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# Direct activation of *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; Bae 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 does not contact DNA directly but interacts with the Runt domain of the α subunit to increase DNA-binding affinity (Kagoshima et al., 1993; Ogawa et al., 1993b). There are two *Drosophila* homologues of mammalian *CBFβ*, the *Brother* (*Bro*) and *Big-brother* (*Bgb*) genes (Golling et al., 1996). The embryonic expression patterns of these genes overlap with *runt* and DNA-binding studies have confirmed that Bro and Bgb stimulate Runt's ability to bind DNA (Golling et al., 1996), supporting the idea that Runt functions as the DNA-binding subunit of a CBF-like factor. These observations strongly suggest that *runt*'s role as a developmental regulator during *Drosophila* embryogenesis will involve DNA binding and transcriptional regulation.

Genetic studies reveal that *runt* acts as a transcriptional activator of some genes and a repressor of others. However, it has not been demonstrated that direct DNA binding accounts for any of these regulatory interactions in vivo. Several lines of evidence indicate that *runt* acts as a transcriptional repressor. In early embryos, ectopic *runt* causes rapid repression of *even-skipped* (*eve*), indicating potential direct regulation (Manoukian and Krause, 1993). 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 (Aronson 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 (Aronson et al., 1997). Finally, in cell culture transfection experiments, Runt can actively repress transcription (Fujioka et al., 1996; Aronson et al., 1997). In contrast, Runt domain proteins are thought to act primarily as activators of transcription in mammals and sea urchins (Bae 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 ($Sxl_{Pe}$). The X:A signal is communicated to $Sxl_{Pe}$ 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 $Sxl_{Pe}$, 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 numerators 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 *daughterless* (*da*), *hermaphrodite* (*her*), *extramachrochaetae* (*emc*) and *groucho* (Cronmiller and Cline, 1986; Paroush et al., 1994; Pultz and Baker, 1995). In addition, zygotic expression 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 $Sxl_{Pe}$. 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/SisB heterodimers cooperatively activate transcription of $Sxl_{Pe}$ which can be repressed by interactions between Dpn and $Sxl_{Pe}$ (Hoshijima et al., 1995). These findings support the idea that the DNA binding of transcription factors determines the on/off state of $Sxl_{Pe}$.

*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 *Sxl* 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 $Sxl_{Pe}$ 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 *Sxl* 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 $Sxl_{Pe}$ in females prior to *runt*'s requirement in segmentation. Runt binds sequence specifically to several sites in $Sxl_{Pe}$ and using an in vivo mRNA injection assay, we demonstrate that DNA binding by Runt is essential for $Sxl_{Pe}$ activation in vivo. These assays also reveal that increasing the dosage of *runt* alone is sufficient for triggering transcriptional activation of $Sxl_{Pe}$ in male embryos. We further show that fusing a heterologous transcriptional activation domain to Runt does not disrupt Runt's ability to activate $Sxl_{Pe}$, arguing against the possibility that regulation of *Sxl* occurs indirectly via repression of a repressor. Finally, we demonstrate that a small segment of $Sxl_{Pe}$ 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 Runt 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′-GATCGTCCCACGAAGCGCAGATC-3′ and 5′-GGGACGCGG-CGCCTCCTTCAGCC-3′, respectively. runtΔRD was made by deleting an internal 345 base pair *Sal*I-*Pst*I fragment from the *runt* cDNA. pCS2$^+$runt constructs were generated using *Bam*HI-*Eco*RI fragments from relevant *runt* cDNAs. To generate runt[VP16], a *Bam*HI-*Xba*I 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 *Bam*HI fragment from runt[CK].

### *Drosophila* stocks

The *runt* alleles that were used in this study include the temperature-sensitive $runt^{YP17}$allele (Gergen and Wieschaus, 1986) and the loss-of-function $runt^{LB5}$ allele (Gergen and Butler, 1988), which produces no detectable mRNA. For X-gal stainings, the allele $runt^{YE96}$ was used, which produces no detectable protein accumulation in blastoderm embryos.

### Genetics

Female embryos homozygous for $Sxl_{Pe}$:*lacZ* $runt^{YP17}$ were generated by mating $Sxl_{Pe}$:*lacZ* $runt^{YP17}$/FM7 females to $Sxl_{Pe}$:*lacZ* $runt^{YP17}$ males carrying the Y-chromosome duplication $y^+Ymal^{102}$. 1/4 of the embryos from this cross will be females homozygous for $runt^{YP17}$. Female embryos homozygous for $Sxl_{Pe}$:*lacZ* $runt^{LB5}$ were generated by mating $Sxl_{Pe}$:*lacZ* $runt^{LB5}$/FM7 females to $Sxl_{Pe}$:*lacZ* $runt^{LB5}$ males carrying the Y-chromosome duplication $y^+Ymal^{102}$.

### 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: $Sxl_{Pe}$:*lacZ* $runt^{YP17}$ embryos were either collected at the permissive temperature (18°C) for 30 minute 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 immunohistochemsitry 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 *Sxl$_{Pe}$: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 $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$ 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 *Eco*RI-*Bgl*II fragments of the respective *Sxl$_{Pe}$* constructs into the *Eco*RI and *Bam*HI sites of the pCaSpeR-AUG-β-galactosidase vector. *Sxl$_{Pe0.4kb}$:lacZ* was previously described in Estes et al. (1995). *Sxl$_{Pe\ S\Delta B}$:lacZ* was created from a pBlueScript *Sxl$_{Pe}$* plasmid which has a *Sna*BI site engineered at –270. This plasmid was digested with *Bam*HI, the overhanging ends were filled using the Klenow fragment of DNA polymerase, and then digested with *Sna*BI. The resulting linear plasmid was re-ligated to reconstruct *Sxl$_{Pe}$* with the desired deletion. The deletion was confirmed by sequence analysis. The *Sxl$_{PeGOF}$:lacZ* promoter construct consists of the 0.4 kb *Sxl$_{Pe}$* sequence with four copies of a PCR-generated fragment inserted into the flanking polylinker *Eco*RV site. The PCR fragment was generated using the primers 5′-CGGATATCAATAGAACTATCACC-3′ and 5′-CGGATATC-GCGGATCTTCGC-3′. The PCR products were digested with *Eco*RV and ligated together. The desired four-copy molecule was isolated by gel purification and subcloned into the *Eco*RV site of a pBluescript *Sxl$_{Pe}$* construct. The correctly oriented clone was identified directly by sequence analysis. The above DNAs were transformed together with the helper plasmid P-turbo (pUChspD2-3wc) into the germ line of *w$^{1118}$ D. melanogaster* stock by standard procedures.

### Transient expression assay

Coding sequences for *runt* derivatives were cloned into the pCS2$^+$ expression vector (Rupp et al., 1994), which was used to synthesize in vitro transcribed capped RNA (Ambion). All pCS2$^+$*runt* plasmids were linearized with *Asp*718. RNA preparations were resuspended in water to a final concentration of 200 ng/ul and quantified by measuring incorporation of α-$^{32}$P-UTP. After dechorionation and dehydration, injection of mRNA was directed to the middle or anterior region of the embryo. After injection, the embryos were aged under halocarbon oil until cellularization was complete, washed off the coverslip with heptane and transferred to vial containing 1 ml of 10% paraformaldehyde solution in PBS and 6 ml of heptane. After fixing for 20 minutes with shaking, embryos were transferred to a slab of agar, affixed to a coverslip with doublestick tape, and submerged in PBS + 0.1% Tween-20. After hand peeling embryos from the vitelline membrane, embryos were transferred to an Eppendorf tube, dehydrated in MeOH and stored in 100% EtOH before subjection to standard in situ hybridization. In these experiments, female embryos were identified based on strong *lacZ* staining at the anterior and posterior poles, regions that do not require *runt* for female-specific expression.

### Electrophoretic mobility-shift assays

Runt and Bro proteins were expressed as 6× His-tagged fusions by cloning relevant cDNA segments into the pQE30 expression vector (Qiagen). Fusion proteins were prepared under denaturing conditions according to the manufacturer's instructions. EMSAs were performed as described in Pepling and Gergen (1995). DNA-protein complexes were electrophoresed on a 8% polyacrylamide (60:1 acrylamide/bisacrylamide) gel in 0.25× TBE at 200 V for 4 hours at 4°C using the polyoma probe or a 6% polyacrylamide gel for probes generated from *Sxl$_{Pe}$*. The wild-type CBF-binding site from the Polyoma enhancer is a 37 bp *Eco*RI-*Hin*dIII fragment referred to as PyA(wt) in Bolwig and Hearing (1991). Competition experiments to demonstrate sequence-specific binding were done with this fragment and with a mutant derivative, PyA(M2) in which the CBF site TGCGGTC is mutated to TGTGCTC (Bolwig and Hearing, 1991). Probes from *Sxl$_{Pe}$* were generated by PCR using the Expand High Fidelity PCR System (Boehringer Mannheim) according to manufacturer's instructions except that 5 μCi of $^{32}$P-dATP was added to each reaction. Following amplification for 15 cycles, probes were electrophoresed through a 5% polyacrylamide gel and eluted using the crush and soak method. Amplification was performed from the plasmid Sxl-Pe(5-1) using the following oligonucleotide pairs to generate four probes spanning the 1.1 kb fragment of *Sxl$_{Pe}$* Region I: 1: 5′-GATCCCAAAGTGTAGGAC-3′ and 5′-GCACGTTTGG-TTCACTCT-3′, 2: 5′-TACTGTTCTTGGCGTTCG-3′ and 5′-TTTCCTGCAGGCCAATTG-3′, 3: 5′-AGCAAAAAGGCA-GCTGCA-3′ and 5′-CTCCATGAGGGCATAACA-3′, 4: 5′-GTGCTGGTATTGCTTCAG-3′ and 5-ATTTCGCGGATC-CCCGAT-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 multimerized in the *Sxl$_{PeGOF}$:lacZ* construct was generated by PCR amplification from plasmid IM16 using the primers 5′-CGGATATCG-CGGATCTTCGC-3′ and 5′-CGGATATCAATACGGCTATCACC-3′.

## RESULTS

### *runt* functions in the initial activation of *Sxl* transcription

Previous work demonstrated a region-specific requirement for *runt* in the activation of *Sxl* 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 *Sxl* early embryonic promoter (*Sxl$_{Pe}$*). Wild-type female embryos containing a *Sxl$_{Pe}$: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 *runt$^{LB5}$* mutation fail to express the *Sxl$_{Pe}$:lacZ* reporter gene within a broad central region of the embryo (Fig. 1D). This defect is observed concomitant with the earliest detectable expression of this reporter gene (Fig. 1C), demonstrating an early requirement for *runt* in *Sxl$_{Pe}$* activation.

The alterations in *Sxl* 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 *Sxl* activation can be temporally separated from its role in segmentation. To test this

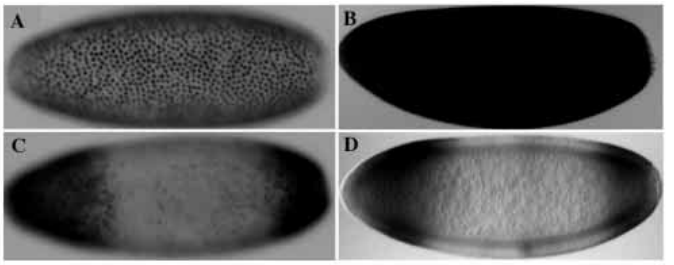


**Fig. 1.** Mutation in *runt* affects early transcriptional activation of $Sxl_{Pe}$. Expression of $Sxl_{Pe}$:*lacZ* is visualized by in situ hybridization with a *lacZ* riboprobe. Embryos in this, and all other figures, are oriented anterior to the left and dorsal side up. In wild-type female embryos, $Sxl_{Pe}$ 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, $Sxl_{Pe}$ expression is at higher levels and seen as uniform staining in the cytoplasm (B). In female embryos homozygous for $runt^{LB5}$, activation of $Sxl_{Pe}$ 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.

idea, we utilized the temperature-sensitive $runt^{YP17}$ mutation. Female embryos homozygous for $runt^{YP17}$ displayed normal $Sxl_{Pe}$ expression when reared and collected at the permissive temperature (18°C, Fig. 2A). At the restrictive temperature of 29°C, these embryos show non-uniform $Sxl_{Pe}$ expression identical to that observed in embryos deleted for *runt* (Fig. 2B). To examine *runt's* effects on segmentation, we examined the expression pattern of the segment polarity gene *engrailed* (*en*) in these embryos. In $runt^{YP17}$ embryos maintained at 18°C, En is expressed in a regular, well-spaced 14-stripe pattern (Fig. 2A), whereas at 29°C this pattern is disrupted (Fig. 2B). In collections of embryos aged at the non-permissive temperature for two hours and then shifted to the permissive temperature, female embryos with the abnormal $Sxl_{Pe}$ expression pattern typical of *runt* mutants showed normal En expression (Fig. 2C). In reciprocal temperature-shift experiments, female embryos, which were aged at the permissive temperature to the cellular blastoderm stage and then shifted to the non-permissive temperature, showed normal $Sxl_{Pe}$ expression and abnormal En expression (Fig. 2D). These results demonstrate that *runt*'s role in the activation of $Sxl_{Pe}$ is temporally distinct from and precedes the requirement for *runt* in segmentation, and provide strong evidence that *runt*'s role as an activator of *Sxl* transcription occurs prior to cellularization, during the earlier syncitial blastoderm stages of *Drosophila* embryogenesis.

## Runt binds to multiple sites in $Sxl_{Pe}$

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 $Sxl_{Pe}$ 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 $Sxl_{Pe}$ 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 $runt^{LB5}$ female embryos, $Sxl_{Pe}$ fails to be activated in the central region of the embryo (Fig. 1C,D). To investigate *runt's* role in $Sxl_{Pe}$ activation, we wished to test *runt* derivatives for their ability to rescue $Sxl_{Pe}$ expression in these

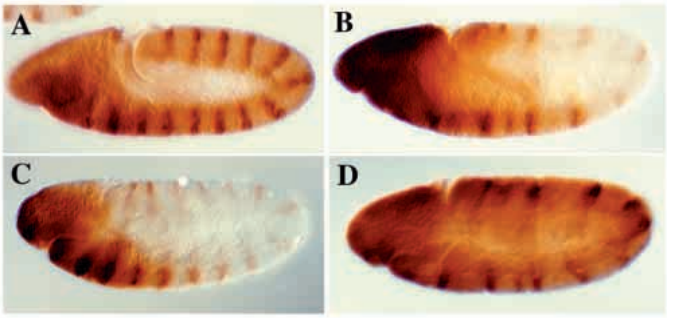


**Fig. 2.** Temporally distinct requirements for *runt* in sex determination and segmentation. Expression of the $Sxl_{Pe}$:*lacZ* reporter gene and *en* are visualized with antibodies that detect the β-galactosidase (β-gal) and En proteins, respectively. The embryos shown are from a cross in which all the embryos carry the $Sxl_{Pe}$:*lacZ* reporter gene and 1/4 of the embryos are homozygous for the temperature-sensitive $runt^{YP17}$ 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 $Sxl_{Pe}$:*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 $Sxl_{Pe}$ expression and abnormal En expression (D).

embryos. As a method for assaying *runt* activity in early embryos, we took advantage of the fact that *Sxl* transcription is activated prior to cellularization. At this stage, all of the nuclei in the embryo share a common cytoplasm. We injected pre-blastoderm stage $runt^{LB5}$ embryos carrying the $Sxl_{Pe}$:*lacZ* transgene with in vitro transcribed *runt* mRNA and assayed the embryos for rescue of $Sxl_{Pe}$ expression by in situ hybridization. In these experiments, 25% of the total number of embryos will be females homozygous for $runt^{LB5}$ (see Materials and Methods). As expected, in control embryos injected with buffer alone, approximately 25% of the total population, or 50% of the females, failed to express $Sxl_{Pe}$ in the central domain (Fig. 4A; Table 1). In a population of similar embryos that were injected with wild-type (WT) *runt* mRNA in 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 $Sxl_{Pe}$:*lacZ* expression pattern typical of wild-type females (Fig. 4B; Table 1). Thus, injection of *runt* mRNA rescues $Sxl_{Pe}$ expression in embryos mutant for *runt*.

## DNA-binding activity of the Runt domain is required for $Sxl_{Pe}$ 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, runtΔRD into the central

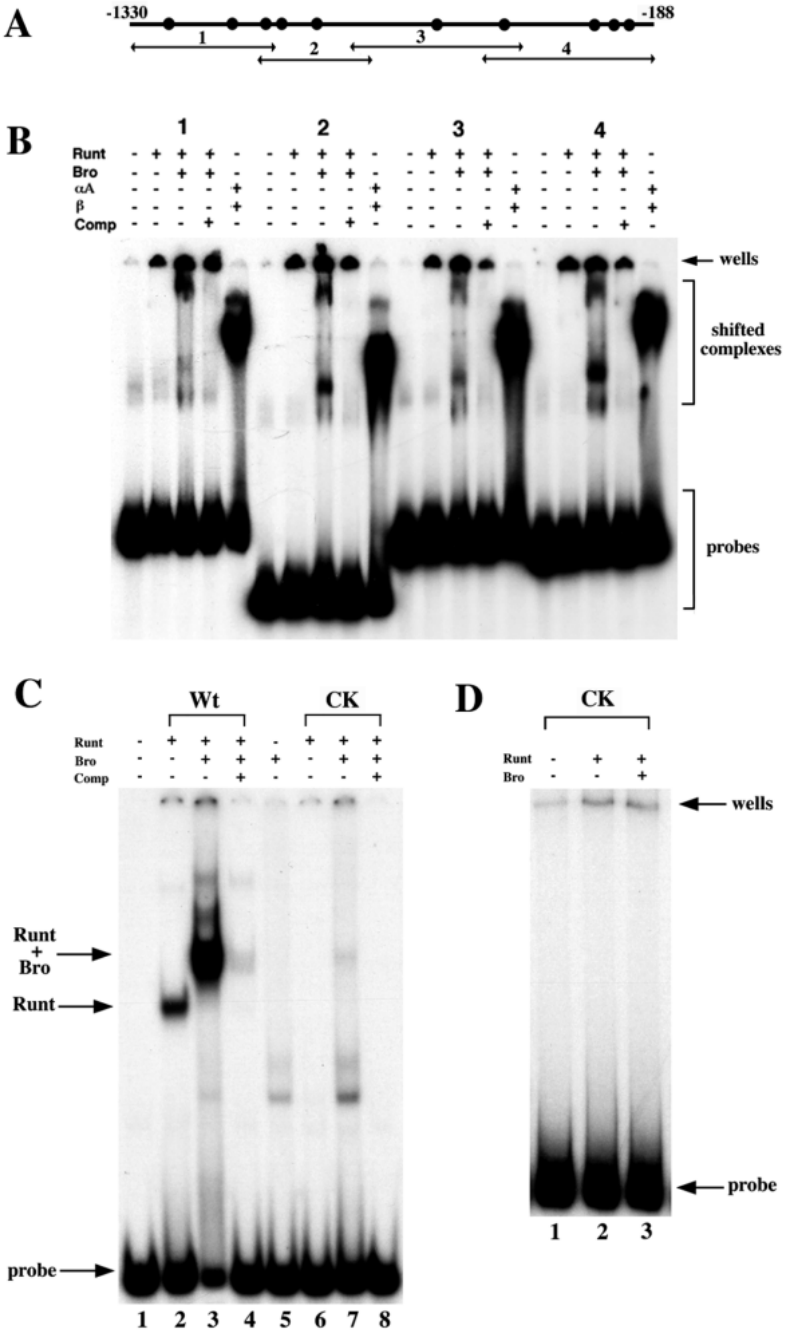


**Fig. 3.** Runt binds specifically to the *Sxl* early embryonic promoter and specific mutations in the Runt domain interfere with this binding. (A) Schematic diagram of a 1.1 kb segment of $Sxl_{Pe}$ containing the essential sequences for full $Sxl_{Pe}$ function (Estes et al., 1995). Arrows indicate the overlapping fragments of this region that were PCR-amplified and used as probes in EMSA. The circles represent the presence of a 5 out of 7 match to the CBF consensus binding site TG(C/T)GGT(C/T). (B) Results of EMSA. Each labeled fragment (between 200 and 300 base pairs in length) was used in binding reactions with Runt and Bro. The presence or absence of recombinant protein in the binding reaction is indicated above each lane. Note the presence of multiple shifted complexes with each fragment tested when incubated with Runt and Bro, indicating the presence of multiple binding sites. These complexes are competed with 50-fold excess DNA containing a binding site for Runt. No binding is observed when Bro is used in the absence of Runt. The final lane for each probe shows that recombinant mouse CBF (PEBP2αA + PEBP2β) also forms complexes with these fragments. (C) EMSA with a radiolabeled 37 bp fragment from the polyoma enhancer virus containing a CBF-binding site. Free probe is shown in lane 1. Wild-type (WT) Runt alone binds weakly (lane 2) and the addition of Bro results in stimulated formation of a novel complex of reduced mobility (lane 3). Addition of 50-fold excess cold competitor effectively competes away this binding. In contrast, DNA binding by a Runt protein that contains two specific point mutations within the Runt domain, Runt[CK] is not detected in the absence of Bro (lane 6), and only weak binding is detected upon addition of Bro (lane 7). As with the WT protein, the weak binding of Runt[CK] is competed away with cold-specific competitor (lane 8). Non-specific complexes that migrate below Runt and the Runt and Bro complexes are background bands due to contaminating proteins in these preparations. (D) EMSA using a DNA probe from the $Sxl_{Pe}$ promoter (corresponding to probe 4 in Fig. 3) and Runt[CK]. We observed no binding of Runt[CK] to this probe.

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

| Total no. of embryos scored[a] | Injected mRNA | % Female embryos with *lacZ* pattern | | |
|---|---|---|---|---|
| | | Uniform[b] | Partial rescue[c] | Mutant[d] |
| 90 | buffer | 53 | 0 | 47 |
| 90 | runtWT | 85 | 12 | 3 |
| 84 | runtΔRD | 51 | 0 | 49 |
| 70 | runt[CK][e] | 47 | 0 | 53 |

[a]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.
[b]These embryos showed dark uniform staining throughout the embryo except for in pole cells.
[c]Partial rescue was identified as strong staining at the poles and lighter staining in the central region.
[d]Mutant embryos had strong staining at the poles but lacked any detectable staining in the central region.
[e]CK refers to the (C127S,K199A) double substitution in Runt.

region of female homozygous *runt*$^{LB5}$ embryos. We saw no evidence of rescue in runtΔRD-injected embryos, indicating that the DNA-binding domain is important for *runt's* function as an activator of *Sxl*$_{Pe}$ (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 *Sxl*$_{Pe}$ 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 *Sxl*$_{Pe}$ promoter.

## Increased *runt* dosage is sufficient for *Sxl*$_{Pe}$ 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 *Sxl*$_{Pe}$ only in the central region of the embryo and were presumed to be males that were inappropriately activating *Sxl*$_{Pe}$ 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*$_{Pe}$*: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*$_{Pe}$*:lacZ* transgene (Table 2). Approximately half of the injected embryos showed uniform expression as expected for females, and approximately half showed broad domains of localized expression centered near the site of mRNA injection (Fig. 5A). This expression is not an artifact due to injection, as it is not observed in embryos injected with buffer alone (Table 2). Further, this novel ectopic activation pattern is not observed in embryos injected with mRNAs encoding the RuntΔRD and Runt[CK] derivatives (Table 2). Based on these results, we conclude that increasing the dosage of *runt* alone is sufficient to trigger the

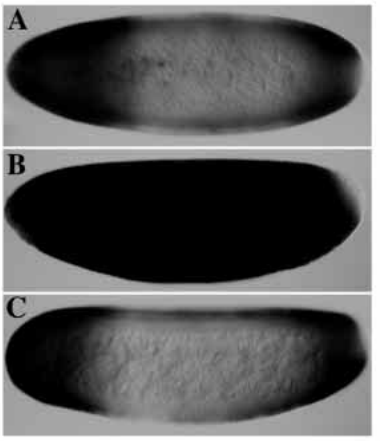


**Fig. 4.** DNA binding is required for activation of *Sxl*$_{Pe}$ in vivo. In situ hybridization was used to detect expression of the *Sxl*$_{Pe}$*:lacZ* reporter gene. (A) Embryo injected with buffer alone. (B) Injection of WT *runt* mRNA rescues the *runt*$^{LB5}$ phenotype. (C) Injection of an mRNA that encodes the mutant Runt[CK] protein is unable to rescue.

**Table 2. Injection of *runt* mRNA activates *Sxl*$_{Pe}$ expression in wild-type males**

| Total no. of embryos scored | Injected mRNA | % Embryos with *lacZ* pattern | | |
|---|---|---|---|---|
| | | Uniform | Partial | No staining |
| 184 | buffer | 51 | 0 | 49 |
| 312 | runtWT | 48 | 47 | 5 |
| 147 | runtΔRD | 52 | 0 | 48 |
| 226 | runt[CK][a] | 49 | 0 | 51 |
| 213 | runtWT (into head) | 52 | 38 | 10 |
| 206 | runt[VP16] | 47 | 25 | 28 |

[a]CK refers to the (C127S,K199A) double substitution in Runt.

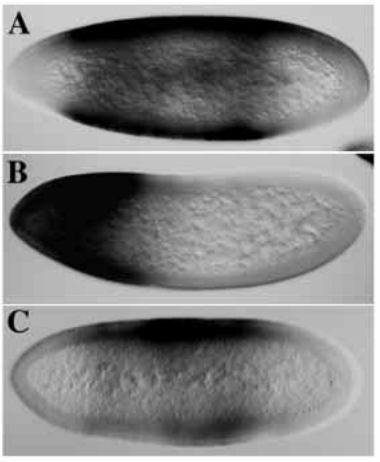


**Fig. 5.** Increasing *runt* dosage triggers activation of $Sxl_{Pe}$ in males. In situ hybridization was used to detect expression of the $Sxl_{Pe}$:*lacZ* reporter gene. (A) Embryo injected with WT *runt* mRNA that shows activation of $Sxl_{Pe}$ in a broad central domain of the embryo. Activation 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).

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 $Sxl_{Pe}$ 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 $Sxl_{Pe}$ expression in the anterior end (Fig. 5B; Table 2). Activation of $Sxl_{Pe}$ 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 $Sxl_{Pe}$

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 $Sxl_{Pe}$. 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 $Sxl_{Pe}$ 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 $Sxl_{Pe}$ expression in females. More importantly, however, this fusion protein should lose the ability to trigger Runt-dependent activation of $Sxl_{Pe}$ expression in injected males. Injection of Runt[VP16] does not affect $Sxl_{Pe}$ expression in females and activates $Sxl_{Pe}$ transcription in injected males (Fig. 5C; Table 2). These results argue strongly against an indirect model and provide evidence in support of

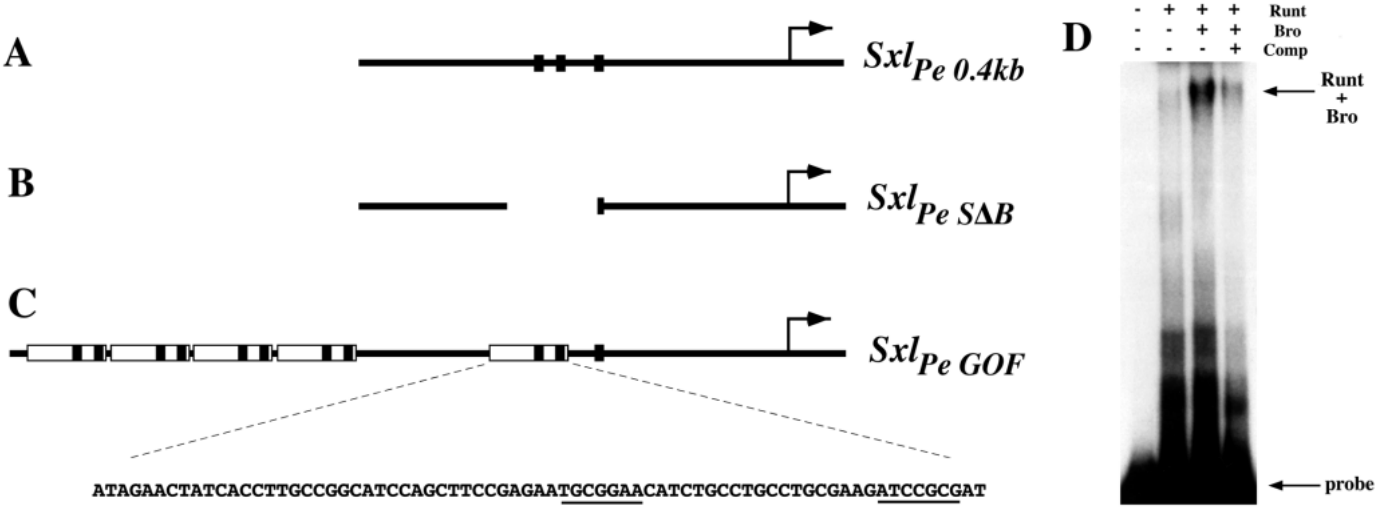


**Fig. 6.** Runt binds to a conserved site in a minimal region of $Sxl_{Pe}$. (A) Schematic diagram of the minimal functional segment of $Sxl_{Pe}$. The reporter $Sxl_{Pe0.4kb}$:*lacZ* is composed of sequences extending from −389 to +47 and contains several potential Runt-binding sites (shaded blocks). (B) A portion of this segment containing two Runt-binding sites is deleted in the construct $Sxl_{PeS\Delta B}$ 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 $Sxl_{PeGOF}$. (D) EMSA using a probe from this fragment demonstrating that Runt binds specifically to sequences within this region of $Sxl_{Pe}$.

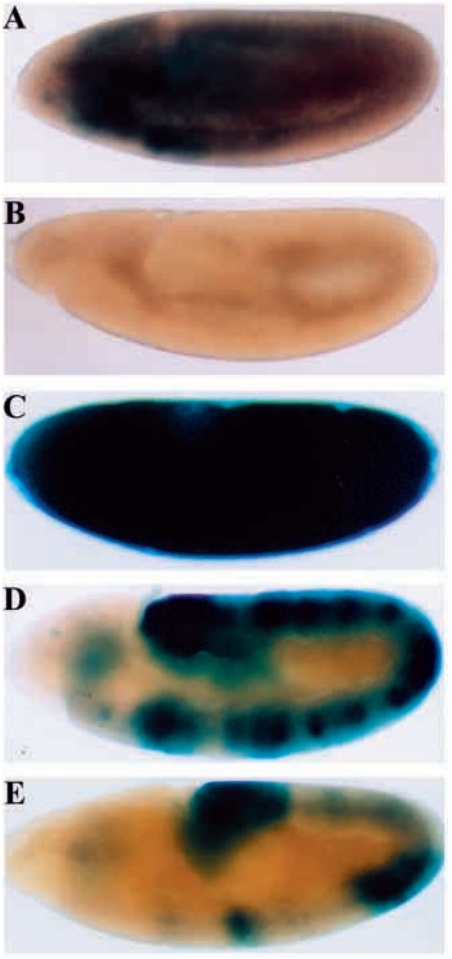


**Fig. 7.** A conserved Runt-binding site mediates Runt-dependent activation. The figure shows *lacZ* expression as visualized by X-gal staining for representative embryos of each genotype. (A) A wild-type female embryo with two copies of $Sxl_{Pe0.4kb}$:*lacZ* exhibits a distinct pattern of staining with higher levels found in the anterior and the posterior. There is a low level of staining throughout the embryo as is evident by the slightly brownish appearance. (B) A segment of $Sxl_{Pe}$ containing at least two Runt-binding sites is necessary for promoter function as shown by expression of $Sxl_{PeS\Delta B}$:*lacZ* in which 73 bp of sequence is deleted. (C) The gain-of-function promoter $Sxl_{PeGOF}$:*lacZ* consists of a multiple array of the segment deleted in $Sxl_{PeS\Delta B}$:*lacZ*, and is expressed at very high levels as one copy in wild-type female embryos. (D) $Sxl_{PeGOF}$:*lacZ* is also expressed in wild-type males, however, not in the anterior region. (E) Expression of $Sxl_{PeGOF}$:*lacZ* in males hemizygous for the *runt*[*YE96*] mutation is reduced in most regions of the embryo.

the hypothesis that Runt acts directly to activate $Sxl_{Pe}$ transcription.

### A small segment of $Sxl_{Pe}$ 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 $Sxl_{Pe}$ promoter. As an alternative, we investigated interactions between Runt and a previously identified proximal 400 basepair (bp) fragment of $Sxl_{Pe}$, ($Sxl_{Pe0.4kb}$:*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 $Sxl_{Pe}$ expression (Fig. 7B). Conversely, a reporter gene that contains multiple copies of this segment, $Sxl_{PeGOF}$:*lacZ* (Fig. 6C), is expressed at high levels in WT female embryos (Fig. 7C). Interestingly, the $Sxl_{PeGOF}$:*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 $Sxl_{Pe0.4kb}$:*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 $Sxl_{PeGOF}$:*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 $Sxl_{PeGOF}$:*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 $Sxl_{Pe}$ in vivo. Furthermore, a derivative of Runt containing a heterologous transcriptional activation domain activates rather than represses $Sxl_{Pe}$ 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.

### Runt as a position-specific numerator element

Numerator elements are defined as X-chromosome-linked factors that have a dose-dependent activating effect on *Sxl* expression. In this study, we demonstrate that increasing the

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*, $Sxl_{Pe}$ 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 $Sxl_{Pe}$ 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 $Sxl_{Pe}$ 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 $Sxl_{Pe}$ 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 $Sxl_{Pe}$ 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 $Sxl_{Pe}$. Alternatively, *runt* may function as a more passive activator of transcription. For example, the binding of Runt to $Sxl_{Pe}$ may interfere with the binding of other transcriptional repressors of $Sxl_{Pe}$ such as Dpn. It is also possible that Runt functions to 'quench' active repression of $Sxl_{Pe}$ 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 $Sxl_{Pe}$ in male embryos (Paroush et al., 1994). It is possible that Runt bound to $Sxl_{Pe}$ 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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