Positioning adjacent pair-rule stripes in the posterior Drosophila embryo

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SUMMARY

We present a genetic and molecular analysis of two hairy (h) pair-rule stripes in order to determine how gradients of gap proteins position adjacent stripes of gene expression in the posterior of Drosophila embryos. We have delimited regulatory sequences critical for the expression of h stripes 5 and 6 to 302 bp and 526 bp fragments, respectively, and assayed the expression of stripe-specific reporter constructs in several gap mutant backgrounds. We demonstrate that posterior stripe boundaries are established by gap protein repressors unique to each stripe: h stripe 5 is repressed by the giant (gt) protein on its posterior border and h stripe 6 is repressed by the hunchback (hb) protein on its posterior border. Interestingly, Krüppel (Kr) limits the anterior expression limits of both stripes and is the only gap gene to do so, indicating that stripes 5 and 6 may be coordinately positioned by the Kr repressor. In contrast to these very similar cases of spatial repression, stripes 5 and 6 appear to be activated by different mechanisms. Stripe 6 is critically dependent upon knirps (kni) for activation, while stripe 5 likely requires a combination of activating proteins (gap and non-gap). To begin a mechanistic understanding of stripe formation, we locate binding sites for the Kr protein in both stripe enhancers. The stripe 6 enhancer contains higher affinity Kr-binding sites than the stripe 5 enhancer, which may allow for the two stripes to be repressed at different Kr protein concentration thresholds. We also demonstrate that the kni activator binds to the stripe 6 enhancer and present evidence for a competitive mechanism of Kr repression of stripe 6.

Key words: hairy, pair-rule, gap proteins, repressors, gradients, Krüppel, Drosophila

INTRODUCTION

The reiterated stripes of expression of the primary pair-rule genes hairy (h) and even-skipped (eve) in the Drosophila embryo present an excellent system in which to examine the spatial control of transcription during development. Pattern along the anteroposterior axis of Drosophila arises from the coordinate expression of a hierarchy of genes, most of which encode transcription factors (reviewed by St Johnston and Nüsslein-Volhard, 1992; Akam, 1987). A cascade of regulatory interactions results in the fairly rapid refinement of the spatial organization of the embryo such that by cellular blastoderm (the fourteenth nuclear cycle), seven sharp, transverse stripes of h and eve mRNA and protein appear (Ingham et al., 1985; Macdonald et al., 1986; Frasch et al., 1987; Carroll et al., 1988; reviewed in Carroll, 1990). These stripes provide the first indication of the segmented body plan of the fruit fly and establish the prepatterning for the metameric expression of downstream genes.

Genetic dissections of this process indicate that the proper formation and positioning of h and eve pair-rule stripes requires multiple regulatory inputs and is under the control of the maternal gene bicoid (bcd), and the zygotic gap genes hunchback (hb), Krüppel (Kr), knirps (kni) and giant (gt) (Frasch and Levine, 1987; Carroll and Vavra, 1989; Hooper et al., 1989). The h and eve loci contain large upstream regulatory regions comprising more or less autonomous stripe-specific elements that act to decode the positional information provided by the gap proteins and to drive the expression of individual stripes (Howard et al., 1988; Harding et al., 1989; Goto et al., 1989; Pankratz et al., 1990; Howard and Struhl, 1990; Riddihough and Ish-Horowicz, 1991). This has allowed the genetic identification of specific regulatory proteins that are required for the establishment of individual stripes, as well as examinations of how these proteins interact with discrete cis-regulatory elements.

A series of experiments has led to a detailed model for the regulation of one pair-rule stripe, even-skipped stripe 2 (Stanojevic et al., 1991; Small et al., 1991; 1992). eve stripe 2 is transcriptionally activated by the maternal morphogen bicoid (bcd), as well as by the gap protein hunchback (hb); these activating proteins both broadly overlap eve stripe 2 in the blastoderm embryo. The sharp limits of eve stripe 2 expression result from repression by the giant (gt) and Krüppel (Kr) proteins that flank this stripe in the blastoderm embryo. Binding sites for all of these DNA-binding proteins are tightly clustered and partially overlapping in the small regulatory fragment that controls eve stripe 2. Disruption of bcd- and hb-binding sites causes reduced stripe expression and disruption of gt and Kr sites causes expanded striped expression; this suggests that the action of these protein is direct and that the binding of repressing factors may inhibit the binding or function of activating
factors. These findings provide a clear and relatively simple mechanism for how crude gradients of gap and maternal proteins produce a sharp stripe of pair-rule gene expression. However, such a clear and simple mechanism may not account for the formation of all pair-rule stripes. Patterning in the anterior of the Drosophila embryo is under the control of the bcd/hb morphogen system (Frohnhöfer and Nüsslein-Volhard, 1986; Struhl et al., 1989) and these are important components of the regulation of eve stripe 2. Posterior pair-rule stripes, forming outside of the bcd/hb gradient system, must be predominately under the control of the gap genes, raising significant questions as to the regulatory interactions that establish these stripes. As with eve stripe 2, posterior h stripes require input from more than one gap gene (Pankratz et al., 1990; Howard and Struhl, 1990; Riddihough and Ish-Horowicz, 1991, Langeland and Carroll, 1993) yet it is unclear what the exact contributions of gap protein-mediated activation and gap protein-mediated repression are in the formation of individual stripes. Additionally, given the small number of potential gap protein regulators, it remains unclear whether each stripe has a completely unique set of activators and repressors, or whether adjacent stripes are coordinate positional by shared activating and/or repressing factors.

In this study, we examine two posterior pair-rule stripes, hairy (h) stripe 5 and 6, in order to elucidate their regulatory inputs and thus to expand our knowledge of mechanisms of stripe formation. By focusing on adjacent stripes, we sought also to clarify the mechanism that establishes sharp expression boundaries in close proximity. We find that the posterior limits of these stripes are established by gap protein repression, but that the repressors are unique for each stripe. In contrast, we find that the gap protein Kr, which occupies the central portion of the embryo, is largely responsible for delimiting the anterior borders of both h stripes 5 and 6. We have defined small regulatory fragments that are critical for the formation of these two stripes and identify Kr-binding sites in both of these fragments. The Kr protein has different affinities for the two stripes and identify Kr-binding sites in both of these regulatory fragments that are critical for the formation of these borders of both embryos. Additionally, given the small number of potential gap protein regulators, it remains unclear whether each stripe has a completely unique set of activators and repressors, or whether adjacent stripes are coordinate positional by shared activating and/or repressing factors.

In situ hybridizations and immunohistochemistry

Reporter gene expression was examined in pooled blastoderm stage embryos using digoxigenin-labelled lacZ antisense RNA following the protocol of Jiang et al. (1991). For double detection of reporter gene message and endogenous h or gap proteins, embryos were first fixed and stained for immunohistochemical detection of protein using affinity-purified rabbit polyclonal gap (Langeland and Carroll, 1993), or h (Carroll et al., 1992) antisera, a peroxidase-conjugated goat anti-rabbit secondary (Boehringer-Mannheim), and developed with diaminobenzidine (DAB) as described by Skeath and Carroll (1992). After DAB development, embryos were stored at −20°C overnight. Reporter gene expression was then detected as described, but without the addition of the final step. Double-immunofluorescent labelling of embryos was performed as previously described (Langeland and Carroll, 1993).

Gap mutant stocks and genetic analyses

The following mutant stocks were used for our genetic analyses: Kr/TM3 (a small deletion deleting the Kr locus), Df(1)62g18/FM7 (deleting the gt locus, provided by E. Eldon), kni/7m48/TM3, hb/7m48/TM3 and kntID49, h7m48/TM3. Either stripe 5 or stripe 6 reporter constructs were crossed into single, double or triple mutant backgrounds constructed from these parental stocks. Homozygous mutant embryos were identified either by co-labelling with appropriate digoxigenin-labelled RNA (for Kr and gt deletions), or with antibodies (kni and hb mutants). Lack of staining with the appropriate probe identifies mutant embryos. To be certain that the loss of h stripe 6 in kni mutant embryos was not indirectly due to ectopic repression by flanking gap proteins, we examined stripe 6 expression in Kr/fknt−, kni−/h− and Kr/kni−/h− embryos. In no case did we observe stripe 6 reporter expression in embryos lacking kni expression. Similarly, to be certain that stripe 5 and 6 anterior expansion in Kr mutant embryos was not indirectly due to Kr effects on kni expression, we also analyzed these stripes in Kr−/gt embryos, which restores the wild-type kni expression pattern (Capovilla et al., 1992). Such embryos displayed the same anterior stripe expansion as in Kr− mutants.

DNaseI footprinting, affinity and competition assays

Templates for DNaseI footprinting were prepared using PCR following the protocol of Krummel (1990). The input DNA was either the 302 bp Draf/IIId stripe 5 fragment, the 205 bp Smal-Dral stripe 6 fragment, or the 327 bp Draf-BglII fragment cloned into pBluescript KSII+. For primers, we used the KS and T7 primers that flank the polylinker and insert (obtained from the UW Biotechnology Center). Primers were end-labelled using the DNA 5′ End-Labelling System (Promega). One 32P end-labelled primer and one cold primer were used per PCR reaction. PCR products were purified using the Magic PCR Preps kit (Promega).

Kr and kni proteins were bacterially expressed from full-length cDNAs cloned into inducible T7 expression vectors (kindly provided by C. Desplan and U. Nauber). Inclusion bodies containing the foreign protein were purified from whole lysates of induced cultures (Williams et al., 1994), solubilized in 8 M Urea as described by Stanojevic et al. (1989), and samples of the preparations quantified using Coomassie Blue stained SDS-PAGE gels. Small amounts of these extracts, comparable to those used by Stanojevic et al. (1989) and
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Hoch et al., 1992, either alone or mixed, were added to approximately 0.5 ng labelled fragment. Binding, DNaseI digestion and sample preparation were performed as in Stanojevic et al. (1989), except that 0.2 µg of DNaseI was used for 1 minute. Samples in loading dye were electrophoresed on a 5% Long Ranger (AT Biochem) polyacrylamide gel for approximately one hour and then dried and exposed to film. In separate reactions using the Sequenase kit (US Biochemicals), the template DNA was sequenced using the same (now unlabeled) primer that was end-labelled to produce the footprint template. When electrophoresed together, the ladder generated from DNaseI digestion can be perfectly matched to its corresponding nucleotide sequence.

**Mutagenesis**

The Kr-binding sites in the stripe 5 minimal enhancer lie in two distinct regions; these regions were deleted using two consecutive rounds of the PCR overlap extension method of Ho et al. (1989). The original template DNA was the Bluescript KSI+ plasmid containing the 302 bp Eagl-PvuI stripe 5 minimal enhancer. The first deletion was made using the following deletion-spanning primer 5’GTGGCATGGCGTGTCAGGACACCGGCACGGG3’, as well as its complement and the flanking T7 and KS primers. The second deletion was made using the deletion-spanning primer 5’CAACGGACACAGGTACTCTCA-GTATTGTT3’ and again its complement and the flanking T7 and KS primers. The resulting fragment (now 227 bp) containing both deletions was recloned into Bluescript KSI+ and then into the transformation vector phsplaCaSpeR and transformed as described above.

**Heat shock**

Embryos were collected for 1 hour from the stock K9A (generously provided by G. Struhl) that is homozygous for an insert carrying the Kr cDNA under the control of the heat-inducible hsp70 promoter. The embryos were caged on their agar-molasses collection caps for 1 hour and then floated on a 37°C water bath for 10-30 minutes. The embryos were allowed to recover for 15 minutes and then fixed and stained for h protein as described above, except using an alkaline phosphatase-conjugated tertiary reagent (Vector).

**RESULTS**

**Definition of minimal stripe enhancers for h stripes 5 and 6**

Dissections of the 5’ regulatory region of the h locus have illustrated that each of the seven pair-rule stripes is under the control of more or less autonomous regulatory fragments (Pankratz et al., 1990, Howard and Struhl, 1990; Riddihough and Ish-Horowicz, 1991). When fused to the lacZ reporter gene, these relatively large fragments give rise to β-galactosidase expression that corresponds to individual stripes. Using a series of deleted h stripe 5 and stripe 6 reporter constructs transformed into fly embryos (Fig. 1), we have further delimited sequences critical for the expression of these stripes. The location of these small stripe 5 and stripe 6 enhancers in the h locus are shown schematically in Fig. 1A, and the various constructs are diagrammed in Fig. 1B. Representative reporter gene expression patterns are shown in Fig. 2.

We were not able to narrow the 526 bp Smal-BglII fragment h stripe 6 minimal element previously identified by Howard and Struhl (1990); smaller constructs (205 bp Smal-Dral and 327 bp DraI-BglII) abolished striped expression. Both the anterior

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**Fig. 1.** Definition of h stripe 5 and stripe 6 regulatory sequences. (A) Map of the h locus indicating the location of restriction fragments that control stripes 5 and 6; see also (Pankratz et al., 1989; Howard and Struhl, 1990; Riddihough and Ish-Horowicz, 1991; Langeland and Carroll, 1993). (B) Summary of h stripe 6-lacZ reporter constructs. The 526 bp Smal-BglII construct drives strong expression corresponding to h stripe 6; this is the same regulatory fragment defined by Howard and Struhl (1990). Further deletions of this element (205 bp Smal-Dral and 327 bp DraI-BglII) abolish stripe expression. (C) Summary of 10 h stripe 5 reporter constructs. Critical stripe 5 sequences were defined using progressive 5’ and 3’ deletions of the previously identified 2.7kb Kpn1-BamHI fragment which produces both stripes 1 and 5 (Pankratz et al., 1989; Howard and Struhl, 1990; Riddihough and Ish-Horowicz, 1991; Langeland and Carroll, 1993). Deletions from the 3’ end remove stripe 1 expression and also cause a posterior spreading of stripe 5 expression. In all cases examined, the 302bp Eag1-PvuI fragment (asterixed) is required for stripe 5 expression. Constructs that contain this fragment (1.9 kb Kpn1-BalI; 1.7 kb Kpn1-EcoRI; 1.0 kb ApaI-EcoRI; 700 bp SacI-EcoRI; 730 bp ApaI-PvuI; 579 bp Eag1-EcoRI) all give rise to very similar stripe 5 expression. Constructs lacking this 302 bp core element (279 bp PvuI-EcoRI; 430 bp ApaI-EagI) do not produce stripes. Furthermore, this core element itself is sufficient to produce weak stripe 5 expression. See Fig. 2 for representative stripe 5 and stripe 6 reporter gene expression patterns.
and posterior borders of this stripe 6 reporter gene expression are sharp (Fig. 2A), and double labelling for reporter gene expression and either endogenous h protein (not shown) or gap proteins (see Figs 3, 4 and 5) indicate that the stripe is faithfully represented. In a much more extensive series, we have delimited sequences critical for stripe 5 expression enhancer to a 302 bp Eagl-PvuI fragment; in all constructs examined, this fragment is necessary for stripe 5 expression. Expression arising from this small stripe 5 construct (Fig. 2E), as well as most larger stripe 5 constructs, (Fig. 2C,D) has an ill-defined posterior border, likely resulting from the removal of sequences necessary for the posterior repression of stripe 5 (Langeland and Carroll, 1993). Again, double labelling for reporter gene expression and either h protein (not shown) or gap proteins (see Figs 3, 4 and 5), indicate that the anterior border of small stripe 5 constructs and both the anterior and posterior borders of the largest stripe 5 construct (Fig. 2B, constructs also makes stripe 1), match those of the native h stripe 5.

**Gap protein repression establishes the posterior borders of stripes 5 and 6**

Examining stripe-specific reporter gene expression in mutant embryos is an established approach for isolating the effects of trans-acting factors on the formation of individual stripes (Pankratz et al., 1990; Riddihough and Ish-Horowicz, 1991; Stanojevic et al., 1991; Small et al., 1991, 1992; Langeland and Carroll, 1993; Hartmann et al., 1994). While individual stripes are clearly under the control of unique combinations of gap proteins, it has remained unclear how a given gap protein may affect different stripes. We have conducted an exhaustive genetic analysis of gap gene regulation of h stripes 5 and 6, involving several single, double and triple gap mutant backgrounds and, in this report, highlight the most pertinent results. As shown in Fig. 3, gap protein repression appears to be responsible for positioning the posterior border of stripes 5 and 6. For these experiments, we used the smallest stripe 6 construct, but a much larger stripe 5 construct which produces a sharp posterior border and also contains regulatory sequences that produce stripe 1. Stripe 5 is normally flanked by the posterior domain of the gap protein gt (Fig. 3A), while stripe 6 is normally flanked by the posterior domain of hb (Fig. 3B). These stripes are posteriorly derepressed in gt− (Fig. 3C) and hb− (Fig. 3D) mutant embryos, respectively. This indicates that a wild-type function of gt is to establish the posterior boundary of stripe 5 and a wild-type function of hb is to establish the posterior boundary of stripe 6, thus supporting the general principle of spatial repression of pair-rule stripes by gap proteins. Note that the anterior represors are unique for each stripe; gt does not affect stripe 6 formation (see Fig. 5) and hb does not affect stripe 5 formation (data not shown).

**Krüppel behaves genetically as a repressor of both h stripes 5 and 6**

In contrast to the establishment of posterior stripe borders, we find that the same gap gene, Kr, affects the anterior border of both stripe 5 and 6. The wild-type Kr protein is closely flanked by h stripe 5 reporter gene expression (Fig. 4A), while it is clearly separated from h stripe 6 reporter gene expression (Fig. 4B); these spatial relationships hold for endogenous h stripes as well (Langeland and Carroll, 1993). Despite these spatial
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differences, we find that lacZ expression arising both from our smallest h stripe 6 and stripe 5 reporter constructs expands anteriorly in Kr mutant embryos (Fig. 4C,D). By also analyzing various mutant combinations with hb, kni and gt (see Materials and Methods), we are able to confirm that the effect of Kr mutants is not mediated by other gap genes and therefore that Kr is very likely directly affecting these stripe borders. Larger stripe 5 and stripe 6 reporter constructs behave in the same manner (data not shown), indicating that a wild-type function of Kr is to establish the anterior limits of both h stripe 5 and stripe 6.

The role of local activators

Given that repression by flanking gap proteins is apparently a general feature of pair-rule stripe regulation, we wanted to determine the relative contribution of local activators in stripe positioning by genetically removing the gap proteins that normally overlap h stripes 5 and 6. h stripe 5 is coincident with peak kni protein expression at cellular blastoderm (Fig. 5A), and stripe 5 reporter gene expression is significantly weaker in kni mutant embryos (Fig. 5B), indicating some role for kni in activating this stripe. However, no gap gene tested (hb, Kr, kni, gt) is absolutely required for the activation of our minimal stripe 5 element or larger stripe 5 elements, and we therefore surmise that more general factors (i.e. non-gap proteins) may be required for activating this stripe. A similar spatial relationship exists between stripe 6 expression and the posterior band of gt protein (Fig. 5C), but surprisingly, stripe 6 is not noticeably affected in gt mutant embryos (Fig. 5D), and thus the most logical candidate for a local activator of this stripe is not essential as such. Instead, although stripe 6 forms within the posterior-most portion of the kni domain (Fig. 5E), no stripe forms in embryos lacking kni function (Fig. 5F). To be certain that the loss of h stripe 6 in kni mutant embryos was not an indirect effect due to repression by other gap proteins, we examined stripe 6 expression in Kr–kni–, kni–hb– and Kr–kni–hb– embryos. In no case did we observe stripe 6 reporter expression in embryos lacking kni expression, suggesting a direct requirement for kni protein.

Fig. 3. Gap protein repression defines the posterior border of h stripes 5 and 6. Blastoderm patterns of embryos double-labelled for specific gap proteins and either stripe 1 and 5 (1(2.7)lacZ), or stripe 6 (6(526)lacZ) reporter gene expression. Expression corresponding to h stripe 5 is flanked on its posterior border by the gt protein (A, arrow). Similarly, reporter gene expression corresponding to h stripe 6 is flanked by the posterior domain of the hb protein (B, arrow). In gt– mutant embryos stripe 5 expands posteriorly (C, arrow), while stripe 6 expands posteriorly in hb– mutant embryos (D, arrow). These observations indicate that spatial repression by the gt and hb proteins establishes the posterior limits of h stripes 5 and 6, respectively.

Fig. 4. Kr repression defines the anterior limits of both h stripes 5 and 6. (A) Embryo double labelled for Kr protein and h stripe 5 reporter gene expression 5(302)lacZ. Note that this construct is the smallest stripe 5 construct we defined, and has an ill-defined posterior border (see Figs 1 and 2). The anterior border of this stripe closely flanks the Kr protein domain, as does the wild-type h stripe 5 (Langeland and Carroll, 1993). (B) Embryo double labelled for Kr protein and h stripe 6 reporter gene expression (6(526)lacZ). This stripe is positioned further posterior of the Kr domain. In Kr– mutant embryos, both stripe 5 (C, arrow) and stripe 6 (D, arrow) reporter gene expression is significantly shifted toward the anterior. Kr is the only gap gene to affect the anterior border of either stripe, suggesting that both stripes are positioned by Kr protein repression.
A molecular switch controlling h stripe 6?

A summary of the spatial and genetic relationships of h stripes 5 and 6 with posterior gap proteins is shown in Fig. 6. In order to begin a molecular understanding of these regulatory relationships, we have located binding sites for the Kr and kni proteins within the stripe 6 enhancer. We find large blocks of sequences protected by kni protein that are overlapped by the Kr-binding sites at the distal end of the stripe 6 enhancer (Fig. 7A,B). The Kr-protected sequences all contain matches for the Kr consensus binding site reported by Stanojevic et al. (1989) and Triesman and Desplan (1989). Within kni protected sequences, we find from one to three matches of the consensus kni-binding sequence reported by Hartmann et al. (1994). We believe that the physical overlap of these sites is functionally
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Fig. 7. Overlapping Kr and kni-binding sites in the stripe 6 control region. (A) The location of Kr (hashed box) and kni (white box)-binding sites in the 526 bp \( SmaI-BglII \) stripe 6 regulatory fragment, as determined by DNaseI footprinting with bacterially expressed Kr and kni proteins. We identified 7 Kr-binding sites; the alignment of these sequences produces a consensus very similar to the consensus Kr-binding sequence reported by Stanojevic et al., 1989 and Triesman and Desplan, 1989. We also identified eight kni-binding sites whose sequence alignment reveals a consensus similar to the kni consensus binding site reported by Hartmann et al., 1994. Note that most of the kni-binding sites lie in blocks containing two or three contiguous sites. Arrows indicate binding site orientation. (B) DNA binding of Kr and kni proteins in the 205 bp \( SmaI-BglII \) stripe 6 fragment containing the highest concentration of binding sites. 4 sites are protected from DNaseI digestion by bacterially expressed Kr protein (hashed boxes; sites Kr1 through Kr 4). Note that the Kr1 site only appears at the higher level of protein used. These Kr sites are overlapped by 3 large sequence tracts that are protected by kni protein (white boxes). These protected sequences each contain 2 or 3 copies of the kni consensus binding sequence (kni1 through kni7). The orientation of the various binding sites are indicated by arrows. Footprint assays were run alongside a dideoxy sequencing reaction primed with the same oligonucleotide which was end-labelled to generate the footprint template (see Materials and Methods). Control binding experiments using bacterial extracts without Kr or kni coding sequences did not produce footprints (data not shown). (C) Wild-type embryo double labelled for Kr (red) and kni (green) proteins. Nuclei in which Kr and kni are coexpressed appear yellow. Note the extent of this region in which these two proteins may interact. (D) Competitive binding of Kr and kni to the stripe 6 control region. When both Kr and kni proteins are mixed in DNaseI protection assays, we find that the Kr sites are preferentially protected. This suggests that Kr protein is able to prevent binding of kni protein to the stripe 6 enhancer, even at roughly 5-fold lower levels.
important because the two proteins are coexpressed over a significant portion of the embryo (Fig. 7C) and when the two proteins are mixed in vitro, low amounts of Kr protein appears to prevent the kni protein from binding to the stripe 6 enhancer (Fig. 7D). This in vitro competitive mechanism correlates very well with the observed genetic behavior in which kni activates and Kr represses stripe 6 expression.

**Kr binds to h stripes 5 and 6 with different affinities**

Given that Kr appears to establish the anterior limits of both stripes 5 and 6, we also located Kr protein-binding sites within the stripe 5 core minimal enhancer. The Kr protein binds to four sites within this enhancer element (diagrammed in Fig. 8A). As with the stripe 6 Kr sites, the stripe 5 Kr sites contain identifiable matches with the Kr-binding consensus, but the

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**Fig. 8.** Low affinity Kr-binding sites in conserved regions of the stripe 5 core element. (A) Location of Kr-binding sites in the 302 bp EagI-PvuI stripe 5 regulatory fragment. We identified 4 sites that are protected from DNaseI digestion by Kr protein, arrows indicate binding site orientation. Upon sequence alignment, these Kr sites appear more degenerate than the stripe 6 Kr-binding sites (compare to Fig. 7A), and do not produce as good of consensus. (B) Increasing amounts of Kr protein were used on parallel footprint assays on equimolar concentrations of the stripe 5 and stripe 6 regulatory fragments. The stripe 5 Kr footprints become evident only at the highest Kr protein concentration, while the stripe 6 Kr protein sites become apparent at a five-fold lower Kr concentration, suggesting that the stripe 5 sites are of significantly lower affinity. (C) The sequence of the stripe 5 core element. Bold nucleotides indicate conserved sequences identified in an interspecific comparison of the h regulatory region in *D. melanogaster* and *D. virilis* (Langeland and Carroll, 1993). Boxes indicate footprinted Kr-binding sites; note that 3 out of 4 of the Kr sites lie within conserved regions. The underlined sequences were deleted from this construct in order to make a construct lacking Kr sites 5(302ΔKr)lacZ. Reporter gene expression arising from this deleted construct (D) is very weak, but also appears derepressed in the Kr domain (arrows) relative to the wild-type construct (E, 5 (302)lacZ).
matches are considerably more degenerate (compare alignment in Fig. 8A with alignment in Fig. 7A). Note, however, that the Kr sites lie within regions that are conserved between Drosophila melanogaster and Drosophila virilis (Fig. 8C; see also Langeland and Carroll, 1993).

Intriguingly, Kr apparently has a lower affinity for the sites within the stripe 5 enhancer than it does for the stripe 6 enhancer (Fig. 8B). Kr-binding to the stripe 5 sites is evident only at the highest concentration of Kr protein that we used, while the stripe 6 Kr sites become evident at a much lower Kr concentration (a five-fold lower Kr concentration in our assay; only the four distal-most stripe 6 Kr sites are shown in this assay). In spatial terms, h stripe 5 closely abuts the posterior of the detectable wild-type Kr domain (Figs 4A, 6A), while stripe 6 lies in a region of much lower Kr protein (Figs 4B, 6A); in fact stripe 6 is in a region below the limits of immunohistochemical detection of Kr at the cellular blastoderm stage. Since Kr apparently represses both h stripes 5 and 6 (Fig. 4), the repressing activity must function at different concentrations of Kr protein. The different Kr-binding site affinities that we observe correlate nicely with the requirement for Kr to position each of these stripes at different concentration thresholds. Accordingly, we believe that the sites we have located may represent functional binding sites through which the Kr protein directly represses these stripes.

In order to test this contention, we deleted Kr-binding sites in the stripe 5 minimal element and reintroduced the mutagenized constructs into embryos. Deletion of all four binding sites (schematized in Fig. 8C) leads to significant spreading of stripe expression into the wild-type Kr domain, as well as a reduction in the level of stripe expression (compare Fig. 8D,E). Note that these deletions are rather large and eliminate not only the Kr sites, but flanking DNA as well. We feel the reduced expression may therefore be due to concomitant disruption of overlapping binding sites for currently unknown activating proteins. However, since the deleted Kr sites have been evolutionarily conserved and the expression arising from this deleted construct is evident across much of the wild-type Kr domain, the Kr-binding sites likely mediate Kr repression of this element.

The effect of ectopic gap protein expression on stripe formation

In all of the genetic observations described above, we have relied on loss-of-function backgrounds to assess the role of gap proteins on stripe formation. As a complementary approach toward testing the validity of our conclusions, we examined native h stripe expression in blastoderm embryos carrying a Kr gene under the control of the heat-inducible hsp70 promoter (kindly provided by G. Struhl). Upon heat induction (1- to 2-hour-old embryos induced up to 30 minutes followed by a 15 minute recovery, see Materials and Methods), the most severe phenotype resulting from ectopic Kr expression is convincingly simple (Fig. 9C): all h stripes are extinguished except stripes 3 and 4, which normally fall within the wild-type Kr domain. By examining large pools of embryos, it is apparent that the h stripes become extinguished in a progressive manner starting from the poles: first stripes 1 and 7, then stripe 6, and then stripes 2 and 5. An intermediate stage embryo with just h stripes 2, 3, 4 and 5 is shown in panel B. Note that the order of stripe disappearance does not correlate with the wild-type order of appearance of h stripes (Hooper et al., 1989), and thus is likely due to the level of Kr induction. Ectopic Kr therefore not only represses stripes outside of its normal domain (including stripes 5 and 6), but since stripes 3 and 4 are neither repressed nor ectopically activated, Kr protein does not appear to be sufficient to activate the stripes within its normal domain. Interestingly, expression of the eve gene parallels that of h in heat shock Kr embryos (data not shown), suggesting that Kr regulates these two overlapping pair-rule genes in a similar fashion.

DISCUSSION

The formation and positioning of pair-rule stripes in Drosophila embryos is dependent upon multiple regulatory inputs from members of the gap class of segmentation genes. In order to
understand how posterior pair-rule stripes are formed and adjacent stripes coordinate their position, we have focused on the regulation of two hairy (h) pair-rule stripes (stripes 5 and 6). We genetically isolated the effects of gap genes on the positioning of these stripes and examined the interactions of key gap proteins with the cis-acting regulatory fragments controlling each of these stripes. We demonstrate that spatial repression by gap proteins is the regulatory mechanism likely common to all primary pair-rule stripes and that activation strategies are more varied. Furthermore, the gap protein Kr appears to have a graded effect on stripes 5 and 6, thus allowing the spacing of adjacent stripes to be established by a common repressor.

**The role of gap proteins in pair-rule stripe positioning**

Our results indicate that the primary means of establishing the sharp boundaries of h stripes 5 and 6 is direct spatial repression by gap proteins. These stripes each display significant anterior expansion when Kr is removed. Additionally, stripe 5 expands posteriorly when gt is removed and stripe 6 expands posteriorly when hb is removed. Similar derepressions have been shown for the second stripe of the primary pair-rule gene even-skipped (eve) (Stanojevic et al., 1991; Small et al., 1991; 1992). The finding that posterior (h 5 and 6) and anterior (eve 2) pair-rule stripes require direct gap protein repression argues that this principle will prove to be general for all pair-rule stripes. This contrasts with the case for stripe activation. eve stripe 2 is jointly activated by hb and bed, both of which clearly overlap this stripe (Stanojevic et al., 1991; Small et al., 1991; 1992). As we show here, h stripe 6 absolutely requires kni to be activated, although it falls on the edge of the kni domain, and h stripe 5 appears to be only partially activated by kni, although it is coincident with peak kni expression. Accordingly, we view the mechanism of pair-rule stripe activation to be more divergent, with varying degrees of gap protein input.

In functioning as repressors, gap proteins appear to be critical at the periphery of their expression domains, where low levels of protein serve to repress transcription arising from stripe-specific enhancers. In each of the cases examined in the most detail (eve stripe 2, h stripes 5 and 6), the expression domains of the repressing proteins flank the affected stripe; this correlation between spatial and regulatory relationships argues that gap protein repression is direct. This conclusion is strengthened by the identification of binding sites for both posterior and anterior repressing gap proteins in the regulatory elements for these stripes. Putative binding sites for the gt protein have been localized in stripe 5 regulatory sequences (Langeland and Carroll, 1993), and several putative hb-binding sites exist in the stripe 6 regulatory sequences (J. Langeland, unpublished observations). Here, we have also directly identified several binding sites for the Kr repressor in both the stripe 5 and 6 regulatory sequences. In the case of stripe 6, these Kr-binding sites overlap extensively with binding sites for the kni protein, an obligate activator of stripe 6. While a detailed mechanistic understanding of stripe 6 regulation awaits the creation of point mutations in each of these overlapping binding sites, our demonstration that Kr can prevent the binding of kni activators is consistent with genetic observations and provides molecular support for a model first proposed by Pankratz et al. (1990) for the regulation of h stripe 6. This result suggests that Kr may be a direct competitive repressor of stripe 6 expression and that low amounts of Kr protein present at the extreme posterior limits of the Kr protein domain compete off activating kni protein, thus establishing the anterior limits of stripe 6.

In contrast to this clear mechanistic explanation for Kr repression of stripe 6, we do not understand how Kr may be repressing stripe 5. The Kr sites are evolutionarily conserved and appear to be required for repression of the stripe 5 element, yet the overall reduction of stripe 5 expression when Kr sites are deleted suggests that overlapping binding sites for activating proteins sites may also have been disrupted. Our genetic analyses have eliminated various gap proteins, but yielded no clues as to what the activator(s) may be. This leaves open the possibility that a competitive mechanism of Kr repression, similar to the one that we postulate for h stripe 6 may exist for h stripe 5.

**Kr coordinately positions stripes**

The genetic and molecular data described here indicate that the hierarchy of positional gradients in Drosophila likely extends to pair-rule gene regulation as well. Positional gradients are employed extensively in early Drosophila embryogenesis. Along the anteroposterior axis, the maternally supplied bicoid (bcd) morphogen gradient activates the gap gene hunchback (hb) above a critical threshold (Driever and Nüsslein-Volhard, 1989; Struhl, 1989). This produces a much steeper protein gradient which, in turn, acts both positively and negatively to help position the gap proteins Kr, kni and gt (Struhl et al., 1992). This system of graded morphogens works because early Drosophila development occurs in a syncytium in which molecules can diffuse from their source positions. Pair-rule stripes also form in a syncyntial environment and, while distinct combinations of gap proteins are clearly required to specify individual pair-rule stripes, there has been no evidence that graded levels of a single protein are critical for positioning different stripes. Kr has a steep bell-shaped distribution in the blastoderm embryo, presumably formed by protein diffusion from more sharply defined transcription boundaries (Gaul et al., 1987). The peak of Kr expression coincides with h stripes 3 and 4, while stripes 2 and 5 closely flank the Kr peak (Langeland and Carroll, 1993); these spatial relationships hold for eve stripes as well (Small et al., 1991). We have shown that h stripe 5 is repressed anteriorly by Kr, and Small et al. (1991, 1992) have shown that eve stripe 2 is repressed posteriorly by Kr. Given that the striped expression pattern of eve is only slightly out of phase with the h pattern (Carroll et al., 1988; Hooper et al., 1989), we strongly suspect that corresponding h and eve stripes will prove to have very similar regulatory mechanisms, and thus that h stripe 2 and eve stripe 5 are also repressed by Kr. Ectopic Kr has very similar effects on endogenous h and eve stripes, which tends to confirm this (J. Langeland, unpublished observations). Since stripes 2 and 5 are essentially mirror images with respect to their relationship to the Kr domain, Kr can position these stripes at nearly identical concentrations; this does not constitute a graded activity. However, as we have shown, Kr also represses h stripe 6 and binds its enhancer at much lower concentration than it binds the stripe 5 enhancer. This differential binding may allow stripe 6 to be repressed in regions of very low Kr (i.e. more posterior) while stripe 5 would require regions of higher Kr (i.e. more central) to be repressed. The fact that ectopic Kr extinguishes h stripe 6 prior to stripe 5 also supports this contention. This points to a new mechanism for pair-rule regulation in which
Kr sets positional values across the entire midrange of the embryo in a concentration-dependent manner, integrating the positions of at least three pair-rule stripes (stripes 2, 5 and 6).

While the mechanistic details of the regulation of several primary pair-rule stripes remain to be worked out, this, and that on eve stripe 2 suggest which features will prove general to the regulation of all pair-rule stripes. Whether organized as tight clusters, or more loose associations of binding sites, the existence of separable cis-elements for each stripe indicates that there is little coordination of stripe positions at the cis-level. Yet, as we have shown, stripes may be coordinated at the trans-level by common repressors. Indeed, direct spatial repression by flanking gap proteins is the most fundamental means of positionining stripes and forming stripe boundaries. Stripe activation is clearly more flexible, depending upon gap protein input to varying degrees. This being the case, a critical area for future mechanistic studies of pair-rule stripe regulation will be the identification of presumably general transcription factors that play a role in the stripe activation.

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