oskar RNA plays multiple noncoding roles to support oogenesis and maintain integrity of the germline/soma distinction

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ABSTRACT
The Drosophila oskar (osk) mRNA is unusual in that it has both coding and noncoding functions. As an mRNA, osk encodes a protein required for embryonic patterning and germ cell formation. Independent of that function, the absence of osk mRNA disrupts formation of the karyosome and blocks progression through oogenesis. Here we show that loss of osk mRNA also affects the distribution of regulatory proteins, relaxing their association with large RNPs within the germline, and allowing them to accumulate in the somatic follicle cells. This and other noncoding functions of the osk mRNA are mediated by multiple sequence elements with distinct roles. One role, provided by numerous binding sites in two distinct regions of the osk 3′ UTR, is to sequester the translational regulator Bruno (Bru), which itself controls translation of osk mRNA. This defines a novel regulatory circuit, with Bru restricting the activity of osk, and osk in turn restricting the activity of Bru. Other functional elements, which do not bind Bru and are positioned close to the 3′ end of the RNA, act in the oocyte and are essential. Despite the different roles played by the different types of elements contributing to RNA function, mutation of any leads to accumulation of the germline regulatory factors in the follicle cells.

Keywords: lncRNA function; oskar; protein sequestration; Bruno

INTRODUCTION
The fundamental role of an mRNA is to serve as a template for translation, and thus encode a protein. To ensure that this protein is produced at the desired level and in the appropriate location, the mRNA may also contain a variety of regulatory signals. These are often located within the non-coding parts of the mRNA, the 5′ and 3′ untranslated regions (UTRs). In particular, the 3′ UTR is a common position for signals that control mRNA translation, stability, localization, and length of the poly(A) tail.

Additional RNAs perform noncoding functions. Some of these RNAs were recognized early, notably the ribosomal and transfer RNAs that work in conjunction with mRNAs during translation. Other noncoding RNAs perform myriad functions, with the breadth and diversity of this group only becoming recognized in recent years. Some of the noncoding RNAs share substantial similarities in structure and function. Examples include certain types of small regulatory RNAs, such as microRNAs (miRNAs) which influence stability and translation of bound mRNAs (Bartel 2009). But other noncoding RNAs defy classification, with many grouped solely by size into the category of long noncoding RNAs (lncRNAs). Some lncRNAs act in cis, influencing transcription close to their site of synthesis. Others act in trans, by a variety of mechanisms including, but not limited to, control of transcription. An lncRNA can act as a scaffold for assembly of multiprotein complexes, and it can act as a decoy to bind and inhibit the action of a protein. Interactions with other RNAs are also possible via base-pairing. Identification of lncRNAs has far outpaced characterization of their roles and how they function, and the range of mechanisms can only expand. Given their large size, there is no reason to assume that all lncRNAs will be constrained to perform a single function (for review, see Cech and Steitz 2014).
The Drosophila oskar (osk) mRNA is unusual, even by these standards, in that it performs both coding and noncoding functions. osk mRNA is expressed at high levels during oogenesis, where it appears only in the germline cells of the ovary, and this maternal mRNA is contributed to the embryo, where it is rapidly degraded at the midblastula transition (Ephrussi et al. 1991; Kim-Ha et al. 1991). A role for osk in axial patterning of the embryo was established with classical mutants affecting the Osk protein. Protein null alleles cause a strong maternal-effect phenotype: There are no obvious defects during oogenesis but all of the resulting embryos fail to develop abdominal segments (Lehmann and Nüsslein-Volhard 1986; Kim-Ha et al. 1991). More recently, new osk alleles were identified in which insertion of a transposon largely abolishes production of osk mRNA (Jenny et al. 2006). Surprisingly, these osk RNA null mutants revealed a further phenotype: Progression through oogenesis was blocked. In wild-type ovaries osk mRNA is present continually from the very earliest stages, but its translation is repressed until the later stages. Because Osk protein is not required earlier in oogenesis, this suggested that the osk mRNA had a separate noncoding role. Indeed, dissection of the osk mRNA revealed that the RNA activity resides in the 3′ UTR (Jenny et al. 2006).

Here we show that there are multiple components to osk RNA function. One role is to sequester the translational regulator Bruno (Bru), an interaction that defines a novel regulatory circuit. In addition, sequences clustered near the 3′ end of the osk 3′ UTR are critical for osk RNA function and appear to comprise multiple different elements. This organization would be consistent with a scaffolding function, with the different elements serving to bind factors. One surprising consequence of the absence of osk mRNA is enhanced accumulation in the somatic follicle cells of proteins thought to be restricted to the germline, a phenomenon that may contribute to the arrest of oogenesis.

RESULTS

Bru binding sites are required for osk RNA function

BREs, one class of binding site for Bru, are clustered in two separate regions of the osk mRNA 3′ UTR, the AB and C regions (Fig. 1A). BREs mediate translational regulation, and mutation of all of the BREs results in precocious expression of Osk protein and disruption of embryonic patterning. This phenotype was first observed when testing an osk ABC BRE− transgene in an osk protein null background (Kim-Ha et al. 1995). More recently, identification of osk RNA null mutants (Jenny et al. 2006) allowed the same transgene to be tested as the only source of osk mRNA (Reveal et al. 2010). In addition to the embryonic patterning defects, a second phenotype was observed: Mutation of all BREs substantially reduced the rate of egg laying (Fig. 1B). Reduced egg laying is consistent with partial disruption of the osk noncoding RNA function, such that progression through oogenesis is impaired but not abolished. Examination of ovaries supported this interpretation: Many ovarioles had egg chambers that arrested development (Supplemental Fig. 1). To explore this phenomenon we compared rates of egg laying for females lacking endogenous osk mRNA (oskA87/Df(3R)osk) and expressing transgenic osk mRNAs with all BREs intact (osk+), with AB or C region BREs mutated (osk AB BRE− and osk C BRE−, respectively), or with all BREs mutated (osk ABC BRE−). Quantitative assays showed that mutation of any set of BREs reduced egg laying, with loss of all BREs having the strongest effect (Fig. 1B). The different activities were not due to different levels of osk mRNA, as the various mRNAs were present at similar levels (Fig. 1C).

To further confirm that provision of Bru binding sites constitutes at least part of osk RNA function, we asked if impairment of osk RNA function caused by loss of Bru binding sites in the osk mRNA could be offset by providing Bru binding sites in another mRNA (Fig. 1D). The UAS-GFP-4xBRE transgene has four copies of an isolated 12 bp BRE-type binding site, but no other osk mRNA sequences, and the GFP-4xBRE mRNA is translationally repressed in ovaries (Reveal et al. 2011). Germline expression of this mRNA with a GAL4 driver resulted in a substantial increase in the rate of egg laying relative to osk ABC BRE− alone. In contrast, expression of a control UAS-GFP transgene lacking Bru binding sites did not increase egg laying (Fig. 1D).

osk mRNA acts to sequester Bru

Two models have been proposed for the noncoding function of osk mRNA (Jenny et al. 2006). In one model, the osk mRNA (and more specifically the osk mRNA 3′ UTR, which is both necessary and sufficient for this function [Jenny et al. 2006]) serves as a scaffold for assembly of an RNP particle that in some manner facilitates progression through oogenesis. In the second model, the osk mRNA 3′-UTR sequesters a regulatory factor that would otherwise inhibit oogenesis. Our data clearly demonstrate a role for Bru binding sites in the function of osk mRNA. In the context of the two models, Bru would either be a component of the assembled RNP particle, or be sequestered and thus limited in activity. For the first model a reduction in the level of Bru would be expected to interfere with RNP assembly, potentially enhancing the osk mRNA null phenotype. In contrast, for the second model a reduction in the level of Bru would serve the same purpose as sequestration of Bru, and should suppress the osk RNA null phenotype.

To test these predictions we adopted two approaches. The first was to reduce the level of Bru activity in ovaries lacking osk mRNA; this approach could reveal a suppression of the RNA null phenotype. In females lacking osk mRNA and heterozygous for either of two aret alleles (aret encodes Bru [Webster et al. 1997]) oogenesis was still arrested, but often progressed further as judged by elongation of the egg chambers (Fig. 1E). The degree of rescue corresponded to the
FIGURE 1. Bru binding sites are required for osk RNA function. (A) Schematic of the osk 3′ UTR indicating the locations of Bru binding sites (BREs, type II and type III). The sites are clustered in the AB and C regions. (B) Rescue of the osk RNA null egg laying defect by osk transgenes. Rates of egg laying (see Materials and Methods) for females lacking endogenous osk mRNA [osk<sup>−/−</sup>/Df(3R)osk] but carrying a single copy of an osk transgene, as indicated. The rate obtained with a single copy of the osk<sup>+</sup> transgene was set at 100%. (C) Transcript levels for osk transgenes. The rp49 mRNA was used as a control to ensure that similar amounts of ovarian RNA were used for each genotype. (D) Addition of Bru binding sites rescues the egg laying defect of osk ABC BRE<sup>−</sup>. In all cases females lack endogenous osk mRNA and carry the nosGAL4::VP16 driver and a single copy of the osk ABC BRE<sup>−</sup> transgene, which provides only partial rescue of egg laying. A UAS-GFP transgene or UAS-GFP-4xBRE transgene was also present, as indicated. (E) Reducing Bru activity partially suppresses the oogenesis progression defect of osk RNA null females. Median lengths of developmentally arrested egg chambers were measured for the genotypes shown (n for osk<sup>−/−</sup>/Df(3R)pXT103, 16; with aret<sup>−/−</sup>, 37; with aret<sup>−/−</sup>, 36). P values derived from the Kolmogorov–Smirnov Test: (*) P < 0.05; (**) P < 0.01. (F) Reducing Bru activity rescues the egg laying defect of osk ABC BRE<sup>−</sup>. Rates of egg laying for females lacking endogenous osk mRNA [osk<sup>−/−</sup>/Df(3R)osk] but carrying an osk<sup>+</sup> or osk ABC BRE<sup>−</sup> transgene were determined, testing the consequences of reducing Bru activity by heterozygosity for aret<sup>−/−</sup>. (G–I) Ovarioles from wild type (G), osk<sup>−/−</sup>/Df(3R)osk (H), or osk<sup>−/−</sup>/Df(3R)osk expressing the osk C all<sup>−</sup> transgene (I). The ovarioles were stained with ToPro for DNA (red) and anti-Hts for Adducin-like (green). (J) UV crosslinking assay of Bru in ovarian extract binding to osk RNA probes. The AB and C regions contain the Bru binding sites, and are as previously defined (Kim-Ha et al. 1995). Deletion of part of the C region enhances Bru binding, perhaps by altering secondary structure that would otherwise limit accessibility. Even with the enhanced binding, the C region binds substantially less Bru than does the AB region. Similar amounts of each probe were used. All lanes are from the same autoradiogram of a single experiment and gel, with irrelevant lanes removed.
severity of the aret allele: aretPA, a missense mutant which retains some activity, had a weaker suppressive effect, while aret201, a stronger nonsense mutant more strongly suppressed the osk RNA null phenotype.

In a second approach, we used females expressing the osk ABC BRE− mRNA, such that partial osk RNA function is provided and either enhancement or suppression of the phenotype is possible. Mutating one copy of aret in this background substantially suppressed the defect in egg laying (Fig. 1F). The results of both approaches support the model (see Discussion) that the noncoding function of osk mRNA is, at least in part, to sequester Bru.

A second component to osk RNA function

Mutation of the BREs reduces but does not abolish osk RNA function. The residual osk RNA activity could be due to residual Bru binding to the osk mRNA, or osk mRNA could perform a function in addition to sequestration of Bru, or both. The BREs are not the only type of Bru binding site, and other classes of binding sites have been identified. The type II and type III sites are, like the BREs, clustered in the AB and C regions of the osk mRNA 3′-UTR (Reveal et al. 2010). Mutation of all AB region sites (the BREs and the single type II site) in osk AB all− caused a moderate disruption of osk RNA function, similar to that for osk AB BRE− (Fig. 1B). In striking contrast, mutation of all C region sites (the BREs, the two type II sites, and the single type III site) in osk C all− eliminated osk RNA function: No eggs were laid (Fig. 1B) and oogenesis was arrested just as in the absence of osk mRNA (Fig. 1H,I). Similarly, the osk ABC all− mRNA (with both AB and C sites mutated), also lacked osk RNA function (Fig. 1B). The dramatic loss of osk RNA function from mutating the C region Bru binding sites was not due to loss of the type III site (found only in the C region), as mutation of this site alone did not affect egg laying (Reveal et al. 2010 and below). Likewise, mutation of the two type II sites in the C region did not interfere with egg laying (Reveal et al. 2010 and below).

That the osk ABC all− mutant was more defective in osk RNA function than the osk ABC BRE− mutant was not surprising, since the all− mutant should be less able to sequester Bru. However, the differences in osk RNA activity of the osk AB all− and osk C all− mutants was unexpected: The AB region binds Bru more strongly than does the C region (Fig. 1J; Kim-Ha et al. 1995), and loss of the AB binding sites should therefore lead to a stronger phenotype if the sole noncoding function of osk mRNA is to sequester Bru. This inconsistency between the strength of Bru binding and strength of osk RNA function suggests that the C all− mutations have consequences beyond reduction of Bru binding. Specifically, they appear to disrupt an additional noncoding function of the osk mRNA. Presumably, this other function is mediated by sequences overlapping with, or close to, the C region Bru binding sites.

A complementary mapping approach also revealed the importance of the osk 3′ region. Portions of the osk 3′ UTR were appended to an egfp mRNA and expressed under UAS/GAL4 control in the germline of osk RNA null ovaries, and assayed for rescue of progression through oogenesis and the ability to lay eggs (Fig. 2A). Because in this UAS-egfp-osk assay the degree of rescue cannot be directly compared with that obtained using genomic osk transgenes (UAS/GAL4 expression does not fully restore egg laying rates to wild type, even with the complete osk 3′ UTR [Jenny et al. 2006; Vazquez-Pianzola et al. 1998]).

FIGURE 2. Mapping regions of the osk 3′ UTR that contribute to osk RNA function. (A) The osk 3′ UTR is shown in schematic form, with regions included in transgenes shown as horizontal gray bars. Defined Bru binding sites are indicated by black rectangles and the OES (the signal that mediates transport of the mRNA to the oocyte) is indicated by a red bar. The K10-TLS, the oocyte entry signal of fs(1)K10, is indicated by a blue bar. The osk sequences were incorporated into UAS-egfp transgenes, except for K10-TLS 119 of 3b, which lacks gfp (Materials and Methods). Each transgene was tested in the osk−/Df(3R)pXT103 background with the pCgo-Gal4:VP16 (Rorth 1998) and nanos-Gal4:VP16 (Van Doren et al. 1998) drivers. Results of the assays are indicated at right. RNA enriched: +, strong enrichment of the RNA in the oocyte; −, no enrichment in the oocyte. For the complementation test the distributions of the two mRNAs were not monitored directly (n.a.), but can be inferred from tests with the individual mRNAs. RNA null rescue: +, eggs laid; −, no eggs laid. The presence of eggs was scored, not the frequency of egg laying. (B) Representative of in situ hybridizations against the GFP portion of the transgenic construct. Transgenic RNA signal in red and DNA in blue (scale bar, 30 µm).
Precise mapping of osk RNA function elements

Both of the approaches described above highlight the importance of osk RNA function of a 3′ part of the osk 3′ UTR, which acts together with sequences elsewhere in the 3′ UTR (e.g., the AB region BREs) to provide full osk RNA function. To more precisely map functional elements near the osk mRNA 3′ end, a scanning mutagenesis of this region was performed (Fig. 3). Fifteen sets of mutations, most having five contiguous nucleotides altered, were each introduced into a genomic osk transgene. The mutants were named osk3′x−y, where x and y refer to the first and last positions of mutations within the osk 3′ UTR.

In this assay, the use of a genomic osk transgene provides, with a wild-type version of osk, full osk function: All defects associated with the absence of osk mRNA are rescued, and the rate of egg laying is restored to that obtained with a wild-type, endogenous osk allele (Fig. 1B and below). This assay is therefore well suited to determine how strongly a mutation interferes with osk RNA function. Unlike the UAS-gfp-osk assay (Fig. 2), the genomic transgenes can make Osk protein. However, this does not interfere with or influence the rescue assays, as either absence or inappropriate expression of Osk protein has no effect on progression through oogenesis (Lehmann and Nüsslein-Volhard 1986; Vanzo and Ephrussi 2002): The osk RNA and Osk protein functions appear to be completely independent.

As an initial test of the scanning mutants for osk RNA function, the egg laying assay was used (Fig. 3A), with a single copy of a mutant transgene in the osknull background. Most of the mutants support osk RNA function: Eggs were laid at a rate similar to that found with a wild-type osk transgene, indicating that progression through oogenesis was efficient. However, mutation of sequences close to the 3′ end of osk mRNA interfered with osk RNA function: Mutants osk3′977-981 and osk3′984-988 failed to lay any eggs, and mutants osk3′990-994 and osk3′1004-1008 had greatly reduced egg laying.

The mutations from the scanning mutagenesis that interfered with osk RNA function do not affect known Bru binding sites, which are all positioned more 5′ in the mRNA (Reveal et al. 2010). Furthermore, Bru does not bind appreciably to the region of the 3′ UTR bearing the defective mutations (Kim-Ha et al. 1995). Nevertheless, the mutations might indirectly affect Bru binding, and thereby disrupt osk RNA function. To address this possibility, RNA binding assays were performed with RNAs containing the most 3′ part of the osk mRNA, which includes the C region Bru binding sites and the sequences critical for osk RNA function (Fig. 3B). Neither of the mutants lacking all osk RNA function (osk3′977-981 and osk3′984-988) affected Bru binding. Similarly, a mutant with impaired osk RNA function (osk3′990-994) retained full Bru RNA binding. In contrast, the osk3′970-974 mutant, in which one of the type II Bru binding sites is disrupted, showed reduced Bru binding. Thus, although one component of the osk RNA function is to bind Bru, mutations near the 3′ end of the osk mRNA define other key elements that contribute to osk RNA function by a different mechanism.

Failure of certain scanning mutants to provide osk RNA function could owe to a defect in the RNA element that provides the function. Alternatively, the RNA could be unstable or not enriched in the oocyte. Although most mutant mRNAs were present at levels similar to the wild-type mRNA, several with mutations near the 3′ end of the osk mRNA were less abundant (Fig. 3A). This group includes mutants with normal osk RNA function, so a lower mRNA level is not by itself sufficient to abrogate this function. Nevertheless, lower levels of mutant osk mRNAs could be partially responsible for osk RNA function defects. To address this possibility, we increased mRNA levels using additional copies of the transgenes. Notably, even when the levels of the osk3′977-981 and osk3′984-988 mutant mRNAs were equal to the wild-type osk mRNA (Fig. 3C), no eggs were laid, and oogenesis was arrested at a stage similar to that when no osk mRNA is present (Fig. 3D,E). Thus, these
FIGURE 3. Fine scale mapping of osk RNA function elements. (A) Mutations in the osk 3′ region. The sequence of the region is shown, with Bru binding sites (BREs, type II, and type III sites) marked in blue. Black bars above the sequence indicate minimal fragments tested in Figure 2 (the final 18 nt of the osk 3′ UTR are not shown, but are present in the minimal fragments along with a further 8 nt of genomic DNA). Beneath the sequence are shown the mutations (lowercase) introduced into genomic osk transgenes. The osk CII− mutant has the mutations of both osk 3′ 920-923 and osk 3′ 970-974. Single copies of each transgene were tested in the osk<sup>Δ57</sup>/Df(3R)osk background for rate of egg laying and mRNA level. At least two independent transgenic lines were tested for mutants with substantial defects. The additional lines of mutants osk3 977-981 and osk3 984-988 also lacked detectable activity. RNA levels were determined as in Figure 1, using rp49 as an internal control. (B) Affinity capture assay of Bru binding to osk C region RNAs, either wild type or with scanning mutations. The osk RNAs are fused to the S1 aptamer (the first two lanes are the aptamer alone). After incubating with ovary extract, the RNAs and bound proteins were recovered by affinity purification to generate supernatent (S; unbound) and pellet (P; bound) fractions, and the presence of Bru was determined by Western blotting. How well each version of osk mRNA supports the osk RNA function is indicated at bottom. (C) Increasing transgene dosage to raise osk mRNA levels for selected mutants. The RNase protection assays are shown at left with transgenic osk mRNAs indicated (all were in the osk<sup>Δ57</sup>/Df(3R)osk background), and the quantitation by phosphorimaging at right (samples in the same order). (D,E) Ovarioles stained with TOPRO-3 to detect nuclei. Both are osk<sup>Δ57</sup>/Df(3R)osk, with E expressing two copies of the osk 3′ 977-981 transgene (same genotype as in panel C). (F) Distribution of mutant osk mRNAs. All egg chambers are from osk<sup>Δ57</sup>/Df(3R)osk females expressing a single copy of the transgene indicated. osk mRNA (green) was detected by in situ hybridization, with DNA (red) labeled with DAPI.
mutants have extreme effects on osk RNA function independent of any effect they may have on osk mRNA stability. We also examined the distribution of representative mutant mRNAs. Of the mutants tested, two provided normal osk RNA function, two provided partial function, and two were most seriously affected. Each of these six mutants displayed the normal pattern of osk mRNA accumulation in early-stage egg chambers, with the mRNA highly concentrated in the oocyte (Fig. 3F). Thus, loss of osk RNA function by the affected mutants cannot be attributed to a defect in nuclear export or transport to the oocyte.

\textbf{osk RNA function and karyosome formation}

During oogenesis the meiotic chromosomes of the oocyte form a single compact cluster—the karyosome—within the nucleus. In the absence of osk mRNA the karyosome does not form properly, and the chromosomes usually appear in multiple zones within the oocyte nucleus (Fig. 4C; Jenny et al. 2006). This phenotype raised the possibility that the oogenesis arrest of osk RNA null mutants might be caused by defects known to affect the karyosome.

\textbf{FIGURE 4.} Karyosome defects of osk RNA null mutants. (A) Suppression of karyosome defects by reducing \textit{aret} activity. Females are all \textit{osk}^{\delta 3}/\textit{Df}(3R)\textit{pXT103}, with the \textit{aret} alleles indicated at top. (B) Frequency of karyosome defects for osk mutants. Egg chambers from \textit{osk}^{\delta 3}/\textit{Df}(3R)\textit{osk} females expressing a single copy of the transgene indicated were scored for karyosome morphology (for osk ABC BRE, the females were \textit{osk}^{\delta 3}/\textit{Df}(3R)\textit{osk}). Mutants \textit{osk}^{3889-893}, \textit{osk}^{3896-900}, \textit{osk}^{3909-913}, \textit{osk}^{3915-919}, \textit{osk}^{3920-923}, \textit{osk}^{3930-954}, \textit{osk}^{3957-961}, and \textit{osk}^{3963-967}, which are not included in the diagram, all had 100\% normal karyosomes with \textit{n} values of 9 or greater. Two independent transgenic lines were tested for the scanning mutants shown. To facilitate comparison, the results of the egg laying tests (Figs. 1B, 3A) are summarized below the graph. (C) Examples of karyosome morphology. Complete egg chambers are shown for examples of wild type (\textit{w}^{-}\textit{118}) and \textit{osk}^{\delta 3}/\textit{Df}(3R)\textit{osk} (left and center images in the top row). For the other panels only the oocyte is shown. Samples were labeled with TOPRO-3 for DNA (red) and anti-lamin (green).

The karyosome phenotype of osk RNA null egg-chambers is strikingly similar to that of mutants defective in repair of DNA damage (Ghabrial et al. 1998). When damaged DNA is not repaired, a checkpoint is activated and karyosome morphology is altered. These karyosome defects can be suppressed by mutation of genes responsible for execution of the checkpoint, such as \textit{mei}41 (Ghabrial and Schüpbach 1999). To ask if the karyosome defects in the absence of osk mRNA are due to activation of this checkpoint, we tested osk RNA null females that were also homozygous for \textit{mei}41\textit{D}3. There was no rescuing effect on either oogenesis arrest or karyosome morphology (Supplemental Fig. 2), suggesting that an ectopically activated DNA damage checkpoint is not the cause for the osk RNA null phenotype.

We have identified two contributions to osk RNA function: Bru binding sites which act, at least in part, to sequester Bru; and 3′ sequences that do not bind Bru and must have a separate role. To ask if the karyosome defects can be assigned to Bru sequestration or to the separate action of the 3′ sequences, we did two types of experiments. In one, the effect of reducing \textit{aret} activity was monitored. Just as for progression through oogenesis (Fig. 1E,F), the karyosome phenotype was suppressed, although to a much larger degree (Fig. 4A). For the second type of experiment, we tested the osk mutants that have defects in progression through oogenesis for karyosome morphology (Fig. 4B). Mutation of either Bru binding sites or the essential 3′ sequences interfered with karyosome formation, just as for progression through oogenesis. However, the severity of the two phenotypes was not perfectly correlated. In particular, mutation of Bru binding sites had a stronger effect on karyosome morphology than mutation of the 3′ sequences, despite the more severe effects of the latter mutations on egg laying.

\textbf{The absence of osk mRNA alters the distribution of germline-specific and –enriched proteins}

Bru protein normally appears in the germline cells of the ovary, where it is enriched in the oocyte at early stages (Weber et al. 1997). Immunodetection of Bru in osk RNA null females by confocal microscopy revealed notable changes in distribution (Fig. 5A–D). The very low level of signal normally detected in the somatic follicle cells, previously assumed to be background, was enhanced. As a quantitative measure of this change, fluorescence intensity levels were determined.
along lines drawn across the follicle cell/nurse cell boundary (Fig. 5E), revealing a consistent increase in follicle cell signal when osk mRNA is missing (Fig. 5F). We also measured average fluorescence intensity in multiple areas of follicle cells, an approach that minimizes variation (see Materials and Methods). The difference in follicle cell signal intensity for wild-type and osk RNA null egg chambers was substantial (Fig. 5G).

Detection of Bru in follicle cells was unexpected, as expression of the protein in the ovary was thought to be restricted to the germline (Webster et al. 1997). This surprising discovery raised a number of questions. One key issue was whether this phenomenon was caused by the absence of osk mRNA. Notably, this defect was fully rescued by addition of an osk transgene retaining full osk RNA function (Fig. 5G). Furthermore, two different osk RNA null genotypes, osk^0/osk^0 (Fig. 5A, C) and osk^{osk/D(3R)osk} (below), showed the same changes in Bru distribution. Therefore, this defect is indeed due to loss of osk mRNA, and cannot be attributed to other mutations present in the osk RNA null flies. A second question was whether the protein detected in the follicle cells is really Bru. Ideally, a mutant lacking Bru protein would be used to confirm the specificity of the immunodetection. However, aret null mutants arrest oogenesis too early to perform the experiment (Schübisch and Wieschaus 1989). As an alternative, the ovaries were stained with different anti-Bru antibodies. The same expanded distribution of Bru was detected (Supplemental Fig. 3).

To determine if the change in protein distribution was exclusive to Bru, or a more general property, two additional proteins were tested. Within the ovary Orb is thought to be expressed exclusively in the germline (Lantz et al. 1994). Immunodetection of Orb in wild-type and osk RNA null ovaries showed that in both genotypes the protein was highly enriched in the germline cells (Fig. 5H, I). However, quantifying signal intensity revealed a small but statistically significant enhancement in the follicle cells of osk RNA null egg chambers (Fig. 5J). This difference between wild-type and

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**FIGURE 5.** Redistribution of Bru and other germline proteins in osk RNA null ovaries. (A–D) Immunodetection of Bru in wild-type (A,B) and osk RNA null (osk^0/osk^0) ovaries (C,D). The higher magnification samples (B,D) show the punctate distribution of Bru, which is reduced in the osk RNA null mutant (see also Fig. 6). The level of Bru is much greater in the oocyte than the nurse cells, as can be seen in egg chambers in which the confocal section includes the oocyte (examples in A, C, D). (E) Organization of the egg chamber. Half of an egg chamber is shown, with the different cell types indicated at left. To monitor Bru signal in nurse cells and follicle cells, signal intensity was measured along multiple lines (e.g., the white lines) in Fiji (Materials and Methods). (F) Traces of signal intensity along four lines are shown for both wild-type and osk RNA null egg chambers. The relative positions of follicle and nurse cells along the lines are indicated below. (G) Quantitation of Bru signal intensity in follicle cells. Each data point represents a region crossing multiple follicle cells (thus including a mixture of nuclei and cytoplasm) and avoiding the inner (adjacent to the germline cells) and outer boundaries of the follicle cell layer. The “rescue” sample was osk^0/osk^0 with an osk transgene providing full rescue of the oogenesis arrest phenotype. (H, I) Immunodetection of Orb in wild-type and osk RNA null ovaries. For H and I, signal intensities were adjusted identically in Photoshop to enhance the signal. (J) Quantitation of Orb signal intensity in areas of follicle cells, as in G. The difference is statistically significant: from an unpaired two-tailed Student’s t test, P < 0.001. (K, L) Quantitation of CG9925 signal intensity in areas of follicle cells, as in G. The difference is statistically significant: from an unpaired two-tailed Student’s t test, P < 0.001.
osk RNA null ovaries could be visualized by enhancing the signal intensity, revealing a halo of Orb staining in the follicle cells. The halo was detectable and restricted to the follicle cell region in the osk RNA null ovaries (Fig. 5I′), but weaker or not visible in wild type (Fig. 5H′). It is uncertain if such a low level of Orb in the follicle cells would have any biological effect. Nevertheless, Orb provides another example of a protein normally thought to be restricted to the germline, but appearing in somatic follicle cells in the absence of osk mRNA.

The CG9925 protein is expressed predominantly in the female germline and is a nuage component (PM Macdonald, unpubl.). Just as for Bru and Orb, the level of immunofluorescence in follicle cells is elevated when comparing wild-type to osk RNA null ovaries (Fig. 5M).

In addition to the elevated levels of Bru, Orb and CG9925 proteins in the follicle cells of osk RNA null egg chambers, there were also changes in the subcellular distribution of each protein. Bru is normally enriched in cytoplasmic particles, most obviously in perinuclear nuage but also in cytoplasmic sponge bodies (Snee and Macdonald 2009). Bru localization to nuage can be seen in Figure 5A, and is prominent at higher magnification (Fig. 5B). In osk RNA null ovaries the enrichment of Bru in nuage was substantially reduced (Fig. 5C,D). Loss of Bru from nuage was consistent (Fig. 6A). Similarly, the enrichment of Bru in sponge bodies was greatly reduced in the absence of osk mRNA (Fig. 6B). Orb protein is also present in sponge bodies and enriched in nuage (Snee and Macdonald 2009), although to a lesser extent than Bru. As for Bru, the particulate distribution of Orb was reduced in the osk RNA null ovaries (Figs. 5H,I, 6C). CG9925 protein was highly enriched in nuage in wild-type ovaries, prominently outlining the nurse cell nuclei (Fig. 5K,K′). Although the protein remained associated with the perinuclear nuage in the osk RNA null mutant, the degree of nuage enrichment was substantially reduced (Fig. 5L,L′).

To determine if the sequences in osk mRNA that mediate other aspects of osk RNA function are also required to ensure the normal distribution of Bru protein, mutant osk transgenes were tested for this phenotype (Fig. 7). The osk ABC BRE− mutant, which retains an intermediate level of osk RNA function as measured by progression through oogenesis, showed an intermediate phenotype in Bru redistribution: The level of Bru in follicle cells was elevated as compared with wild type (Fig. 7A,G,G), but less so than in the absence of any osk mRNA (Fig. 7B,G). Mutants of the osk 3′-UTR C region with stronger defects in osk RNA
DISCUSSION

A striking property of the osk mRNA is its dual function. In the guise of an mRNA it encodes the Osk protein, which is essential for axial patterning of the oocyte and embryo. Acting as an lncRNA, the osk mRNA is required for progression through oogenesis. Here we have characterized the non-coding role of osk mRNA. This has revealed a surprising feature of what goes wrong when osk mRNA is not present, and shows that osk mRNA performs multiple noncoding functions using different sequence elements. The work we have described has three overlapping components. One is the discovery of new phenotypes associated with loss of osk mRNA. The second is the identification of sequences in the 3’ UTR important for osk RNA function. The third is the elucidation of the mechanism by which some of the cis-acting sequences function.

The initial characterization of mutants lacking osk mRNA revealed three defects: At stage 2 of oogenesis the karyosome fails to form, appearing in fragments; Stau protein fails to become enriched in the oocyte, consistent with its transport there in a complex with osk mRNA; and oogenesis is arrested at stage 7, after which the egg chambers degenerate (Jenny et al. 2006). We have found two additional defects. One is the infrequent appearance of egg chambers with too many nurse cells, suggesting an extra round of cell division (Fig. 6A). More notably, we discovered that Bru and other germline-specific or -enriched proteins appeared in the somatic follicle cells when the flies lacked osk mRNA. These proteins also changed their distribution within germline cells, with reduced enrichment in nuage and/or sponge bodies, two different but related types of germline RNPs.

The mechanism by which these proteins accumulate in the follicle cells is not clear, as none of them are secreted proteins that could be delivered by endocytosis. We observed egg chambers in which vesicles or regions of Bru-rich nurse cell cytoplasm appear in the follicle cell layer (e.g., Fig. 7D,E), a phenomenon that could contribute to this change in protein distribution. However, the same phenomenon is also seen in wild-type egg chambers (Fig. 5A). Ring canals allow for movement of macromolecules between nurse cells and from nurse cells to the oocyte (Robinson and Cooley 1996), as well as between subsets of follicle cells (McLean and Cooley 2013). In contrast, portals for exchange of components between germline and somatic cells have been thought to be limited to gap junctions, which allow only for transit of small molecules (Bohrmann and Haas-Assenbaum 1993; Zhu et al. 2007). A recent report of germline-derived proteins appearing in the follicle cell epithelium independent of endocytosis raises the possibility of alternate pathways of protein exchange, although in this example the germline proteins do not appear to actually enter into individual follicle cells (Furriols and Casanova 2014).
The displacement, in the absence of osk mRNA, of the Bru, Orb and CG9925 proteins from large cytoplasmic RNP complexes raises the possibility that these proteins can move between the different cell types, but that inclusion in the large RNPs normally limits this behavior. A less provocative explanation is that the known pathways for transfer of small molecules between the germline and somatic cells could mediate transfer of information, which then results in ectopic or enhanced transcription of certain germline genes in the somatic cells. Attempts to monitor levels of the mRNAs in follicle cells by in situ hybridization have been inconclusive. This approach produces significant background staining, making it difficult to distinguish between background and what would very likely be an extremely low level of signal from transcription (based on the observed levels of proteins in the follicle cells).

Independent of how germline proteins appear in the somatic follicle cells in the absence of osk mRNA, and why these proteins are less enriched in germline RNPs, these changes in protein distribution could underlie the other osk RNA null phenotypes. The protein distribution defects are present from the earliest stages of oogenesis, and are completely penetrant. Changes in the germline RNPs could affect the proper regulation of germline transcripts, and thereby cause some or all of the osk RNA null phenotypes. Similarly, the inappropriate presence of Bru and other post-transcriptional regulators could alter the follicle cell proteome, perhaps causing the arrest of oogenesis. Discovery of this novel phenotype provides a new option for a mechanism by which osk RNA facilitates progression through oogenesis.

Insight into a different mechanism for the noncoding function of the osk mRNA came from identification of required sequence elements. Mutation of the BREs, the primary Bru binding sites in osk mRNA, caused a partial disruption of osk RNA function with defects in multiple features of the osk RNA null phenotype: progression through oogenesis, the distribution of Bru, and karyosome formation. The established role of the Bru binding sites in translational repression of osk mRNA (Kim-Ha et al. 1995) cannot account for these defects, as unregulated translation of an osk mRNA lacking the 3′ UTR does not interfere with progression through oogenesis (Vanzo and Ephrussi 2002). Instead, the role of Bru binding sites in osk RNA function can be explained by either of two likely models: sequestration of a factor—Bru—that would otherwise disrupt oogenesis; and formation of a critical RNP required for progression through oogenesis. The models make very different predictions for the consequences of reducing the level of Bru, and our data argue very strongly for the sequestration model. Although there are examples of RNAs functioning as decoys to bind and inhibit proteins (for review, see Cech and Steitz 2014), the osk Bru interplay is unusual in that the known activity of Bru—to control osk translation—is not inhibited. Instead, association of Bru with osk mRNA is expected to constrain the binding of Bru to lower affinity targets. In the absence of osk mRNA, promiscuous binding by Bru may occur, presumably leading to misregulation of those mRNAs.

Other elements required for osk RNA function are positioned in a region at the extreme 3′ end of the osk mRNA. This region of the RNA is only functional when the RNA is in the oocyte. The 3′ region does contain Bru binding sites, but other included elements play an even more important role: While mutation of the main Bru binding sites (the BREs) substantially reduces egg laying, certain mutations near the 3′ end have more drastic effects, with the most severe abolishing egg laying and closely resembling a complete loss of osk mRNA. The tight proximity of these strongly inhibitory mutations—all are contained within a 30 nt region—might suggest that a single element is disrupted, but all indications argue for two or more different functional elements.

One type of functional element in the 3′ region has already been reported. Vazquez-Pianzola et al. (2011) showed that a cluster of A-rich sequences (ARS) at the extreme 3′ end of osk mRNA are bound by poly(A) binding protein (PABP). In a rescue assay similar to that used here for one set of experiments (Fig. 2), UAS/GAL4 expression of the wild type osk 3′ UTR restored some egg laying to osk RNA null flies, but ΔAR mutants lacking parts of the ARS region did not. Although this was taken as evidence for a role for the ARS elements and PABP in osk RNA function, two issues complicate that interpretation. First, the osk 3′ UTR ΔAR RNAs are present at reduced levels relative to the wild type. Because even the wild-type osk 3′ UTR does not fully rescue egg laying to the wild-type level in the UAS/GAL4 assay, a reduced level of the RNA might disrupt activity. Second, the deletions in the ΔAR mutants impinge on the other region we have found to be critical for osk RNA function (below), with a 1-nt overlap between the ΔAR deletions and the positions mutated in the very strongly defective osk3′984-988 mutant. Thus, an effect on the element defined by osk3′984-988 could disrupt egg laying in this assay. Despite these issues, two lines of evidence do support a role for the ARS elements and PABP in osk RNA function. First, the partial loss of osk RNA activity by our mutant osk3′1004-1008 would be consistent with a role for the ARS elements in osk RNA function. This mutant disrupts part of one ARS: Five of a run of 10 A nucleotides are altered. Because this mutant has both egg laying and karyosome defects in the robust osk genomic transgene assay (in which a wild-type transgene provides complete rescue and fully restores the normal rate of egg laying), there is clearly a disruption of osk RNA function. Whether mutating more of the ARS elements would completely abolish osk RNA function remains uncertain. Second, reducing levels of both osk mRNA and PABP activity substantially enhances karyosome defects similar to those due to absence of osk mRNA (Supplemental Fig. 4).

The scanning mutants with the strongest defects in osk RNA function are osk3′977-981 and osk3′984-988, which are positioned adjacent to one another and do not alter A-rich sequences. Because each of these mutants almost
completely lacks osk RNA function, they must define a critical element (or elements). Like the Bru binding sites, this element could act in sequestration of one or more factors. Alternatively, it could play a scaffolding function. We have assayed for proteins that bind this region, but have not detected any whose binding to the scanning mutants correlates with their effects on osk RNA function (YH Ryu and PM Macdonald, unpubl.).

The other mutant with such severe defects is osk C all−, in which all of the C region Bru binding sites are mutated. None of these mutations have a similar effect when tested individually. The most obvious explanations for the severity of this mutant invoke redundant binding sites or a critical RNA structure. While the C all− mutations do affect Bru binding sites, consistent with redundancy, the strong phenotype cannot be attributed to loss of Bru sequestration, since mutation of the higher affinity AB region Bru binding sites does not have an equivalent effect. However, Bru bound to sites in the C region might facilitate the binding or action of another factor or factors brought into close proximity by binding to the nearby essential osk RNA function element. The combined effects of the multiple mutations of the osk C all− mutant could also alter the structure of the RNA, even if an individual mutation alone has this effect. Folding predictions do differ for the wild type and mutants, but none are predicted to form highly stable structures in this region of the 3′ UTR.

A precedent for an mRNA with dual functions comes from the Xenopus oocyte, where the VegT mRNA both encodes a protein and plays a structural role in organization of the cytokeratin cytoskeleton (Heasman et al. 2001; Kloc et al. 2005, 2007). Different regions of the VegT mRNA have different effects on cytokeratin polymerization and depolymerization (Kloc et al. 2011). Loss of either VegT or osk mRNAs perturbs the subcellular distribution of other molecules—certain localized mRNAs for VegT, and proteins in RNPs for osk—but at present there are no clear parallels in how the osk and VegT mRNAs perform their noncoding roles. This is not surprising given the mechanistic diversity for RNAs with strictly noncoding functions (Cech and Steitz 2014).

In conclusion, we have discovered a striking consequence of loss of osk mRNA—the altered distribution of germline regulatory factors, including enrichment in somatic follicle cells and displacement from large cytoplasmic RNPs—and we have identified sequence elements required for osk RNA function. Although different sequence elements have very different functions, one set acting to sequester Bru and others acting in different but as yet incompletely defined roles, mutation of either type is sufficient to bring about the changes in protein distribution. Although misregulation of transcripts in both somatic and germline cells resulting from these changes in protein distribution could account for the other osk RNA null phenotypes, it remains a puzzle how the different types of osk RNA functional elements elicit the same effect, and whether a common underlying mechanism is responsible.

MATERIALS AND METHODS

Flies and transgenes

aret22286 was from Mary Lilly. The osk0 allele (RNA null) was generated by homologous recombination (Gong and Golic 2003), and has sequences R3:8935117-8938212 (r6.01) deleted. Details are available on request. Transgenes of genomic osk DNA with mutated Bru binding sites are from previous studies (Kim-Ha et al. 1995; Reveal et al. 2010). All of the genomic osk transgenes have the complete, wild-type coding region. Scanning mutations in the osk C region were introduced by PCR, and incorporated into genomic osk transgenes essentially the same as for the Bru binding site mutations. Transgenes UAS-GFP and UAS-GFP-4xBRE (four copies of the BRE sequence TGGTTTATATGTT) have been described (Reveal et al. 2011). For these transgenes the GFP is mGFP6 (Haseloff 1999). Some of the UAS-egfp transgenes with osk 3′-UTR sequences (Fig. 2) are from Jambor et al. (2014). For the new transgenes, the osk 3′-UTR segments used are provided in the Supplemental Materials. Transgene K10-TLS 119 of 3b differs from the others in Figure 2 in that it lacks egfp.

Egg laying assays

Newly eclosed flies were collected, aged for 2–3 d (when the first eggs appeared) and placed in cages with yeasted apple juice plates. Collections were performed over 60 h, and the total number of eggs scored. All assays were performed at least twice. The females were typically oskK10-Df(3R)osk with one copy of an osk transgene, unless otherwise noted. A wild-type osk transgene was used as a standard, and egg laying rates are all relative to that standard. Other genetic elements (UAS-GFP transgenes, the nosGAL4::VP16 driver, and aret22286) were included as indicated in the figures. For the results in Figure 2, a simplified assay was used, scoring for the presence of any laid eggs.

RNA detection

To measure RNA levels, ovaries were dissected from 3–4 d old females, RNA purified using TRizol according to the manufacturer’s instructions, probed for osk and rp49 mRNAs by RNase protection assay (Ambion RPAIII), and quantified by phosphorimaging. Assays were performed three or more times. The rp49 signal was used to normalize for the amount of RNA in each preparation.

In situ hybridization to detect osk or egfp mRNA in ovary whole-mount preparations was performed as previously described (Jambor et al. 2014). For the K10-TLS 119 of 3b transcripts, the probe was from transcribed UASP vector sequences (Supplemental Materials). Ovaries were imaged with a confocal microscope (DMR-E, Leica; TCS-SP2, AOBS scan head; Leica) or a wide-field microscope (Axioplan imaging2, Zeiss) equipped with an optical sectioning device (DSD1, Andor) in Figures 2 and 3, respectively.

Measurement of egg chamber length

Newly eclosed flies were collected and incubated with fresh yeast at 25°C until eggs were laid or for a maximum of 4 d. The ovaries were then dissected and fixed. To determine egg-chamber length, we measured the length of the major axis of the oldest egg-chamber
RNA binding

To monitor Bru binding to osk 3′-UTR segments by UV crosslinking, the RNA probes, ovary extract preparation, and assays were all as described previously (Kim-Ha et al. 1995). For the affinity capture assays, a segment of the osk gene 3′ UTR (the final 150 nt of the 3′ UTR) in wild-type or mutant forms was fused to DNA encoding the S1 RNA aptamer which binds streptavidin (Walker et al. 2008). Transcripts were mixed with ovary extract and streptavidin beads. After washing, the beads were recovered, associated proteins separated by SDS-PAGE and Bru detected by western blotting. Details available on request.

Whole mount immunodetection

Immunostaining of ovaries was largely as described previously (Kim-Ha et al. 1995), except that secondary antibodies were labeled with Alexafluor 488 (Invitrogen). In addition, for analysis of ovaries with arrested oogenesis the ovarioles were teased apart with a tungsten ultramicro needle (Ted Pella, Inc.). Primary antibodies were used at the following dilutions: mouse anti-Lamin Dm0 (ADL84.12), 1:100; mouse anti-Orb (4H8), 1:1; mouse anti-Hts (1B1), 1:1; rabbit anti-CGG9925, 1:2000; rabbit anti-Bru 4005 S2789-1 and -2, 1:1000. Samples were mounted on slides with Vectashield Mounting Medium (Vector Labs) and imaged with a Leica TCS-SP laser scanning confocal microscope.

Quantitation of levels made use of samples fixed and processed in parallel from flies grown in parallel and of the same age. Signal intensities along lines crossing the nurse cell/follicle cell boundary and avoiding nuclei in both nurse cells and follicle cells (Bru and the other proteins tested are predominantly cytoplasmic) were measured in Fiji. Because of the granularity in the signal, there is substantial variation along in signal intensity along the lines. Measurements of mean intensity in follicle cells were made in Fiji, on regions from the central portion of the follicle cell layer. Each region tested covered the central portion of the follicle cell layer. Each region tested covered

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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