Sex-lethal transcription by the Drosophila Runt protein

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SUMMARY

Runt functions as a transcriptional regulator in multiple developmental pathways in Drosophila melanogaster. Recent evidence indicates that Runt represses the transcription of several downstream target genes in the segmentation pathway. Here we demonstrate that runt also functions to activate transcription. The initial expression of the female-specific sex-determining gene Sex-lethal in the blastoderm embryo requires runt activity. Consistent with a role as a direct activator, Runt shows sequence-specific binding to multiple sites in the Sex-lethal early promoter. Using an in vivo transient assay, we demonstrate that Runt’s DNA-binding activity is essential for Sex-lethal activation in vivo. These experiments further reveal that increasing the dosage of runt alone is sufficient for triggering the transcriptional activation of Sex-lethal in males. In addition, a Runt fusion protein, containing a heterologous transcriptional activation domain activates Sex-lethal expression, indicating that this regulation is direct and not via repression of other repressors. Moreover, we demonstrate that a small segment of the Sex-lethal early promoter that contains Runt-binding sites mediates Runt-dependent transcriptional activation in vivo.

Key words: Runt domain, Sex-lethal, Sex determination, Transcriptional activation, AML1

INTRODUCTION

The Drosophila runt gene was the first recognised member of the Runt domain family of transcriptional regulators. These proteins function in developmental processes extending from pattern formation in the Drosophila embryo and eye, to the formation of blood and bone in mammals (Kania et al., 1990; Daga et al., 1996; Okuda et al., 1996; Speck and Stacy, 1996). A hallmark of this protein family is the Runt domain, a conserved 128 amino acid region that mediates sequence-specific DNA binding (Kagoshima et al., 1993). The DNA-binding function of the Runt domain was revealed upon the isolation of cDNAs for a murine transcription factor referred to as PEBP2 and/or CBF (Wang and Speck, 1992; Baé et al., 1993; Ogawa et al., 1993a). This factor, referred to here as CBF, is a heterodimer of two unrelated subunits. The CBFα subunit contains the Runt domain and mediates sequence-specific DNA binding while the CBFβ subunit contains the Runt domain and mediates interaction with a co-repressor protein Groucho. In further support of this, a reciprocal effect is observed on eve when a Runt fusion protein containing a VP16 activation domain is expressed during these same stages of embryogenesis (Jimenez et al., 1996). Repression of eve requires a C-terminal VWRPY motif that is conserved in all Runt domain proteins and mediates interaction with a co-repressor protein Groucho (Arason et al., 1997). Interestingly, only a subset of the genes that are repressed by runt require an intact VWRPY motif and the recruitment of Groucho for their repression (Arason et al., 1997). Finally, in cell culture transfection experiments, Runt can actively repress transcription (Fujioke et al., 1996; Arason et al., 1997). In contrast, Runt domain proteins are thought to act primarily as activators of transcription in mammals and sea urchins (Baé et al., 1994; Kurokawa et al., 1995; Tanaka et al., 1995; Coffman et al., 1996). The issue of
whether Runt can directly activate transcription in Drosophila has yet to be addressed.

One context in which runt is genetically identified as an activator is in the developmental pathway of sex determination. The target of runt in this pathway is the sex-determining gene Sex-lethal (Sxl) which is activated in female embryos during early embryogenesis in response to the X chromosome to autosome (X:A) ratio (Keyes et al., 1992). Embryos that have an X:A ratio of 1:1 (2X:2A) develop as females while 1X:2A embryos follow the male pathway of development (reviewed in Parkhurst and Meneely, 1994; Cline and Meyer, 1996). The decision to activate Sxl occurs at its own early promoter (SxlPc). The X:A signal is communicated to SxlPc through X-linked numerator genes, which have a dosage-dependent activating effect on Sxl transcription. The double dose of numerator genes present in female embryos results in activation of SxlPc, while the single dose in males is insufficient for activation leading to the male pathway of development. Genes that have been identified as X chromosome-linked numerator include sisterless-a (sisA), sisterless-b (sisB), sisterless-c (sisC) and runt (reviewed in Parkhurst and Meneely, 1994; Cline and Meyer, 1996). Numerator dosage is measured against a background of maternally supplied positive and negative regulators of Sxl, which include daughtercless (da), hermaphrodite (her), extramauchroaetae (emc) and groucho (Crommiller and Cline, 1986; Paroush et al., 1994; Pultz and Baker, 1995). In addition, zygotically expressed of the autosomal gene deadpan (dpn) acts as a negative regulator of Sxl and is required for repression of Sxl transcription in male embryos (Younger-Shepherd et al., 1992).

Evidence suggests that the transcriptional regulation of Sxl is due to direct interactions between different components of the X:A signaling system and SxlPc. For example, the dpn, sisB, da and sisA genes encode proteins with sequence homologies to the helix-loop-helix (HLH) or bZIP family of DNA-binding proteins. Cell transfection studies reveal that Da/Sis/B heterodimers cooperatively activate transcription of SxlPc which can be repressed by interactions between Dpn and SxlPc (Hoshijima et al., 1995). These findings support the idea that the DNA binding of transcription factors determines the on/off state of SxlPc.

runt was discovered to have an important role in the sex determination pathway due to vital dose-dependent interactions between runt and other components of the X:A signaling pathway (Duffy and Gergen, 1991; Torres and Sanchez, 1992). Loss of runt function results in the failure to activate SxlPc expression in the central region of female embryos (Duffy and Gergen, 1991). This is in contrast to other activators of Sxl, which are required throughout the embryo (Erickson and Cline, 1993; Deshpande et al., 1995). Although reducing runt dosage affects the activation of SxlPc in females (Estes et al., 1995), it has not yet been demonstrated that an increase in the dose of runt alone can lead to activation of SxlPc transcription in males. Duplications of runt have a limited ability to induce Sxl-dependent male-specific lethality (Torres and Sanchez, 1992). However, the interpretation of this result is obscured by the dose-dependent effects that runt has on segmentation (Gergen and Wieschaus, 1986).

In this study, we provide several lines of evidence that Runt directly activates Sxl transcription. We show that runt is required for the initial activation of SxlPc in females prior to runt’s requirement in segmentation. Runt binds sequence specifically to several sites in SxlPc and using an in vivo mRNA injection assay, we demonstrate that DNA binding by Runt is essential for SxlPc activation in vivo. These assays also reveal that increasing the dosage of runt alone is sufficient for triggering transcriptional activation of SxlPc in male embryos. We further show that fusing a heterologous transcriptional activation domain to Runt does not disrupt Runt’s ability to activate SxlPc, arguing against the possibility that regulation of Sxl occurs indirectly via repression of a repressor. Finally, we demonstrate that a small segment of SxlPc containing Runt-binding sites is required for promoter function and mediates runt-dependent activation. We discuss the significance of these results in demonstrating the function of Run domain proteins as context-dependent regulators of gene expression.

MATERIALS AND METHODS

Plasmid constructs

runt[CK] was made using the Exsite PCR-Based Site-Directed Mutagenesis Kit (Stratagene). The oligonucleotides used for the C127S and K199A substitutions were 5'-GATCGTCCCAAGGCGAGAGATCCGTAGTCAGC-3' and 5'-GGAGACGCGCCGCTTCTCCAGCC-3', respectively. rantrAD was made by deleting an internal 345 base pair Sall-PstI fragment from the runt cDNA. pCS2+runt constructs were generated using BamHI-EcoRI fragments from relevant runt cDNAs. To generate runt[VP16], a BamHI-XhoI fragment from pCS2+runt was cloned into pCS2+VP16-N resulting in VP16 (nucleotides 1681-1923) fused in frame to the N terminus of runt. pQE30runt and pQE30bro were described previously (Pepling and Gergen, 1995; Golling et al., 1996). pQE30runt[CK] was generated from a BamHI fragment of runt[CK].

Drosophila stocks

The runt alleles that were used in this study include the temperature-sensitive runt[VP17] allele (Gergen and Wieschaus, 1986) and the loss-of-function runt[85] allele (Gergen and Butler, 1988), which produces no detectable mRNA. For X-gal stainings, the allele runt[1296] was used, which produces no detectable protein accumulation in blastoderm embryos.

Genetics

Female embryos homozygous for SxlPc-lacZ runt[85] were generated by mating SxlPc-lacZ runt[1297]FM7 females to SxlPc-lacZ runt[85] males carrying the Y-chromosome duplication y+Ymal102. 1/4 of the embryos from this cross will be females homozygous for runt[85]. Female embryos homozygous for SxlPc-lacZ runt[85] were generated by mating SxlPc-lacZ runt[85]FM7 males to SxlPc-lacZ runt[85] males carrying the Y-chromosome duplication y+Ymal102.

Embryo manipulation

The in situ hybridization protocol used was described in Klingler and Gergen (1993). A digoxigenin-labeled riboprobe to detect lacZ mRNA was synthesized as described in Tsai and Gergen (1994). Immunohistochemistry of whole embryos was performed as in Kania et al. (1990). The β-gal antibody (Cappel) was used at a dilution of 1:5000. The Engrailed antibody used in these experiments was a mouse monoclonal (Patel et al., 1989).

Temperature-shift experiments were carried out as follows: SxlPc-lacZ runt[1297] embryos were either collected at the permissive temperature (18°C) for 30 minutes intervals and shifted to the non-permissive temperature (29°C) or collected at 29°C and shifted to 18°C. After dechorionation and fixation, embryos were subjected to
whole-mount immunohistochemistry as described above. Embryos were double labeled with an antibody against the Engrailed and β-galactosidase proteins and developed by HRP staining. The persistence of the β-galactosidase protein allows SxlPe :lacZ reporter gene activity to be examined at the germ-band-extended stage.

For X-gal staining, embryos were collected on apple juice agar plates at 25°C for 2 hours and maintained at 25°C for 5 hours. The embryos were washed extensively in water and dechorionated in a 2.5% solution of NaClO for 5 minutes. Following a PBS wash, the dechorionated embryos were fixed in a heptane solution saturated with 25% glutaraldehyde/PBS for 15–20 minutes at 21°C, and washed with PBS and 0.3% Triton X-100 (PBT) until they settled to the bottom of the tube without sticking together. The embryos were then washed with 5 mM K3Fe(CN)6 and 5 mM K4Fe(CN)6 solution and incubated with the same solution containing 0.24% X-gal for ~5 hours at 21°C. The stained embryos were washed with two changes of PBT and mounted in 50% glycerol/PBS.

Generation of transgenes

All transgenes were produced by cloning EcoRI-BsgII fragments of the respective Saxl constructs into the EcoRI and BamHI sites of the pCaSpeR-AUG-β-galactosidase vector. SaxlPe, saxl :lacZ was created from a pBlueScript Saxl plasmid which has a SnoBI site engineered at ~270. This plasmid was digested with BamHI, the overhanging ends were filled using the Klenow fragment of DNA polymerase, and then digested with SnoBI. The resulting linear plasmid was re-ligated with the desired deletion. The deletion was confirmed by sequence analysis. The SaxlPeGOF :lacZ promoter construct consists of the 0.4 kb Saxl sequence with four copies of a PCR-generated fragment inserted into the flanking polylinker EcoRV site. The PCR fragment was generated using the primers 5¢-CGGATACAATAAGAAGTACACC-3¢ and 5¢-CGGATACGGGATCCTCAG-3¢ and 5¢-TTTCTCTGAGGCAAATTG-3¢, 3¢-AGCAGAAAAAGGCA-GCTGCA-3¢ and 5¢-CITCCATGAGGCGATACAAC-3¢, 4¢-GGTGCGTGATTTGCTCAG-3¢ and 5¢-ATTTGGACCCGATCGCCTAG-3¢. The sizes of the amplified probes are 337, 253, 356 and 361 base pairs, respectively. Relative binding of WT and Runt[CK] proteins was quantified using the Molecular Dynamics ImageQuant software. The probe containing the DNA segment that is transcribed was generated by PCR amplification from plasmid IM16 using the primers 5¢-CGGATACCGGATCCTTCGC-3¢ and 5¢-CGGATACCATACGGGCTATCC-3¢.

RESULTS

runt functions in the initial activation of Saxl transcription

Previous work demonstrated a region-specific requirement for runt in the activation of Saxl expression in female embryos and indicated that this effect is at the transcriptional level (Duffy and Gergen, 1991; Estes et al., 1995). Here, we used in situ hybridization to define more precisely the earliest effects of runt on transcription from the Saxl early embryonic promoter (SxlP). Wild-type female embryos containing a SxlP :lacZ reporter gene begin to express lacZ transcripts during the syncitial nuclear division cycles preceding formation of the cellular blastoderm. Expression at nuclear division cycle 12 is observed in punctate dots distributed throughout the embryo except in pole cells (Fig. 1A). Later, this expression is seen as uniform staining throughout the embryo except in pole cells (Fig. 1B). Females homozygous for the amorphic runtLBS mutation fail to express the SxlP :lacZ reporter gene within a broad central region of the embryo (Fig. 1D). This defect is observed concomitantly with the earliest detectable expression of this reporter gene (Fig. 1C), demonstrating an early requirement for runt in SxlP activation.

The alterations in Saxl expression observed in runt mutants correspond well to the initial expression of runt in a broad central domain of syncitial blastoderm stage embryos ( Klingler and Gergen, 1993). This expression precedes the formation of the seven-stripe pair-rule pattern during cellularization, suggesting that runt’s function in Saxl activation can be temporally separated from its role in segmentation. To test this
Expression is most abundant within the nuclei (A). Later, during the earliest stages, this expression is most abundant within the nuclei (A). Later, during the earliest stages, this expression is most abundant within the nuclei (A). Later, during the earliest stages, this expression is most abundant within the nuclei (A). Later, during the earliest stages, this expression is most abundant within the nuclei (A).

**Fig. 1.** Mutation in *runt* affects early transcriptional activation of *SxlP*. Expression of *SxlP-lacZ* is visualized by in situ hybridization with a *lacZ* riboprobe. Embryos in this, and all other figures, are oriented anterior to the left and dorsal side up. In wild-type female embryos, *SxlP* is activated around cycle 12 throughout the embryo except within the pole cells. During the earliest stages, this expression is most abundant within the nuclei (A). Later, during cellularization, *SxlP* expression is at higher levels and seen as uniform staining in the cytoplasm (B). In female embryos homozygous for *runt*^4BS*, activation of *SxlP* is absent from the central region of the embryo in both early (C) and late (D) embryos. The embryo shown in C is slightly older than A as evident from the increased staining in the cytoplasm.

The early regulation of *Sxl* transcription by *runt* is readily explained if Runt interacts directly with the *Sxl* early promoter to activate transcription. Previous work identified a 1.1 kb fragment of the *SxlP* promoter that contains sequences essential for sex-specific transcriptional activation (Estes et al., 1995). We tested for direct interactions between Runt and these DNA sequences. Probes that span this DNA fragment were generated (Fig. 3A) and tested in electrophoretic mobility-shift assays (EMSAs). Runt binds only weakly to each of these DNA fragments (Fig. 3B). However, upon addition of the Bro partner protein, multiple complexes are obtained with each of these probes. These complexes are Runt-dependent, as they are not detected when only Bro protein is added (data not shown).

Competition with a bona fide CBF-binding site from the Polyoma enhancer prevents detection of these complexes (Fig. 3B). Competition was not observed when a mutant CBF-binding site was used (data not shown), indicating that the binding is sequence specific. Recombinant mammalian CBF also recognizes multiple sites within these fragments from the *SxlP* promoter (Fig. 3B). Inspection of the sequence for matches to the consensus CBF-binding sequence TG(T/C)GGT(T/C) (Melnikova et al., 1993) identifies ten sites that match this consensus at positions two through five that also match at least one of the three other, less critical positions. (Fig. 3A). Interestingly, no perfect matches to the consensus were found. The presence of multiple binding sites is consistent with the hypothesis that activation of *Sxl* transcription involves direct interactions between Runt and the *Sxl* promoter. One prediction of this hypothesis is that Runt’s DNA-binding activity should be required for *Sxl* activation. In order to test this hypothesis, it was necessary to develop an in vivo assay for *runt* function.

**An in vivo assay for *runt* function**

In homozygous *runt*^4BS* female embryos, *SxlP* fails to be activated in the central region of the embryo (Fig. 1C,D). To investigate *runt*’s role in *SxlP* activation, we wished to test *runt* derivatives for their ability to rescue *SxlP* expression in these

**Fig. 2.** Temporally distinct requirements for *runt* in sex determination and segmentation. Expression of the *SxlP-lacZ* reporter gene and *en* are visualized with antibodies that detect the β-galactosidase (β-gal) and En proteins, respectively. The embryos shown are from a cross in which all the embryos carry the *SxlP-lacZ* reporter gene and 1/4 of the embryos are homozygous for the temperature-sensitive *runt*^VP17* mutation. The embryo in A is from a collection of embryos that were reared and collected at the permissive temperature (18°C). All of the embryos in this collection show the wild type, regularly spaced pattern of En expression and 50% of the embryos show the normal, uniform expression of the *SxlP-lacZ* reporter gene as in wild-type females. In contrast, when embryos from this cross are reared and collected at the non-permissive temperature (29°C), 1/4 of the embryos show abnormal expression of both *Sxl* and En (B). In collections of embryos aged at 29°C for 2 hours and then shifted 18°C, female embryos with the abnormal *Sxl* expression pattern typical of *runt* mutants showed normal En expression (C). Female embryos which were aged at 18°C to the cellular blastoderm stage and then shifted to 29°C showed normal *SxlP* expression and abnormal En expression (D).
of the embryo, the number of females that show the typical mutant expression pattern is dramatically reduced. Instead, nearly 50% of the embryos, or nearly all of the females, show the uniform Sxlp⁻lacZ expression pattern typical of wild-type females (Fig. 4B; Table 1). Thus, injection of runt mRNA rescues Sxlp expression in embryos mutant for runt.

**DNA-binding activity of the Runt domain is required for Sxlp activation**

The 128 amino acid Runt domain confers sequence-specific DNA binding as well as heterodimerization with Brother (Golling et al., 1996). As an initial test of the importance of Runt’s DNA-binding domain, we injected a form of runt that was deleted for its Runt domain, runt∆RD into the central region of the embryo, the number of females that show the typical mutant expression pattern is dramatically reduced. Instead, nearly 50% of the embryos, or nearly all of the females, show the uniform Sxlp⁻lacZ expression pattern typical of wild-type females (Fig. 4B; Table 1). Thus, injection of runt mRNA rescues Sxlp expression in embryos mutant for runt.

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region of female homozygous *runt*Δ*BD* embryos. We saw no evidence of rescue in *runt*ΔRD-injected embryos, indicating that the DNA-binding domain is important for *runt*’s function as an activator of *Sxl* (Table 1). However, as this is a large deletion, the effects could be attributed to improper folding and/or protein stability.

Random- and site-directed mutagenesis experiments have identified several amino acids within the Runt domain that specifically affect DNA binding without disrupting association with the partner protein CBFβ. (Lenny et al., 1995; Kurokawa et al., 1996; Akamatsu et al., 1997a,b). Two conserved amino acids that are important for DNA binding correspond to a cysteine at position 127 and a lysine at position 199 in Runt. In order to obtain a DNA-binding-defective form of Runt, we generated a protein containing mutations at both of these sites (C127S, K199A). We first compared the DNA-binding activity of this mutant with that of wild-type Runt in EMSAs with the high-affinity CBF-binding site from the Polyoma virus enhancer. The mutant protein, Runt[CK] shows only very low levels of complex formation on this DNA, and this is only in the presence of Bro (Fig. 3C). Similar experiments with a DNA probe from the *Sxl* promoter confirm the reduced DNA-binding activity of Runt[CK] (Fig. 3D). We estimate that these mutations reduce DNA-binding affinity at least 50-fold. The observation that Bro stimulates DNA binding by Runt[CK] suggests that the two mutations do not disrupt interaction between the Runt and Bro proteins. We confirmed this in the yeast two-hybrid system (data not shown). Thus, these two mutations specifically impair DNA binding without affecting the overall structure of the Runt domain. We used the mRNA injection assay to examine the in vivo activity of this DNA-binding-defective form of Runt and found no evidence for rescue of *SxlPv* expression in *runt* mutant female embryos (Fig. 4C; Table 1). These results are consistent with the hypothesis that Runt activates *Sxl* transcription by binding to sequences in the *SxlPv* promoter.

**Increased *runt* dosage is sufficient for *SxlPv* activation in males**

In the course of performing the above experiments, we noted that a subset of embryos injected with WT *runt* mRNA displayed a novel expression pattern. These embryos expressed *SxlPv* only in the central region of the embryo and were presumed to be males that were inappropriately activating *SxlPv* due to increased *runt* dosage. The interpretation of this observation is complicated by the fact that the males in the above experiments all have a Y chromosome duplication containing a wild-type copy of *runt*, as well as a number of other X-chromosome-linked genes. In order to determine if this effect was due solely to the increase in *runt* dosage, we performed injection experiments in embryos that carry the *Sxl*::*lacZ* transgene but that are wild-type with respect to the dosage of X-chromosome-linked numerator elements. We found that nearly all of the embryos injected with WT *runt* mRNA expressed the *Sxl*::*lacZ* transgene in vivo. In situ hybridization was used to detect expression of the *Sxl*::*lacZ* reporter gene. (A) Embryo injected with buffer alone. (B) Injection of WT *runt* mRNA rescues the *runt*ΔRD phenotype. (C) Injection of an mRNA that encodes the mutant Runt[CK] protein is unable to rescue.

**Table 1. DNA-binding activity of Runt is required for *Sxl* activation in females**

<table>
<thead>
<tr>
<th>Total no. of embryos scored&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Injected mRNA</th>
<th>% Female embryos with <em>lacZ</em> pattern</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Uniform&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>90 buffer</td>
<td>53</td>
<td>0</td>
</tr>
<tr>
<td>90 <em>runt</em>WT</td>
<td>85</td>
<td>12</td>
</tr>
<tr>
<td>84 <em>runt</em>ΔRD</td>
<td>51</td>
<td>0</td>
</tr>
<tr>
<td>70 Runt[CK]&lt;sup&gt;e&lt;/sup&gt;</td>
<td>47</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Only female embryos that represent approximately 50% of a total population of embryos are represented here. These embryos were identified based on their *lacZ* staining pattern as described in Materials and Methods.

<sup>b</sup>These embryos showed dark uniform staining throughout the embryo except for in pole cells.

<sup>c</sup>Partial rescue was identified as strong staining at the poles and lighter staining in the central region.

<sup>d</sup>Mutant embryos had strong staining at the poles but lacked any detectable staining in the central region.

<sup>e</sup>CK refers to the (C127S,K199A) double substitution in Runt.

**Table 2. Injection of *runt* mRNA activates *SxlPv* expression in wild-type males**

<table>
<thead>
<tr>
<th>Total no. of embryos scored</th>
<th>Injected mRNA</th>
<th>% Embryos with <em>lacZ</em> pattern</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Uniform</td>
</tr>
<tr>
<td>184 buffer</td>
<td>51</td>
<td>0</td>
</tr>
<tr>
<td>312 <em>runt</em>WT</td>
<td>48</td>
<td>47</td>
</tr>
<tr>
<td>147 <em>runt</em>ΔRD</td>
<td>52</td>
<td>0</td>
</tr>
<tr>
<td>226 Runt[CK]&lt;sup&gt;e&lt;/sup&gt;</td>
<td>49</td>
<td>0</td>
</tr>
<tr>
<td>213 <em>runt</em>WT (into head)</td>
<td>52</td>
<td>38</td>
</tr>
<tr>
<td>206 <em>runt</em>[VP16]</td>
<td>47</td>
<td>25</td>
</tr>
</tbody>
</table>

<sup>e</sup>CK refers to the (C127S,K199A) double substitution in Runt.
transcriptional activation of the Sxl gene in male embryos. Further, similar to the results obtained in the rescue experiments in runt mutant females, transcriptional activation of the Sxl gene in males requires DNA binding by Runt.

The requirement for runt in activating Sxl transcription only in the central region of female embryos is unique amongst the X-chromosome-linked numerators. The experiments above show that injection of runt mRNA into this central region is sufficient for ectopic activation of Sxl transcription in males. To test whether runt can activate SxlPe outside of this central domain, we injected runt mRNA near the anterior end of male embryos. In this population, approximately half of the embryos show the normal female pattern of expression and 38% showed strong SxlPe expression in the anterior end (Fig. 5B; Table 2). Activation of SxlPe was also observed upon injection near the posterior end (data not shown). This demonstrates that runt can act in regions outside its normal expression domain.

Runt[VP16] activates SxlPe

The above experiments demonstrate that runt plays a positive role in activating Sxl transcription, and indicate that this activation requires DNA binding by the Runt protein. Although these results are consistent with direct activation by Runt, they can also be explained if Runt acted indirectly by repressing the expression of other repressors of SxlPe. In order to distinguish between direct and indirect models, we used our in vivo assay to examine the activity of a Runt derivative containing a heterologous activation domain from the VP16 transcriptional regulator (Triezenberg et al., 1988). This experiment is analogous to those done by Jimenez et al. (1996) to provide evidence that Runt acts to repress transcription in the pathway of segmentation. In these experiments, a runt VP16 fusion activated genes that are normally repressed by runt. If Runt is a dedicated repressor that signals SxlPe indirectly, then the regulatory effects of injecting this Runt[VP16] derivative would be opposite to the effects of injecting Runt and could lead to repression of SxlPe expression in females. More importantly, however, this fusion protein should lose the ability to trigger Runt-dependent activation of SxlPe expression in injected males. Injection of Runt[VP16] does not affect SxlPe expression in females and activates SxlPe transcription in injected males (Fig. 5C; Table 2). These results argue strongly against an indirect model and provide evidence in support of

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**Fig. 5.** Increasing runt dosage triggers activation of SxlPe in males. In situ hybridization was used to detect expression of the SxlPe::lacZ reporter gene. (A) Embryo injected with WT runt mRNA that shows activation of SxlPe in a broad central domain of the embryo. Activation is also observed when runt is injected near the anterior end (B). Injection of a Runt fusion protein containing the VP16 activation domain also results in ectopic activation in male embryos, although this activation is slightly weaker than that seen with WT runt (C).

**Fig. 6.** Runt binds to a conserved site in a minimal region of SxlPe. (A) Schematic diagram of the minimal functional segment of SxlPe. The reporter SxlPe::lacZ is composed of sequences extending from −389 to +47 and contains several potential Runt-binding sites (shaded blocks). (B) A portion of this segment containing two Runt-binding sites is deleted in the construct SxlPeΔB as indicated by the gap in the schematic. (C) The nucleotide sequence of the fragment between −284 and −212 is shown with the potential Runt sites underlined. This fragment is the sequence multimerized SxlPeGOF. (D) EMSA using a probe from this fragment demonstrating that Runt binds specifically to sequences within this region of SxlPe.
the hypothesis that Runt acts directly to activate SxlPe transcription.

A small segment of SxlPe mediates Runt-dependent activation

A prediction of the model that Runt interacts directly with the Sxl promoter to activate transcription is that mutation of the Runt-binding sites will lead to a loss of expression. However, execution of this conceptually straightforward experiment is impeded by the presence of numerous putative binding sites within the full-length SxlPe promoter. As an alternative, we investigated interactions between Runt and a previously identified proximal 400 basepair (bp) fragment of SxlPe, SxlPe0.4kb: lacZ (Estes et al., 1995) (Fig. 6A). Although this truncated reporter gene exhibits an abnormal pattern of expression in wild-type females with higher levels found in the anterior and posterior (Fig. 7A), the expression is sex-specific. There are several putative Runt-binding sites found within this 400 bp fragment (Fig. 6A). Deletion of a small 70 bp segment within this fragment, which contains at least two putative binding sites for Runt (Fig. 6B), results in a loss of SxlPe expression (Fig. 7B). Conversely, a reporter gene that contains multiple copies of this segment, SxlPeGOF: lacZ (Fig. 6C), is expressed at high levels in WT female embryos (Fig. 7C). Interestingly, the SxlPeGOF: lacZ reporter gene is also expressed in males, however, at much lower levels and not in the anterior regions of the embryo (Fig. 7D). EMSA with Runt and Bro proteins demonstrates that Runt binds to sequences within this small segment (Fig. 6D). This interaction is sequence specific as it is competed by a DNA fragment from the Polyoma enhancer containing a wild-type CBF-binding site, but not by a similar DNA fragment with a mutant CBF-binding site (data not shown). The differential expression in female and male embryos indicates that this reporter gene retains the ability to respond to numerator gene dosage. The observation that this transgene is expressed in males suggests that the activation mediated by multimerization of this small segment of DNA is sufficient to overcome the repression that is normally established in males for the parental SxlPe0.4kb: lacZ reporter gene. Furthermore, the preferential expression within the segmented region of the embryo strongly suggests that this reporter gene is responding to runt. To test this, we examined SxlPeGOF: lacZ expression in embryos mutant for runt. Expression is reduced in most, but not all, regions of runt mutant male embryos (Fig. 7E). Thus, the region that is multimerized in the SxlPeGOF: lacZ reporter gene mediates runt-dependent transcriptional activation.

DISCUSSION

In this study, we provide several lines of evidence indicating that Runt activates Sxl transcription by interacting directly with the Sxl early promoter. There are several binding sites for Runt within the regulatory elements that are responsible for the early sex-specific regulation of Sxl transcription and a mutant form of Runt that is impaired for DNA binding fails to activate SxlPe in vivo. Furthermore, a derivative of Runt containing a heterologous transcriptional activation domain activates rather than represses SxlPe in males. This result argues strongly against a model where runt activates Sxl expression solely by repressing the expression of other repressors. Finally we demonstrate that a small segment of the Sxl promoter that contains several putative binding sites for Runt is required for promoter function and mediates runt-dependent transcriptional activation.
level of runt activity is sufficient for triggering the activation of Sxl transcription in male embryos that contain the normal dosage of the other X-chromosome-linked numerator elements. This result unequivocally establishes runt’s identity as a numerator element. runt’s role in Sxl activation is unique in that its requirements are region specific. In female embryos deleted for runt, SxlPe fails to be expressed in the central region of the embryo, while expression is still observed at the poles (Duffy and Gergen, 1991). Interestingly, runt is capable of activating SxlPe transcription at the poles of male embryos, where runt expression is normally not observed. This indicates that these regions of the embryo, which normally do not require runt for Sxl activation, are in fact-sensitive to runt dosage. We previously demonstrated that Sxl transcription is regulated by other position-specific activating and/or repressing cues (Duffy and Gergen, 1991). These could be either additional positive regulators that act at the poles of the embryo, or negative regulators with higher levels of activity in the central regions of the embryo. A candidate for one such negative regulator is the product of the dpn gene. Male embryos mutant for dpn show ectopic activation of Sxl expression, preferentially within the central, pre-segmented region of the embryo (Younger-Shepherd et al., 1992; S. G. K. and J. P. G., unpublished observations). Thus, it is possible that a major role of runt in the regulation of Sxl transcription is to counteract repression by dpn. However, the observation that runt can trigger activation of Sxl at the poles indicates that Runt must also be able to interact in a more general way with other positive and negative regulatory components of the X:A signaling system.

**Mechanism of transcriptional activation by Runt**

Here, we have provided several lines of evidence indicating that Runt functions directly to activate Sxl transcription. The question that remains however, is the mechanism by which Runt activates SxlPe transcription, especially in light of several observations indicating that Runt also functions as a transcriptional repressor (Manoukian and Krause, 1993; Tsai and Gergen, 1994; Jimenez et al., 1996; Aronson et al., 1997). One possibility is that Runt bound to SxlPe acts directly as a transcriptional activator. Full-length Runt does not activate in yeast. However, experiments using the yeast two-hybrid system suggest that Runt contains an activation domain that is masked in the context of the full-length protein (G. Golling and J. P. G., unpublished). Perhaps this putative activation domain is unmasked when Runt is bound to the Sxl promoter in the embryo.

Previous studies on the structure and regulation of the Sxl embryonic promoter led to the proposal that dose-dependent transcriptional activation is obtained through the cooperative binding of numerator proteins to multiple, low-affinity binding sites (Estes et al., 1995). Consistent with this, inspection of the SxlPe sequence reveals several potential bindings sites for Runt, all of which appear to be low-affinity sites based on studies done with the mammalian Runt domain proteins. Further studies are required to reveal whether Runt binds DNA cooperatively with either SisA and/or SisB. Our injection experiments demonstrate that increasing the dosage of runt alone is sufficient for triggering transcriptional activation in males. A key question is whether this activation is due solely to an increase in the concentration of Runt bound to the Sxl promoter, or whether the increased concentration of Runt in these male embryos drives the cooperative assembly of complexes that also contain other numerator proteins.

The above models are based on Runt contributing directly to the transcriptional activation of SxlPe. Alternatively, runt may function as a more passive activator of transcription. For example, the binding of Runt to SxlPe may interfere with the binding of other transcriptional repressors of SxlPe such as Dpn. It is also possible that Runt functions to ‘quench’ active repression of SxlPe in a manner analogous to the quenching mechanism exhibited by the zinc finger repressor proteins Kruppel and snail (Gray and Levine 1996). For example, Runt has been shown to interact with Gro to mediate transcriptional repression in the segmentation pathway (Aronson et al., 1997). Gro is also required to repress SxlPe in male embryos (Parouch et al., 1994). It is possible that Runt bound to SxlPe interacts with Gro in a manner that blocks Gro-mediated repression.

In any event, the activity of Runt must be regulated to account for its role as an activator, rather than a repressor, of Sxl transcription. For example, either Runt’s interaction with the co-repressor protein Groucho must be blocked, or the activity of the Runt-Groucho complex must be prevented from repressing transcription of the Sxl promoter. This regulation is not simply developmental, as the transcription of other target genes is repressed in the same cells in which Runt activates Sxl expression. Specific interactions with other DNA-bound regulatory proteins are almost certain to account for the contrasting effects of Runt on the transcription of different target genes. The genetic advantages of the Drosophila system, in conjunction with the accessibility of the blastoderm embryo to experimental manipulation, make Sxl regulation a powerful model system for further investigating the molecular mechanisms used by Runt domain proteins to regulate gene expression during development.

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