Rapid ATP-dependent Deadenylation of *nanos* mRNA in a Cell-free System from *Drosophila* Embryos*

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Shortening of the poly(A) tail (deadenylation) is the first and often rate-limiting step in the degradation pathway of most eukaryotic mRNAs and is also used as a means of translational repression, in particular in early embryonic development. The *nanos* mRNA is translationally repressed by the protein Smaug in *Drosophila* embryos. The RNA has a short poly(A) tail at steady state and decays gradually during the first 2–3 h of development. Smaug has recently also been implicated in mRNA deadenylation. To study the mechanism of sequence-dependent deadenylation, we have developed a cell-free system from *Drosophila* embryos that displays rapid deadenylation of *nanos* mRNA. The Smaug response elements contained in the *nanos* 3′-untranslated region are necessary and sufficient to induce deadenylation; thus, Smaug is likely to be involved. Unexpectedly, deadenylation requires the presence of an ATP regeneration system. The activity can be pelleted by ultracentrifugation, and both the Smaug protein and the CCR4-NOT complex, a known deadenylase, are enriched in the active fraction. The same extracts show pronounced translational repression mediated by the Smaug response elements. RNAs lacking a poly(A) tail are poorly translated in the extract; therefore, SRE-dependent deadenylation contributes to translational repression. However, repression is strong even with RNAs either bearing a poly(A) tract that cannot be removed or lacking poly(A) altogether; thus, an additional aspect of translational repression functions independently of deadenylation.

Eukaryotic mRNA precursors undergo a 3′ processing reaction in the cell nucleus in which almost all mRNAs receive a poly(A) tail. Most newly made poly(A) tails are of a more or less uniform, species-specific length, about 250 nucleotides in mammals (1) and 70–90 nucleotides in yeast (2). After export to the cytoplasm, poly(A) tails are gradually shortened. This shortening reaction, termed deadenylation, is the first step in mRNA decay (3, 4). Subsequent decay reactions take place only after the poly(A) tail has been shortened beyond a certain critical limit (5, 6).

Different mRNAs decay at different rates, which are largely determined by the rates of deadenylation; stable RNAs are deadenylated slowly, and unstable RNAs are deadenylated rapidly (4, 7–9). The rapid deadenylation of unstable RNAs is caused by destabilizing elements, for example the so-called AU-rich elements found in the 3′-UTRs of messages like those encoding c-fos or granulocyte-macrophage colony stimulating factor (10). A number of proteins have been identified that bind to destabilizing RNA sequences and accelerate mRNA decay (11–14). Such proteins can promote deadenylation as well as subsequent steps of decay (14–18). MicroRNAs also contribute to accelerated deadenylation (19–21).

In yeast the most prominent deadenylase is the multisubunit CCR4-NOT complex (22, 23), and this complex plays a similar role in *Drosophila* (24–26) and in mammalian cells (27, 28). The PAN2-PAN3 complex, another conserved deadenylase, is thought to act before the CCR4-NOT complex (28, 29) but at least in yeast makes only minor contributions to the total deadenylation activity (23). A third enzyme, the poly(A)-specific ribonuclease (PARN) (30) is not present in all eukaryotes. PARN is involved in an oocyte-specific, sequence-independent deadenylation reaction in *Xenopus* (31) and also seems to act on certain mRNAs in plants (32), but the general role of the enzyme in poly(A) tail metabolism remains poorly defined.

Poly(A) tail shortening also plays an important role in the translational control of mRNAs in oocytes and early embryonic development. Oocytes accumulate large amounts of maternal RNAs that are translationally silent until they are activated at specific stages of oocyte maturation or early development, before the onset of zygotic transcription (33). It has been demonstrated in several different biological systems that translational silencing depends on the shortening of poly(A) tails, whereas translational activation requires poly(A) tail extension (34–36). This function of regulated changes in poly(A) tail length is probably related to the fact that the poly(A) tail and the associated cytoplasmic poly(A) binding protein play a role in the initiation of translation (34, 36, 37). In maturing *Xenopus* oocytes, poly(A) tail shortening is a sequence-independent default reaction that appears to be catalyzed by PARN, as mentioned above. In contrast, deadenylation is message-specific and sequence-dependent during embryogenesis (38–40). This reaction has been reproduced *in vitro* in extracts from *Xenopus* eggs and embryos (41, 42) and has led to the identification of the protein EDEN-BP as an activator of deadenylation (38, 43), but the responsible deadenylase has not been identified.

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2 The abbreviations used are: 3′-UTR, 3′ untranslated region; PARN, poly(A)-specific ribonuclease; SRE, Smaug response element; ATPγS, adenosine 5′-O-(thiotriphosphate); AMPPNP, adenosine 5′-O-(β,γ-imino)triphosphate; AMPPCP, adenosine 5′-(β,γ-methylene)triphosphate; nt, nucleotide(s).
Translational regulation of maternal mRNAs is essential in the formation of the anterior-posterior body axis in the Drosophila embryo. The posterior determinant, the Nanos (Nos) protein (44), together with other proteins binds the maternal hunchback (hb) mRNA, repressing its translation and limiting the expression of Hb protein to the anterior part of the embryo (45, 46). Nanos and its partners induce deadenylation of the hb message (47, 48). Deadenylation contributes to but is not essential for translational repression of hb (46, 49). Nos itself is also subject to translation regulation; a small fraction of nos RNA is localized to the posterior pole of the embryo, and it is only here that the RNA is translated. A Nos protein concentration gradient then develops by diffusion in the embryonic syncytium (50, 51). The large majority of the nos RNA is distributed uniformly throughout the embryo (52, 53). Translation of this unlocalized RNA is repressed by the protein Smaug, which binds two Smaug response elements (SREs) in the proximal part of the nos 3′-UTR (54–57).

Bulk nos RNA has a relatively short poly(A) tail of up to about 50 nucleotides (58, 59). Non-localized nos RNA decays gradually; its degradation is complete at 2–3 h of embryonic development. Importantly, degradation depends on the SREs (53, 56, 57). Several lines of evidence indicated that the regulation of nos RNA might involve poly(A) tail shortening induced by Smaug; a Saccharomyces cerevisiae homolog of Smaug, Vts1p, also binds SREs and leads to accelerated decay of SRE-containing reporter RNAs, which requires the deadenylase subunit Ccr4p (60). In Drosophila, Smaug induces the deadenylation of the unstable hsp83 mRNA by the CCR4-NOT complex (25).

A mechanistic understanding of sequence-dependent accelerated deadenylation will require studies in a cell-free system. Therefore, we have used extracts of Drosophila embryos to examine the regulation of nos mRNA in vitro. We confirm that the nos 3′-UTR causes a very strong translational repression in vitro. We have discovered that the same extracts exhibit a pronounced SRE-dependent deadenylation activity, supporting the prediction of Smaug-induced deadenylation. The activity requires ATP hydrolysis and is associated with rapidly sedimenting particles. Translation is strongly enhanced by the poly(A) tail in the embryo extract, so SRE-dependent deadenylation represses translation. However, a second aspect of repression functions independently of deadenylation.

**EXPERIMENTAL PROCEDURES**

Extracts—Cytoplasmic extract of 0–2-h-old Drosophila embryos (Canton S) was prepared according to Tuschl et al. (61). Nuclease-treated rabbit reticulocyte lysate (62) was either obtained from Promega or donated by A. Ostareck-Lederer and N. Flach.

**DNA Templates for In Vitro Transcription**—Nucleotides 7–161 of the nos 3′-UTR (kindly supplied by D. Curtis and R. Lehmann) were PCR-amplified with oligonucleotides that introduced a 5′ BglIII site and a 3′ BamHI site. The PCR product was cloned into the BglIII and BamHI sites of a BSK vector containing the Photinus pyralis luciferase coding region upstream of the BglIII site and a poly(A) tract of 72 residues downstream of the BamHI site (a gift of F. Gebauer). RNA obtained by transcription of this plasmid is called luciferase nos. Nucleotides 286–433 of the hunchback 3′-UTR were PCR-amplified from the hunchback cDNA (obtained from D. Curtis and R. Lehmann), and the resulting fragment was cloned in an inverted orientation into the BglIII-BamHI site of the luciferase cassette. Transcription resulted in the RNA luciferase control. The two SREs of the nos 3′-UTR were changed by site-directed mutagenesis from CTGGC to CTCGCC (luciferase nos-SRE−RNA). The AU-rich stem region of the nos 3′-UTR (nt 50–62) was changed from TTTATATAACATG to TTA-AAAAAACATG (luciferase nos-IIIA RNA). The same mutation was also introduced into the plasmid already carrying the SRE− double mutation (luciferase nos-SRE−IIIA RNA). For the generation of shortened RNAs to be used in deadenylation assays, most of the luciferase coding region was removed from the plasmids described above by digestion with ScaI and EcoRV and religation. Upon digestion of the DNA templates with HindIII, BamHI, or KpnI, transcription resulted in RNAs carrying a poly(A) tail, lacking a poly(A) tail, or carrying an internal poly(A) region followed by 40 residues of mixed sequence, respectively. The template for the “SRE only” RNA was generated with the help of the synthetic oligonucleotide CAGAGGCCTCTGGCAAGCTTTTGAGCGTTACTAGCGCCTGGC GTTCGAG. The underlined sequences correspond to the two authentic SREs from the nos 3′-UTR, with the loop sequences in bold. A complementary oligonucleotide added overlaps corresponding to a ScaI site upstream and a BamHI site downstream, and the double strand was cloned into the vector described above from which the luciferase coding sequence had been removed by ScaI-BamHI digestion. For the “SRE only” RNA, DNA oligonucleotides were used that generated SRE loops with the mutant sequence CTGCAG. All constructs were confirmed by DNA sequencing. RNAs derived from these templates are shown schematically in Fig. 1.

**In Vitro Translation**—RNAs used for translation assays were synthesized with T3 RNA polymerase (Stratagene) in the presence of 7 mM m7GpppG (New England Biolabs or KEDAR, Warsaw, Poland), 1 mM concentrations each of ATP, CTP, and UTP, and 10 μCi of [α-32P]UTP (Amersham Biosciences). After a 5-min preincubation, GTP was added to a final concentration of 1 mM, and the reaction was left at 37 °C for 2 h. After a 20-min digestion with 1.5 units of RNase-free DNase I (Roche Applied Science), the RNA was phenol-extracted, ethanol-precipitated, dissolved, and purified over a Sephadex G50 spun column. RNA substrates used for deadenylation assays were synthesized as above with UTP reduced to 0.2 mM. When desired, ApppG (New England Biolabs) was used instead of m7GpppG. For later assays, cap analogues were omitted.

**In Vitro Translation Assay**—In vitro translation assays contained 40% (v/v) extract, 2 mM luciferase RNA, 16 mM Heps-KOH, pH 7.4, 100 μM concentrations of an amino acid mixture (Promega), 250 ng/μl S. cerevisiae tRNA (Roche Applied Science), 50 mM potassium acetate, 2.5 mM magnesium acetate, 100 μM spermidine, 20 mM creatine phosphate (Roche Applied Science), 80 ng/μl creatine kinase (Roche Applied Science), 800 μM ATP, and 100 μM GTP. The reaction, containing Drosophila embryo extract or rabbit reticulocyte lysate, was incubated at 26 or 37 °C, respectively, for the
times indicated. Luciferase activity was determined with the luciferase assay system (Promega).

**Deadenylation Assays**—

$^{32}$P-Labeled RNA constructs lacking the luciferase coding region were incubated at 26 °C under conditions similar to the *in vitro* translation assays. 20% (v/v) of embryo extract was used, and the amino acid mixture, and in later experiments, GTP, were omitted. After incubation the RNA was phenol-extracted, precipitated, resolved in a 5% polyacrylamide/urea gel, and detected by phosphorimaging. Quantitative evaluation was as follows; radioactivity corresponding to the deadenylated product and total radioactivity in each lane (the gel area from just above the substrate RNA to just below the deadenylated product) were calculated from the phosphorimaging data, numbers were obtained for the corresponding areas of an empty gel lane were subtracted as background, and the amount of product was expressed as % of total. The apparent amount of product present in unreacted substrate RNA was usually about 1%, and this number was again subtracted as background from deadenylation products generated during the incubation. For ATP depletion, creatine kinase was omitted from the deadenylation reaction, 0.1 units/μl of hexokinase (Roche Applied Science) and 10 mM glucose were added, and the sample was precipitated for 5 min at 26 °C. ATP depletion was confirmed by the addition of a trace amount of [α-$^{32}$P]ATP at the beginning of the preincubation and analysis by thin layer chromatography using polyethyleneimine-cellulose (Merck) and 1 M formic acid. The resulting pellet was resuspended in 15 mM Hepes-KOH, pH 7.4, 50 mM potassium acetate, and 1 mM magnesium acetate. The buffer for RNase H digestions was 50 mM Tris-HCl, pH 7.9, 20 mM dithiothreitol, 75 mM KCl, 3 mM MgCl$_2$, 1 unit/μl of RNasin (Promega).

**Ultracentrifugation**—Embryo extract was centrifuged for 1 h at 4 °C and 50,000 rpm (equivalent to $\times 103,000$) in a Beckman Optima$^\text{TM}$ TLX Ultracentrifuge and a TLA 100.3 rotor. The resulting pellet was resuspended in 15 mM Hepes-KOH, pH 7.4, 50 mM potassium acetate, and 1 mM magnesium acetate.

**Antibodies and Western Blots**—Antibodies to Smaug, Pacman, and Me31B were kindly supplied by R. Wharton, S. Newbury, and A. Nakamura, respectively. Antibodies to CCR4 and POP2/CAF1 have been described (24). Bases 1912–3355 of the NOT1 (CG1884) coding sequence were amplified by reverse transcription-PCR from total RNA prepared from *Drosophila* S2 cells and cloned into the PvuII site of the pRSET C expression vector (Invitrogen). The NOT2 (CG2161) and NOT3 (CG8426) coding sequences were cloned in-frame with an N-terminal His tag. The proteins were expressed in Escherichia coli BL21 Codon+ cells and purified by solubilization from inclusion bodies and chromatography on a Ni$^{2+}$-nitrilotriacetic acid column in 8 M urea. Polyclonal rabbit antibodies were produced by Eurogentec (Belgium). If necessary, SDS-polyacrylamide gel slices containing the antigen of interest were used for immunization. Further details are available upon request. Western blots were done essentially as described (24).

**RESULTS**

**SRE-dependent Deadenylation of nos mRNA in Vitro**—For experiments designed to test a possible SRE- and Smaug-dependent deadenylation, extracts were made from 0–2-h-old *Drosophila* embryos. In this interval of development, translation of non-localized nos mRNA is repressed by Smaug (56). In translation experiments with luciferase reporters, the extracts displayed a very strong repression by the nos 3′-UTR (see below). Three substrate RNAs were initially generated; the nos RNA carried a segment of nucleotides 7–161 from the nos 3′-UTR. This segment contains both SREs and is sufficient for translational control *in vivo* (56, 57). In the control RNA, the nos sequence was replaced by an irrelevant sequence (antisense to nucleotides 286–433 of the *hb* 3′-UTR). The nos-SRE RNA differed from the nos RNA by two point mutations changing the CUGGC loop sequence to CUCGCC in each of the two SREs of the nos 3′-UTR. These point mutations abolish translational regulation and Smaug binding (56). All RNAs were capped and contained a poly(A) tail of 72 nucleotides, encoded in the transcription template, 22 nucleotides downstream of the inserted *Drosophila* sequences. These RNAs and all others used in this work are shown schematically in Fig. 1.

The RNAs were incubated in the embryo extract under conditions similar to those used for translation and then examined by denaturing gel electrophoresis. The nos RNA was rapidly shortened to about the length expected for the completely deadenylated product; the shortened product remained stable (Fig. 2A). Mutation of the two nos SREs abolished deadenylation almost completely (∼20-fold reduction in deadenylation rate). Shortening of the control RNA was also barely detectable, and very little deadenylated product accumulated during the 60-min incubation (Fig. 2A). An RNA in which only the upstream SRE had been inactivated by point mutation was still rapidly deadenylated, although a slight reduction in rate cannot be excluded (data not shown). In additional experiments, rapid shortening of the nos RNA was competed by the addition of an excess of unlabeled RNA corresponding to the nos 3′-UTR but not by the addition of similar amounts of an unrelated RNA (data not shown). In experiments as shown in Fig. 2A, total recoveries of RNA did not decrease significantly with time and were similar for all RNAs. Thus, the SRE-dependent accumulation of the shortened RNA was due to selective shortening of the substrate rather than to selective stabilization of the product. This was confirmed by an experiment showing that RNAs
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FIGURE 1. Deadenylation substrates and translation templates. A, the luciferase plasmid with a DNA-encoded poly(A) tail was the basis of all constructs. The various 3'-UTR sequences were inserted between the BglII and BamHI sites. For deadenylation substrates, much of the luciferase open reading frame (ORF) was removed by digestion with SacI and EcoRV and religation. Linearization of the plasmids by HindIII led to RNAs carrying a poly(A) tail 3' nucleotides upstream of the 3' end (A72N40), whereas linearization by KpnI yielded RNAs in which the poly(A) tract was followed by 40 random nucleotides upstream of the 3' end (A72N40).

B = A72N40
control RNA

- SRE A72N3
nos RNA

- SRE A72N3
nos SRE RNA

- SRE A72N3
nos IIIA RNA

- SRE A72N3
nos SRE IIIA RNA

- SRE A72N40
nos N40 RNA

- SRE A72N40
nos SRE N40 RNA

C = SRE A72N3
SRE only

- SRE A72N3
SRE only

as above lacking a poly(A) tail were stable in extract (data not shown).

Deadenylation of the reaction products was probed by RNase H digestion in the presence of oligo(dT). RNase H/oligo(dT) digestion of the polyadenylated substrate RNA provided a control and a size marker for completely deadenylated RNA (Fig. 2B). Digestion of the reaction products purified after a 60-min incubation in extract (extract product) resulted in two bands. The major RNA species was resistant to RNase H; thus, it had been completely deadenylated in the extract. In contrast, a minor population of partially shortened RNA species was further shortened by RNase H to the size of completely deadenylated RNA; thus, they represented partially deadenylated reaction intermediates. The main extract product was slightly shorter than the fully deadenylated RNA produced by RNase H. For further analysis, substrate RNA and extract product were digested with RNase H in the presence of both oligo(dT) and an oligonucleotide complementary to an internal sequence of the nos RNA. Under these conditions, the 5' fragment obtained by internal cleavage of the extract product was indistinguishable from the 5' fragment originating from the substrate RNA. In contrast, the 3' fragment derived from extract product was slightly shorter than the corresponding fragment generated from the substrate RNA (Fig. 2C). Thus, during incubation in extract, the RNA substrate had been shortened by an additional ~10 nucleotides beyond removal of the poly(A) tail.

The SREs Are Sufficient to Induce Deadenylation—The nos 3'-UTR region responsible for translational repression contains a third predicted stem-loop structure (stem-loop III in the nomenclature of (63)), which is bound by the translational repressor Glorund, active during oogenesis (64). A triple point mutation disrupting three AU base pairs in this stem (U52A, U54A, U56A; mutation IIIA of Crucs et al. (63)) interferes with nos regulation in oocytes (63, 65). Introduction of the IIIA mutation into the nos construct did not affect deadenylation (Fig. 2D). An additional pair of substrate RNAs was produced (SRE only and SRE only) in which nos sequences were limited to two segments of 21 and 19 nt, forming a pair of SRE stem-loop sequences (wild type or mutant as above) separated by an 8-nt spacer compared with a sequence of 75 nt separating the SREs in the nos 3'-UTR. Upon incubation in embryo extract, the wild-type SRE-only RNA was deadenylated with a rate comparable with that of the nos RNA, whereas the SRE only RNA was stable. Thus, the SREs are sufficient to induce rapid deadenylation (Fig. 2E). Three copies of the SRE are sufficient for nos regulation in embryos (56).

Deadenylation Is Catalyzed by a 3' Exonuclease—The partially deadenylated reaction intermediates were consistent with either exonucleolytic shortening of the RNA or digestion by an endonuclease cutting randomly within the poly(A) tail. When the substrate RNAs were modified by the inclusion of 40 additional nucleotides of mixed sequence downstream of the poly(A) tail, they were almost completely stable in the extract (Fig. 3). Thus, deadenylation of the regular substrate is catalyzed by a 3' exonuclease.

Deadenylation Is Cap-independent—Initially, all RNA substrates contained a regular m7G cap at their 5' ends. This cap is recognized by translation initiation factors and also facilitates deadenylation by vertebrate PARN (66–68). Deadenylation of nos RNA with a m7G cap was compared with deadenylation of RNA carrying an A cap. The latter will prevent 5' exonucleolytic RNA degradation but is not recognized by cap-binding proteins or by PARN. The rates of deadenylation of both RNAs were indistinguishable (Fig. 4). RNAs bearing no cap at all
behaved in the same manner as the capped RNAs (data not shown). Therefore, the translation initiation complex does not affect deadenylation, and the reaction is not catalyzed by a cap-dependent enzyme. In later experiments, uncapped RNAs were used routinely.

Deadenylation Depends on ATP—Incubation conditions employed for initial deadenylation assays involved the addition of ATP, GTP, and of creatine phosphate and creatine kinase as an ATP regenerating system, as used for translation assays. Omission of GTP had no effect on SRE-dependent deadenylation (data not shown). In the following experiments, designed to test the ATP requirement of the reaction, creatine phosphate was always maintained at 20 mM. This avoided potential complications due to effects unrelated to the maintenance of the ATP level, e.g. ionic effects. For ATP depletion, creatine kinase was omitted from the reaction, hexokinase and glucose were

![FIGURE 2](http://www.jbc.org/)

**FIGURE 2.** The nos 3′-UTR induces rapid deadenylation. A, the 32P-labeled RNA substrates indicated above the gel lanes were incubated with Drosophila embryo extract. At the times indicated, the RNA was isolated and separated on a denaturing polyacrylamide gel. S, unreacted substrate RNA. The percentage of fully deadenylated product is shown below each lane. The migration of DNA markers is indicated on the left. B, the shortened RNA is deadenylated. Both the nos RNA substrate (S) and the product isolated after 60 min of incubation in extract (60') were analyzed by RNase H digestion in the presence of dT12 followed by denaturing gel electrophoresis. The migration of DNA markers is indicated on the left. C, 3′ exonuclease digestion continues into the 3′-UTR. Both the nos RNA substrate (S) and the product isolated after 60 min of incubation in extract (60') were analyzed by RNase H digestion in the presence or absence of dT12 and an oligonucleotide hybridizing to an internal sequence of the nos RNA. The 5′ fragment generated by internal cleavage is indicated on the left. The asterisks (*) indicate the corresponding 3′ fragments. DNA markers are indicated on the right. D, stem-loop III is not required for rapid deadenylation. The experiment was carried out as in A with the RNAs indicated above the gel lanes. E, the SREs are sufficient for rapid deadenylation. The experiment was carried out as in A with the RNAs indicated above the gel lanes. The RNAs carried A caps. In a second experiment, the SRE only RNA was tested side by side with the nos RNA; the rates of deadenylation were indistinguishable.

![FIGURE 3](http://www.jbc.org/)

**FIGURE 3.** The SRE-mediated deadenylation is carried out by a 3′ exonuclease. The 32P-labeled RNA substrates indicated above the gel lanes, carrying an internal poly(A) sequence, were incubated with Drosophila embryo extract. At the times indicated, the RNA was isolated and separated on a denaturing polyacrylamide gel. DNA markers are indicated on the left. S, unreacted substrate RNA. The positive control for this experiment (rapid deadenylation of nos RNA) was the reaction shown in Fig. 4, which was carried out in parallel.

![FIGURE 4](http://www.jbc.org/)

**FIGURE 4.** The SRE-mediated deadenylation is cap-independent. 32P-Labeled nos and nos-SRE RNA substrates carrying either a 7-methylguanosyl (m7G) or an adenosyl (A) cap structure were incubated with Drosophila embryo extract. At the times indicated, the RNA was isolated and analyzed on a denaturing polyacrylamide gel. DNA markers are indicated on the left. S, unreacted substrate RNA. The percentage of fully deadenylated product is given below each lane.
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Upon ATP depletion, the nos RNA was almost completely stable (Fig. 5A). Thus, rapid deadenylation requires either ATP or a nucleoside triphosphate in equilibrium with ATP. When, under conditions of ATP depletion, the reactions were supplemented with the nonhydrolyzable or poorly hydrolyzable ATP analogs ATPγS, AMPPNP, or AMPPCP, rapid deadenylation was not restored (data not shown). Thus, the SRE-dependent deadenylation reaction most likely requires ATP hydrolysis. However, the nonhydrolyzable ATP analogs were not inhibitory in the presence of ATP; therefore, their failure to support the reaction in the absence of ATP might be caused by an inability of the ATP-dependent protein(s) to bind them.

The SRE Affects Deadenylation, Not Polyadenylation—An induction of deadenylation by the SREs is the simplest explanation for the results described so far. However, because the assays were done in the presence of ATP, the data are at first glance also consistent with an alternative model whereby the control RNAs would remain stable due to a balance between a sequence-independent default deadenylation and polyadenylation, whereas shortening of the nos RNA would be the result of default deadenylation combined with an SRE-dependent inhibition of polyadenylation. This model is excluded by three experimental observations. First, when polyadenylation was prevented by ATP depletion, both RNAs remained stable; no default deadenylation became visible (Fig. 5A). Second, when nos RNA and control RNAs lacking poly(A) tails were incubated in the extract in the presence of ATP, neither RNA was polyadenylated; the addition of 10 AMP residues would have been detectable in the electrophoretic analysis (data not shown). Third, ATP could be substituted by 3′-dATP in the deadenylation assay without any effect on SRE-dependent deadenylation (Fig. 5B). Because 3′-dATP acts as a chain terminator in polyadenylation, this result provides strong evidence that polyadenylation does not play a role in SRE-dependent poly(A) tail shortening.

The Deadenylating Activity Can Be Pelleted—Centrifugation of embryo extracts for 1 h at 100,000 × g in a table-top ultracentrifuge resulted in three fractions; a clear upper supernatant, a turbid lower supernatant, and a pellet. The latter was dissolved in buffer, and all three fractions were assayed for SRE-dependent deadenylation. Whereas the upper supernatant had a weak SRE-independent deadenylation activity and the lower supernatant had no detectable deadenylation activity at all, essentially full SRE-dependent activity was recovered in the resuspended pellet (Fig. 6A). The sedimentation of candidate proteins that might be involved in the SRE-dependent deadenylation was examined by Western blot assays of the three fractions. These

FIGURE 6. The SRE-mediated deadenylation activity can be sedimented. A, 32P-labeled nos RNA or control RNA were incubated with the three fractions obtained by centrifugation of the extract; that is, the upper and lower supernatant and the resuspended pellet. Equivalent volumes of the three fractions were used for the assay. The RNA was isolated at the time points indicated and analyzed on denaturing polyacrylamide gels. The percentage of fully deadenylated product is indicated below each lane. DNA markers are indicated on the left. S, unreacted substrate RNA; B, 3′-dATP can substitute for ATP. Deadenylation time courses were carried out with the substrate RNAs indicated. For the two time courses on the right, 3′-dATP was added instead of ATP. Migration of DNA markers is indicated on the left. Numbers below the gel lanes indicate the amount of fully deadenylated RNA. DNA markers are shown on the left. S, unreacted substrate RNA.

The SRE-mediated deadenylation activity was sedimented. A, 32P-labeled nos RNA was incubated with Drosophila embryo extract. At the times indicated, the RNA was isolated and separated on a denaturing polyacrylamide gel. The time course shown on the left was carried out under standard deadenylation reaction conditions, i.e. in the presence of creatine kinase (CK). The time course on the right was done in the absence of creatine kinase and in the presence of hexokinase (HK) and glucose (for details, see “Experimental Procedures”). Note that both reactions initially contained (80) μM ATP. The percentage of fully deadenylated product is indicated below each lane. DNA markers are indicated on the left. S, unreacted substrate RNA. B, 3′-dATP can substitute for ATP. Deadenylation time courses were carried out with the substrate RNAs indicated. For the two time courses on the right, 3′-dATP was added instead of ATP. Migration of DNA markers is indicated on the left. Numbers below the gel lanes indicate the amount of fully deadenylated RNA. DNA markers are shown on the left. S, unreacted substrate RNA.
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revealed that the Smaug protein was quantitatively recovered in the pellet. The NOT1, NOT2, NOT3, and NOT4 subunits of the CCR4-NOT complex were also recovered in the pellet, whereas the CCR4 and CAF1 subunits were enriched in the pellet and present to a lesser degree in the lower supernatant (Fig. 6B). Partial separation of the subunits of the CCR4-NOT complex suggests either the presence of an excess of the CCR4 and CAF1 proteins or a relatively loose association. Two enzymes involved in the 5′ degradation of mRNA, the 5′ exonuclease Pacman (69) (orthologue of yeast Xrn1p) and the helicase Me31B (70) (orthologue of yeast Dhh1p), were also pelleted under the same conditions (Fig. 6B).

The Role of Deadenylation in Translational Control—RNAs similar to those used for deadenylation assays were synthesized that contained the luciferase coding sequence. Capped and polyadenylated RNAs were incubated in extract under translation conditions, and translation efficiencies were quantified by luciferase activity measurements. The RNA concentration (2 nM) was within the range that gave a linear dependence of luciferase yield on RNA concentration. Under these conditions translation of the luciferase nos RNA was repressed at least 4-fold in all batches of extract compared with the luciferase control RNA. A much stronger repression was reproducibly obtained with the best batches of extract (Fig. 7A); luciferase nos RNA was translated with a yield of 7.3 ± 0.2% (n = 3) compared with luciferase control RNA, whereas the translation yield of the luciferase nos-SRE− RNA was 60.6 ± 6.2% (n = 3) (relative translation yields refer to the 1-h time point). Thus, the extracts reproduce the translational regulation of nos RNA. Point mutations in the SREs relieve the translational repression to a large but not the full extent. All RNA preparations were controlled by translation in a rabbit reticulocyte lysate; no significant differences were observed. All RNAs had similar stabilities in the embryo extracts; thus, differential decay does not account for the differences in luciferase yields (data not shown).

FIGURE 7. The SRE-dependent translational repression involves deadenylation and a second, deadenylation-independent mechanism. A, polyadenylated luciferase RNAs carrying the nos 3′-UTR fragment, the point-mutated fragment (nos-SRE−), or a control sequence were incubated with Drosophila embryo extract under translation conditions. At the times indicated aliquots from the translation reaction were transferred to a luciferase activity assay. B, the nos 3′-UTR inhibits the translation of mRNAs lacking a poly(A) tail. The same experiment as in A was carried out with a similar set of RNAs lacking poly(A) tails. The curves in A and B represent individual experiments, which were repeated two more times with very similar results.

FIGURE 8. The SREs inhibit the translation of RNAs that cannot be deadenylation. Translation assays were carried out in embryo extract with the luciferase RNAs indicated. Luciferase activity was measured after 1 h. For ease of comparison luciferase yield of the control RNA was set to 100% for each set of five RNAs. Absolute values were between 2.1 × 10^6 and 2.5 × 10^6 relative light units for the control RNA with a poly(A) tail, between 0.22 × 10^6 and 0.44 × 10^6 relative light units for the control RNA lacking a poly(A) tail, and between 1.4 and 3.5 × 10^5 relative light units for the control RNA with an internal poly(A) tract. The data represent the average ± S.D. of four independent translation experiments with four batches of extract and two batches of transcripts. In each experiment all 15 RNAs were tested in parallel. The data of Fig. 7 were not included here because the complete set of RNAs was not tested in those experiments.
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Deadenylation was only partially relieved by the same mutations. The IIIA mutation, which did not affect deadenylation, relieved translational repression to a small extent on its own, and the combination of the SRE mutations with the IIIA mutation completely abolished translation repression (Fig. 8). Thus, it appears that Glorund, the binding site that is affected by the IIIA mutation, plays a role in translational regulation but not in deadenylation in the embryo extracts.

In these experiments differential polyadenylation during the translation assay can be excluded as an explanation for differences in translational yield. One set of RNAs was stably polyadenylated anyway, and polyadenylation activity in the extracts was not detectable with the short RNA substrates, as noted above.

DISCUSSION

The data reported in this paper provide direct evidence supporting the prediction (25) that Smaug induces deadenylation of the nanos RNA. The system that we describe here should be amenable to biochemical resolution and reconstitution and also offers the promise of combining biochemistry with Droso-philica genetics. Thus, a detailed mechanistic analysis of this reaction should become possible. Because the CCR4-NOT complex is implicated in the deadenylation not just of nanos but of many RNAs in all eukaryotes, the results should be relevant for sequence-dependent rapid deadenylation in general.

Regulation of deadenylation has usually been discussed in terms of the simplest possible model. A sequence-specific RNA-binding protein associates with destabilizing sequences and then recruits a deadenylating enzyme. As suggested by the effect of the SRE mutations, the sequence-specific RNA-binding protein responsible for in vitro deadenylation of the nanos RNA is Smaug. Both the CCR4 dependence of the yeast Vts1 protein effect on RNA stability (60) and the association of Smaug with the Droso-philica CCR4-NOT complex (25) argue that the CCR4-NOT complex is responsible for the in vitro reaction described here, and strong genetic evidence for the function of both Smaug and the CCR4-NOT complex in nanos deadenylation has been obtained by Martine Simonelig and co-workers.3 Consistently, both Smaug and the CCR4-NOT complex are found in the enriched fraction obtained by centrifugation. However, the ATP dependence of the in vitro reaction is inconsistent with the simple model that SRE-bound Smaug merely recruits the CCR4-NOT complex. The activity of the CCR4-NOT complex is ATP-independent in vitro (23)4 and so is RNA binding by Smaug (60). Thus, the mechanism is likely to include at least one additional, ATP-requiring step and one or more additional proteins. Very recently, Wickens and co-workers (71) reported that the yeast Mpt5 protein, a member of the RNA binding family of PUF proteins, recruits the CCR4-NOT complex via interaction with the Pop2p subunit and promotes deadenylation of an RNA containing an Mpt5p binding site. Reconstitution of the reaction from affinity-purified proteins in vitro suggested that no other proteins were required and the reaction was ATP-independent (71). Thus, the crude embryo extract may contain other components that impose an ATP requirement on the deadenylation reaction.

Resistance of internal poly(A) to degradation clearly indicates that a 3’ exonuclease is responsible for deadenylation. The reaction is sensitive to EDTA (data not shown). However, no conclusion regarding the metal dependence of the exonuclease can be drawn since ATPases also depend on divalent cations. The enzyme is unlikely to be absolutely poly(A)-specific, since transcription of the HindIII site used for linearization of the DNA template is expected to result in three non-A residues at the 3’ end of the DNA-encoded poly(A) tail (Fig. 1). Whether the additional ~10 nucleotides digested beyond the poly(A) tail are removed by the deadenylase or by a different enzyme remains unknown. However, partial invasion of the non-poly(A) part of the RNA can be seen in some published experiments with affinity-purified yeast CCR4-NOT complex (71, 72). The POP2/CAF1 subunit of the CCR4-NOT complex is able to digest sequences other than poly(A) (73, 74).

In the cell-free system, the nanos RNA is rapidly deadenylated to completion. In contrast, the nanos mRNA carries a poly(A) tail of up to about 50 nt in length in vivo. The reason for this discrepancy is unknown. A balance between deadenylation and polyadenylation in vivo may play a role. It is also possible that the association of repressed nanos mRNA with polysomes (75) prevents complete deadenylation. In most batches of extract, the deadenylated product was stable. Possibly, enzymes responsible for the degradation steps subsequent to deadenylation are not fully active at early times of development (53). Accumulation of the deadenylated product is a distinct technical advantage for the biochemical analysis. Deadenylation, as opposed to other (nonspecific) pathways of RNA degradation, becomes obvious, and reaction rates can be calculated from the appearance of the specific product, not just from the disappearance of the substrate.

The deadenylation activity can be pelleted by centrifugation. Preliminary results from centrifugations under different conditions suggest that the activity sediments at ~90 S or more. It is possible that the activity is associated with ribosomes. A perhaps more interesting possibility would be that it is associated with P bodies or related complexes. P bodies, also called GW bodies or Dcp1 bodies, are cytoplasmic structures, identified by immunofluorescence experiments that contain most of the proteins involved in 5’-3’ decay of mRNA and also mRNA decay intermediates (76–78). Two proteins found in P bodies of yeast and mammals are the Dhh1 helicase and the 5’ exonuclease Xrn1. The CCR4-NOT complex is associated with P bodies in mammalian cells (76) and under some conditions also in yeast (78, 79). In Droso-philica, co-localization studies of P body constituents have not been reported, but subunits of the CCR4-NOT complex exist in cytoplasmic foci in oocytes and early embryos (24). In oocytes, the Dhh1 orthologue Me31B is found in cytoplasmic foci that also contain localizing, translationally repressed mRNAs such as nanos. Translational repression is compromised when Me31B is lost (70). All P body compo-

3 M. Simonelig, personal communication.
4 C. Temme and E. Wahle, unpublished data.
5 C. Temme and M. Simonelig, unpublished data.
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dents tested, Me31B, the Xrn1-orthologue Pacman, and the CCR4-NOT complex, are enriched in the pellet fraction active in SRE-dependent deadenylation.

As reported before (55), SRE-dependent repression of translation is also reproduced in the embryo extract. Because translation in the extract is strongly poly(A)-dependent, SRE-dependent deadenylation would suffice for translational repression. However, repression also functions independently of deadenylation. Point mutations in the SREs largely eliminate translational repression of RNAs that cannot be deadenylated, as is particularly evident with RNAs carrying an internal poly(A) tract (Fig. 8). Thus, Smaug is almost certainly responsible not only for deadenylation but also for the deadenylation-independent aspect of repression. Translational repression of the nos RNA by both deadenylation and a second mechanism is reminiscent of the situation with the hunchback RNA (49) and is also consistent with earlier data. One mechanism appears to act during elongation, as suggested by the involvement of a subunit of the nascent polypeptide associated complex, Bicaudal, (80) and by polysome analysis (75). Although it remains to be elucidated how this aspect of translational repression operates, there is no known mechanism by which the poly(A) tail would affect translational elongation. A second mode of repression is believed to affect initiation and depends on the interaction of Smaug with the protein Cup. Cup can simultaneously bind the initiation factor eIF4E, disrupting the interaction between eIF4E and eIF4G, which is important for translation initiation. Therefore, translation initiation may be inhibited by the recruitment of Cup through Smaug bound to the 3′-UTR (81–83). A similar mechanism has recently been directly confirmed by measurements of translation preinitiation complex formation for the oskar mRNA, which is also regulated in a Cup-dependent manner (84). This mechanism is similar to the way in which the protein Maskin has been proposed to repress mRNAs in Xenopus (85). Repression by Maskin is sensitive to the length of the poly(A) tail (86). Cup is an orthologue of the mammalian protein eIF4E-T (81), which has recently been found in P bodies and also plays a role in mRNA decay (87, 88). Thus, it is tempting to speculate that an additional function of Cup may lie in the deadenylation and/or degradation of nos RNA.

A weak effect of the IIIA mutation on the initiation of translation by the nos 3′-UTR in embryo extracts has been reported before (65). The Glorund protein is present during the first hours of embryogenesis, and it has been proposed that its repressive effect may be relevant at the very beginning of embryogenesis, whereas Smaug accumulates (64). The very early stages of embryogenesis were represented among the embryos used for extract preparation. After the experiments reported here were complete, it was reported that the IIIA mutation is not as disruptive to Glorund binding in vitro as one would have expected from the phenotypic effect of the mutation in development (64). It remains to be seen whether use of a more severe mutation will reveal a more prominent role of Glorund.

Our data together with the observation that the SREs are responsible for the instability of the nos RNA (53, 56, 57) suggest that the biological function of deadenylation is not only a direct translational repression of nos but also to prepare the non-localized RNA for degradation. This in turn contributes to the localization of translation to the posterior pole of the embryo.

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