In most animal species, transcription from the zygotic genome does not commence immediately after fertilization, and early development is controlled and supported by maternally provided mRNAs and proteins stored in the oocyte (Gray and Wickens, 1998). While most of these are uniformly distributed in the oocyte, some essential activities involved in axis determination, body patterning and cell fate specification must be tightly localized and restricted to specific sub-cellular domains for normal embryonic development to proceed. This is achieved in many cases by prelocalization of the mRNAs encoding these activities to the specific regions where their function is required (St Johnston, 1995). In Drosophila, several mRNAs involved in determination and development of oocyte polarity, and later, in specification of the embryonic body pattern and establishment of the germ line, are localized asymmetrically within the developing germ cell during oogenesis. Accumulation of bicoid (bcd), oskar (osk) and gurken (grk) mRNAs at specific locations within the oocyte cytoplasm is crucial for the establishment of anteroposterior and dorsoventral polarity in the embryo.

osk mRNA is localized to the posterior pole of the oocyte, where its activity is required for the organization of the posterior pole cytoplasm (Ephrussi et al., 1991; Kim-Ha et al., 1991). The posterior pole plasm contains the determinants of the germ line and of the abdomen, and thus regulates posterior body patterning (Lehmann and Nüsslein-Volhard, 1986). Both the generation of OSK activity and its restriction to the posterior pole are critical for proper embryonic patterning. Ectopic expression of osk mRNA results in a disruption of the anteroposterior body plan of the embryo and, consequently, in lethality (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992; Smith, 1992). The deleterious expression of unlocalized osk mRNA is prevented by the tight control of its translation. Prior to its localization, osk mRNA is translationally repressed (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). This is mediated, at least in part, by specific sequences present in the 3′ untranslated region (UTR) of osk mRNA. We have established a cell-free translation system derived from Drosophila ovaries, which faithfully reproduces critical features of mRNA translation in vivo, namely cap structure and poly(A) tail dependence. We show that this ovary extract, containing endogenous Bruno, is able to recapitulate osk mRNA regulation in a BRE-dependent way. Thus, the assembly of a ribonucleoprotein (RNP) complex leading to the translationally repressed state occurs in vitro. Moreover, we show that a Drosophila embryo extract lacking Bruno efficiently translates osk mRNA. Addition of recombinant Bruno to this extract establishes the repressed state in a BRE-dependent manner, providing a direct biochemical demonstration of the critical role of Bruno in osk mRNA translation. The approach that we describe opens new avenues to investigate translational regulation in Drosophila oogenesis at a biochemical level.

Key words: osk mRNA, Bruno, Drosophila cell-free translation, Oogenesis
posterior pole of the oocyte, but whose translation occurs precociously. This results in ectopic OSK activity throughout the oocyte and, consequently, in the formation of ectopic pole plasm and posterior structures. Hence, BRE-dependent translational repression of osk mRNA prior to its localization is essential.

Localization of osk mRNA at the posterior pole is a prerequisite for translation: no OSK protein is detected in mutants that fail to localize osk mRNA (Markussen et al., 1995; Rongo et al., 1995). Nevertheless, posterior localization per se is not sufficient to allow translational activation of osk mRNA. A cis-acting element located in the 5′ region of the osk transcript is required to relieve the BRE-mediated repression (Gunkel et al., 1998). This cis-acting derepressor element functions only at the posterior pole, suggesting that an active derepressor machinery or a limiting component of it is located in this region of the cytoplasm.

Genetic experiments have provided evidence for the involvement of Bruno and many other factors in different steps of osk RNA translational control. While mutations in arrest (aret), the locus encoding Bruno protein, are either unsuitable for analysis or show no defect in regulation of endogenous osk mRNA, a chimeric osk-bcd mRNA containing the Bruno binding sites showed increased activity when the gene dosage of Bruno was reduced (Webster et al., 1997), consistent with the idea that Bruno is a translational repressor. Mutations in Bicaudal-C (Bic-C) cause translation of unlocalized osk mRNA, suggesting a potential role for the Bic-C protein as a translational repressor (Saffman et al., 1998), staufen (stau), aubergine (aub) and vasa (vas), on the other hand, appear to play a role in the translational activation of osk. In stau mutants, osk mRNA localization is disrupted and neither wild-type osk mRNA nor osk mRNAs lacking BREs are efficiently translated (Kim-Ha et al., 1995). aub and vas mutants show normal levels of osk RNA, but produce only a reduced amount of protein (Markussen et al., 1995; Rongo et al., 1995; Wilson et al., 1996).

While genetics has allowed the identification of some of the molecular components involved in the translational control of osk mRNA, a mechanistic understanding of this regulatory process requires a complementary biochemical approach. Encouraged by recent progress in establishing an in vitro translation system from Drosophila embryos (Gebauer et al., 1999), we have now developed a cell-free translation system from Drosophila ovaries. Using both systems, we study the translational repression of osk mRNA by Bruno.

### MATERIALS AND METHODS

**DNA constructs**

m1m2lacWT has been described previously (Gunkel et al., 1998). m1m2lacAABC is derived from m1m2lacWT by deletion of an EcoRI/Dral fragment containing the AB repressor region, and additional deletion of the C region by PCR-directed mutagenesis. This construct lacks nucleotides 3660-3778 and 4416-4487 of the chimeric osk-lacZ transcript.

The luciferase (luc) (lizuka et al., 1994) and the chloramphenicol acetyl transferase (CAT) (Preiss and Hentze, 1998) plasmids were described previously.

A Bruno cDNA-containing fragment was obtained by digestion of a Bluescript plasmid containing the cDNA (gift from Paul Macdonald) with EcoRI/Dral, and was cloned into pQE32 (QIAGEN) to yield plasmid pQE32-BruF. Bruno protein expressed from this plasmid lacks the first 24 amino acids and was fused to a 6His-GIRMARV peptide at the amino terminus.

**In vitro transcription**

Capped chimeric osk-lacZ mRNAs were synthesized from m1m2lacWT and m1m2lacAABC plasmids that were linearized with NotI, using an SP6 Message Machine kit from Ambion. After a 2-hour reaction, the template DNA was eliminated by digestion with DNaseI, and the RNA purified using RNeasy columns from QIAGEN. Luc and CAT mRNAs were synthesized as described by Gray and Hentze (1994). The RNAs were trace-labelled with [α-32P]UTP to facilitate assessment of their concentration and integrity. All RNAs used in the same experiment were synthesized in parallel.

**Recombinant Bruno protein purification**

Plasmid pQE32-BruF was transformed into competent E. coli M15[pREP4]. Culture conditions were as described in the QAexpressionist handbook (QIAGEN) with the exception that induction was performed with 0.3 mM IPTG for 3 hours at 30°C. Cells were harvested after the culture reached 0.6 OD600, and lysed in a buffer containing 50 mM naphosphate pH 8.0, 300 mM NaCl, 20 mM imidazole and 1× protease inhibitor cocktail (Roche). The recombinant protein was purified using the recommendations of the QAexpressionist handbook, and was eluted in 250 mM imidazole. The protein was further purified by FPLC on a Highload Superdex-200 column to remove the imidazole.

**UV cross-linking assay**

UV cross-linking assays were performed essentially as described by Gunkel et al. (1998) except that 0.6 mM DTT was included in the reaction. An α-32P-labelled RNA fragment corresponding to the BRE region (corresponding to nt 3652-3739 of the chimeric RNA) was incubated with 10-20 μg of ovary or embryo extract for 30 minutes at room temperature. After UV cross-linking (Gunkel et al., 1998, method 2), samples were treated with RNase A and the proteins separated on a 10% SDS-polyacrylamide gel.

**Ovary extract preparation**

Females kept at 25°C on yeast for 2-3 days were manually dissected in cold PBS to obtain the ovaries. All steps after the collection were performed at 4°C. Ovaries were allowed to settle by gravity, and the volume of settled material was measured. Ovaries were washed twice with 12 volumes of a (1:1) mix of PBS:DEI (10 mM Hepes pH 7.4, 5 mM DTT, 1× COMPLETE-Protease Inhibitors Cocktail EDTA-free from Roche), and quickly washed twice with 12 volumes of DEI. Excess buffer was removed, and the ovaries were homogenized using a pestle directly in the microfuge tube. The homogenate was spun for 10 minutes at 14000 rpm in a microcentrifuge. The pellet was discarded and the two remaining phases were mixed and used for the translation reaction.

**In vitro translation**

Translation assays were performed as described in Gebauer et al. (1999), with conditions optimized for the osk chimeric mRNAs. Briefly, 50 ng of template osk mRNA were translated in a 12.5 μl reaction containing 60 mM amino acids, 16.8 mM creatine phosphate, 80 ng/μl creatine kinase, 24 mM Hepes pH 7.4, 0.6 mM magnesium acetate, 60 mM potassium acetate, 0.1 mM Spermidine, 1.2 mM DTT, 100 ng/μl calf liver tRNA and 40% ovary extract. 20 ng of luciferase mRNA were co-translated as an internal control. The reaction was incubated for 90 minutes at 25°C. The translation efficiency of the osk chimeric mRNAs was quantified using the chemiluminescent β-Gal Reporter Gene Assay (Roche). Luciferase activity was measured according to the method of Brasier et al. (1989). In the RNA
competition experiments, 5 μl of ovary extract were incubated for 15 minutes on ice with increasing amounts of competitor RNA before setting up the translation reaction. Prior to its addition to the embryo extract, Bruno protein was preincubated with the RNA for 15 minutes on ice.

RESULTS

Establishment of a cell-free translation system from Drosophila ovaries

osk mRNA exists in at least two translational states, dependent on its localization within the egg chamber: unlocalized and translationally silent, or localized and translationally active. Translational repression of osk mRNA prior to its localization at the posterior pole of the oocyte is mediated, at least in part, by the ovarian protein Bruno, which binds to discrete elements in osk mRNA 3′UTR, the BREs. Transcription, localization and translation of osk mRNA occur during oogenesis within the egg chamber. We reasoned that a cell-free translation system from Drosophila ovaries should in principle recapitulate most faithfully the normal physiological environment for osk mRNA translational regulation.

An ovarian extract was prepared according to a protocol previously established for the preparation of Drosophila embryo translation extracts (Gebauer et al., 1999). Unfortunately, this approach did not yield a translationally active ovary extract (data not shown). We then modified the procedure, taking into account the characteristics of the ovarian tissue (see Materials and Methods for details), to obtain an ovary extract that was competent for translation.

We next tested the ability of the extract to recapitulate basic features of translation in vivo, such as 5′ cap-dependence, or its capability to reproduce the synergism between the cap and the poly(A) tail to promote translation (Preiss and Hentze, 1999). Luciferase reporter mRNAs bearing a m7GpppG cap or a poly(A) tail were approximately 10-fold better translated than a similar mRNA bearing no end-modification (Fig. 1, upper panel, compare – with c and a RNAs), indicating that the cap and the poly(A) tail could stimulate translation independently in this system. Importantly, translation of the mRNA bearing both a cap and a poly(A) tail (c-a) was approximately 40-fold more efficient than that of those mRNAs containing only one of the end-modifications. However, the lower stabilities of the unmodified mRNAs (Fig. 1, middle panel) precluded the interpretation that the higher translational efficiency of the c-a mRNA reflected a bona fide synergism between the mRNA ends for translation. To address this question, we analyzed the translation of templates bearing an ApppG instead of a m7GpppG cap, which stabilizes the mRNA but does not support translation because it is unable to interact with the translation initiation factor eIF4E. Although the RNAs bearing the ApppG cap structure were stable (middle panel, compare c* with –, and c*-a with a), the translational efficiency of these RNAs did not increase (upper panel). A cooperative enhancement of translation of the luciferase reporter mRNA was observed only when both a canonical m7GpppG cap and a poly(A) tail were present. Therefore, the ovarian extract is able to recapitulate the synergism between the 5′ cap structure and the poly(A) tail as observed in vivo for many RNAs.

BRE-dependent assembly of a repressed osk mRNP in ovarian extracts

The ovarian cell-free translation system described above displays similar characteristics for basic translation as the recently established embryo extract (Gebauer et al., 1999). However, with regard to osk mRNA regulation, the two extracts differ in one important aspect. Whereas the repressor protein Bruno is readily detected in the ovarian extract by UV-crosslinking to a BRE-containing RNA probe, it is not detected in the embryo extract (Fig. 2A). Both extracts, however, contain p50, a factor that recognizes both the repressor and derepressor elements of osk mRNA (Gunkel et al., 1998). This pair of extracts is therefore ideally suited to study the mechanism of translational regulation of osk mRNA and, in particular, its translational repression by Bruno.

osk chimeric transcripts were prepared that contained (m1m2lacWT) or lacked (m1m2lacΔABC) the BREs (Fig. 2B). The m1m2lacWT construct contained the 5′ and 3′UTRs of osk mRNA, and encoded the amino-terminal 136 amino acids of OSK fused in frame with β-galactosidase. This

![Fig. 1. Translational synergism between the cap and the poly(A) tail in Drosophila ovary extracts. Luc mRNAs containing the following end-modifications were translated in ovary extracts at a concentration of 3.2 ng RNA/μl reaction: –, no end-modification; c, m7GpppG cap; c*, ApppG cap; a, A98 tail; c-a, m7GpppG cap and A98 tail; c*-a, ApppG cap and poly(A) tail. 12 μl aliquots were taken at 0, 20 and 90 minutes. Of these, 3 μl were used to measure the luc activity (upper panel) and 9 μl were employed to extract total RNA and to assess mRNA stability by northern blot (middle panel). As an RNA recovery control, 20 ng of CAT mRNA were added to the reactions before extraction (lower panel). The estimated half-lives (t1/2)of the different RNAs are indicated below the middle panel. The increased Luc activity of the mRNA bearing an ApppG cap (c*) compared to the unmodified RNA (–), can be fully accounted for by increased RNA stability.](imageurl)
construct has been shown to reproduce the localization and translational regulation of endogenous osk RNA in vivo (Gunkel et al., 1998). The translational efficiency of the osk chimeric transcripts was then analyzed in ovary and embryo extracts. In spite of the strong poly(A) tail-dependence observed for translation of luc RNA in both types of extract, only a two-fold increase in translation was observed when an A\textsubscript{73} tail, sufficient for full stimulation of luc mRNA, was added to the 3' end of capped transcripts bearing the full-length osk 3'UTR (data not shown). Interestingly, a similar lack of poly(A) tail-dependence for translation has been observed for another developmentally controlled Drosophila mRNA, msl-2, in the embryo extract (Gebauer et al., 1999).

In ovary extracts, m1m2lacWT mRNA was poorly translated, while m1m2lac\textDelta ABC was translated efficiently (Fig. 3A). The two RNAs incubated in extract were equally stable over time, ruling out that the difference in translation was due to a differential stability of the transcripts (data not shown). This result suggested that a repressive mRNP could be assembled on the 'naked' BRE-containing transcript, and that a factor binding to the BREs was responsible for its poor translation. To exclude the possibility that the wild-type mRNA had an intrinsically reduced translational efficiency irrespective of the binding of a titratable factor, translation reactions were performed in the presence of increasing amounts of BRE-containing competitor RNA (Fig. 3B). A four-fold molar excess (5 ng) of competitor RNA relative to the wild-type reporter RNA resulted in twice the amount of translation in the ovarian extract. Increasing amounts of competitor caused a further increase in translation, reaching a five-fold stimulation of translation of the wild-type template. Translation of the BRE-deleted reporter RNA was essentially unaffected by addition of increasing amounts of competitor RNA (Fig. 3B). At the highest levels of competitor, the wild-type reporter was translated as efficiently as the BRE-deleted reporter, indicating that a repressor was titrated out and no longer available to bind the BREs in the m1m2lacWT transcript. These results confirm the previous in vivo observation that osk translational repression is mediated, at least in part, through the BRE elements present in the osk 3'UTR. They also suggest that translation of the wild-type reporter RNA in ovary extracts is inhibited by Bruno.
Recombinant Bruno silences oskar mRNA translation in vitro

To directly test whether Bruno can cause the poor translation of the BRE-containing mRNA, the oskar chimeric mRNAs were translated in the embryo extract, which lacks Bruno. In this system, both mRNAs were translated with a similar efficiency (Fig. 4A). We then added increasing amounts of purified recombinant Bruno to the embryo extract and measured the translation of the osk chimeric mRNAs. The addition of Bruno protein caused a significant (4-fold) decrease in translation of the BRE-containing transcript (Fig. 4B). Translational repression of the wild-type transcript was already evident at a 2:1 protein:RNA molar ratio (corresponding to the addition of 4 ng of Bruno). This repression was specific, because translation of a luciferase mRNA bearing no osk sequences that was used as an internal control was not affected by Bruno (data not shown). Furthermore, the translation of m1m2lacΔABC mRNA, lacking the BREs, was unaffected (Fig. 4B). These results provide direct biochemical evidence that Bruno mediates translational repression of osk mRNA in vitro by interacting with the BREs.

DISCUSSION

Genetic experiments have shown that discrete regions in the osk mRNA 3′UTR are required for its translational repression before the RNA is localized (Kim-Ha et al., 1995). Biochemical evidence indicated that these regions were bound by an ovarian protein called Bruno, and hence the regions were referred to as Bruno Response Elements (BREs). Further experiments demonstrated that Bruno protein is encoded by the aret locus (Webster et al., 1997). Interestingly, the strong phenotypes observed for aret mutants, in which oogenesis arrests at an early stage, suggest that Bruno is involved in the regulation of other mRNAs, in addition to osk. However, the early arrest of oogenesis in the mutants, much before the onset of osk RNA localization and translation, have prevented a direct assessment of Bruno function on osk mRNA in vivo. Weaker aret mutants that allow development of an oocyte beyond stage 9 when osk mRNA is localized and translated, have revealed no defects in osk regulation. The evidence that Bruno protein is a repressor of osk translation has been based on the observation that a chimeric transcript consisting of the osk coding region fused to the bcd mRNA 3′UTR into which the Bruno binding sites were inserted, showed increased activity when the gene dosage of Bruno was reduced (Webster et al., 1997). We have now established a cell-free translation system from Drosophila ovaries for the purpose of studying osk mRNA translational regulation. Using this system, as well as Drosophila embryo extracts, we provide the first direct biochemical evidence for the role of Bruno in the repression of osk mRNA translation.

Similar to the situation in vivo, the BREs are required for translational repression of osk chimeric mRNAs in both the ovarian and embryo cell-free systems (Figs 3A and 4B). The ovarian extract, but not the embryo extract from which Bruno is absent, mediates the assembly of a silenced mRNP on a naked BRE-containing template transcribed in vitro (compare Figs 3A and 4A). This process involves one or several titratable factors in the ovarian extract (Fig. 3B). The self-assembly of a translationally silent osk mRNP in the ovarian extract implies that all necessary factors must be present in the soluble phase. This might be biologically important to silence those osk mRNAs that escape the localization process in the oocyte. Importantly, addition of purified recombinant Bruno to embryo extracts results in specific translational inhibition of the BRE-containing mRNA (Fig. 4B).

Translational control plays an important role during early embryonic development in Drosophila. Numerous examples have been described in which a single type of cis-element in the mRNA 5′ and/or 3′UTR is bound by a trans-acting factor to effect regulation. nanos mRNA translation is inhibited at the anterior of the embryo by Smaug, an RNA-binding protein that recognizes a hairpin in the 3′UTR of the message (Smibert et al., 1996; Dahanukar et al., 1999). Similarly, translation of caudal mRNA is repressed at the anterior by binding of the Bicoid homeo-domain protein to a specific region in the 3′UTR (Dubnau, 1996; Rivera-Pomar, 1996). Translation of hunchback mRNA, on the other hand, is inhibited at the posterior by the binding of the Pumilio/Nanos complex to two NREs (Nanos Response Elements) in the 3′UTR (Murata and Wharton, 1995; Sonoda and Wharton, 1999). In yet another example, Sex-lethal inhibits msl-2 mRNA translation by binding to sites located in both UTRs of the transcript (Bashaw and Baker, 1997; Gebauer et al., 1999; Kelley et al., 1997). However, the translational regulation of osk mRNA seems to

Fig. 4. Bruno-dependent repression of osk translation in vitro. (A) Translation of osk chimeric transcripts in embryo extracts. The data represent the average of 5 independent experiments. (B) Translational efficiency of osk chimeric transcripts in embryo extracts, in the presence of increasing amounts of purified recombinant Bruno. The data represent the average of 4 experiments. The values were normalized for those obtained for a luciferase RNA that was co-translated as an internal control. The experimental error of the different data points ranged between 2.19% of the plotted values. The absolute values in the absence of Bruno (defining 100% translation) were approx. 2-19% of the plotted values. The absolute values in the presence of increasing amounts of Bruno (defining 100% translation) were approx. 1-5x103 arbitrary β-galactosidase units/μl reaction. Translation of the luc internal control was unaffected by addition of recombinant Bruno.
be more complex, in that silencing of osk translation may require other factors in addition to Bruno (Gunkel et al., 1998; Lie and Macdonald, 1999; Saffman et al., 1998). Some factors have been identified genetically, others like p50 have been implicated biochemically, but their exact biochemical roles are undefined. Furthermore, once osk mRNA is localized at the posterior pole, its translational derepression requires a cis-acting element in the 5' region of the transcript that is distinct from the BRES (Gunkel et al., 1998). An additional level of complexity is added through the coupling of the translational control of osk mRNA to its localization.

The cell-free system described here provides the first biochemical tool to dissect the complex translational regulation of osk mRNA. Future experiments will validate the utility of this system in the study of the translational control of osk as well as other mRNAs.

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Note added in proof

While this manuscript was under review, Lie and Macdonald reported findings similar to those described in this manuscript (Lie, Y. S. and Macdonald, P. M. Translational regulation of oskar mRNA occurs independent of the cap and poly(A) tail in Drosophila ovarian extracts. Development (1999), 126, 4989-4996).