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Intron and upstream sequences regulate expression of the *Drosophila* β3-tubulin gene in the visceral and somatic musculature, respectively

(mesoderm/differentiation/tissue-specific regulation)

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ABSTRACT **The morphogenetic programs involved in the differentiation of internal organs, such as the muscle system, during *Drosophila* embryogenesis have remained largely obscure. β3-tubulin has proved to be a good marker for mesoderm development as this tubulin isotype is detectable soon after mesoderm formation and during the process of mesoderm differentiation. The β3-tubulin gene is expressed in the somatic and pharyngeal musculature, the dorsal vessel, and the visceral musculature. To learn more about the programs underlying mesodermal differentiation, we have started to dissect the regulatory elements of the β3-tubulin gene by means of *P*-element-mediated transformation experiments. We show that expression of the β3-tubulin gene in the somatic muscles, the pharyngeal muscles, and the dorsal vessel is mediated by far upstream sequences. We also demonstrate that the first intron of the β3-tubulin gene bears a tissue-specific enhancer element that is required for expression in the visceral muscles and that also functions efficiently when cloned downstream of an indicator gene. The separability of elements driving β3-tubulin expression in the somatic and visceral mesoderm facilitates the investigation of the different programs involved in regulating the early differentiation of this germ layer.**

In comparison to ectoderm differentiation, little is known about the differentiation of the mesodermal germ layer in *Drosophila* (for review, see refs. 1–4). The mesoderm of *Drosophila* is formed during gastrulation. The β3-tubulin gene has been cloned (5, 6) and was recently shown to be specifically expressed in the mesodermal germ layer during embryogenesis (7). Antibodies against this β-tubulin isotype enable the differentiation of the muscle system to be followed (8). The gene is expressed in all detectable mesodermal derivatives, notably the somatic and visceral musculature, pharyngeal musculature, and dorsal vessel. The question arises if all these different mesodermal cell types express β3-tubulin under a common simple control mechanism or if each cell type is guiding β3-tubulin expression in an individual and highly specific mode. The β3-tubulin gene may provide a model system to investigate the programs underlying mesodermal differentiation and to determine the steps between mesoderm formation and downstream-regulated genes. As a first step, we have started to dissect the regulatory elements of the β3-tubulin gene by means of *P*-element transformation experiments. This analysis has revealed that β3-tubulin gene expression in individual mesodermal derivatives is regulated by distinct cis-acting sequences. Expression in the somatic musculature, dorsal vessel, and pharyngeal musculature is controlled by upstream sequences. In contrast, the β3-tubulin expression in the visceral musculature is dependent on the major intron; the latter guides the expression in an enhancer-like manner.

EXPERIMENTAL PROCEDURES

Construction of *lacZ* Fusion Genes. Fusion between the *lacZ* gene and the upstream region of the β3-tubulin gene was performed in the 5′ untranslated leader following addition of *Hin*dIII linkers (Boehringer) at the *Ava* II site (+116) in the first β3 exon. This newly created *Hin*dIII site served for fusing the β3 upstream region to the *Hin*dIII site upstream of the *lacZ* gene in pCH110 (9), which contains a simian virus 40 (SV40) polyadenylylation signal downstream of the *lacZ* gene (for details see ref. 30). In this way, four different 5′ upstream fragments [−6.0, −1.2, −0.23, and −0.08 kilobases (kb)] were fused to the *lacZ* gene. Only the largest of these constructs is shown in Fig. 1*B*. Fusion to the *lacZ* gene in the second β3 exon of a genomic clone (β3/lac-6.0I) was performed as follows. The *Sau*3A site within the codon for amino acid 25 was used for the in-frame fusion by means of the *Sma* I site in pMC 1871 (10). Sequences downstream of the *Cla* I site within the *lacZ* gene were exchanged by the equivalent sequences of pCH110, thereby adding the SV40 polyadenylylation signal. A similar strategy was used to construct β3/lac-6.0c. Genomic sequences from −6.0 kb to +0.224 (*Pst* I site at codon for amino acid 8) were fused to the corresponding *Pst* I site of a cDNA clone (7). Fusion in-frame to the *lacZ* gene was performed in the codon for amino acid 25 (*Sau*3A site) as described for β3/lac-6.0I. To construct the β3/lac-6.0cDI construct, the 4.3-kb *Xma* III (+305)/*Bgl* II (+4600) fragment, which contains most of the first β3 intron, was cloned downstream of the SV40 polyadenylylation site in the β3/lac-6.0c construct such that orientation of this intron fragment relative to the start site of transcription was maintained.

***P*-Element-Mediated Transformation Experiments.** The fusion genes were cloned between the *Eco*RI and *Xba* I sites of pW8 (11) such that the direction of transcription was the same as that of the 70-kDa heat shock protein/white (*w*) fusion gene. To establish transgenic lines, injections of w^1 embryos were performed by using 0.5 mg of the β3/lac*P* DNA per ml and 0.1 mg of pπ25.7wc DNA (12) per ml essentially as described (13). For each construct a minimum of three independent transformed G_1 adults served for establishing transformed lines [upstream deletion mutants: β3/lac-6.0 (7 strains), β3/lac-1.2 (10 strains), β3/lac-0.23 (4 strains), β3/lac-0.08 (7 strains); intron and cDNA containing mutants: β3/lac-6.0I (6 strains), β3/lac-6.0c (5 strains), β3/lac-6.0cDI (3 strains)]. *In situ* hybridization to polytene chromosomes and Southern blots showed single insertion sites in all strains analyzed.

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Abbreviation: SV40, simian virus 40.

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Immunostaining of Embryos. Preparation of embryos for horseradish peroxidase immunochemistry was essentially as described (8) with a monoclonal antibody against β-galactosidase (Promega) and the Vectastain ABC kit (Vector Laboratories). Staining was performed in the presence of 0.03% $NiCl_2$. After dehydration, embryos were embedded in Epon and photographed with Nomarski optics.

RESULTS

To study the control of expression, various 5′ fragments containing the β3-tubulin promoter, ranging from −6.0 kb to −0.08 kb were fused to a *lacZ* indicator gene in the 5′ untranslated leader region and then used for germ-line transformation experiments to determine the cis-acting sequences responsible for tissue specificity (see Fig. 1*A* for the genomic structure of the β3-tubulin gene and Fig. 1 *B–E* for the *lacZ* fusion genes). We inserted the fusion genes into the *P*-element vector pW8 (11). For each of these constructs, β-galactosidase expression was then monitored in embryos of several independently transformed strains by immunostaining. The mode of expression was compared to the expression pattern of the endogenous gene as revealed by a β3-tubulin-specific antibody, which, after germ-band extension, stains the developing visceral and somatic muscles, the dorsal vessel, and macrophages (8).

Sequences Between −1.2 Kb and −6.0 Kb Direct β3-Tubulin Expression in the Somatic and Heart Musculature. One major result concerns the cis-acting sequences necessary for the expression of the β3-tubulin gene in the somatic muscles and in the dorsal vessel. We constructed fusion genes containing 6.0 kb of upstream sequences and named this construct β3/lac-6.0 (Fig. 1*B*). By looking at the expression of different strains transformed with this construct, two conclusions can be drawn (Fig. 2). Each of these strains expresses β-galactosidase in the somatic muscles (Fig. 2*A*), the pharyngeal muscles, and the dorsal vessel (Fig. 2*B*). However, in contrast to the pattern obtained with the β3-tubulin antibody, we never observed staining of the visceral muscles (Fig. 2*C*), indicating that not all the elements regulating β3-tubulin expression are present in 6.0 kb of upstream sequences. In contrast to the analysis of β3/lac-6.0 strains, the different constructs carrying between 1.2 kb and 0.08 kb of 5′ sequence were not able to direct β-galactosidase expression in a pattern similar to that of the endogenous β3-tubulin. In the majority of the strains analyzed, different tissues of all three germ layers were stained with the β-galactosidase antibody with no obvious similarities between the strains. Thus, sequences close to the transcription initiation site are extremely sensitive to the influence of regulatory elements in the region flanking the integration site, which results in expression of the indicator gene in different tissues (data not shown). The potential of such constitutive promoter elements fused to an indicator gene for isolating enhancer elements has recently been reported (15). The feature that in β3/lac-6.0 strains the influence of elements in the region flanking the integration site was strongly reduced could be explained simply by an unspecific buffering effect of the upstream region, but one

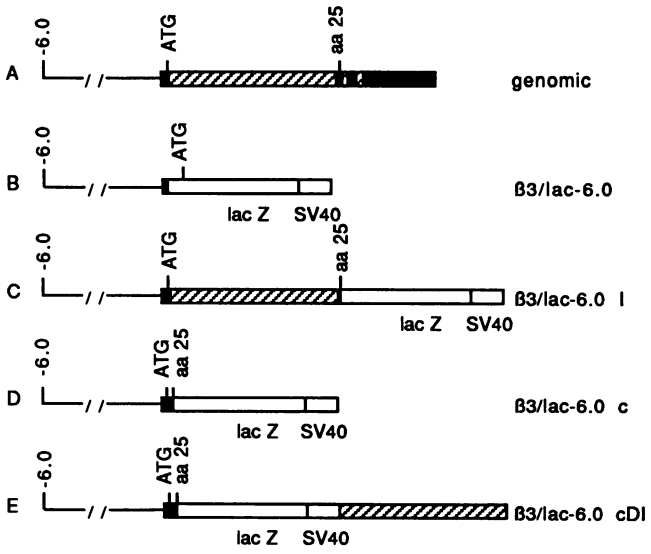

FIG. 1. Construction of β3/lacZ fusion genes. (*A*) Genomic region of the β3-tubulin gene. Upstream sequences (line), regions contained in the mRNA (filled boxes) and introns (hatched boxes) are indicated. (*B*) Fusion between the *lacZ* gene (open box) and the β3 upstream region was performed in the 5′ untranslated leader after addition of *Hin*dIII linkers (Boehringer) at the *Ava* II site (+116) in the first β3 exon. (*C*) Fusion to the *lacZ* gene in the second β3 exon of a genomic clone in the codon of amino acid 25 resulted in the construct β3/lac-6.0I. (*D*) The *lacZ* gene was fused in-frame to a β3 cDNA clone at amino acid 25 to yield construct β3/lac-6.0c. (*E*) The intron was placed 3′ to the β3/lac-6.0c construct, giving rise to the clone β3/lac-6.0cDI. aa, Amino acid.

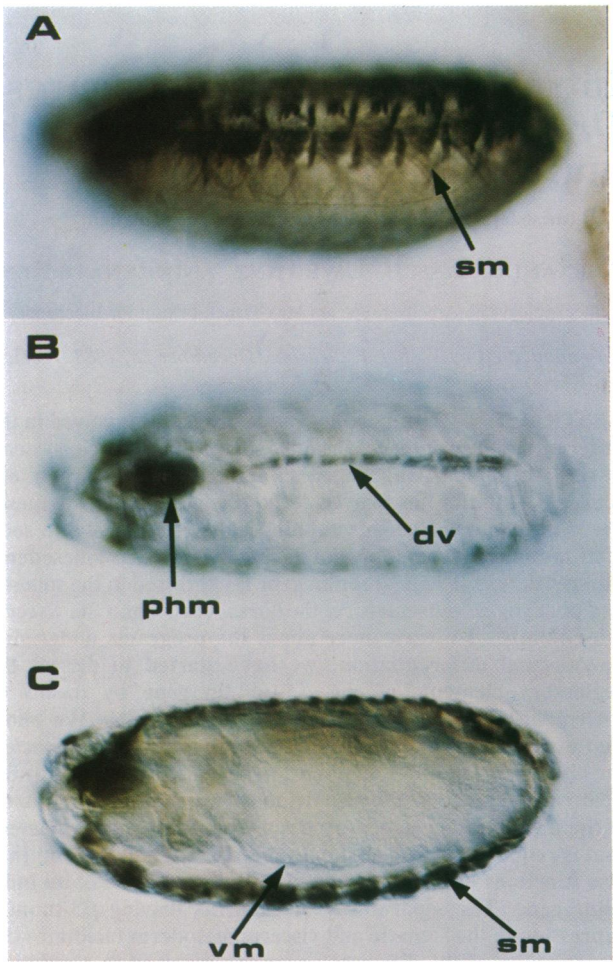

FIG. 2. Antibody staining of β3/lac-6.0 transgenic embryos reveals expression of β-galactosidase in the somatic musculature and the dorsal vessel but not in the visceral musculature. Anterior is to the left. Staging follows Campos-Ortega and Hartenstein (14). (*A*) Lateral view of a stage-16 embryo stained with a monoclonal antibody against β-galactosidase (Promega). Expression of the fusion gene in the somatic muscles (sm) is evident. (*B*) Specific staining of the dorsal vessel (dv) and the pharyngeal musculature (phm), which is out of the plane of focus, in a stage-17 embryo (horizontal view). (*C*) Slightly oblique lateral view of a stage-15 embryo clearly shows that the visceral musculature (vm) surrounding the gut is not stained.

could also postulate the presence of an attachment site to the nuclear scaffold in the region upstream of −1.2 kb. These sites have been localized in the vicinity of several strongly transcribed *Drosophila* genes and have been shown to co-map with enhancer elements (for review, see ref. 16).

In regards to the regulation of the β3-tubulin gene, our results show that, upstream of −1.2 kb relative to the transcription initiation site, at least one element is present in the β3-tubulin upstream sequences capable of efficiently driving expression in the somatic muscles and the dorsal vessel. Nevertheless the region analyzed, −6.0 kb to +0.12 kb, does not contain all the regulatory elements for β3-tubulin expression, as expression in the visceral musculature is clearly missing (Fig. 2*C*).

Intron Sequences Drive the Expression of the β3-Tubulin Gene in the Visceral Musculature. Bearing in mind that the different β3/lac-6.0 strains seemed insensitive to the influence of further upstream elements, we speculated that this might reflect the 5′ border of the gene limited by nuclear matrix attachment sites. The β3-tubulin gene contains a rather large intron between the first and second exons in comparison to other *Drosophila* genes coding for structural proteins. This intron of 4.5 kb is localized between codons 19 and 20 (7). Taking these two features of the β3-tubulin gene into consideration, we searched for the element required for expression in the visceral muscles downstream of the transcriptional start site. We tested for the presence of a regulatory element in this intron by using the two fusion constructs β3/lac-6.0I and β3/lac-6.0c (Fig. 1 *C* and *D*). Both constructs are fused in the codon for amino acid 25 in-frame to the *lacZ* gene. In the first construct, β3/lac-6.0I, a genomic β3-tubulin clone including the major intron was used. In the second construct, β3/lac-6.0c, a genomic clone (−6.0 kb to +0.224 kb) was fused to a β3 cDNA clone (7) such that all leader sequences and the codons for the first 25 amino acids correspond to the β3-tubulin mRNA. Thus the *lacZ* gene fusion point in the second β3 exon is identical in these plasmids as well as all regions of the mature message. These constructs differ only by the presence or absence of the 4.5-kb intron (compare Fig. 1*C* to Fig. 1*D*). Both constructs contain 6.0 kb of upstream sequences in order to stabilize against the influence of chromosomal integration sites. Embryos of strains transformed with these constructs were stained with an anti-β-galactosidase antibody and compared to embryos stained with anti-β3 antibody (Fig. 3). As can be clearly seen, strains transformed with the intron-containing construct (β3/lac-6.0I) efficiently express β-galactosidase in the visceral muscles (Fig. 3*B*; compare to Fig. 3*A*, which shows the expression of the endogenous β3-tubulin gene). In contrast, each of the five different lines containing the construct without intron failed to do so (Fig. 3*C*). As the β3/lac-6.0I and β3/lac-6.0c constructs should both direct formation of the same mature mRNA, an effect at the translational level can be ruled out. Also, a regulatory element in the leader region or the codons for the first 25 amino acids is not responsible for expression in the visceral musculature. We conclude that the presence of the first intron is necessary to guide efficient expression in this mesodermal derivative, an effect that may be explained by a cis-acting element in the intron conferring tissue specificity.

To decide if the observed effect occurs at the level of transcription, we tested the regulatory capacity of the intron inserted downstream of the polyadenylylation site in the β3/lac-6.0cDI construct (Fig. 1*E*). Embryos containing this β3/lac-6.0cDI fusion gene also clearly show expression in the visceral muscles (Fig. 3*D*). These results demonstrate the presence of a tissue-specific enhancer element in the first intron of the β3-tubulin gene and argue against the involvement of any posttranscriptional regulation.

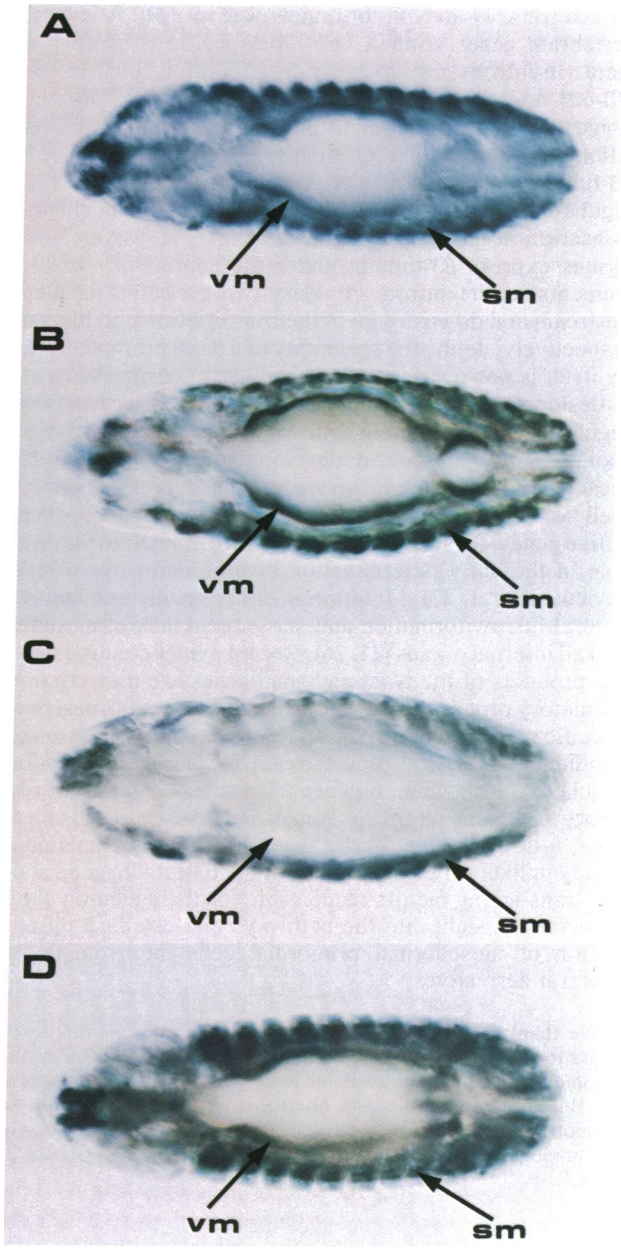

FIG. 3. The first intron of the β3-tubulin gene cloned within or downstream of a β3/lac-6.0 fusion gene enhances expression of β-galactosidase in the visceral muscles. Horizontal views of stage-15 embryos are shown. Anterior is to the left. Staging follows Campos-Ortega and Hartenstein (14). (*A*) Expression of β3-tubulin in a wild-type embryo monitored by staining with a β3-tubulin-specific antibody (8). Staining of the visceral musculature (vm) and the somatic musculature (sm) is observed. (*B*) An embryo, transgenic for the β3/lac-6.0I construct (see Fig. 1*C*), stained with a monoclonal antibody against β-galactosidase. β-galactosidase is expressed in the somatic as well as in the visceral musculature. (*C*) The β3/lac-6.0c-bearing embryos, which differ from the β3/lac-6.0I embryos only by the absence of the 4.5-kb intron (see Fig. 1*D*), do not express β-galactosidase in the visceral musculature. (*D*) Transgenic embryos carrying the β3/lac-6.0cDI construct (where the intron is cloned downstream of the SV40 polyadenylylation signal in β3/lac-6.0c, see Fig. 1*E*) again show expression in the visceral musculature.

DISCUSSION

Enhancer elements in introns have been reported for a few vertebrate genes of which the B-cell-specific enhancers within the introns of immunoglobulin genes are best charac-

terized (refs. 17 and 18; for review, see ref. 19). A few other vertebrate genes contain cell type-specific regulatory elements in introns, as has been described for collagen genes (20–22) and the δ1-crystalline gene (23). It has also been suggested that introns might prove important in gene regulation in *Drosophila* (24). In the case of the *Drosophila* β3-tubulin gene, our results present strong evidence that a regulatory element in the intron mediates enhancement of transcription in a tissue-specific manner. However, not all tissues express β3-tubulin under the control of regulatory elements in the intron. At least two cis-acting elements, upstream and downstream of the transcription initiation site, respectively, define the specificity of a basal promoter, which by itself is not restricted in its activity to particular tissues.

Besides providing a model for studies on tissue-specific regulation, the β3-tubulin gene may prove to be an excellent tool to study mesoderm development. Formation of the mesoderm is dependent on maternally expressed genes as well as on zygotically active genes. The two zygotically active genes, twist and snail (25, 26), are thought to play a key role in the early determination of mesoderm (for a recent review, see ref. 27). Mutation in either results in a failure of ventral furrow formation and subsequent lack of mesoderm and all internal organs (25, 26). Recent evidence suggests that the products of the twist and snail genes are transcriptional regulatory proteins (28, 29), presumably affecting mesoderm-specific morphogenetic genes. The β3-tubulin gene is a likely candidate for a gene downstream of these transcriptional regulators. This gene, however, is subject to different regulatory mechanisms in the somatic and visceral mesoderm; thus, a direct regulation by twist or snail proteins alone is highly unlikely. Further studies of the β3-tubulin gene and of the trans-acting factors responsible for its regulation should give some insight into the pathways that establish differentiation of mesodermal primordial cells into somatic and visceral derivatives.

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1. Martinez-Arias, A. & Lawrence, P. A. (1985) *Nature (London)* **313,** 639–642.
2. Lawrence, P. A. (1985) *Trends Neurosci.* **1,** 267–269.
3. Ingham, P. W. (1988) *Nature (London)* **335,** 25–34.
4. Akam, M. (1987) *Development* **101,** 1–22.
5. Bialojan, S., Falkenburg, D. & Renkawitz-Pohl, R. (1984) *EMBO J.* **3,** 2543–2548.
6. Natzle, J. E. & McCarthy, B. J. (1984) *Dev. Biol.* **104,** 187–198.
7. Gasch, A., Hinz, U., Leiss, D. & Renkawitz-Pohl, R. (1988) *Mol. Gen. Genet.* **211,** 8–16.
8. Leiss, D., Hinz, U., Gasch, A., Mertz, R. & Renkawitz-Pohl, R. (1988) *Development* **104,** 525–531.
9. Hall, C. V., Jacob, P. E., Ringold, G. M. & Lee, F. (1983) *J. Mol. Appl. Genet.* **2,** 101–109.
10. Casadaban, M. J., Martinez-Arias, A., Shapira, S. K. & Chou, J. (1983) *Methods Enzymol.* **100,** 293–308.
11. Klemenz, R., Weber, U. & Gehring, W. J. (1987) *Nucleic Acids Res.* **15,** 3947–3959.
12. Karess, R. E. & Rubin, G. M. (1984) *Cell* **38,** 135–146.
13. Rubin, G. M. & Spradling, A. C. (1982) *Science* **218,** 348–353.
14. Campos-Ortega, J. A. & Hartenstein, V. (1985) *The Embryonic Development of Drosophila Melanogaster* (Springer, Berlin).
15. O'Kane, C. J. & Gehring, W. J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 9123–9127.
16. Gasser, S. M. & Laemmli, U K. (1987) *Trends Genet.* **3,** 16–22.
17. Gillies, S. D., Morrison, S. L., Oi, V. T. & Tonegawa, S. (1983) *Cell* **33,** 717–728.
18. Banerji, J., Olson, L. & Schaffner, W. (1983) *Cell* **33,** 729–740.
19. Maniatis, T., Goodbourn, S. & Fischer, J. A. (1987) *Science* **236,** 1237–1245.
20. Rossi, P. & de Crombrugghe, B. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 5590–5594.
21. Horton, W., Miyashita, T., Kohno, K., Hassel, J. R. & Yamada, Y. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8864–8868.
22. Bornstein, P., McKay, J., Morishima, J. K., Deravarajalu, S. & Gelinas, R. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8869–8873.
23. Hayashi, S., Goto, K., Okada, T. S. & Kondoh, H. (1987) *Genes Dev.* **1,** 818–828.
24. Bingham, P. M., Chou, T., Mims, I. & Zachar, Z. (1988) *Trends Genet.* **4,** 131–138.
25. Nüsslein-Volhard, C., Weischaus, E. & Kluding, H. (1984) *Wilhelm Roux's Arch. Dev. Biol.* **193,** 267–282.
26. Simpson, P. (1983) *Genetics* **105,** 615–632.
27. Levine, M. (1988) *Cell* **52,** 785–786.
28. Thisse, B., Stoetzel, C., Gorostiza-Thisse, C. & Perrin-Schmitt, F. (1988) *EMBO J.* **7,** 2175–2183.
29. Boulay, J. L., Dennefeld, C. & Alberga, A. (1987) *Nature (London)* **330,** 395–398.
30. Michiels, F., Gasch, A., Kaltschmidt, B. & Renkawitz-Pohl, R. (1989) *EMBO J.* **8,** in press.