The *Drosophila* segmentation gene *runt* encodes a novel nuclear regulatory protein that is also expressed in the developing nervous system

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Generation of the anterior–posterior body pattern in the *Drosophila* embryo requires the activity of the segmentation genes. The segmentation gene *runt* has been classified as one of the primary pair-rule genes because of the pivotal role it plays in regulating the expression of other pair-rule genes. Here, we present the structure of this gene and describe the pattern of *runt* protein expression during embryogenesis. The deduced protein sequence shows no obvious overall homology with any sequences in the data base. The absence of an identifiable transcription factor motif (e.g., homeo box, zinc finger, leucine zipper, or helix-loop-helix) makes *runt* different from the other early-acting segmentation proteins. A *runt*-specific polyclonal antibody was generated and used to demonstrate that the subcellular location of the protein is in the nucleus. Double-staining immunolocalization experiments were used to determine the overlap of the *runt* protein pattern with the patterns of the pair-rule genes *hairy* (*h*), *even-skipped* (*eve*), and *fushi tarazu* (*ftz*). We found that the patterns of *runt* and *hairy* are complementary. Their phasing is shifted anteriorly by two cell diameters with respect to the complementary *eve* and *ftz* patterns. Experiments with the *runt* antibody also indicated that the protein is present throughout embryogenesis and is expressed extensively in the developing central and peripheral nervous system.

[Key Words: *Drosophila*; segmentation gene; nuclear protein; neurogenesis]

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Segmentation in *Drosophila* offers a unique opportunity to investigate embryonic pattern formation. Extensive genetic studies have identified many genes that are required for normal segmentation, and both molecular and genetic approaches have yielded significant insight into this process. The segmental primordia are established by the action of the gap and pair-rule segmentation genes during the blastoderm stage of embryogenesis. These genes respond to coarse gradients of maternal information present in the egg and act to subdivide the body of the embryo into its initial metameric pattern. Molecular analyses have indicated that fundamental aspects of this process are accomplished through a hierarchy of regulatory interactions between genes encoding transcriptional regulatory proteins. All of the gap gene proteins characterized to date encode nuclearily localized proteins. The proteins of the gap gene Krüppel (Kr), *hunchback* (*hb*), and *knirps* (*kni*) all contain zinc finger DNA-binding motifs (Rosenberg et al. 1986; Tautz et al. 1987; Nauber et al. 1988). Several studies demonstrated that these gap proteins bind to DNA in vitro. The gap proteins act to regulate the expression of each other (Jäckle et al. 1986; Mohler et al. 1989; Pankratz et al. 1989) and also play a critical role in generating the periodic expression patterns of the pair-rule genes (Carroll and Scott 1986; Frasch and Levine 1987; Howard 1988; Ingham and Gergen 1988). In vitro binding sites for the Kr, hb, and kni gap proteins have been identified in the promoter regions of some of the relevant genes (Pankratz et al. 1989, 1990; Stanojevic et al. 1989; Treisman and Desplan 1989). These several observations suggest strongly that the important regulatory roles the gap genes play in segmentation are mediated by their proteins acting as DNA-binding transcription factors.

Similar arguments suggest strongly that the functions of the pair-rule genes also involve the regulation of transcription. The pair-rule genes act to regulate each other (Carroll and Scott 1986; Howard and Ingham 1986; Ingham and Gergen 1988), as well as to regulate the expression of the later-acting segment–polarity genes (DiNardo and O’Farrell 1987). Several lines of evidence suggest that these regulatory effects are mediated at the transcriptional level. The *even-skipped* (*eve*), *fushi tarazu* (*ftz*), and *hairy* (*h*) proteins are localized in the nuclei of expressing cells (Carroll and Scott 1985; Frasch et al. 1987; Carroll et al. 1988; Hooper et al. 1989). The
Kania et al.

eve and ftz proteins, as well as that encoded by the paired (prd) gene, all contain the homeo box DNA-binding domain (Laughon and Scott 1984; Frigerio et al. 1986; Macdonald et al. 1986; Frasch et al. 1987). In vitro-expressed forms of all three of these homeo box-containing pair-rule proteins bind DNA (Desplan et al. 1988; Hoey and Levine 1988; Treisman et al. 1989). An additional important piece of evidence that these proteins function as transcriptional regulators comes from the observations that ftz activates transcription from promoters containing consensus homeo domain-binding sites both in Drosophila cells (Jaynes and O'Farrell 1988) and in yeast (Fitzpatrick and Ingles 1989). The h protein, which plays an important role in regulating the expression of eve and ftz, does not have a homeo box but, instead, has homology to an amphipathic helix-loop-helix motif present in a variety of other transcription factors (Rushlow et al. 1989).

Here, we investigate the protein encoded by the pair-rule gene runt. This gene plays a key role in regulating the expression of all other pair-rule genes examined to date (Carroll and Scott 1986; Frasch and Levine 1987; Ingham and Gergen 1988; Carroll and Vavra 1989; Hooper et al. 1989). Therefore, defining the function of runt and the mechanism by which it acts to regulate other genes is central to understanding the process of segmentation. In this paper, we show that runt encodes a protein without obvious homology to recognized motifs for DNA binding or protein–protein interaction. However, consistent with a potential role as a transcriptional regulator, we found that this protein is localized in the nucleus. We determined the pattern of runt expression relative to other pair-rule genes. These results are discussed in the context of the known regulatory interactions between these genes. Interestingly, we also found that the runt protein is expressed extensively in both the central nervous system (CNS) and peripheral nervous system (PNS) of the developing embryo.

Results

Organization of the runt transcription unit

The runt locus encodes a 2.6-kb poly(A)+ RNA that is most abundantly expressed at the blastoderm stage of embryogenesis (Gergen and Butler 1988). We used S1 nuclease protection, primer extension, and DNA sequence analyses to determine the structure of this transcription unit. A schematic summary of these results is shown in Figure 1. S1 nuclease protection experiments done with embryo RNA indicate that runt RNA is comprised of two principal exons, 1.5 and 0.95 kb in size (Fig. 2A). The localization of the intron was determined from the sequence of the genomic and cDNA clones. The intron is 409 nucleotides in length, is flanked at its 5′ and 3′ boundaries by the consensus GT...AG nucleotides, respectively, and agrees well (21 of 23 positions; data not shown) with a consensus sequence for intron boundaries (Mount 1982).

The sequence spanned by the cDNA clone inserts (2454 nucleotides; see below) should represent almost all of the 2.6-kb runt mRNA. The 3′-most cDNA clone (E20) contains the consensus AATAAA signal for poly(A) tail addition 18 nucleotides upstream of a string of 17 As [Fig. 3]. This suggests that this cDNA extends to the 3′ end of the transcription unit. Primer extension experiments were carried out to determine the extent of 5′ sequence not contained within the cDNAs. The results reveal a single predominant reverse transcription product that extends 30 nucleotides beyond the end of the sequence contained in the 5′-most cDNA clone (D1, see Fig. 2B). Figure 2B also shows the genomic DNA sequence in this region. There are no good consensus splice acceptor sites within this 30 nucleotides of upstream sequence, suggesting that transcription initiates at the position indicated by the reverse transcription product. The sequence around the putative cap site is in perfect concordance (7 of 7) with a consensus derived from other Drosophila genes (Hultmark et al. 1986). There is an AT-rich TATA box-like region [Fig. 3] 30 nucleotides upstream of the putative cap site. Further evidence that this cap site corresponds to the site of transcript initiation comes from in vitro transcription experiments with Drosophila embryo extracts. These reveal a single run-off transcription product that initiates at or near the site indicated by our primer extension analysis (J. Topol and C. Parker, pers. comm.). On the basis of these observations, we conclude that the runt mRNA is comprised of only two exons and that transcription initiates at the nucleotide designated +1 in Figure 3.

Deduced sequence of the runt protein

We have used both cDNA and genomic clones to determine the sequence of the runt gene. This sequence is shown in Figure 3, where we included 67 nucleotides of
Comparison of the in vitro translation product with a full-length, nonfusion protein made in bacteria provided stronger evidence that the predicted first AUG is utilized. The recombinant DNA construction used to generate this bacterial form of the runt protein was designed to initiate translation at the predicted first AUG. These proteins both migrated at 68 kD on SDS gels and also gave identical partial digestion patterns with V8 protease (data not shown). On the basis of these results, we conclude the runt mRNA encodes the ORF shown in Figure 3. Experiments described below demonstrate that antibodies directed against this ORF recognize the runt protein in Drosophila embryos. The anomalous migration of this protein [68 vs. 53 kD predicted] is most likely due to its unusual amino acid composition (e.g., 16% alanine, 14% serine, 9% proline). Similar observations have been made for many other proteins, including several involved in segmentation [Carroll and Scott 1986; Gaul et al. 1987; Ollo and Maniatis 1987; Krause et al. 1988].

A search of the Swiss Protein Data Base (release 11) with the FASTP algorithm revealed no sequences of obvious overall homology or relatedness to the deduced runt protein sequence. We searched carefully for and failed to find any homology between the runt sequence and the zinc finger and homeo box DNA-binding motifs found in other segmentation genes. We also found no evidence of homology to the leucine zipper or the amphipathic helix-loop-helix motifs similar to those described for a variety of transcriptional regulatory proteins.

A significant difference between runt and other characterized gap and pair-rule proteins is the prediction that the runt protein has an amino-terminal signal sequence, which could target it for translocation across the membrane of the endoplasmic reticulum [see Fig. 3]. An unusual aspect of this predicted signal sequence is that 15 of the 23 hydrophobic residues are alanine. Twelve of these are in a homopolymeric run, extending from position 18 to position 29 in the protein sequence. This type of arrangement is unprecedented in known signal sequences; however, there is precedent for homopolymeric stretches similar to this in the nonsecreted segmentation proteins eve and engrailed [en]. Therefore, the possibility exists that the amino-terminal localization of this element is coincidental and that the predicted signal sequence might not be functional.

Localization of runt protein in the blastoderm embryo

We generated a runt-specific antiserum to characterize the in vivo localization of the protein. This antiserum was raised against a β-galactosidase fusion protein containing the entire runt ORF (for details of construction and for purification of runt specific antibodies, see Materials and methods). This antibody reveals a pair-rule pattern of seven stripes of staining in blastoderm-stage Drosophila embryos (Fig. 4A). This staining is runt-dependent, as it is eliminated in embryos mutant for a transcript null allele runt(L8B1) (Fig. 4B). The staining observed in blastoderm-stage embryos suggests strongly
that the subcellular localization of the \textit{runt} protein is in the nucleus. To distinguish more definitively between the possibility of intranuclear versus nuclear membrane localization, we compared the \textit{runt} staining pattern with that of \textit{Drosophila} lamin, which is nuclear membrane associated. As a control for another intranuclear protein, we also examined the localization pattern of the \textit{eve} protein. Surface views of staining patterns with these three antisera show clearly that the \textit{runt} pattern [Fig. 5A] resembles that of \textit{eve} [Fig. 5C] more closely than that of \textit{lamin} [Fig. 5B]. We therefore conclude that the \textit{runt} protein is localized within nuclei of blastoderm-stage embryos.

We performed double-label immunolocalization experiments to determine the phasing of the pattern of \textit{runt} expression relative to other segmentation genes. In blastoderm embryos double-stained for \textit{runt} and \textit{h}, the stripes of \textit{h} accumulation [indicated by brown] are anterior to the stripes of \textit{runt} accumulation [blue, Fig. 6A]. These two patterns are complementary, and no clear
Figure 4. Pattern of runt expression. Photomicrographs of Drosophila embryos stained with affinity-purified rabbit anti-runt antibodies are shown. Immunoreactivity is indicated by a brown staining reaction. This was obtained by use of a biotin-conjugated anti-rabbit secondary antibody and the HRP version of the ABC immunodetection kit (Vector Laboratories). All embryos are oriented with their anterior end to the left. (A) and (B) are both lateral views of blastoderm-stage embryos. The embryo in (A) is wild type, that in (B) is hemizygous mutant for a runt allele that produces no mRNA (LBS). Lateral views of wild-type embryos during mid-germ-band extension, fully extended, and after germ-band retraction, are shown in (C), (E), and (G), respectively. (D) and (F) are dorsal views of mid- and fully germ band-extended embryos. (H) is a ventral view of a germ band-retracted embryo.

gaps of nonexpressing cells are evident within the region of the embryo that contains the stripes. This complementarity is retained until the stage at which h expression disappears. A different result is obtained in embryos double-stained for runt and eve. The stripes of eve accumulation (blue) lie anterior to those of runt, but overlap them partially (brown; Fig. 6B). Normal eve and runt stripes would be four nuclei wide at this stage. Two rows of eve-expressing (blue) nuclei are evident anterior to an overlap region comprised of two rows of nuclei that express both of these proteins. Posterior to this are two rows of runt-expressing (brown) nuclei, followed by two rows of nuclei located between the posterior margin of each runt stripe and the anterior margin of the next eve stripe that do not express detectable levels of either protein. We also performed double-staining experiments to determine the relative phasings of runt and ftz. These patterns also overlap, but the runt stripes are more ante-
Kania et al.

Figure 5. The runt protein is localized within blastoderm nuclei. Surface views of blastoderm-stage embryos stained with antibodies that detect runt (A), nuclear lamin (B), and eve proteins (C) are shown. All three antisera are rabbit polyclonals. Immunoreactivity was detected with biotin-conjugated anti-rabbit secondary antibodies and the HRP version of the Vector Laboratories ABC kit.

rior (Fig. 6C). Two rows of runt-expressing nuclei (brown) lie anterior to each region of overlap, and two rows of ftz-expressing nuclei (blue) lie posterior to each region. The gaps of unstained nuclei consistently appeared larger in the runt-ftz double-stained embryos than in the runt-eve embryos. This probably reflects the fact that for ftz, the nuclei not contained in the overlap are those from the posterior half of each stripe. These are the nuclei that initially stain more weakly for ftz and lose ftz expression when the stripes narrow (Carroll and Scott 1985). The opposite is true for eve, where eve-expressing nuclei not in the runt overlap region stain more strongly and retain expression as the eve stripes narrow (Frasch and Levine 1987). In conclusion, the runt and h patterns are complementary and are phase-shifted anteriorly with respect to the complementary eve and ftz patterns. The relative phasing of these pair-rule patterns of expression is maintained along the length of the anterior–posterior axis.

Postblastoderm expression of the runt protein

In early germ band-extending embryos, the pattern of runt mRNA accumulation undergoes a transition from a pair-rule to a segmental pattern of expression and is also expressed in a dorsal patch of cells in the head (Gergen and Butler 1988). These patterns are clearly evident in embryos stained with the runt antibody (Fig. 4). The expression of runt in the head of early germ band-extending embryos is composed of two regions of staining. There is a dorso–ventral group of ~10 cells and a ventral group of 7 less intensely stained cells (Fig. 4C,D). Expression in this region is much more complex by the time of full germ-band extension, with four pairs of clusters of staining cells [Fig. 4E,F]. At this stage in the segmented regions of the body, the staining intensifies within individual cells just off the ventral midline and fades laterally in each segment. The stained cells along the ventral midline (approximately six per segment) are of different sizes, have varied staining intensities, and probably correspond to neuroblasts (Fig. 4F). During germ-band retraction, this putative neural expression of runt becomes more evident, with accumulation in regions corresponding to both the CNS and PNS. Upon completion of germ-band retraction, ~50 cells per hemisegment, located ventrally in a region corresponding to the developing CNS, are stained with the runt antibody (Fig. 4H). Three more laterally situated clusters of runt-expressing cells are found in regions that would correspond to PNS (Fig. 4G). These postblastoderm patterns of antibody staining are due to accumulation of runt protein as they are eliminated in runtLBs mutant embryos. At these later stages, the protein appears to be accumulating primarily in the nuclei of expressing cells.

We examined the pattern of accumulation in embryos mutant for the Notch gene to demonstrate that this later runt expression is in neural cells. In these mutant embryos, the cells in the neurogenic region that would normally take on epidermal fates instead become neuroblasts. The presence of more runt-expressing cells in these embryos in regions corresponding to both CNS and PNS (Fig. 7A) provides evidence that runt is being expressed in cells of neural origin. The expanded runt expression in these embryos is still organized into a fairly coherent pattern. This is most evident in the PNS. For example, runt is normally expressed in three cells in a region corresponding to the V’ cluster (Fig. 7B). In Notch− embryos, a cluster of runt-expressing cells is still evident in this region but is now expanded to include from 12 to 18 cells [Fig. 7C]. This observation is consistent with an “equivalence group” model of neuroblast determination. In this model, groups of cells are prespecified such that they have the potential to become different types of neuroblasts (e.g., runt expressing). The neurogenic genes normally act to ensure that the proper number of neuroblasts is produced from these groups and that other cells in the group are inhibited from en-
runt sequence and protein distribution

Figure 6. Phasing of patterns of pair-rule protein accumulation. Blastoderm-stage embryos double-stained with antibodies against runt and h [A], eve [B], and ftz [C] are shown. (A) runt accumulation is indicated by blue, and h by brown. (B and C) The runt pattern is indicated by brown staining, and eve and ftz, respectively, are indicated by blue. An enlarged view of these patterns is shown at right of (A–C). Our interpretation of the phasings of each of the other pair-rule genes relative to runt is indicated just above each enlargement. The h antiserum is a rat polyclonal. The other antisera are all rabbit polyclonals. (For details of the double-staining protocols, see Materials and methods.)

itering this pathway but, instead, differentiate into epi-
dermblasts. When this regulation is removed, other cells in the equivalence group with the potential to become runt-expressing neuroblasts do so.

Discussion

Function of the runt protein

We determined the sequence of the Drosophila segmentation gene runt. Antibodies raised against the ORF deduced from this sequence recognize a nuclear protein that shows the anticipated pair-rule pattern of expression at the blastoderm stage. The regulation of the pair-rule genes eve, ftz, and h depends on runt function (Carroll and Scott 1986; Frasch and Levine 1987; Ingham and Gergen 1988). Investigations of the cis-regulatory regions of all three of these genes (Hiromi and Gehring 1987; Howard et al. 1988; Dearolf et al. 1989; Goto et al. 1989; Harding et al. 1989; Hooper et al. 1989), as well as RNA-labeling experiments (Edgar and Schubiger 1986; Edgar et al. 1986), indicate that significant aspects of the regulation of these genes are mediated at the level of transcription. Given these observations, we might expect runt to encode some type of transcriptional regula-
tory protein. This expectation is consistent with the observed nuclear localization of the protein.

The sequence of the runt protein provides no obvious clues as to how it might be involved in transcriptional regulation. It does not contain one of the known types of DNA-binding domains nor does it contain one of the recognized protein–protein interaction motifs for transcriptional regulatory proteins. This stands in dramatic contrast to other segmentation genes that act at the blastoderm stage (Table 1). The products of three of the gap genes contain zinc finger DNA-binding motifs (Rosenberg et al. 1986; Tautz et al. 1987; Nauber et al. 1988). Among the pair-rule genes, the eve, ftz, and prd proteins all contain a DNA-binding homeo domain (Laughon and Scott 1984; Macdonald et al. 1986; Frigerio et al. 1986; Frasch et al. 1987). The pair-rule gene h, which, like runt, also plays a key role in regulating other pair-rule genes, is a member of the helix–loop–helix family of transcription factors (Rushlow et al. 1989). If runt plays a direct role in regulating transcription, it would therefore represent a novel type of regulatory protein within the segmentation gene hierarchy.

There are several examples of DNA-binding transcriptional regulatory proteins for which no identifiable
structural motif has yet been defined. In a recent review on transcriptional regulatory proteins, nine transcription factors fell into this category (Johnson and McKnight 1989). However, a feature that is common to all of these proteins, as well as to the other characterized gap and pair-rule proteins [Table 1], is the presence of regions containing clusters of basic residues [a cluster of basic residues being defined as a region containing three basic residues within a 5-amino-acid stretch]. Most of these proteins contain multiple clusters of basic residues [mean = 4]. In contrast, there are no regions in the runt protein where three basic residues are clustered within a 5-amino-acid stretch. The basic regions of transcription factors have been implicated as playing roles in DNA binding. The original motif defined for the helix–loop–helix family of proteins contains a basic region that is frequently contain several β-strands, which are thought to form a stable hydrophobic binding pocket (Bradley et al. 1987). Secondary structure predictions indicate five potential β-strands in the putative runt ATP-binding site. One is just before the glycine-rich A box, and the other four occur within the next 60 residues [three before and one after the B box]. Although the consensus motif for ATP-binding domains is not absolutely predictive, these several observations suggest that runt has the potential to be an ATP-binding protein. In this regard, it is worth noting that runt does not have homology to the related (and more predictive) consensus motifs that exist for either GTP-binding proteins or protein kinases.

Pattern of runt expression

The blastoderm expression pattern of the runt protein is consistent with expectations from analysis of RNA expression (Gergen and Butler 1988). We found the protein to be expressed in a pattern complementary to that of the pair-rule gene h. This phasing agrees well with the interpretation that these two genes play complementary and mutually antagonistic roles in regulating the expression of each other and the other pair-rule genes (Frasch and Levine 1987; Ingham and Gergen 1988; Carrol and Vavra 1989). We found that the stripes of runt accumulation overlap with the posterior half of each eve stripe, and the anterior half of each ftz stripe. Once the
Table 1. Features of segmentation proteins

| Gap genes  | Transcription factor motif | Number of clusters of basic residues | PEST regions |
|------------|----------------------------|-------------------------------------|--------------|
| Krippel    | zinc fingers (4)           | 4                                   | 0            |
| hunchback  | zinc fingers (6)           | 3                                   | 2            |
| knirps     | zinc fingers (2)           | 5                                   | 0            |
| giant      | leucine zipper             | 5                                   | 1            |

| Pair-rule genes | Transcription factor motif | Number of clusters of basic residues | PEST regions |
|-----------------|----------------------------|-------------------------------------|--------------|
| even-skipped    | homeo box                  | 3                                   | 3            |
| fushi tarazu    | homeo box                  | 5                                   | 2            |
| hairy           | helix−loop−helix           | 3                                   | 1            |
| paired          | homeo box                  | 2                                   | 1            |
| runt            | —                          | 0                                   | 2            |

This table was compiled by analyzing available sequence data. The references are Rosenberg et al. (1986) (Kr); Tautz et al. (1987) (hb); Nauber et al. (1988) (kni); B. Eldon and V. Pirrotta (pers. comm.) (get); Macdonald et al. (1986) (eve); Laughon and Scott (1984) (ftz); Rushlow et al. (1989) (h); and Frigerio et al. (1986) (pred). Clusters of basic residues were defined as regions where at least three of five consecutive residues are basic (i.e., either lysine or arginine). PEST regions were identified and scored as good or poor by using the PESTFIND program in the PC/GENE sequence analysis package. This program utilizes the original PEST algorithm of Rogers et al. (1986). The selection parameters specified minimal regions of 10 residues containing at least one proline, one glutamic or aspartic acid, and one serine or threonine. Good PEST regions have scores of 5 or better.

The initial four-cell-wide stripes of eve and ftz are established, they evolve rapidly by narrowing. This occurs by the elimination of expression in the more posterior rows of cells in each stripe. Our results show that those cells that lose eve expression are also those that express runt. This is exactly what is expected given the negative regulatory effect that runt has on eve expression (Fraser and Levine 1987; Ingham and Gegen 1988). Our results indicate a reciprocal role for runt in the regulation of ftz. The cells in the anterior region of each ftz stripe, in which the expression of ftz perdures, are those that express runt. The regulatory effects of runt could be direct, or they could be indirect and due to the repressing effects of runt on h. In this paper, we show that this gene is normally expressed in a pattern complementary to runt. It is negatively regulated by runt (Ingham and Gegen 1988) and regulates both ftz and eve in a manner reciprocal to runt. Elucidation of the mechanisms of these regulatory effects requires more knowledge on the function of runt.

We found that the runt protein is expressed throughout embryogenesis. The protein is expressed in both the CNS and the PNS. Several other segmentation proteins have also been found to be re-expressed in the embryonic nervous system (Carroll and Scott 1985; DiNardo et al. 1985; Fraser et al. 1987; Gaul et al. 1987). For the pair-rule proteins eve and ftz, there is evidence that this expression plays a role in the specification of neural cell fates (Doc et al. 1988a,b). The extensive neural expression of runt suggests that it may play an important role in the developing nervous system. This suggestion is supported by experiments with a runt temperature-sensitive allele, which indicate that runt is involved in regulating the expression of eve in the CNS [J.B. Duffy and J.P. Gegen, unpubl.]. These observations are particularly intriguing in the context of the recent hypothesis that the neural functions of the Drosophila segmentation genes are the ancestral ones that have been conserved throughout evolution (Patel et al. 1989). This suggests that investigating the function of the runt protein and the mechanism by which it regulates the expression of other genes will have significance beyond contributing to our understanding of the formation of body pattern in the early Drosophila embryo.

Materials and methods

Plasmid DNAs

The three original runt cDNA clones (D1, E20, and E25) were isolated from libraries provided by the laboratory of T. Kornberg [University of California, San Francisco; see Poole et al. 1985]. The composite cDNA referred to as ED was constructed by joining a fragment that corresponds to the 5' end of the mRNA from clone D1 to a fragment that corresponds to the 3' end of the mRNA from clone E20. These two fragments were joined at a unique XhoI site present in their region of overlap (map position 492 in Fig. 3). The construct used to generate the runt–β-galactosidase fusion protein used for immunization was made by cloning an ORF-spanning fragment of the E25 cDNA into the pWRS90-1 vector (Guo et al. 1984). Sequences from the region corresponding to the 5'-untranslated leader were removed by using oligonucleotide-site-directed mutagenesis to insert a BamHI recognition site starting 9 nucleotides upstream of the ATG at the beginning of the runt ORF. A T7 expression system (Studier and Moffatt 1986) was used to express a nonfusion form of the runt protein in bacteria. Site-directed mutagenesis was used to create an Ndel cleavage site at the position of the first ATG in the E25 and ED cDNAs. ORFs were then cloned into the pT7-7 expression vector using this site (S. Tabor, pers. comm.; see Tabor and Richardson 1985).

A nested set of unidirectional deletions were generated for sequencing, using the exonuclease III/mung bean nuclease strategy (Henikoff 1984, Stratagene kit). Deletion series were made from both ends of cDNA E20 and from the 5' end of...
Kania et al.

cDNA D1. The genomic DNA clone used for S1 nuclelease protection contained an 8.5-kb fragment extending from a BamHI site, 2.5 kb upstream of the transcription unit, to a downstream HindIII site. Genomic sequence information was obtained from this template and/or subcloned derivatives of it.

**Nucleic acid manipulation**

Isolation of plasmid DNAs, restriction digestions, and analytical and preparative gels were all done under standard conditions (Maniatis et al. 1982). S1 nuclease protection experiments were done essentially as described in Maniatis et al. (1982). Hybridizations were done with 1 µg of the cloned genomic DNA and 50 ng of labeled oligonucleotide. DNA isolation of plasmid DNAs, restriction digestions, and analyses (Maniatis et al. 1982). S1 nuclease protection experiments were done essentially as described in Maniatis et al. (1982). Hybridizations were done with 1 µg of the cloned genomic DNA and 50 ng of labeled oligonucleotide. DNA isolation of plasmid DNAs, restriction digestions, and analyses (Maniatis et al. 1982). S1 nuclease protection experiments were done essentially as described in Maniatis et al. (1982). Hybridizations were done with 1 µg of the cloned genomic DNA and 50 ng of labeled oligonucleotide. DNA isolation of plasmid DNAs, restriction digestions, and analyses (Maniatis et al. 1982). S1 nuclease protection experiments were done essentially as described in Maniatis et al. (1982).

**Homology searches**

Homology searches were done by using the FASTP algorithm with this package and the Compact Disc version of the Swiss Protein Data Base (release 11, 10,856 sequences). Homology searches were also done on the NBRF data base (12,476 sequences). Homology searches were done by using the FASTP algorithm with this package and the Compact Disc version of the Swiss Protein Data Base (release 11, 10,856 sequences). Homology searches were done by using the FASTP algorithm with this package and the Compact Disc version of the Swiss Protein Data Base (release 11, 10,856 sequences).

DNA sequence analysis

The PCGENE sequence analysis package was used for analysis. Homology searches were done by using the FASTP algorithm with this package and the Compact Disc version of the Swiss Protein Data Base (release 11, 10,856 sequences). Homology searches were done by using the FASTP algorithm with this package and the Compact Disc version of the Swiss Protein Data Base (release 11, 10,856 sequences). Homology searches were also done on the NBRF data base (12,476 sequences). Homology searches were also done on the NBRF data base (12,476 sequences). Homology searches were done by using the FASTP algorithm with this package and the Compact Disc version of the Swiss Protein Data Base (release 11, 10,856 sequences). Homology searches were done by using the FASTP algorithm with this package and the Compact Disc version of the Swiss Protein Data Base (release 11, 10,856 sequences). Homology searches were done by using the FASTP algorithm with this package and the Compact Disc version of the Swiss Protein Data Base (release 11, 10,856 sequences).

**Immunization and antibody purification**

Four young male rabbits (New Zealand) were immunized with 200 µg of gel-purified pWR/runt protein emulsified in complete Freund’s adjuvant. Subsequent monthly boosts were done with the same amount of antigen in incomplete adjuvant. Serum was collected 10 days after a boost. One rabbit gave a useful immune response (staining of embryos with a 1:25 dilution of sera) after the fifth boost. Affinity purification of antibodies from this sera was required to reduce nonspecific background staining. The procedure used for affinity purification was adapted from Guerriero et al. (1986). Purified nonfusion—runt protein made with the T7 expression system was spotted onto nitrocellulose and dried. Filters were blocked in Blotto [50 mM Tris (pH 7.5), 0.9% NaCl, 0.05% Tween 20, 3% Carnation nonfat dry milk powder, 0.05% sodium azide], and rinsed in TBS plus sodium azide [TBS is 20 mM Tris (pH 7.5), 500 mM NaCl]. Rabbit serum was diluted 1:1 in TBS and incubated with the filter overnight at 4°C with gentle agitation. Nonspecifically bound antibodies were removed with several washes of TBS over a 2-hr period at room temperature. runt-specific antibodies were eluted from the filter by washing it in 0.5 ml of 0.1 M glycine (pH 2.7) and 75 mM NaCl for 2 min. This eluant was pooled with the eluant from one subsequent wash of the filter with 6 mg/ml BSA and dialyzed against TBS at 4°C. Affinity-purified antibody was used undiluted for immunohistochemistry.

The h antibody was obtained from Ken Howard and Gary Struhl (Columbia College of Physicians and Surgeons, New York). This polyclonal serum was generated in rats and used at a 1:200 dilution. The eve antibody was obtained from Manfred Frasch (Max Planck Institut für Entwicklungsbiologie, Tübingen). This rabbit polyclonal was used at a dilution of 1:10,000. The fitz antibody was obtained from Henry Krause [Banting and Best Institute, Toronto] and was used at a dilution of 1:500. The lamin antibody was provided by Paul Fisher [SUNY at Stony Brook, New York] and was used at a dilution of 1:800. The biotin conjugated anti-rabbit IgG (Vector labs) and anti-rat IgG (Sigma) antibodies were used at 5 µg/ml. The alkaline phosphatase-conjugated anti-rabbit IgG antibody (Jackson) was used at 2 µg/ml. All secondary antibodies were preadsorbed against random embryos at twice the concentrations listed above.

**Drosophila embryos and immunohistochemistry**

The wild-type embryos were from a stock homozygous for yellow and white embryos were collected from a stock in which the FMR7 X-chromosome balancer carries a P-element that expresses β-galactosidase under the control of the fitz promoter. This chromosome was generated by use of 62-3 (Robertson et al. 1988) to move fitz/lacC transposons [Hiromi et al. 1985] onto FM7c. In this stock, the male embryos mutant for runt are the only embryos that do not express β-galactosidase in the typical fitz pattern. Therefore, the mutant runt embryos can be identified unambiguously by double staining with antibodies against β-galactosidase. Because of the stability of the β-galactosidase protein in the embryo, this marker is useful for all stages of embryogenesis subsequent to the initial appearance of the fitz/lacC pattern at the late cellular blastoderm stage. The runt mutation is a null allele that produces no transcript [Gergen and Butler 1988]. The Notch2.44 mutation is an embryonic lethal allele with an intermediate phenotype. It was obtained from the stock collection at Princeton University.

Embryos for immunohistochemistry were permeabilized, fixed, and devitelilized, essentially as per Mitchison and Sedat [1983]. The aqueous fixative phase was 4% formaldehyde and contained 10 mm potassium phosphate (pH 6.8), 45 mM KCl, 15 mM NaCl, and 13 mM MgCl2. Fixed embryos were rehydrated in PBS [137 mM NaCl, 3 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4] and blocked with 2% goat serum [Cappel] in PBS for 30 min at room temperature. Primary antibody was diluted in PBS/MT [PBS containing 1% milk powder and 0.1% Tween 80] and incubated with embryos either for 4 hr at room temperature or overnight at 4°C. Embryos were then washed 10 times with 0.4 ml of PBS/MT and incubated with preadsorbed sec-
orary antibodies for either 2 hr at room temperature or overnight at 4°C. After this incubation, the embryos were washed 10 times in PBS/T (PBS plus 0.1% Tween 80). The HRP (Horseradish peroxidase) version of the Vectastain kit (Vector) was used with biotinylated secondary antibodies to obtain brown immunohistochemical staining signals. The biotinylated HRP/Avidin-DH complex was formed according to the supplier's protocol and incubated with the relevant embryos for 30 min. After 10 washes in PBS/T, the embryos were postfixed for 10 min in ethanol and rehydrated in HRP buffer [50 mM citric acid, 50 mM ammonium acetate (pH 5.0)]. The first step of this rehydration was done in 1:1 ethanol/HRP buffer. Embryos were stained in HRP buffer containing 2.7 mg/ml diaminobenzidine, 0.02% NiCl₂ and CoCl₂, and 0.006% hydrogen peroxide. The staining reaction was stopped by addition of sodium azide to 2%. Prior to mounting, the embryos were washed in PBS plus azide, dehydrated in ethanol, cleared in Histoclear (National Diagnostics), and mounted in either Histomount (National Diagnostics) or Permount.

The procedure for double staining embryos was adapted from a protocol provided by Nipam Patel (University of California, Berkeley). Embryos were fixed and rehydrated as above and then blocked in PBT + N (0.2% BSA, 0.1% Triton X-100, 0.5% goya protein in PBS). When the primary antibodies were derived from different species (i.e., runt–β double), they were simultaneously incubated with the embryos in PBT + N. In this case, after washing the embryos as above, the two secondaries were then also incubated simultaneously with the embryos. The HRP color reaction was done first. The embryos were incubated for 10 min in PBT containing 0.3 mg/ml diaminobenzidine. Hydrogen peroxide was then added to 0.01% to start the staining reaction. After development of the HRP color reaction, the embryos were rinsed in PBT and transferred to AP buffer [100 mM NaCl, 5 mM MgCl₂, 100 mM Tris (pH 9.5), 0.1% Tween 20, 1 mM levamisole]. The alkaline phosphatase color reaction was started by adding the B-CIP and X-phosphate substrates (Boehringer). In the cases where the two primary antibodies were from the same species, the antibody to be visualized with HRP was carried through the entire staining procedure. The embryos were then reblocked, as described above, and the entire set of incubations was repeated with the other antibody and the AP staining protocol.

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