The Nanos gradient in *Drosophila* embryos is generated by translational regulation

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Abdominal segmentation in the *Drosophila* embryo is governed by a gradient of Nanos (Nos) emanating from the posterior pole. This gradient is derived from translation of the *nos* mRNA that is localized in the pole plasm; in contrast, unlocalized *nos* mRNA is translationally repressed. Here we define the essential signals in the 3' untranslated region (UTR) of *nos* mRNA. Deletion of a 184-nucleotide translational control element (TCE) from the 3' UTR leads to the derepression of *nos* mRNA in the bulk cytoplasm and the development of lethal anterior defects. Furthermore, a minimal mRNA containing essentially only the TCE in its 3' UTR rescues *nos*⁻ embryos to adulthood. The TCE is also sufficient to confer on maternal *torso* mRNA all three aspects of *nos* mRNA regulation: translational repression in the bulk cytoplasm, localization to the pole plasm, and translational activation at the posterior pole. These three phenomena are coupled intimately, as mutations in a pair of CUGGC pentamers within the TCE simultaneously abrogate all three regulatory events. This coupling suggests a model in which the polarized distribution of *nos* protein is generated primarily by translational control and that *nos* mRNA localization is a byproduct of this regulation, at least in part.

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The initial steps in specification of the antero-posterior axis of the *Drosophila* embryo are regulated by graded signals emanating from each pole of the egg (Nüsslein-Volhard et al. 1987; Driever 1993; St. Johnston 1993). These graded signals, Bicoid (Bcd) protein at the anterior and Nos protein at the posterior, are generated by translation of localized mRNA precursors. Thus, genesis of these morphogenetic gradients depends critically on the mechanisms that restrict the subcellular localization and translation of mRNA.

bcd mRNA is tightly localized to the anterior pole of the prospective embryo during oogenesis such that none is detectable elsewhere in the egg (Macdonald and Struhl 1988; St. Johnston et al. 1989). As is the case for many maternal messages, translation of *bcd* mRNA is repressed during oogenesis. Following fertilization, the poly(A) tail of *bcd* mRNA is extended (Sallés et al. 1994), translation commences, and Bcd protein accumulates in a gradient in the anterior of the preblastoderm embryo (Driever and Nüsslein-Volhard 1988a). Although the signals that mediate the localization and translation regulation of *bcd* mRNA have not been defined precisely (but see Macdonald et al. 1993; Ferrandon et al. 1994; Sallés et al. 1994), it is clear that these two regulatory events

can be uncoupled. Genetic analyses have shown that de-localized *bcd* mRNA is translated normally (Driever and Nüsslein-Volhard 1988b) and that *bcd* mRNA that is not derepressed following egg activation remains localized (Lieberfarb et al. 1996).

In contrast, localization and translational regulation are coupled for two mRNA determinants that govern posterior development. Normal abdominal segmentation is governed (in part) by the successive action of Oskar (Osk) and Nos. During the early stages of oogenesis, *osk* mRNA is distributed throughout the cytoplasm of the maturing oocyte; shortly thereafter, a subset of the *osk* mRNA is localized to the posterior pole of the maturing oocyte (Ephrussi et al. 1991; Kim-Ha et al. 1991, 1993). Recent experiments have shown that the unlocalized *osk* mRNA is subject to translational repression that is mediated by sites within its 3' untranslated region (UTR) (Kim-Ha et al. 1995). This repression is relieved at the posterior pole with the result that *osk* protein is found only in the prospective pole plasm (Kim-Ha et al. 1995; Markussen et al. 1995; Rongo et al. 1995).

nos mRNA is localized to the posterior pole of the maturing oocyte during the final stages of oogenesis, after the polarity of the egg chamber has broken down (Theurkauf 1994; Wang et al. 1994). This localization is inefficient; at fertilization, much of the *nos* mRNA is

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distributed throughout the bulk cytoplasm of the egg and the remainder is localized to the specialized pole plasm at the posterior pole at a somewhat higher concentration. Only the *nos* mRNA at the posterior pole is translated (Gavis and Lehmann 1994), giving rise to a transient gradient of Nos protein emanating from the pole. The Nos gradient is thought to initiate abdominal patterning by regulating the translation of *hunchback* (*hb*) mRNA in collaboration with other factors (Wharton and Struhl 1991; Murata and Wharton 1995).

The localization and translational regulation of *nos* mRNA depends both on components of the pole plasm such as *osk* and on signals in its 3' UTR. Study of various hybrid mRNAs has revealed that the *nos* 3' UTR contains signals that can direct localization to the posterior pole or translational repression in the bulk cytoplasm (Gavis and Lehmann 1992, 1994; Gavis et al. 1996). These observations have led to the view that the anchoring of a subset of *nos* mRNA to components of the pole plasm plays a key role in the generation of embryonic polarity. Specifically, the pole plasm has been described as a privileged compartment in which a repressor that otherwise turns off translation of *nos* mRNA (e.g., in the bulk cytoplasm) is inactive (Gavis and Lehmann 1994). According to this view, the polarized distribution of Nos is generated by the localization-dependent translation of *nos* mRNA in the pole plasm. However, critical aspects of this model have not been tested. In particular, the relative contributions of localization and translational regulation in restricting Nos activity to the posterior have not been assessed. In addition, it has not been clear whether the translational repression of unlocalized *nos* mRNA is essential, as is the case for *osk* mRNA (Kim-Ha et al. 1995).

To address these and other issues, we have defined the regulatory signals in the 3' UTR of *nos* mRNA that are required for embryonic development. These studies identify a translational control element (TCE) that is necessary and sufficient (in conjunction with a polyadenylation signal) for *nos* function in the embryo. Mutational analysis reveals that the critical sites within the TCE consist of redundant, tandem CUGGC pentanucleotides. These pentamer-containing signals are sufficient to mediate the three major aspects of *nos* mRNA regulation: repression in the bulk cytoplasm, activation in the pole plasm, and localization to the pole plasm. Taken together, these results support a model in which the Nos gradient is generated primarily by differential translational control and not by mRNA localization.

Results

The 184-nucleotide TCE is necessary and sufficient for nos function

To identify the essential regulatory signals in the *nos* 3' UTR, transgenic flies expressing each of the deletion derivatives shown in Figure 1 were prepared. One of the transgenes, Δ TCE, causes dominant female sterility. All embryos derived from otherwise wild-type females bear-

Figure 1. Essential signals within the *nos* 3' UTR are confined to the 184-nucleotide TCE. Shown schematically (not to scale) are the structures of mRNAs encoded by various *nos* derivatives as well as the results of complementation tests at the right. The restriction enzyme sites at the top indicate deletion endpoints, the squares represent the CUGGC-containing pentamers referred to in the text, and the two mRNAs referred to repeatedly in the text (Δ TCE and Δ BX) are named at the left. The open bar on the sixth line indicates that *nos* sequences downstream of *Xho*I were replaced with sequences encoding part of the 3' UTR and polyadenylation signals of α tub84B (Theurkauf et al. 1986). Each transgene was tested to determine whether it complements the segmentation defects and embryonic lethality of homozygous *nos*^{BN}, *osk*¹⁶⁶, and *vas*^{PD} embryos, as indicated. The asterisk indicates that derepression of unlocalized mRNA also causes embryonic lethality and head defects, as described in Fig. 2.

ing the Δ TCE transgene die with minor head defects. These defects apparently result from the deregulation of *nos* mRNA such that ectopic *nos* activity accumulates in the anterior; evidence supporting this interpretation is discussed below in detail (Fig. 2). An additional indication that the *nos*(Δ TCE) mRNA is deregulated comes from analysis of its activity in mutant embryos. Translation of wild-type *nos* mRNA in the embryo is absolutely dependent on components of the pole plasm, including *osk* and *vasa* (*vas*) (Gavis and Lehmann 1994). However, the *nos*(Δ TCE) mRNA evidently is translated in the absence of either *osk* or *vas* function; homozygous *osk*⁻ or *vas*⁻ embryos, which develop no abdominal segments if they contain only wild-type *nos* mRNA, develop a normal complement of 8 abdominal segments if they contain *nos*(Δ TCE) mRNA (Fig. 1). Thus, the TCE is necessary for the normal regulation of *nos* mRNA.

The results of Figure 1 indicate that, with the exception of the TCE, the remainder of the sequences in the 3' UTR are dispensable for *nos* function in the embryo. As described above, the ability of various deletion-bearing transgenes to complement the abdominal segmentation defects associated with a *nos*⁻ mutation was assayed. In the absence of *nos* function, no abdominal segments develop. As shown in Figure 1, a single copy of each transgene (except Δ BX) restores a full complement of 8 abdominal segments. A single copy of the Δ BX transgene partially rescues abdominal development and two copies do so fully. As is the case for *nos*⁺ mRNA, translation of *nos*(Δ BX) mRNA is completely dependent on both *osk*

Figure 2. The TCE is required for translational repression of unlocalized *nos* mRNA and for embryonic viability. Shown are the distributions of *nos* mRNA (top), *nos* protein (middle) and *hb* protein (bottom) in embryos bearing either *nos*⁺ mRNA (left) or *nos*(Δ TCE) (right) mRNA. For *nos* RNA and protein, early and late cleavage stage embryos are shown, above and below, respectively. For *hb* protein, the younger embryos (above) are in nuclear cycle 10 whereas the older embryos (below) are in late cleavage stage. The *nos*⁺ embryos on the left are *w*¹¹⁸ and thus contain two copies of the wild-type *nos* gene. The *nos*(Δ TCE) embryos are derived *nos*^{BN} females bearing a single copy of the appropriate transgene; as a result, all of the *nos* mRNA and protein in the embryo is derived from the transgene (Wang et al. 1994). In these and all subsequent figures, the anterior of each embryo is to the left and its dorsal surface is at the top. At the bottom are shown phase-contrast micrographs of the heads of *nos*⁺ (g) and *nos*(Δ TCE) (n) embryos. In the latter, note that structures derived from the most anterior regions of the wild-type fate map (e.g., labral tooth, dorsal bridge) are missing. Also note that the head defects are indistinguishable in *nos*(Δ TCE) embryos that are otherwise wild-type, *nos*[−], *osk*[−], or *vas*[−].

and *vas* (Fig. 1). The 3' UTR of the Δ BX mRNA consists only of the TCE, 32 nt flanking the polyadenylation signal, and the poly(A) tail itself. Furthermore, the polyadenylation signal of *nos* apparently contains no special-

ized signals, as it can be substituted by the corresponding sequence from an unregulated mRNA (Fig. 1, line 6; see also Gavis and Lehmann 1994). Thus, the TCE contains all the signals within the 3' UTR required for normal regulation of *nos* mRNA; and sequences outside the TCE are not required for its function in the embryo.

The TCE mediates translational repression of unlocalized nos mRNA

The observation that translation of *nos*(Δ TCE) mRNA is independent of the usual upstream requirements (e.g., *osk* function) suggested that it is deregulated. To investigate this further, standard genetic manipulations were performed to prepare homozygous *nos*^{BN} mutant females that also contain the *nos*(Δ TCE) transgene; in embryos from such females, all the *nos* mRNA and protein is derived from the transgene (Wang et al. 1994).

We first examined the consequences of removing the TCE on the mRNA. At the beginning of embryogenesis, some of the *nos*(Δ TCE) mRNA is localized to the pole plasm whereas the remainder is distributed throughout the bulk cytoplasm, much as is the case for *nos*⁺ mRNA (Fig. 2h). However, in comparison to *nos*⁺ mRNA, a somewhat lower proportion of *nos*(Δ TCE) mRNA appears to be present in the pole plasm, consistent with the idea that the TCE plays a role in normal localization. This is supported by recent results (Gavis et al. 1996) showing that redundant signals for localization exist throughout the *nos* 3' UTR. As development proceeds in wild-type embryos, the translationally inert unlocalized *nos*⁺ mRNA is degraded while the translationally active *nos*⁺ mRNA localized to the pole plasm persists (Fig. 2b). In contrast, unlocalized *nos*(Δ TCE) mRNA persists throughout the embryo with the result that *nos*(Δ TCE) mRNA is detectable throughout the embryo at stages when *nos*⁺ mRNA is present only in the pole plasm (Fig. 2b,i). Based in part on the results described below, we believe the TCE-dependent destabilization of *nos* mRNA to be a consequence of translational repression, as is the case for other maternal mRNAs (e.g., *caudal* and *hb*) (Macdonald and Struhl 1986; Tautz 1988).

Next, we examined the consequences of removing the TCE on translation of the mRNA. As shown in Figure 2k, the unlocalized *nos*(Δ TCE) is translated, and a high level of ectopic Nos accumulates uniformly along the antero-posterior axis. At a similar developmental stage, no Nos is detectable in wild-type embryos except within the pole cells at the posterior (Fig. 2d). Earlier in embryonic development, no ectopic Nos is detectable in *nos*(Δ TCE) embryos; however, the localized *nos*(Δ TCE) mRNA is translated, albeit poorly, giving rise to a weak gradient (Fig. 2c,j). This inefficient translation of localized *nos*(Δ TCE) mRNA suggests that the TCE may also mediate translational activation in the pole plasm; however, we cannot rule out the possibility that TCE-mediated repression is simply relieved in the pole plasm (Fig. 7, see below). The main conclusion derived from these observations is that the TCE is required to prevent the translation of unlocalized *nos* mRNA.

The ectopic Nos in *nos*(Δ TCE) embryos interferes with anterior development by inhibiting the translation of *hb* and *bcd* mRNAs. Previous work has shown that, in wild-type embryos, Nos inhibits the translation of maternal *hb* mRNA in the posterior (Tautz 1988). If expressed at high levels in the anterior of the embryo, Nos blocks the translation of both anterior *hb* and *bcd* mRNAs (Wharton and Struhl 1989; Wharton and Struhl 1991). Accordingly, we examined the distributions of Hb (Fig. 2) and Bcd (data not shown) in *nos*(Δ TCE) embryos. Evidently, the level of ectopic Nos in *nos*(Δ TCE) embryos is sufficient only to partially inhibit translation of *bcd* and *hb* mRNAs. Thus, although Hb is virtually absent in cycle 9–10 *nos*(Δ TCE) embryos (Fig. 2l), the continuous accumulation of *hb* mRNA and protein in subsequent nuclear cycles leads to the formation of an attenuated Hb gradient (Fig. 2m). We believe that the reduced levels of Hb (and Bcd) in such embryos ultimately cause anterior defects and death. In support of this idea, *nos*(Δ TCE) embryos that also bear a Nos-resistant *bcd* mRNA (Wharton and Struhl 1991) survive to adulthood (data not shown).

In summary, we find that: (1) repression of *nos*(Δ TCE) mRNA in the bulk cytoplasm is essential for viability, and (2) the TCE contains signals that are required for the repression of unlocalized mRNA. The data in Figure 2 are also consistent with the idea that the TCE plays a role in the activation of localized mRNA; the experiments described below and in Figure 4 (see below) show this to be the case.

The TCE also mediates the destabilization of *nos* mRNA; however, the following result indicates that this destabilization is a consequence of translational repression. We prepared transgenic flies bearing two different *nos-bcd* hybrid genes (Gavis and Lehmann 1992) in which the protein coding region of *nos* is fused to the RNA-localization signals in the 3' UTR of *bcd* (Macdonald and Struhl 1988). In addition, one of the hybrid mRNAs (*nos-TCE-bcd*) bears the TCE whereas the other (*nos-bcd*) does not. As shown in Figure 3, the *nos-bcd* mRNA is localized to the anterior and efficiently translated, giving rise to a high level of Nos which leads to the development of an extreme bicaudal body plan. In contrast, the *nos-TCE-bcd* hybrid is not detectably translated, and embryos bearing this hybrid develop normal body plans. Significantly, the TCE-bearing hybrid is stable throughout the first several hours of embryogenesis, even though it is not detectably translated. Thus, untranslated *nos-TCE-bcd* mRNA is relatively stable whereas untranslated *nos*⁺ mRNA is not. We conclude that the TCE mediates primarily translational control, and that signals elsewhere in the untranslated mRNA determine its stability.

The TCE mediates osk-dependent activation and localization

As described above and in Figure 1, the minimal Δ BX *nos* mRNA derivative, which contains essentially only the TCE in its 3' UTR, rescues *nos*[−] embryos to fertile

Figure 3. TCE-dependent repression of *nos-bcd* mRNA. (top) Embryonic distributions of the *nos-bcd* (left) and *nos-TCE-bcd* (right) chimeric mRNAs. Note that these chimeric *nos* mRNAs are localized strictly to the anterior pole (Gavis and Lehmann 1992), and that the endogenous *nos*⁺ mRNA, which is present throughout the bulk cytoplasm and concentrated at higher levels at the posterior pole, is also visible in these preparations. Translation of this endogenous *nos*⁺ mRNA at the posterior pole gives rise to the posterior protein gradients evident in (b,e), whereas translation of the *nos-bcd* chimera gives rise to the anterior gradient in (b). The segmentation defects in *nos-bcd* embryos (c) are somewhat greater than those caused by a related transgene (Gavis and Lehmann 1992). We assume this more severely posteriorized phenotype results from the production of higher levels of Nos, much as described by Kim-Ha et al. (1995). Note that a minority of the *nos-TCE-bcd* embryos develop head defects and die, suggesting that translation of this message is not completely repressed.

adulthood. This raised the possibility that the TCE mediates not only the repression of unlocalized mRNA but that it is also sufficient to mediate the localization and translational activation of *nos* mRNA in the pole plasm. To test this idea, we constructed a chimeric *torso* (*tor*) gene that bears sequences encoding the TCE inserted into those encoding the *tor* 3' UTR. Germ-line transformants of the resulting *tor*(TCE) chimeras were generated, and their properties were assayed in the absence of endogenous Tor function as described below.

Tor activity is required at each pole of the embryo for the normal development of the unsegmented terminalia (Casanova and Struhl 1989; Sprenger et al. 1989; Sprenger and Nüsslein-Volhard 1992). Thus, we anticipated that TCE-mediated translational repression might reduce the level of Tor protein below the threshold required for normal development and that, in consequence, *tor*(TCE) embryos would die. Although, a control *tor*⁺ transgene rescues the lethality of embryos derived from *tor*[−] females, the *tor*(TCE) transgene does not. Moreover, examination of the relative levels of RNA and protein in these embryos supports the idea that the TCE mediates translational repression. As shown in Figure 4A, the level of mRNA encoded by two representative *tor*(TCE) transgenes is similar to the level encoded by a *tor*⁺ transgene; however, the level of protein in the *tor*(TCE) embryos is substantially lower. Serial dilution

Figure 4. A *torso*(TCE) chimeric mRNA is regulated in a manner similar to *nos* mRNA. (A) TCE-mediated translational repression. Shown are the results of a Northern blot (above) and a Western blot (below) of embryonic samples derived from *tor^{XRI}* females (lane 1), or samples derived from *tor^{XRI}* females that also bear a *tor⁺* transgene (lane 2) or one of two representative *tor*(TCE) transgenes (lanes 3,4). *tor^{XRI}* is a deletion of the entire RNA-coding region of *tor*. The Northern blot was hybridized with sequences homologous to *tor* (arrow) and, as a control, *nos* (arrowhead). Note that the *tor*(TCE) mRNA, which bears 184 additional nucleotides, migrates more slowly than the *tor⁺* mRNA. The arrow at the bottom indicates the position of mature Tor protein; the protein detected by the antiserum that comigrates with the 120-kD marker (indicated at the right) is present in the *tor^{XRI}* extract, and hence is not derived from *tor*. (B) Differential rescue of the terminalia by *tor*(TCE). Shown are high magnification views of the heads (left) and cuticles secreted by embryos bearing either no *tor* mRNA (from *tor^{XRI}* homozygous females), *tor⁺* mRNA from one copy of a transgene (e.g., *tor^{XRI}/tor^{XRI}*; *tor⁺*), or *tor*(TCE) mRNA from one copy of a transgene. In the phase contrast photographs on the left, note that only the *tor⁺* embryo contains structures of the anterior terminalia: labral tooth (arrow), dorsal bridge (upper arrowhead), ventral process (lower arrowhead). In the dark field photographs on the right, note that both the *tor⁺* and *tor*(TCE) embryos have normal posterior terminalia including a normal eighth abdominal segment, anal pads, and filzkörper (arrow). We also constructed a *tor*(TCE-A) transgene (see Fig. 5) that encodes a mRNA bearing the proximal-half of the TCE. The phenotype of *tor*(TCE-A) embryos is similar to that shown for *tor*(TCE) embryos, except that some exhibit parital rescue of the anterior terminalia and some have deletions of thoracic and abdominal segments. Thus, a single copy of the CUGGC pentamer apparently is sufficient to confer differential rescuing activity on *tor* mRNA. Although we have not investigated the *tor*(TCE-A) phenotype further, our results are consistent with the idea that a single copy of the CUGGC element confers less repression than do two copies. We imagine that *tor* protein accumulates to intermediate levels throughout such embryos, sufficient to partially rescue anterior development but insufficient to completely trap the extracellular ligand. As a result, some of the ligand presumably diffuses toward the middle of the embryo where it ectopically activates Tor, leading to the dominant *tor* (or *splice*) phenotype. A similar phenotype arises in *tor*(NRE) embryos in which translation of the mRNA is inhibited in the posterior (Casanova and Struhl 1993). (C) Tor-dependent transcription of *tailless* (*tll*) is blocked at the anterior, but not at the posterior, of *tor*(TCE) embryos. Shown are the distributions of *tll* mRNA in various embryos as revealed by in situ hybridization. Embryos on the left bear wild-type mRNA from both endogenous *tor⁺* genes, whereas embryos on the right bear *tor*(TCE) mRNA from a single copy of a representative transgene. The embryos in the top row are otherwise wild-type, whereas those in the middle and bottom rows lack *bcd* and *osk* function, respectively. Note that the distribution of anterior *tll* in (d) is not identical to that observed in the complete absence of Tor function (Pignoni et al. 1992), suggesting that translation of *tor*(TCE) mRNA is not completely inhibited. In support of this interpretation, a trace of Tor-dependent *tll* transcription is evident in the absence of *bcd* function at the anterior of the embryo in (e). Note also that the level of posterior *tll* mRNA in (d,e) is somewhat less than that in (a,b). This difference is attributable (at least in part) to a lower level of *tor*(TCE) mRNA in the embryo as compared with wild-type (see 3D). (D) Localization of *tor*(TCE) mRNA to the pole plasm. Shown are the results of in situ hybridization with a probe homologous to *tor*. The wild-type embryo in (a) contains *tor⁺* mRNA from both endogenous *tor⁺* genes, and the embryo in (b) contains *tor*(TCE) mRNA from a single copy of a representative transgene. Both are early cleavage stage embryos. No signal is detectable in control *tor^{XRI}* embryos. The higher magnification views at the right are of the posterior termini of later cleavage stage embryos. Note the preferential stabilization of *tor*(TCE) mRNA in the pole cells.

of the protein and RNA samples suggests that translational repression reduces the level of *tor* protein 10- to 15-fold. Thus, the TCE is sufficient to mediate translational repression in a mRNA bearing no other *nos*-derived sequences.

Examination of the cuticle secreted by *tor*(TCE) embryos suggested that the chimeric mRNA is translation-

ally repressed at the anterior, but not at the posterior. As shown in Figure 3B, *tor*(TCE) embryos exhibit developmental defects of the anterior terminalia that are indistinguishable from those in *tor⁻* embryos. In contrast, the posterior terminalia of *tor*(TCE) embryos are normal. We tested the ability of several *tor*(TCE) and *tor⁺* transgenes to rescue the *tor⁻* phenotype; all of the former differen-

tially rescue the mutant phenotype whereas all of the latter rescue both anterior and posterior defects to the same extent.

To determine whether the differential rescue of *tor*(TCE) embryos is the result of differential translation of the chimeric mRNA at the opposing poles of the embryo, the following experiments were performed. We were unable to visualize the distribution of *tor* protein directly in fixed embryos using the antibody described in Figure 3A. Therefore, expression of the zygotic gap gene *tailless* (*tll*) which is regulated by Tor activity was examined. At the posterior, transcription of *tll* is absolutely dependent on Tor activity, whereas at the anterior, both Bcd and Tor regulate *tll* transcription (Pignoni et al. 1992).

As shown in Figure 4C, insertion of the TCE into *tor* mRNA differentially affects *tll* transcription at the opposing poles of the embryo. At the anterior of *tor*(TCE) embryos, the level of *tll* mRNA is diminished and it is distributed in a broader zone, as compared with the pattern in wild-type embryos. In contrast, *tll* transcription at the posterior is similar in *tor*(TCE) and *tor*⁺ embryos. These results are consistent with the idea that translation of *tor*(TCE) mRNA is repressed at the anterior but not the posterior of the embryo; but because of the confounding contribution of Bcd, the magnitude of this repression is difficult to assess. Accordingly, we examined the transcription of *tll* in *tor*(TCE) embryos bearing no functional Bcd. Whereas *tll* is transcribed in similar domains and to similar levels at both poles in *tor*⁺; *bcd*[−] embryos, we observe a strong reduction of anterior, but not posterior, *tll* transcription in *tor*(TCE); *bcd*[−] embryos (Fig. 3C). Thus, the translational regulation of the *tor*(TCE) and *nos* mRNAs apparently is similar; both are repressed in the bulk cytoplasm and activated at the posterior pole.

We next determined whether the preferential activation of *tor*(TCE) mRNA at the posterior pole is dependent on Osk activity, as is the case for *nos* mRNA (Ephrussi and Lehmann 1992, Smith et al. 1992). In the absence of Osk function, no posterior *tll* transcription is observed in *tor*(TCE) embryos, whereas posterior *tll* transcription under the control of *tor*⁺ is independent of Osk function (Fig. 4C). Thus, translation of *tor*(TCE) mRNA is repressed in the bulk cytoplasm at the anterior and is activated in the pole plasm at the posterior; moreover, the posterior activation is Osk-dependent.

Consistent with the Osk-dependence of Tor activity in *tor*(TCE) embryos, *tor*(TCE) mRNA is concentrated in the pole plasm, much as is *nos*⁺ mRNA (Fig. 3D). In contrast, *tor*⁺ mRNA is distributed uniformly throughout the bulk cytoplasm (Sprenger et al. 1989). The *tor*(TCE) mRNA at the posterior pole appears to be specifically associated with the pole plasm, as it is preferentially stabilized and incorporated into the pole cells; no such preferential accumulation of *tor*⁺ mRNA into the pole cells is observed (Fig. 3D).

In summary, inclusion of the TCE in *tor* mRNA subjects it to all three major aspects of *nos* mRNA regulation: translational repression in the bulk cytoplasm,

Osk-dependent relief of repression (or activation) at the posterior, and localization to the pole plasm.

Tandem, redundant CUGGC pentamers mediate all three activities of the TCE

The evidence described above demonstrates that the 184-nucleotide TCE governs translational repression, translational activation, and localization. To understand the mechanisms underlying these events, we chose to define more precisely the essential sites within the TCE. Two derivatives, *nos*(ΔTCE-A) and *nos*(ΔTCE-B), were first constructed; these encode otherwise wild-type mRNAs lacking the proximal and distal half, respectively, of the TCE. Several lines of evidence support the idea that either half of the TCE is sufficient to repress the translation of unlocalized *nos* mRNA. First, embryos bearing either *nos*(ΔTCE-A) or *nos*(ΔTCE-B) mRNA are viable and exhibit none of the head defects associated with *nos*(ΔTCE). Second, no ectopic Nos is detectable in such embryos, and the distribution of Hb is essentially indistinguishable from that in wild-type embryos (data not shown). Third, biologically significant levels of ectopic Nos activity do not accumulate in *osk*[−] embryos bearing these derivatives, by the criterion that no abdominal segments develop (Fig. 5A). In contrast, the ectopic Nos activity arising from translation of unlocalized *nos*(ΔTCE) mRNA is sufficient to support development of a normal complement of 8 abdominal segments (Fig. 5A). Thus, the TCE apparently contains at least two redundant signals that regulate translational repression.

To further define signals within each half-TCE, we constructed five linker-scanning mutations in the region encoding TCE-A and four such mutations in the region encoding TCE-B (Fig. 5B). Sequences encoding each mutant half-TCE were substituted for those encoding the wild-type TCE, and transgenic flies were prepared. As deletion of the entire TCE causes abnormal head development and embryonic lethality (Fig. 2), the activity of each TCE mutant was assayed simply by scoring these phenotypes in embryos from the appropriate transgenic females. As summarized in Figure 5B, of the nine mutations tested, only one in TCE-A (TCE2) and two in TCE-B (TCE7 and 8) disrupt repression activity. Thus, discrete sites, one in the center of each half-TCE, appear to mediate translational repression.

Examination of the sequence in the centers of TCE-A and TCE-B reveals that each can fold into a hairpin bearing CUGGC in the loop (Fig. 5C). To test whether this pentamer is a key element in the repression signal, we constructed otherwise wild-type *nos* derivatives in which the sequences encoding the CUGGC pentamer in either half-TCE are substituted with sequences encoding UGCG; this sequence was chosen because it folds into a stable tetraloop in the hairpins of other RNA molecules (Woese et al. 1990), and therefore might be expected to minimally disrupt the structure of each TCE hairpin. When assayed in vivo as described above, the tetraloop mutations abolish the activity of TCE-A and TCE-B, respectively; moreover, the developmental defects in em-

Figure 5. Tandem, redundant signals in the TCE mediate its repression activity. (A) Each half of the TCE is sufficient to repress the translation of unlocalized *nos* mRNA. Shown are dark field micrographs of the cuticles secreted by *osk*[−] embryos that also bear the *nos* mRNA indicated in each panel. *osk*-independent rescue of abdominal segmentation constitutes a sensitive bioassay for derepression of the unlocalized *nos* mRNA. Note that the *nos*(ΔTCE) embryos (and none of the others) also have head defects not visible in the photograph. Abdominal segmentation in *nos*(ΔTCE) embryos presumably is governed by a *bcd*-generated Hb gradient, with the ubiquitous Nos serving to block the accumulation of excess Hb in the posterior. The mechanisms underlying such redundant specification of abdominal segmentation are discussed elsewhere (Hülskamp et al. 1990; Struhl et al. 1992; Wharton and Struhl 1991). (B) Sequences of TCE-A and TCE-B as well as the structures of the linker-scanning mutations referred to in the text. In each case, the sequence within the bracket is substituted by the eight bases encoded by an *Eco*RI linker. The mutations indicated above each line abolish repression activity of the relevant half-TCE by the criterion that at least three independently derived transgenes cause dominant female sterility and head defects similar to those shown in Fig. 2 for *nos*(ΔTCE). In contrast, mutations below each line have no effect on TCE function, by the same criterion. The conserved CUGGC pentamer is highlighted. (C) Critical portion of each half-TCE that can fold into a hairpin with the CUGGC pentamer comprising the loop. These hypothetical structures were identified by inspection, with guidance from the foldrna program of the GCG sequence analysis package (University of Wisconsin). Intriguingly, two copies of the CUGGC pentamer are found in the 3' UTR of the maternal CyclinB mRNA that, like *nos* mRNA, is localized to the pole plasm and subject to translational regulation (Dalby and Glover 1993). Perhaps the germ-line cells use a common pathway to regulate translation of the *nos* and CycB mRNAs.

bryos bearing a *nos* mRNA derivative containing both tetraloop mutations, *nos*(tetra²), are essentially indistinguishable from those in embryos bearing *nos*(ΔTCE) mRNA (data not shown). Thus, although we have no direct evidence for the existence of these hairpins, the CUGGC pentamers appear to be essential components of the redundant signals that mediate translational repression in *nos* mRNA.

We next wished to determine whether the other activities mediated by the TCE—translational activation and localization—are affected by the tetraloop mutations. To this end, we constructed a derivative of *nos*(ΔBX), which encodes a minimal mRNA lacking the nonessential portion of the 3' UTR (Fig. 1). (As described above, the dispensable portion of the 3' UTR contains redundant signals for activation and localization which would otherwise interfere with the experiment.) This derivative, named *nos*(ΔBX, tetra²), contains mutations in the se-

quences encoding both of the conserved CUGGC pentamers. The properties of these mRNAs were then examined following the preparation of transgenic flies, as described above.

Surprisingly, the tetraloop mutations also eliminate TCE-mediated translational activation and localization. As shown in Figure 6a, *nos*(ΔBX) mRNA is localized in a manner essentially indistinguishable from wild-type *nos* mRNA and is weakly translated at the posterior pole, giving rise to a very low level of protein which is barely detectable in the immediate vicinity of the posterior pole (Fig. 6b,c). In contrast, the *nos*(ΔBX, tetra²) mRNA is uniformly distributed throughout the cytoplasm from the beginning of embryogenesis (Fig. 6e). Moreover, we never detect an asymmetric distribution of Nos protein arising from translation of this mRNA; instead, Nos accumulates uniformly throughout the embryo (Fig. 6f,g). Apparently either the graded Nos in *nos*(ΔBX) embryos

Figure 6. The CUGGC-containing signals of the TCE mediate all three of its activities. As indicated at the top, embryos on the left are derived from *nos*^{BN} mutant females bearing a *nos*(Δ BX) transgene and embryos on the right are derived from *nos*^{BN} females bearing a *nos*(Δ BX, *tetra*²) transgene. Thus, all of the *nos* mRNA and protein in these embryos is derived from the transgene. The first row shows the distribution of *nos* mRNA in early cleavage stage embryos. The second and third rows show the distribution of Nos protein in early and late cleavage stages, respectively. The fourth row shows representative cuticles secreted by these embryos. Interestingly, the *nos*(Δ BX, *tetra*²);*nos*^{BN}/*nos*^{BN} embryos do not have head defects and develop into fertile adults. Apparently, the level of ectopic Nos in *nos*(Δ BX, *tetra*²) embryos is lower than that in *nos*(Δ TCE) embryos (Fig. 2); this lower level evidently is sufficient to allow abdominal segmentation but insufficient to interfere with anterior development. Note that the extremely low level of protein in (b) is difficult to visualize, and that high concentrations of antibody and long reaction times were used in these experiments. As a result, the level of protein cannot be compared to that in embryos of Figures 2 or 3.

or the ubiquitous Nos in *nos*(Δ BX, *tetra*²) embryos is sufficient to support abdominal segmentation (Fig. 6d,h). In summary, we conclude that all three regulatory events governed by the TCE—translational repression, activation, and localization—are mediated by the same CUGGC-containing elements.

Discussion

The tethering of *nos* mRNA to some component of the pole plasm generally has been considered to play a pre-eminent role in restricting Nos activity to the posterior of the embryo. In this report, we show that three apparently distinct regulatory events—repression, activation, and localization—are intimately coupled. Within the essential portion of the *nos* 3' UTR, each is mediated by a pair of small, redundant signals. These observations support a model in which the Nos gradient is generated primarily by translational control and in which localization

of *nos* mRNA is a consequence of this translational regulation rather than the converse.

A model for regulatory events at the *nos* TCE is presented in Figure 7. In the bulk cytoplasm, we suggest that a repressor binds to the tandem CUGGC pentamers of the TCE and thereby turns off translation of the mRNA. As development proceeds, this translationally inert mRNA is degraded. In the pole plasm, the repressor is inactivated by a localized antagonist. This antagonist may act directly by binding to the repressor (either on or off the RNA); alternatively, it may bind directly to the same pentamer-containing signals and displace the repressor. In either case, Nos protein accumulates in the posterior and the translationally active mRNA is protected from degradation. In this model, the mRNA is localized not because it is tethered to an anchor in the pole plasm, but because it is translationally activated and thereby spared from degradation. Maternal Hsp83 mRNA is thought to be localized to the posterior of the embryo by a similar mechanism: preferential degradation of mRNA in the bulk cytoplasm (Ding et al. 1993).

Critical aspects of the model described above are unt-

Figure 7. A model for TCE-mediated regulation of *nos* mRNA. The main features of the model are discussed in the text. In the bulk cytoplasm at the left, an unknown repressor (○) binds to the CUGGC-containing signals in the TCE (■) and shuts off translation. The dashed lines indicate degradation of the RNA. In the pole plasm, an unknown activator antagonizes the activity of the repressor, and *nos* mRNA is translated by ribosomes (diagonal hatching) as a result. This leads to the stabilization of the mRNA and the accumulation of *nos* protein (shaded ellipsoids). The mechanism of activator action is unclear, and three possibilities are indicated. At the top, activation is simply relief of repression, with the liberated mRNA translated by default. In the next two scenarios, the activator simultaneously antagonizes repressor function and stimulates translation above a basal level. These latter scenarios are weakly supported by the evidence in Fig. 2.

ested. In particular, the model assumes that translational repression is coupled to degradation of the mRNA. Consistent with this idea, we find that *nos*(Δ BX) mRNA bearing an early amber codon is undetectable, presumably because the untranslated mRNA is unstable and rapidly degraded; in contrast, *nos*(Δ BX) mRNA bearing an amber codon in the middle of the protein coding region is stable and localized normally (data not shown). Thus, premature translation termination apparently destabilizes *nos*(Δ BX) mRNA, much as has been observed in other cases (for review, see Theodorakis and Cleveland 1996). Perhaps TCE-mediated repression in the bulk cytoplasm activates the same degradation pathway, thereby contributing to the localization of the mRNA in the pole plasm.

Further tests of the regulatory model described above await the identification of the repressor and its putative antagonist, which at present are unknown. One candidate factor has been identified by Smibert and colleagues (pers. comm.), who detected in embryonic extracts a 135-kD protein that binds specifically to the TCE. Their results, together with those presented here, are consistent with the model presented in Figure 7. However, alternative models cannot be ruled out until the 135-kD protein, or other *trans*-acting factors, have been identified.

Given that the TCE appears to contain all the signals required for the generation of a normal pattern of Nos protein in the embryo, what then is the role of the remainder of the 3' UTR? Gavis et al. (1996) recently have mapped RNA localization elements throughout much of the 3' UTR, including regions that are not required for Nos function (Fig. 1). Consistent with their observations, we find that *nos*(Δ TCE) is localized (albeit somewhat less efficiently than is *nos*⁺ mRNA). Thus, the remainder of the 3' UTR apparently contains redundant signals for the activation and localization of the mRNA. The major difference between the properties of the minimal *nos*(Δ BX) mRNA, which contains essentially only the TCE in its 3' UTR, and *nos*⁺ mRNA is the amount of protein produced per transcript; thus, the remainder of the 3' UTR may act simply as a nonspecific translational enhancer. Whatever their role, the signals elsewhere in the 3' UTR are subordinate to TCE-mediated repression and are not required for abdominal patterning.

For both *nos* and *osk* mRNAs, localization and translational regulation are coupled. In this report, we show that the signals that mediate repression of unlocalized *nos* mRNA are inseparable from signals that mediate its localization and relief of repression at the posterior pole. In contrast, the translational control and localization signals in *osk* mRNA are separable (Kim Ha et al. 1995). *nos* mRNA is localized later in oogenesis than is *osk* mRNA, after the antero-posterior polarity of the cytoskeleton within the egg chamber is thought to have broken down (Theurkauf 1994). Thus, it is perhaps not surprising that the mechanisms underlying regulation of these two mRNAs are somewhat different.

The importance of TCE-mediated repression is underscored by the observation that the attenuated Nos gradient generated from translation of the minimal *nos*(Δ BX)

mRNA is sufficient to allow abdominal segmentation (Fig. 6). Apparently, wild-type embryos contain a large excess of Nos beyond the amount necessary to repress translation of maternal *hb* mRNA. Taken together, these results suggest that Nos is a potent translational inhibitor. Further, they suggest that TCE-mediated repression (and the consequent degradation of *nos* mRNA in the bulk cytoplasm) is extremely efficient at preventing the accumulation of ectopic Nos.

A large body of evidence supports the idea that *bcd* mRNA is the critical localized determinant for anterior embryonic patterning. By analogy, others have argued that *nos* mRNA is the corresponding localized determinant for posterior patterning (Gavis and Lehmann 1992; Wang et al. 1994) and that RNA localization is the primary event in establishing asymmetry (Gavis et al. 1996). The results described in this report suggest that translational regulation may play the major role in generating a restricted distribution of Nos. According to this idea, the activator of *nos* mRNA translation that is sequestered in the pole plasm, and not *nos* mRNA itself, is the key localized determinant.

Materials and methods

Strains, reagents, and techniques

The following mutant alleles were used: *nos*^{BN}, *tor*^{XR1}, *bcd*^{E1}, *vas*^{PD}, and *osk*¹⁶⁶. Wild-type flies were either Oregon R or *w*¹¹⁸. Rat anti-Nos sera were described by Murata and Wharton (1995). The rabbit anti-Tor serum was a gift from F. Sprenger (University of California at San Francisco). Whole mount in situ hybridization and immunohistochemical detection of proteins were performed by standard methods. Northern blot analysis was performed using samples of total embryonic RNA; quantification was carried out using a PhosphorImager and Imagequant software (Molecular Dynamics). Total protein extracts (Sprenger and Nüsslein-Volhard 1992) were subjected to Western analysis using the ECL Detection system (Amersham).

Construction of nos and tor derivatives

The *nos*⁺ DNA used was a 5.7-kb *EcoRI*–*DraIII* genomic fragment that rescues the maternal *nos* mutant phenotype. A unique *Bam*HI site was engineered using PCR immediately downstream of the stop codon. The TCE is encoded by a 184-nucleotide *Bam*HI–*Bgl*III fragment derived from this gene. TCE-A and TCE-B are the proximal and distal halves, respectively, obtained on cleavage of the TCE coding sequences with *Hpa*I. The *EcoRI*–*Bam*HI fragment from the *nos* gene described above was fused to the *Mlu*I–*Xba*I fragment from the *bcd* 3' UTR that contains the RNA localization signals (Macdonald and Struhl 1988). *nos*–TCE–*bcd* was created by the insertion of TCE coding sequences in the *Bam*HI site present at the junction in the *nos*–*bcd* gene. The *nos*(Δ TCE) was generated by deleting the 184-nucleotide *Bam*HI–*Bgl*III fragment from the *nos*⁺ gene described above. The *tor*⁺ DNA was an 11.5-kb *EcoRI* fragment that complements *tor*[–] maternal effect lethality. Two versions of *tor*(TCE) that exhibited similar properties were created. In one case, TCE coding sequences were inserted into the unique *Spe*I site of p2599 (Wharton and Struhl 1991). In the other, TCE coding sequences were inserted at an *Asp*718 site that was first generated using PCR immediately downstream of the Tor stop codon. For the generation of TCE1–9, linker scanning muta-

tions were generated using *ExoIII* and Mung Bean Nuclease essentially as described by (Henikoff 1987), with deleted sequences substituted by an 8-nucleotide *EcoRI* linker (GGAAT-TCC). All of these genes were cloned into derivatives of pCaSpeR and transgenic flies were generated by P element-mediated transformation by standard methods. With a few exceptions, the phenotypes associated with at least three independent lines were examined.

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The Nanos gradient in *Drosophila* embryos is generated by translational regulation.

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