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Joint action of two RNA degradation pathways controls the timing of maternal transcript elimination at the midblastula transition in *Drosophila melanogaster*

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Maternally synthesized RNAs program early embryonic development in many animals. These RNAs are degraded rapidly by the midblastula transition (MBT), allowing genetic control of development to pass to zygotically synthesized transcripts. Here we show that in the early embryo of *Drosophila melanogaster*, there are two independent RNA degradation pathways, either of which is sufficient for transcript elimination. However, only the concerted action of both pathways leads to elimination of transcripts with the correct timing, at the MBT. The first pathway is maternally encoded, is targeted to specific classes of mRNAs through *cis*-acting elements in the 3′-untranslated region and is conserved in *Xenopus laevis*. The second pathway is activated 2 h after fertilization and functions together with the maternal pathway to ensure that transcripts are degraded by the MBT.

Keywords: *Drosophila*/midblastula transition (MBT)/localization/stability/*Xenopus*

Introduction

In animal embryos as diverse as echinoderms, insects, amphibians and mammals, the earliest stages of development are programed by maternally synthesized RNAs and proteins (reviewed in Davidson, 1986). Subsequent phases of embryogenesis require products encoded by zygotically synthesized transcripts. The transition from maternal to zygotic control of development is referred to as the midblastula transition (MBT) which is defined by the first developmental processes that require zygotic products. The MBT should not be confused with the onset of zygotic transcription, which initiates earlier. Prior to the MBT, a subset of the maternally synthesized transcripts is degraded. In mammals, maternal transcript degradation is complete by the two-cell stage, while in echinoderms, amphibians and insects it occurs prior to the MBT, when either several hundred cells (echinoderms), several thousand cells (amphibians) or several thousand syncytial nuclei (insects) are present.

Genetic and molecular studies in *Drosophila melanogaster* have demonstrated that a subset of the maternal mRNAs encode proteins controlling the cell cycle, positional specification of cells or the morphogenetic movements that drive gastrulation. These transcripts are eliminated during the syncytial blastoderm stage, shortly after zygotic transcription commences (reviewed in Cooperstock and Lipshitz, 1997; Bashirullah *et al*., 1998). The subsequent cellularization of the blastoderm is the first developmental process requiring zygotic contributions and thus marks the *Drosophila* MBT (Edgar *et al*., 1986; Merrill *et al*., 1988; Sibon *et al*., 1997).

During early *Drosophila* embryogenesis, from egg-laying until the MBT, there are 13 synchronous syncytial nuclear divisions (Zalokar and Erk, 1976; Foe and Alberts, 1983; Campos-Ortega and Hartenstein, 1998). Subsequently, the nuclei enter interphase of nuclear division cycle 14 and the somatic blastoderm cellularizes by concerted invagination of cell membranes from the periphery. Blastoderm cellularization is followed by the first gastrulation movements and the first zygotically programed mitosis, mitosis 14, which is no longer synchronous (Foe, 1989). It has been shown that this transition from synchronous maternally driven mitoses to asynchronous zygotically programed mitoses requires degradation of maternal *string* transcripts (*string* encodes a homolog of the cell cycle regulator, CDC25) (Edgar and O'Farrell, 1989, 1990; Edgar and Datar, 1996).

While turnover of maternal transcripts was first reported more than 20 years ago, little is known of the degradation machinery or of the mechanisms by which specific classes of transcripts are targeted to it. Here we show that two transcript degradation pathways function in the early *Drosophila* embryo. The first, 'maternal', pathway is driven by maternally encoded factors that are recruited by *cis*-acting RNA degradation elements independently of whether the transcript is translationally active or translationally repressed. This maternal degradation apparatus is conserved in *Xenopus* oocytes and early embryos. The second 'zygotic' pathway becomes active 2 h after fertilization. Either pathway acting alone is sufficient to eliminate maternal transcripts; however, the joint action of both pathways is necessary for elimination of transcripts prior to the MBT. This dual degradation system is likely

to coordinate developmental events by ensuring the elimination of maternal transcripts prior to the MBT.

Results

Degradation of maternally synthesized transcripts begins shortly after fertilization in Drosophila

We analyzed the dynamics of transcript degradation in the early embryo focusing on three maternally encoded RNAs: *Hsp83*, *string* and *nanos*. *Hsp83* transcripts are particularly abundant, representing 1% of the polyadenylated transcripts in the early embryo (Zimmerman *et al*., 1983). RNA tissue *in situ* analyses demonstrated that maternal *Hsp83* and *string* transcripts are no longer detectable by late stage 5, after completion of blastoderm cellularization (Figure 1A–F); *nanos* transcripts are undetectable by stage 4 (Figure 1G–I). RNA tissue *in situ* analyses are useful for defining when very low levels of transcripts are reached, but they are not sufficiently quantitative to be used to define the time course of degradation. Consequently, we carried out Northern blot analyses of RNA samples prepared from embryo collections spanning 30 min intervals during the first 5 h after fertilization. These blots were probed for *Hsp83*, *string*, *nanos* and *rpA1* transcripts. *rpA1* served as an internal control since maternal *rpA1* transcripts are stable beyond the MBT (Riedl and Jacobs-Lorena, 1996) (Figure 1J). Maternal *Hsp83*, *string* and *nanos* transcript levels decrease throughout the pre-MBT stages initiating within the first hour after fertilization (Figure 1K). By the MBT (2.5–3.0 h after fertilization), >96% of the maternal *Hsp83* transcripts loaded into the embryo have disappeared (Figure 1K). The *Hsp83* transcripts present at and after this stage comprise undegraded maternal transcripts in the pole cells and zygotic transcripts. Ninety-five percent of long isoform *string* transcripts (Figure 1J and K; only the long isoform is strictly maternally synthesized and is shown in K, although quantitation was done on each isoform) and >99% of the *nanos* transcripts have been eliminated by the MBT (Figure 1J and K). The remaining *nanos* transcripts represent undegraded maternal transcripts in the pole cells (Figure 1H and I). These results demonstrate that maternal transcript degradation initiates at or shortly after fertilization, and that the vast majority of these transcripts have been eliminated by the MBT.

Maternally encoded products direct transcript degradation, even in unfertilized eggs

We used unfertilized eggs to ask whether maternally encoded products are sufficient to direct transcript degradation in the absence of both fertilization and zygotic transcription. In *Drosophila*, egg activation occurs when the mature oocyte passes from the ovary into the uterus. This process does not require fertilization. Egg activation releases the female pronucleus from meiotic arrest at metaphase I, results in translation of various maternally encoded transcripts deposited in the oocyte, and induces

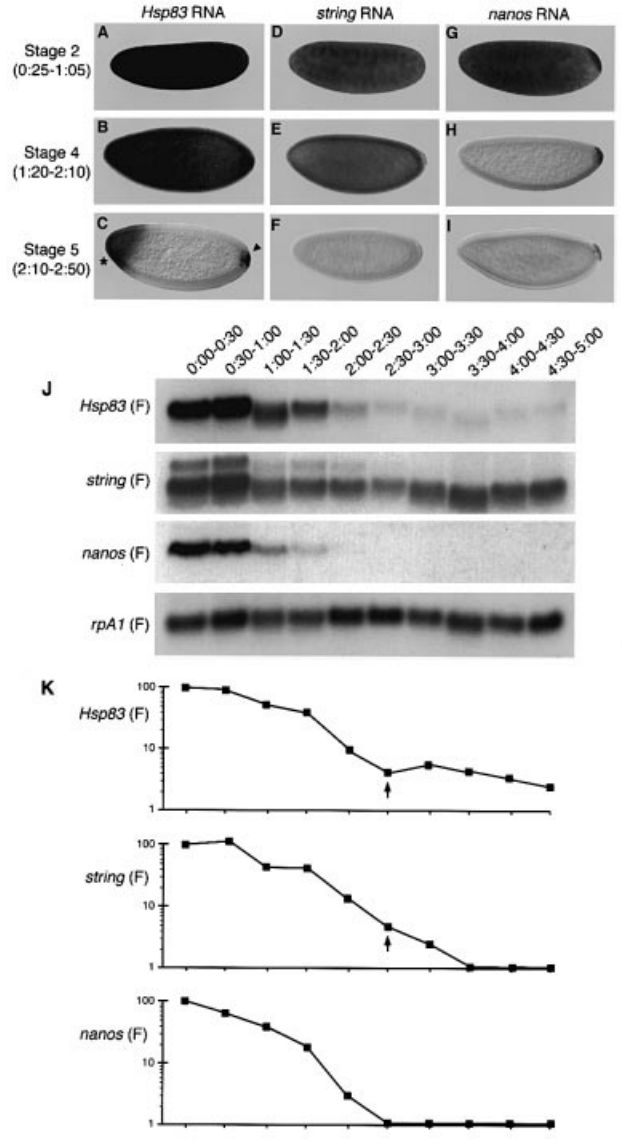

Fig. 1. Degradation of maternal *Hsp83*, *string* and *nanos* transcripts begins at fertilization, and >95% of the transcripts are eliminated by the MBT. Whole-mount *in situ* analysis of transcript distributions in wild-type early embryos are shown in (A–I) while Northern blots and quantitative analysis are shown in (J) and (K). All three species of transcripts are present throughout the embryo at late stage 2 (**A**, **D** and **G**; at about nuclear cycle 8; 25 min–1 h 5 min after fertilization). By stage 4 (nuclear cycle 13, 1 h 20 min–2 h 10 min after fertilization), *nanos* transcripts have been degraded in the somatic region (**H**) while *Hsp83* and *string* transcripts are still present (**B** and **E**). By the cellular blastoderm stage (nuclear cycle 14, 2 h 10 min–2 h 50 min after fertilization), *Hsp83* and *string* transcripts have been degraded throughout the somatic region (**C** and **F**) while *Hsp83* transcripts (arrowhead, C) and *nanos* transcripts (**I**) remain undegraded in the pole cells. The previously reported zygotic expression of *Hsp83* RNA (Ding *et al*., 1993a) can be seen in the presumptive head region (asterisk). Stages are according to Campos-Ortega and Hartenstein (1998). Embryos are oriented with the anterior to the left and dorsal up. This convention will be followed throughout. (**J**) Northern blots are shown for *Hsp83*, *string*, *nanos* and *rpA1* transcripts in developing embryos. For quantitation (**K**), amounts were normalized relative to *rpA1* levels since *rpA1* transcripts are stable. The time points shown in (J) also apply to (K). The ordinate in (K) presents [RNA] plotted on a logarithmic scale. A total of 96% of the maternally encoded *Hsp83* transcripts, 95% of the strictly maternally encoded *string* transcripts ('long isoform', upper band in J) and >99% of the *nanos* transcripts have disappeared by 2.5 h after fertilization (arrows). The *Hsp83* transcripts present after 2.5 h in embryos comprise maternal transcripts present in pole cells and zygotically synthesized transcripts. The larger *string* transcript, which is strictly maternal in origin (Edgar and Datar, 1996), was quantified. Details of the number of repetitions of each Northern blot experiment are given in Materials and methods.

depolymerization of cortical microtubules and cross-linking of the vitelline membrane (Mahowald *et al*., 1983).

The transcripts which we analyzed, i.e. maternal *Hsp83* (Figure 2A–C), *string* (Figure 2D–F), *nanos* (Figure 2G–I) and *Polar granule component* (*Pgc*; Figure 7G), are degraded in unfertilized eggs. Northern blot analyses (Figure 2J) indicated that 99% of the *Hsp83* transcripts and 90% of the long isoform *string* transcripts have been degraded by 4–5 h after egg activation (Figure 2K), 1–2 h later than in developing embryos (Figure 2J and K). Over 99% of the *nanos* transcripts have been degraded by 3 h after egg activation (Figure 2J and K), 0.5 h later than in developing embryos but >1 h earlier than *Hsp83* and *string* transcripts. *rpA1* transcripts are stable throughout this period (Figure 2J). We conclude that maternally encoded factors are sufficient for degradation of *Hsp83*, *string*, *nanos* and *Pgc* transcripts. A second, superimposed, degradation pathway causes the more rapid elimination of transcripts in embryos than in unfertilized eggs (see below).

Cis-acting elements in the 3′-UTR target transcripts for degradation

To identify *cis*-acting sequences essential for transcript degradation, we focused on the 3′-untranslated regions (3′-UTRs) of *Hsp83* and *nanos* transcripts. If an element targets transcripts for degradation, then absence of this element would be expected to stabilize the transcript. Unfertilized eggs were used for the analysis in order to identify those elements necessary for maternally encoded transcript degradation.

For *Hsp83*, a series of reporter transgenes was constructed that contained a fragment of the *Escherichia coli lacZ* gene and either the full 407 nucleotides of the *Hsp83* 3′-UTR or deleted versions (see Figure 3A for sequence, Figure 3B for transgenic constructs). By this analysis, a region in the 3′-UTR from nucleotide 253 to 349, which we refer to as the *Hsp83* degradation element (HDE), proved necessary for maternally encoded *Hsp83* transcript degradation (Figure 3C–F). Transcripts lacking the HDE (252Δ350) are stable in unfertilized eggs for at least 6 h (Figure 3F). This contrasts with transcripts that include the HDE (endogenous *Hsp83* transcripts or transgenic transcripts that include the HDE) which are fully degraded in unfertilized eggs by 4 h after activation (see Figure 2C, J and K for endogenous transcripts; data not shown for transgenic transcripts). Elimination of either the 5′ or the 3′ half of the HDE (252Δ301 or 300Δ351) results in incomplete degradation (data not shown; see Figure 3B).

The first 186 nucleotides of the *nanos* 3′-UTR, designated the translation control element (TCE), have also been implicated in control of transcript stability (Dahanukar and Wharton, 1996; Smibert *et al*., 1996). In unfertilized eggs, this element is shown here to target the maternally encoded degradation machinery to *nanos* transcripts. Transcripts lacking the first 186 nucleotides (ΔTCE) are not degraded by 2–4 h after egg activation (Figure 4C and D) whereas endogenous *nanos* transcripts have completely disappeared (Figure 4A and B).

To study whether the *Hsp83* HDE and the *nanos* TCE are functionally interchangeable, two additional transgenic constructs were made. In one, the HDE was added to a *nanos* transgene lacking the TCE (*nos[ΔTCE+HDE]*)

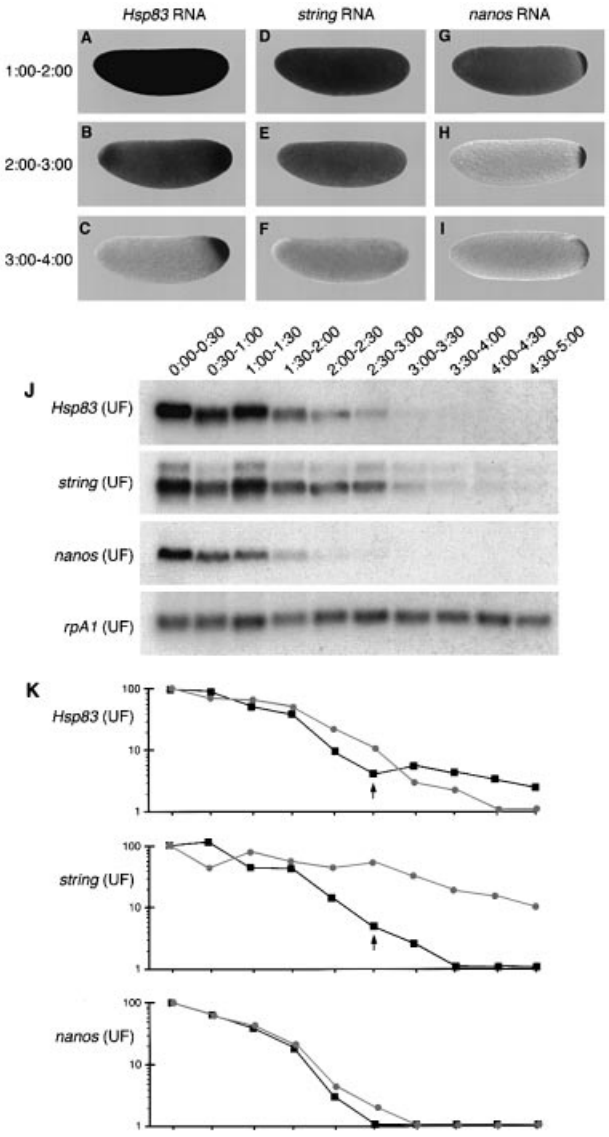

Fig. 2. *Hsp83*, *string* and *nanos* transcripts are degraded in unfertilized eggs. Whole-mount *in situ* analysis of transcript distributions in unfertilized eggs are shown in (A–I) while Northern blots and quantitative analysis are shown in (J) and (K). *Hsp83*, *string* and *nanos* transcripts are present throughout the egg from 0 to 2 h after egg activation (**A**, **D** and **G**). Between 2 and 3 h after egg activation, levels of *Hsp83* and *string* transcripts have decreased (**B** and **E**), *nanos* transcripts are no longer detectable outside the posterior polar plasm (**H**) and *Hsp83* transcripts can be seen to be concentrated in the posterior polar plasm (B). By 3–4 h, all three classes of transcripts are undetectable (**C**, **F** and **I**) except for the posterior-localized *Hsp83* and *nanos* transcripts (C and I). (**J**) Northern blots are shown for *Hsp83*, *string*, *nanos* and *rpA1* transcripts in unfertilized eggs. *Hsp83* and *string* transcripts (strictly maternal, upper band) reach minimal levels by 4–5 h, whereas *nanos* transcripts have disappeared by 3 h after egg activation. For quantitation (**K**), amounts were normalized relative to *rpA1* levels since *rpA1* transcripts are stable. The time points shown in (J) also apply to (K). The ordinate in (K) presents [RNA] plotted on a logarithmic scale. In unfertilized eggs, >99% of the *nanos* transcripts, 99% of the *Hsp83* transcripts and 90% of the *string* transcripts (strictly maternal, upper band in J) are degraded. Transcripts from unfertilized eggs are shown in gray (●) while transcripts from embryos (taken from Figure 1) are shown in black (■) for comparison. Details of the number of repetitions of each Northern blot experiment are given in Materials and methods.

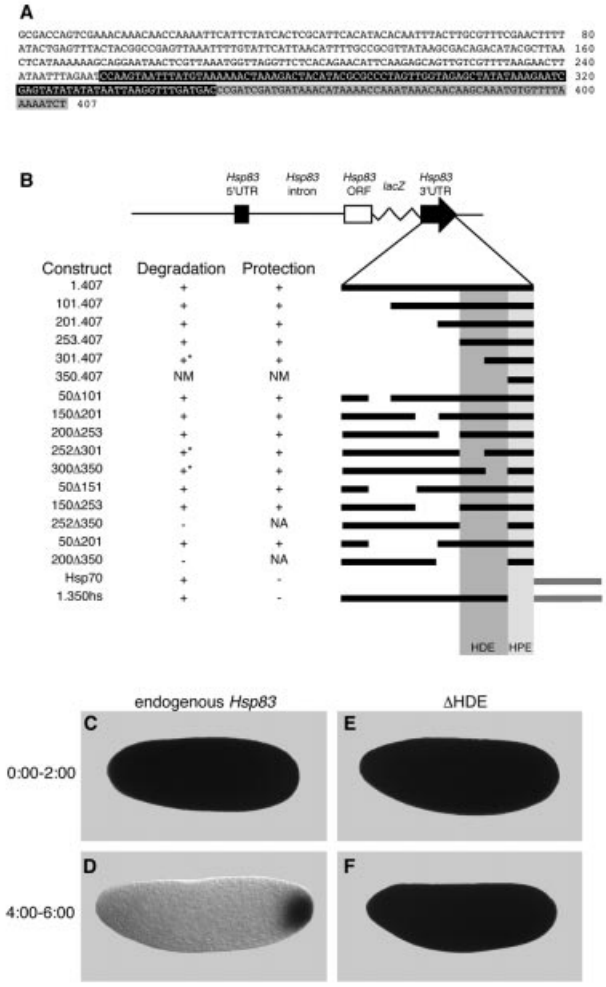

Fig. 3. Mapping of the *Hsp83* degradation (HDE) and protection (HPE) elements. (**A**) Sequence of the *Hsp83* 3′-UTR with highlighted HDE (black) and HPE (gray). (**B**) Transcription of the transgenes is under the control of *Hsp83* regulatory sequences fused to a 603 bp *lacZ* tag and followed by the 407 nucleotide *Hsp83* 3′-UTR (1.407) or the *Hsp70* 3′-UTR (‘*Hsp70*’) (see Materials and methods). 3′, 5′ and internal deletions within the *Hsp83* 3′-UTR are shown as a diagram. +, degradation or protection occur as in wild-type; +*, degradation incomplete; –, degradation or protection fail; NA, protection cannot be assayed in the absence of degradation; NM, no maternal RNA present in the egg (Halsell, 1995). Black bars represent *Hsp83* 3′-UTR sequences; gray bars represent *Hsp70* 3′-UTR sequences. The HDE which maps from nucleotide 253 to 349 and the HPE which maps from nucleotide 350 to 407 are highlighted. (**C** and **D**) Endogenous *Hsp83* transcripts visualized with an antisense *Hsp83* RNA probe. (**E** and **F**) Transgenic 252Δ350 reporter transcripts visualized with an antisense β-galactosidase RNA probe. The age of eggs or embryos in hours is shown to the left. In contrast to endogenous transcripts (C and D), the 252Δ350 transcripts persist throughout the egg until at least 6 h after activation (E and F).

(Figure 4E and F); in the other, the *nanos* TCE was added to an *Hsp83–lacZ* reporter construct lacking the HDE (*Hsp83[ΔHDE+TCE]*) (Figure 4G and H). Addition of the *Hsp83* HDE restores degradation to the otherwise stable *nos[ΔTCE]* transcript (Figure 4E and F) while addition of the *nanos* TCE similarly targets the stable *Hsp83–lacZ[ΔHDE]* transcript for degradation (Figure 4G and H). While our data demonstrate that the HDE and TCE are functionally interchangeable, we have been unable to identify any significant primary sequence or secondary structure conservation between these elements.

These data support a model in which specific *cis*-acting elements recruit a maternally encoded degradation machinery to both the *Hsp83* and *nanos* transcripts.

Relationship between translation and RNA degradation

Our next goal was to study whether translational repression is a prerequisite for transcript degradation. Such a requirement had been postulated for *nanos* transcripts in early embryos since relief of translational repression (by deletion of the TCE) correlated with an increase in transcript stability (Dahanukar and Wharton, 1996; Smibert *et al*., 1996). Translation of *nanos* in the head region of the embryo causes repression of *bicoid* translation and thus head skeleton defects (Dahanukar and Wharton, 1996; Smibert *et al*., 1996). The severity of the defect serves as a sensitive readout of the Nanos protein level (Wharton and Struhl, 1991; Gavis and Lehmann, 1994) in the absence of a direct assay using anti-Nanos antibodies, which are no longer available.

We examined the head skeleton of transgenic embryos carrying *nos[ΔTCE]* or *nos[ΔTCE+HDE]* transcripts. As previously reported (Dahanukar and Wharton, 1996; Smibert *et al*., 1996), *nos[ΔTCE]* transcripts are translated and produce head skeleton defects (Figure 4J). Surprisingly, even though *nos[ΔTCE+HDE]* transcripts are degraded (Figure 4F), they too produce head skeleton defects (Figure 4K). Furthermore, the severity of the head skeleton defects was greater in the case of *nos[ΔTCE+HDE]* than *nos[ΔTCE]*. In five independent *nos[ΔTCE]* transgenic lines, an average of 72 ± 29% of the embryos exhibited defects and these were restricted to the dorsal bridge. In contrast, all four independent *nos[ΔTCE+HDE]* lines resulted in 100 ± 0% of the embryos exhibiting severe defects (loss of dorsal bridge, dorsal arms, vertical plates and ventral arms).

Thus, not only are the *nos[ΔTCE+HDE]* transcripts translated, but they are translated at higher levels than the *nos[ΔTCE]* transcripts, perhaps because the HDE includes a translational enhancer. We conclude that translational repression and transcript degradation are not causally linked; even highly translated transcripts are targeted for degradation. Moreover, non-protein-coding transcripts (e.g. *Pgc*) are also degraded in unfertilized eggs (Figure 7G). Together, these data indicate that the transcript degradation machinery acts on transcripts that contain a degradation element independently of whether they are untranslatable, translationally repressed or translated.

Discovery of a second, zygotically encoded or activated degradation pathway

We next addressed why *Hsp83* transcripts are eliminated more rapidly in embryos than in unfertilized eggs. We asked whether embryos contain an additional degradation machinery that acts independently of the HDE, by comparing the stability of transcripts containing or lacking the HDE. Degradation of endogenous *Hsp83* transcripts as well as HDE-containing transgenic transcripts is initiated shortly after fertilization, and minimal transcript levels are reached by 2.5 h (Figure 5A, B and F for endogenous transcripts; transgenic transcripts not shown). In unfertilized eggs, transcripts lacking the HDE are stable for well over 5 h (Figures 3E and F, and 5E).

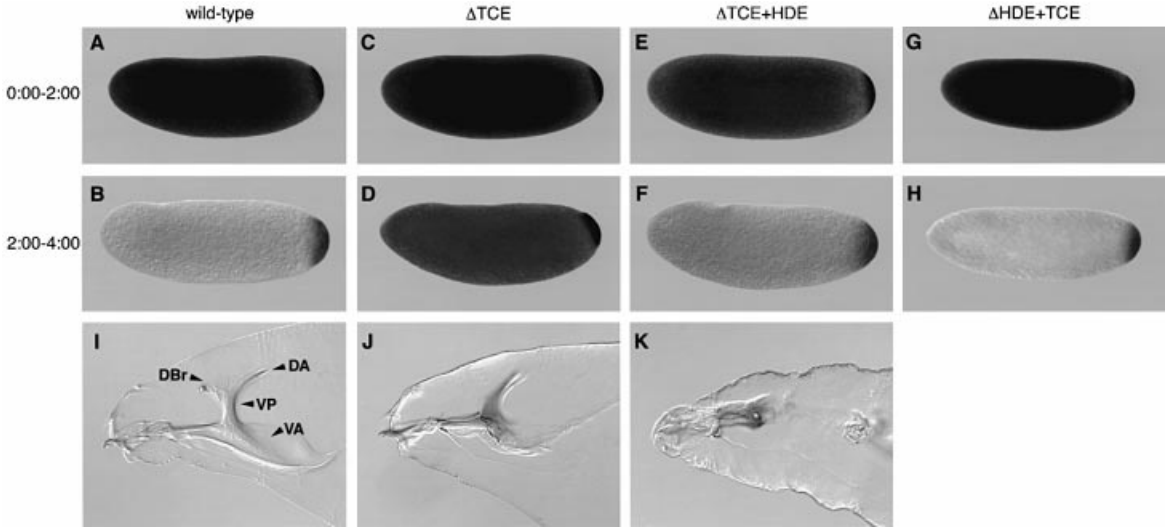

Fig. 4. The *Hsp83* HDE and the *nanos* TCE are interchangeable with respect to maternal transcript degradation, which is independent of translational repression. (**A** and **B**) Endogenous *nanos* transcripts are degraded by 2 h after egg deposition. (**C** and **D**) Deletion of the *nanos* TCE (*nanos* 3′-UTR nucleotides 1–185) results in transcript stabilization. (**E** and **F**) Replacement of the *nanos* TCE with the *Hsp83* HDE (*Hsp83* 3′-UTR nucleotides 253–349) restores transcript degradation. (**G** and **H**) Replacement of the *Hsp83* HDE with the *nanos* TCE restores transcript degradation. *nanos[ΔTCE+HDE]* transcripts are not translationally repressed even though they are degraded. Head skeletons are shown from wild-type (**I**), embryos from *nanos[ΔTCE]* transgenic females (**J**) and embryos from *nanos[ΔTCE+HDE]* females (**K**). As previously reported (Dahanukar and Wharton, 1996; Smibert *et al*., 1996), there is a reduction of the dorsal bridge in *nanos[ΔTCE]* embryos (J). Surprisingly, although *nanos[ΔTCE+HDE]* transcripts are degraded (F), they are not translationally repressed, as evidenced by the severe head skeleton phenotype (K) (note the loss of dorsal bridge, dorsal arms, vertical plate and ventral arms). DBr, dorsal bridge; DA, dorsal arms; VP, vertical plate; VA, ventral arms.

HDE-deleted (252Δ350) transcripts are stable throughout the first 2 h of embryogenesis, after which degradation initiates, and 95% of these transcripts are gone by 4 h after fertilization (Figure 5C, D, F and G). Thus, in addition to the maternally encoded degradation machinery, a zygotically synthesized or zygotically triggered degradation machinery is active starting 2 h after fertilization. Furthermore, since transcript degradation occurs at this stage in the absence of the HDE, this 'zygotic' degradation is independent of elements in the 3′-UTR that mediate the maternally encoded degradation. Zygotic transcript degradation occurs for all 3′-UTR reporter constructs listed in Figure 3.

Taken together, our analyses demonstrate that the joint action of both the 'maternal' and the 'zygotic' degradation pathways is needed to eliminate the bulk of maternal transcripts by the onset of the MBT.

Elements in the 3′-UTR protect transcripts from degradation in the pole cells

Certain maternal transcripts fail to degrade in pole cells of developing embryos or in the posterior polar plasm of unfertilized eggs. These include *Hsp83* (Figures 1C and 2C), *nanos* (Figures 1H and I, and 2H and I) and *Pgc* (Figure 7G) (Wang and Lehmann, 1991; Ding *et al*., 1993a; Nakamura *et al*., 1996). Our time course analysis shows that 1% of the total maternally loaded *Hsp83* transcripts and $<1\%$ of the total maternally loaded *nanos* transcripts remain in the posterior polar plasm of unfertilized eggs (Figure 2K). Two possible mechanisms could lead to the absence of transcript degradation at the posterior: the degradation machinery might be excluded from the posterior polar plasm and thus also from the pole cells. Alternatively, the machinery could be active in the posterior polar plasm and pole cells, but certain classes of transcripts could be masked from the machinery by components of the polar plasm.

To distinguish between these possibilities, we replaced the *Hsp83* 3′-UTR in our reporter construct with the 3′-UTR from the *Hsp70* transcript (Figure 3B). Transgenic reporter transcripts carrying the *Hsp83* 3′-UTR are degraded normally in the soma but are not degraded in the pole cells (Figure 6A–C), mimicking the distribution of the endogenous *Hsp83* transcripts (Figure 1A–C). The reporter transcripts carrying the *Hsp70* 3′-UTR are taken up into the pole cells when they bud (Figure 6D and E) but are unstable and are degraded by the cellular blastoderm stage (Figure 6F; see figure legend for details). These results demonstrate that the maternally encoded degradation machinery is present throughout the egg and embryo.

To identify 'protection' elements within the *Hsp83* 3′-UTR, we assayed posterior protection of our reporter transcripts in unfertilized eggs (Figure 3B). Sequences sufficient for protection reside within the 3′-most 107 nucleotides of the *Hsp83* 3′-UTR since transgenic RNA produced by the 301.407 construct is protected (Figure 3A and B). Since transgenic RNA carrying only nucleotides 351–407 is unstable during oogenesis (Halsell, 1995), it was not possible to determine whether the 3′-most 57 nucleotides alone are sufficient for protection. However, replacement of nucleotides 351–407 with the *Hsp70* 3′-UTR results in failure of protection (Figure 3B), showing that nucleotides 351–407 are necessary for protection. We refer to nucleotides 351–407 as the *Hsp83* protection element or HPE. Thus elements necessary for degradation versus protection map to distinct regions within the *Hsp83* 3′-UTR.

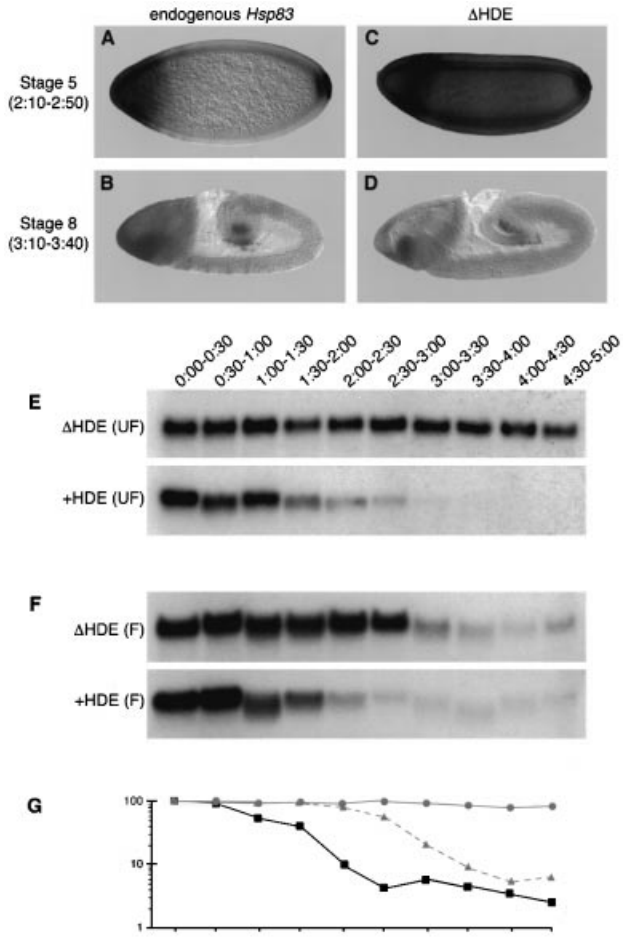

Fig. 5. *Hsp83* transcripts lacking the HDE are degraded in embryos by a zygotically synthesized or activated degradation mechanism. (A and B) Endogenous *Hsp83* transcripts visualized with an antisense *Hsp83* RNA probe. (C and D) Transgenic 252Δ350 reporter transcripts visualized with an antisense β-galactosidase RNA probe. The stage of embryos is shown to the left. In contrast to endogenous transcripts (**A** and **B**) which are degraded by the MBT and so are absent by stage 5, the 252Δ350 transcripts persist beyond stage 5 (**C**) and are degraded by stage 8 (**D**). Zygotically synthesized transcripts accumulate in the head region and in the germ band (B and D). Northern blots are shown of unfertilized eggs ('UF' in **E**) and developing embryos ('F' in **F**). Quantitation is shown in (**G**). In unfertilized eggs, transgenic transcripts lacking the HDE (●, solid gray line) are stable, whereas they are degraded starting after 2 h and are gone by 4 h after fertilization in embryos (▲, dashed gray line). Endogenous transcripts in embryos are shown for reference (■, solid black line; taken from Figure 1K). For quantitation, amounts were normalized as in Figures 1 and 2. In embryos, the *Hsp83* transcripts that persist after 2.5 h (black, endogenous) or after 4 h (dashed line, ΔHDE) comprise maternal transcripts present in pole cells and zygotically synthesized transcripts. Details of the number of repetitions of each Northern experiment are given in Materials and methods.

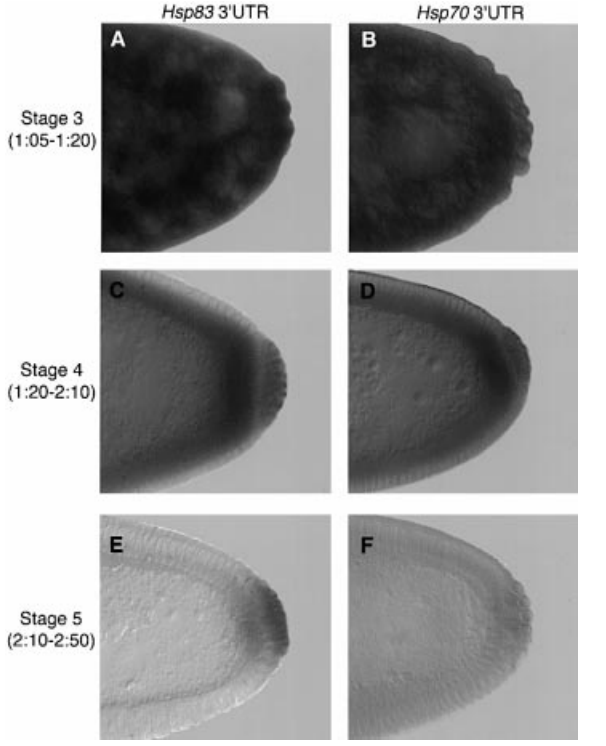

Fig. 6. Degradation activity is present in pole cells but *Hsp83* transcripts are normally protected from it. (**A** and **B**) Stage 3 pole cells; (**C** and **D**) stage 4 pole cells; (**E** and **F**) stage 5 pole cells. (A–C) *Hsp83* 1.407 transgenic transcripts or (D–F) transgenic transcripts that replace the *Hsp83* 3′-UTR with the *Hsp70* 3′-UTR (see Figure 3A), visualized with an antisense β-galactosidase probe. Transcripts that include the *Hsp83* 3′-UTR are protected from degradation in pole cells, while transcripts with the *Hsp70* 3′-UTR are degraded in both the pole cells and the soma by stage 5, indicating that degradation activity is present in pole cells. At stage 5, 82% of the embryos with the *Hsp70* 3′-UTR transgenic RNA had little or no detectable transcript in pole cells ($n = 39$). At stages 6–8, 95% of the embryos with the *Hsp70* 3′-UTR transgenic RNA had little or no detectable transcript in pole cells ($n = 42$). In contrast, 100% of the embryos carrying full-length *Hsp83* 3′-UTR transgenic RNA had high levels of transcript in their pole cells at these stages ($n = 50$).

Identification of mutants that fail to undergo maternally encoded RNA degradation

Since our data demonstrated that maternal factors are sufficient for transcript degradation prior to the MBT, we tested a collection of female sterile mutant lines (Schupbach and Wieschaus, 1989) to identify maternal effect mutants that fail to undergo degradation (see Materials and methods for list). Mutants were assayed for *Hsp83* RNA degradation in unfertilized eggs aged well beyond the stage at which transcripts would have disappeared in the wild-type. The presence of protected transcripts at the posterior served as an internal control for the *in situ* hybridizations (if degradation occurs, then protected *Hsp83* transcripts are visible at the posterior). Mutations in two loci, *cortex* and *grauzone*, result in failure of *Hsp83* transcript degradation (Figure 7B and C). Failure of degradation was not specific to *Hsp83* since *nanos* (Figure 7E and F), *Pgc* (Figure 7H and I) and *string* (Figure 7K and L) transcripts also failed to degrade. The *cortex* and *grauzone* mutants exhibit defective egg activation and cytoplasmic polyadenylation of transcripts (Lieberfarb *et al*., 1996; Page and Orr-Weaver, 1996).

The maternally encoded degradation pathway is conserved in Xenopus

Several maternal transcripts are localized during *Xenopus* oogenesis although it is not yet known whether the type of degradation–protection system described here for *Drosophila* functions in their localization (reviewed in Bashirullah *et al*., 1998). Furthermore, as in *Drosophila*, many maternal transcripts are degraded prior to the *Xenopus* MBT (reviewed in Davidson, 1986). Thus, it was of interest to determine whether *Xenopus* oocytes and early embryos have a maternal RNA degradation

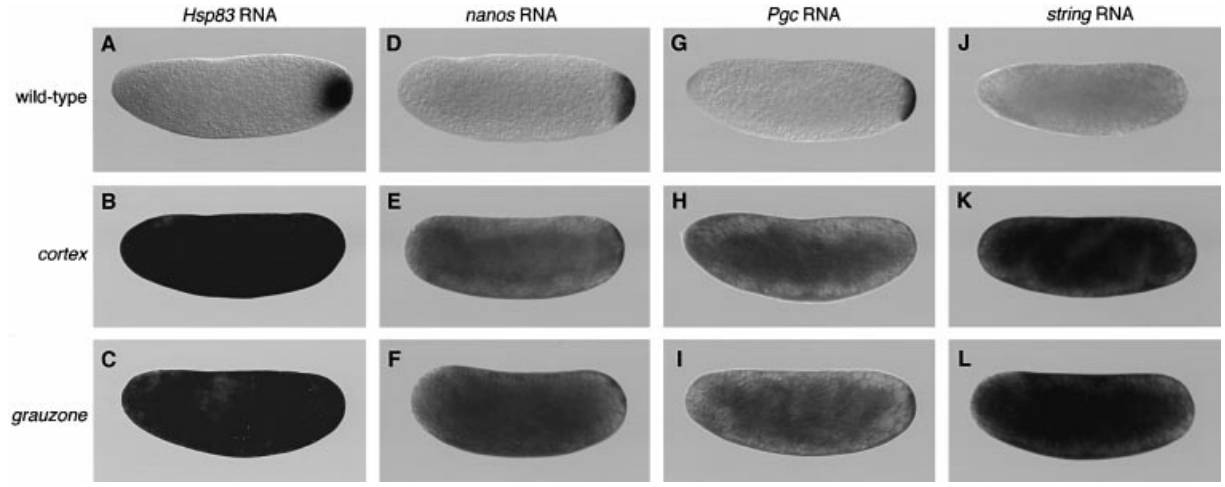

Fig. 7. The *cortex* and *grauzone* mutants result in failure of maternally encoded transcript degradation. Assays were conducted in unfertilized eggs, aged for at least 3 h, from wild-type females (A, D, G and J), *cortex* females (B, E, H and K) or *grauzone* females (C, F, I and L). Degradation of *Hsp83* (**A**–**C**), *nanos* (**D**–**F**), *Pgc* (**G**–**I**) and *string* (**J**–**L**) transcripts fails in eggs produced by the mutants. The defects in transcript localization (E, F, H and I) may result from cytoskeletal abnormalities (Lieberfarb *et al*., 1996; Page and Orr-Weaver, 1996).

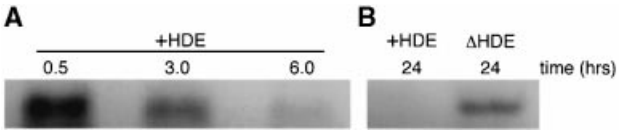

Fig. 8. The maternal transcript degradation pathway is conserved in *Xenopus* and *Drosophila*. (**A**) *In vitro* synthesized *Drosophila Hsp83* 3′-UTR transcripts (1.407) that carry the HDE (‘+HDE’) are highly unstable in *Xenopus* oocytes and are almost completely degraded within 6 h of injection. (**B**) In contrast, *in vitro* synthesized *Drosophila Hsp83* 3′-UTR transcripts that lack the HDE (‘ΔHDE’; 252Δ350) are stable and can still be readily detected 24 h after injection.

machinery similar to that identified here in *Drosophila*. We injected *in vitro* synthesized, digoxigenin-labeled transcripts comprising the full-length *Drosophila Hsp83* 3′-UTR into stage 6 *Xenopus* oocytes or recently fertilized embryos. These transcripts are unstable and are degraded rapidly after injection (Figure 8A for oocytes; data not shown for embryos). We then injected *in vitro* transcribed *Drosophila Hsp83* 3′-UTR ΔHDE transcripts into stage 6 oocytes; these transcripts are extremely stable and are still readily detectable 24 h after injection (Figure 8B). We conclude that the *Drosophila* HDE functions as a degradation element in *Xenopus* oocytes and embryos. Thus, *Xenopus* must contain a transcript degradation activity highly related to the *Drosophila* maternal activity and capable of recognizing the *Drosophila* HDE.

Discussion

Passage from maternal to zygotic control of development is thought to depend on the elimination of a subset of maternal transcript classes from the early embryo. For example, degradation of ubiquitous maternal *string* transcripts is necessary to permit zygotically driven, patterned domains of cell division after the MBT (Edgar and Datar, 1996). Here we have shown that the joint action of two RNA degradation pathways controls maternal transcript degradation and its timing in the early *Drosophila* embryo, and that at least one of these pathways (the ‘maternal’ pathway) is conserved in *Xenopus*.

Two genetic pathways ensure RNA degradation prior to the Drosophila MBT

The first degradation pathway is maternally encoded and does not require fertilization or zygotic gene transcription for its function. This ‘maternal’ pathway begins to function at or shortly after egg activation, and is sufficient for degradation of even abundant maternally loaded transcripts by 4–5 h after egg activation (Figure 9). The second ‘zygotic’ pathway requires fertilization and becomes active 1.5–2 h after fertilization. Acting alone, the zygotic pathway eliminates transcripts by 4 h after fertilization. Since the zygotic pathway can remove *Hsp83* transcripts in 2 h, while the maternal pathway takes 4–5 h, the zygotic machinery is approximately twice as efficient as the maternal machinery. Under normal circumstances, relatively rare transcripts are degraded almost exclusively by the maternal pathway (e.g. *nanos*). In contrast, more abundant transcripts require the action of both pathways in order to be eliminated by the MBT (e.g. *Hsp83*, *string*). The maternal pathway initiates transcript degradation at or shortly after egg activation, while the zygotic pathway ensures degradation of abundant transcripts prior to the MBT.

Our results predict four classes of maternal RNAs in the early embryo. The first class lacks both maternal and zygotic degradation elements and is stable (e.g. *rpA1*). The second class (if it exists—there are no known examples) would be degraded exclusively by the maternal machinery either because these transcripts lack zygotic degradation elements or because their low abundance enables them to be fully degraded by the maternal machinery prior to activation of the zygotic factors. A third class (if it exists—there are no known examples) would be degraded exclusively by the zygotic machinery, possibly because of a lack of elements that target the maternal factors. The fourth class (e.g. *Hsp83*, *string* and *nanos*) is degraded by the combined action of the maternal and zygotic machinery.

The dual mechanism for transcript degradation discovered here sheds light on two previously reported observations. First, it has been shown that modulation

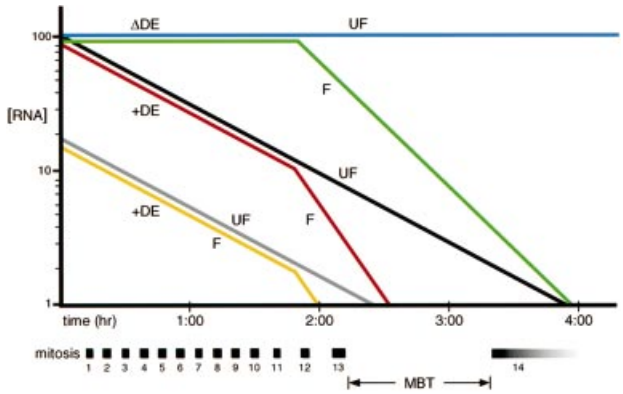

Fig. 9. Maternal and zygotic degradation activities cooperate to eliminate maternal transcripts by the MBT. Below the time scale on the *x*-axis are shown the maternally driven mitoses (1–13) followed by interphase 14 (the MBT) during which zygotic products take over control of the cell cycle before mitosis 14, the first patterned and asynchronous mitosis. The *y*-axis represents the [RNA] plotted on a logarithmic scale. In unfertilized eggs, an abundant transcript (e.g. *Hsp83*) lacking a maternal degradation element but carrying a zygotic degradation element is stable (ΔDE, blue). In contrast, such a transcript is degraded in fertilized, developing embryos by the zygotic degradation apparatus, starting ~2 h after fertilization, such that transcripts are fully degraded by 4 h after fertilization (ΔDE, green). In the presence of both maternal and zygotic degradation elements, transcript degradation initiates at or shortly after activation of unfertilized eggs and is complete by 4 h after fertilization (+DE, black). In fertilized, developing embryos, transcripts carrying both maternal and zygotic degradation elements initially are degraded solely by the maternal apparatus (<2 h after fertilization) and then cooperatively by both the maternal and zygotic factors (>2 h after fertilization) (+DE, red). Only in this last case do abundant transcripts disappear by the MBT (2.0–2.5 h after fertilization). Rarer transcripts (e.g. *nanos*) are eliminated almost exclusively by the maternal machinery (yellow and grey). ΔDE, no maternal degradation element; +DE, maternal degradation element present; UF, unfertilized egg; F, developing embryo.

of the nuclear:cytoplasmic ratio affects the timing of disappearance of maternally synthesized *string* transcripts (Yasuda *et al*., 1991). Changing the nuclear:cytoplasmic ratio would not affect the maternal degradation machinery since it would be pre-loaded into the embryo and its activity/concentration should be independent of that ratio. In contrast, changes in the nuclear:cytoplasmic ratio might be expected to alter the synthesis or local concentration of the zygotic degradation machinery, particularly if activation of this machinery is dependent on zygotic transcription. Thus, altering the nuclear:cytoplasmic ratio would alter the exact timing of *string* transcript disappearance by modulating the zygotic pathway. Secondly, our results explain why α-amanitin injection into embryos prior to nuclear cycle 6 causes a delay in degradation of maternal *string* transcripts (α-amanitin inhibits RNA polymerase II and thus zygotic transcription) (Edgar and Datar, 1996). If production or activation of the zygotic degradation machinery requires zygotic transcription, α-amanitin would be expected to inhibit it while leaving the maternal degradation machinery unaffected. This would result in a delay in *string* degradation since only the maternally encoded degradation apparatus would be functional.

In summary, while previous results suggested that turnover of transcripts at the MBT might be controlled by zygotic products (O'Farrell *et al*., 1989; Yasuda *et al*., 1991), we have uncovered a dual maternal–zygotic degradation system that is likely to coordinate developmental events by ensuring the elimination of maternal transcripts prior to the MBT.

Cis-acting elements target transcripts for degradation or for protection

We have shown that the maternal transcript degradation activity is present throughout the embryo and unfertilized egg. Deletion of specific *cis*-acting 3′-UTR sequences results in failure of maternally encoded transcript degradation. These elements do not share any obvious primary sequence or secondary structure, nor do they include any previously defined degradation tags (reviewed in Jacobson and Peltz, 1996). Despite the lack of sequence conservation, our data demonstrate that the degradation elements are interchangeable (the *Hsp83* HDE can substitute for the *nanos* TCE, and vice versa). The *cis*-acting elements defined here are not necessary for targeting transcripts to the zygotic degradation pathway since deletion of these elements has no detectable effect on the zygotic degradation process; in fact, none of our *Hsp83* 3′-UTR deletions abrogates zygotic transcript degradation. There are three plausible explanations for this result: (i) there are redundant 'zygotic' degradation elements in the *Hsp83* 3′-UTR; (ii) such elements reside in another part of the *Hsp83* transcript [given the structure of the reporter transgenes, this could be the 5′-UTR or the first 333 nucleotides of the open reading frame (ORF)]; or (iii) zygotic degradation is not a targeted event but is the default state.

For RNAs such as *rpA1*, which are stable in the early embryo, we do not yet know whether there are specific stabilization elements or whether transcript stability is a default state. Indirect evidence in support of the latter possibility comes from analyses of a transcript instability element in the *ftz* 3′-UTR (Riedl and Jacobs-Lorena, 1996). Addition of this element (FIE3) to *rpA1* transcripts destabilizes them, suggesting that for *rpA1* transcripts, stability is either the default state or that instability elements such as FIE3 are dominant to as yet undefined stability elements.

Our data indicate that it is possible to differentially stabilize transcripts carrying degradation tags in different regions of the cytoplasm and/or in different cell types. Specifically, we have shown that the maternal degradation apparatus is active throughout the egg and embryo, but that transcripts such as *Hsp83*, *nanos* and *Pgc* are protected from degradation in the pole plasm and pole cells. We have also been able to map sequences in the *Hsp83* 3′-UTR that are necessary for this protection. The most likely candidate organelles for protecting transcripts in the polar plasm and pole cells are the polar granules themselves. First, one of the RNAs we have studied (*Pgc*) has been shown to be an integral component of the polar granules (Nakamura *et al*., 1996). Secondly, it has been shown that disruption of the polar granules results in failure of *Hsp83*, *nanos* and *Pgc* transcript protection in the polar plasm and pole cells, while ectopic assembly of polar granules at the anterior of the embryo results in ectopic protection of these transcripts anteriorly (Ephrussi and Lehmann, 1992; Ding *et al*., 1993a; Nakamura *et al*., 1996).

Role of transcript degradation and protection in cytoplasmic RNA localization

Differential stabilization of transcripts in specific regions of the cytoplasm represents one of several RNA localization mechanisms (reviewed in Bashirullah *et al*., 1998). *Hsp83* is the only example of a transcript localized by a degradation–protection mechanism (Ding *et al*., 1993a; St Johnston, 1995). However, we have shown that *nanos*, *Pgc* and *Hsp83* transcript elimination in the somatic region of the embryo is accomplished by the same RNA degradation machinery. Further, our data suggest that 99% of the *Hsp83* transcripts and >99% of the *nanos* transcripts reside in somatic region. This last result is very similar to the 96% value reported recently for somatic *nanos* transcripts (Bergsten and Gavis, 1999). We speculate that the differences in localization patterns of transcripts that are obviously localized prior to egg activation and those which only appear localized well after egg activation may be largely quantitative.

Relationship of transcript degradation to translation and polyadenylation status

The literature contains numerous examples of a correlation between translational repression of mRNAs and their degradation (reviewed in Jacobson and Peltz, 1996; Cooperstock and Lipshitz, 1997). In the early *Drosophila* embryo, the best studied example is *nanos*: *nanos* transcripts outside of the posterior polar plasm are translationally repressed and are also unstable (Figure 4, and see also Dahanukar and Wharton, 1996; Gavis *et al*., 1996; Smibert *et al*., 1996). Deletion or mutation of *cis*-acting elements that mediate translational repression results in transcript stabilization (Dahanukar and Wharton, 1996; Smibert *et al*., 1996; this study). Despite this correlation, our data demonstrate that there is no obligatory link between translational repression and transcript degradation. Replacing the element from the *nanos* 3′-UTR that mediates translational repression and degradation (the TCE) with the *Hsp83* degradation element (the HDE) is sufficient to allow maternal degradation of *nanos [ΔTCE+HDE]* transcripts. However, at the same time, the presence of severe head defects demonstrates that this transgenic RNA is in fact highly translated. Thus, transcript degradation can be unlinked from translational repression.

There are several examples of a correlation between polyadenylation status and transcript stability (reviewed in Jacobson and Peltz, 1996). In particular, long poly(A) tails are associated with translational activation and transcript stabilization, while short poly(A) tails are associated with translational repression and transcript destabilization. In the *Drosophila* embryo, it has been shown that several classes of maternal transcripts are cytoplasmically polyadenylated after fertilization, correlating with initiation of their translation (Salles *et al*., 1994). The *cortex* and *grauzone* mutations perturb cytoplasmic polyadenylation (Lieberfarb *et al*., 1996), but transcript stability was not assayed in those studies. We have shown here that maternal transcripts are stabilized by *cortex* and *grauzone* mutations. The primary cause of the defects in embryos produced by *cortex* and *grauzone* females is unknown; their phenotypes are complex and several aspects of egg activation are abnormal (Lieberfarb *et al*., 1996; Page and Orr-Weaver, 1996). Thus it is not yet possible to draw firm conclusions from these mutants regarding a possible link between cytoplasmic polyadenylation status and transcript stability.

Evolutionary conservation of transcript degradation pathways

We have shown that *Xenopus* oocytes and early embryos contain an activity highly related to the maternal transcript degradation activity we have defined in *Drosophila*. Remarkably, the *Drosophila* HDE is recognized by this activity, and *in vitro* sythesized, injected transcripts are degraded rapidly, disappearing within a few hours. Removal of the HDE stabilizes these transcripts for at least 24 h. These results suggest that evolutionarily highly conserved pathways may control transcript degradation and localization throughout metazoa, opening up the possibility of exploiting the particular advantages of different model systems for future mechanistic and functional analysis.

Materials and methods

Drosophila whole-mount RNA in situ hybridization

RNA *in situ* hybridization with DNA probes was performed as described previously, with minor modifications (Tautz and Pfeifle, 1989; Ding *et al*., 1993a,b). Digoxigenin-labeled antisense RNA probes were made using the Megascript RNA transcription kit (Ambion, Inc.) according to the manufacturer's instructions, and included digoxigenin-labeled UTP (Boehringer Mannheim). The RNA probes were subjected to limited alkaline hydrolysis by incubation in an equal volume of 100 mM $NaCO_3$ pH 10.2 for 1 h at 60°C followed by ethanol precipitation. Prehybridization and hybridization were carried out at 55°C for RNA probes and at 48°C for DNA probes, and detection of the probe was as previously described (Tautz and Pfeifle, 1989). After hybridization, eggs or embryos were cleared in 50% glycerol followed by 70% glycerol. For exact staging, nuclei were visualized with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in phosphate-buffered saline (PBS) for 5 min prior to clearing of the embryos. Eggs or embryos were then individually mounted in 70% glycerol and images were captured using a Spot cooled-CCD camera (Diagnostic Instruments, Inc.) mounted on a Zeiss Axioplan microscope. Adobe Photoshop software was used to process the images. For transgenic lines, at least two independently derived lines were analyzed for each construct.

Drosophila RNA extraction and analysis

RNA samples were isolated from staged egg collections by phenol extraction using Trizol (BRL). Equal amounts of total RNA were electrophoresed in 1.2% agarose/formaldehyde/MOPS gels (Sambrook *et al*., 1989), and transferred onto nylon membranes (Amersham). Filters were pre-hybridized for at least 1 h at 63°C in hybridization buffer containing 0.5 M Na-phosphate/7% SDS. ^{32}P-Labeled random primed DNA probes were added and hybridization was performed overnight at the same temperature. Filters were rinsed twice at 60°C in 30 mM Na-phosphate/0.1% SDS, and exposed to Kodak XAR5 film at –80°C with an intensifying screen. Films were also exposed to a Molecular Dynamics phosphor screen, and quantitation was performed using ImageQuant software. *Hsp83*, *string* and *nanos* transcript levels were normalized to *rpA1* mRNA levels to control for variations in loading. The number of repetitions of each experiment was as follows: *Hsp83* endogenous transcripts, fertilized = 3; *Hsp83* endogenous transcripts, unfertilized = 2; *Hsp83* ΔHDE transcripts, fertilized = 2; *Hsp83* ΔHDE transcripts, unfertilized = 1; *string* transcripts, fertilized = 2; *string* transcripts, unfertilized = 2; *nanos* transcripts, fertilized = 1; *nanos* transcripts, unfertilized = 1.

Construction of transgenes for Drosophila germline transformation

Standard protocols were used (Sambrook *et al*., 1989).

Hsp83–lacZ transgenes. The following approach was taken to generate the series of *lacZ–Hsp83* 3′-UTR constructs that include the 5′-*Hsp83* enhancer sequences and promoter (Figure 3B). Previous reports (Kim-Ha *et al*., 1993) suggested that a full-length *lacZ* ORF might not

be completely transcribed *in vivo*. Therefore, we inserted the *Hsp83* 3′-UTR fragments downstream of a truncated *lacZ* tag. We accomplished this by constructing pB83Z, a Bluescript subclone that contains all of the *Hsp83* 5′ upstream region, the first exon, the intron and the first 111 codons of the ORF fused to 603 bp of *lacZ* sequence (Halsell, 1995). At the 3′ end of the *lacZ* sequence are *Aat*II, *Hin*dIII and *Kpn*I cloning sites for inserting the *Hsp83* 3′-UTR fragments. The template for the PCR was Eco9, an 8 kb *Eco*RI fragment subcloned in Bluescript that contains the full-length *Hsp83* transcription unit. 5′ Fragments of the 3′-UTR were PCR amplified and were flanked with *Aat*II and *Hin*dIII restriction sites, while 3′ fragments of the 3′-UTR were flanked with *Hin*dIII and KpnI restriction sites. The position of the *Hin*dIII restriction site within the amplified fragments allowed us to generate nested 5′ deletion constructs, the 1.350hs 3′ deletion construct and a series of internal deletions within the 3′-UTR. 3′-UTR constructs with internal deletions (the 'Δ' series) were generated by first subcloning an *Aat*II–*Hin*dIII 5′ fragment into pB83Z and subsequently adding a *Hin*dIII–*Kpn*I 3′ fragment. After subcloning into pB83Z, a *Not*I–*Kpn*I fragment was isolated and subcloned into *Not*I–*Kpn*I-digested CaSpeR4 (Thummel and Pirrotta, 1991). As a control, the *Hsp70* 3′-UTR was also cloned independently of any *Hsp83* 3′-UTR sequence (Figure 3A, '*Hsp70*'). The *Hsp70* fragment was generated by PCR from a CaSpeR-hs template (Thummel and Pirrotta, 1991).

nanos–Hsp83 hybrid transgenes. (i) *nanos[ΔTCE]*: a genomic 3′ fragment (*Bgl*II–*Eco*RI) containing a *nanos* 3′-UTR lacking the TCE was subcloned into pCaSpeR2 (Thummel and Pirrotta, 1991). Subsequently a genomic 5′ fragment (*Bam*HI) extending from the *nanos cis*-regulatory sequences to the end of the ORF was inserted. (ii) *nanos[ΔTCE + HDE]*: a genomic 3′ fragment (*Bgl*II–*Eco*RI) containing a *nanos* 3′-UTR lacking the TCE was subcloned into pCaSpeR2. The *Hsp83* HDE was PCR amplified incorporating 5′-*Bam*HI and 3′-*Bgl*II sites. This fragment was subcloned upstream of the *nanos* 3′ fragment. Subsequently a genomic 5′ fragment (*Bam*HI) extending from the *nanos cis*-regulatory sequences to the end of the ORF was inserted. (iii) *Hsp83[ΔHDE + TCE]*: the *nanos* TCE was amplified by PCR incorporating *Hin*dIII sites at the ends, and inserted into *lacZ*-tagged pBS *Hsp83[ΔHDE]* (253Δ350) at the *Hin*dIII site.

Drosophila germline transformation

Germline transformation was carried out using standard procedures (Rubin and Spradling, 1982). w^{1118} embryos were co-injected with 500 μg/ml of the *Hsp83–lacZ* construct and 100 μg/ml of pHSπ helper plasmid (Steller and Pirrotta, 1985). All transgenic insertions were homozygosed or balanced over *CyO* or *TM3*.

Drosophila stocks and egg collection

Drosophila melanogaster Oregon-R and w^{1118} stocks are described in Lindsley and Zimm (1992). The following 'class 2' and 'class 3' maternal effect lethal mutants (Schupbach and Wieschaus, 1989) were assayed for transcript degradation in unfertilized eggs: *cell-PK42*, *cell-RH36*, *cortex*, *cribble*, *grauzone*, *luckenhaft*, *syn-Hi10*, *presto*, *scraps*, *valois*. For collection of unfertilized eggs, males of genotype $T(Y;2)bw^{DRev\#11}$, $cn\ bw^{DRev\#11}$ *mr2/SM6a* were crossed to appropriate females (Reed and Orr-Weaver, 1997). Terminal duplication segregants are male sterile (Reed and Orr-Weaver, 1997).

Drosophila embryo cuticle preparations

For cuticle preparations, wild-type and mutant embryos were dechorionated, devitellinized and mounted as described previously (Raz and Shilo, 1993).

RNA analysis in Xenopus

Drosophila Hsp83 full-length 3′-UTR (1.407, see above and Figure 3) was transcribed from a pBluescriptSK subclone using T3 RNA polymerase after linearization at the *Kpn*I site. ΔHDE 3′-UTR (252Δ350, see above and Figure 3) was transcribed from a PCR-amplified fragment that included the T3 RNA polymerase promoter at the 5′ end of the 3′-UTR sequence. Transcription was carried out in the presence of digoxigenin-11-UTP (Kloc *et al*., 1996). For oocyte injections, ovaries were surgically removed from anaesthetized *X.laevis* females, stage 6 oocytes were manually defolliculated in calcium-deficient buffer (1× OR2) and were stored, injected and cultured for 0.5, 3, 6 or 24 h in 1× modified Barth's buffer. For embryo injections, recently fertilized eggs were collected, injected and allowed to develop to stage 12 (gastrula stage, ~24 h). For the time course in stage 6 oocytes (Figure 8A), 500 ng of synthetic labeled RNA was injected into the cytoplasm whereas for comparative analysis of +HDE and ΔHDE 3′-UTRs (Figure 8B), ~100 ng of RNA was injected. Total RNA was prepared from injected oocytes or embryos, separated on a formaldehyde gel and blotted as previously described (Kloc *et al*., 1989). The blot was blocked for 1.5 h in 2% blocking solution (BMB, Genius kit) and was then incubated for 1.5 h with a 1:5000 dilution of anti-digoxigenin–alkaline phosphatase antibody in 2% blocking buffer. Subsequently blots were washed twice for 15 min each wash in G1 buffer and stained in NBT/BCIP solution. All of the preceding used BMB Genius kit reagents and protocols.

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