Stability of RNA in developing *Xenopus* embryos and identification of a destabilizing sequence in TFIIIA messenger RNA

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Summary

Synthetic capped RNA transcripts injected into fertilized eggs of *Xenopus laevis* have a half-life of 3–4 h. Addition of a long (~200 nucleotide) poly(A) tail increases the half-life to 6–8 h which approaches the half-life of natural polyadenylated globin RNA injected into embryos. Since exonucleolytic action alone could account for the degradation of RNA, we tested whether circular RNA is stable after injection and find that circles are exceptionally stable (half-life greater than 40 h). After the midblastula transition, polyadenylated chloramphenicol transferase (CAT) mRNAs transcribed from injected plasmids have a half-life of 2.5 h. Insertion of a 1000 nucleotide 3' untranslated region from the *Xhox-36* gene into the transcripts does not affect the half-life. In contrast to the finding that internal sequences do not affect stability, we find that sequences from the TFIIIA message reduce the half-life of CAT mRNA from 2.5 h to less than 30 min. We conclude that most RNAs are degraded exonucleolytically from the 3' end, but specialized internal sequences can greatly destabilize the RNA, possibly by acting as a site for an endonuclease.

Key words: RNA stability, *Xenopus* development, TFIIIA, poly(A).

Introduction

The regulation of mRNA stability plays an important part in the control of gene expression; the numerous examples where stability is controlled have been recently reviewed (Shapiro et al. 1987). It is of particular interest to us to identify mechanisms that control RNA stability in the developing *Xenopus* embryo, not only in general, but also in special cases where stability of individual RNAs is controlled.

A mechanism for differential RNA stability must be in effect throughout early *Xenopus* development since there are cases of RNAs that were stable in the oocyte being degraded either early in the blastula (King et al. 1986) or at different times around the beginning of gastrulation (Rebagliati et al. 1985; Dworkin et al. 1985). In these examples, total RNA was examined, thus excluding the possibility that deadenylation led to apparent absence of mRNA on the basis of oligo(dT) selectability. In *Drosophila*, it has been proposed that one mechanism for the generation of asymmetry in the homogeneously distributed caudal (cad) mRNA is differential RNA stability (Macdonald & Struhl, 1986; Mlodzik & Gehring, 1987).

The possibility that differential RNA stability is important to regional specification in the amphibian embryo is also attractive. Although prelocalized RNAs exist along the animal–vegetal axis (Rebagliati et al. 1985; Melton, 1987; Weeks & Melton, 1987), the capacity for dorsal and ventral development is not prelocalized in the radially symmetrical amphibian egg (reviewed by Gerhart & Keller, 1986). The transduction of early cytoplasmic reorganization and inductive interactions into changes in gene expression may well involve the differential stabilization of maternal RNA in different regions of the egg.

Despite the work that has been done, we still do not understand what RNA sequences and nucleolytic enzymes control RNA stability in *Xenopus* oocytes and embryos. Interpretations from earlier work, employing natural RNAs, stressed the importance of a poly(A) tail for stability in oocytes (reviewed by Littauer & Soreq, 1982; Nevins, 1983) though in some cases a fraction of nonadenylated RNA was known to be stable for an extended period (Woodland & Wilt,
Stability of RNA has also been related to its presence on polysomes (Allende et al. 1974; Richter & Smith, 1981; Audet et al. 1987). More recently, well-defined transcripts synthesized from bacteriophage SP6 and T7 promoters have been used to address questions of stability and translatability. A consensus is emerging from these experiments that nonadenylated RNAs are much more stable in oocytes than formerly believed (Harland & Weintraub, 1985; Drummond et al. 1985; Kruys et al. 1987). Even antisense RNAs, which are poorly translated, are much more stable in oocytes than would have been expected from earlier work (Harland & Weintraub, 1985). Furthermore, a poly(A) tail is not absolutely required for translation of the RNA (Harland & Weintraub, 1985; Kruys et al. 1987) though it does have an effect on long-term stability and the efficiency of translation of at least some messenger RNAs in oocytes (Drummond et al. 1985).

In this paper, we confine our experiments to developing Xenopus embryos. The metabolism of RNA in the egg is in many respects different from oocytes; changes in adenylation of endogenous RNAs occur during maturation (see discussion in Dworkin & Dworkin-Rastl, 1985) and a double-stranded RNA unwinding activity appears (Bass & Weintraub, 1987; Rebagliati & Melton, 1987). There is now a clear consensus that synthetic RNA injected into embryos is less stable than in oocytes (Rebagliati & Melton, 1987; Colman & Drummond, 1986; this work) though previous experiments had suggested that injected globin RNA may be stable for several days as monitored by the continual translation of globin protein (Gurdon et al. 1974). The role of the poly(A) tail is not clear in embryos and at least one group has failed to find any effect on stability (Rebagliati & Melton, 1987).

We have used injection and direct quantification of RNA to address the effect of short and long poly(A) tails on the stability of RNA. To address the relative importance of exo- and endonucleases to degradation we have injected RNA with no ends; this circular RNA was prepared by exploiting the self-splicing properties of the Tetrahymena pre-rRNA intron (Been & Cech, 1986).

Our conclusions from RNA injection experiments have been extended by experiments in which we injected plasmids encoding defined polyadenylated transcripts. We have measured the stability of these transcripts and tested whether the presence of a long 3' untranslated sequence destabilizes the RNA. To complement our results, which suggest that endonucleases are relatively unimportant in degrading most RNAs, we have asked what is the effect of a sequence from an unstable RNA when inserted into another transcription unit. These experiments identify a sequence in the TFIIIA mRNA (Ginsberg et al. 1984) which destabilizes RNA and may act as a target for a site-specific endonuclease.

**Materials and methods**

(A) **Plasmid construction and RNA preparation**

Plasmids with poly(dA) · poly(dT) tracts

In principle, the simplest way to synthesize polyadenylated RNA is to encode the poly(A) tail in the transcription template. We therefore prepared plasmids that could be transcribed to make chloramphenicol acetyl transferase (CAT) mRNA, but which contained a poly(dA) · poly(dT) tract 3' of the CAT coding sequences. This was done by preparing a CAT fragment with a poly(dA) tail and a plasmid fragment with a poly(dT) tail. The non-tailed end of both fragments was generated by restriction enzyme digestion.

In order to prepare the appropriate DNA fragments, the plasmid pSP6SCATS (Harland & Weintraub, 1985) was digested with XbaI (3' of the CAT gene relative to the SP6 promoter) and this site was filled in with reverse transcriptase. The reaction was controlled by availability of nucleotide to yield tails of length 100–400 nucleotides. The DNAs were then digested 5' of the CAT insert with SalI and both the A-tailed CAT fragment and T-tailed plasmid fragments purified from an agarose gel. Following selection of successfully tailed molecules on oligo(dA) or oligo(dT)-cellulose, the DNAs were annealed and ligated. Transformants were picked and DNA minipreps analysed for poly(A) tail length.

To measure the size of the poly(dA),poly(dT) insert, DNAs were digested on each side of the tail with HindIII and XbaI. The ends were labelled by filling in with radioactive nucleotides and the size of the fragments determined on a sequencing gel. Out of 24 colonies, 16 contained plasmids with tails of greater than 50 nucleotides. The longest tails were of about 400 nucleotides, but constituted a minority of a population from an individual colony. Even clonal isolates of such lines derived by restreaking or retransformation continued to yield heterogeneous tails. A line with a reasonably homogeneous length of poly(A) (80 nucleotides) was amplified from the original culture to yield a transcription template. We therefore prepared plasmids that could be transcribed to make chloramphenicol acetyl transferase (CAT) mRNA, but which contained a poly(dA) · poly(dT) tract 3' of the CAT coding sequences. This was done by preparing a CAT fragment with a poly(dA) tail and a plasmid fragment with a poly(dT) tail. The non-tailed end of both fragments was generated by restriction enzyme digestion.

Enzymic addition of poly(A) tails

The enzyme poly(A) polymerase (BRL) was used according to the manufacturer's instructions to yield RNA with poly(A) tails of approximately 200 nucleotides. The length of the tails was controlled by adding the chain terminating
The plasmid pBGST7 (Been & Cech, 1986) was used as a transcription template under standard conditions but in the absence of the cap reagent (Harland & Weintraub, 1985). For splicing, the RNA was resuspended in 200 mM-NaCl with GTP and MgCl₂ and incubated at 42°C for 1 h (Sullivan & Cech, 1985). The identity of the different species was confirmed by their migration on 4%, 6% or 8% polyacrylamide gels with respect to linear DNA markers. Circular RNAs have anomalous mobility under these conditions. To prepare larger circles containing the CAT sequences, the CAT gene was isolated as a BamHI fragment and inserted in both orientations in the BglII site of the intron. In this case, capped transcripts were prepared and spliced prior to injection.

**Preparation of circular RNA**

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**Plasmids with promoters**

The plasmid containing a CAT gene and the SV40 promoter (EMSV CATS) has been described previously (Harland & Weintraub, 1985). For testing other promoters, we constructed a new vector, PT-CAT, which is potentially more versatile. This exploits the ‘blueprint’ (Stratagene Inc.) polylinker and plasmid. The CAT gene is present as a BamHI fragment and can therefore be removed easily after a promoter has been tested. Multiple single-cut restriction sites are present in the polylinker for insertion of promoter sequences or cDNAs to be expressed. The SV40 late polyadenylation signal is located downstream of the CAT gene. Single-stranded DNA can be generated by superinfection with M13 helper phage for confirmation of insert identity and orientation by sequencing. As a primer for DNA sequencing, we used a synthetic oligonucleotide complementary to the region around the CAT gene ATG initiator codon (gift of H. Weintraub). The derivative containing a heat-shock promoter is diagrammed in Fig. 3A; the other promoter-containing plasmids are the same except for the different promoter.

For all the promoters tested here, we inserted promoter fragments into a filled-in XhoI site upstream of the CAT gene in order to leave an extensive choice of restriction sites downstream of the transcription initiation site. These plasmids and other details are available on request. Concentrations of DNA determined from optical density were routinely checked by gel analysis to confirm that contaminating oligonucleotides were not contributing to apparent concentration. We find that plasmids containing the CAT gene, although resistant to chloramphenicol, respond particularly well to amplification by chloramphenicol with yields of 2–10 µg plasmid per litre of culture. The following promoters have been tested.

**Heat-shock promoter.** Plasmids were obtained from Mariann Bienz (Bienz, 1984) and Jay T'so (pHSTF10). A promoter fragment from a deletion endpoint at -196 to the PvuII site at +90 was used to make the plasmid HS-CAT. pHSTF10 was modified by digesting the plasmid with BglII which removes a part of the heat-shock 5' untranslated sequence to position +29 (Bienz, 1984) and some of the TFIIA sequence to position +343 (Ginsberg et al., 1984). A BamHI fragment coding for CAT was inserted in its place to make the plasmid HS-CAT TF10. These plasmids are further diagrammed below in Fig. 3A.

**L14 ribosomal protein promoter.** A subclone of the L14 gene (Bozozon et al., 1984) containing the promoter and the promoter sequence were generously provided by Elena Beccari. A fragment from -880 to +12 was used to generate L14 CAT. The L1 promoter was also tested and had similar properties (not shown); however, since the L1 promoter initiates at two well-separated positions and yields two distinct RNAs whereas the L14 promoter yields one RNA the L14 promoter was used for subsequent experiments.

Xenopus borealis cytoskeletal actin promoter. The plasmid pSC9 and the sequence around the cap site were generously provided by Gareth Cross and Hugh Woodland (Cross et al., 1988). A fragment from the HindIII site approximately 1670 nucleotides upstream of the transcription start site to an Mspl site at +10 was initially tested. Subsequently, we found that the fragment from the EcoRI site (approx. -950) to the Mspl site gave identical transcriptional efficiency. This fragment was used to make CSK-CAT.

**(B) Embryo injections**

Some detail is given here since different workers find that DNA injection yields quite different results with respect to the amount of amplification of injected DNA.

Eggs were obtained and fertilized by standard procedures. After dejellifying in 2% cysteine dissolved in 100 mM-NaCl, 1.8 mM-KCl, 1 mM-MgCl₂, 2 mM-CaCl₂, adjusted to pH 8 with NaOH, the embryos were rinsed in iMR (modified Ringer’s 100 mM-NaCl, 1.8 mM-KCl, 1 mM-MgCl₂, 2 mM-CaCl₂ buffered to pH 6.9 with 5 mM-Hepes). Batches of 20–60 embryos were transferred to Ficoll (2–5% Pharmacia) in iMR in plastic dishes to which a Nitex grid (1000 µm) had been fixed and just prior to injection excess buffer was removed. Injection apparatus and needles are described by Gurdon (1974) except that a Narishige MM3 manipulator was used to minimize lateral tearing motion. The surface tension of buffer above the eggs was used to hold the eggs in the grid during injection. For embryo injection, it is important to minimize the injection volume. The needles that make this possible are sufficiently fine that microfocusing of the tip is not necessary. 5–10 nl of solution containing 50–100 pg DNA (except where noted) or up to 20 ng RNA was injected at any time from 60 min postfertilization (at 22°C) to the 4-cell stage. We have also injected DNA during the first hour after fertilization (see also Rusconi & Schaffner, 1981; see below) and find similar results except that survival of the eggs is reduced. Injection volume was measured as described by Gurdon (1974). In any one experiment, eggs of similar stage were injected. To provide eggs of different stages, dishes of dejellied eggs were placed on a large aluminum plate at one end of which was a tray of ice. This provides a temperature gradient over the range 15–22°C. Eggs were injected in a variety of positions, though some
care was taken to avoid known positions of nuclei. Slight positive pressure was maintained in the needle so that continuous flow of solution occurred after penetration of the embryo.

After injection, the dish was refilled with JMR and embryos allowed to develop at either 15°C, 19°C or room temperature to enable harvesting of the appropriate stages. Control experiments showed that temperature did not affect the half-life of RNA with respect to developmental stage. Throughout this paper, times of incubation have been normalized to 23°C in order to simplify comparison with Nieuwkoop & Faber (1967) stages.

In the case of DNA injections, we found that the amount of DNA remains approximately constant until it gradually declines in the tailbud stage. This result is like that of Krieg & Melton (1985) but quite different to that of others, who have found amplification of DNA up to the gastrula stage followed by degradation of the DNA (Bendig, 1981; Rusconi & Schaffner, 1981; Etkin et al., 1984; Wilson et al., 1986). We have deliberately tried to achieve amplification of injected DNA by following the injection protocols of Schaffner and colleagues, but so far have not seen gross amplification. However, the amount of DNA injected is critical to amplification (Rusconi & Schaffner, 1981) and we always find such doses to be toxic even with highly purified DNA. With all of the promoters tested, we found no difference in transcription efficiency between circular and linear injected templates (not shown) though we did confirm that linear DNA is always ligated into long concatemers in eggs (Harland, 1980; Rusconi & Schaffner, 1981; Bendig, 1981).

(C) RNA preparation

Batches of five embryos were homogenized with a Pipemax in 0-5 ml of 1% SDS, 20 mM-Tris-Cl, pH 7-5, 100 mM-NaCl and 30 mM-EDTA prior to freezing at -80°C. Embryos were thawed at 37°C and supplemented with 0-2 ml proteinase K in the above buffer (250 μg ml⁻¹ final). After digestion at 37°C for 30 min, the tubes were mildly sonicated in a cup horn attachment of a Branson sonicator. This shears the DNA to ease extraction steps. The embryos were extracted once with aqueous phenol, and nucleic acids were precipitated by addition of sodium acetate (pH 5-5) to 0-3 M and an equal volume of isopropanol. The precipitate was resuspended in 50 μl diethyl-pyrocarbonate-treated water and reprecipitated by addition of 25 μl 10% LiCl for 1 h on ice. The RNA pellet was washed with 80% ethanol and resuspended in water. In some cases, the supernatant was saved and reprecipitated with ethanol for analysis of DNA by Southern blotting. Northern blotting was done as described (Condie & Harland, 1987) using an RNA probe generated from pSP65CATA (Harland & Weintraub, 1985). Direct autoradiographs were scanned to quantify CAT RNA. Control experiments in which known amounts of synthetic CAT RNA were mixed with oocyte RNA and subjected to the above procedure show that this method is quantitative over a range of 100 fg-1 ng CAT RNA. In some cases, bands were excised from the filter and counted directly. For removal of the background (as seen in Fig. 3), blots were incubated at room temperature in 2xSSC with 1 μg ml⁻¹ RNAse A and 1 unit ml⁻¹ RNAse T₁ prior to rinsing and stringent washing to yield clean blots (see Fig. 4).

Radioactive RNAs were visualized by fluorography of fixed and dried gels (Harland & Weintraub, 1985). For quantification, direct autoradiographs were scanned with a densitometer. For these experiments, the amount of [³²P]RNA injected into each sample of embryos was determined by Cerenkov counting of the homogenate. We find that even though RNA may be degraded in the embryo the radioactivity is not lost and is eventually recycled into new embryo RNA and DNA. Such quantification revealed small variations in the amount of RNA injected. The samples loaded onto the gels were adjusted to reflect the same original amount of radioactivity in the homogenate.

Results

Stability of injected adenylated or nonadenylated RNA

We wished to assay the stability of various RNAs in the developing Xenopus embryo. Accordingly, capped RNA was synthesized from templates containing an SP6 promoter (Harland & Weintraub, 1985). The transcripts were radiolabelled during synthesis by including [³²P]-GTP in the reaction mixture so that their fates could be monitored easily after injection into fertilized eggs. After incubation for various times the embryos were lysed and RNA purified for electrophoresis on denaturing agarose gels followed by autoradiography. We believe it to be important to use such a direct assay for full-length RNA since occasional nicks in the starting RNA may not show up in a nuclease protection assay and thereby would cause difficulties in interpreting experiments on stability. The use of a denaturing gel assay monitors both the amount and the size of the RNA.

The results of an experiment to determine the stability of various mRNAs are presented in Fig. 1. In agreement with others, we find that transcripts have a much shorter half-life in embryos than in oocytes (Colman & Drummond, 1986; Melton & Rebagliati, 1986; Bass & Weintraub, 1987). Whereas a transcript coding for CAT (chloramphenicol acetyl transferase) had a half-life of greater than 12 h in oocytes (Harland & Weintraub, 1985), the experiment presented in Fig. 1B shows that the same transcript is comparatively short lived in embryos. The synthetic mRNA injected into fertilized eggs is no longer detectable by the end of gastrulation. From quantification of the autoradiograph, we estimate the initial half-life of the RNA as 3-4 h.

Gurdon et al. (1974) reported that mammalian globin RNA is stable after injection. The assay available at the time was based on the production of labelled globin protein at intervals following injection. We examined the stability of natural frog a-globin RNA by a more direct Northern blotting
RNA stability in Xenopus embryos

Fig. 1. Stability of injected RNA. 10–20 ng RNA was injected into embryos at the 2-cell stage. Embryos were homogenized at the times indicated, RNA extracted and fractionated on denaturing agarose gels. Times are given as hours after fertilization; the first time point was taken less than 5 min after injection. (A) Frog reticulocyte mRNA selected by oligo(dT)-cellulose chromatography was injected into embryos. Adult α-globin RNA was detected by hybridizing a Northern blot with an RNA probe complementary to adult frog α-globin. (B) Synthetic capped RNA made from pSP65 CAT S was injected into embryos and detected by autoradiography. (C) Synthetic RNA made from a template with a homopolymer tract encoding a poly(A) tail of 80 nucleotides. (D) Polyadenylated CAT RNA; the same RNA as B, but containing an enzymically added poly(A) tail of about 200 nucleotides. (E) Synthetic human β-globin (oligo(A))

Method (Fig. 1A). Essentially we have confirmed that globin RNA is fairly stable after injection, though with a half-life of 10–15 h rather than greater than 8 days as reported by Gurdon et al. (1974). The discrepancy is probably due to the assay. Since the polysome content of early embryos is low (Woodland, 1974), RNAs may be in competition for translation (Laskey et al. 1977; Richter & Smith, 1981); later in development, polysome content is high and the competition may not be so severe. Therefore, early estimates of mRNA amount based on translation are likely to be low and the apparent half-life will be overestimated. A second possibility is that a small proportion of globin RNA was recruited onto polysomes and thereby stabilized (Richter & Smith, 1981; Audet et al. 1987); in this case, the apparent half-life of mRNA as judged by the translation product would be considerably longer than the half-life of total injected RNA.

In any case, natural globin RNA is considerably more stable than the CAT transcript described above. The difference in stability could be due to the sequence of the RNA or the difference in polyadenylation. We therefore examined the effect of different poly(A) length on CAT RNA stability. Initially we hoped that a poly(A) tail could be encoded in the transcription template, so we obtained a human globin cDNA clone with a long poly(dA) tract (Lang & Spritz, 1985; Lang et al. 1985) and, in addition, constructed plasmids with long poly(dA) tracts. A major problem with this approach, however, was the instability of such long homopolymer tracts during propagation in bacterial plasmids. The globin poly(A) tract was originally reported to be 225 nucleotides in length (Lang & Spritz, 1985) but we found that it had stabilized at approximately 65 nucleotides. Furthermore, the new plasmids we constructed had unstable homopolymer tracts. Although many of the original clonal isolates had homopolymer tracts of greater than 100 nucleotides, the size usually stabilized at 20–30 nucleotides (see Materials and methods for details). For experiments reported here, we used an isolate in which the bulk of the population of plasmids still had homopolymer tracts of about 80 nucleotides. As is evident from Fig. 1C, the transcripts synthesized from such templates and containing a short poly(A) tail are not significantly more stable than nonadenylated RNA.

Because of the limitations of poly(A) length that could be encoded in the transcription template, we resorted to enzymic addition of poly(A) tails to the transcripts using poly(A) polymerase after transcription. Although the tails added were of variable length we found that long tails of 200–300 nucleotides could be added. The main difficulty we encountered was partial degradation of the RNA in some reactions as was also observed by Drummond et al. (1986). Extensively degraded preparations were discarded since the analysis of such RNAs is complex. Fig. 1D shows the analysis of RNA with a tail of 200 nucleotides. We find that a long poly(A) tail stabilizes the transcript approximately twofold to yield a half-life of 6–8 h. In all other respects, this transcript is identical to the transcript tested in Fig. 1B; therefore, the increase in stability must be due to the increased length of poly(A). Although a doubling of half-life
does not appear dramatic, it is developmentally significant; instead of being completely degraded by the end of gastrulation a significant proportion of the RNA survives through neurulation.

Given that a long poly(A) tail can stabilize CAT RNA, it is possible that the stability of injected globin RNA could be accounted for solely by the length of its poly(A) tail. However, this experiment does not rule out a contribution of globin primary sequence to stability. We therefore synthesized a globin transcript with a short poly(A) tail encoded in the template (Lang et al. 1985). In three out of four experiments, this transcript was no more stable than nonadenylated CAT RNA (Fig. 1E). We conclude that the globin primary sequence does not add greatly to its stability. (This conclusion is supported by experiments presented below which show that endonucleases do not contribute to the instability of most RNAs). Although three out of four experiments showed that synthetic globin RNA was no more stable than CAT RNA, in one experiment (not shown here) the transcript was much more stable than CAT RNA injected into the same embryos and was as stable as natural globin RNA (surviving until the tailbud stage). This result raises the untidy possibility that different batches of embryos have different degradative activities on poly(A) tails; in this isolated case, the short tail of 65 A residues may have been sufficient to stabilize the globin RNA. Our general conclusion, however, is that a longer poly(A) tail is required to stabilize injected RNA.

One further observation from the gel analysis is that the polyadenylated RNA starts as a fairly heterogeneous population but the size distribution sharpens and the size decreases with time. This has been observed before, most clearly with transcripts that are transiently expressed (see for example Restifo & Guild, 1986). The fact that the size diminution does

![Figure 2](image-url)

**Fig. 2.** Stability of *Tetrahymena* rRNA intron circles and linears. (A) Autoradiograph of a 4% polyacrylamide gel showing the analysis of radioactive circular and linear RNA injected into embryos and harvested at the indicated times. In this experiment, the RNA was not capped. The exon fragments show the stability characteristic of uncapped RNA. The circular species is the most stable, but the linear intron is more stable than expected for uncapped RNA. The radioactive nucleotide released from degraded RNA is reincorporated into RNA and DNA. The position of rRNA in this gel is indicated. Size markers are pBR322 *Mspl* fragments. (B) Plot of stability of synthetic *Tetrahymena* RNAs containing the CAT sequence. The experiment is similar to that shown in A, but was quantified by densitometric scanning of gel lanes. In this experiment, splicing is less efficient than in A because of the CAT insert in the intron. The RNA was capped during synthesis (though only the unspliced linear remains capped). RNA amount is in arbitrary units. For plotting the lines decay kinetics were assumed to be exponential. No detectable unspliced linear RNA was detectable after 12 h.
not continue below the size of full-length CAT RNA suggests that this may be due to slow deadenylation followed by rapid degradation of the nonadenylated species. Evidence will be presented below that this may be due to slow deadenylation and not continue below the size of full-length CAT RNA.

Circular RNA is stable in embryos
The finding that a cap at the 5' end and a poly(A) tail at the 3' end increase the stability of RNA raises the question of whether cellular RNA is in general degraded by endonucleases, or whether all degradation can be accounted for by exonucleases. A simple test of this is to use RNA with no termini, namely circular RNA. Circular RNA was made by exploiting the self splicing pre-rRNA intron of Tetrahymena thermophila (Been & Cech, 1986). Transcripts including the intron will self splice in vitro in the presence of GTP and Mg\(^{2+}\); subsequently the linear intron will circularize. The experiment shown in Fig. 2A shows that, indeed, the circular RNA was extremely stable after injection. The simple conclusion that this is due to absence of termini was complicated by the observation that the linear form was somewhat stable, even though it contains no capped 5' end. We suspect that the stability is analogous to that of RNA and is due to the extensive secondary structure that the intron adopts (Price et al. 1985). In contrast, the linear exon fragments were quickly degraded, as expected for uncapped linear RNA.

To test the stability of a less-structured region of RNA within a circle we included the CAT coding sequences in the pre-rRNA intron. This was achieved by cloning the CAT fragment in the BgII site of the Tetrahymena pre-rRNA template. Transcripts of the new template self splice at reduced efficiency, presumably because the large CAT insert interferes with the formation of secondary structure necessary to the intron's enzymic activity; nevertheless, sufficient circles can be generated to test stability. We find that such large circles are stable in embryos, whereas all the linear species are progressively degraded. Densitometric quantification from the gel is presented in Fig. 2B. For simplicity, we only present the quantification of circle and unspliced linear. The unspliced molecule, which in this case was capped, has stability expected for a capped but nonadenylated RNA (cf. Fig. 1). The spliced linear (not shown) is intermediate in stability between the circle and unspliced linear, again suggesting that the ends may be protected by secondary structure of the whole intron. The circular form is extremely stable, with an estimated half-life of 40 h. Even this measurable rate of decay of the circle could be accounted for by slow back reaction of the circle to a linear form (Sullivan & Cech, 1985) and instability of this linear form. It is possible, therefore, that no endonucleolytic degradation of circular RNA occurs at all.

The main result of this experiment is that circular RNA is extremely stable after injection. We take these results as strong evidence that nonspecific endonucleases do not degrade cellular RNA and suggest that the primary route of instability is through deadenylation followed by 3' exonucleolytic degradation.

Use of injected DNA to measure RNA stability
A different way of testing RNA stability, and one which may be considered more physiological, is to generate transcripts in the embryo from an injected DNA template. If the promoter in the DNA is inducible, or only transiently active, a pulse of defined RNA can be generated whose decay kinetics can be measured. In all the following cases, the transcribed sequence included a marker CAT gene and the SV40 late polyadenylation signal. The SV40 late polyadenylation signal had previously been shown to function well in oocytes to produce polyadenylated RNA (Wickens & Gurdon, 1983), and we have confirmed that it functions well in embryos. The CAT gene provides a marker for quantitative detection of RNA by Northern blotting. In repeated reconstruction experiments, we find that CAT RNA yields quantifiable data over the range from 100 fg to 1 ng, a 10,000-fold range.

We have tested a variety of promoters for their activity in injected embryos and an example of the results is presented in Fig. 3. The tests were done with transcription templates similar to HS CAT (shown in Fig. 3A); different promoters were inserted upstream of CAT as described in Materials and methods. A more quantitative analysis of transcription results is given in Table 1. We estimate the time of activity of promoters as those stages when the longest species of RNA is being produced (before deadenylation occurs). We have also analysed the amount of DNA in the embryos at different stages by Southern blotting (not shown). Our results resemble those of Krieg & Melton (1985), who found that injected DNA did not amplify to a great extent, but persisted into late tailbud stages. The inactivation of the promoters at different times cannot therefore be attributed to the gross absence of DNA.

Graves et al. (1985) had previously shown that the murine sarcoma virus (MSV) LTR promoter was active in injected oocytes and we expected it to be constitutively active at all stages of development. Surprisingly, however, it is only transiently active in embryos from the midblastula transition until gastrulation (Fig. 3B, lanes 9–11; Table 1). The L1 and L14 ribosomal protein gene promoters are active from the
midblastula transition until neurula stages (Fig. 3, lanes 12–14; Table 1). The X. borealis cytoskeletal actin promoter is active until the latest times, being on at low levels prior to gastrulation, but reaching a peak of activity from gastrulation until early tailbud stages (Fig. 3, lanes 15–17; Table 1).

The most useful promoter for studying RNA stability is the Xenopus heat-shock promoter. This injected promoter is inactive at 18°C, but is readily inducible by heat shock in embryos, producing detectable transcripts at 25°C and reaching maximal activity at 33°C (not shown). Fig. 3 lanes 18–20 show the inducibility of the promoter at different stages. RNA was harvested after 30 min of heat shock at the early gastrula, neurula or tailbud stages. The injected promoter is inducible from the midblastula transition onward, but its induced activity declines from the neurula onward such that it is only weakly induced in the tailbud tadpole (Fig. 3, lanes 18–20; Table 1). The amount of transcript made in a 30 min heat shock is related to the ratio of DNAs injected; in this case pHS CAT TF10 was injected in excess over HS CAT.

We have done most experiments with the heat-shock promoter and have determined that the half-
RNA stability in Xenopus embryos

Table 1. Stage and dose dependence of promoter efficiency

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Key: 0 not detected in 15 h exposure (+) detectable but less than 1 pg + 1–5 pg ++ 5–25 pg +++ 25–125 pg ++++ 125–625 pg

Batches of 40 embryos were injected with the amounts of DNA indicated in a volume of 10 nl. Groups of embryos were harvested at the indicated times and processed for Northern blotting. Amounts of RNA were estimated compared to a standard curve of synthetic CAT RNA serially diluted in oocyte RNA so that the same amount of total RNA was loaded in each lane. Abnormal embryos were harvested as long as no signs of cytosis were present. Embryos injected with 1 ng DNA always cleaved abnormally in the blastula stage. Such embryos do not develop beyond gastrulation. If fewer than five noncytolytic embryos remained the table shows ‘dead’. In our hands, only embryos injected with less than 100 pg DNA are viable indefinitely. The absolute amount of transcript produced by the promoters is quite respectable when compared to an abundant mRNA like muscle actin, which produces 10–100 pg RNA (10^7–10^8 transcripts, Mohun et al. 1984); the heat-shock promoter, which is the most active we have tested, makes up to 25 pg RNA in a 30 min heat shock when injected at nontoxic levels. In this context, it is worth noting that the amount of RNA made appears to be proportional to the amount of template injected. In the case of the cardiac actin gene, Wilson et al. (1986) found that ten times more transcript was made from the injected DNA (which amplified 10-fold) than was made from endogenous genes (i.e. 100–1000 pg RNA).

One notable feature of the decline in size of RNAs is that the decline does not continue beyond the size of nonadenylated RNA, rather the RNA is degraded completely. This observation highlights the importance of a poly(A) tail for RNA stability; once the tail...
is removed degradation is complete. In this paper, we do not address any connection between the adenylation state of an RNA and its translation. We do know, however, that all these plasmids transcribe translatable RNA by performing enzyme assay for CAT activity (not shown).

It is easiest to measure the stability of RNA produced from the promoters that are most transiently active; however, it is clear that even for long-acting promoters that produce RNA of more heterogeneous size the stability is of the same order. Although we cannot rule out the possibility that a promoter can affect RNA stability, perhaps by modifying the transcriptional machinery, the variety of examples reported here suggests that the possibility is not generally true. Most importantly for the subsequent experiments, the heat-shock promoter does not encode RNA of unusual stability or instability (data presented below).

**Long 3' untranslated regions do not affect RNA stability**

Results presented above show that a circular RNA is stable and we concluded that nonspecific endonucleases are not active in the embryo even though the RNA is not translated. Another way of testing this possibility is to examine the stability of RNA with different-sized 3' untranslated regions. This method uses substrates with normal 5' and 3' ends, in contrast to the injection of circular RNA. In prokaryotes, it is thought that untranslated RNA is extremely sensitive to endonucleases (Cannistraro et al. 1986); however, here we show that the presence of a 1 kb untranslated region has no effect on RNA stability. We have tested four different sequences for their effect on RNA stability, the longest of which is from a *Xenopus* homeobox-containing gene, *Xhox-36* (clone 36.1, Condie & Harland, 1987). This cDNA clone spans the entire 3' untranslated region of 1000 nucleotides. A 1 kb fragment of the cDNA was inserted downstream of the CAT gene in the heat-shock/CAT plasmid. The new DNA construct was coinjected into embryos with the unmodified CAT construct serving as a control. Because there is experimental variation in the amount of transcript made in different samples this control is important; it allows the comparison of RNA amounts within a lane rather than between lanes. At various times after heat shock, RNA was examined by Northern blotting and the results are presented in Fig. 4A. The RNA containing the *Xhox-36* 3' untranslated region is 1 kb larger than the original CAT transcript and easily distinguished. Two transcripts of intermediate size appear in the autoradiograph; the size of these is consistent with polyadenylation at two AAUAAA sequences that we
have identified in the Xhox-36 sequence. These RNA species have consistently been found to be slightly less stable than the transcripts that terminate at the SV40 polyadenylation sequence but the reasons for this have not been investigated further. Neither of the Xhox-36 polyadenylation signals works quantitatively in this context thus leaving an ample amount of transcript which extends all the way to the SV40 polyadenylation site. Data presented in Fig. 4A show that transcripts either containing or lacking the Xhox-36 sequences and that terminate at the SV40 polyadenylation site have equivalent stability. We therefore conclude that the 3' untranslated sequence does not act as a target for degradation. Inspection of the DNA sequence (not shown) reveals that the Xhox-36 3' untranslated sequence contains numerous stop codons in all three reading frames; therefore, even if ribosomes were to reinitiate translation after the CAT open reading frame, translation would not proceed far (see review by Kozak, 1986). We therefore assume that this stretch of RNA cannot be protected from endonucleases by ribosomes.

Although we initially considered that the Xhox-36 transcript might be particularly unstable, it is clear from these results that the normal level of instability conferred by deadenylation and exonucleolytic degradation is adequate to account for the disappearance of the transcript between the late neurula and tadpole stages (Condie & Harland, 1987).

**Fig. 5.** Plasmids used to locate destabilizing sequences. The TFIIIA cDNA clone used as starting point for these constructs is illustrated with relevant sites (Bg, BglII; S, Sau3A; X, XbaI; B, BamHI). This sequence terminates at the polyadenylation point identified by T'so et al. (1986) at nucleotide 1328 of the sequence of Ginsberg et al. (1984). The plasmid pHSCATTFl0 contains a heat-shock promoter, 800 nucleotides of CAT sequence and TFIIIA sequences from nucleotide 342 to 1328. A short polylinker containing BamHI and XbaI sites precedes the late polyadenylation signal of SV40. The TFIIIA sites shown are at 870 (Sau3A); 1052 and 1212 (XbaI). A 21-nucleotide stretch of AT containing two ATTTA motifs is present from 1088 to 1109. ΔXba3 and 4 remove sequences from 1212 to 1328 and 1052 to 1328, respectively. The HSCAT TFSau3A constructs were derived from HSCAT by inserting the Sau3A fragment from pHSCATTFl0 (a Sau3A fragment from 870 to the BamHI site in the linker between TFIIIA sequence and SV40 sequence). The overlap of TFIIIA sequence between ΔXba4 and HSCAT TFSau3A is from nucleotide 870 to 1052.
Fig. 6. Quantification of stability of RNA expressed from injected plasmids. Densitometry of the films shown in Fig. 4 was used to generate some of the data. Additional data came from other injection samples from the same batch of embryos, using the plasmids diagrammed in Fig. 5 coinjected with control HS CAT DNA. Lines were drawn assuming that the RNA decayed exponentially. Open squares show results with RNA expressed from HS-CAT; triangles show results from RNA expressed from HS-CAT TF10. Half-lives are 3 h (CAT) and 15 min (CAT TF10).

The main conclusion of the experiments where the length of 3' untranslated sequence is altered is that nonspecific endoribonucleases are not active in the embryo, supporting the conclusions made earlier from experiments with circular RNA.

A sequence in TFIIIA mRNA that causes instability

The data presented so far suggest that only exonucleases act to degrade mRNA and that nonspecific endonucleases are not responsible. However, we have not yet addressed the question of why some transcripts are relatively unstable. We therefore tested a likely candidate sequence which may contain a destabilizing sequence, in order to find out whether such a sequence may act as a site for a sequence-specific endonuclease. An example of a mRNA that may be particularly unstable is the mRNA coding for the transcription factor TFIIIA. This maternal transcript is degraded some time before the end of gastrulation (Taylor et al. 1986). We found that, in contrast to other sequences, a model transcript-containing TFIIIA sequence is extremely unstable when made in the embryo. We inserted the CAT gene into the plasmid pHSTF10 (gift of Jay T'so and Laurence Korn) and monitored the fate of the RNA after injecting this plasmid with the control HS-CAT plasmid into embryos. In contrast to the control CAT mRNA, the RNA containing the TFIIIA sequences has a half-life of less than 30 min, and in this particular batch of embryos the RNA had a half-life of 15 min (Figs 4B, 6). The difference cannot simply be due to size since we have already shown in Fig. 4A that an extended 3' untranslated sequence does not affect RNA stability. From the size of the RNA produced by pHs CAT TF10, we can be sure that polyadenylation is occurring at the late SV40 polyadenylation site. We can therefore rule out the possibility that the unusual ATTAAA polyadenylation signal of TFIIIA mRNA (T'so et al. 1986) is responsible for the instability; rather, we conclude that the TFIIIA sequences affect stability when they are internal in the RNA, possibly by acting as a target for a site-specific endoribonuclease.

An obvious candidate for the destabilizing sequence in a 21-nucleotide stretch of AU which contains two repeats of the AUUUA motif identified by Shaw & Kamen (1986) in a variety of growth factor RNAs. In order to characterize the destabilizing sequence further, we constructed the plasmids shown in Fig. 5. Using the enzyme XbaI we deleted sequences from pHSCATTF10, and found that the RNA from both the deleted plasmids AXba3 and AXba4 was as unstable as RNA from the parent plasmid. Of these AXba4 deletes the 21-nucleotide AU sequence, showing that it is not required in the RNA to mediate instability. Furthermore we took a 500-nucleotide Sau3A fragment from pHSTF10 and inserted it in both orientations downstream of the CAT gene in HS CAT. Only one of these plasmids, in which the TFIIIA sequences were inserted in the sense orientation, synthesized unstable RNA. The overlap of common sequence between AXba3 and HS CAT TFSau3A is only 180 nucleotides which is contained within the protein-coding sequence for TFIIIA.

In order to obtain a more accurate time course of decay of RNA containing or lacking TFIIIA sequence we quantified the data from the experiment described above, where the plasmids diagrammed in Fig. 5 were assayed (the experiment shown in Fig. 4B is included in the analysis). The combined results are presented in Fig. 6. Assuming the RNA decays exponentially, this analysis yields a half-life for the CAT RNA of 2-3 h at 23°C; in this batch of embryos inclusion of TFIIIA sequences reduces the half-life to 15 min. Whereas the half-life for CAT RNA is fairly consistent, the destabilizing sequence had a particularly dramatic effect in this batch of embryos, the more typical half-life being closer to 30 min.
Discussion

Contribution of polyadenylation to RNA stability
We have tested the stability of a variety of injected RNA sequences and conclude that a long poly(A) tail of 200 nucleotides has a stabilizing effect on RNA in the developing Xenopus embryo. Without a tail, the RNA has a half-life of 3–4 h and with a tail the half-life is doubled to 6–8 h. Thus, if endogenous transcripts behave in the same way, we would expect nonadenylated RNAs to be degraded by the end of gastrulation whereas adenylated transcripts would persist at some level through neurulation. We do not know to what extent polyadenylation is used by the embryo to effect changes in the stability of maternal mRNA. Clearly many transcripts undergo deadenylation during early development (see discussion in Dworkin & Dworkin-Rastl, 1985) but these changes may not be sufficient to account for the differing stability of different maternal RNAs.

Poly(A)-binding proteins interact with stretches of poly(A) of greater than 30 nucleotides (Baer & Kornberg, 1980). Poly(A) tails of greater than this length might therefore be expected to have a stabilizing effect as suggested by the injection of native, deadenylated and readenylated RNAs into oocytes (reviewed by Littauer & Soreq, 1982; Nevins, 1983). However, we find that poly(A) tails of 60–80 nucleotides encoded in the plasmid template do not reliably stabilize injected RNAs in embryos. Since we see that the poly(A) tails on injected RNAs are progressively removed it may be that short tails are removed sufficiently rapidly that any short-term stabilization is not seen in the assay. Alternatively, the stabilizing effect may be cooperative and require several poly(A)-binding proteins to affect stability significantly.

Our results are consistent with results obtained in tissue culture cells, where the drug corticosterone (3’dATP) was used to inhibit polyadenylation (Zeevi et al. 1982). Transport of RNA to the cytoplasm was not affected, but the half-life of RNA was markedly reduced. The experiments reported here have the advantage that the RNA was introduced into a normal, viable cellular environment so that we can exclude any indirect effects that a drug may have.

Stability of endogenously transcribed sequences
We have tested the stability of RNA produced from four different promoters. We can estimate stability in all cases because the promoters are either inducible (heat shock), or only transiently active (MSV LTR, L14 and cytoskeletal actin). The clearest cases are for MSV or the heat-shock promoters which make a brief pulse of RNA. Decay of the transcripts follows a half-life of about 2.5 h (normalized to development at 23°C). We also see that the RNAs produced by these plasmids are polyadenylated. When first made we estimate the poly(A) tail length to be approximately 250 nucleotides; the length progressively declines until the length of nonadenylated RNA is reached. At this time no further decrease in size is seen, rather the RNA disappears completely. We take this as supporting evidence that a poly(A) tail is required for stability. The actin and L14 promoters are active for longer than MSV and heat-shocked promoters and so decay kinetics are best estimated when they turn off. We can estimate this time because RNA of greatest poly(A) tail length is no longer present. We estimate that these promoters produce RNA of equivalent half-life to RNA transcribed from the MSV or heat-shock promoters (Fig. 3; Table 1 and data not shown).

The half-life of these endogenously made RNAs is shorter than that of polyadenylated RNA injected into the egg even though its tail is about the same size (200–250 nucleotides). Since injected plasmids are only expressed after the midblastula transition, it is difficult to compare stability of injected RNA and RNA expressed from injected plasmids at the same developmental times. The difference might reflect a difference in the metabolism of newly synthesized RNA compared to RNA that has been in the cytoplasm since early cleavage stages.

Stability of RNA to endonucleases
Two kinds of experiments support the idea that internal sequences in RNA are not susceptible to general endonucleolytic cleavage. First, we find that circular RNA is quite stable in embryos even when it contains CAT sequences that are normally degraded as linear (Fig. 2A,B). Second, when a long 3' untranslated sequence (from Xhox-36) is introduced into a transcription unit driven by the heat-shock promoter, it has no effect on stability (Fig. 4A). These results confirm that eukaryotic RNA stabilization is quite different from that of prokaryotes, in which untranslated RNA is exquisitely sensitive to nuclease (Cannistraro et al. 1986). Superficially these results would indicate that RNA stability would be regulated by the length of the poly(A) tail and the stability of the cap. We have argued that the poly(A) tail is subject to removal and we would therefore expect that no RNA would be more stable than any of the model CAT transcripts. However, this model is difficult to reconcile with evidence that some RNAs are unusually stable. In many cases, RNAs are specifically stabilized (reviewed by Shapiro et al. 1987) but there are no clear cases of endogenous RNAs in developing Xenopus embryos that have unusually high stability. However, inspection of the data presented by Krieg & Melton (1985) would
suggest that a GS17/globin fusion transcript expressed from an injected plasmid in unusually stable. Although the promoter is only active until gastrulation, the transcripts persist until the tailbud stage; in contrast, we find that CAT transcripts from the similarly active MSV promoter are completely absent at the tailbud stage.

A destabilizing sequence in TFIIIA mRNA

An AU-rich sequence in the 3' untranslated region of a growth factor RNA has a destabilizing effect on mRNA (Shaw & Kamen, 1986). We have found a similarly acting sequence in a transcription factor RNA and assayed its activity in the developing embryo. The maternal mRNA, for TFIIIA, does not persist beyond gastrulation and is thought to be specifically targeted for degradation (Ginsberg et al., 1984; Taylor et al. 1986). We have shown that a specific mechanism exists to destabilize the RNA. It is likely that the destabilizing activity is induced during embryogenesis, since the half-life of TFIIIA mRNA is much more than 30min in the oocyte and early embryo (Ginsberg et al. 1984; Taylor et al. 1986). In this respect, the destabilizing activity resembles that for GM-CSF, which is induced in cells by phytohaemagglutinin but not phorbol ester (Shaw & Kamen, 1986).

By using the plasmid diagrammed in Fig. 5, we have shown that a 23-nucleotide AU sequence similar to that shown by Shaw & Kamen (1986) to destabilize growth factor RNAs is not required in this example of an unstable RNA. This sequence is in any case polymorphic in Xenopus laevis TFIIIA genes and is absent in the genomic sequence of T'so et al. (1986). From the series of plasmids we have tested (Fig. 5), it appears likely that a 180-nucleotide sequence in TFIIIA mRNA may contain all the information necessary to destabilize it. This 180-nucleotide sequence is contained within the coding region for TFIIIA protein in the normal mRNA, though in the constructs we have tested, the sequence is downstream of the CAT open reading frame. We therefore would expect the 180-nucleotide sequence to be poorly translated, if, indeed, it is translated at all. Interestingly, a destabilizing sequence in tubulin mRNA is within a protein-coding region of the mRNA (Gay et al. 1987). Experiments to characterize the mechanism of destabilization further are currently in progress.

We thank M. Bienz and J. T'so for heat-shock plasmids, G. Cross and H. Woodland for actin DNA and E. Beccari for the L14 DNA. We thank C. James for help in constructing and testing pH5S CAT TF10. We are grateful to our colleagues for their comments on the manuscript and in particular to J. Gerhart and F. Wilt. This work was supported by grants from the NIH.

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(Accepted 5 January 1988)