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Maternal Groucho and bHLH repressors amplify the dose-sensitive X chromosome signal in *Drosophila* sex determination

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ABSTRACT

In *Drosophila*, XX embryos are fated to develop as females, and XY embryos as males, because the diplo-X dose of four X-linked signal element genes, XSEs, activates the *Sex-lethal* establishment promoter, *SxlPe*, whereas the haplo-X XSE dose leaves *SxlPe* off. The threshold response of *SxlPe* to XSE concentrations depends in part on the bHLH repressor, Deadpan, present in equal amounts in XX and XY embryos. We identified canonical and non-canonical DNA-binding sites for Dpn at *SxlPe* and found that *cis*-acting mutations in the Dpn-binding sites caused stronger and earlier *Sxl* expression than did deletion of *dpn* implicating other bHLH repressors in *Sxl* regulation. Maternal *Hey* encodes one such bHLH regulator but the *E(spl)* locus does not. Elimination of the maternal corepressor Groucho also caused strong ectopic *Sxl* expression in XY, and premature *Sxl* activation in XX embryos, but *Sxl* was still expressed differently in the sexes. Our findings suggest that Groucho and associated maternal and zygotic bHLH repressors define the threshold XSE concentrations needed to activate *SxlPe* and that they participate directly in sex signal amplification. We present a model in which the XSE signal is amplified by a feedback mechanism that interferes with Gro-mediated repression in XX, but not XY embryos.

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Introduction

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; 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; Ashburner et al., 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; 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 Zelda, bind directly to *SxlPe* to facilitate expression (Yang et al., 2001; Bosch et al., 2006; Avila and Erickson, 2007; Liang et

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al., in press). 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, 1998; 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, 1998; 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, 1998). 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, 1998; 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.

Materials and methods

Plasmids, mutagenesis, and P-element transformation

The GST-Dpn bHLH plasmid was made by inserting a PCR fragment encoding amino acids 1–108 into the Bam H1 and Eco RI sites of PGEX-2TK. To make the MBP-Dpn plasmid the entire *dpn* coding sequence was cloned as a PCR fragment into the Eco RI and Hin dIII sites of pMAL-c2. The *dpn*-VP16 cell culture expression plasmid carried *dpn* codons 1–108 fused to the VP16 activation domain (residues 410–490) plus a Kozak sequence in PAct5CPPA (Han et al., 1989). The minimal −94 bp *SxlPe*-luciferase plasmid has been described (Yang et al., 2001). The 4× Dpn site-firefly luciferase reporters have the following sequences between the Xho I and Eco RI sites of the −94 bp Sxl_{Pe}-*Fluc* plasmid: 1,2-CCCACGCGACGCCCACGCGAGCCCACGCGACGCCCACGCGAC; 3-GGCACACTTCTGGCACACTTCCGCACACTTCTGGCACACTTC (3m has CACcCT); 4-GCCACGTTCCAGCCACGTTCCGCCACGTTCCTGCCACGTTCC (4m has CAaGcT).

P-element transformation vectors were based on pCaSpeR-AUG-βgal and carried *SxlPe* sequences from −1.45 kb to +44 bp derived from wild-type or mutated variants of plasmid pG01 (Yang et al., 2001). Point mutations were made by site-directed oligonucleotide mutagenesis and confirmed by DNA sequencing. Mutated sequences were as follows: site 1, CACtgG; site 2, CtCGaG; site 3, CACcCT; site 4, CAaGcT. P-element transformants were obtained from w^{1118} flies by co-injection with the pTurbo transposase source.

Protein expression and purification

To produce GST-Dpn bHLH and MBP-Dpn proteins, BL21(DE3) cells carrying the corresponding expression plasmid were grown in LB at 21° to an OD_{600} of 0.3 and induced with 0.1 mM IPTG for 1–2 h. Cell pellets were suspended in 1/40 culture volume of 20 mM Hepes, 0.6 M NaCL, 0.5 mM EDTA, 1%(v/v) NP-40, 2mM DTT, pH=7.9 and lysed by sonication. After 10 min centrifugation at 10,000 ×*g*, supernatants

were diluted with one volume of 20 mM Hepes pH=7.9. GST-Dpn bHLH was purified to homogeneity using glutathione-agarose beads (Sigma) and MBP-Dpn using amylose affinity resin (New England BioLabs).

DNase I footprinting and electrophoretic mobility-shift assays (EMSA)

For DNase I footprinting, probes were made by PCR amplification with one ^{32}P end-labeled primer, and gel-purified. Approximately 10^4 cpm of probe was included in a 20 µl reaction containing: 15 mM Hepes, 50 mM KCL, 1 mM EDTA, 2mM DTT, 7.5% (v/v) glycerol, 0.1% (v/v) NP-40, 1 µg polydI:polydC, 5 µg bovine serum albumin pH=7.9 and indicated units of Dpn fusion protein. One Dpn unit equaled 15 nM (~10 ng of GST-Dpn bHLH domain or 20 ng of MBP-Dpn). After 30 min at 21° 0.05 U of DNase I (Epicentre) was added. After 2 min 80 µl 0.1 M EDTA, 1.0 M NaCl was added to stop the reaction. Samples were phenol:$CHCl_3$ extracted, ethanol precipitated, dissolved in 80% formamide, 0.01 N NaOH, 1 mM EDTA, and heated to 90° for 5 min before loading on 6% polyacrylamide/8 M urea gels. *Msp* I-cut ^{32}P-labeled pBR322 served as size standards. For EMSA, double-strand oligonucleotides were ^{32}P-5′-end-labeled with polynucleotide kinase and then filled in using unlabeled dNTPs. Competitor oligonucleotides were blunt-ended, but not labeled. The indicated units of GST-Dpn bHLH were incubated for 30 min with 5×10^4 cpm probe and then electrophoresed on pre-run 0.25× TBE/4% polyacrylamide gels at 21 °C. In competition experiments, unlabeled probes were added immediately after labeled probes. Probe sequences are listed in Table 1.

Cell culture, 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, digoxigenin-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 corresponds to the number of X chromosomes. Embryo cell cycles were determined by nuclear 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.

Table 1
SxlPe oligonucleotides tested for Dpn DNA-binding in EMSA

	Oligo	Dpn-binding
Sites 1 and 2		
TTAGGTAGCCCACGCGACTGGCACGCGCACCTT	(1+2)	+
TTAGGTAGCCCcCGGGACTGGCACagGCACCTT	(1+2)m	−
Site 3		
GAAAGTACGCCTGGCACACTTCCTAGCG	3	+
AAGAAAGTACGCCTGGCACA	3L	−
CCTGGCACACTTCCTAGCGGGAT	3R	+
CCTGGCACACTTCCT	3C	+
CCTGGCACcCTTCCT	3Cm	−
Site 4		
ATAACATGCAGCTTGCCACGTTCCACC	4	+
CGTTCCACCTTTCGGCGT	4R	−
TAACATGCAGCTTGCCACG	4L	−
TTGCCACGTTCCAC	4C	+
TTGCCAaGcTCCAC	4Cm	−
Site 5		
GAACCAAAACGTGCGATTAGAG	5	+

Sequences and names of oligonucleotides used in electrophoretic mobility shift assays. Underlined sequences represent inferred hexameric core binding sites and lower case bases indicate mutated residues. Dpn DNA-binding capability is indicated as binding (+) or non-binding (−). Oligonucleotides carried an added GATC at each end.

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: Δdpn^2 (*Df(2R)dpn-2*) (Barbash and Cline, 1995), gro^{E48} (Jennings et al., 2006), *Df(3R)E(spl)*^{P11}, *E(spl)*$^-$, *HLHmγ*$^-$, *HLHmβ*$^-$, *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 or Sxl^{f1} males and their gro^{mat-} or hey^{mat-} progeny analyzed. Crosses between gro^{mat-} females and males with the deletion allele Sxl^{f7bO} produced too few embryos for analysis. 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.

Results

Dpn binds canonical and non-canonical sites at SxlPe

To identify Dpn-binding sites at *SxlPe* we expressed 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-

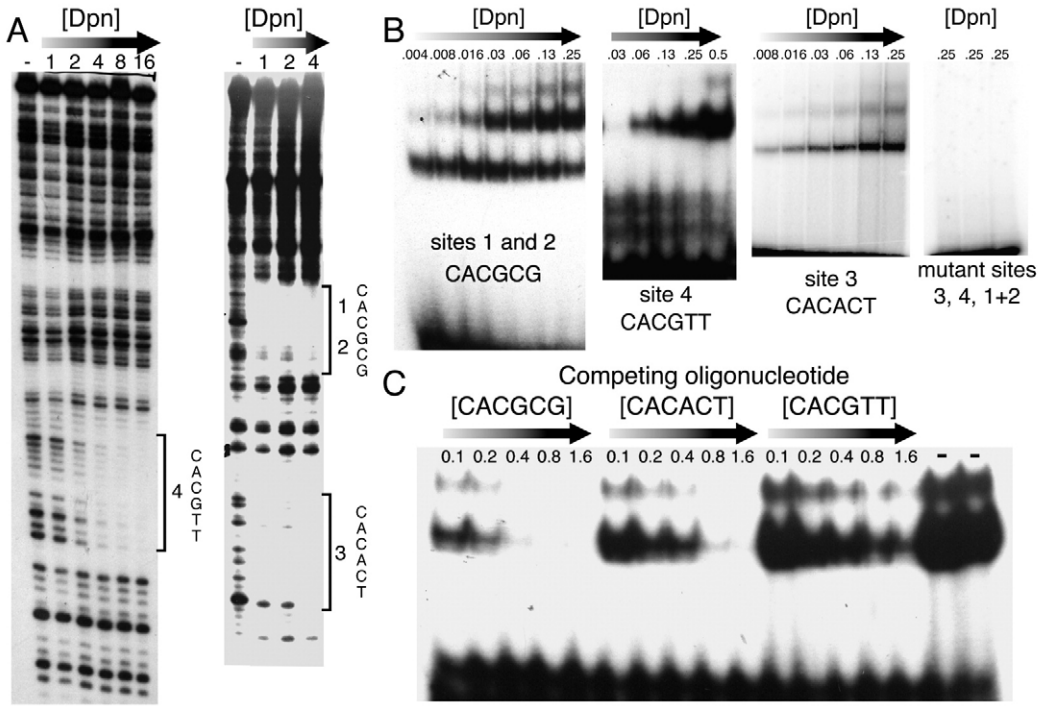

Fig. 1. Binding of Dpn to canonical and non-canonical DNA sequences at *SxlPe*. (A) DNase I footprinting with the indicated units of full-length MBP-Dpn fusion protein. One unit equaled 0.3 pmol (15 nM) MBP-Dpn. Left panel, protection of Dpn-binding site 4, right panel, protection of Dpn-binding sites 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 pmol (15 nM) GST-Dpn bHLH protein. Core sequences for the Dpn-sites are shown. Sequences of probes (1+2), 4a, 3, 3Cm, 4Cm and (1+2)m are in Table 1. (C) Binding site competition in EMSA. Complexes were formed between GST-Dpn bHLH protein (0.02 U) 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.

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 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 Dpn:DNA complexes (Winston et al., 1999) and mutations in the site 1 and 2 core sequences eliminated Dpn binding (Fig. 1B, Table 1). Consistent with the quantitative 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 (Table 1). 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 CACcCT (Fig. 1B, Table 1). 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 CAaGcT prevented Dpn binding in the gel-shift assay (Fig. 1C, Table 1). 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 (Table 1). 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

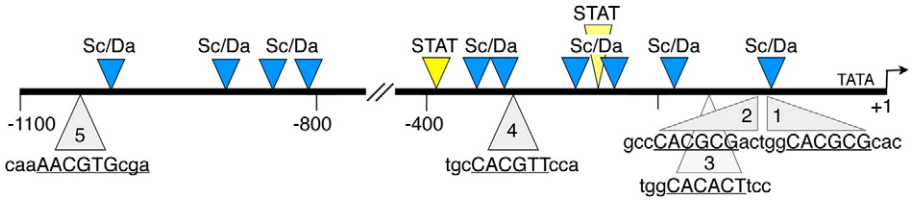

Fig. 2. Location of protein-binding sites at *SxlPe*. Diagram represents sequences from −1.1 kb to +1 relative to start of transcription. Triange apices denote positions of identified protein-binding sites. Ten binding sites for the activator Sc/Da (Yang et al. 2001) and two binding sites for the activator 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).

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), when used at the same concentration as Dpn, bound the same sequences with similar relative affinities (unpublished data).

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 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 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).

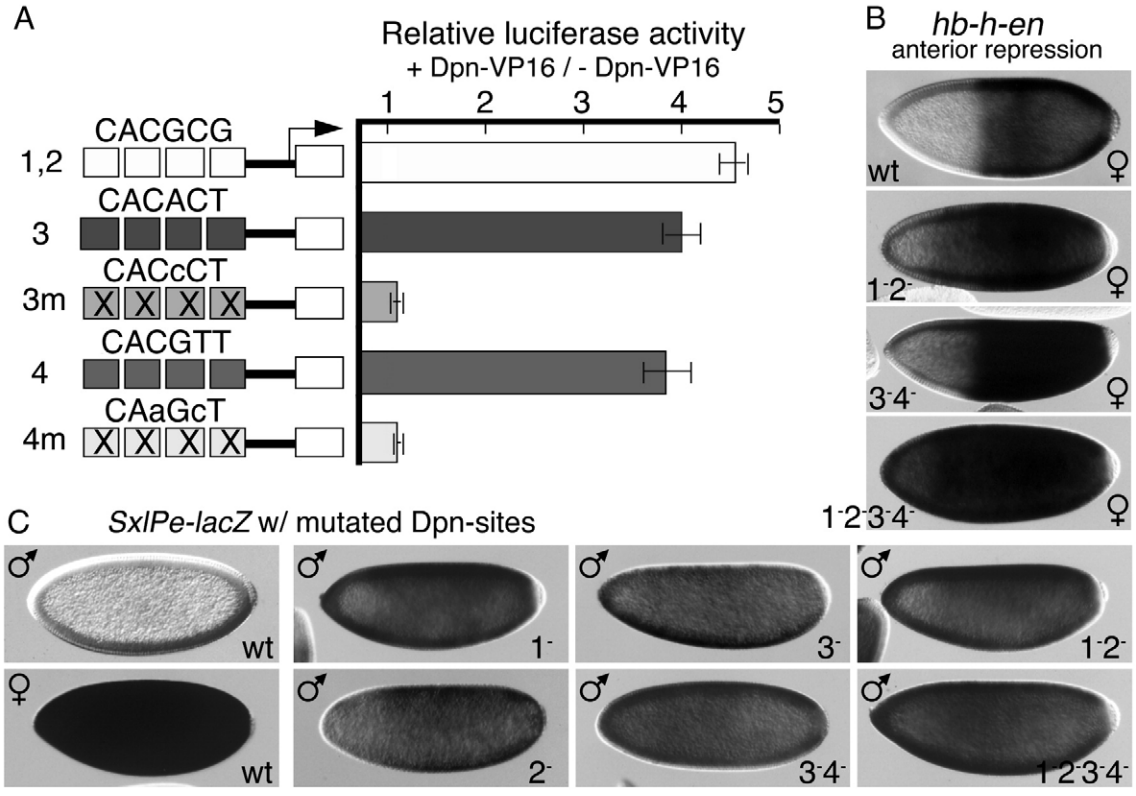

Fig. 3. Canonical and non-canonical DNA sequences mediate HES protein-binding at *SxlPe*. (A) Dpn-VP16 activates transcription in SL-2 cells via predicted Dpn-binding site 1, 2, 3, and 4 sequences. Four copies of Dpn-binding sequences were joined to a −95 bp *SxlPe*-luciferase reporter and co-transfected 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.

Table 2
Summary of expression of 1.4 kb *SxlPe-lacZ* transgene lines

SxlPe-lacZ	Embryo genotype	Onset lacZ expression (# transgene lines)	Comments
wt	+/+	XY cycle 13 (4) XX cycle 11 (4)	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) 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	Δdpn^2	XY cycle 13 (1) 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) 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) 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) XX cycle 11 (4)	XY, XX: like wt *SxlPe-lacZ* in Δdpn^2 genotype.
4^-	+/+	XY cycle 13 (2) XX cycle 11 (2)	XY, XX: indistinguishable from wt transgenes in +/+ genotype.
1^-2^-	+/+	XY cycle 12 (2) XX cycles 10 (2)	XY, XX: nuclei like 2^-. mRNA like 1^- but elevated.
3^-4^-	+/+	XY cycle 12 (3) XX cycle 11 (3)	XY, XX: indistinguishable from 3^- transgenes.
$1^-2^-3^-4^-$	+/+	XY cycle 12 (3) 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.

Abbreviations: 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 Δdpn^2. XY male, XX female chromosome complement.

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 2), 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, 1998; 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 implications for 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 (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*

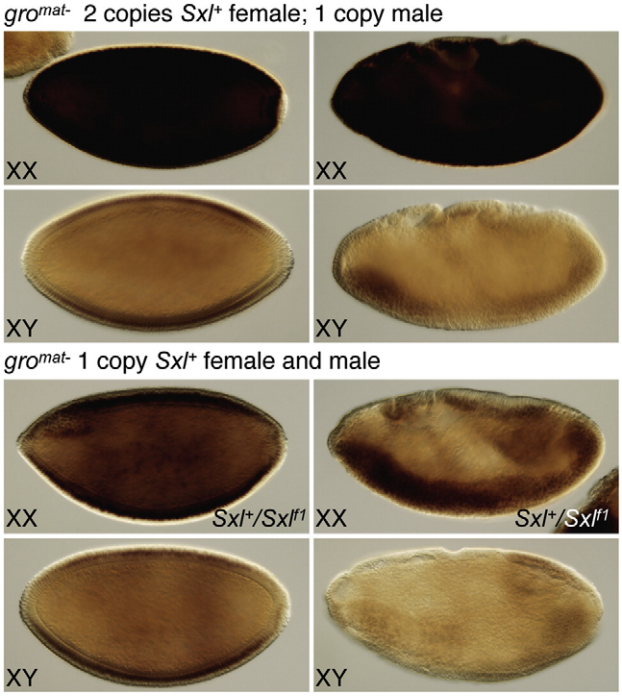

Fig. 4. *Sxl* protein in gro^{mat-} embryos. Embryos from mothers bearing gro^{E48} germline clones were immunostained stained for Sxl. Embryonic stages are mid-cellularization (left) and gastrulation (right). (Top panels) XX and XY embryos bearing normal doses of the X-linked *Sxl* gene. (Bottom panels) 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.

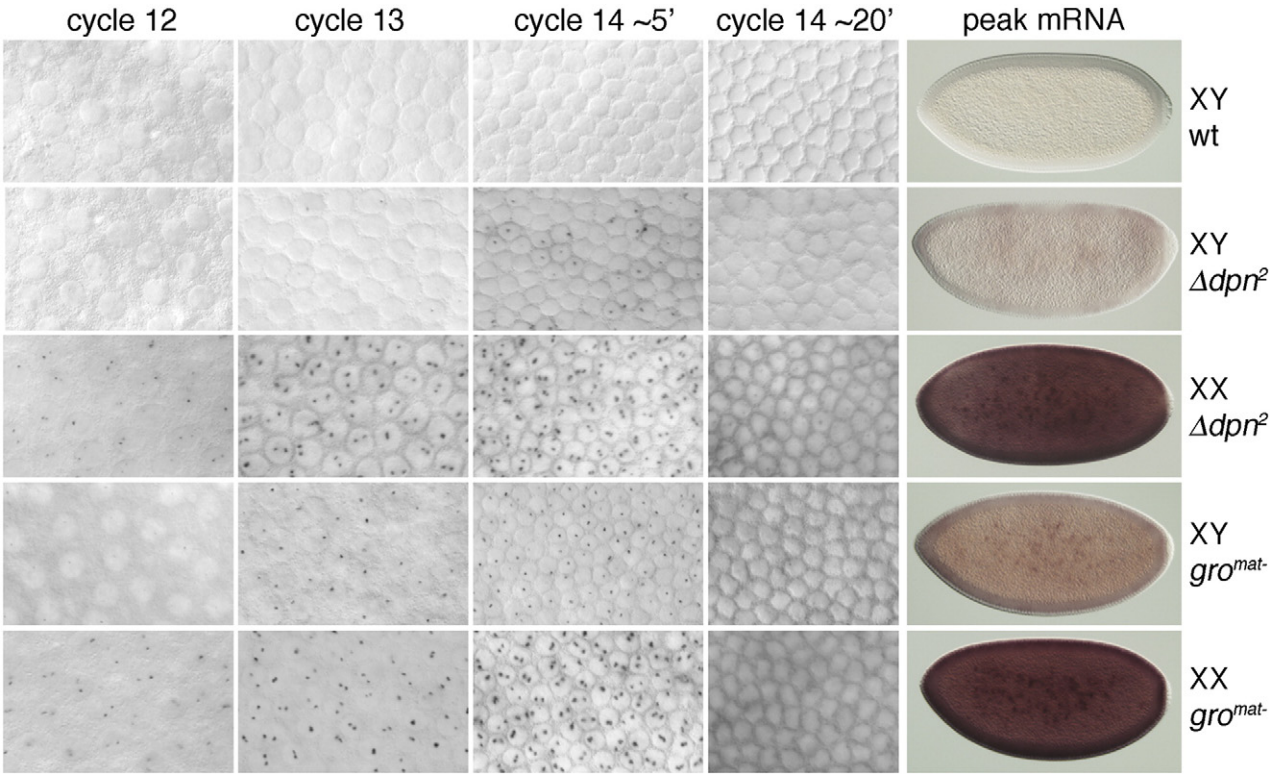

Fig. 5. Time course of *SxlPe* activation in wild-type, Δdpn^2, and maternal gro^{E48} mutant embryos. Wild-type and mutant embryos were stained following in situ hybridization. Black and white panels show surface views of embryonic nuclei at indicated nuclear cycles. Dots represent nascent transcripts 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}; Δdpn^2/CyO females and males, or females with *FRT82B* gro^{E48} germ lines and w^{1118}/Y males. Cycle 12 embryos from Δdpn^2/crosses could not be distinguished from Δdpn^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 Δdpn^2 heterozygotes.

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 Δdpn^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 2). We observed ectopic *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 Δdpn^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 2). 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 expression of a typical *SxlPe-lacZ* reporter more than it did 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 2). 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*)*m*γ, *E*(*spl*)*m*β, *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 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

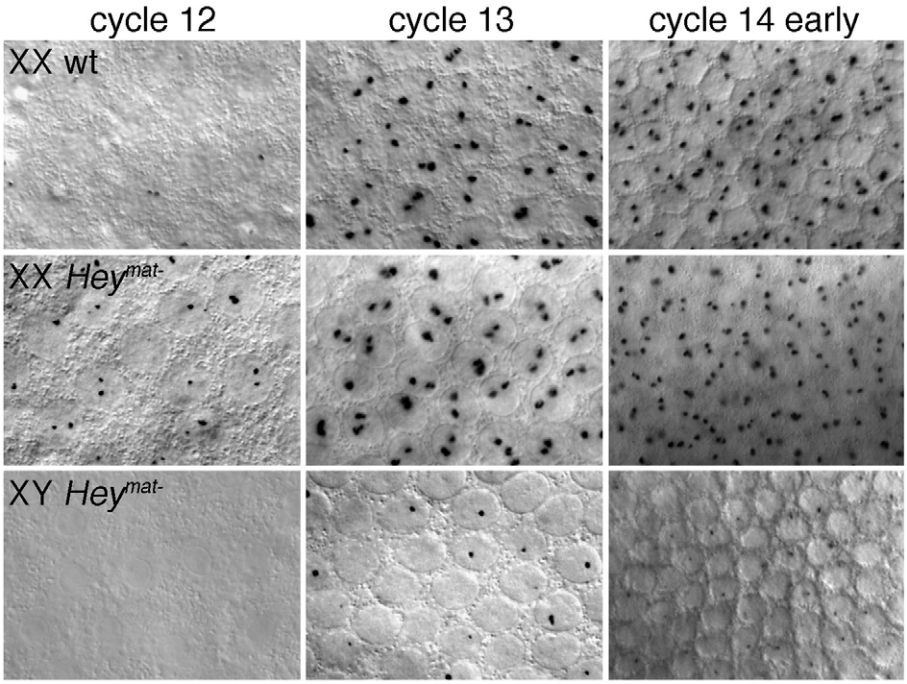

Fig. 6. Maternal *Hey* negatively regulates *SxlPe*. Surface views of embryos stained after in situ hybridization to detect nascent transcripts from the X-linked *SxlPe*. Top row: wild-type XX embryos. Middle and bottom rows: XX and XY progeny of mothers carrying Hey^{f06656} germline clones (Hey^{mat-}).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

explained by partial *Hey* protein function, or whether yet other HES family repressors regulate *SxlPe* remains to be determined.

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 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 2). 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, 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 2).

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 2). While the somewhat derepressed 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

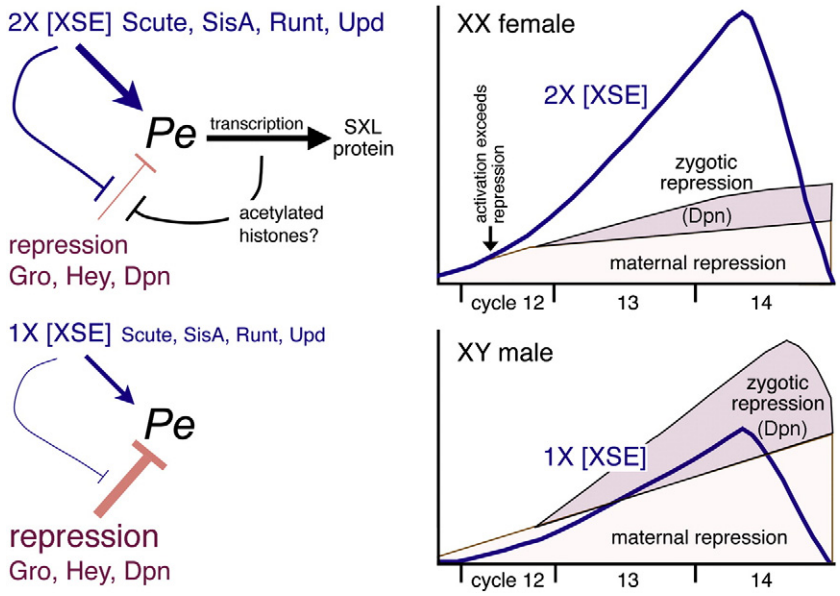

Fig. 7. Model for dose-sensitive regulation of *SxlPe*. (Top) In XX embryos Gro and other products of maternally supplied mRNAs establish initial threshold XSE concentrations by binding to *SxlPe*. XX embryos exceed threshold [XSE] in cycle 12. Increased histone acetylation, arising from the activation of *SxlPe*, inhibits Gro-mediated repression allowing the XSE proteins to more effectively stimulate transcription from *SxlPe* during cycles 13 and 14. (Bottom) In XY embryos continued translation of *gro* and other maternal mRNAs maintains repression potential above XY XSE concentrations until cycle 13. Zygotic expression of Dpn combined with maternal gro thereafter maintains repression potential above XY [XSE]. The amounts of XSEs differ by two-fold in XX and XY embryos. The amounts of Gro and other maternal or autosomal regulators are equal in both sexes, but their repressive potentials differ because of the proposed feedback mechanism. Y axis represents XSE concentrations and repressor/corepressor function. 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).

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. 7).

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-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 of 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.

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