was replaced with 10 mM KCl, and the acrylamide concentration of the gel was increased to 10%. The amounts of shifted ([HMG · DNA]) and unshifted ([DNA]) probe were determined from quantitative analyses of dried gels on a Molecular Dynamics phosphorimager. The concentrations of free HMG1 ([HMG]) were obtained by subtracting [HMG · DNA] from the concentrations of HMG1 added to the binding reactions. The dissociation constant K_d was calculated at three different protein concentrations (9×10^{-8}, 1.8×10^{-7}, and 3.6×10^{-7} M) and at a DNA probe concentration of 6 to 9 nM by using the equation K_d = [HMG][DNA]/[HMG · DNA].

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Positive Control of Pre-mRNA Splicing in Vitro

Ming Tian and Tom Maniatis

Positive control of the sex-specific alternative splicing of *doublesex* (*dsx*) precursor messenger RNA (pre-mRNA) in *Drosophila melanogaster* involves the activation of a female-specific 3′ splice site by the products of the *transformer* (*tra*) and *transformer-2* (*tra-2*) genes. The mechanisms of this process were investigated in an in vitro system in which the female-specific 3′ splice site could be activated by recombinant Tra or Tra-2 (or both). An exon sequence essential for regulation in vivo was shown to be both necessary and sufficient for activation in vitro. Nuclear proteins in addition to Tra and Tra-2 were found to bind specifically to this exon sequence. Therefore, Tra and Tra-2 may act by promoting the assembly of a multiprotein complex on the exon sequence. This complex may facilitate recognition of the adjacent 3′ splice site by the splicing machinery.

Somatic sex determination in *Drosophila melanogaster* involves a cascade of regulated splicing events (*1*). The last step in this hierarchy is the alternative splicing of *dsx* pre-mRNA. The *dsx* gene contains six exons. In females, *dsx* mRNA consists of exons 1, 2, 3, and 4 (*2*) (Fig. 1A) and encodes a protein that represses the expression of male-specific genes (*3, 4*). In males, exons 1, 2, 3, 5, and 6 comprise the alternative *dsx* mRNA (*2*) (Fig. 1A) whose translation product blocks female sexual differentiation (*3, 4*).

In *Drosophila*, the female-specific processing pathway of *dsx* pre-mRNA depends on the activities of both the *tra* and *tra-2* genes (*2, 5*). The polypyrimidine tract (6) of the female-specific 3′ splice site is interrupted by purine residues and is inefficiently recognized by the splicing apparatus (*5, 7–10*). When the purines are mutated to pyrimidines, the *dsx* female-specific 3′ splice site is constitutively used both in vivo (*9*) and in vitro (*11*). Genetic studies in flies (*7*) and transfection experiments with cells in culture (*8–10*) indicate that products of *tra* and *tra-2* serve as activators of the female-specific 3′ splice site. Male flies produce functional *tra-2* (*12*) but not *tra* gene products (*5*). As a result, *dsx* pre-mRNA undergoes default processing to generate the male *dsx* mRNA.

The primary structures of Tra (*13*) and Tra-2 (*14*) are consistent with their roles as splicing regulators; Tra-2, but not Tra, contains a ribonucleoprotein-consensus (RNP-CS) RNA binding domain, a 90-residue motif implicated in protein-RNA interactions (*15*). Recombinant Tra-2 produced in *Escherichia coli* binds specifically to a region in the female-specific exon of *dsx* pre-mRNA (*8*). This region contains six copies of a 13-nucleotide (nt) sequence and is required for female-specific splicing (*7–10*). Both Tra and Tra-2 contain arginine-serine–rich regions (RS domain) that are present in several splicing factors (*13, 14, 16–18*). Although the function of this motif is not known, recent studies indicate that it can direct proteins to speckles (*19*), subnuclear regions where several splicing components are preferentially localized (*20*).

To study the mechanisms involved in the positive control of *dsx* female-specific splicing by Tra and Tra-2, we established an in vitro splicing system that consisted of HeLa cell nuclear extracts and recombinant Tra and Tra-2 produced in a baculovirus expression system. Female-specific splicing was not observed when an in vitro–synthesized transcript of the *dsx* minigene was incubated under splicing conditions in HeLa cell nuclear extracts in the absence of Tra and Tra-2 (Fig. 1B). The low level of splicing observed resulted from the use of a cryptic 3′ splice site (*21*). Addition of recombinant Tra or Tra-2 (or both) (*22*) activated female-specific splicing (Fig. 1B, lanes 2 to 5 and 12 to 26). The highest level of female-specific splicing was observed when both Tra and Tra-2 were added (Fig. 1B, lanes 22 to 26). Activation of female-specific splicing by Tra or Tra-2 alone has also been observed in transfection studies with *Drosophila* tissue culture cells (*9, 10*). In flies, female-specific splicing requires both Tra and Tra-2. This difference may result from the use of minigene substrates and altered ratio of regulators and basic splicing components in vitro and in transfection systems.

The repeat region in exon 4 is required for female-specific splicing in vivo (*7–10*). The deletion of most or all of the repeat sequence from the minigene transcript severely impaired or abolished activation in vitro (Fig. 2). In addition, the splicing of both human β-globin (hβ-globin) (*23*) and *fushi taruzu* (*ftz*) pre-mRNA (*24*) was unaffected by Tra and Tra-2 (*11*). These results demonstrate that Tra and Tra-2 function in a sequence-specific manner in vitro.

To test the possibility that the repeat region is sufficient for regulation, we introduced this sequence downstream of the 3′ splice site of hβ-globin intervening sequence 1 (*23*). By analogy with the suboptimal female-specific 3′ splice site of *dsx* pre-mRNA, we used hβ-globin constructs that contained mutations that adversely affected 3′ splice site recognition. The polypyrimidine tract in G(py) (*25*) is interrupted by purines. The branchpoint sequence in G(bps) (*26*) deviates significantly from the consensus. Both mutations substantially decrease the efficiency of hβ-globin splicing in vitro (*25, 26*).

In splicing reactions with the hybrid substrates, the 3′ splice sites from G(py) and G(bps) were not utilized in the absence of Tra and Tra-2 (Fig. 3, lanes 2 and 13). Instead, splicing to cryptic splice sites was

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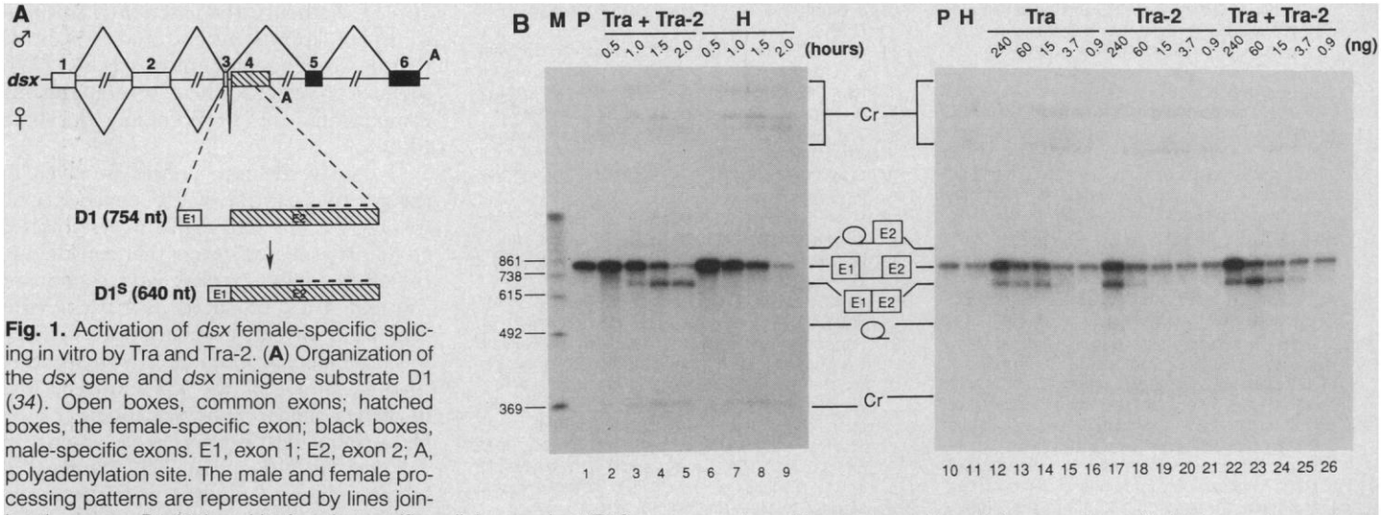

Fig. 1. Activation of *dsx* female-specific splicing in vitro by Tra and Tra-2. (**A**) Organization of the *dsx* gene and *dsx* minigene substrate D1 (*34*). Open boxes, common exons; hatched boxes, the female-specific exon; black boxes, male-specific exons. E1, exon 1; E2, exon 2; A, polyadenylation site. The male and female processing patterns are represented by lines joining the exons. D1 (*34*) and its female-specific splicing product ($D1^S$) are depicted below the diagram of *dsx*. The short lines above E2 in D1 represent the location of the 13-nt repeats. (**B**) Analysis of ^{32}P-labeled D1 splicing in vitro (*35*) by denaturing polyacrylamide gel electrophoresis (PAGE). Structures of the RNAs are illustrated between the two autoradiograms. The lariat, released intron, and excised exon 1 for the female-specific splicing can be observed more clearly with longer exposure and on higher percentage gels. Cr indicates splicing product and intermediates involving cryptic splice sites. Each lane is specified at the top: M, 123-bp ladder (Bethesda Research Laboratories) as molecular size markers; P, precursor; H, splicing reaction with HeLa nuclear extract alone; Tra, Tra-2, Tra+Tra-2, splicing reactions with Tra, Tra-2, or both added, respectively. For lanes 1 through 9, the time of incubation is specified at the top of each lane. In reactions containing Tra and Tra-2, 60 ng of each was added. In lanes 10 through 26, the amount of each protein added is indicated at the top of each lane. Because both Tra and Tra-2 went through denaturation and renaturation during purification, the amount of active protein is unknown. The amount given for each protein serves mainly as an indication of relative amounts of protein for comparison.

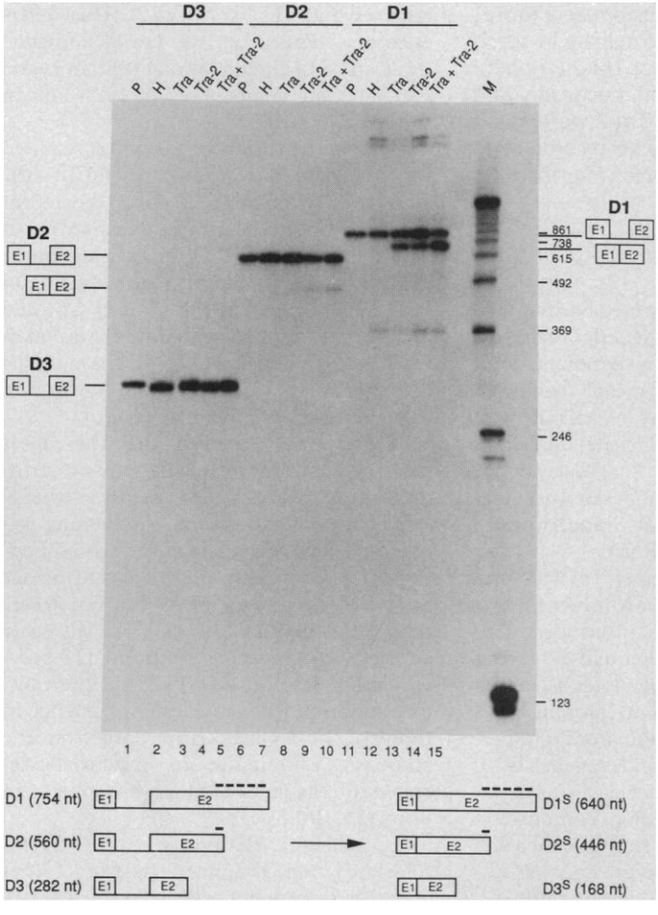

Fig. 2. RNA sequence requirements for the activation of *dsx* female-specific splicing by Tra and Tra-2 in vitro. Splicing reactions of ^{32}P-labeled substrates were performed and analyzed as in Fig. 1B. Precursors and female-specific splicing products are indicated on both sides of the autoradiogram. The lanes are specified as in Fig. 1B. Where indicated, 60 ng of Tra, 240 ng of Tra-2, or 60 ng of each were added. M, P, and H are the same as in Fig. 1B. Substrates (*36*) and their expected female-specific splicing products are illustrated below the autoradiogram. E1, exon 1; E2, exon 2; short lines above exons, location of 13-nt repeats. Arrow indicates splicing.

observed as in splicing reactions with the *dsx* minigene transcript. The mutant 3′ splice sites in the hybrid substrates were activated by Tra or Tra-2 (or both) (Fig. 3). As expected, activation by Tra or Tra-2 was not observed with RNAs lacking the repeat sequence (*11*). These results demonstrate that the repeat region is both necessary and sufficient for mediating activation of a 3′ splice site by Tra and Tra-2. Similar observations have been made in vivo (*10*). The data presented here further indicate that Tra and Tra-2 can overcome mutations in either the branchpoint sequence or the polypyrimidine tract.

Previous studies have shown that Tra-2 binds specifically to the repeat region (*8*). In contrast, Tra, either alone or together with Tra-2, binds indiscriminately to all the RNAs tested in ultraviolet (UV) cross-linking experiments (*27*) (Fig. 4, lanes 1 to 3). However, an adduct corresponding in size to Tra (29 kD) was specifically formed on RNA containing the repeat sequence when the binding reactions contained nuclear extract in addition to Tra and Tra-2 (Fig. 4, lanes 7 to 9) and this adduct was not observed in the absence of Tra (Fig. 4, lanes 4 to 6).

These results suggest that the 29-kD adduct corresponds to Tra specifically bound to the repeat region. In addition, adducts of 25 and 33 kD (p25 and p33) were also specifically formed on the repeat-containing RNA (Fig. 4, lanes 7 to 9). In the absence of Tra and Tra-2 (Fig. 4, lanes

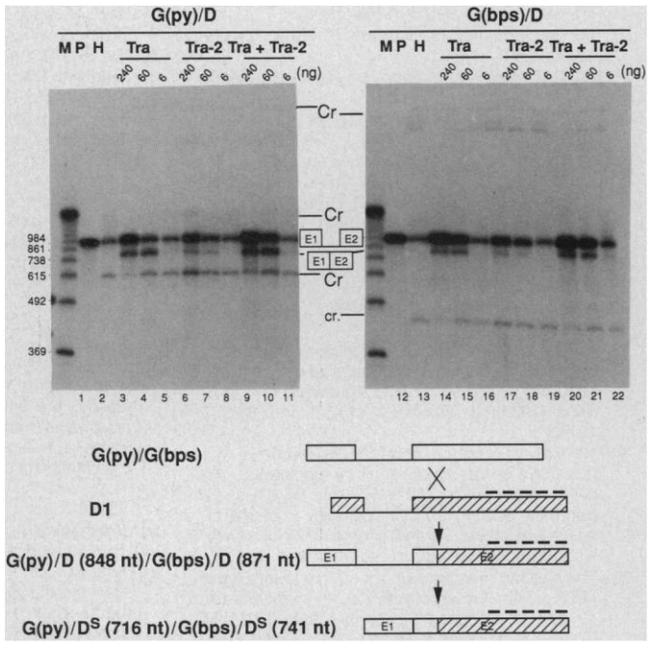

Fig. 3. Activation of mutant hβ-globin 3′ splice sites by Tra and Tra-2 in vitro. Splicing reactions of ^{32}P-labeled G(py)/D and G(bps)/D (*37*) splicing in vitro were carried out and analyzed as in Fig. 1B. Structures of the RNAs are indicated on the sides. The lariats, the excised introns, and exon 1 for the activated splicing cannot be seen in this autoradiogram but are detectable with longer exposure time. Lanes 1 through 11 and 12 through 22 are with G(py)/D and G(bps)/D as substrates, respectively; lanes are specified as in Fig. 1B. G(py)/D, G(bps)/D (*37*), and their splicing products [G(py)/D^s and G(bps)/D^s] are illustrated below the autoradiogram. E1, exon 1; E2, exon 2; short lines above exons, location of 13-nt repeats. X and the upper arrow iilustrate the construction of the hybrid substrates; the lower arrow indicates splicing.

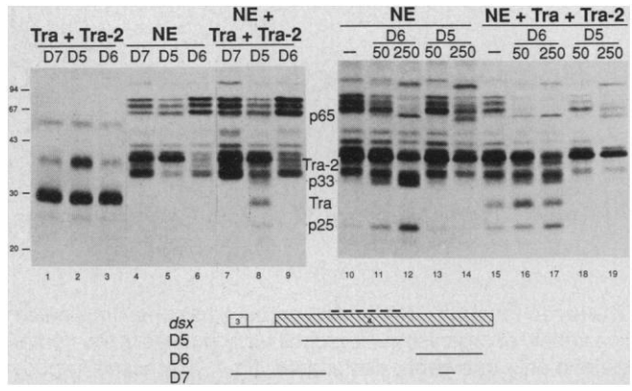

Fig. 4. Interactions of Tra, Tra-2, and nuclear proteins with the repeat region. Shown are autoradiograms of UV cross-linking products fractionated by SDS-PAGE. Binding reactions were set up as splicing reactions (*35*), except that the reaction volume was 10 μl, and polyvinyl alcohol was omitted from the reactions. Cross-linking of proteins to ^{32}P-labeled RNA probes by irradiation with UV was carried out as described (*28*). The sizes of the molecular markers (Pharmacia) are indicated at the left in kilodaltons. The left autoradiogram (lanes 1 through 9) shows the reactions with different probes [D5, D6, and D7 (*38*) as indicated at the top], under different binding conditions. The right autoradiogram (lanes 10 through 19) shows competition experiments with different competitors (D5 and D6) under different conditions. NE, reactions containing nuclear extract only; NE+Tra+Tra-2, reactions containing both nuclear extract and Tra and Tra-2; Tra+Tra-2, reactions containing only Tra and Tra-2. D5 is the probe for all of the reactions in the autoradiogram on the right; numbers represent the molar ratio of the unlabeled competitor RNA to the labeled probe, and dashes indicate reactions without competitors. Between the two autoradiograms, the positions of the adducts corresponding to Tra, Tra-2, p25, p33, and p65 are indicated. The structures of the probes and competitors are shown below the autoradiograms. Broken line indicates the 13-nt repeat; lines indicate the probes or competitor RNAs.

4 to 6), the formation of the 25- and 33-kD adducts was much weaker, and longer exposure was required for clear visualization of them. All of these adducts could be specifically abolished by excess unlabeled RNAs containing the repeat (Fig. 4, lanes 10 to 19). The presence of competitor RNAs that lacked the repeat not only stimulated the cross-linking of these proteins but also revealed two more adducts (at 38 and 65 kD) specific to the repeat (Fig. 4, lanes 10 to 19). The 38-kD adduct most likely corresponds to Tra-2; the identity of the 65-kD (p65) protein is unknown. The stimulatory effect is more pronounced for the cross-linking of p25, p33, and p65 in the absence of Tra and Tra-2. Our interpretation is that the binding of these nuclear proteins to the repeat is weak in the absence of Tra and Tra-2 and is therefore blocked by the abundant nonspecific RNA binding proteins in the extract. The competitor RNA that lacked the repeat titrated away these nonspecific RNA binding proteins, which allowed specific interactions of these nuclear proteins with the repeat.

These experiments show that Tra, a nonspecific RNA binding protein, either by itself or with Tra-2, interacts specifically with the repeat region in the presence of nuclear extract. In addition, several factors from the nuclear extract specifically recognize the repeat region. It is likely that Tra achieves RNA binding specificity through cooperative interactions with these nuclear factors. A characteristic feature of Tra is an extended RS domain, which may function in both protein-RNA and protein-protein interactions. In addition to the RS dipeptide that is a hallmark of this motif (*16*), there are clusters of residues resembling the basic RNA binding domain of Tat and Rev (*28*). These basic domains may be the site of contact with RNA. The remainder of the RS domain—that is, the RS dipeptides—may interact with other proteins; these interactions could change the conformation of the closely linked basic domains to achieve RNA binding specificity.

The binding of Tra, Tra-2, and these nuclear proteins to the regulatory sequence may lead to the formation of an activation complex. This complex could associate directly with splicing components and facilitate their interaction with the 3′ splice site or it could change the secondary or higher order structure of the pre-mRNA in a way that renders the 3′ splice site more accessible to splicing components. The observation that Tra and Tra-2 can activate heterologous 3′ splice sites through the repeat region does not support the latter possibility.

The direct targets of the activation complex are unknown at present. Both Tra and Tra-2 can suppress mutations in either the branchpoint sequence or the pyrimidine tract, and these elements interact with distinct components of the splicing apparatus. The branchpoint sequence binds directly to U2snRNP (*29*) and the pyrimidine tract interacts with a number of proteins including the splicing factor U2AF (*30*). Recent studies indicate that additional factors are involved in the recognition of the 3′ splice site (*31*). A detailed understanding of the interactions between Tra, Tra-2, and these factors should provide insight into the mechanisms of regulated splicing and 3′ splice site recognition.

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21. The use of cryptic and authentic 3′ splice sites was determined by ribonuclease protection assays (*32*).
22. The cDNAs of *tra* (*13*) and *tra-2* (*14*) were cloned into the baculovirus expression vector pJVP10Z. Recombinant viruses producing Tra or Tra-2, respectively, were generated and purified (*33*). After 72 hours of infection, cells were lysed in buffer A [20 mM tris-Cl (pH 7.5), 150 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% NP-40, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, leupeptin (1 μg/ml), pepstatin (1 μg/ml), and aprotinin (1 μg/ml)] at 4°C for 30 min. The lysate was centrifuged at 8000 rpm (HB-4 rotor) for 30 min. The pellet containing Tra and Tra-2 was extracted in buffer B [10 M urea, 20 mM tris-Cl (pH 7.5), 100 mM KCl, 0.2 mM EDTA, and 1% β-mercaptoethanol] at room temperature for 1 hour. The extract was centrifuged at 50,000 rpm (SW55 rotor) for 1 hour. The supernatant containing Tra and Tra-2 was chromatographed on P11 columns (Whatman, Maidstone, Kent, England) equilibrated with buffer B100 [6 M urea, 20 mM tris-Cl (pH 7.5), 100 mM KCl, 0.2 mM EDTA, and 14.4 mM β-mercaptoethanol]. Tra was eluted with B300 [6 M urea, 20 mM tris-Cl (pH 7.5), 300 mM KCl, 0.2 mM EDTA, and 14.4 mM β-mercaptoethanol]. The flowthrough of P11 containing Tra-2 was chromatographed on DEAE-Sepharose (Pharmacia, Uppsala, Sweden) equilibrated with B100. Tra-2 was eluted with B300. The P11 B300 fraction of Tra and the DEAE B300 fraction of Tra-2 were dialyzed against BC850 [20 mM tris-Cl (pH 7.5), 850 mM KCl, 0.2 mM EDTA, and 0.5 mM DTT] at 4°C overnight. Both Tra and Tra-2 were quantified by Coomassie staining on SDS–polyacrylamide electrophoresis gels. As a control, lysate from a recombinant baculovirus expressing the *Drosophila bicoid* protein was subjected to the same fractionation procedures as Tra and Tra-2, respectively. These control protein preparations did not affect splicing.
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34. D1 was transcribed from T7S/R linearized with Dra I. We constructed T7S/R by cloning into the SP73 vector (Promega) a 1775-bp Sty I–Eco RI fragment starting 90 bp upstream from the 5′ splice site of exon 3 and ending 1175 bp downstream from the 3′ splice site of exon 4.
35. The splicing reaction was set up in a total volume of 25 μl with 100 μg of HeLa cell nuclear extract, 12 mM tris-Cl (pH 7.5), 72 mM KCl, 0.12 mM EDTA, 4% glycerol, 3.2 mM $MgCl_2$, 1 mM adenosine 5′-triphosphate, 20 mM creatine phosphate, 3% polyvinyl alcohol, 1 ng of pre-mRNA, Tra, and Tra-2 in the amounts specified elsewhere. The reactions were incubated at 30°C for 2 hours unless otherwise specified, and the RNAs were subsequently analyzed on 6% polyacrylamide gels containing 8 M urea (*24*).
36. D2 and D3 were transcribed from T7S/R linearized with Mlu I and Fsp I, respectively.
37. G(py)/D and G(bps)/D were transcribed, respectively, from SP6G(py)/D and SP6G(bps)/D linearized with Dra 1. SP6G(py)/D and SP6G(bps)/D were constructed by replacing the Sac I–Hinc II fragment in SP6RV/RI with the Hind III–Sfa NI fragments from G(py) and G(bps), respectively. SP6RV/RI was constructed by cloning into the pGEM7Z vector (Promega) a 2075-bp fragment starting 300 bp upstream from exon 3 and ending 1578 bp downstream from the female 3′ splice site.
38. D5, D6, and D7 were transcribed from T7F540 linearized with Eco RI, T7Afl linearized with Eco RI, and T7S/R-M/B linearized with Cla I, respectively. We constructed T7F540 and T7Afl by inserting the 540-bp Fsp I and the 360-bp Afl II fragments from *dsx* exon 4 into the Bluescript SK^+ vector (Stratagene). T7S/R-M/B was constructed by deletion of a 585-bp fragment containing all six copies of the 13-bp repeat sequence from the T7S/R.
39. We thank H. Amrein, M. L. Hedley, J. Bruzik, X.-D. Fu, R. Reed, C.-M. Fan, and S. Abmayr for discussions and readings of this manuscript; A. Barberis, L. Barberis-Maino, and T. Vik for the baculovirus expression system and advice on procedures; K. Diepold for *bicoid* recombinant baculovirus; and R. Reed for G(py) and G(bps) constructs. Supported by NIH grant GM42231 (T.M.).

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Body-Wall Muscle Formation in *Caenorhabditis elegans* Embryos That Lack the MyoD Homolog *hlh-1*

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The myoD family of DNA binding proteins has been implicated in the control of myogenesis in a variety of organisms. Searches for homologs in the nematode *Caenorhabditis elegans* yielded only one gene, designated *hlh-1*, expressed in body-wall muscle cells and their precursors. To assess the role of *hlh-1* in *C. elegans* myogenesis, genetic deficiencies spanning the *hlh-1* locus were isolated after gamma irradiation. Embryos homozygous for these deficiencies exhibited extensive body-wall muscle differentiation, including expression of several characteristic myofilament proteins and weak contracile behavior. Thus, zygotic *hlh-1* expression was not required for body-wall muscle precursors to adopt muscle cell fates.

The myoD family of DNA binding proteins, normally expressed early in vertebrate muscle differentiation, can induce a variety of nonmuscle and premuscle cells to adopt striated muscle characteristics when ectopic expression is engineered (*1*). Whether the myoD family also initiates myogenesis during normal development can be addressed with invertebrate organisms amenable to genetic analysis: *Caenorhabditis elegans* and *Drosophila melanogaster*. To date, just one close myoD homolog has been found in each species [*hlh-1* in *C. elegans* (*2*) and *nau* in *Drosophila* (3)]. Similarities to vertebrate myoD family members in both sequence and muscle expression patterns suggest equivalent functions in development. Consistent with this hypothesis, the *hlh-1* product can induce myogene-

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