Degradation of a Developmentally Regulated mRNA in *Xenopus* Embryos Is Controlled by the 3’ Region and Requires the Translation of Another Maternal mRNA

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By injecting the appropriately constructed plasmids into one-cell *Xenopus* embryos, we determined that the 3’ region of the maternal *Xenopus* Eg2 mRNA confers instability on the chimeric mRNA transcribed from these plasmids. This instability, like that of the maternal Eg2 transcript, was abolished by treatment of the embryos with cycloheximide. Analysis of the polysome distribution of the maternal Eg2 mRNA in cycloheximide-treated and untreated embryos showed that Eg2 mRNA was released from polysomes after fertilization and that the stabilization caused by cycloheximide treatment was not due to a reloading of ribosomes onto the mRNA. Insertion of a stable hairpin loop (ΔG = −50 kcal/mol) 5’ to the reporter gene in the injected plasmid caused a 10- to 20-fold decrease in translation from the transcribed mRNAs. This decrease in translation did not abolish the instability conferred by the 3’ Eg2 region. Therefore, the degradation of these chimeric mRNAs in *Xenopus* embryos requires the translation of another maternal mRNA coding for a trans-acting factor involved in mRNA degradation. Further restriction of the 3’ Eg2 region, placed 3’ to the reporter gene, showed that a cis-acting instability-conferring sequence is contained in a 497-nucleotide fragment.

cis-acting sequence elements that confer instability on mRNA by a nonautogenous feedback mechanism have been characterized in several mRNAs (13, 22, 33, 34, 40). However, the proteins which bind to these cis-acting elements have only been identified for the motifs present in transferrin receptor mRNA (18, 23) and the AU-rich instability motifs (20; see reference 5 for a review). This lack of information about the potential trans-acting factors involved in RNA stability suggests that new experimental models will be required for their identification and purification. Differential regulation of mRNA stability is a characteristic of early embryonic development. In most species, the first hours of embryonic development, which progress in the absence of transcription, are dependent on a store of maternal mRNA accumulated during oogenesis (8).

In *Xenopus laevis*, certain of the oocyte mRNAs, such as those coding for ribosomal proteins (29), zinc finger protein Xfin (31), the U1 70-kDa (U1 70K) small nuclear ribonucleoprotein particle protein (10), and α1 gap junction proteins (11), are degraded during the meiotic maturation of the fully grown oocyte into the egg. Others are retained, at least partially, until transcription of the zygotic genome is initiated. These include the tumor growth factor β-related protein (Vg1) (30), c-mos (32), histone H1-like protein (B4) (36), D7 protein (37), and lamin LIII (39).

The store of maternal mRNA in *Xenopus* eggs allows the embryo to perform the first 12 mitotic cell cycles in the absence of gene transcription (24). Hence, only posttranscriptional modes of gene regulation are effective during this period. Transcription is initiated during the 12th cell cycle at the midblastula transition (MBT) (35). This means that the factors (trans-acting) which control posttranscriptional regulation in early embryos must be stored as proteins or maternal RNAs in the egg, a situation which should facilitate their identification and purification.

We have recently described several maternal mRNAs which are deadenylated after fertilization (26) but remain stable as poly(A)+ mRNA until the MBT (25), after which they are degraded (9). Therefore, the deadenylation and the degradation of the body of these maternal *Xenopus* mRNAs are uncoupled. This is a particularly interesting situation allowing the two processes to be studied separately. The uncoupling of deadenylation and degradation is illustrated by the observation (9) that treatment of the embryos with the inhibitor of protein synthesis cycloheximide leads to a stabilization of these already deadenylated mRNAs. This also suggests that either the translation of these mRNAs is a prerequisite for their degradation or the trans-acting factors which trigger their degradation only accumulate after the MBT.

In the work described here, we studied the destabilization of one of these mRNAs (Eg2) (26). The region of an mRNA that confers instability on a transcript in post-MBT *Xenopus* embryos can be studied either by measuring the degradation kinetics of an injected vitro transcript after the MBT or by measuring the steady-state level of mRNA transcribed in the embryos from injected plasmids. Due to constraints associated with microinjection into embryos, we found that it was not technically possible to inject a sufficiently large number of embryos to define correctly the degradation kinetics of the transcripts studied here. Therefore, plasmids coding for different chimeric mRNAs were injected into one-cell embryos and the steady-state amount of the mRNAs was analyzed. In this way, we delimited a region of Eg2 mRNA which targets the chimeric mRNAs for rapid degradation in post-MBT embryos. Characterization of the mechanism by which cycloheximide stabilizes the Eg2 mRNA showed that degradation of this mRNA does not require its own translation. As a control, we also analyzed the stability of a chimeric mRNA containing the 3’ region of *Xenopus* ome-

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thine decarboxylase (ODC), a maternal mRNA which is stable during early Xenopus development (9, 25).

MATERIALS AND METHODS

Plasmid constructions. Plasmids were constructed by standard procedures (21). The two parental plasmids used were MSV-CAT (16) and HS-CAT (3), in which the chloramphenicol acetyltransferase (CAT) reporter gene is under the control of the Moloney sarcoma virus (MSV) and the heat shock (HS) promoters, respectively.

MSV-CAT 3' Eg2 was constructed as follows. The HindIII site of Eg2 cDNA, situated one nucleotide after the polyadenylation signal, was filled in and ligated with BamHI linkers. The 380-bp BglII-BamHI fragment containing the last 129 bp of the coding region, the entire 3' untranslated region, and the BamHI linker site was then isolated and subcloned into the BamHI site of MSV-CAT. This places the 3' Eg2 sequence between the cat gene and the simian virus 40 (SV40) polyadenylation signal.

For MSV-CAT 3' ODC, the 380-bp BglII-BamHI fragment of Xenopus ODC cDNA (2) containing the last 89 bp of the coding region, the complete 3' untranslated region, and the EcoRI-BamHI poly linker sequence from the Bluescript plasmid was similarly subcloned into MSV-CAT linearized by BamHI.

Plasmid HS-CAT 3' Eg2 was constructed by using the same strategy with the parental HS-CAT plasmid. Plasmid HS-CAT XbaI 3' Eg2 was constructed by blunt ending the BglII-BamHI 3' Eg2 fragment and subcloning it into the filled-in XbaI site downstream of the SV40 polyadenylation signal in the HS-CAT plasmid.

Eg2 deletions (see Fig. 7A) were obtained as follows. The Eg2 EcoRI-HindIII fragment was digested with StuI, and the resulting fragments EcoRI-StuI (497 bp) and StuI-HindIII (410 bp) were blunt ended and subcloned into the BamHI site of MSV-CAT as above, to give MSV-CAT Eg2(497) and MSV-CAT Eg2(410).

Plasmids Hp-CAT, 17a-CAT, 17b-CAT, Hp-CAT 3'Eg2, 17a-CAT 3'Eg2, and 17b-CAT 3'Eg2 were constructed as follows. The EcoRI-HindIII fragments containing the SV40 promoter associated with the hairpin structure (Hp) or one-half of the hairpin (17a and 17b) were isolated from plasmids B35-Hp, B35-17a, and B35-17b, respectively (19). These fragments were subcloned into the EcoRI-HindIII site of pSPT19 to give pSPT19-Hp, pSPT19-17a, and pSPT19-17b. Then, the EcoRV-XbaI fragment containing the CAT gene and the SV40 polyadenylation signal was isolated from the HS-CAT plasmid, and the SmaI-XbaI fragment containing the CAT gene, the 3' Eg2 region previously described, and the SV40 polyadenylation signal was isolated from HS-CAT 3'Eg2. These fragments were subcloned into pSPT19-Hp, pSPT19-17a, and pSPT19-17b, linearized by BamHI, and blunt ended to give HP-CAT, 17a-CAT, 17b-CAT, Hp-CAT 3'Eg2, 17a-CAT 3'Eg2, and 17b-CAT 3'Eg2.

Plasmids were purified through cesium chloride gradients for embryo injections.

Embryo injections. Eggs were obtained and fertilized by standard procedures and incubated in F1 solution as previously described (25). Embryos were injected with 20 nl of DNA solutions containing 125 pg of plasmid (except when noted) at any time from 60 min postfertilization at 22°C to the two-cell stage and incubated for the indicated times. Pools of 5 to 10 embryos at the appropriate stages were collected, rapidly frozen, and stored at −70°C.

For polysome analysis, cycloheximide treatment of embryos dissociated in Ca²⁺/Mg²⁺-free medium (14) was performed as previously described (9). Briefly, dissociated embryos were incubated with cycloheximide (10 μg/ml) from 6 to 7 h postfertilization and then transferred to fresh medium without cycloheximide. They were left to develop for 1 h and then homogenized as described below. For injected embryos, incubation in the Ca²⁺/Mg²⁺-free medium led to inhibition of the MSV and HS promoters, and in addition, the blastomers of the injected embryos lysed in this medium. Therefore, cycloheximide treatment of injected embryos was achieved by their direct incubation in F1 medium supplemented with 200 μg of cycloheximide per ml.

RNA and DNA analysis. Each batch of embryos, still frozen, was homogenized in 0.25 M Tris-HCl (pH 8) (50 μl per embryo). When CAT activity was to be assayed, a 50-μl sample was withdrawn and treated as described below. To the remaining material, 1 volume of 2% sodium dodecyl sulfate–0.2 M NaCl–60 mM EDTA was added, and nucleic acids were extracted by the proteinase K-phenol method (15). Northern (RNA) blot analysis was performed as previously described (9). For DNA slot blots, DNA was denatured for 15 min at 70°C in 0.2 M NaOH and neutralized by adding ammonium acetate.

Poly(A)⁺ RNA was isolated by oligo(dT)-cellulose chromatography (1). Polysomal and nonpolysomal RNAs were separated as described by Bassez et al. (2). The recovery of total RNA in the samples was verified by ethidium bromide staining of the samples electrophoresed in nondenaturing agarose gels.

CAT assays. One volume of 0.45 M Tris-HCl (pH 8) was added to the embryo homogenate (see above), heated for 5 min at 55°C, and centrifuged for 5 min at 13,000 × g, and the yolk-free supernatant was stored at −70°C. Enzyme assays were performed as described previously (12).

RESULTS

Role of 3' region in destabilization of Eg2 mRNA. To determine whether the 3' region of Eg2 mRNA is implicated in the destabilization of this mRNA after the MBT, we constructed plasmids which contained (in order 5' to 3') the MSV promoter, the CAT reporter gene, a 3' sequence of either Eg2 or ODC maternal mRNAs, and the SV40 polyadenylation signal (respectively, MSV-CAT 3'Eg2 and MSV-CAT 3'ODC). The plasmid (MSV-CAT) did not contain any sequences corresponding to the 3' region of maternal mRNAs. To show that the eventual effects of the 3' sequences were not promoter specific, we also made similar constructions using the heat shock (HS) promoter. Comparable results were obtained with both series of plasmids.

Initially, we determined the time course for the accumulation in the embryo of the mRNAs transcribed from these plasmids after the MBT. Northern analyses of a typical experiment, in which the embryos had been injected with a mix of either MSV-CAT and MSV-CAT 3'ODC or MSV-CAT and MSV-CAT 3'Eg2, are shown in Fig. 1A and B, respectively. ODC mRNA does not decrease in amount after the MBT (9), which in these experiments occurred at about 6 h postfertilization, and therefore the signals from this mRNA in the upper part of the figure show that approximately equivalent amounts of mRNA were loaded for the different samples. The mRNAs transcribed from both the MSV-CAT and MSV-CAT 3'ODC plasmids were not detected in the 6-h-old embryos but had started to accumulate in the 9-h-old embryos (3 h after the MBT). The amount of these two chimeric mRNAs attained a plateau between 12
FIG. 1. (A) Kinetics of CAT and CAT 3’ODC mRNA accumulation, endogenously transcribed in Xenopus embryos. Embryos were injected with 125 pg of each plasmid, MSV CAT and MSV CAT 3’ODC, at the one-cell stage, and the embryos were then left to develop at 22°C. Total RNA was isolated from batches of 5 to 10 embryos at the indicated times after fertilization and subjected to Northern analysis. One embryo equivalent was loaded for each time point. The same membrane was first hybridized with a 3P-labeled CAT cDNA probe (bottom panel) and then with a 3P-labeled Xenopus ODC cDNA probe (top panel). (B) Kinetics of CAT and CAT 3’Eg2 mRNA accumulation. Experimental conditions were as described for panel A except that a mixture of MSV CAT and MSV CAT 3’Eg2 was injected into embryos and the autoradiogram exposure time was three times longer.

and 14 h of development and did not change afterward. The first two lanes in Fig. 1A correspond to RNA extracted from uninjected embryos and show that in the absence of the plasmids, no RNAs hybridizing with the CAT probe were detected.

In these experiments, the two chimeric mRNAs (CAT and CAT 3’ODC) were visualized by a probe to the CAT gene which is common to both transcripts. As the same amounts of both plasmids were co-injected, the similarity of the Northern signals shows that the 3’ sequence of the maternal ODC mRNA does not affect the accumulation of the chimeric mRNA. This was confirmed by densitometer quantification of the autoradiograms (data not shown).

For the embryos co-injected with the MSV-CAT and MSV-CAT 3’Eg2 plasmids (Fig. 1B), the 3P-labeled probe to the CAT gene clearly visualized the mRNA transcribed from the MSV-CAT plasmid but only produced a faint signal for the CAT 3’Eg2 mRNA visible in the samples taken 14 h after fertilization (note that the autoradiogram exposure time for the CAT probe is three times longer in Fig. 1B than in Fig. 1A).

As mentioned above, the lesser accumulation of CAT 3’Eg2 mRNA relative to CAT 3’ODC mRNA was also observed when the mRNAs were transcribed from the HS promoter (for example, see Fig. 4). This indicates that the lesser accumulation of the chimeric mRNA containing the 3’Eg2 sequence is not promoter specific. Also note that with both the MSV-CAT 3’Eg2 and HS-CAT 3’Eg2 plasmids, no transient accumulation and subsequent degradation of the CAT 3’Eg2 transcript was observed. All the mRNAs transcribed from the MSV promoter (CAT, CAT 3’ODC, and CAT 3’Eg2) accumulated gradually after initiation of transcription at the MBT, to attain a plateau value about 6 h later. This was about 14 h after fertilization in the experiments described here. Therefore, in the majority of the experiments described below, 14-h-old embryos were used.

The small amount of CAT 3’Eg2 mRNA relative to CAT 3’ODC or CAT mRNA that accumulates in the embryos could be caused by several independent mechanisms: (i) the CAT 3’Eg2 plasmid is not stable or the CAT and CAT 3’ODC plasmids are amplified to a greater extent; (ii) the Eg2 sequence negatively affects transcription; or (iii) the Eg2 sequence confers instability on the chimeric mRNA.

To determine whether the plasmids containing the different 3’ sequences were equally stable, we injected embryos with increasing amounts (between 62 pg and 1 ng) of either MSV-CAT 3’ODC or MSV-CAT 3’Eg2 and quantified the amount of plasmid DNA present in the 14-h-old embryos by slot-blot analysis (Table 1). When 250 pg or less of these plasmids was injected, neither was amplified and the MSV-CAT 3’Eg2 plasmid was at least as stable as the MSV-CAT 3’ODC plasmid. However, when 500 pg or 1 ng of these plasmids was injected, they were both amplified on average about 10-fold, although there was considerable variation in the degree of amplification between experiments. Therefore, the small amount of CAT 3’Eg2 mRNA relative to the CAT 3’ODC or CAT mRNA that accumulates in 14-h-old embryos injected with 125 pg of plasmid is not due to differences in the amounts of the corresponding templates.

If the lesser accumulation of the CAT 3’Eg2 transcript was due to a silencer-type action of the 3’ Eg2 sequence on transcription, then this effect would not require the 3’ Eg2 fragment to be actually transcribed. To test this possibility, we constructed plasmids (HS-CAT XbaI 3’Eg2) in which the 3’ Eg2 fragment was situated 3’ to the SV40 polyadenylation signal. In this plasmid, the 3’ Eg2 sequence is only 243 nucleotides downstream of its position in the HS-CAT 3’Eg2 or MSV-CAT 3’Eg2 plasmid, but it is absent from the mRNA transcribed.

Embryos were injected with either HS-CAT 3’ODC and HS-CAT plasmids or HS-CAT 3’ODC and HS-CAT XbaI

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<tr>
<th>Amt injected (pg)</th>
<th>Amt (pg) per embryo at 14 h</th>
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<tr>
<td></td>
<td>MSV-CAT 3’ODC</td>
</tr>
<tr>
<td>62</td>
<td>30 ± 15</td>
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<tr>
<td>125</td>
<td>90 ± 40</td>
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* Embryos were injected at the one-cell stage with the indicated amounts of either MSV-CAT 3’ODC or MSV-CAT 3’Eg2 plasmid. After 14 h of development, plasmid DNA was extracted from the different batches of embryos and quantified by serial dilution as described in Materials and Methods. The values given are average ± standard deviation.
3' Eg2 plasmids and heat shocked (33°C) between 8.5 and 9 h of development. Northern analyses of the RNAs extracted from these embryos at the beginning of the heat shock and at various times afterwards are shown in Fig. 2. The signals obtained when the membrane was hybridized with a probe to the endogenous ODC mRNA (upper panels) show that similar amounts of total RNA were present in all the samples. At the beginning of the heat shock, no transcripts were detected with the 32P-labeled probe to the CAT gene in either batch of embryos (Fig. 2A, lower panel). One hour later, however, CAT 3’ODC and CAT mRNAs were clearly detected in the RNA extracted from both batches of embryos (Fig. 2B and C, lower panels). The ratio of CAT mRNA to CAT 3’ODC mRNA, determined by densitometer quantification, was approximately constant for the three time points shown in Fig. 2B and C and was unaffected by the presence of the 3’ Eg2 sequence downstream of the SV40 polyadenylation signal: CAT/CAT 3’ODC = 1.3 ± 0.1 (average ± standard deviation) for plasmids HS-CAT and HS-CAT 3’ODC; CAT/CAT 3’ODC = 1.35 ± 0.25 for plasmids HS-CAT XbaI 3’Eg2 and HS-CAT 3’ODC. Similar results were obtained when the 3’ Eg2 fragment was situated upstream of the HS promoter (data not shown). These results indicate, therefore, that the small amount of CAT 3’Eg2 mRNA that accumulates relative to the CAT or CAT 3’ODC mRNA is not due to a silencer-type sequence contained in the 3’ Eg2 fragment.

Taken together, these two sets of experiments indicate that the 3’ region of the maternal Eg2 mRNA confers an instability on the chimeric mRNA.

To ascertain that the amount of accumulated chimeric mRNA was proportional to the amount of template, RNA was extracted from the embryos, injected with increasing amounts of plasmid, and subjected to Northern analysis using a 32P-labeled probe to the CAT gene (Fig. 3). To normalize the different RNA samples, we also hybridized these membranes with probes to both the DG 42 and the maternal Eg2 mRNAs. Densitometer quantification of these autoradiograms showed that for the MSV-CAT 3’ODC plasmid, there was a good correlation between the amounts of chimeric CAT 3’ODC mRNA and plasmid DNA actually present in the embryos at 14 h of development. For the embryos injected with 500 pg or 1 ng of the MSV-CAT 3' Eg2 plasmid, a signal for the CAT 3’Eg2 mRNA was easily detected (Fig. 3, middle panel). However, when 250 pg was injected, a faint signal was only detected on overexposed autoradiograms (Fig. 3, bottom panel). This difference in the amount of CAT 3’Eg2 mRNA between the embryos injected with 250 or 500 pg and 1 ng of plasmid is probably at least partially due to the amplification of the injected plasmids (Table 1). Note that for a given amount of plasmid present in the embryo, CAT 3’Eg2 mRNA was always less abundant than the CAT 3’ODC mRNA (Fig. 3, middle panel).

Since large amounts of chimeric CAT 3’Eg2 mRNA could eventually saturate the degradation system of the embryo for this mRNA, the amount of injected plasmid was limited to 125 pg per embryo.

**Effect of cycloheximide on chimeric mRNA stability.** One of the characteristics of maternal Eg2 mRNA destabilization is its inhibition by cycloheximide (9). It was not possible to use the MSV-CAT 3’Eg2 plasmid to analyze this effect of cycloheximide on the stability of the chimeric CAT 3’Eg2 mRNA, as the MSV promoter is not active in the presence of this drug (data not shown). The HS promoter, however, is active in the presence of cycloheximide. Therefore, the plasmids in which the transcription of the chimeric mRNA was under the control of the HS promoter were used. Embryos were co-injected with these plasmids, and one-half were treated with cycloheximide between 8 and 9 h of development. Northern analysis (Fig. 4) of the RNA extracted from these embryos, heat shocked (33°C) between 9 and 9.5 h of development, showed that CAT mRNA transcribed from the HS-CAT plasmid accumulated in both the presence and absence of cycloheximide. For the CAT 3’Eg2 mRNA, the faint signal observed in the untreated embryos had increased importantly in the cycloheximide-treated embryos. As shown in Fig. 2, no mRNA transcribed from these plasmids was detected in non-heat-shocked embryos. Densitometer quantification of the autoradiograms determined

![Image](http://mcb.asm.org/)
that the cycloheximide treatment caused CAT 3'Eg2 mRNA to increase about 50-fold, whereas CAT mRNA only increased about 3-fold. In addition, in the cycloheximide-treated embryos, the amount of CAT 3'Eg2 mRNA was only three to four times less than the amount of CAT mRNA. Therefore, the 3' region of Eg2 mRNA is sufficient to confer the stability characteristics of the maternal Eg2 mRNA on a chimeric molecule.

Note that the degrees to which cycloheximide stabilizes the CAT 3'Eg2 mRNA and the maternal Eg2 mRNA are not comparable. The amount of CAT 3'Eg2 mRNA in post-MBT embryos is the result of an equilibrium between the rates of transcription and degradation. If the rate of degradation was decreased many fold, so that the mRNA became very stable, the ongoing transcription would cause the CAT 3'Eg2 mRNA to increase continuously. For the maternally inherited Eg2 mRNA, even complete stabilization by the cycloheximide treatment could not increase the amount of the maternal transcript above that present in the egg.

Role of translation in Eg2 destabilization. The inhibition by cycloheximide of maternal Eg2 mRNA destabilization suggests either that the translation of Eg2 mRNA is a necessary part of its destabilization or that the translation of at least one other mRNA is required to provide a necessary trans-acting factor.

Eg2 mRNA is deadenylated after fertilization (26), and the deadenylation of two other Eg mRNAs is associated with their release from polysomes (24b, 26). To verify that maternal Eg2 mRNA has a similar behavior, we analyzed the polysome recruitment of this mRNA in embryos at different times after fertilization. The recovery of total RNA in the different samples was verified by ethidium bromide staining of aliquots electrophoresed in native agarose gels. The data given in Fig. 5A illustrate the progressive loss of Eg2 mRNA from the polysomal fraction after fertilization. At the MBT, the majority of maternal Eg2 mRNA was in the nonpolysomal fraction. A similar polysome analysis, performed on samples prepared from cycloheximide-treated and untreated embryos (Fig. 5B), showed that cycloheximide did not increase the amount of this mRNA in the polysomal fraction. Cycloheximide therefore does not stabilize Eg2 mRNA by an artifactual reloading of ribosomes onto the mRNA, as has been observed in Dictyostelium discoideum (17, 38). This result is in agreement with the observation that pactamycin treatment of Xenopus embryos, which inhibits initiation of translation, also stabilized the maternal Eg2 mRNA (data not shown). In these experiments, the 8-h-old embryos had passed through the MBT and therefore the degradation of maternal Eg2 mRNA had already begun in the untreated embryos. For the cycloheximide-treated embryos, the more intense signal for the nonpolysomal Eg2 mRNA showed that it was this population of Eg2 mRNA that was stabilized by the cycloheximide treatment.

The poly(A)+ and poly(A)− fractions of total RNA extracted from 10-h-old (about 4.5 h after the MBT) cycloheximide-treated or untreated embryos were also prepared and subjected to Northern analysis. To verify that any effect of cycloheximide on the distribution of Eg2 mRNA between the poly(A)+ and poly(A)− fractions was not a general effect of this drug on adenylation, we also used a cDNA probe to one of the CI mRNAs (26) in these Northern analyses (Fig. 5C). The CI mRNAs (C11 and C12) are, in majority, present in the poly(A)+ and polysomal fractions of mRNA in embryos (25, 26), that is, the opposite distribution to Eg2 mRNA. Quantification of the autoradiograms showed, in agreement with our previously published results (9), that the total amount [poly(A)+ plus poly(A)−] of Eg2 mRNA was 3.5 ± 0.5-fold greater in the cycloheximide-treated embryos than in the untreated embryos. The total amount of CI2 mRNA was not affected by the cycloheximide treatment. The poly(A)+ fraction from untreated embryos contained 35% ± 5% of the Eg2 mRNA and 77% ± 3% of CI2 mRNA. When poly(A)+ RNA was prepared from cycloheximide-treated embryos, it contained only 20% ± 4% of Eg2 mRNA but 90% ± 8% of CI2 mRNA. These results show that the cycloheximide treatment causes a decrease rather than an increase of Eg2 mRNA in the poly(A)+ fraction of 10-h-old embryos. This is not a general effect of cycloheximide on the adenylation of all mRNAs as the proportion of CI2 mRNA in the poly(A)+ fraction was either unchanged or slightly increased.

These analyses of the maternal Eg2 mRNA showed that the cycloheximide treatment primarily stabilizes the nonpolysomal poly(A)+ fraction of this mRNA. They did not, however, determine whether translation of this mRNA is required for its degradation. We used chimeric CAT 3'Eg2 mRNAs to answer this question.

Kozak (19) has shown that in COS cells the presence of a stable hairpin (ΔG = −50 kcal/mol) in the 5' untranslated region of an mRNA (just before the initiation ATG codon) reduces the translation of the chimeric mRNAs by about 95%. mRNAs with the half-hairpin sequences, which, when similarly positioned in the mRNA, do not affect translation, served as controls in these experiments. Plasmids were therefore constructed in which the CAT-3'Eg2-SV40 polyadenylation signal cassette was flanked on its 5' side either by the hairpin structure (Hp-CAT 3'Eg2) or by the sequences of the 5' or 3' half of the hairpin (respectively, 17b-CAT 3'Eg2 and 17a-CAT 3'Eg2). Similar plasmids were also constructed in which the 3' Eg2 sequence was omitted (Hp-CAT, 17a-CAT, and 17b-CAT). The accumulation in Xenopus embryos of the CAT mRNA transcribed from the SV40 promoter in this plasmid was at least fivefold less than...
that transcribed from either the MSV or HS promoter. Therefore, the embryos were injected with 500 pg of these plasmids (separately), and total RNA and cytosol extracts were prepared after 18 h of development.

The CAT activity in the cytosol extracts, which is a direct measure of the translation from the chimeric mRNA, was determined, and the values are given in Fig. 6. The results showed that translation of both Hp-CAT and Hp-CAT 3'Eg2 mRNAs (which contain the hairpin) is reduced by about 95% compared with the controls which contain one-half of the hairpin. The amounts of the different mRNAs were quantified by Northern blot analysis (Fig. 6) using a 32P-labeled probe to the CAT gene, and the membranes were also hybridized with a DG 42 probe to normalize the amount of RNA in the different samples (Fig. 6, upper panel). Variations in the amounts of plasmid in the embryos were also verified by slot-blot analysis. In five separate experiments, the amounts (average ± standard deviation) of 17a-CAT 3'Eg2 and 17b-CAT 3'Eg2 relative to Hp-CAT 3'Eg2 were 1.1 ± 0.6 and 2.2 ± 1.9, respectively. At present, we have no explanation for the larger variation in the amount of 17b-CAT 3'Eg2 than for the other mRNAs. The amounts of these three 3'Eg2-containing mRNAs were about 4-fold (4.5 ± 1.0) less than that of the Hp-CAT, 17a-CAT, or 17b-CAT mRNA. Therefore, although translation of the chimeric Hp-CAT 3'Eg2 mRNA was reduced by about 95% compared with the control 17a-CAT 3'Eg2 and 17b-CAT 3'Eg2 mRNAs, this mRNA was not stabilized to the level of Hp-CAT mRNA. Hence, the degradation of the chimeric Hp-CAT 3'Eg2 does not require its own translational elongation.

Delimitation of the 3' region required for destabilization of chimeric mRNAs. We showed above that the Eg2 3' region is sufficient to destabilize chimeric mRNAs containing the CAT reporter mRNA. To more precisely delimit the sequence motif(s) involved in this process, we made constructions including different parts of this 3' region (Fig. 7A). These plasmids were injected separately into embryos, and 14 h later, RNA, DNA, and CAT activity were extracted. A typical Northern analysis using a CAT probe is shown in Fig. 7B. These blots were also hybridized with a probe to the Xenopus ODC mRNA to normalize the amount of RNA in the different samples. As already described above, only trace amounts of the mRNA transcribed from the CAT 3'Eg2 plasmid were detected. For the mRNAs containing the different parts of Eg2 3' region, the mRNA CAT Eg2(410) accumulated to levels comparable to that of the CAT mRNA. However, only small amounts of CAT Eg2(497) were present. Quantification of autoradiograms from these Northern analyses confirmed that the mRNAs transcribed from the MSV-CAT and MSV-CAT Eg2(410) plasmids accumulated to quantitatively similar amounts. However, the

**FIG. 5.** (A) Polysomal distribution of maternal Eg2 mRNA in fertilized eggs (E) and in 2- and 6-h-old embryos. Polysomal (P) and nonpolysomal (S) RNAs were separated as indicated in Materials and Methods by centrifugation through 15 to 40% (wt/wt) sucrose gradients containing Mg2+ to retain polyosome integrity and subjected to Northern analysis with a 32P-labeled Eg2 cDNA probe. Shown below is the ethidium bromide staining of the different RNA samples. Due to the large proportion of free nonpolysomal ribosomes in *Xenopus* embryos, the amounts loaded for the nonpolysomal RNA samples were less than those for the polysomal samples. For polysomal RNA, lanes a contain one embryo equivalent and lanes b contain two embryo equivalents. For nonpolysomal RNA, lanes a contain 0.25 embryo equivalents and lanes b contain 0.5 embryo equivalents. (B) Polysomal distribution of maternal Eg2 mRNA in 8-h-old cycloheximide-treated (Cy) or untreated (C) embryos. Embryos were treated with cycloheximide as described in Material and Methods. The RNA extracted from each batch was separated into polysomal (P) and nonpolysomal (S) fractions on Mg2+-containing sucrose gradients and subjected to Northern analysis as described for panel A. The distribution of Eg2 mRNA in gradients containing EDTA, which dissociates the polyosome structure, is also shown. In the lower panels is shown the ethidium bromide staining of the RNA in the different samples (0.33 embryo equivalents for all the samples). (C) Analysis of Eg2 mRNA polyadenylation in cycloheximide-treated embryos. Embryos were treated as described for panel B except that to embryos were allowed to develop until 10 h after fertilization. Total RNA (10 μg) from cycloheximide-treated embryos (Cy) or untreated embryos (C) was then separated into poly(A)+ and poly(A)− fractions and subjected to Northern analysis with 32P-labeled cDNA probes to the Eg2 and C12 mRNAs.

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FIG. 6. Stable hairpin (Hp) 3′ to the initiation codon efficiently reduces translation but does not change chimeric mRNA stability. Embryos were injected with 500 pg of the indicated plasmids and left to develop until 18 h after fertilization. Cytosol extracts and RNA preparation were performed as described in Materials and Methods from batches of 5 to 10 embryos. With these plasmids, CAT activity was usually analyzed with 0.5 embryo equivalent. The CAT activity for the hairpin-containing plasmid of each series (CAT or CAT 3′Eg2) was arbitrarily set to unity. For Northern analysis, one embryo equivalent was loaded in each lane. The bottom panel shows the hybridization with a 32P-labeled CAT cDNA probe, and the upper panel shows the hybridization of the same membrane with a DG 42 cDNA probe.

amounts of mRNAs transcribed from MSV-CAT 3′Eg2 and MSV-CAT Eg2(497) were three- to fourfold less than that of CAT mRNA. Therefore, the sequence motif(s) in the 3′ region of Eg2 which confers instability on chimeric mRNAs is contained within the 497-bp fragment. When this fragment was digested with EcoRII, which cuts 12 nucleotides before the stop codon, the two partial fragments produced did not confer instability on the chimeric CAT mRNA (data not shown). The CAT activity extracted from the different batches of embryos was not significantly different when normalized by the amount of chimeric CAT mRNA present (Fig. 7C).

**DISCUSSION**

The purpose of the experiments described in this report was to determine the role of the 3′ region and translation on the degradation of a maternal mRNA, Eg2, whose expression is developmentally controlled. The role of the Eg2 3′ region on mRNA degradation was tested by injecting appropriately constructed plasmids into one-cell *Xenopus* embryos. The steady-state amount of the endogenously transcribed chimeric mRNA containing a CAT reporter gene and a 3′ Eg2 sequence was at least three- to fourfold less than that of CAT or CAT 3′ODC mRNA. In some experiments an even greater difference was observed.

This small amount of CAT 3′Eg2 mRNA relative to CAT 3′ODC or CAT mRNA could be caused by differences in the amounts of template, a silencer sequence in the 3′ Eg2 fragment, or instability of the chimeric mRNA. Control experiments showed that for all the plasmids used, their stability and their amplification (when it occurred) were similar. In addition, mRNAs transcribed from plasmids in which the 3′ Eg2 sequence was situated just downstream of the SV40 polyadenylation signal accumulated to a level similar to those transcribed from plasmids which did not contain a 3′ Eg2 sequence. Therefore, these results indicate that the Eg2 sequence introduced 3′ to the CAT-coding region confers an instability on the chimeric mRNA. We are currently developing a protocol for *Xenopus* embryo extracts that retain the in vivo stability characteristics of the maternal ODC and Eg2 transcripts. Preliminary data obtained by incubating the in vitro-synthesized CAT 3′ODC and CAT 3′Eg2 RNAs in these extracts confirm that the 3′ Eg2 fragment confers instability on the chimeric mRNA (24a).

When a large amount of the MSV-CAT 3′Eg2 plasmid was injected, the chimeric transcript was easily detected, suggesting that it is possible to saturate the degradation system as has been observed for c-fos mRNA (34). Nonsaturating concentrations of plasmids were therefore used for all the experiments described here.

The fact that the amount of the maternal Eg2 mRNA did not increase concomitantly with the greater accumulation of the chimeric mRNA does not preclude a saturation of the degradation system. The degradation of the maternal mRNA starts at the MBT (9) second destabilizing motif in the chimeric mRNA is accumulating over several hours (Fig. 1). Therefore, an eventual saturation of the degradation system by the high level of chimeric mRNA would only occur when the maternal mRNA had already decreased importantly. Under these conditions, it would not be possible to observe a significant stabilization of the maternal mRNA.

Deletions of various parts of the 3′ Eg2 sequence showed that the Eg2(497) segment reproduced the compartment of the large fragment used initially. The Eg2 sequence present in Eg2(497) is the EcoRI-StuI fragment that contains the last 239 nucleotides of the coding region and the first 258 nucleotides of the 3′ untranslated region. The EcoRI-BglII fragment (110 bp) present in Eg2(497) was absent from the 3′ Eg2 sequence used initially. This suggests that the common 385-bp fragment, BglII-StuI, contains a cis-acting sequence required for destabilization of the chimeric CAT 3′Eg2 mRNA. Indeed, restriction of the Eg2(497) fragment with EcoRII, which cuts 12 nucleotides before the stop codon, led to a loss of the destabilization activity. However, we cannot formally exclude that a second destabilizing motif exists in the 110-bp EcoRII-BglII fragment. We have also observed that the instability conferred on a chimeric mRNA by the 3′ Eg2 region was independent of its orientation (3a), suggesting that it is a complex secondary structure in this fragment that confers instability on the chimeric mRNA.

In many cases the degradation of an mRNA is preceded by a shortening of its poly(A) tail (4, 6, 41). The constructions used in the present study all contained a SV40 polyadenylation signal, and the chimeric mRNAs possessed a poly(A) tail of 150 to 200 nucleotides (3a, 15). Due to the very small amount of chimeric CAT 3′Eg2 mRNA, we were not able to determine whether deadenylation of this mRNA precedes destabilization. The fact that on Northern blots we obtained a short smear for the chimeric CAT 3′Eg2 transcripts rather than a distinct signal could indicate that these mRNAs have different lengths, implying that shortening of the poly(A) tail of this mRNA is occurring more rapidly than for the other mRNAs. Furthermore, the CAT Eg2(497) chimeric mRNA is smaller than expected, suggesting that this chimeric mRNA is totally deadenylated. This is being analyzed in more detail and will allow us to define the role of deadenylation in the
Eg2 degradation pathway and the identity of the cis-acting regulatory elements involved.

We have previously described (9) three motifs that were perfectly or partially conserved with respect to their relative position and sequence between three Eg mRNAs, denoted as Eg1, Eg2, and Eg9 (26). In Eg2 mRNA, these motifs are situated 3' to the Stul site. The experiments described here showed that these motifs are not responsible for the instability conferred by the 497-bp EcoRI-Stul fragment. They could still, however, be cis-regulatory elements for other posttranscriptional modifications such as deadenylation and release from polysomes after fertilization. The Eg family of mRNAs are all deadenylated after fertilization (26), and for three of these mRNAs, which have been studied in more detail (Eg5 [26], Eg1 [24b], and Eg2 [this study]), this deadenylation is correlated with a release from polysomes. Work is in progress to study this possibility.

An increasing number of examples of regulated RNA instability in eukaryotes show that translation is a necessary part of this process. This may involve translation elongation, as for the mRNAs encoding tubulin (42), histone (28), and MATa1 protein in yeasts (27), or synthesis of a labile factor (7). The facts that the presence of a 3' Eg2 sequence is sufficient to produce a chimeric mRNA with the behavior of the intact Eg2 mRNA and that this chimeric mRNA was stabilized, like the maternal Eg2 mRNA, by cycloheximide allowed us to study the potential role of translation elongation in the Eg2 mRNA degradation pathway. We first verified that the cycloheximide effect was not artifactual: stabilization by reloading and blockage of ribosomes on the mRNA or by readenylation of the poly(A)+ Eg2 mRNA. Indeed, cycloheximide stabilized the nonpolysomal and poly(A)+ fraction rather than the polysomal and poly(A)− mRNA.

To determine specifically whether the translation elongation of the chimeric CAT 3'Eg2 mRNA was required for its degradation, we constructed plasmids with a stable hairpin structure 5' to the initiation codon (19). The advantage of this approach is that it allowed us to modify the translation of only the chimeric mRNA without affecting that of the endogenous mRNAs, as occurs when embryos are treated with drugs. The hairpin structure reduced the translation of the chimeric CAT and CAT 3'Eg2 mRNA by about 95% in Xenopus embryos compared with the control plasmids containing one-half of the hairpin. However, the chimeric CAT 3'Eg2 mRNA which contained the hairpin structure was not stabilized compared with the control plasmids. Therefore, the destabilization of this chimeric mRNA does not require its translation elongation. This implies that the cycloheximide-induced stabilization of maternal Eg transcripts (9) is due to the effect of this drug on the translation of at least one other mRNA which presumably codes for a trans-acting factor involved in the process of mRNA degradation. We have previously shown that inhibition of transcription does not prevent the degradation of the maternal Eg mRNAs in Xenopus embryos after the MBT (9). Therefore, the RNA(s) whose translation is inhibited by cycloheximide must be a maternal transcript. In addition, the encoded protein is either only synthesized at or just before the MBT or only accumulates to a sufficient amount at this time.

It is tempting to speculate that a family of maternal mRNAs exists whose expression is regulated similarly to that of these trans-acting factor(s) involved in mRNA stability. The recruitment of these mRNAs into polysomes toward the end of the rapid growth phase could then provide some of the trans-acting factors necessary for the change in gene expression (e.g., initiation of gene transcription) at the MBT.

In conclusion, we described the destabilization of a Xen-
pus maternal mRNA which may require a complex secondary structure in its 3' region. Furthermore, the trans-acting factor(s) necessary for the degradation of this mRNA after the MBT is translated from a maternal mRNA(s). The facility with which chimeric mRNA can be expressed in Xenopus embryos and the use of a cell-free system will allow us to characterize and purify the proteins involved in this process.

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