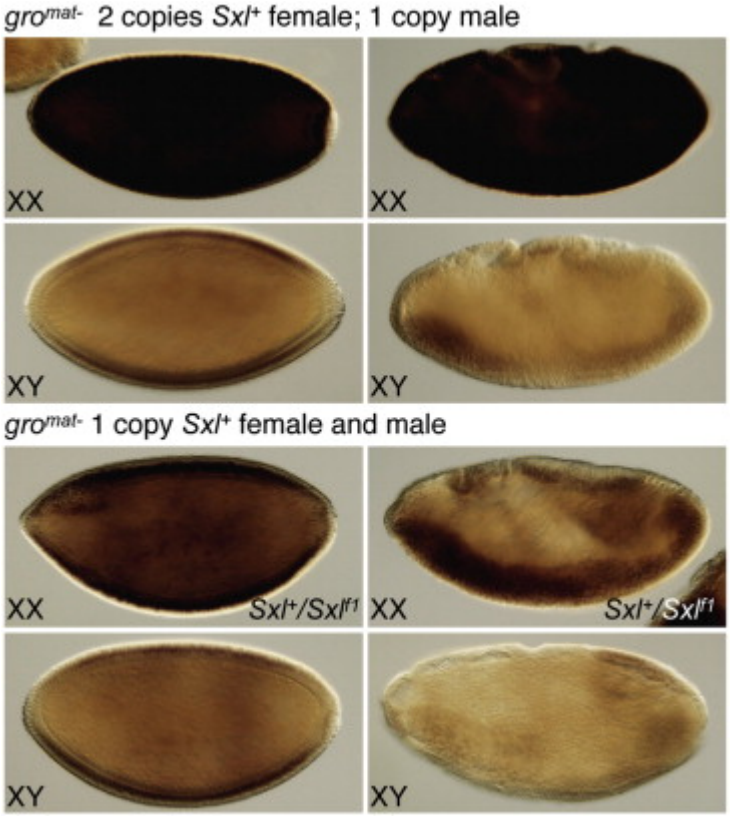



Fig. 4. Sxl protein in *gro*$^{mat-}$ embryos. Embryos from mothers bearing *gro*$^{E48}$ germline clones were immunostained stained for Sxl. Embryonic stages are mid-cellularization (left) and gastrulation (right). (Top panels) XX and XY embryos bearing normal doses of the X-linked *Sxl* gene. (Bottom panels) XX and XY embryos each with one functional copy of *Sxl*$^{+}$ were the progeny of females with *FRT82B gro*$^{E48}$ germ cells and *y w cm Sxl*$^{f1}$ *ct*/Y males.

(hereafter *gro*$^{mat-}$), express *Sxl* protein in most or all cells, but also revealed, contrary to the initial report, that Sxl levels were higher in XX than in XY embryos at all stages (Fig. 4). The observed sex differences in Sxl staining could not be accounted for by gene copy number as *Sxl*$^{f1}$*/Sxl*$^{+}$ females carrying only one functional *Sxl* allele still stained more darkly than their *Sxl*$^{+}$*/Y* brothers (Fig. 4). We found similar effects on *SxlPe*-derived mRNA, with females always staining more intensely than males (Fig. 5),

demonstrating that *Sxl* retains some ability to differentiate between male or female XSE gene doses in the absence of maternal *gro*.

**_Gro_ has a stronger and earlier effect on _SxlPe_ than does _dpn_**

Loss of maternal *gro* raises ectopic male *Sxl* protein levels well above those present in *dpn* mutants (Younger-Shepherd et al., 1992; Paroush et al., 1994; Barbash and Cline, 1995; Fig. 4 and unpublished data). To understand the differential effects of *gro* and *dpn* on *Sxl* transcription, we asked how elimination of maternal *gro* and zygotic *dpn* functions altered the timing of *SxlPe* activation and the levels of mRNA using in situ hybridization to measure nascent and mature *Sxl* transcripts (Fig. 5).

In wild-type XX embryos, *SxlPe* is expressed from nuclear cycle 12 through the first minutes of cycle 14 (Barbash and Cline, 1995; Avila and Erickson, 2007; Erickson and Quintero, 2007). In normal XY embryos the promoter remains silent. We observed that the $\Delta dpn^2$ deletion had no detectable effect on *SxlPe* activity in XX embryos, but that the *dpn* deletion caused sporadic and weak ectopic *Sxl* expression in XY embryos beginning in cycle 13 (Fig. 5; Barbash and Cline, 1995). During early cycle 14, *SxlPe* became active in more XY nuclei, but this caused only a modest and non-uniform accumulation of *SxlPe*-derived mRNA, consistent with the low-level accumulation of ectopic SXL (Barbash and Cline, 1995).

In contrast, loss of maternal *gro* function caused earlier, stronger, and more uniform effects on *SxlPe* than did deletion of *dpn* (Fig. 5, Table 1). We observed ectopic

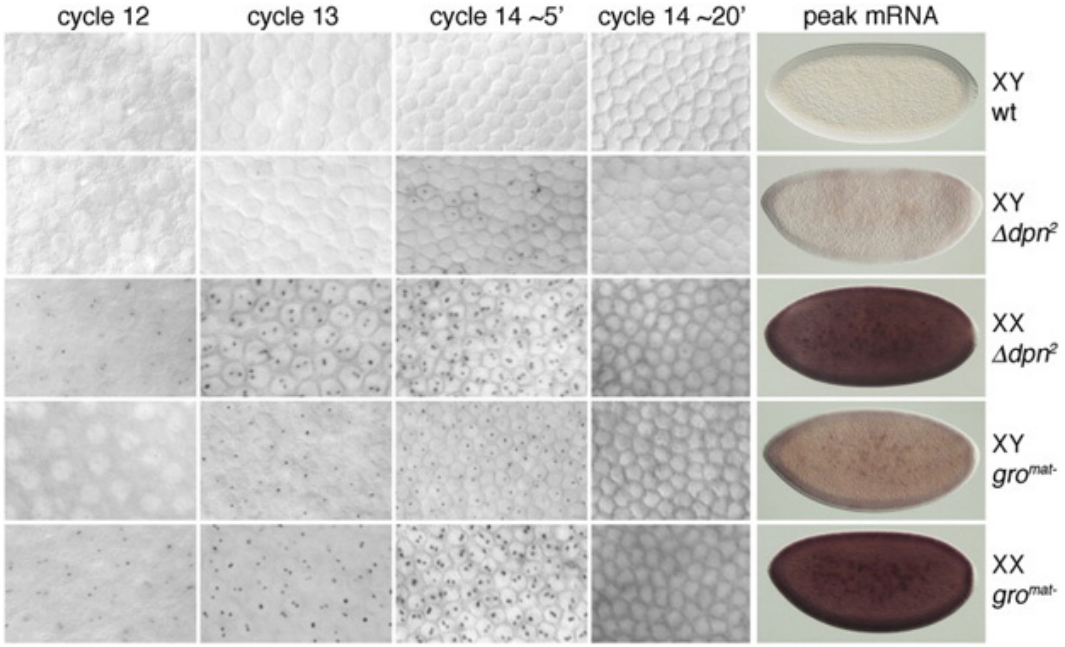



Fig. 5. Time course of *SxlPe* activation in wild-type, Δ*dpn*$^{2}$, and maternal *gro*$^{E48}$ mutant embryos. Wild-type and mutant embryos were stained following in situ hybridization. Black and white panels show surface views of embryonic nuclei at indicated nuclear cycles. Dots represent nascent transcripts from the X-linked *Sxl* locus. Cycle 12 nuclei were illuminated with UV and visible light to enhance DAPI-stained nuclei. Color panels show peak accumulation of *SxlPe*-derived mRNA in early cycle 14. Embryos were progeny of wild-type (wt, *w*$^{1118}$) females and males, *w*$^{1118}$; Δ*dpn*$^{2}$/CyO females and males, or females with *FRT82B gro*$^{E48}$ germ lines and *w*$^{1118}$/Y males. Cycle 12 embryos from Δ*dpn*$^{2}$/crosses could not be distinguished from Δ*dpn*$^{2}$ heterozygotes or wildtype of the same sex. Time courses are representative of repeated stainings of embryos from four separate inductions of *gro*$^{E48}$germline clones and five series of embryo collections from crosses between Δ*dpn*$^{2}$ heterozygotes.

*Sxl* expression in many nuclei in cycle 11 XX embryos and in occasional nuclei in cycle 12 XY embryos. Every XX *gro*$^{mat-}$ nucleus expressed *SxlPe* throughout cycle 12, and every XY nucleus expressed *SxlPe* by the end of cycle 13. As a consequence, *Sxl* mRNA was present at relatively low, but uniform, levels in XY embryos and at slightly elevated levels in XX females. Nacent *SxlPe* transcripts were detected until

about 15 min into cycle 14 in both sexes suggesting that maternal *gro* does not significantly affect the timing of the shut-off of *SxlPe*.

The finding that maternal *gro* has stronger and earlier effects on *SxlPe* than does *dpn* could be explained in several ways: by the involvement of additional HES-related proteins, by the involvement of yet other types of Gro-interacting proteins, or by indirect effects of the pleiotropic *gro* gene in the germline or early zygote. One way to distinguish between these possibilities is to ask what effects mutations in the Dpn-binding sites have on *SxlPe* activity. If Dpn is the only HES-type repressor to regulate *SxlPe*, or if Gro acts indirectly, then the effects of mutations in the Dpn-binding sites should equal those of *dpn* null alleles. On the other hand, if additional HES proteins repress *SxlPe*, the cis-acting changes should exert a stronger effect than *dpn* mutations because they would block the actions of all repressors utilizing those DNA-binding sites.

**Dpn-binding site mutations affect SxlPe more than the loss of Dpn protein**

Comparison of the male embryos carrying Dpn-site mutant *SxlPe-lacZ* reporters shown in Fig. 3C with the ectopic expression of endogenous *Sxl* in the $\Delta dpn^2$ male in Fig. 5 immediately suggests that the cis-acting binding site mutations have stronger effects on *SxlPe* than does loss of *dpn*. However, this simple comparison is potentially misleading because wild-type 1.4 kb *SxlPe-lacZ* transgenes do not precisely mimic the normal promoter. Specifically, wild-type *SxlPe-lacZ* transgenes exhibit low-level activation in XY embryos and are expressed earlier in XX embryos than is endogenous *SxlPe* (Bosch et al., 2006) Table 1). To determine if the relatively strong *lacZ* expression from the Dpn-binding site mutant transgenes implicated other bHLH

repressors in *Sxl* regulation, or if it was instead caused by the loss of Dpn-binding to already derepressed transgenes, we compared the effects of the *Δdpn*$^{2}$ mutation on *SxlPe-lacZ* expression with those of the Dpn-binding site mutations. We found, that while *Δdpn*$^{2}$ elevated *SxlPe-lacZ* reporter expression more than the endogenous *Sxl* locus, the effects of most Dpn-binding site mutations were stronger still. The 1$^{-}$, 2$^{-}$, 1$^{-}$2,$^{-}$ and 1$^{-}$2$^{-}$3$^{-}$4$^{-}$ Dpn-site mutations caused *SxlPe-lacZ* to be expressed in more nuclei at earlier times and at higher overall levels than did *Δdpn*$^{2}$ (Table 1). Transgenes carrying Dpn-binding site 3$^{-}$ and 3$^{-}$4$^{-}$ mutations expressed ectopic *lacZ* at levels and times similar to the wild-type *SxlPe-lacZ* reporter in *Δdpn*$^{2}$ mutants. This could indicate that Dpn binds only to site 3, but we favor the simpler idea that this non-canonical sequence is less effective at mediating repression than are sites 1 and 2.

**A search for other bHLH repressors of SxlPe**

Our findings that mutations in the cis-acting Dpn-binding sites led to earlier and higher levels of *SxlPe-lacZ* expression than did loss of *dpn* protein, suggests that other bHLH proteins bind these sequences to repress *SxlPe*. We used a genetic approach to identify the missing proteins by examining mutants with defects in known, or predicted, bHLH repressors for alterations in *SxlPe* expression (Moore et al., 2000; Ledent and Vervoort, 2001).

We began with *E(spl)m3*, a maternally supplied HES-family repressor previously cited as a negative regulator of *SxlPe* (Dawson et al., 1995; Poortinga et al., 1998). We found that embryos derived from mothers whose germlines lacked *E(spl)m3,* expressed *SxlPe* in a completely wild-type pattern (data not shown). There was no ectopic

activation of *SxlPe* in XY embryos, and XX embryos expressed *SxlPe* at normal levels with normal timing. Homozygous mutant embryos were also wild-type for *Sxl* expression indicating that any zygotically expressed *E(spl)m3* was without effect on *SxlPe*. The deletion allele we used, *Df(3R)E(spl)*$^{P11}$, also removes the *E(spl)*, *E(spl)mγ*, *E(spl)mβ*, *E(spl)m5*, *E(spl)m7*, and *E(spl)m8* loci (Nagel et al., 2004), eliminating seven HES proteins as maternal or zygotic regulators of *SxlPe*. The protein most similar to Dpn is Side (CG10446) (Moore et al., 2000). We examined several *Side* deletion mutants for dominant maternal and recessive zygotic effects on *SxlPe*, but found none, consistent with reports that *Side* is not expressed maternally, or in the early embryo (Tomancak et al., 2002; Chintapalli et al., 2007; but see Moore et al., 2000). We did not analyze *Side* for recessive maternal effects because we expected the relatively large *Side* deletions to be cell lethal in germline clones.

**Maternal *Hey* negatively regulates *SxlPe***

The *Hey* gene encodes a protein related to Dpn, Hairy, and E(spl), but which lacks the characteristic C-terminal Gro-binding WRPW motif (Kokubo et al., 1999; Leimeister et al., 1999). Instead, Hey and its mammalian homologs posses a YRPW motif that appears not to interact with Gro/TLE proteins (Davis and Turner, 2001; Iso et al., 2001; Fischer and Gessler, 2007; Kageyama et al., 2007). Nonetheless, Hey proteins can potentially interact with Gro as they form heterodimers with several different HES proteins, including Dpn (Iso et al., 2001; Giot et al., 2003; Chintapalli et al., 2007). The resulting Hey/HES heterodimers appear to bind DNA with higher affinity than the individual homodimers (Iso et al., 2001). The single available mutation, *Hey*$^{f06656}$, is a

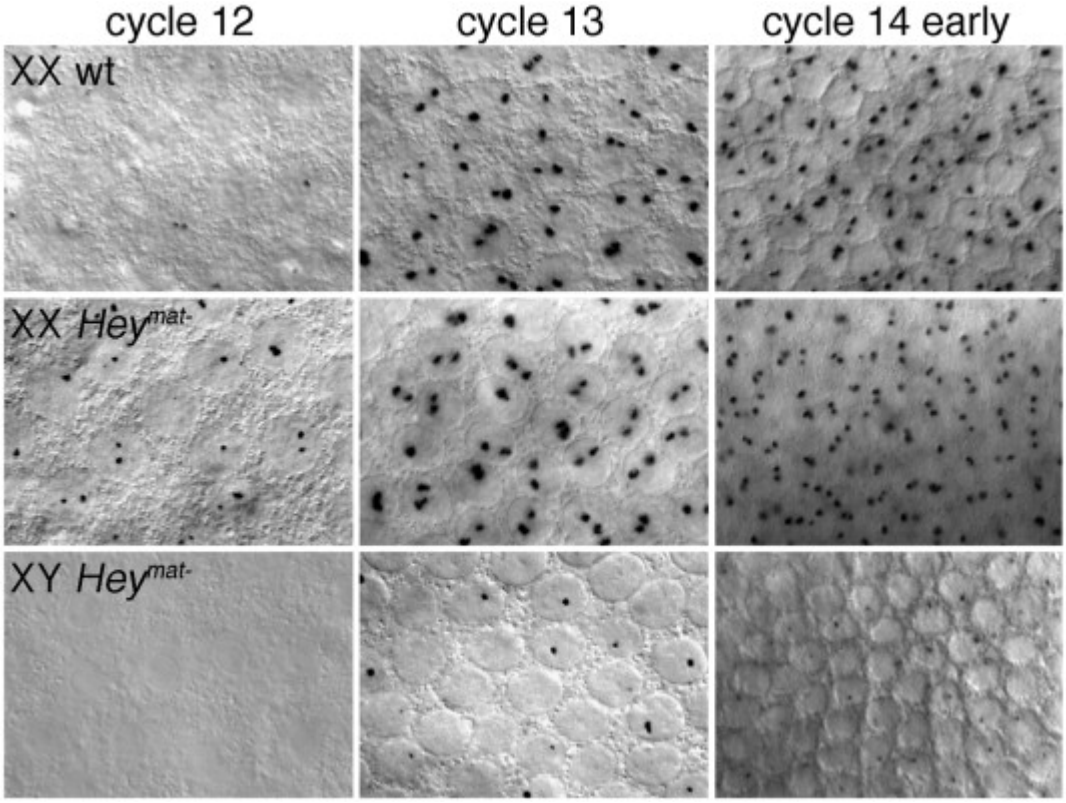



Fig. 6. Maternal *Hey* negatively regulates *SxlPe*. Surface views of embryos at indicated nuclear cycles stained after in situ hybridization to detect nascent transcripts from the X-linked *SxlPe*. Top row: wild-type XX embryos. Middle and bottom rows: XX and XY progeny of mothers carrying $Hey^{f06656}$ germline clones ($Hey^{mat-}$). Wild-type XY embryos do not activate *SxlPe*.

recessive lethal caused by a P(Bac) insertion in the 1st intron. To examine the effects of *Hey* on *SxlPe*, we recombined $Hey^{f06656}$ onto an FRT-containing chromosome and generated $Hey^{f06656}$ germline clones (Chou and Perrimon, 1996). We found that 100% of $Hey^{mat-}$ XY progeny expressed *SxlPe* ectopically during cycles 13 and 14 but that *Sxl* expression was spatially variable, with about half the nuclei in each XY embryo expressing *SxlPe* (Fig. 6). There was no observable accumulation of *Sxl* mRNA in XY embryos, consistent with the lack of a dominant maternal effect on male viability. *SxlPe*

activity also appeared to be affected in XX *Hey*$^{mat-}$ progeny as we noticed an increase in the proportion of cycle 12 XX embryos that expressed *SxlPe*, and an increase in the proportion of active nuclei in the expressing embryos (Fig. 6, see Materials and Methods).

The identification of Hey as a maternally-supplied bHLH repressor of *SxlPe*, fulfills an important prediction of our experiments: that bHLH repressors in addition to Dpn regulate the on-or-off control of *SxlPe*. The involvement of maternal *Hey* and *gro* are also in keeping with the hypothesis that maternal repressors are integrated parts of the mechanism by which XSE concentrations, rather than X:A ratios, are sensed in the embryo (Cline, 1993; Erickson and Cline, 1993; Barbash and Cline, 1995, Wrischnik et al., 2003; Erickson and Quintero, 2007). Whether the relatively weak effects of *Hey*$^{f06656}$ are explained by partial *Hey* protein function, or whether yet other HES family repressors regulate *SxlPe* remains to be determined.

# 4. *CLOCKWORK ORANGE* AND MYC ARE ADDITIONAL REPRESSORS OF *SXL*

## *Clockwork orange* is a transcription factor that represses expression of *SxlPe*

*Clockwork orange* (CG 17100) is a bHLH transcription factor most closely related to the *Gridlock and E(spl)-hairy* families. In flies, these families include *Hey, cwo, hairy, deadpan, E(spl)* complex*, Side* and *CG5927 (*Ledent et al., 2001). *Clockwork orange (cwo)* lacks the tetrapeptide WRPW present in most of these proteins and is responsible for interaction with corepressor *Gro*.

Despite the absence of WRPW Gro - interacting domains, *cwo* is thought to be a transcriptional repressor (Matsumoto et al., 2007). It recognizes "type B" E box CACGTG (Matsumotoet al., 2007), two of which are present at 3220 bp downstream from ATG site of *SxlPe* promoter. It is possible that *cwo* regulates *Sxl* not from these but other noncanonical E-boxes including Dpn binding sites.

## Expression of *SxlPe* in *cwo* mutants begins 2 cycles earlier than in wild-type

To determine if *cwo* regulates *Sxl* expression, we examined nascent RNA transcripts from *SxlPe* in the homozygous viable $cwo^{f05073}$ mutant. $cwo^{f05073}$ is a *PBac{WH}* insertion into the first intron of *cwo* gene.

Expression of *SxlPe* in $cwo^{f05073}$ mutant began 2 cycles earlier than in wild-type (Table 2). In wild-type, embryo expression begins in cycle 12. This data implies that *cwo* acts as a transcriptional repressor of *Sxl* and, in the *cwo* mutant, we observed derepression of the *Sxl* promoter. It is not possible to determine from our data if it is the maternal or

zygotic contribution of *cwo* that negatively regulates *Sxl*. Published data do not include *cwo* among the earliest zygotic genes, other data against its early zygotic expression is

Table 2. Expression of *SxlPe* in *cwo*$^{f05073}$ mutant. Nuclei from each embryo were scored as expressed or not expressed *Sxl* (columns "expression"): the first number is the number of nuclei that expressed *Sxl*; the second number is the overall number of counted nuclei. Nuclei that expressed in one dot (from one X chromosome) and two dots (from two X chromosomes) were counted. Columns "expression in one dot": the first number is the number of nuclei that expressed *Sxl* in one dot; the second number is the overall number of nuclei that expressed *Sxl*. The next column is a graphic representation of column "expression".

| **Cycle** | **Expression** | **Expression in one dot** | **Fraction of nuclei that express *Sxl* in each embtyo** |
|---|---|---|---|
| Cycle 9 | (All embryos are at mitosis) | | |
| | | | |
| Cycle 10 | 11/30 | 10/11 | |
| | 0/30 | 0 | |
| Cycle 11 | 25/41 | 8/25 | |
| | 15/35 | 15/15 | |
| | 15/42 | 15/15 | |
| | 3/33 | 3/3 | |
| Cycle 12 | 19/20 | 10/19 | |
| | 21/21 | 6/21 | |
| | 27/27 | 12/27 | |
| | 27/27 | 2/27 | |
| | 21/50 | 2/21 | |
| | 13/24 | 13/13 | |
| | 15/25 | 15/15 | |
| | 9/27 | 9/9 | |
| | 7/41 | 7/7 | |
| | 0/37 | 0 | |
| | 0/20 | 0 | |
| | 1/37 | 1/1 | |
| | 0/40 | 0 | |

the absence of TAGteam motif in its promoter region (Erickson and Cline, 1998). So, most probably, it acts as maternal gene.

Ectopic expression in males and earlier expression at a higher level in female embryos at cycle 11 support our hypothesis about repressor function of *cwo* during the earliest stages of development.

Expression at cycle 12 also supports our hypothesis about the repressive function of *cwo* on *SxlPe*. I observed embryos that expressed *Sxl* from only one X chromosome. Nevertheless, it is not enough data to conclude entopic expression in male embryos at cycle 12. Embryos that are expressed in one dot can be as male as female embryos that expressed only from one out of two X chromosomes. Most likely c*wo* is more important as a *Sxl* repressor at the earlier stages of development. At cycle 12, other repressor(s) can play a more significant role at *Sxl* repression. *Sxl* stayed repressed at cycle 12 in presumably male embryos (#10-#13, Table 2) even without (or at a significantly reduced level of) Cwo protein in the homozygous mutant.

Complex mechanism allows maintaining repressed status of *Sxl* in wild-type males and activation in females during 40 min at cycle 12 – early 14. At the beginning of each cycle (telophase phase of mitosis), expression was repressed in all nuclei in female as well as in male embryos (data not shown). Then expression reactivated in females but stayed repressed in males. A very similar mode of telophase repression can be observed at cycles 12, 13 and 14. Although repression at early cycle 14 is not absolutely completed or very short, *cwo* repression may prevent activation in early male embryos in the beginning of each cycle. Later other repressors may play this role.

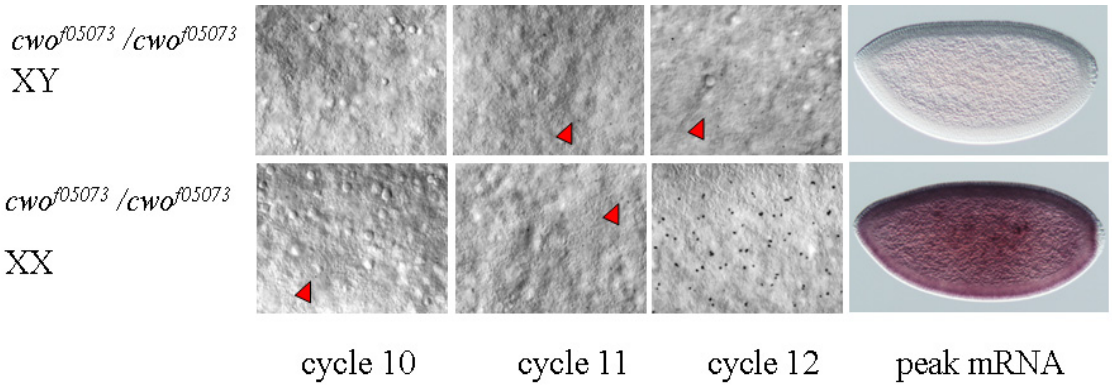



Fig. 7. Cwo represses *SxlPe* in early embryos. *SxlPe* is expressed approximately 20 min or 2 cycles earlier in homozygous *cwo*$^{f05073}$ mutants.
Mutant *cwo*$^{f05073}$ embryos were stained following in situ hybridization. Black and white panels show surface views of embryonic nuclei at indicated nuclei cycles. Dots represent nascent transcripts from the X-linked *Sxl* locus. Color panels show peak accumulation of *SxlPe*-derived mRNA in early cycle 14. Embryos are homozygous *cwo*$^{f05073}$ mutants

**There is no difference in the level of mRNA cytoplasmic accumulation of *SxlPe* in *cwo* mutants**

Expression at cycle 13 and cycle 14 in *cwo* mutant was indistinguishable from wild-type expression. There was also no difference in the level of mRNA cytoplasmic accumulation of *Sxl* (Fig. 7). This observation supports our hypothesis about *cwo* repressing specifically at the earliest stages of development.

Expression of Sxl protein was not very different from wild-type embryos. Half embryos (females) expressed Sxl. Fifteen of the 486 embryos had abnormal patterns of Sxl expression. Most of them (10 out of 15) looked like morphologically normal embryos in the germband elongation stage with either very low levels of Sxl

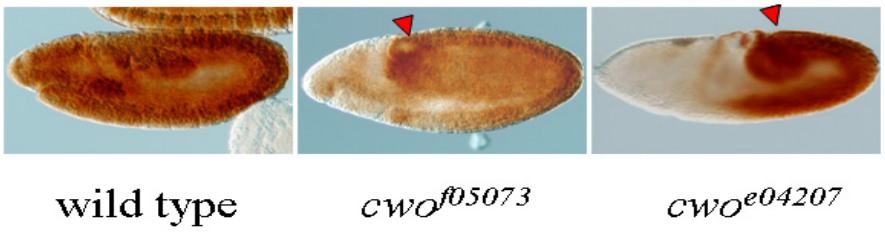



Fig. 8. Sxl level are normal: *cwo* mutant, but occasional embryos display abnormal staining patterns. Two different homozygous mutant *cwo*$^{f05073}$ and *cwo*$^{e04207}$ express Sxl mostly in the central and posterior part of the embryos in germline elongation stage. Arrows point expression in more posterior half of the embryos.

expression or expression localized only in the central and posterior part of the embryo (Fig. 8). All the other female embryos expressed Sxl at normal levels. Another homozygous viable *cwo* mutant *cwo*$^{e04207}$ had a similar pattern of Sxl expression. In this mutant, 6 out of 118 counted female embryos had abnormal patterns of Sxl expression (Fig. 8) but all but one of these 6 embryos were also morphologically abnormal. The male/female ratio of the adult flies is close to 1:1 (58 females to 73 males from 131 counted adult flies). So *cwo* can be one of the regulators of *Sxl* expression only on the early stages of development.

**Myc is a maternal repressor of *Sxl***

Myc is a bHLH-leucine zipper activator transcription factor that binds "class B" E-box sequences CA(C/T)GTG (Fisher and Caudy,1998b; Dang et al., 1992). It also can bind non-canonical CA(C/T)GCG, CACGAG, CACGTTG sites (Yang et al.,2001; Blackwell et al., 1993). Myc DNA binding requires the cofactor Max. Max can also bind to the related Mnt protein to form a repressor complex. Myc regulates cell growth

by controling ribosome biogenesis and controls the progression through the G1 phase of the cell cycle (Grandori et al., 2000, De la Cova and Johnston, 2006). In *Drosophila*, Myc is supplied maternally to the embryo (Gallant et al., 1996).

The *SxlPe* promoter contains 2 non-canonical sequences CA(C/T)GCG for Myc protein that also function as Dpn binding sites (Lu et al., 2008).

To determine if Myc regulates *Sxl* expression, we examined nascent transcripts from *SxlPe* in the progeny of mothers heterozygous for the weak Myc $dm^{1}$, $dm^{P0}$ mutants, and strong $dm^{P1}$ mutant. It is not possible to induce homozygous dm germline clones because Myc is essential for oogenesis. The *dm1* allele is a gypsy insertion into the first intron, 418 nucleotides upstream of the translation initiation site. $dm^{P0}$ is caused by the insertion $P\{P\text{-}Sal\}dm^{P0}$, which is less than 100 bp upstream of putative transcription start site; $dm^{P1}$ is caused by insertion in the same place $P\{P\text{-}Sal\text{-}\ P1\}dm^{P1}$, which is internal deletion of *P{P-Sal}* element. Mutation $dm^{P1}$ is considered to be the strongest mutation.

**Expression of *SxlPe* in *Myc* mutants begins 2 cycles earlier than in wild-type**

I investigated expression of *SxlPe* in *Myc* mutant embryos that were progeny from the cross $dm^{P1}$/FM7 x $dm^{P1}$/Y. Female embryos from this cross have genotype either $dm^{P1}/dm^{P1}$ or $dm^{P1}$/FM7 and male embryos have genotype $dm^{P1}$/Y or FM7/Y. All embryos have heterozygous $dm^{P1}$/FM7 mothers.

I did not observe any entopic *SxlPe* expression at cycle 8 (approximately 80 min after fertilization) and only very weak (possibly background) level of expression in one embryo at cycle 9 (Table3). At cycle 10 I observed ectopic expression of *SxlPe* in most

or all embryos. Early expression implies that Myc acts as a repressor for *SxlPe* at this time of development. But there are two possible modes of action: it can be direct repression by occupation of Dpn-binding site or it can act indirectly. We favor the indirect mode because Myc usually acts as transcriptional activator presumably by activation of a repressor(s). It may be sequence - specific transcription factor repressor of *SxlPe* or repressor that keeps almost all *Drosophila* genes silenced at early stages of embryo development. It is known that during oocyte and sperm formation chromosomes become tightly condensed and transcriptionally silenced. After fertilization zygotes keep transcriptionally silenced status for most of genes up to cycle 14. There are some genes that express earlier then cycle 14. They included *SxlPe* and genes for activation transcription factors for *SxlPe*. If maternal Myc is responsible for activation of repressor(s) (that keep all genes silenced at early cycle of development) then mutation of *Myc* would lead to ectopic activation of zygotic *SxlPe* in all embryos, as mutant for *Myc* as wild-type for *Myc* zygotic gene.

I observed activation in all but one embryo at cycle 10. That one embryo expressed *SxlPe* in only one out of 24 counted nuclei and expressed only in one dot. This level of expression I estimated as background negative level. Three embryos expressed *SxlPe* in approximately 10% of the nuclei, all in one dot. They are presumably male embryos. They have *Myc*/FM7 heterozygous mothers and *Myc*/Y or FM7/Y zygotic genotype, where the only X chromosome that expressed *SxlPe* comes from mother. If our assumption about the function of Myc as indirect maternal activator of repressor is correct, then derepression in maternal $dm^{P1}$/FM7 progeny leads to ectopic

Table 3. Time course of *SxlPe* activation in $dm^{P1}$ mutant embryos (cycles 9-11). Nuclei from each embryo were scored as expressed or not expressed *Sxl* (columns "expression"): the first number is the number of nuclei that expressed *Sxl*; the second number is the overall number of counted nuclei. Nuclei that express in one dot and two dots were counted (columns "expression in one dot"): the first number is the number of nuclei that expressed *Sxl* in one dot; the second number is the overall number of nuclei that expressed *Sxl*. The next column is graphic representation of column "expression".

| **Cycle** | **Expression** | **Expression in one dot** | **Fraction of nuclei that express *Sxl* in each embryo** |
|---|---|---|---|
| Cycle 8 | 0/20 | | |
| | 0/14 | | |
| Cycle 9 | 0/16 | | |
| | 0/23 | | |
| | 0/14 | | |
| | 3/34 | 3/3 | |
| Cycle10 | 1/24 | 1/1 | |
| | 6/32 | 6/6 | |
| | 7/34 | 7/7 | |
| | 3/20 | 3/3 | |
| | 10/33 | 9/10 | |
| | 25/37 | 24/25 | |
| Cycle 11 | 0/24 | | |
| | 0/24 | | |
| | 0/22 | | |
| | 0/25 | | |
| | 0/20 | | |
| | 3/40 | 3/3 | |
| | 10/40 | 10/10 | |
| | 21/43 | 21/21 | |
| | 15/52 | 15/15 | |
| | 17/42 | 16/17 | |
| | 14/44 | 13/14 | |
| | 19/53 | 16/19 | |
| | 34/38 | 22/34 | |
| | 29/49 | 25/29 | |

activation in all maternally inherited X-chromosomes. Our data fitted well into that hypothesis.

Other two (presumably female) embryos expressed *SxlPe* in about 30% of the nuclei, but only some nuclei expressed in two dots that corresponded to two X chromosomes in females.

Female embryos have $dm^{P1}$/FM7 heterozygous mothers and $dm^{P1}$/ $dm^{P1}$ (maternal), $dm^{P1}$ (maternal)/FM7, $dm^{P1}$/FM7 (maternal) genotype, where "maternal" corresponds to maternally inherited X-chromosome. Only one of X – chromosomes expressed *SxlPe* at that cycle of early *Drosophila* development. If our assumption of indirect repression function of Myc is correct, then only one of X-chromosomes would be derepressed in progeny embryos from cross $dm^{P1}$/FM7 x $dm^{P1}$/Y. As mentioned above, the only X chromosome in males, that are maternally inherited, derepressed in males. So it is reasonable to assume that the one of two female X-chromosome that expresses *SxlPe* is also maternally inherited. In order to prove directly which of X-chromosome is Myc regulated (maternally inherited, paternally inherited or both), we plan a set of experiments in the future.

There is a second explanation of observed data. We hypothesize that zygotic Myc directly represses *Sxl* or activates zygotic repressor(s) of *SxlPe*. If it is correct, then in $dm^{P1}$/FM7 or $dm^{P1}$/ $dm^{P1}$ female embryos we expect derepression of *SxlPe* expression. In this case we would expect derepression in both X-chromosomes but our data showed expression in only one of two X chromosomes in females. So our data do not support importance of function of synchronically expressed Myc at this cycle of development.

At this cycle female embryos are expressed at higher level than male embryos, most probably because of 2 times difference in amount of activator proteins such as scute and sisA located on X-chromosome.

**Expression at cycle 11.** In wild-type embryos expression of *SxlPe* shuts down each cycle at telophase and very early interphase, just after mitosis. But in wild-type

embryos only small portion of embryos stayed at telophase and early interphase and do not express *Sxl*. Reestablishment of *SxlPe* expression at cycle 11 in $dm^{P1}$ embryos reveal different mode of expression. Approximately half of the embryos did not expressed *SxlPe* and half expressed in 10 – 90% of the nuclei.

Embryos that did not express *SxlPe* were all at very early interphase stage. I have observed unproportionally large number of embryos at very early interphase stage in $dm^{P1}$ mutants. It is very unlikely that it is just random fluctuation of sample collection. More direct explanation is based on function of Myc protein. It is known that Myc plays important role in progression through G1 phase of cell cycle. Without proper amount of Myc protein G1 progression is delayed. Most probably that is why we have observed unproportionally large number of nuclei at very early interphase stage, when *SxlPe* (as I mentioned earlier) is not expressed. Why did we observe this phenomena starting at cycle 11 but not earlier? Does maternal/zygotic transition of Myc protein play role in this phenomenon? The *Drosophila* cell cycle is regulated differently in preblastoderm and blastoderm embryos. Is Myc regulation of cell cycle also stage-dependent? We do not have answers to those questions yet.

Embryos that did express *SxlPe* are at interphase and prophase stage of cell cycle. Most of the embryos at this cycle still expressed only from one of X-chromosomes.

**Expression in cycle 12.** At cycle 12 I still could see ectopic expression in male (Fig. 9) embryos and only one embryo does not express *SxlPe* at all (Table 4 ). Some

female embryos expressed mostly in one dot. So early in interphase one of X chromosome (presumably maternally inherited) are more derepressed up to cycle 12.

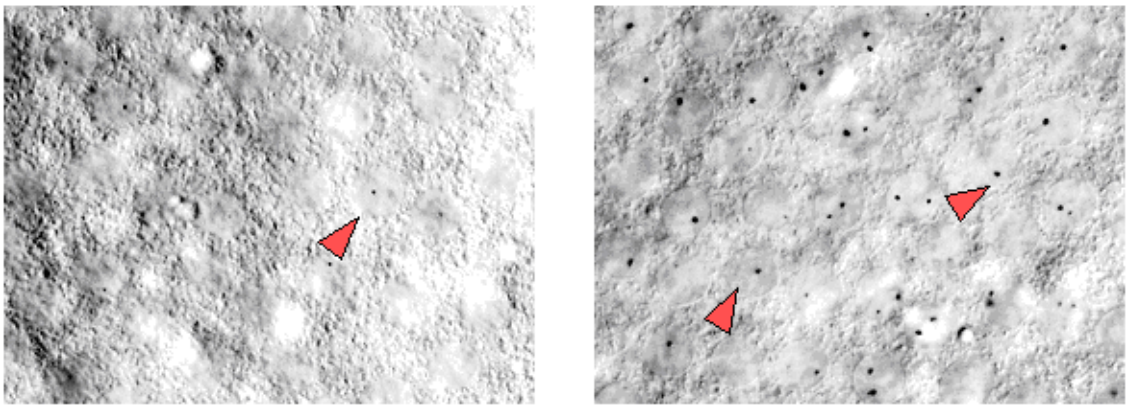
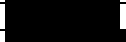
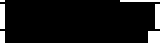
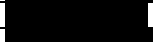
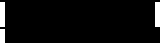
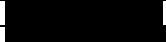
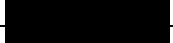
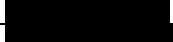
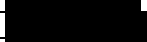
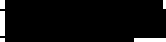
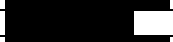
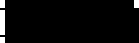
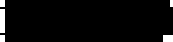
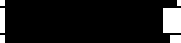


Fig 9. Ectopic expression of *SxlPe* at cycle12 in *Myc* mutant male embryos. Many female *Myc* mutant embryos expressed *SxlPe* from only one X chromosome.

Expression in later interphase and prophase of *Myc* embryos was higher than in wild-type (WT) embryos. At cycle 12 embryos were able to express Sxl earlier in interphase than in the previous cycle. On the other hand, zygotic Myc may start to accumulate by this time and became responsible for proper progression through G1 stage of cell cycle.

**Expression at cycle13.** At cycle 13 expression was rather similar to expression in wild-type embryos. I observed very low level ectopic expression in males, almost undistinguishable from some background expression in WT embryos.

Table 4. Time course of *SxlPe* activation in $dm^{P1}$ mutant embryos (cycles 12-13). Nuclei from each embryo were scored as expressed or not expressed *Sxl* (columns "expression"): the first number is the number of nuclei that expressed *Sxl*; the second number is the overall number of counted nuclei. Nuclei that express in one dot and two dots were counted (columns "expression in one dot"): the first number is the number of nuclei that expressed *Sxl* in one dot; the second number is the overall number of nuclei that expressed *Sxl*. The next column is graphic representation of column "expression".

| **Cycle** | **Expression** | **Expression in one dot** | **Fraction of nuclei that express *Sxl* in each embryo** |
|---|---|---|---|
| Cycle 12 | 0/30 | | |
| | 3/30 | 3/3 |  |
| | 6/43 | 6/6 |  |
| | 17/50 | 17/17 |  |
| | 18/50 | 18/18 |  |
| | 24/42 | 15/24 |  |
| | 27/42 | 23/27 |  |
| | 25/32 | 15/25 |  |
| | 28/34 | 18/28 |  |
| | 55/57 | 27/55 |  |
| | 51/53 | 12/51 |  |
| | 40/41 | 20/40 |  |
| | 28/29 | 11/28 |  |
| | 47/47 | 5/47 |  |
| Cycle 13 | 8/52 | 8/8 |  |
| | 0/52 | | |
| | 1/52 | 1/1 | |
| | 1/50 | 1/1 | |
| | 8/56 | 8/8 |  |
| | 8/72 | 8/8 | |
| | 2/56 | 2/2 | |
| | 1/52 | 1/1 | |
| | 1/52 | 1/1 | |
| | 3/52 | 3/3 | |
| | 0/52 | | |
| | 15/60 | 15/15 |  |
| | 25/33 | 18/25 |  |
| | 38/39 | 10/38 |  |
| | 37/40 | 12/37 |  |
| | 17/32 | 13/17 |  |
| | 25/28 | 10/25 |  |
| | 56/56 | 4/56 |  |
| | 31/41 | 19/31 |  |
| | 46/54 | 17/46 |  |

In females I observed several embryos at early interphase that expressed *SxlPe* at lower level than WT embryos. Most probably that can be explained by some delay in progression through G1 stage of cell cycle in heterozygous $dm^{P1}$/FM7 or homozygous $dm^{P1}$/ $dm^{P1}$ mutants. But embryos at later interphase and prophase looked absolutely

normal, undistinguishable from WT embryos. So when cell cycle progresses up to mid interphase, *Sxl* accumulation became absolutely normal.

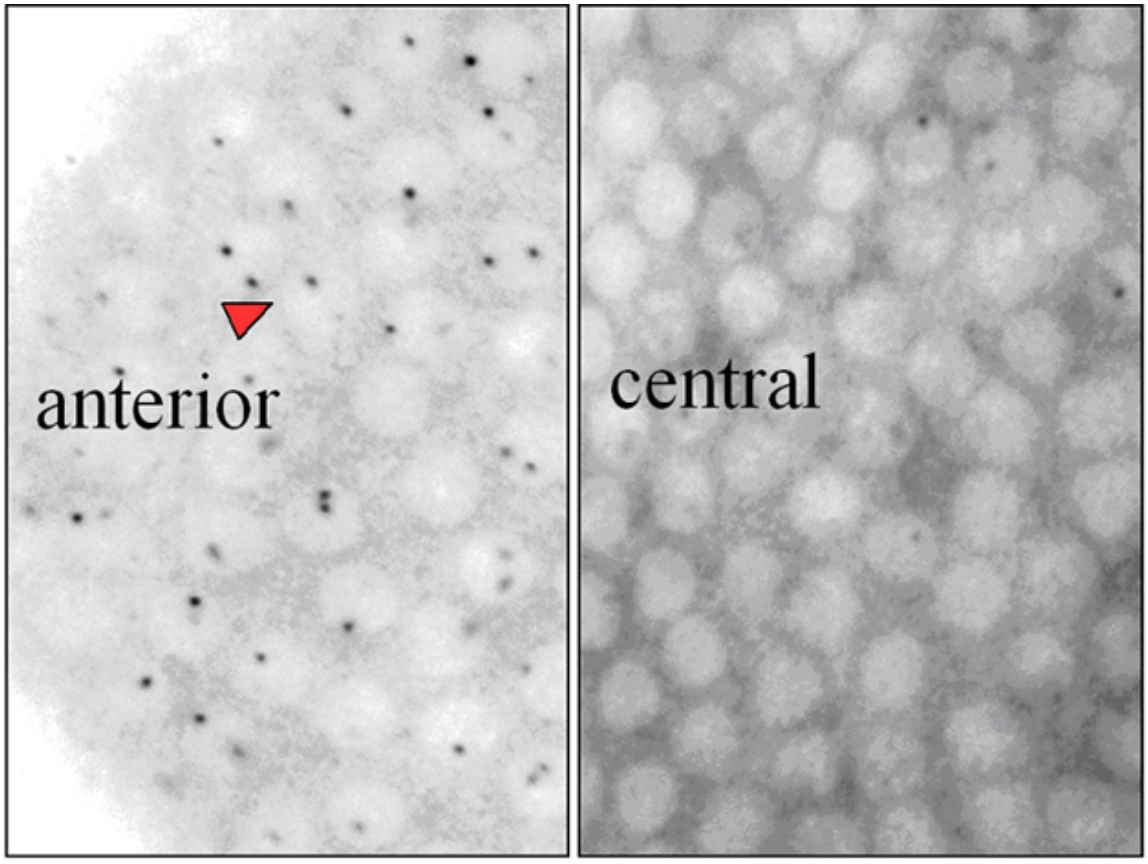



Fig. 10. Very early at cycle 14 $dm^{P1}$ mutant embryos do not express *Sxl* mRNA in the central part of the embryo.

**Expression at cycle 14.** At cycle 14 very rarely I observed ectopic expression in males in 2-3 nuclei per embryo, which is almost identical to WT background expression in males. In females I observed embryos at early interphase stage that did not express *SxlPe* at all or expressed it only in the most anterior part of the embryo (Fig 10). The

same pattern of expression I observed at $dm^{1}$/FM7, which is another Myc mutant. At the same time, I never observed absence of *SxlPe* expression at cycle 14 in wild-type female embryos. Most probably in $dm^{P1}$/ $dm^{P1}$ or $dm^{P1}$/ FM7 embryos I observed delay of progression through G1 stage of cell cycle and more embryos stall at very early interphase stage. In WT embryos G1 stage lasted for a short period of time. Only during this period *SxlPe* expression shuts down and then reestablishes at cycle 14. Later at cycle 14 *Sxl* expression is undistinguishable from wild-type expression.

**Myc is sensitive to mutation in the known regulator of *SxlPe***

In order to answer if Myc is sensitive to mutation in the known regulator of *SxlPe,* I made double mutant *sisA* $dm^{P1}$/FM7, investigated expression *SxlPe* in this mutant and compared it with expression in each of the mutants *sisA*/FM7, $dm^{P1}$/FM7 and wild-type.

*SisA* is an activator of *SxlPe* expression and without this transcription factor expression is absent:

Stock *sisA*/FM7 x *sisA*/Y produce progeny female embryos *sisA/sisA*, *sisA*/FM7 and males *sisA*/Y, FM7/Y. Expression of *SxlPe* in *sisA*/FM7 females was undistinguishable from wild-type and absent *in sisA/sisA* females. Expression in *sisA*/Y and FM7/Y males was absent which is identical to wild-type expression in males. Quarter of all embryos, which is *sisA*/FM7, expressed *SxlPe*. In double mutant *sisA* $dm^{P1}$/FM7 expression reestablished in *sisA* $dm^{P1}$/*sisA* $dm^{P1}$females (Fig 11). Those results confirmed our previous observation of repressor function of Myc protein on *SxlPe*. I observed expression only in the middle part of the embryo.

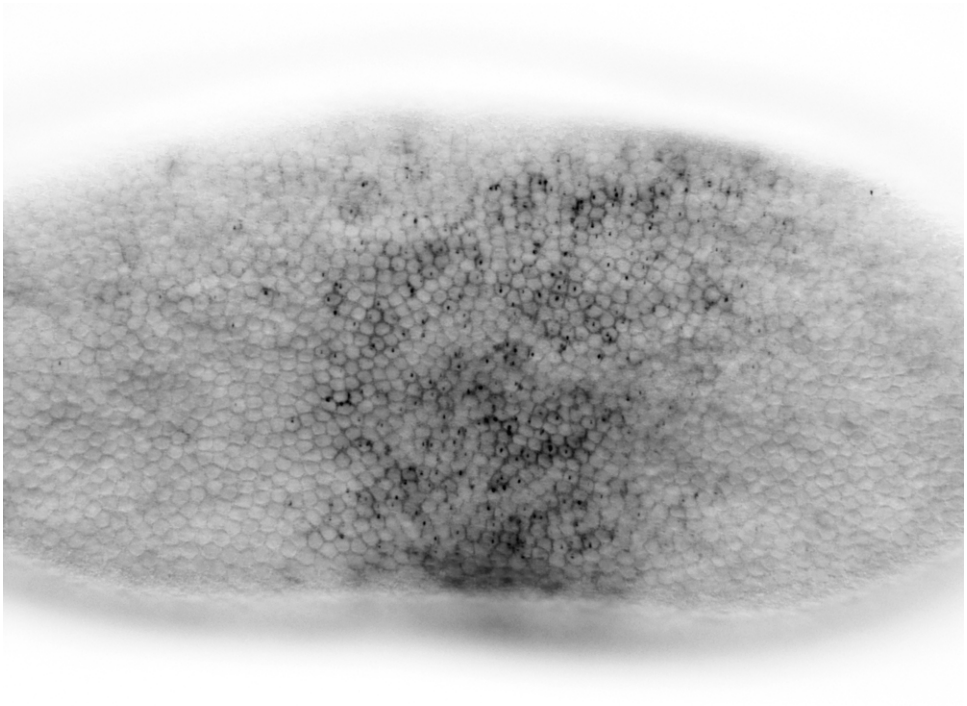


Fig. 11. Myc represses *SxlPe* expression at early cycle 14. Restoration of *SxlPe* expression in homozygous *sisA/sisA* mutants with additional second mutation $dm^{P1}$.

These results suggest that Myc most probably takes part in sex determination in *Drosophila*. It is not a strong repressor but more sensitive approach of double mutant reveals its influence on *SxlPe* expression.

We can conclude that:

1) Myc does influence sex determination. The mode of regulation needs to be further investigated.
2) Myc affects the reestablishment of *SxlPe* activity at the beginning of each interphase, perhaps by affecting the G1 stage of cell cycle progression.

3) Myc may provide a tool for investigating differences in expression from maternally and parentally inherited homologs at early stages of development.

## 5. PREDICTION OF NONCLASSICAL ORGANIZATION OF *SXLPE* AND *SXLPM* AND ITS POSSIBLE ROLE IN REGULATION OF *SXL* EXPRESSION

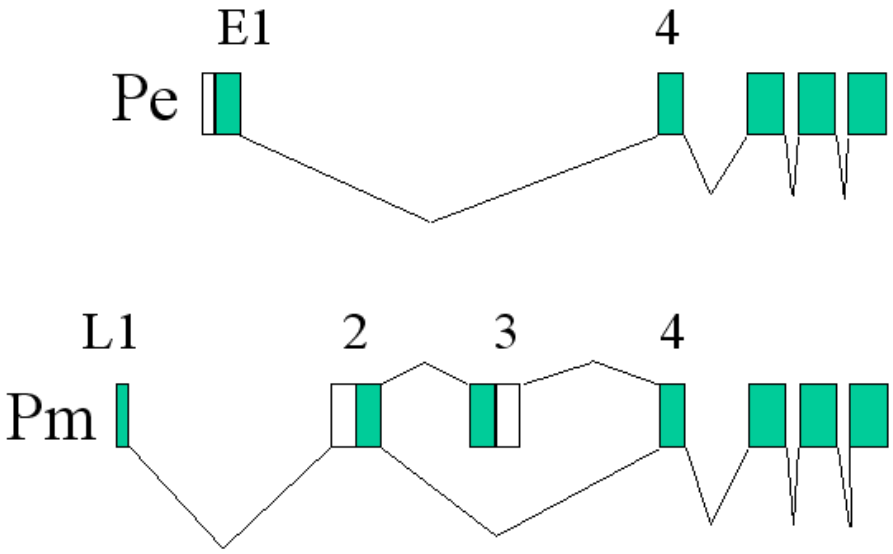



Fig. 12. Early promoter *SxlPe* is located 5 kb downstream from maintenance promoter *SxlPm*.

### Two of *Sxl* promoters are TATAless according to Ohler/Fitzgerald criteria

Two *Sxl* promoters are located 5 kb apart (Fig. 12). I have found that both promoters may be TATAless promoter according to criteria for TATA consequence TATAAA.

*SxlPm* is TATAless Motif1 type of promoter (Fig. 13). It also has two Motif6-like sequences at position –405 and –470 which is relatively far from TSS and less possible to be core promoter motifs. Motif1 TGcaCACTG in *SxlPm* is located at position –4 and almost perfectly fit to Motif1 consensus (C/T)GGTCACTG. Computer program McPromoter confirmed existence of Motif1 in *SxlPm* promoter (data not

shown). Dr. Ohler performed ETS clustering and individual motif search separate from McPromoter results. He confirmed our finding of Motif1 in *SxlPm* promoter and did not find TATA motif in *SxlPm* (non published data).

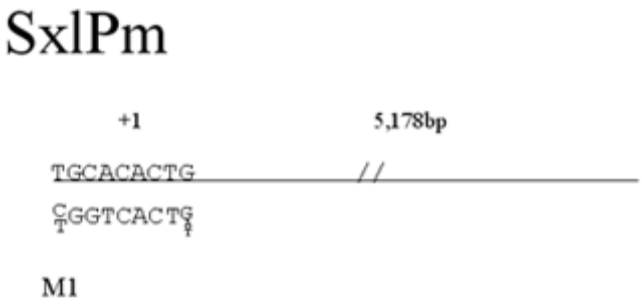



Fig. 13. TATAless promoter *SxlPm* has Motif1 sequence at –4 position.

*SxlPe* is DRE/Inr type of promoter and has -30 sequences TTAAATA that was thought to be a TATA box (Fig. 14). In order to conform or eliminate this possibility we will need to make mutation in those sequences and monitor changes in *SxlPe* expression

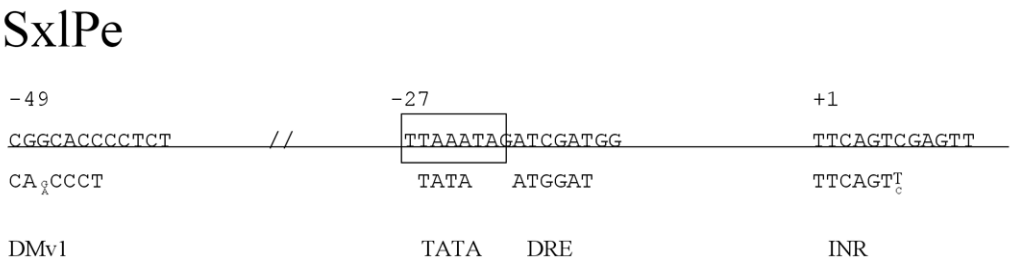



Fig. 14. *SxlPe* is DRE/Inr promoter. Presence of TATA-like sequence in *SxlPe*.

in mutants. We still should consider possibility that Polymerase II uses both TATA and DRE/Inr prompter.

Motif DRE ATcGAT is located at position –22 in *SxlPe* and fits consensus sequence ATGGAT for DRE. Initiator motif TTCAGTCGAGTT is located at –1 position at *SxlPe* promoter and perfectly fits to consensus. Dr. Ohler confirmed our finding of DRE/Inr type of promoter by ETS clustering and individual motif search. Consensus sequence for TATA box is TATAAA, and according to Ohler/Fitzgerald criteria sequence at position –29 TTAAATA is not a TATA box. We need to use experimental approach to confirm or deny this possibility. Dr. Ohler also found DMv1 motif at position –49 at *SxlPe* promoter.

Classical TATA promoter recruits Pol II via TBP, which recruits other TAFs. DRE core promoter is different because it recruits not TBP but TBP-related factor Trf2 or Dref. Other factors that involved in those core TATAless promoter are remodeling factors such as Nurf. Later we are going to investigate importance of Trf2, Dref and Nurf in regulation of *Sxl*.

**Problems that arise from structure of alternative *Sxl* promoter**

Two promoters are located 5 kb apart. Expression of *Sxl* starts at cycle 12 from downstream promoter *SxlPe* and continues till early minutes of cycle 14. Expression from *SxlPm* starts at embryonic cycle 13 and continues through adulthood.
How is this promoter switch regulated? How does the structure of promoter does helps in alternative regulation?

There are at least three levels in regulation of gene expression. First, I will discuss the condensation of chromosomes. If chromosomes are highly condensed (heterochromatin), then almost no genes are expressed or some genes are expressed only during replication phase of cell cycle (S-phase). The same event takes place during formation of gametes. Chromosomes become highly condensed and there is no (or almost no) gene expression. After fertilization several rounds of synchronic replication take place. After each cycle chromosomes become less condensed (most probably because distribution of repressor factors along all new nuclei) and then some genes begin to express. They express like heterochromatin genes only in S-phase of cell cycle. At cycle 12 there is expression from *SxlPe* starts, but not from *SxlPm*. Why?

Is there regulation *SxlPe*/ *SxlPm* on the level of chromosome condensation? Is the concentration of heterochromatic factors on chromosome region of *SxlPe* less in this region in this period of time than in other (including *SxlPm*) regions? What heterochromatic factors are involved in this regulation? Is it HP1, the main heterochromatic protein? Does piRNA regulate the difference in *SxlPe* versus *SxlPm* activation? Is expression from heterochromatin takes place mostly during replication? Is the difference in expression due to the fact that *SxlPe* is located near replication start site? All these hypotheses have to be tested. We can plan to compare distribution of heterochromatic factors on *SxlPe* and 5 kb apart on *SxlPm*. What character of promoter structure does influence on this distribution?

Second, I will discuss the domain/loop organization. If on same period of time TSS (with core promoter) and activator binding sites are in different functional domains,

then expression is suppressed. If remodeling takes place and brings TSS and activator sites in the same functional domain, then transcription takes place. In *SxlPe* most of *sisB/Da*, the main activators, and binding sites are located rather near the TSS, so special looping organization is not necessary in this case. At the same time, several *sisB* binding sites are located about 1500 bp upstream of *SxlPm*. So loop/domain organization may be necessary for initiation of expression from *SxlPm*. Is the presence of specific structural/scanfold proteins necessary for activation of expression from *SxlPm*? Do they become abundant only after cycle 14 when *SxlPm* is expressed?

Other aspect of possible necessity of loop/domain organization for *SxlPm* expression is connected to possible Motif1 structure of *SxlPm* TATAless promoter. Usually Motif1 is accompanied with Motif6 at about -60 positions. In the case of *SxlPm* two Motif6-like sequences are located at about –400 position. So loop/domain organization may be necessary for activation of *SxlPm*. How can we determine the validity of this hypothesis? What factors are necessary for this process?

Third, I will discuss the difference in primary sequence of *SxlPe* and *SxlPm* promoter. *SxlPe* has binding sites for TF, which are already present in embryos by cycle 12. At the same time, *SxlPm* does not have as many as *SxlPe scute/Da* binding sites and may depend on other factors, which are not present by cycle 12, for its activation. *SxlPe* is DRE/Inr promoter according to Ohler criteria. DRE/Inr promoter is different from TATA box promoter in these ways:

Activation from TATA box promoter starts from recruitment TBP, then other TAFs, then polII.

Activation from DRE/Inr promoter starts from recruitment Trf2 or Dref, then alternative TAFs (which are also different from canonical TAFs and include Nurf, remodeling factors), then PolII.

If *SxlPe* is activated earlier because of DRE/Inr function, then it implies that Trf2/Dref and alternative TAFs are more available for *SxlPe* promoter at this time than core promoter elements for *SxlPm*, which is TATAless, Motif1 promoter. It is highly probable because during gametogenesis classical TAFs are substituted by alternative TAFs and they are probably maternally supplied to *Drosophila* embryos. Additional experiments have to be done to confirm that hypothesis. Do those alternative TAFs regulate cascade of gene activation in early *Drosophila* embryos and allow only a small set of TATAless promoters to become active and expressive before cycle 14? Do they, together with maternally supplied mRNA, provide a blueprint for *Drosophila* development? We are planning to compare promoter of early *Drosophila* genes and test if they are rich in DRE elements compared to other majority of the genes that are silent till cycle 14 (onset of cellularization).

Why expression from *SxlPe* shuts down abruptly early at cycle 14? Which factors are involved in this process? How Trf2, Dref and remodeling factors are involved in alternative expression from different promoters? Is there a specific repressor that effect only on DRE/Inr *SxlPe* promoter? What repressor is there?

I propose to perform a set of experiments to answer all the questions mentioned earlier.

**Unusual expression in mutants**

Mutant *Trf2*#12254 have three X chromosomes in 4 out of 34 counted embryos (males and females) (Fig 15). Is there any connection between this phenomenon and sex determination? Is it a feature of genetic background? Does *Trf2* mutant survive better in 3X? These are questions for future research.

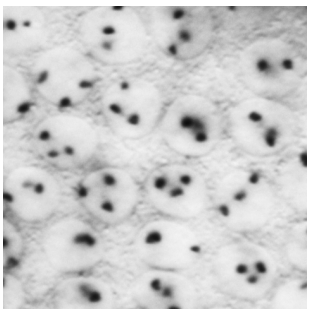


Fig.15. Expression of *SxlPe* is observed from three X chromosomes in *Trf2* mutant#12254.

# 6. DISCUSSION*

*SxlPe* switches on in females because XX embryos have twice the amount of XSE activators as XY embryos. How this two-fold difference in XSE proteins is converted into an all-or-nothing transcriptional response at *SxlPe* is the central question in primary sex determination. The traditional concept of the sex determination signal as the X chromosome to autosome ratio, X:A, led to the hypothesis that the male/female difference in XSE proteins is amplified through the actions of inhibitors encoded by autosomal signal elements, or ASEs (see Schutt and Nothiger, 2000; Gilbert, 2006). In this view, Dpn and other ASE proteins amplify the signal by preferentially titrating XSE proteins in XY embryos and by competing with XSE proteins for binding to *SxlPe* (Parkhurst et al., 1990; Paroush et al., 1994; Schutt and Nothiger, 2000; Louis et al., 2003). An alternative idea, based on the thesis that XSE dose is the sex-determining signal, and on the finding that *dpn* is the only significant ASE, is that signal-amplification might occur primarily through combinatorial interactions between XSE activators and their maternally-supplied cofactors at *SxlPe* (Cline, 1993; Erickson and Cline, 1993; Barbash and Cline, 1995; Yang et al., 2001; Wrischnik et al., 2003; Erickson and Quintero, 2007). Repression by DNA-binding proteins is important in combinatorial schemes, but as a kind of fine-tuning control, rather than as the primary cause of dose-sensitivity.

A full understanding of the role of negative regulators in the dose-sensitive

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control of *SxlPe* requires the identification and characterization of the cis-regulatory sequences controlling repressor binding as well as the trans-acting factors working through those sites. In the following paragraphs we discuss our findings that Dpn, and other, presumably maternal, bHLH proteins bind *SxlPe* and act in conjunction with the corepressor, Gro, to define and maintain the threshold concentrations of XSE proteins needed to activate *SxlPe*. Our data suggest that neither the classical notion of amplification by titration, nor the activator-centered alternative, adequately explain how XSE dose is assessed. Rather they indicate that repression at the level of DNA, or chromatin, is a central aspect of XSE signal amplification. We conclude with a model for how Gro-mediated repression could be modulated by XSE function to generate the dose-sensitive control of *SxlPe*.

**Canonical and non-canonical bHLH repressor-binding sites at *SxlPe***

Although *SxlPe* has two typical DNA-binding sites for HES family proteins (Hoshijima et al., 1995; Winston et al., 1999), their role in *Sxl* regulation in vivo had not been examined. Our analysis confirmed that the canonical CACGCG sites centered at -108 and -119, bind HES-family repressors in the embryo, but also revealed that a non-canonical site 3, CACACT, at -160 mediates repression in its normal promoter context. Although CACACT had not been previously reported as a HES-binding site, considerable evidence points to the in vivo importance of DNA-binding sites with less than optimum binding affinity. N-boxes, CACNAG, bind HES proteins with lower affinity than the optimal CACG(T/G)G sequences, but are known to mediate repression of several genes in mammalian cells, and the variant CACGCA appears to bind control

repression of *Math1* in mice (Iso et al., 2003). The same applies to bHLH activators as illustrated by our finding that the bHLH activator Sc/Da exerts most of its dose-sensitive effects at *SxlPe* through non-canonical DNA-binding sites (Yang et al., 2001).

**Cis-acting mutations implicate additional bHLH repressors in *Sxl* regulation**

We found that mutations in the Dpn-binding sites had stronger and earlier effects on *SxlPe* activity than did complete loss of *dpn* function (Table 1). The simplest explanation for this finding is that additional bHLH repressors work through the same sequences as Dpn to control *SxlPe*. The additional repressors seem likely to be maternally supplied. This argument is based on timing, the cis-acting Dpn-site mutations can affect *SxlPe-lacZ* expression in XX embryos as early as nuclear cycle 10 or 11, when few zygotic genes are active (albeit those active include the XSEs *scute* and *sisA)*. And on the results of sensitive and unbiased genome-wide genetic screens that showed *dpn* to be the only zygotically expressed inhibitor of *SxlPe* of any significance (Barbash and Cline, 1995; Wrischnik et al., 2003).

**Hey is a maternal repressor of *SxlPe***

The prediction that bHLH repressors other than Dpn regulate *SxlPe* was confirmed by our discovery that maternal *Hey* functions as a negative regulator of *SxlPe*. Befitting its maternal origins, *hey* acts earlier than *dpn,* as evidenced by increased *Sxl* expression in cycle 12 XX embryos and ectopic activation in cycle 13 XY $Hey^{mat-}$ mutant embryos. However, $Hey^{mat-}$ mutants, unlike $dpn^{-}$ embryos, accumulate no detectable *Sxl* protein in males suggesting either that the single available *Hey* mutation is not a null allele or that still other bHLH repressors regulate *SxlPe*. The later possibility

is also suggested by the finding that mammalian Hey homologs do not appear to interact directly with Gro/TLE proteins (Iso et al., 2001). One promising candidate bHLH repressor is Her (Hes-related, CG5927). *Her* protein is encoded on the X chromosome and the gene is maternally expressed (Moore et al., 2000), placing this WRPW-containing HES family member in the correct cellular context to regulate *SxlPe*. Unfortunately no *Her* deletions or point mutations are currently available to test its possible function at *Sxl*.

**Gro-dependent repression predominates at *SxlPe***

The first indication that repression is likely to be a quantitatively important part of primary sex determination was the finding that XY $gro^{mat-}$ embryos expressed high-levels of ectopic *Sxl* protein (Paroush et al., 1994). This initial study of *gro* and *Sxl* was limited in scope because X-ray induction of germline clones could generate only a limited number of $gro^{mat-}$ embryos. Using high efficiency FLP/FRT-mediated recombination (Chou and Perrimon, 1996) we analyzed in detail the effects of maternal *gro* on *Sxl* protein and on *SxlPe* activity. Our findings confirmed that loss of maternal *gro* leads to ectopic SXL in XY embryos and showed that this is caused by activation of *SxlPe* in XY embryos. Our results differed from the initial study in one important respect. Whereas Paroush et al., (1994) reported that SXL levels were indistinguishable in XY and XX $gro^{mat}$ embryos, we found that *Sxl* mRNA and protein were expressed at higher levels in XX embryos at all stages of embryogenesis, even when corrected for the copy number of the X-linked *Sxl* gene. This has important implications for function, as it means that *SxlPe* responds differently to the one-X and two-X doses of XSEs even in

the absence of *gro*-mediated repression. The ability of the promoter to distinguish XX from XY is also evident from our finding that *SxlPe* was always activated at least one cycle earlier in female than in male embryos when repression was compromised or eliminated (Figs. 5 and 6, Table 1).

The best evidence that the pleiotropic *gro* protein acts directly at *SxlPe,* rather than on than other maternal or zygotic genes that influence *SxlPe* activation, is that maternal $gro^{E48}$ and the $1^{-}2^{-}3^{-}4^{-}$ Dpn-binding site mutations have nearly identical effects on *SxlPe*, eliciting premature activity in XX embryos and ectopic expression in XY cells (Table 1). While the somewhat depressed state of the 1.4 kb *SxlPe-lacZ* transgenes prevented precise comparisons, our data suggest that most, if not all, of the repressive effects of maternal *gro*, and of the cis-acting repressor sites, can be explained by the recruitment of Gro to *SxlPe* by bHLH proteins. This suggests that several other hypothesized methods of HES-mediated repression, including competition between Dpn and Sc/Da for DNA-binding (Louis et al., 2003), or orange-domain dependent inhibition of Scute function by Dpn (Dawson et al., 1995) are likely to have little quantitative importance at *SxlPe*, unless such interactions are also directly related to Gro function (see below). The predominant corepressive role of Gro is also consistent with the findings that the corepressors dCtBP and Sir2, which can associate with HES proteins, do not influence *Sxl* expression (Poortinga et al., 1998; Zhang and Levine, 1999; Astrom et al., 2003).

**Inhibition by sequestration of activators?**

A means of repression that is independent of Gro and DNA binding is titration, or the sequestration of activators into non-functional heterodimers. Long a staple of models for how the the X:A ratio might be read (see Parkhurst et al., 1990; Schutt and Nothiger, 2000; Gilbert, 2006), titration schemes have found mathematical corroboration (Louis et al., 2003), but little experimental support. To our knowledge, the only evidence for sequestration of an XSE by an ASE protein is a non-reciprocal two-hybrid interaction between Dpn and SisA (Liu and Belote, 1995; Louis et al., 2003). An interaction that we did not observe with a different two-hybrid system (Fields and Song, 1989; unpublished data). Negative regulation at the level of DNA, in contrast, is supported by the known functions of the proteins, by the initial stochastic activation pattern of each copy of *SxlPe* (Erickson and Cline, 1998), and by the strong effects of maternal *gro* and the Dpn-binding site mutations. Nonetheless, our data do leave open the possibility that some XSE signal amplification could occur via sequestration of activators. If so, we suggest that maternally-supplied Emc, the sole example of an inhibitor with a demonstrated ability to heterodimerize with an XSE protein (Campuzano, 2001), is likely the amplifying factor, rather than Dpn or an undiscovered ASE.

**Groucho and the control of the *SxlPe* switch**

*SxlPe* responds to threshold concentrations of XSE activators. Loss of maternal *gro*, or of Dpn-binding site function, causes premature onset of *Sxl* transcription in XX embryos and strong ectopic expression in XY embryos. Loss of *dpn* protein function, in

contrast, has virtually no effect on *Sxl* in females while causing relatively late, and low-level, *Sxl* expression in males. These findings suggest that Gro and associated maternal repressors directly mediate the initial activation threshold at *SxlPe*, and that the same factors, plus the ASE protein Dpn, then act to maintain the threshold at appropriate values throughout the X-counting process (see Erickson and Cline, 1993; Barbash and Cline, 1995). An important mechanistic point is that while Gro is not needed for *SxlPe* to sense male/female differences in XSE doses, it is required to convert the differences into a robust all-or-nothing transcriptional response. How might Gro, acting at the level of DNA, or chromatin, amplify the XSE signal and ensure proper operation of the *SxlPe* switch?

The predominant model for Gro corepressor function; recruitment to DNA by repressors, oligomerization, spreading, and recruitment of histone deactylases, to generate extended regions of inactive chromatin explains how Gro can function as a dominant long-range repressor (Barolo and Levine, 1997; Chen and Courey, 2000; Martinez and Arnosti, 2008). The notion of potent long-range silencing, however, fits poorly with our understanding of *Sxl* regulation. First, short-range repression should suffice at *SxlPe*. The repressor-binding sites are located close to the transcription initiation site, and they can mediate effective repression of *Sxl* by ectopic derivatives of Hairy that carry Gro-independent 'short-range' repression domains (Jimenez et al., 1997). Second, Gro-mediated repression at *SxlPe* is dynamic, reversible, and relatively weak. Established early in both sexes, repression is overcome in XX embryos during cycle 12. Even in XY embryos, where *SxlPe* normally remains inactive, loss of *dpn*

function causes a partial reversal of repression during cycles 13 and 14. Transient repression by Gro is not unique to *Sxl*. As discussed by Jennings et al., (2007), and Martinez and Arnosti, (2008) reversible Gro-mediated local repression is commonly found at loci that are expressed in dynamic developmental contexts suggesting that Gro likely represses transcription by more than one mechanism.

Models for Gro-mediated repression invoking interactions with the mediator complex or RNA polymerase (see Buscarlet and Stifani, 2007) fit better with aspects of *Sxl* regulation, but, like the dominant-silencing model, do not offer ready explanations for how Gro might control the switch-like response of *SxlPe*. In contrast, a recent model for Gro function invoking direct associations between Gro and chromatin as a necessary step in repression (Sekiya and Zaret, 2007) appears to be both compatible with transient local repression and suggestive of a means by which Gro might 'amplify' the XSE signal.

Sekiya and Zaret's (2007) key finding was that the mammalian Gro/TLE protein, Grg3, represses transcription by creating a 3 to 4 nucleosome region of poorly accessible chromatin that inhibits binding by transcriptional activators. Surprisingly, Grg3 is not recruited directly by DNA-binding repressors. Instead, Grg3 first associates with chromatin via interactions with histones to form an open nucleosome array. Gro-interacting transcription factors, including the bHLH protein Hes-1, then bind their DNA sites in the array enabling Grg3 recruitment and formation of the repressive chromatin complex (Sekiya and Zaret, 2007). We propose that the requirement that Gro bind nucleosomal histones, combined with Gro's low affinity for highly acetylated chromatin

(Edmondson et al., 1996; Chen and Courey, 2000) provides the elements of a possible feedback mechanism that could work in the early embryo to amplify the female/male difference in XSE proteins into a reliable developmental signal (Fig. 16).

**A model for Gro-mediated amplification of dose-sensitive signals**

The basic tenets of our model for *SxlPe* regulation are: 1) The initial threshold XSE concentration needed to activate *SxlPe* is set by the translation products of maternally-supplied *gro* mRNA acting in conjunction with the products of maternally-

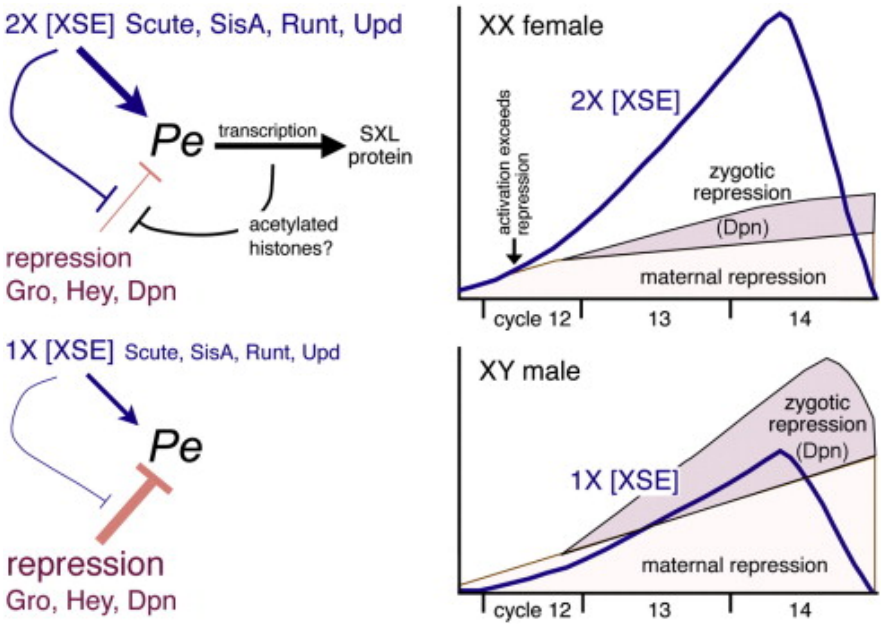


Fig. 16. Model for dose-sensitive regulation of *SxlPe*. (Top) Gro and other products of maternally supplied mRNAs establish initial threshold XSE concentrations for *SxlPe* activation. XX embryos exceed threshold [XSE] in cycle 12. Activation of *SxlPe* inhibits Gro-mediated repression leading to increased *Sxl* mRNA in cycles 13 and 14. (Bottom) Gro and other maternal products maintain repression potential above XY XSE concentrations until cycle 13. Zygotic expression of Dpn combined with maternal Gro and other products thereafter maintains repression potential above XY [XSE]. XSE mRNAs are degraded early in cycle 14. Time scale; cycle 13 is 18 min long and begins 112 min after fertilization (Foe et al., 1993).

supplied mRNAs encoding bHLH repressors. 2) The initial *SxlPe* activation threshold is crossed first in XX embryos because they possess twice the amount of XSE proteins present in XY embryos. 3) Activation of *Sxl* transcription leads to acetylation of histones at *SxlPe*. Histone acetylation decreases the ability of Gro to bind chromatin reducing Gro's 'repression potential' and allowing the XX dose of XSE proteins to more effectively stimulate transcription from *SxlPe*. 4) In XY embryos, continued translation of maternal mRNAs and the activation of zygotic *dpn* adjust the *SxlPe* activation threshold upward so that it remains above the XSE concentrations present in male embryos in cycles 13 and 14 (Fig. 7). The net result is a form of signal amplification via positive feedback. Once initiated in XX embryos, *Sxl* transcription gains in strength from the interacting effects of rising XSE levels and decreased potential for Gro-mediated repression. The initial failure to activate *SxlPe* in XY embryos, in contrast, leaves Gro function unabated, so that the single-X dose of XSEs can never exceed the growing *SxlPe* activation threshold.

Our model for operation of the *SxlPe* switch, with its emphasis on signal amplification by modulation of corepressor function, is distinct from traditional titration schemes (Parkhurst et al., 1990; Schutt and Nothiger, 2000; Gilbert, 2006), and from composite models invoking titration, DNA-binding site competition, or interactions between multiple activators (Yang et al., 2001; Louis et al., 2003). Its most novel aspect is the feedback mechanism in which high XSE protein concentrations and transcription from *SxlPe* inhibit Gro function in females. The specific proposal that histone acetylation, occurring as a consequence of transcription and XSE activator binding

(reviewed in (Shahbazian and Grunstein, 2007) inhibits Gro-mediated repression is speculative but based on the finding that the yeast Gro/TLE protein Tup1 does not bind highly acetylated histones (Edmondson et al., 1996; see Chen and Courey, 2000; Sekiya and Zaret, 2007). Feedback regulation, however, need not be limited to chromatin modifications. XSE proteins could also decrease the repression potential of Gro by competing with Dpn, Hey, and other repressors for overlapping DNA-binding sites, or by direct interference with Gro or repressor function. The C-terminal VWRPY motif of the XSE protein Runt can interact with Gro, raising the possibility that much of Runt's positive role at *SxlPe* is due to its ability to directly antagonize repression (Aronson et al., 1997).

One question our model does not directly address is what prevents stochastic fluctuations in XSE levels from causing stable activation of *SxlPe* in some XY nuclei? The one nuclear cycle lag in *Sxl* activation seen in XY compared to XX nuclei when repression is compromised by mutation (Figs. 5 and 6, Table 2), hints that literal two-fold differences in XSE concentrations may be sufficient to reliably signal an on-or-off response for a limited period of time. The regulatory scheme may also provide a kind of double-check against activation due to random variations in XSE levels. Stable expression of *SxlPe* would require not only that the promoter be activated, but also that it be turned on at sufficiently high levels to establish the feedback mechanism. XX cells meet both criteria, but the occasional XY nucleus that surpassed threshold XSE levels would likely fail to reinforce the initial event because the single Xs of it and its neighbors would supply insufficient XSE products to do so (Gregor et al., 2007). On the

other hand, the discriminatory power of the system would likely be increased by even a small increase in the relative female/male XSE signal prior to the onset of feedback regulation. Plausible early amplification mechanisms include titration of Scute by maternal Emc, and combinatorial effects due to multiple XSE activator-binding sites (Wang et al., 1999; Louis et al., 2003; Veitia, 2003).

Although our focus here is on *Sxl*, the idea that transcriptional activation could be a kind feedback control of Gro-activity may be applicable to other genes and systems that respond to small or transient changes in regulatory proteins. As discussed by (Jennings et al., 2007) Gro acts in a dynamic fashion to sharpen spatial expression boundaries during segmentation and to precisely control periodic patterns expression in neuroblast multiplication and during vertebrate somitogenesis. A reversible feedback mechanism relying on general properties of transcriptional activation rather than specific interactions might have considerable evolutionary flexibility.

Our colleague Hong Lu was killed in automobile crash December 1 2002. Her friendship, humanity, and insight are greatly missed. We thank the Bloomington *Drosophila* stock center, G. Jimenez (IBMB-CSIC-PCB, Barcelona), T. Cline (University of California, Berkeley), A. Preiss (University of Hohenheim), and P. Simpson (University of Cambridge) for providing fly stocks. K. Maggert and A. Gonzalez provided stimulating discussions and helpful comments on the manuscript. This work was supported in its early stages by American Cancer Society Grant RPG-97-079-01-DB, and later, by National Institutes of Health Grant GM063606 to J.W.E.

# 7. SUMMARY AND CONCLUSIONS

1. Maternally supplied Gro is responsible for amplification difference in dose of activator transcription factors.

2. Three new repressors of *SxlPe* are identified:

Hey, which is bHLH maternal transcription factor

bHLH Cwo

Prooncogene Myc

3. *SxPe* promote is predicted to be TATAless DRE/Inr promoter. Importance of core TF Trf2 and Dref for regulation of *SxlPe* is discussed. *SxlPm* is predicted to be Motif1 (Motif6?) TATAless promoter.

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# APPENDIX

## Lack of maternally contributed Big brother does not influence *SxlPe* expression

Two closely related (Fig. 17) proteins Bro (Brother) and Bgb (Big Brother) are transcriptional co-activators, analogs to human core binding factors, beta subunit. They interact with runt domain proteins Runt and lozenge (Canon and Banerjee, 2000). Runt domain is also found in p53 protein, one of the core proteins that prevent cancer development in human.

```
Bgb         mmNEAALANMIP-YDTIGLYEQPKPRFIFKMPRVVPDQKSKFESDELFRRLSRESEVRYTGY  61
              + AA+  MIP Y+ + +YEQPKPRFIFKMPRVVPDQ+SKF+SDELFRRLSRESEVRYTGY
Bro   mhhhqnlgDAAAMNGMIPPYEAMAMYEQPKPRFIFKMPRVVPDQRSKFDSDELFRRLSRESEVRYTGY  68

Bgb           RERSIEERQVRFMNGCREGHTEASFVASGTNLQLVFNANQNPYLHDKECDFDKEHGKVHI  121
              RER++EER++RF+N CR+G+ E S VASGTNLQL FNAN NPY  +++CDF++E GKVH+
Bro           RERAMEERRMRFVNDCRKGYAEISMVASGTNLQLYFNANHNPYAQEQDCDFERERGKVHL  128

Bgb           KSYFIMNGVCVRFRGWIDLERLDGVGCLEYDERRAMHEDAILRDQIDRYNQRLREFEDTK  181
              +S FIMNGVCVRFRGW+DL+RLDG  CLE+DE+RA  EDA L++QI  YNQR+ E   ++
Bro           RSSFIMNGVCVRFRGWVDLDRLDGAACLEFDEQRAQQEDAQLQEQIQSYNQRMAE---SR  185

Bgb           RAYRDNRQDEMEAVRRGVASGGIGVGASMW  211
              R Y   +    +   RG    G+  G   W
Bro           RIYHTPQTPPEDHHHRG--GPGLPRGPMGW  213
```

Fig. 17. Bro and Bgb are highly similar proteins. Alignment of Bro(AAF47538) and Bib (AAF47533) proteins. NCBI BLAST score is 77% Positive163/210.

Runt domain proteins can act as activators or as repressors. We speculate that this phenomenon can be explained by the fact that runt DNA-binding domain acts to clamp or encircle the DNA target in order to stabilize the protein-DNA complex. This domain has an immunoglobulin-like fold consisting of a beta-sandwich of 9 strands in two sheets with a Greek key topology (Berardi et al., 1999). We speculate that in the

case of *SxlPe* expression Runt/Bgb or Runt/Bro complex are most important for stabilization of activator factors and so that is how it works as transcriptional activator.

Bgb is a maternally supplied protein that is distributed ubiquitously during stages 1-3. According to (Golling et al., 1996) data *Bro* is also expressed maternally which is contradicted to (Fujioka et al., 1996) data who confirmed that only *Bgb* is expressed maternally. Both *Bro* and *Bgb* are expressed also zygoticaly and Bro function only in early embryos. *Bgb* mRNA (zygotic) begins to accumulate ubiquitously during blastoderm stage and then can be seen only in embryonic brain and ventral nerve cord from stage 11 (flybase, BDGP, SD 08175). This pattern of expression implies that function of these two closely related genes can be partly redundant.

**Expression of *SxlPe* in germline *Bgb* clones**

To determine if only one of the maternally supplied co-activator Bgb take part in regulation of *SxlPe* expression we generated germline clone $Bgb^{mat-}$ and assayed its effect in embryos derived from $Bgb^{germline}$ x o♂*Bgb*/+ .

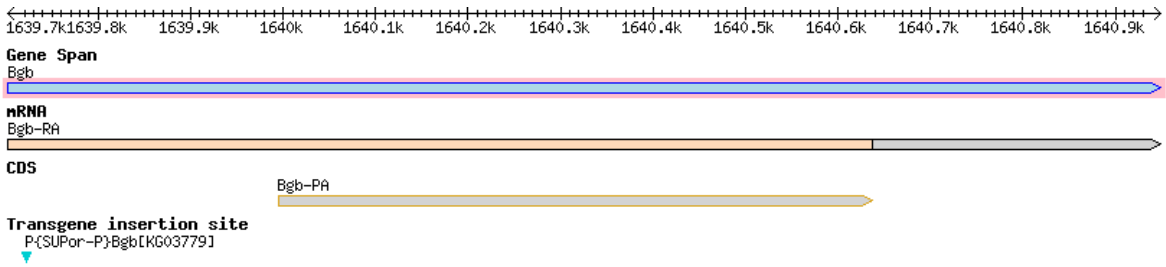


Fig. 18. *Bgb* mutant (Bloomington#13509) is a P element insertion *P{SUPor-P}Bgb[KG03779]* at about 20 bp downstream transcriptional start site and about 300 bp upstream the first ATG start codon.

I used *Bgb* mutant (Bloomington#13509) which is a P element insertion at about 20 bp downstream transcriptional start site and about 300 bp upstream the first ATG start codon (Fig 18.) To create *Bgb* FRT line I crossed

# Bloomington5750 *Gl,st,*FRT*(w+)*/TM3 x o♂*Bgb(w+)*/TM3

↙

and selected progeny *Gl,st,*FRT(*w+*)/*Bgb*(w+) x o♂ #5750 *Gl,st,*FRT(*w+*)/TM3

↙

for recombinant o♂ *Bgb*(w+)FRT(*w+*)/TM3 which has similar to wild-type red *w(+)* eyes in compare to o♂*Bgb*(w+)/TM3 brownish or o♂ FRT(*w+*) orange eyes.

I confirmed o♂*Bgb*(w+)FRT(*w+*)/TM3 genotype by crossing them with wild-type o♂$w^{1118}$ flies and obtained all type of progeny o♂ *Bgb*(w+)/TM3 brown eyes o♂ FRT(w+)/TM3 orange eyes o♂ *Bgb*(w+) FRT*(w+)*/TM3 red eyes and +/+ white eyes.

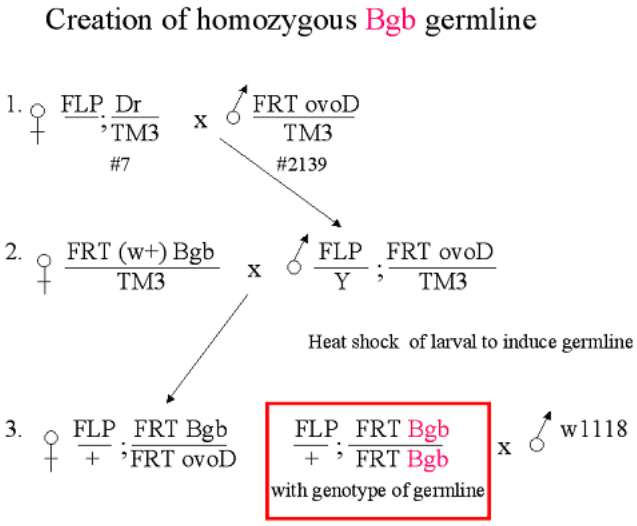


Fig. 19. Creation of homozygous *Bgb* germline clones.

To create germline clone I followed the protocol of (Chou and Perrimon, 1996) (Fig. 19). Germline clones were generated following heat treatment of female larvae of following genotype P{hsFLP}, $y^1w^{1118}/w^{1118}$; P{*w(+)*FRT 2A} ,*Bgb*/P{*w(+)*FRT}2A, *ovoD*. Females bearing recombinant germlines were crossed to *Bgb*/TM3 males.

In germline clone expression of *SxlPe* starts at appropriate time (Fig. 20) at cell cycle 12. There was no ectopic expression of *SxlPe* during earlier cycle 9, 10 or 11.

| | | |
|---|---|---|
| Cycle 12 | | 1embryo"–" just after mitosis |
| | o♂ | 3 embryos "–" |
| | o♂ | 2 embryos "–" about 6 dots per embryo |
| | female | 4 embryos "+" more the 30% of nucleus |

In some of the male embryos at cycle 12 there were 3-6 nuclear with ectopically expressed *SxlPe*, which is less then 2% of the nuclear. If negative regulator are able to

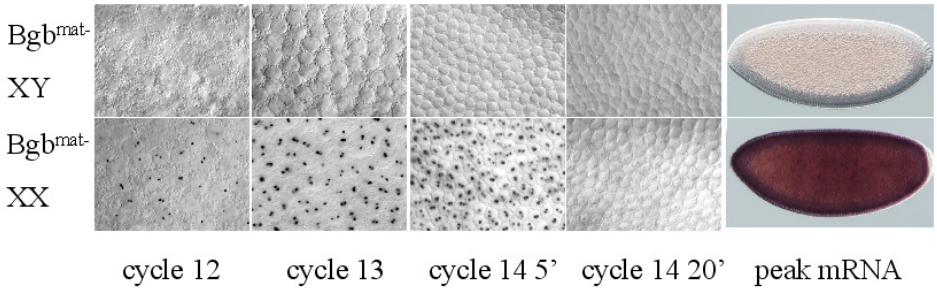


Fig. 20. Time course of *SxlPe* activation in maternal $Bgb^{KG03779}$ mutant embryos. $Bgb^{mat-}$ do not influence on *SxlPe* time course. Mutant $Bgb^{mat-}$ embryos were stained following in situ hybridization. Black and white panels show surface views of embryonic nuclei at indicated nuclear cycles. Dots represent nascent transcripts from the X-linked *Sxl* locus. Color panels show peak accumulation of *SxlPe*-derived mRNA in early cycle 14. Embryos were progeny of females with $Bgb^{KG03779}$ FRT/ $Bgb^{KG03779}$ FRT germline and $Bgb^{KG03779}$ /TM3 males.

amplify the female/male difference during the time of cycle12 – early cycle 14 then these misexpressed male nuclear are subject for complete later repression.

At cycle 13 half of male embryos (9 out of 19) expressed *SxlPe* in 3-6 nuclei, which is less then 2% of the nuclei. In female embryos (15 female embryos of cycle 13 were examined) *SxlPe* is expressed at obviously normal level at cycle 13. Some of female embryos expressed *SxlPe* not in all but in 80% of the embryo, and about 20 express *SxlPe* in only one dot, which is correspondent to only one out of two female X chromosomes. This pattern of expression is similar to wild-type variation of expression. Because both Bro and Bgb are able to bind Runt (Li and Gergen, 1999) and both are expressed during blastoderm stage (Fujioka et al., 1996), the stage that is critical for *SxlPe* activation, it is reasonable to speculate that their function is redundant. So zygoticay expressed Bro completely compensate lack of maternally supplied Bgb in this case.

At cycle 14 *SxlPe* in $Bgb^{mat-}$ is expressed at normal level in male and female embryos. This fact is one additional conformation of the model that both proteins act as transcriptional co-activator of *SxlPe* and their function is mostly redundant. Accumulation of mRNA in *Bgb* germline started to become visible at cycle 13 and got it's maximum level by 5 min cycle 14 (Fig 20), the same as in wild-type embryos.

There was no difference in *Sxl* expression on the protein level also. In the progeny of germlene *Bgb*/*Bgb* with cross of wild-type males *w1118*, expression of Sxl protein started at cycle 14 in female embryos. Approximately half of embryos, which

were 43 out of 36, did not express Sxl, which is normal for male embryos (data not shown).

So our data support the conclusion that lack of maternally contributed Bgb do not influence onto *Sxl* expression as on protein, as on level of nascent transcripts, as on level of mRNA expression.

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