Endonucleolytic cleavage of a maternal homeo box mRNA in *Xenopus* oocytes

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We have identified a messenger RNA (mRNA) sequence from a *Xenopus* homeo box-containing gene that is the target for a sequence-specific endoribonuclease in vivo. Synthetic RNA transcribed from an allele of the maternal gene *Xlhbox2B* is efficiently cleaved when injected into *Xenopus* oocytes. The cleavage sequence lies between the protein-coding region and a 600-base 3'-untranslated region. Intermediates in degradation are readily observed: Both the 5' and 3' products of cleavage are recovered, thus showing that the cleavage activity is an endonuclease. When a 90-base region of the *Xlhbox2B* sequence is inserted into a second homeo box RNA that is normally stable, it is sufficient to confer an identical cleavage reaction on the hybrid RNA. The cleaved region contains a repeated sequence motif and is cut at multiple sites. Inhibition of translation does not affect the rate or extent of cleavage, while the coinjection of antisense RNA complementary to the 90-base region completely blocks the reaction. Because most mRNAs are not found on polysomes during oogenesis, translation-independent cleavage at such sites may provide a novel post-transcriptional mechanism to regulate the amount of mRNA available for embryogenesis.

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The stability of messenger RNA (mRNA) plays a crucial role in the regulation of gene expression, but as yet the determinants of differential mRNA stability in eukaryotic cells have not been characterized in biochemical detail (for review, see Brawerman 1987, 1989; Shapiro et al. 1987; Cleveland and Yen 1989). Attempts to identify the initial events in RNA turnover have been hampered by the problem that intermediates in degradation are usually difficult to detect because cells contain exonucleases that rapidly degrade RNA after initial destabilizing events (Ross 1988).

Protection of intact mRNA against general exonucleases is afforded by specialized structures at both the 5' and 3' ends of the molecules. A 5' cap on most mRNAs, with the exception of some viruses (for review, see Banerjee 1980), is crucial for mRNA stability because it blocks the activity of 5' to 3' exonucleases on both natural and synthetic RNAs (Furuichi et al. 1977; Green et al. 1983). Poly(A) tails, found at the 3' end of most eukaryotic mRNAs, have been shown to be resistant to 3' to 5' nucleases when bound by poly(A)-binding proteins (Bergmann and Brawerman 1977; Bernstein et al. 1989). Further evidence that the ends of RNA are the main target for cellular nucleases comes from injections of circular RNAs into *Xenopus* embryos. Such RNAs, lacking either 5' or 3' ends, are found to be exceptionally stable when compared with linear RNA molecules (Harland and Misher 1988).

It is not surprising, then, that removal of the poly(A) tail appears to be a crucial event in the degradation of

many mRNAs. During selective turnover of *c-myc* and *c-fos* mRNAs, deadenylation occurs prior to the rapid degradation of the remainder of the molecules (Brewer and Ross 1988; Wilson and Treisman 1988). Deadenylation prior to degradation has also been reported in *Xenopus* oocytes and embryos (Colot and Rosbash 1982; Hyman and Wormington 1988). Although the specific effect of the poly(A) tail on stability and translation in many systems remains controversial (for review, see Sachs and Davis 1989; Jackson and Standart 1990), it is clear that enzymatic addition of a poly(A) tail to mRNA synthesized in vitro stabilizes it after injection into both oocytes and developing embryos (Drummond et al. 1985; Harland and Misher 1988).

The relative influences of endonucleases and exonucleases and their interactions with the translation apparatus of the cell to destabilize mRNAs differentially are not well understood. Destabilization of mRNA in vivo is usually associated with either translation of the target mRNA or translation of total cellular mRNA (for review, see Ross 1988; Brawerman 1989). For example, tubulin mRNA degradation caused by an increase in free tubulin subunits is based on the recognition of the nascent amino termini on polysomes, and not strictly on the tubulin mRNA sequence (Yen et al. 1988), thus accounting for the fact that only polysomal mRNA is subject to degradation (Pachter et al. 1987). During histone mRNA degradation ribosomes must translate to the vicinity of the destabilizing sequence for rapid degradation to occur (Graves et al. 1987). In addition, translational

inhibitors generally stabilize mRNAs (for review, see Shaw and Kamen 1986; Ross 1988; Brawerman 1989). One mechanism to account for this is that the nucleases that initiate degradation are associated with the ribosome and translation inhibition prevents access of the nucleases to the mRNA. However, an alternative hypothesis is that the nuclease activity is itself unstable and must continually be replenished by translation; this is suggested by experiments in which the selective destabilizing activity in a cell-free extract was measured and found to be depleted in extracts from cycloheximide-treated cells (Brewer and Ross 1989).

Xenopus oocytes offer a simple system in which to study RNA stability because most mRNAs that have been examined in these oocytes are unusually stable, and the absolute levels of most endogenous mRNA species are established very early in oogenesis (Golden et al. 1980; Dworkin and Dworkin-Rastl 1985). Many RNAs persist throughout oogenesis (Ford et al. 1977; for exceptions, see Dworkin and Dworkin-Rastl 1985; Dworkin et al. 1985). Furthermore, synthetic RNAs without poly(A) tails persist for many hours after injection (Drummond et al. 1985; Harland and Weintraub 1985; Galili et al. 1988; Kruijs et al. 1989). For these reasons, and because of the low level of RNases in oocytes (or the presence of nuclease inhibitors; Xing and Worcel 1989), intermediates in the degradation of specifically destabilized mRNAs may be detectable. The identification of such intermediates should allow the detailed characterization of the initial cleavage events in mRNA turnover.

In this work we have examined the turnover of synthetic *Xlhbox2* mRNA. *Xlhbox2* is a homeo box gene of the *Antennapedia* class whose transcripts are found in *Xenopus* oocytes, eggs, and embryos (Müller et al. 1984; Wright et al. 1987; A. Hemmati-Brivanlou and B.G. Condie, unpubl.). Absolute *Xlhbox2* mRNA levels in *Xenopus* oocytes are quite high in previtellogenic oocytes, but decrease to a low level in later stages (Wright et al. 1987), thus posing the question of how *Xlhbox2* mRNA is specifically destabilized in a population of RNAs that are normally extremely stable. In addition, two *Xlhbox2* genes have been found in the *Xenopus* genome; these are referred to as type A (formerly MM3) and type B (formerly p52) (Müller et al. 1984; Wright et al. 1987; Fritz et al. 1989). Small sequence differences in the coding regions of the type A and type B genes result in 11 substitutions and two deleted residues out of 220 amino acids, but the largest sequence difference is a 123-base deletion in the 3'-untranslated region (3' UTR) of *Xlhbox2A* (Fritz et al. 1989). As we show below, this sequence that is deleted from *Xlhbox2A* may be critical in regulating the stability of *Xlhbox2B* mRNA.

To investigate the mechanism of turnover, we injected synthetic *Xlhbox2B* mRNA into oocytes and show that sequence-specific endonucleolytic cleavages are detectable within 30 min after injection. Both the 5' and 3' products of cleavage are recovered and directly observed. Cutting occurs when the translation of the *Xlhbox2B* RNA is blocked by injecting it as an uncapped

molecule or when total cellular translation is inhibited. We have also identified the sequence responsible for conferring cleavage on *Xlhbox2B* by transferring it into a normally uncleaved, synthetic mRNA that encodes *Xhox36* (Condie and Harland 1987). We are able to recover both 5' and 3' products of cleavage; therefore, this is an unambiguous description of endonucleolytic cleavage of a cellular mRNA in vivo.

Results

Xhox45 RNA injected into oocytes is cleaved

To study the degradation of *Xlhbox2* RNA, we made use of cDNA clones isolated in a screen for *Antennapedia* class homeo boxes (Condie and Harland 1987). The cDNA clone *Xhox45* is related to *Xlhbox2B*. Synthetic RNAs, diagrammed in Figure 1A, were transcribed in vitro in the presence of [α - 32 P]CTP and then injected into the cytoplasm of *Xenopus* oocytes. After incubation and reisololation the RNAs were analyzed by denaturing gel electrophoresis and autoradiography, as seen in Figure 1B.

Figure 1. Injection of RNA into *Xenopus* oocytes and detection of mRNA cleavage. (A) Structures of the RNAs used in injection experiments. Boxed regions indicate protein-coding sequences. The bar below the constructs is 100 bases in length. (B) Autoradiograph of mRNAs injected into *Xenopus* oocytes, reisolated with total cellular RNA, and run on a denaturing agarose gel at one oocyte per lane. The time of reisololation after injection is given in hours above each lane. Denatured λ HindIII/EcoRI markers from 2 kb to 0.125 kb are shown. Lanes 2 and 4 do not show any discrete degradation products from *Xhox36* or CAT RNAs. Lanes 6, 8, and 10 show the appearance, after 4 hr, of RNA species shorter than originally injected. (x) The approximate positions of the 3' ends of these shortened RNAs are shown in A.

As controls, we injected two RNAs: Chloramphenicol acetyltransferase (CAT), which has been shown previously to be stable in oocytes (Harland and Weintraub 1985) and *Xhox36*. The amount of full-length *Xhox36* transcripts decreases, but no distinct degradation products are detected (Fig. 1B, lane 2). Similarly, no distinct degradation products are observed for the CAT RNA (Fig. 1B, lane 4). In contrast, the amount of full-length *Xhox45* transcript, 1342 bases in length, is seen to decrease after 4 hr and a cleavage product of ~800 bases in length appears below it (Fig. 1B, lane 6). To characterize the cleavage reaction further, we injected truncated derivatives of *Xhox45* RNA. The d21 construct is a large 5' deletion of *Xhox45* in which only 53 bases of the coding sequence and the 650-base 3'-untranslated region remain (see Fig. 1A), producing a transcript with a length of 752 bases. This RNA is cleaved to yield a product of ~200 bases, although somewhat longer material can be seen in a smear above the most intense band (Fig. 1B, lane 8). To test the effect of altering 3' sequences we extended the d21 transcript by linearizing the d21 plasmid with *PvuII*. This modified template produces a d21 transcript lengthened at its 3' end by 270 bases of vector sequence, for a total length of 1022 bases. This transcript is also cleaved to yield a product that is identical in length to that from d21 (Fig. 1B, lane 10).

The length of the degradation product bands observed for the *Xhox45* RNAs shown in Figure 1B is dependent only on the length of RNA on the 5' side of the cleavage site (cf. Fig. 1B, lanes 6, 8, and 10); furthermore, the observed size decrease of each injected RNA species corresponds precisely to the length of RNA on the 3' side of the putative cleavage site. These results, and the results of injections using other 5' and 3' deletion and insertion constructs (Fig. 2B, and data not shown), demonstrate that the shortened bands observed in Figure 1B are the capped 5' ends of the RNA molecules. The unlikely possibility that the shortening is due to splicing is also eliminated, because a splicing event would remove a constant length of sequence from each RNA.

Because changes to the 5' and 3' ends of *Xhox45* RNAs do not alter the efficiency of cleavage, the recognition of the *Xhox45* message does not require sequences at the termini of the RNA. We also find that the addition of ~200-base poly(A) tails to the 3' end of the RNA using poly(A) polymerase has no effect on the cleavage reaction (data not shown).

Confirmation that the *Xhox45* cleavage activity is an endonuclease

Sequence-dependent targeting of a 3' to 5' exonuclease to the *Xhox45* mRNA could produce shortened 5' fragments if the exonuclease were to stall in a few specific regions or if it were to release the RNA after degrading the targeting sequence itself. Therefore, an unequivocal demonstration of an endonuclease activity requires the observation of both the 5' and 3' RNA fragments, as shown in Figure 2.

An autoradiograph of radiolabeled d21 RNA at high

resolution is shown in Figure 2a. The bands marked B (Fig. 2a, lanes 2–6) correspond with the capped 5' fragments of d21 seen at lower resolution in Figure 1B. In this short time course, analyzed by polyacrylamide gel electrophoresis, these 5' fragments are resolved into four major bands (Fig. 2a, arrowheads), revealing that cleavage occurs at several sites. Most importantly, however, bands corresponding in size to putative 3' cleavage fragments (A in Fig. 2a, lanes 2–4) are also detected. Because of the structure of the d21 RNA, the 3' fragments are longer than the capped 5' fragments (Fig. 1A).

The size spread of the two sets of bands is consistent with endonucleolytic cleavage of d21 RNA: Their approximate respective lengths add up to the total 752-base length of d21. Note that the uncapped 3' fragments decay more quickly than the shorter capped 5' ends, explaining why these bands were not detected in the longer time course shown in Figure 1. CAT mRNA was used as the control, and although the level of this RNA decreases after 8 hr and despite the long exposure, no distinct degradation products are detected (Fig. 2a, lane 8).

To confirm the identity of the bands shown in Figure 2a, unlabeled RNAs were injected and the cleavage products were visualized with 5'- and 3'-specific probes in a Northern blot experiment shown in Figure 2b. In lanes 1–4, d21 and d133 RNAs (diagramed in Fig. 2d) were probed with the 3'-most 280 bases of d21. Specific bands below the full-length injected RNA were detected in the 80-min samples (A in Fig. 2b, lanes 2 and 4). These cleavage products, detected by the 3' probe, are identical in size even though full-length d21 and d133 RNAs differ in length by 81 bases at their 5' ends, thus confirming their identity as 3' cleavage fragments.

A probe complementary to the 5' 201 bases of d21 was used to reprobe the blot, and the results are shown in Figure 2b, lanes 5–8. As expected, small and diffuse RNAs corresponding to the 5' cleavage products were detected (B_{d21} in lane 6 and B_{d133} in lane 8), and they differ in size according to the size difference of the injected full-length RNAs (cf. with Fig. 1, lanes 8 and 10). The 5' probe does not detect the 3' species labeled A (Fig. 2b, lanes 2 and 4).

As a final confirmation of the identity of the bands shown in Figure 2, a and b, 5'- and 3'-specific DNA oligonucleotides were used with RNase H to separately destroy the d21 RNA cleavage fragment sets, as shown in Figure 2c. Three samples of cold d21 RNA reisolated 80 min after injection were treated with RNase H, plus or minus specific DNA oligonucleotides (diagramed in Fig. 2d), and analyzed by Northern blotting. The blot was probed with full-length d21. The control shows that RNase H treatment without DNA oligonucleotides leads to some general RNA degradation, but the cleavage products are still visible (Fig. 2c, lane 9). The 3' fragments are again labeled A, and the 5' fragments are labeled B.

An identical RNA sample, after treatment with RNase H and the 3'-specific oligonucleotide, is shown in lane 10 (Fig. 2c). The 3' fragments are destroyed, but the 5'

Figure 2. Detection of both the 5' and 3' cleavage products. (a) Capped RNA from the d21 construct (Fig. 1A) was injected and reisolated after the times indicated in minutes above the lanes. The gel was 4% acrylamide/8 M urea, with total RNA from one oocyte per lane. Bracket A marks the uncapped 3' cleavage fragments; bracket B marks the separately resolved 5'-capped fragments. Arrowheads mark the most intense 5' cleavage product bands (lanes 3–6). Note the rapid disappearance of the uncapped 3' fragments relative to the 5' fragments (lanes 3 and 4). In spite of the overexposure of the CAT samples (lanes 7 and 8), no distinct degradation products of CAT mRNA are seen. Because of the exposure, a small fraction of full-length d21 appears to resist cleavage (cf. lane 1 with lane 6), but completion of the reaction is seen in longer time courses. The DNA markers are denatured pBR322 *Hae*III fragments sized 587, 540, 504, 458, 434, 267, 234, 213, 192, and 184 bases from top to bottom. Full-length d21 RNA is 752 bases in length. (b) Northern blot analysis of d21 and d133 RNA cleavage; 81 bases were deleted from the 5' end of the d21 construct to produce d133. Times in minutes are given above each lane. Lanes 1–4 were probed with a 3'-specific probe and in the 80-min time points (lanes 2 and 4), distinct cleavage product bands of equal length can be seen (A). Lanes 5–8 are 1–4 reprobed with a 5'-specific probe, and 5' cleavage products of unequal lengths are detected (lane 6, B_{d21}; lane 8, B_{d133}). The size difference of the 5' cleavage products corresponds to the size difference of d21 and d133 RNAs at their 5' ends. (c) A Northern blot probed with full-length d21 showing specific oligodeoxynucleotide/RNase H identification of the cleavage fragments in 80-min time point d21 RNA. A sample of the same d21 RNA used in b (lanes 2 and 6) shows some increased background due to general degradation when treated with RNase H alone, but the cleavage products are both visible and indicated as A and B for 3' and 5' fragments, respectively (lane 9). Treatment with RNase H and a 3'-specific complementary DNA oligonucleotide destroys only the full-length RNA and the 3' cleavage bands (bracket A, cf. lane 10 with lane 9), while an identical treatment using a 5'-specific oligonucleotide destroys only the full-length RNA and the 5' cleavage bands (bracket B, cf. lane 11 with lane 9). (d) A diagram of the constructs used in a–c. d133 RNA is identical to d21 except for an 81-base deletion near its 5' end. The positions of the 5'- and 3'-specific Northern blot probes are shown as heavy bars. The positions of the 5'-specific (1) and 3'-specific (2) DNA oligonucleotides used for the RNase H assay are shown as solid black boxes. (x) The cleavage region.

fragments remain unchanged (cf. lane 9 with lane 10). The result of the complementary experiment using a 5'-end-specific DNA oligonucleotide is shown in lane 11 (Fig. 2c). The 5'-specific oligonucleotide and RNase H destroy the 5' fragments but not the 3' fragments (cf. lane 9 with lane 11). New bands that appear in lane 10 (heavy pair of bands between A and B) and lane 11 (heavy bands above A and below B) result from oligonucleotide/RNase H treatment of the full-length injected d21 RNA, with small contributions from the 3' or 5' cleavage products (see Fig. 2d).

The results presented in Figure 2 confirm that *Xhox45* cleavage is endonucleolytic. Both 5' and 3' RNA frag-

ments of transcripts derived from *Xhox45* are clearly detectable and identifiable.

Localization of the endonuclease recognition sites

In an attempt to localize the end points of the cleavage fragments more closely, we carried out S1 nuclease protection analysis on the total RNA isolated from injected oocytes. Figure 3a shows the results by use of a 5' fragment-specific probe. Lanes g–k show an accumulation of 5' cleavage products (arrowheads) corresponding to those seen in Figure 2. The probe length and position relative to the new 3' ends of these fragments allow only

Figure 3. Localization of the 5' and 3' cleavage product ends by S1 nuclease protection. M and H indicate pBR322 *Msp*I and *Hae*III markers, respectively. (a) A probe specific to the 5' fragments was used to analyze RNA from injected oocytes. RNA was isolated after the times of incubation shown in hours above lanes g–k. Strong protection bands (arrowheads) result from 5' cleavage fragments that accumulated during the course of the injection experiment (lane k). These bands correspond to the three shortest 5' cleavage product bands, whereas the band of increasing intensity just below the full-length probe corresponds to the fourth and longest 5' cleavage product (Fig. 2a, arrowheads). Lanes a–d are controls of uninjected d21 RNA added to the S1 reaction in the amounts given above the lanes. G and G + A, (lanes l and m) are sequencing ladders of the 5' probe. (b) A probe specific to the 3' fragments was used to analyze oocyte RNA from the same injection experiment shown in a. The brackets mark groups of bands resulting from the uncapped 3' fragments of d21 RNA. These correspond to the positions of the two smallest 5' fragment bands (Fig. 2a, arrowheads). Lanes t–w are dideoxy sequencing ladders of the 3' probe.

three of the four bands indicated by arrows in Figure 2 to be seen clearly, although the fourth and longest is visible just below the full-length probe band (lane k). S1 nuclease protection, using a probe specific for 3' cleavage products, is shown in Figure 3b. As expected, due to much faster degradation of these uncapped fragments, the protected probe fragments appear as groups of faint bands. Two sets of fragments complementary in size to the two smallest 5' cleavage products are indicated by brackets. The probe positions relative to d21 are shown in Figure 4b.

On the basis of the sizes of the arrowed 5' cleavage fragments in Figure 2, and the 5' protection bands in Figure 3a, the approximate areas of the d21 sequence into which the four distinct cleavage product bands map

are shown in Figure 4a (horizontal brackets above the sequence). Most of the region is a repeat of a 4-base unit, ACCT, but with slight variations that may break the sequence into larger functional repeat units (see Discussion).

The Xhox45-cleaved sequence is necessary for recognition and is also sufficient to confer endonucleolytic cleavage on another mRNA species

We determined whether the region into which the endonuclease sites map is necessary and sufficient for the cleavage of *Xhox45* mRNA by using the constructs diagrammed in Figure 5. An autoradiograph from a typical set of injection experiments with these RNAs is shown in Figure 6.

Two *Spe*I restriction sites were utilized to internally delete 336 bases from *Xhox45* to produce the clone 45Δ *Spe*I. This deletion completely removes the cleavage sites and results in an RNA ~1 kb in length with a 314-base 3'-untranslated region. As can be seen in Figure 6A, 45Δ *Spe*I is at least twice as stable as intact *Xhox45* (see also Fig. 5) and shows no sign of the endonucleolytic cleavage. This region is therefore required for *Xhox45* cleavage.

Shaw and Kamen (1986) have demonstrated an instability sequence of fused ATTTA repeats in GM-CSF (granulocyte macrophage colony-stimulating factor) mRNA. Because a general motif of fused ACCTA repeats in the *Xhox45* cleavage region is faintly reminiscent of this sequence, RNA transcribed from a human GM-CSF clone was tested as an additional control (Fig. 6A). GM-CSF is extremely stable in oocytes, confirming

Figure 4. Sequence of the d21 clone derived from *Xhox45*. (a) The sequence of the *Xhox45* deletion subcloned to produce BS-d21, (see Materials and Methods). Base numbering is according to Wright et al. (1987). The 90-base region subcloned into *Xhox36* to produce 36x1 is underlined. The cleavage site ranges, as mapped by the arrowed fragments in Fig. 2a and by the 5' fragment S1 analysis in Fig. 3a, are indicated by the horizontal brackets above the sequence. (b) Diagram of the d21 RNA and the S1 probes used in Fig. 3. The heavy bar represents d21 RNA, the lighter bars represent the probes, and the stars indicate the position of the label in each probe. The vertical arrowheads indicate the relative positions of the cleaved areas of d21 shown in a.

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Figure 5. Structure and half-lives of the RNAs shown in Fig. 6. Boxes indicate coding sequences. The heavy black bar indicates the region in which cleavages of *Xhox45* occur; 45 is the RNA from the *Xhox45* cDNA used in previous figures. A 347-base internal *SpeI* deletion of 45, including the cleavage region, was used to produce 45Δ*SpeI*. GM-CSF is RNA from a clone of granulocyte macrophage colony-stimulating factor. 36x1 has 200 bases replaced by the 126-base PCR fragment, containing 90 bases of *Xhox45* sequence, as described in Materials and methods. 36x2 has two 126-base insertions separated by the inverted 200-base fragment of *Xhox36*. The construct half-lives were determined by densitometric scanning of lighter exposures of the autoradiographs shown in Fig. 6. The bar below the constructs is 100 bases in length.

the results of Kruys et al. (1989), and is not susceptible to *Xhox45*-like cleavage.

To determine whether the region to which the cleavage sites map is also sufficient to confer cleavage on another RNA, a 126-base fragment containing 90 bases from *Xhox45* (Fig. 4, underlined) was inserted into *Xhox36*, as described in Materials and methods. This yielded two plasmids: 36x1, containing one insert, and 36x2, containing two inserts separated by a small region of inverted *Xhox36* sequence (Fig. 5).

When the modified *Xhox36* RNAs from these new constructs were injected and reisolated, they were cut in a manner indistinguishable from that of *Xhox45* (Fig. 6B). 36x1, with one copy of the cleavage region insert, has a half-life of 3 hr, equal to that of *Xhox45*, and significantly less than the 4.9-hr half-life of *Xhox36*. 36x2, with two inserts, has a half-life of only 1.7 hr. The observed cleavage patterns and half-lives of the constructs shown in Figures 5 and 6 confirm that the sequence in Figure 4a (underlined) is both necessary and sufficient for the endonucleolytic cleavage of mRNA.

Xhox45 cleavage is completely independent of translation and the nuclease activity is stable in the absence of translation

We tested the effects of both message-specific and global-translation inhibition on *Xhox45* RNA degradation, because all previously characterized mRNA degradation systems appear to involve translation of either total cellular RNA or the individual RNA being degraded. Message-specific translation inhibition was accomplished by injecting uncapped RNA, whereas total translation was blocked by incubating oocytes in cycloheximide.

The translation of uncapped, nonviral RNA in oocytes is very inefficient, being reduced by at least 90% relative

to capped RNA (for exceptions, see Lockard and Lane 1978; Banerjee 1980; Drummond et al. 1985). In addition, the stability of uncapped RNA in oocytes has been found to be variable, with half-lives ranging from minutes (Green et al. 1983; Harland and Weintraub 1985) to several hours (Drummond et al. 1985). A large part of these differences appear to be due to the immediate 5' sequence of the RNA (B.D. Brown, unpubl.). Fortunately, we find that uncapped, full-length *Xhox45* RNA is almost as stable as capped *Xhox45*, unlike the 3' fragment resulting from endonucleolytic cleavage. As

Figure 6. Determination of a region of *Xhox45* that is necessary and sufficient for cleavage. Autoradiograph of RNAs, which were injected, reisolated, and separated on a denaturing agarose gel. The time of reisolation is shown in minutes above the lanes. The half-lives of these RNAs are shown in Fig. 5. The markers represent λ *HindIII*-*EcoRI* fragments from 2 kb to 0.125 kb. (A) Deletion of the cleavage sequence from *Xhox45* eliminates the formation of a discrete cleavage product, as shown in the 45Δ*SpeI* lanes. The half-life of 45Δ*SpeI* is more than doubled: 7.5 hr for 45Δ*SpeI* compared with 3.3 hr for *Xhox45*. GM-CSF mRNA was found to be very stable, and even overloading of the RNA does not reveal any distinct degradation products. The small band running slightly below GM-CSF at ~830 bases is a premature transcription termination product seen in uninjected GM-CSF RNA. (B) Insertion of 90 bases of the *Xhox45* cleavage region into *Xhox36* causes it to be cleaved in a manner identical to that of *Xhox45* (see also Fig. 8). The open arrowhead marks a premature transcription termination product that was seen in the uninjected 36x1 and 36x2 RNAs used in this experiment. The half-lives of *Xhox36*, 36x1, and 36x2 were 4.9, 3.0, and 1.7 hr, respectively. The solid arrowheads mark the bands resulting from cleavage in the *Xhox45* insert that are still visible 480 min after injection. In the 36x2 sample cleavage at both of the inserts is detectable.

shown in Figure 7, the uncapped species (lane 6) is cleaved as efficiently as the capped message (lane 2), demonstrating that the cleavage reaction is not dependent on the presence of ribosomes on the *Xhox45* RNA.

Cycloheximide was used to block total protein synthesis both prior to and after mRNA injections. The conditions used were sufficient to inhibit protein synthesis by 97% (see Materials and methods). In the extreme treatment, oocytes were preincubated in buffer containing cycloheximide for 4 hr, injected, and incubated for 4 hr more in the presence of cycloheximide. Cleavage of *Xhox45* is found to be completely unaffected by the inhibition of total protein synthesis (Fig. 7, lanes 9–11). These results support the results of procedures using uncapped RNA and also indicate that the cleavage activity is not labile.

Antisense RNA coinjection blocks the endonuclease activity

RNA duplexes between coinjected complementary RNAs form rapidly and are stable in *Xenopus* oocytes, unlike eggs and embryos (Bass and Weintraub 1987; Rebagliati and Melton 1987). Therefore, antisense RNAs to regions of *Xhox45* (diagramed in Fig. 8a) were coinjected with both the *Xhox45* and 36x1 RNAs to test the effect

of duplex formation on the endonuclease activity. As seen in Figure 8b, α d251, an antisense RNA that hybridizes to the cleavage region, completely blocked cleavage of *Xhox45* (lane 4), whereas α d191 did not affect the reaction at all (lane 6). α d251 is also complementary to 36x1, but only in the 90-base region derived from *Xhox45*, and therefore provides a more specific duplexed region. Coinjection with α d251 with 36x1 also completely blocked cleavage (lane 10). These results indicate that single-stranded RNA is required for recognition by the endonuclease.

Discussion

We have presented results showing unequivocally that an endoribonuclease activity cleaves the 3'-untranslated region of the *Xhox45* isolate of the *Xenopus* homeo box-containing gene *Xlhbox2B* in a sequence-specific manner. We have also identified a 90-base sequence from *Xhox45* that is both necessary and sufficient for this endonucleolytic cleavage to occur. A very simple consensus sequence for this region appears to be repeats of a 4-base sequence: ACCT. The functional unit of the cleavage region may be some minimum number of this simple repeat, or it may be as complex as a 19-base repeat that is formed by minor variations in the short repeat: CTACCTACCTACCCACCTA (located around the overlined regions in Fig. 4). To determine the exact recognition site, mutant sequences have been synthesized and inserted into a test transcription template and assayed for biological activity (B.D. Brown, in prep.).

Potential mechanisms of *Xhox45* cleavage

The detection of both the 5' and 3' fragments resulting from endonucleolytic cleavage of d21 RNA eliminates a targeted 3' to 5' exonuclease as the explanation for the appearance of shortened *Xhox45* RNA fragments. Another possibility is that the sequence contains all of the information necessary for self-cleavage. In some cases, self-cleavage activity is inhibited by the formation of alternate secondary structures in RNA and is therefore enhanced in smaller fragments from larger RNAs (Wu et al. 1989). We have never observed self-cleavage of *Xhox45* RNA in vitro under conditions where other self-cleaving RNAs react efficiently (for review, see Cech 1987), even when small RNA fragments containing the entire destabilizing region are incubated in vitro.

Sequence analysis suggests that the destabilizing region does not form any stable Watson-Crick, base-paired secondary structure either internally or with any other region of *Xhox45*. Therefore, cleavage of the RNA appears to be based on recognition of a single-stranded molecule, a hypothesis that is also supported by results showing that a small region of *Xhox45* supports cleavage when transferred to another molecule and by results showing that hybridization of an antisense RNA to the cleavage region completely blocks cleavage. This is in strong contrast to histone and transferrin receptor mRNAs, where base-paired RNA structures have been

Figure 7. Injection of uncapped RNA or incubation in cycloheximide shows that translation is not required for *Xhox45* cleavage. *Xhox45* and *Xhox36* RNAs were injected and analyzed as in Fig. 1. The times at which the RNA samples were reisolated are given in hours above each lane. (Lanes 1 and 2) Capped *Xhox45* RNA; (lanes 3 and 4) capped *Xhox36* RNA injected into untreated control oocytes (–). Lanes 5–8 are identical to lanes 1–4, respectively, except the injected RNAs were uncapped (unc) to specifically inhibit translation of the injected RNA. (Lanes 9–11) *Xhox45* RNA injected into oocytes that were incubated in cycloheximide to block global translation (+). The lengths of preincubation in cycloheximide is given in hours above the plus marks. The solid arrowhead marks the 5' fragments that result from cleavage; the open arrowhead marks the faint band caused by the 3' RNA fragments.

Figure 8. Coinjection of antisense RNA can block the endonuclease activity. (a) Diagram of the constructs injected and shown in b. The antisense transcription product of the BS⁻d251 clone (α d251) is complementary to 321 bases of *Xhox45*, and completely covers the endonuclease cleavage region (large open box). α d191 hybridizes to 283 bases at the extreme 3' end of *Xhox45*, but is almost identical in total length to α d251. The antisense transcripts hybridize to *Xhox45* RNA in the positions shown. α d251 hybridizes to 36x1 only at the 90-base insert (small open box), and α d191 does not hybridize to 36x1 at all. (b) Autoradiograph of RNA injections plus or minus small antisense RNAs. The numbers immediately above each lane indicate hours after injection. The smallest species visible in lanes 3–6, 9–12, and 14 and 15 are due to antisense RNAs (as marked). Normal cleavage of *Xhox45* RNA injected without antisense RNA (lane 2). Coinjection of *Xhox45* with a 10-fold molar excess of α d251 completely blocks the endonuclease (lane 4), while coinjection with α d191 has no effect on the cleavage reaction (lane 6). Injections using the cleavable 36x1 hybrid RNA (see also Figs. 5 and 6) give the same pattern of results as with *Xhox45*: 36x1 alone is cleaved efficiently (lane 8), α d251 blocks cleavage (lane 10), and α d191 has no effect (lane 12). (Lane 13) *Xhox36* RNA injected alone; (lanes 14 and 15) unmodified *Xhox36* coinjected with α d251 and α d191, respectively; (lane 16) α d251 and α d191 injected together. The time after injection is given in hours above each lane. (M) λ HindIII/EcoRI markers.

proposed to play critical roles in regulating the stability of these messages (Graves et al. 1987; Casey et al. 1988; Müllner and Kühn 1988). *Xhox45* recognition may resemble more closely that of mRNAs such as those encoding growth factors, whose stability is controlled by AU-rich sequences without well-defined secondary structures (Shaw and Kamen 1986; Kabnick and Housman 1988), even though the AUUUA motif is not recognized in oocytes.

The result that *Xhox45* mRNA degradation lacks any requirement for translation is unusual and suggests that this putative endoribonuclease is not associated with either polysomes or individual ribosomes. The presence of the endoribonuclease activity after sustained incubations in cycloheximide also implies that the molecules responsible for this activity are not labile, unlike the

c-myc mRNA destabilizing factor reported by Brewer and Ross (1989). These results should greatly simplify the detailed analysis of the cleavage reaction and the biochemical fractionation and characterization of the activity.

Sequence dependence of *Xhox45* mRNA cleavage

Beutler et al. (1989) have speculated that the UA dinucleotide content of the AU-rich sequence identified by Shaw and Kamen (1986) causes instability due to the presence of a UA-specific ribonuclease. We find that this mechanism cannot account for the cleavage of the *Xhox45* cDNA transcript in *Xenopus* oocytes. The total UA dinucleotide content of the cleavage region of *Xhox45* is even less than that of the destabilizing region of GM-CSF (19% vs. 28%, respectively), and we have confirmed the result of Kruijs et al. (1989) that GM-CSF mRNA is extraordinarily stable in oocytes. Therefore, based on the UA content alone, *Xhox45* should be more stable than GM-CSF, but we find quite the opposite result. *Xenopus* oocytes therefore lack an active UA-specific nuclease but do contain an endoribonuclease of a higher sequence specificity.

The cleavage of *Xhox45* in *Xenopus* oocytes differs from GM-CSF degradation in human cell lines in two significant ways: (1) *Xhox45* cleavage does not require any translation, unlike GM-CSF degradation; and (2) endonucleolytic cleavage of *Xhox45* is the first detectable step, as opposed to the initial poly(A)-shortening event seen with mRNAs that contain the AU-rich sequence (Brewer and Ross 1988; Wilson and Treisman 1988). However, the resemblance between the *Xhox45* and GM-CSF instability sequences, as APyPyUA, raises the question of whether the structural recognition of these sequences may be somewhat similar.

Bandyopadhyay et al. (1990) report the detection in vitro of what may be dinucleotide-specific endonucleases on mRNAs from mammalian cells, but the sequences that are apparently required for recognition and cleavage are as yet undefined. The evidence presented suggests that the recognition sequence of the endoribonuclease that attacks *Xhox45* is higher than a dinucleotide. Recognition almost certainly requires more than a tetramer: All of the variations of the tetramer repeat found in the *Xhox45* endonuclease recognition region appear randomly in single copies in the other RNAs analyzed in this work, and no corresponding cleavage products were detected (see below).

Stoeckle and Hanafusa (1989) have reported endonucleolytic cleavage of 9E3 mRNA in vivo. Unlike *Xhox45*, 9E3 RNA is stabilized by cycloheximide treatment, and the authors speculate that degradation may be due to a self-cleavage reaction that is modulated by labile factors.

Comparison of *Xlhbox2A* and *Xlhbox2B* cleavage

Two copies of the *Xlhbox2* gene are present in the *Xenopus* genome, designated *Xlhbox2A* and *Xlhbox2B* (Fritz et al. 1989). The *Xhox45* cDNA is a representative of *Xlhbox2B*. It is interesting to note that the 123-base

deletion in the 3' UTR of *Xlhbox2A*, described by Fritz et al. (1989), removes the cleavage region that we have defined in the *Xhox45* isolate (90 bases) but that *Xlhbox2A* retains the 15-base sequence CTACCTACCCACCT around the deletion site. We have tested both *Xlhbox2* type A and type B RNA (clones obtained from C. Wright and E. DeRobertis) in oocytes and find that only *Xlhbox2B* RNA is cleaved and that the reaction is indistinguishable from the cleavage of RNA transcribed from the *Xhox45* cDNA (data not shown). The 15-base sequence remaining in *Xlhbox2A*, though similar to the cleavage region, is insufficient for recognition.

The existence of such a deletion in *Xlhbox2A*, resulting in a gene capable of producing cleavage-resistant transcripts, poses the question of whether or not the transcription of these two genes is differentially regulated at a spatial and/or temporal level. *Xlhbox2A* could be transcribed whenever the *Xlhbox2* protein is needed continuously, whereas the *Xlhbox2B* mRNA could be transcribed when rapid regulation of the level of protein is needed. The promoter function of these two genes is therefore of particular interest, because *Xlhbox2* RNA levels are known to be regulated during oogenesis (Wright et al. 1987).

Because nonadenylated RNAs are measurably less stable in oocytes than polyadenylated RNAs (Drummond et al. 1985), loss of the poly(A) tail and almost all of the 3'-untranslated region may lead to more rapid turnover of the *Xlhbox2B* transcript, thus accounting for the changes in *Xlhbox2* mRNA levels during oogenesis. Cleavage at this site would almost certainly eliminate *Xlhbox2B* mRNA from the translatable maternal mRNA pool, regardless of the decay rate of the body of the RNA, because poly(A) tails have been shown to be crucial for long-term maintenance of polysomes in oocytes (Galili et al. 1988). Cleavage would also eliminate the possibility of readenylation of *Xlhbox2B* upon maturation of the oocyte, because both the polyadenylation signal itself and any potential hyperadenylation sequence nearby (McGrew et al. 1989) are completely removed.

How general is this potential mechanism of gene expression control? We have screened oocyte-specific and neurula-specific cDNA libraries for other genes containing this cleavage sequence and have found another clone from oocytes with an RNA half-life <1 hr (B.D. Brown, in prep.), in contrast to the already short half-life of *Xlhbox2B*. Comparison between this new clone and the *Xlhbox2B* instability sequences may allow a consensus sequence to be found and a determination of how this motif is used to generate mRNAs with different half-lives.

RNA stability can make a critical contribution to the level of expression of genes (for a theoretical discussion, see Hargrove and Schmidt 1989). Although a considerable amount is known about the general determinants of mRNA stability, far less is known about the enzymes and mechanisms involved in preferentially destabilizing specific mRNAs. In the cases where the instability of

individual RNAs has been studied in detail, internal sequences are responsible for the observed rates of turnover, and multiple, independent destabilizing sequences have been found in different regions of mRNAs (Kabnick and Housman 1988; Shyu et al. 1989). The identification of the sequence-specific cleavage of *Xlhbox2B* RNA by an endonuclease may lead to a greater understanding of the mechanisms by which the decay of cellular mRNAs in eukaryotes is initiated and regulated.

Materials and methods

Construction of transcription templates

The *Xhox45* cDNA clone used in this work was 1342 bases in length, including a 15-base 5'-untranslated region, a coding region of 660 bases, and a 3' UTR of 650 bases plus 17 bases of polylinker sequence. The BS-d21 clone consists of the 3'-most 703 bases of the *Xhox45* cDNA clone inserted between the *Bam*HI and *Eco*RI sites of Bluescript KS⁻ (Stratagene), and when linearized with *Eco*RI, produces an RNA 752 bases long including polylinker sequence. d21PvuII is RNA produced by linearizing this construct with *Pvu*II, resulting in a 270-base extension of the 3' end. Exonuclease III was used to remove the 489 and 381 3'-most bases of BS-d21 to produce BS-d272 and BS-d251, respectively. A 420-base exonuclease III deletion of BS-d21 from the 5' end was used to produce BS-d191. The *Xhox36* clone used, 36/12, has been described previously (Condie and Harland 1987) and produces an RNA 1460 bases long, including a coding region of 626 bases and a 3' UTR of 550 bases. 36x1 and 36x2 were constructed as follows: A polymerase chain reaction (PCR; Saiki et al. 1988; *Taq* DNA polymerase from Cetus) was then used to amplify a 126-base fragment containing 90 bases of *Xhox45* sequence out of BS-d272. The first primer, 5'-GCTCTAGAGGGTTAGCTACCTACCTACC-3', contains the *Xba*I restriction site and hybridizes to BS-d272 at the 5' end of the cleavage region. The second oligonucleotide, containing the *Pst*I site, is 5'-CGCTGCAGTAAAGGGAACAAAAGCTGGG-3' and hybridizes to the Bluescript vector just 3' of the end of the 272 fragment. The PCR fragment was then cloned into an *Xba*I/*Pst*I-cut 36/12 5' deletion clone named 36.12Δ5'09. This produced the single-insert clone called 36x1 and the double-insert clone called 36x2. The two inserts in 36x2 are separated by the inverted 206-base *Xba*I-*Pst*I fragment of 36.12Δ5'09. For consistency, the *Xhox36* RNA shown in Fig. 5 was transcribed from 36.12Δ5'09 and is therefore 218 bases shorter than the *Xhox36* RNAs shown in Figs. 1 and 2.

In vitro transcriptions and oocyte injections

Transcriptions were carried out with linearized templates, according to Green et al. (1983), with GpppG (Pharmacia) included in the transcription reaction at a concentration five times greater than the GTP concentration. From 40 to 50 μCi of [α -³²P]CTP was also included in each transcription. All reactions were carried out at 37°C for 2 hr, regardless of the RNA polymerase used. The reactions were then diluted with 0.1 M sodium chloride, 1 : 1 phenol/chloroform-extracted, and precipitated with 0.25 volumes of 10 M ammonium acetate/0.6 volume of isopropanol at room temperature for 30 min. The RNAs were then resuspended in 50 μl DEP (diethylpyrocatechol)-treated water and reprecipitated with 0.5 volume of 10 M lithium chloride at -20°C for 1 hr, followed by an 80% ethanol wash; they were finally resuspended at 1 mg/ml in DEP-treated water for injection. Antisense RNAs from BS-d251 and

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d191 were labeled to one-tenth of the specific activity of sense transcripts. Mixtures of 1:10 molar sense/antisense RNA were injected directly without any prehybridization step. Ovaries were obtained from frogs that were from 1.5 to 2 years old. Oocytes were prepared by treating the ovaries with collagenase (2 mg/ml) in modified Barth's saline until the oocytes were released. Stage IV and V oocytes were microinjected with up to 10 ng (20 ng for antisense experiments) of RNA and then incubated at from 20°C to 22°C. Protein synthesis in oocytes was inhibited by incubating oocytes in modified Barth's saline containing 25–30 µg/ml of cycloheximide. As determined by [³⁵S]methionine incorporation into TCA-precipitable material, this concentration was sufficient to inhibit protein synthesis by 97%.

RNA analysis

Five oocytes per sample were homogenized in 500 µl of lysis buffer (100 mM NaCl, 20 mM EDTA, 20 mM Tris, and 1% SDS) containing 200 µg/ml proteinase K and incubated at 37°C for 30 min. They were then frozen and stored at –20°C or processed immediately. After the addition of one-tenth volume of 3 M sodium acetate, the homogenates were extracted with phenol and 1:1 phenol/chloroform, and then precipitated with 2 volumes of ethanol. These total RNA samples were resuspended in 50 µl of DEP-treated water and reprecipitated with 0.5 volume of 10 M lithium chloride at –20°C for 1 hr. The samples were then resuspended in 50 µl of the appropriate gel sample buffer and run on denaturing 1.4% agarose/formaldehyde gels, or denaturing 4% acrylamide/8 M urea gels, which were then dried. Autoradiography was at room temperature.

Northern blot RNA analysis

RNA injected for Northern analysis was initially trace-labeled with ³²P but was then stored for 14 half-lives so that the label was no longer detectable. Reisolated total RNA, at one oocyte per lane, was run out on a 2.2% agarose/formaldehyde gel and blotted onto a nylon membrane (Hybond-N, Amersham). The blot was UV-irradiated (Stratalinker, Stratagene), vacuum baked for 2 hr, prehybridized for 24 hr, and probed with the 3'-specific probe and exposed to film. After stripping and reexposure to confirm the removal of the 3' probe, the blot was prehybridized again and probed with the 5'-specific probe. Probes labeled with ³²P were made by random oligo-primed synthesis by use of the appropriate gel-purified DNA fragments as described in the text.

Oligonucleotide/RNase H RNA analysis

The same d21 RNA isolated 80 min after injection shown in the Northern blot in Figure 2 and described above was used for DNA oligonucleotide-mediated RNase H cleavage of specific RNA fragments. The 5'-specific oligomer 5'-TAGTCCATTGGGGCCACTTCATTC-3' hybridizes to d21 RNA from bases 681 to 658 (Fig. 4), and the 3'-specific oligomer 5'-ACATA-GAAAGTGGTTGTACAGAGG-3' hybridizes from bases 888 to 865. Total injected oocyte RNA samples from one oocyte each (minus oligomers, plus 5' oligomer, and plus 3' oligomer) were denatured at 90°C for 5 min and incubated for 2 hr at 42°C in 300 mM NaCl, 10 mM Tris, 1 mM EDTA (final pH of 7.5), and precipitated. Each sample was then resuspended in 50 µl of 100 mM KCl, 10 mM MgCl₂, 20 mM Tris, 0.1 mM dithiothreitol, 5% sucrose (final pH of 7.5), and 0.5 units RNase H (Bethesda Research Laboratories) and incubated at 37°C for 30 min. Glycogen was then added as a carrier, and the samples were precipitated, resuspended in sample buffer, run out on a 2.2% agarose/formaldehyde gel, blotted onto nylon, and probed as described

above, except that the probe was synthesized from full-length d21 DNA fragments.

S1 nuclease protection

The DNA probe to detect 5' fragments was labeled by filling the *Bam*HI site of BS-d272 with 3000 Ci/mmol [α-³²P]dCTP (Amersham) and reverse transcriptase (Life Sciences), and cutting with *Kpn*I in the Bluescript polylinker. The 5'-sequencing ladder was generated using Maxam–Gilbert sequencing reactions on the 5' probe. The 3' fragment probe was made by phosphorylating an oligonucleotide complementary to bases 865–888 of the d21 sequence with >5000 Ci/mmol [γ-³²P]ATP and polynucleotide kinase, extending it with the Klenow fragment, and cutting with *Bam*HI. The 3' sequence ladder was generated by using the same labeled oligonucleotide in T7 DNA polymerase/dideoxynucleotide sequencing reactions (Pharmacia). The 5'- and 3'-specific probes were purified on strand-separating and denaturing acrylamide gels, respectively. For the S1 nuclease protection reactions, the total RNA reisolated from one-fifth to one-tenth of an oocyte was mixed with an approximately 10-fold molar excess of probe and 40 µg of torula RNA and hybridized at 65°C for 3 hr. The reactions contained from 60 to 90 units of S1 nuclease and were incubated at 37°C for up to 45 min.

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