The instability of the *fushi tarazu* (*ftz*) mRNA is essential for the proper development of the *Drosophila* embryo. Previously, we identified a 201-nucleotide instability element (FIE3) in the 3′ untranslated region (UTR) of the *ftz* mRNA. Here we report on the identification of two additional elements in the protein-coding region of the message: the 63-nucleotide-long FIE5-1 and the 69-nucleotide-long FIE5-2. The function of both elements was position-dependent; the same elements destabilized RNAs when present within the coding region but did not when embedded in the 3′ UTR of the hybrid mRNAs. We conclude that *ftz* mRNA has three redundant instability elements, two in the protein-coding region and one in the 3′ UTR. Although each instability element is sufficient to destabilize a heterologous mRNA, the destabilizing activity of the two 5′-elements depended on their position within the message.

*Drosophila* embryonic development depends on the precise temporal and spatial expression of maternal and zygotic pattern-forming genes (1). Maternal pattern-forming genes are transcribed during oogenesis, and their mRNA abundance decreases rapidly in the early embryo. Moreover, many mRNAs encoded by zygotic pattern-forming genes undergo dramatic changes in abundance and spatial distribution during early embryogenesis. To achieve these rapid changes, especially for rapid down-regulation, transcriptional control alone is insufficient, and regulation at the level of mRNA stability is essential. For instance, the maternal *bicoid* mRNA is completely stable during the first 2 h of embryogenesis but is rapidly destabilized at cellularization of the blastoderm (2). As discussed in the following text, the zygotic *fushi tarazu* (*ftz*) mRNA is one of the most unstable eukaryotic mRNAs known. Given that most mRNAs in the *Drosophila* embryo are constitutively stable (30), the question arises how selected mRNAs in the same embryo cytoplasm are targeted for degradation. Recognition of the targeted RNAs by the RNA degrading machinery must involve cis-acting sequences. These sequences are the focus of the present study.

*ftz* is a member of the pair-rule class of segmentation genes and one of the best characterized early zygotic genes. In early embryos *ftz* mRNA is detected only from about 1.5 to 4.5 h after fertilization. When first expressed, *ftz* mRNA is uniformly distributed through the embryo (4). As development progresses, its distribution first becomes restricted to a region comprising from 15 to 65% of egg length, then to four broad bands, and finally to seven narrow stripes that encircle the embryo (4–6). The seven stripes are short-lived, and no *ftz* mRNA is detected by 5 h after fertilization. This rapid change of expression pattern and formation of stripes in a short time span can be attributed to the termination of transcription in interstripe regions coupled with rapid mRNA turnover. The need for rapid mRNA turnover is emphasized by the fact that the FTZ protein activates its own transcription in a positive feedback loop (7). Thus, it is important during the evolution of the spatial pattern of *ftz* expression that both mRNA and protein be rapidly cleared from the interstripe regions. Edgar et al. (8) measured *ftz* mRNA stability in embryos and found that its half-life changes from 14 min at 2.5 h to 6 min at 4 h after fertilization. This makes *ftz* mRNA one of the shortest-lived mRNAs among higher eukaryotes (3). Stabilization of *ftz* mRNA and FTZ protein results in developmental delay and defects, suggesting that *ftz* mRNA and FTZ protein instability are crucial for normal development (9–11).

Earlier studies from this laboratory using hybrid genes that fuse *ftz* sequences to the stable *ribosomal protein* A1 (*rpA1*) mRNA provided evidence for at least two destabilizing elements in *ftz* mRNA (12). One consisted of a 201-nucleotide element, including an essential 68-nucleotide sequence, located in the 3′ UTR (2) and termed FIE3 (*ftz* instability element 3′). The other element(s) were assigned to the 5′-one-third of the *ftz* mRNA but remained otherwise uncharacterized. Here we report on the identification of two separate 5′-instability elements within the first 600 nucleotides of the *ftz* mRNA. Both 5′-elements are located within the protein-coding region of the message. Each 5′-instability element is sufficient to destabilize the stable *rpA1* mRNA, but the destabilizing activity is dependent on their position within the mRNA.

**EXPERIMENTAL PROCEDURES**

**Construction of Transgenes**

The first letter of the name of each construct designates the promoter that drives it. Thus, *F = ftz*, *r = rpA1*, and *S = sgs-3* promoter attached to the hsp26 nurse cell enhancer.

**Discrepancies in the *ftz* Nucleotide Sequence**

Numbering in the following text uses +1 as the position of transcription initiation, which corresponds to position 901 in the sequence deposited in GenBank™ (accession numbers X00854 and K01951). We found that *ftz* genomic clone AA439 (13) had a nine-nucleotide deletion in the protein-coding region (from 280 to 286) when compared with the sequence deposited in the database. To resolve this discrepancy, the *ftz* 5′-region was amplified by PCR using DNA from *yellow-white* flies, the recipient strain for *P*-element transformation. Sequencing revealed that the same nine nucleotides were missing. Moreover, with the exception of the nine-base deletion and four one-base polymorphisms (C...
instead of T at 126 and 222, C instead of A at 516, and G instead of C at 636) matched the sequence deposited in GenBank™.

Ffigr3 and Rr5r3 (see Fig. 1A)
The 0.7-kb fragment (from 856 to 1557, where +1 is the transcription initiation site) of rat aldolase B (aldB) cDNA (15) was synthesized by PCR with primers ald1 and ald2 (Table I). This PCR fragment contained the 3′-one-third of the protein-coding region and entire 3′ UTR of aldB. A fragment of ftz and the rpA1 gene was combined reciprocally and subcloned into pGEM3 (Promega).

Ffftz + aldB and Ffftz + aldB-FIE3 (see Fig. 1B)
The 0.7-kb fragment (from 856 to 1557, where +1 is the transcription initiation site) of rat aldolase B (aldB) cDNA (15) was synthesized by PCR with primers ald1 and ald2 (Table I). This PCR fragment contained the 3′-one-third of the protein-coding region and entire 3′ UTR of the aldB cDNA, including the polyadenylation signal (16), generated by the primer ald2. The PCR fragment was cloned into the pGEM-T Easy vector (Promega), with EcoRI and inserted into the EcoRI site of the 3′ UTR of f3. In turn, this f3 + aldB fragment was fused to Ff5 to produce Ffftz + aldB. A FIE3 fragment that lacks the 201-base pair FIE3 sequence was obtained as previously described (12). Ffftz + aldB-FIE3 was constructed as described for Ffftz + aldB, except that FIE3-FIE3 was used instead of f3.

Deletion Constructs
Rr Constructs (see Fig. 1C)—The PCR products synthesized from F5r3 with primers 1 and 2, 1 and 4, and 3 and 2 (Table I) were digested with NgoMI and inserted into the NgoMI site in the 3′ UTR of the rpA1 gene in plasmid pD5. They were named Rr-abc, Rr-ab, and Rr-bc, respectively.

Ss Constructs (see Fig. 1D)—PCR products were synthesized with the following primers (Table I): Ss-abc, primers 5 and 6; Ss-ab, primers 5 and 8; Ss-bc, primers 7 and 6; Ss-a, primers 5, 10 and 11; Ss-p, primers 7 and 8; Ss-c, primers 9 and 6; Ss-b1, primers 7 and 12; Ss-b2, primers 11 and 8; Ss-b3, primers 15 and 16; Ss-c1, primers 9 and 14; Ss-c2, primers 13 and 6; Ss-c3, primers 9 and 18; Ss-c4, primers 13 and 14; Ss-c5, primers 17 and 6. For PCR fragments that lacked a translation initiation site, s-bc, b, c, b1–3, and c1–5, a seven-base sequence (GATATGG) was added by the primer to obtain the same efficient translation initiation site as intact ftz mRNA (17, 18). The PCR products were fused to r3. All constructs were sequenced to confirm that the PCR fragments had no errors and that the reading frame was maintained.

P-element-mediated Transformation
Constructs Rr-abc, ab, bc, Ffftz + aldB, and Ffftz + aldB-FIE3 were introduced into CaSpeR vector (19). To provide Ss constructs with a promoter, they were introduced into the CaSpeR4/GERM4 vector (2), which contained CaspER, polyliner from Gehring’s pW8, a nurse cell-specific enhancer from the heat shock 26 gene hsp26, and the sgs3 promoter. All constructs (500 µg/ml) were mixed with the phs-1 helper (20) (100 µg/ml) and injected into yellow-white mutant embryos (19, 21).

Synchronized Embryo Collections
Embryos were collected on fresh yeast plates at 25 °C for 1 h and left at 25 °C for different lengths of time. Embryos were collected, washed with embryo washing buffer (0.5% Triton X-100, 300 mM NaCl, 10 mM Tris, pH 7.5), frozen in liquid nitrogen, and kept at −80 °C. Embryo developmental synchrony was checked by staging an aliquot of each collection at 2–3 h of development. Collections containing more than 10% older embryos (retained) or unfertilized eggs were discarded.

RESULTS
Rationale—The stability of transgenic mRNAs in developing embryos was measured by a strategy previously developed for this purpose (12). The critical feature is the use of promoters that are strongly expressed during oogenesis but silent in early embryogenesis. The two promoters used in this study are rpA1 (14) and hsp26/sgs3 (24, 25). The latter promoter contains the nurse cell-specific enhancer from the hsp26 gene linked to the basal sgs3 promoter. Another key feature is that the ftz mRNA is stable in oocytes and is destabilized at fertilization.2 As a consequence, transcripts containing ftz sequences accumulate to high abundance during oogenesis and start decaying when the egg is fertilized.2 Because the rpA1 and hsp26/sgs3 promot-
ers are silent in early embryos, decay of transgenic mRNA abundance (as measured by Northern blot analysis) serves as a direct measure of mRNA stability. This strategy avoids the use of drugs that may cause artifacts (27). The rpA1 mRNA is stable in both ovaries and embryos and served as an internal loading control.

The ftz mRNA Contains Multiple RNA-destabilizing Sequences—In an earlier study (12), the stability of the 5'-one-third (here called f5; nucleotides 1–636) and the 3'-two-thirds (here called f3) of the ftz mRNA was investigated separately. One of these fragments (f5) was fused to the 3'-two-thirds (f2) and the other (f3) to the 5'-one-third (f5) of the stable rpA1 mRNA to yield the Ff5r3 and Rr5f3 hybrid mRNAs, respectively (Fig. 1A). Both hybrid transcripts were unstable (Fig. 2A). Rr5f3 is transcribed maternally, and its mRNA decayed rapidly after fertilization. Ff5r3 is transcribed only transiently from the ftz promoter during early embryogenesis, and its rapid decay after 4 h of development (compare 3–4 h and 4–5 h in Fig. 2A) indicates that this mRNA is highly unstable. We concluded that in addition to the previously characterized 201-nucleotide FIE3 in the 3'-UTR, the 5'-one-third of the ftz mRNA also contains destabilizing sequences.

The Ff5r3-ald-FIE3 construct provided further evidence for the presence of RNA-destabilizing sequences in f5. This construct consisted of a deletion of FIE3 from the intact ftz mRNA (Fig. 1B). This construct also contained a rat aldolase B mRNA (aldB) tag, to allow the transgenic mRNA to be distinguished on Northern blots from the endogenous ftz transcript. To verify that insertion of the aldolase tag does not affect ftz mRNA stability, a control construct (Fftz+ald; Fig. 1B) containing the intact ftz mRNA tagged with the aldB fragment was analyzed in parallel. As shown in Fig. 2B, both constructs were unstable in Drosophila embryos, indicating that ftz mRNA contains instability elements other than FIE3. The destabilizing element of f5 is FIE3 (12). The identification and characterization of the f5-destabilizing sequences (termed FIE5) was the object of this study.

The Destabilizing Activity of FIE5 Is Position-dependent—Previously, we have inserted FIE3 into the 3'-UTR of the stable rpA1 gene to demonstrate that this element is sufficient for mRNA destabilization (12). We created similar constructs to investigate whether FIE5 is also sufficient for RNA destabilization. The entire f5 sequence (1–636), the 5'-two-thirds (1–423), and the 3'-two-thirds (211–636) were inserted into the 3'-UTR of rpA1 to yield constructs Rr-abc, Rr-ab, and Rr-be, respectively (Fig. 1C). Transcription in all constructs was driven by the rpA1 promoter. Surprisingly, the resulting three transcripts were stable in transgenic embryos (Fig. 3). These results suggest that FIE5 destabilizing activity is position-dependent and that unlike FIE3, FIE5 is not functional when located in the 3'-UTR.

Initial Mapping of the 5'-Destabilizing Sequences—Next, we initiated a deletion analysis by placing the f5 fragment at its original position 5' of the rpA1 sequences. Construct Sa-ab contained the whole f5 sequence (from 1 to 636), Sa-abc contained the 5'-two-thirds (from 1 to 423), and Sa-be contained the 3'-two-thirds (217 to 636) (Fig. 1D). All transcripts decayed rapidly during early embryogenesis (Fig. 4). These results suggest that FIE5 destabilizing sequences are likely to be present in the Sa-ab/Sa-be overlap region. Alternatively, more than one destabilizing sequence may occur within f5. The results also indicate that the maternal hsp26/sgs3 promoter can be used for measurement of mRNA stability despite the presence of 41 additional sgs3-encoded nucleotides at the 5'-end of all transcripts driven by this promoter. Separate experiments showed that these 41 nucleotides contain no destabiliz-

![Fig. 1. Summary of constructs used to identify ftz mRNA instability elements.](image-url)

Deletion Analysis Identifies Two Separate Destabilizing Elements in the 5'-Coding Region—A second generation of constructs placed each third of f5 next to 5'-rpA1 sequences (Sa-a, Sa-b, and Sa-c; Fig. 1D). As shown in Fig. 5, s-a mRNA was stable, whereas s-b and s-c mRNAs decayed rapidly during early embryogenesis. These results suggest that f5 has at least
two destabilizing elements, one (FIE5-1) within fragment b (from 217 to 423) and the other (FIE5-2) within fragment c (from 406 to 636). Each element is sufficient for destabilization of an otherwise stable mRNA, and both elements are located in the protein-coding region of ftz mRNA.

**Further Mapping of FIE5-1**—Additional deletion constructs (Ss-b1 to Ss-b3; Fig. 1D) were generated to further map FIE5-1. Initial analysis of embryos carrying two overlapping constructs, Ss-b1 and Ss-b2, indicated that the s-b1 mRNA was unstable whereas s-b2 mRNA was stable (Fig. 6). These results suggested that FIE5-1 is located in a region of Ss-b1 (250–300) that does not overlap with the two stable sequences, SS-a and SS-b2 (Fig. 1D). This assumption was confirmed with a third construct, Ss-b3 (235–306), which encodes an unstable mRNA (Fig. 6). Thus, FIE5-1 is located within a 63-nucleotide sequence of the ftz protein-coding region (the stated length takes into account that our construct and yellow-white flies have nine fewer nucleotides than the sequence deposited in the database; cf. “Experimental Procedures”).

**Further Mapping of FIE5-2**—Additional deletion constructs (Ss-c1 to Ss-c5; Fig. 1D) were also generated to further map FIE5-2. As shown in Fig. 7, the s-c1 and s-c2 mRNAs were both unstable in early embryos, indicating that an instability element is located in the region of overlap or that multiple instability elements are located in ftz fragment c. To clarify these issues, three more constructs (Ss-c3 to Ss-c5; Fig. 1D) were analyzed. Of the three constructs, only Ss-c4 (from 520 to 588) encoded an unstable mRNA (Fig. 7), indicating that FIE5-2
must reside within this 69-nucleotide region. In some experiments, the s-c3 mRNA was observed to decline slightly from 0–1 to 1–2 h but remained stable during the remaining time points (data not shown).

**DISCUSSION**

In an earlier study (12), we developed a new method for the in vivo analysis of mRNA stability in early *Drosophila* embryos that does not require the use of drugs or any experimental interference. This method led to the identification of a mRNA-destabilizing element (FIE3) in the *ftz* 3′ UTR. In this work the method was used to identify two additional instability elements, both in the 5′-protein-coding region. Hence, *ftz* mRNA has three redundant destabilizing elements, each of which is sufficient to promote mRNA degradation in early embryos. Although the significance of the occurrence of three redundant elements in the same message can only be speculated on, redundancy may be tied to the fact that *ftz* mRNA instability is crucial for normal embryonic development (see the Introduction).

The half-life of a hybrid mRNA containing FIE3 was previously estimated to be about 50 min (12). In this study we estimated the half-life of the FIE5-1-containing s-b mRNA and the FIE5-2-containing s-c mRNA to be about 51 and 65 min, respectively. This thus, each element appears to have similar “strength.” Each element can act independently, and we found no evidence that the destabilizing activity of these elements is additive or synergistic. The estimated half-lives cited above are significantly longer than the 14–6 min half-life reported previously for the endogenous *ftz* mRNA (8). One reason for this difference may be that our measurements started early during embryonic development (from fertilization to 4 h), whereas endogenous *ftz* transcription occurs between 1.5 and ~4.5 h. The gradual decrease of *ftz* mRNA half-life from 14 to 6 min between 2.5 and 4 h of development (8) suggests that degrading activity increases as embryonic development progresses. Thus, the degrading activity may be low at the very beginning of embryogenesis. Another conceivable reason for the difference in estimated half-lives might be the cytoplasmic localization of the mRNAs (28, 29). Endogenous *ftz* mRNA is located in the...
Determinants of ftz mRNA Instability

Apical cytoplasm, whereas the distribution of the hybrid mRNAs in the transgenic embryos is unknown (30). Apical localization requires the last 53 nucleotides of the ftz 3’ UTR (31, 32), 43 nucleotides of which overlap with FIE3. Deletion of FIE3 (which comprises most of the apical localization sequence) in ftz-aldB-FIE3 did not seem to substantially affect stability when compared with the FIE3-containing ftz-aldB mRNA, suggesting that mRNA stability and localization are independent of each other.

The r-abc and s-abc mRNAs both contain the identical 5’-ftz sequence; yet their stability in the early embryo differs dramatically. The main difference between the two mRNAs is the position of the ftz sequences within the mRNA: in the 3’ UTR for r-abc and in the original 5’-position for s-abc. Therefore, the structure and sequence of the RNA elements are not sufficient for destabilizing activity, and position within the mRNA is crucial. Note that when FIE3 was inserted at the same position in the rpaI 3’ UTR as were the FIE5s in r-abc, FIE3 had full destabilizing activity. Thus, FIE3 is active, and FIE5s are inactive when inserted at the identical position of the rpaI mRNA. These results suggest that FIE5s and FIE3 destabilize mRNAs by different mechanisms. This position dependence of the FIE5 elements suggests that translation is required for degradation to occur. Precedents exist for a connection between mRNA stability and translation (30, 33–36). The suggested dependence of FIE5 activity on mRNA translation is consistent with the results of Edgar et al. (8), who reported that general inhibition of embryonic protein synthesis by cycloheximide inhibits the ftz mRNA. However, these results do not rule out the alternative possibility that cycloheximide prevents the synthesis of an unstable protein required for mRNA degradation.

The three cis-acting ftz instability elements are likely to act by providing a binding site for a factor or a protein complex that mediates mRNA degradation. A sequence comparison among the three elements and a search for similarity with sequences deposited in data bases did not yield any significant homologies. Binding sites could be recognized as secondary structures rather than primary sequences (37). However, no stable secondary structure that has more than a four-base straight stem or a common secondary structure among the three elements was predicted when a computer program of Zuker (26, 38) was used to fold these sequences. Moreover, site-directed mutagenesis of certain nucleotides within FIE3 did not alter the destabilizing activity of this element. Thus, it is presently unclear how these cis-acting elements are recognized in the embryo. Recently, a protein that binds to the ftz apical localization element was identified (32). The identification of proteins that recognize the three instability elements characterized in this and in previous work will bring significant insights to the questions of sequence recognition and mechanism of RNA degradation.

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REFERENCES
1. Ingham, P. W. (1988) Nature 335, 25–34
2. Surdej, P., and Jacobs-Lorena, M. (1998) Mol. Cell. Biol. 18, 2892–2900
3. Deleted in proof
4. Weiner, M. P., and Kuroiwa, T. (1985) Nature 318, 433–439
5. Hafen, E., Kuroiwa, A., and Gehring, W. J. (1984) Cell 37, 833–841
6. Yu, Y., and Pick, L. (1995) Mech. Dev. 50, 163–175
7. Hiroi, Y., and Gehring, W. J. (1987) Cell 50, 963–974
8. Deleted in proof
9. Edgar, B. A., Odell, G. M., and Schubiger, G. (1987) Genes Dev. 1, 1226–1237
10. Kefferman, K. A., Mattson, D. M., and Duncan, I. (1999) Genes Dev. 13, 2852–2855
11. Welte, M. A., Deucan, I., and Lindquist, S. (1995) Genes Dev. 9, 2240–2250
12. Riedl, A., and Jacobs-Lorena, M. (1996) Mol. Cell. Biol. 16, 3047–3053
13. Weiner, A. J., Scott, M. P., and Kaufman, T. C. (1986) Cell 37, 843–851
14. Qian, S., Zhang, J.-Y., Ray, M. A., and Jacobs-Lorena, M. (1987) Nucleic Acids Res. 15, 987–1003
15. Tsutsumi, K., Mukai, T., Tsutsumi, R., Mori, M., Dalimon, M., Tanaka, T., Yatauki, H., Horii, K., and Ishikawa, K. (1984) J. Biol. Chem. 259, 14572–14575
16. Tsutsumi, K., Mukai, T., Tsutsumi, R., Hitaka, S., Ariyai, Y., Horii, K., and Ishikawa, K. (1985) J. Mol. Biol. 181, 153–160
17. Cavener, D. R. (1987) Nucleic Acids Res. 15, 1353–1361
18. Cavener, D. R., and Ray, C. S. (1991) Nucleic Acids Res. 19, 3185–3192
19. Pirrotta, V. (1986) in Vectors: A Survey of Molecular Cloning Vectors and Their Uses (Rodriguez, R., and Denhardt, D., eds) pp. 437–456, Butterworth, Boston
20. Steller, H., and Pirrotta, V. (1986) Mol. Cell. Biol. 6, 1640–1649
21. Rubin, G. M., and Spradling, A. C. (1982) Science 218, 348–353
22. Ito, J., Kuzumaki, T., Otou, K., Iuchi, Y., and Ishikawa, K. (1998) Arch. Biochem. Biophys. 350, 291–297
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Garfinkel, M. D., Pruitt, R. E., and Meyerowitz, E. M. (1983) J. Mol. Biol. 166, 755–789
25. Serano, T. L., Cheung, H. K., Frank, L. H., and Cohen, R. S. (1994) Gene 138, 181–186
26. Zuker, M. (1989) Science 244, 48–52
27. Shyu, A.-B., Greenberg, M. E., and Belasco, J. G. (1989) Genes Dev. 3, 60–72
28. Mason, J. O., Williams, G. T., and Neuberger, M. S. (1988) Genes Dev. 2, 1003–1011
29. Ziff, R. D., Stein, J., and Stein, G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2683–2687
30. Fontes, A. M., Ito, J., and Jacobs-Lorena, M. (1999) Curr. Top. Dev. Biol. 44, 171–202
31. Davis, I., and Ish-Horowicz, D. (1991) Cell 67, 927–940
32. Lall, S., Francis-Long, H., Flamant, A., Norvell, A., Schuhbaff, T., and Ish-Horowicz, D. (1999) Cell 99, 171–180
33. Hennigan, A. N., and Jacobsen, A. (1990) Mol. Cell. Biol. 16, 3833–3843
34. Oliveira, C. C., and McCarthy, J. E. G. (1995) J. Biol. Chem. 270, 8336–8343
35. Zwick, W. D., and Belasco, J. G. (1990) J. Biol. Chem. 265, 3441–3448
36. Veyrne, J. L., Carillo, S., Vie, A., and Blanchard, J. M. (1995) Oncogene 11, 2127–2134
37. Ross, J. (1995) Microbiol. Rev. 58, 423–450
38. Mathews, D. H., Sabino, J., Zuber, M., and Turner, D. H. (1999) J. Mol. Biol. 288, 911–940 presence of 41 additional sg3-encoded nucleotides at the 5’-end of all