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Selective elimination of mRNAs *in vivo*: Complementary oligodeoxynucleotides promote RNA degradation by an RNase H-like activity

(*Xenopus* oocyte/reverse genetics)

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ABSTRACT **Oligodeoxynucleotides lead to translation arrest of complementary mRNAs in the wheat germ translation system by a degradation of the mRNA. In an attempt to develop an effective reverse genetic approach *in vivo*, we demonstrate that injection of short (15- to 30-nucleotide) oligonucleotides into *Xenopus* oocytes leads to complete degradation of both injected and endogenous mRNAs by means of an RNase H-like activity.**

Recombinant DNA techniques have increased greatly the number of organisms accessible to genetic analysis. Nevertheless, the inability to disrupt gene expression in living cells still hampers the detailed understanding of gene function in most organisms. To overcome this limitation, several laboratories have developed a reverse genetic approach based upon antisense- or hybrid-arrested translation (1–7). However, the degree of inhibition achieved by antisense RNA has proven to be variable and to depend upon the particular gene selected for study. Moreover, some eukaryotic cells contain endogenous helicase activity that unwinds RNA·RNA hybrids and thereby relieves the inhibition of translation achieved by the antisense RNA (8, 9).

In the first experimental use of hybrid arrest, Patterson *et al.* (10) used complementary DNA sequences to block the *in vitro* translation of corresponding mRNA sequences. Complementary DNA fragments and oligodeoxynucleotides have since been used successfully to block the translation of injected mRNA coding for interleukin 2 (11), to inhibit growth of herpes simplex virus 1 (12), and to inhibit the replication of human T-cell lymphotropic virus type III (13). Since the early *in vitro* studies (10), it has been assumed that complementary DNA sequences inhibit gene expression by blocking ribosome translocation on the mRNA. However, it was recently found in the wheat germ translation system (14) that complementary DNA sequences actually lead to a degradation of target mRNA sequences. Since RNase H is capable of hydrolyzing the RNA partner of an RNA·DNA hybrid (15, 16), Minshull and Hunt (14) suggested that an endogenous RNase H-like activity in the wheat germ extracts may be responsible for hybrid-arrested translation by complementary DNAs.

With the aim of developing an effective reverse genetic technique that could be used in eukaryotic cells, we have explored the mechanisms of action of complementary DNA blockade *in vivo*. Using the *Xenopus* oocyte, we found that oligonucleotides of 15 and 30 nucleotides in length and complementary to any part of the target mRNA are effective in stimulating mRNA degradation. We demonstrate that this degradation is sequence-specific and can be accounted for by an RNase H-like activity. Since this method works for both injected and endogenous mRNAs, we believe it can be used to study the phenotypic roles of both induced and constitutively expressed mRNAs.

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METHODS

Microinjection of Oocytes with RNA and Oligonucleotides. Stage 5 oocytes were prepared from *Xenopus laevis* by the method of Dascal *et al.* (17). After defolliculation by collagenase treatment, the oocytes were incubated in ND96 medium for a minimum of 4 hr prior to injection. Twenty to thirty microliters of rabbit globin mRNA (Bethesda Research Laboratories) was injected into each oocyte, at a concentration of 0.1 mg/ml in 0.1% diethyl pyrocarbonate-treated 50 mM NaCl, using a glass electrode of 5- to 10-μm tip diameter (18). After RNA injection the oocytes were incubated in ND96 medium at 20°C. Twenty to twenty-five nanoliters of oligonucleotide (0.06–2 mg/ml) was injected using similar glass electrodes. Oligonucleotides were synthesized by the phosphotriester linkage technique (19).

Blot Hybridization Analysis of mRNA. Total oocyte RNA was extracted by the hot phenol method (20). Ten to fifteen oocytes were rinsed with extraction buffer (50 mM Tris·HCl, pH 7.6/100 mM NaCl/10 mM EDTA) in an Eppendorf tube before homogenization. For blot analysis (21), samples (≈10 μg) of total oocyte RNA were denatured with formaldehyde, electrophoresed in 1.4% agarose gels, transferred to nitrocellulose, and hybridized to primer-extended probes using the conditions of Maniatis *et al.* (22).

Recombinant DNA Methods. We used *Escherichia coli* JM101 as host strain throughout these experiments and introduced plasmid DNA by the transformation procedure of Hanahan (23). Plasmid vectors pBSM13+, pKSM13+, and pBSM13−, which permit the recovery of plasmid DNA as a single strand (24), were obtained from Stratagene (La Jolla, CA). Single strands were rescued from bacteria containing these plasmids essentially as suggested by the vendor.

cDNA clones of α- and β-globin were prepared from rabbit erythrocyte mRNA (Bethesda Research Laboratories) by a modification of the method of Gubler and Hoffman (25). Globin clones were first identified by restriction enzyme analysis of plasmids isolated from the resulting transformants and then confirmed by DNA sequence analysis (26). The α-globin probe contained a portion of the 5′ untranslated region, all of the coding region, all of the 3′ untranslated region, and a short poly(A) tail (27). The β-globin probe contained most of the 5′ untranslated region as well as the coding region as far as the internal *Bam*HI site (28).

The *Xenopus* calmodulin clone p11G2 (29) was kindly provided by I. Dawid (National Institutes of Health). A *Kpn* I–*Pst* I fragment was then subcloned into the vector pBSM-13− for probe synthesis. Random primer-extended probes

were synthesized from double-stranded DNA fragments by use of a kit purchased from Pharmacia and the reactions were performed according to the recommendations of the supplier.

Nuclease S1 Mapping Analysis. Half an hour after injection of the oligonucleotides, RNA was extracted from oocytes for S1 mapping analysis, which was carried out using the techniques of Berk and Sharp (30). ^{32}P-labeled single-stranded probes were made as follows: ≈1 μg of single-stranded template was primed with an oligonucleotide (oligonucleotide 2 for α-globin, oligonucleotide 9 for calmodulin; see Table 1) and extended across the insert with DNA polymerase I small (Klenow) fragment for 30 min at 37°C in the presence of 1 mM dGTP, 1 mM dTTP, 1 mM dCTP, 10 μM dATP, and 50 μCi of [α-^{32}P]dATP (800 Ci/mmol; 1 Ci = 37 GBq). After enzyme inactivation at 65°C for 15 min, the double strands were digested at a restriction site located at the distal end of the insert. The resulting fragment was denatured in 80% (vol/vol) formamide at 95°C for 5 min, and the labeled single strand was purified by electrophoresis in 8 M urea/5% polyacrylamide gel and recovered by electroelution. A portion of the probe was hybridized to ≈10 μg of total oocyte RNA in 80% formamide/0.4 M NaCl/40 mM Pipes, pH 6.4/1 mM EDTA in a final volume of 10 μl. The hybridization was carried out at 50°C overnight under mineral oil. Ice-cold S1 mixture (0.1 ml) containing nuclease S1 (Boehringer Mannheim) at 1800 units/ml was added to the hybridization mixture (22) and the digestion reaction was allowed to continue for 1 hr at 37°C. Digestion was stopped by extraction with phenol/chloroform (1:1, vol/vol) and the nucleic acid products were recovered by ethanol precipitation.

The same oligonucleotides used to prime synthesis of the single-stranded probes were also used for dideoxynucleotide sequencing reactions. In this way, the S1-protected 5-terminal cDNA fragments would migrate alongside the products representing the sequence of the cleavage site.

RESULTS

Complementary Oligodeoxynucleotides Promote Degradation of Injected Globin mRNA. We initially studied the sensitivity in *Xenopus* oocytes of injected globin mRNA to complementary oligonucleotide-mediated degradation by using RNA blot analysis. Oligonucleotides complementary to either α-globin or β-globin mRNA sequences (Table 1) were injected into oocytes 4 hr after injection of globin mRNA in order to test the effects of *in vivo* hybridization. Fourteen hours later, we recovered the injected globin mRNA with total oocyte RNA, electrophoresed it in agarose gels, and transferred it to nitrocellulose (21) for hybridization to either α-globin or β-globin cDNA probes. Injection of oligonucleotides complementary to internal sequences of β-globin mRNA stimulates the degradation of injected β-globin mRNA in the *Xenopus* oocyte (Fig. 1*A*, lanes 5 and 6). Similar results were obtained by Cazenave *et al.* (31). The degradation is sequence-specific. β-Globin mRNA degradation was not stimulated by injection, at similar concentrations, of nonspecific oligonucleotides (lanes 3 and 4) or two oligonucleotides complementary to α-globin mRNA (lanes 7 and 8).

Table 1. Oligodeoxynucleotide sequences

No.	Sequence (5′→3′)	Position*
	Rabbit α-globin mRNA-specific	
1	CACCAGCAGGCAGTGGGACAGGAGCTTGAA	330–360
2	TGGCCAGGAACTTGTCCAGGGAGGCATGCA	400–430
3	TCTTGGTGGTGGGGAAGCCCAAGAACATCC	130–160
4	GGACAGGAGCTTGAA	330–345
	Rabbit β-globin mRNA-specific	
5	TGAGGTTGTCCAGGTGACTCAGACCCTCAC	270–300
6	GACTCAGACCCTCAC	270–285
	Xenopus *calmodulin mRNA-specific*	
7	TTCATTGATCATATCCTGCAATTCTGCTTC	202–232
8	CCATCCTTGTCAAAAACACGGAATGCTTCT	327–357
9	AACATCAATATCTGCTTCCCTTATCATTTC	436–466
	Nonspecific	
10	AATATTACAGTCTCCAGCGGAACTAC	
11	ATGGCTGATCAACTGACAGAAGAGCAGATT-GCAG	
12	CTTTACTATTCCCTTCGTCTATAACTACAA	

*The positions of complementarity in the RNA sequences (27–29) are given from the 5′ end of the corresponding mRNAs.

The degradation of β-globin mRNA is dependent on the concentration of the complementary oligonucleotides. Increasing amounts of the injected 30-mer (Fig. 1*B*, lanes 2–6) and 15-mer (lanes 7–10) caused increased degradation of β-globin mRNA, with complete mRNA degradation occurring when the molar ratio of oligonucleotide to complementary mRNA was ≈200. The high concentration of oligonucleotide required to cause mRNA degradation may be due to rapid degradation of oligonucleotides in the oocyte, which both we (data not shown) and others (8) have observed.

Complementary Oligodeoxynucleotides Promote Degradation of Endogenous Calmodulin mRNA. Since it is conceivable that injected mRNAs might be especially susceptible to oligonucleotide-mediated degradation, we next tested whether this approach could be used to inactivate endogenously expressed mRNAs. For these experiments we chose to analyze *Xenopus* calmodulin mRNA because the sequence of this mRNA has been determined (29) and because the mRNA is abundant enough within oocytes for relatively easy detection.

From the published sequence, we chose several complementary sequences for phosphotriester synthesis (Table 1). Complementary oligonucleotides 7 and 8 were injected into oocytes and RNA was extracted for blot analysis with a radioactively labeled *Xenopus* calmodulin cDNA fragment (Fig. 1*C*). Oligonucleotides 7 and 8, complementary to two different regions of calmodulin mRNA, caused degradation of calmodulin mRNA in a concentration-dependent manner (Fig. 1*C*, lanes 3–6). The effectiveness of these oligonucleotides does not, however, appear to be identical. The reasons for this difference are not clear. Injection of unrelated oligonucleotides at even higher concentrations had no effect on the calmodulin mRNA (lanes 7–9).

Appropriate Cleavage Sites Can Be Mapped by Nuclease S1 Protection. Although the sequence specificity of mRNA degradation demonstrated by the RNA blot hybridizations is suggestive of an RNase H-like activity in the *Xenopus* oocyte, it is conceivable that hybridization to DNA could render a mRNA available to other cellular nucleases. Since RNase H cleaves RNA only at sequences hybridized to a complementary DNA sequence (15, 16), a more compelling indication of this activity in the oocyte would be the detection of RNA fragments resulting from cleavage at the specific sequences of oligonucleotide hybridization.

To detect the initial cleavage products, we extracted oocyte RNA 30 min (rather than 14 hr) after injection of the oligonucleotides and used more sensitive S1 mapping methods to reveal the precise sites of cleavage in the target mRNA molecules (29). The single-stranded DNA probes that we used for this S1 mapping contained the sequences of the oligonucleotides used to stimulate mRNA degradation. Fig. 2 illustrates the positions of the six oligonucleotides (black boxes) used (*i*) for generating cDNA probes (oligonucleotides 2 and 9) and (*ii*) for oocyte injection (oligonucleotides 1, 3, 7, and 8). This figure also illustrates the cDNA sequences (shaded boxes) that should be protected from S1 digestion by hybridization to either the predicted "intact mRNA" (dark lines) extracted from control oocytes or the "mRNA frag-

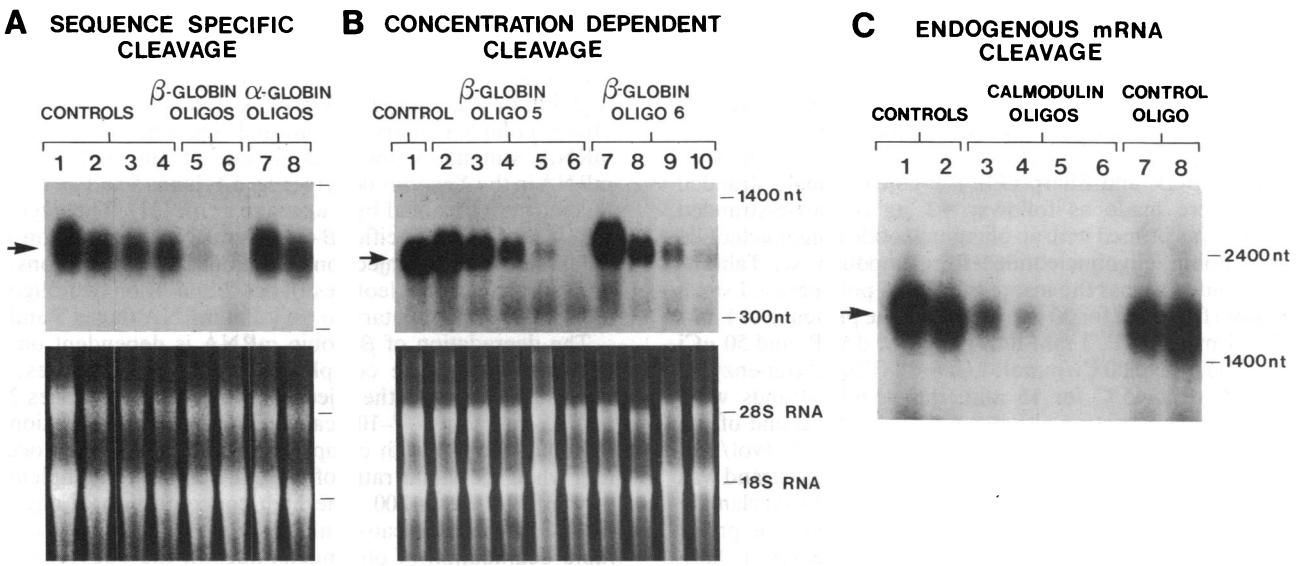

FIG. 1. Blot hybridization analysis of β-globin and calmodulin mRNAs. (*A*) Specificity of β-globin mRNA degradation after injection of oligonucleotides. Approximately 10 μg of total oocyte RNA extracted from a set of injected oocytes was run in each lane of a formaldehyde/1.4% agarose gel. The gel was stained with ethidium bromide to verify relative amounts of RNA (*Lower*) and then transferred to nitrocellulose and hybridized with radioactive β-globin cDNA probe (*Upper*). Arrow indicates position of β-globin mRNA. Oocytes were injected with 3 ng of globin mRNA and challenged 4 hr later with no second injection (lane 1), 50 mM NaCl (lane 2), nonspecific oligonucleotide 11 or 10 (lanes 3 and 4), β-globin oligonucleotide 6 or 5 (lanes 5 and 6), or α-globin oligonucleotide 4 or 1 (lanes 7 and 8). (*B*) Degradation of β-globin mRNA as a function of concentration of injected oligonucleotides. Various amounts of β-globin complementary oligonucleotides were injected into oocytes 4 hr after globin mRNA injection. The extracted oocyte RNAs were electrophoresed, stained, blotted, and probed with a β-globin cDNA as described for *A*. Oocytes were injected with 50 mM NaCl (lane 1), oligonucleotide 5 (0.4, 2.3, 3.6, 10.8 or 32.4 ng; lanes 2–6), or oligonucleotide 6 (0.6, 1.8, 5.4, or 16.2 ng; lanes 7–10). (*C*) Sequence-specific degradation of oocyte calmodulin RNA after injection of oligonucleotides. Oocytes were injected with 50 nl of solution containing either complementary or unrelated oligonucleotides. Total oocyte RNA was extracted 5 hr after injection, and ≈10 μg of RNA from each set of injections was run per lane of a formaldehyde/1.2% agarose gel and transferred to nitrocellulose as described for *A* and *B*. The immobilized RNA was hybridized with a primer-extended *Xenopus* calmodulin probe. Arrow indicates position of *Xenopus* calmodulin mRNA. Oocyte injections carried out for each set, and therefore for each lane, were as follows: no injection (lane 1); 50 mM NaCl (lane 2); calmodulin oligonucleotide 7 (25 or 85 ng; lanes 3 and 4); calmodulin oligonucleotide 8 (25 or 80 ng; lanes 5 and 6); nonspecific oligonucleotide 12 (25 or 100 ng; lanes 7 and 8).

ments" (broken lines) that would be generated by an RNase H-like activity of the oocytes after injection with specific complementary oligonucleotides.

When the labeled cDNA products of S1 digestion were run alongside the products of sequencing reactions primed with the same oligonucleotide used to make the S1 probe, the predicted cDNA products of an RNase H-like cleavage could be detected (Fig. 3). RNA extracted from uninjected oocytes (Fig. 3*A*, lane 1) did not protect the α-globin cDNA probe. However, as expected, RNA extracted from oocytes injected with rabbit globin mRNA (lane 2) protected primarily the full-length 430-nucleotide α-globin cDNA, as well as additional shorter sequences, presumably corresponding to nonspecific degradation products of α-globin mRNA.

The RNA from oocytes injected first with globin mRNA and then 4 hr later with oligonucleotide 1 (Fig. 3*A*, lanes 3 and 3′) protected approximately the same amount of the α-globin cDNA probe as did the RNA from oocytes injected with globin mRNA alone (lanes 2 and 2′). However, more than 50% of the protected probe could be found in two new sets of radioactive DNA products (lanes 3 and 3′) whose lengths (355 nucleotides and 70 nucleotides) approximated the ones predicted for RNase H digestion of the α-globin mRNA at the site of the hybrid (Fig. 2). Furthermore, as would be expected if the protecting α-globin RNA molecule had been digested at the site of the hybrid formed with oligonucleotide 1, the S1 nuclease products of ≈70 nucleotides comigrated with DNAs in the sequence "ladder" corresponding to the sequence of oligonucleotide 1. The S1 mapping analysis of RNA from oocytes injected first with rabbit globin mRNA and then with

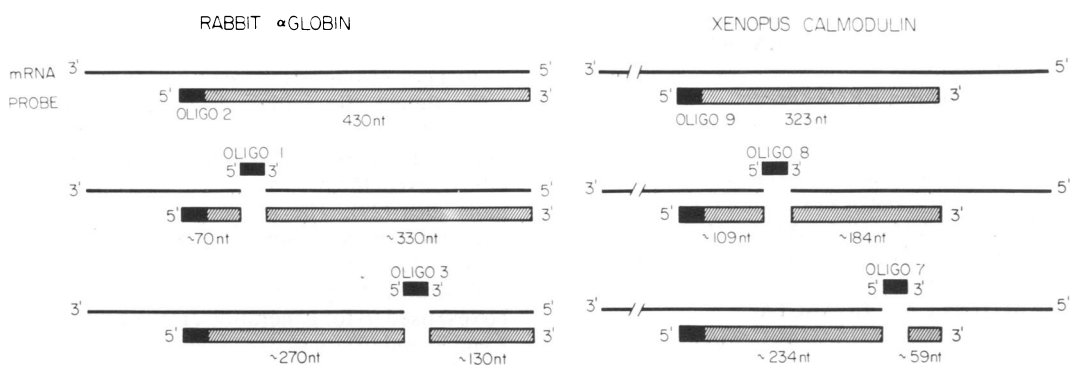

FIG. 2. Schematic representation of the expected α-globin and calmodulin mRNA fragments and the corresponding S1 nuclease-protected cDNA probe fragments. The intact or degraded RNA fragments are shown by lines. The radioactively labeled probe and probe fragments are illustrated as hatched boxes. The primers and oligonucleotides used for injection are illustrated as black boxes.

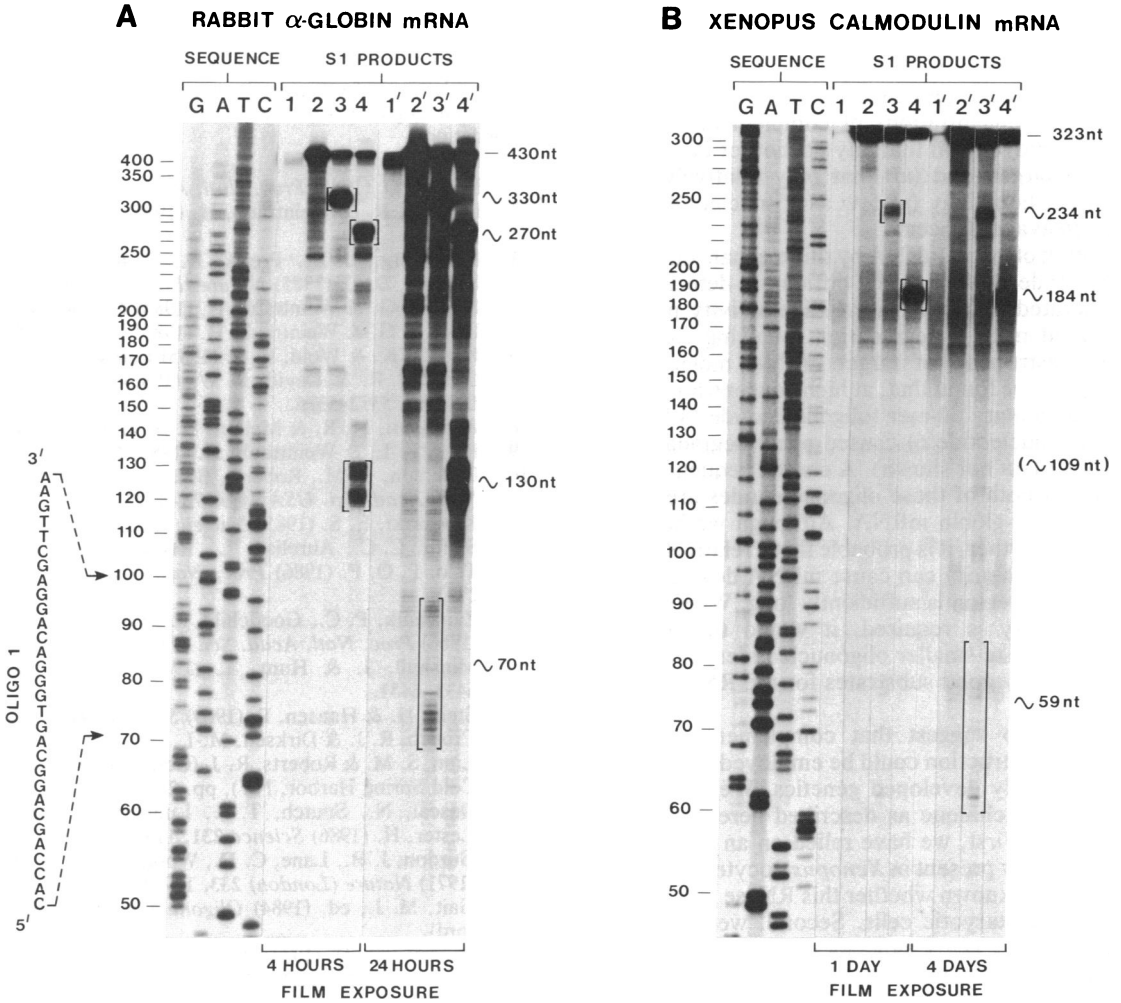

FIG. 3. Nuclease S1 mapping of the oligonucleotide cleavage sites in the α-globin and calmodulin mRNAs. Oligonucleotide primers used to generate probes for S1 mapping were also used for dideoxynucleotide sequencing of α-globin and calmodulin cDNA clones. Approximately 10 μg of total RNA, extracted from several oocytes 30 min after injection with a single oligonucleotide, was hybridized to the appropriate single-stranded probe. The hybrids were then digested with nuclease S1 and loaded onto 6% polyacrylamide gels alongside the products of the sequencing reactions ("sequencing ladder", lanes G, A, T, and C). Nucleotide lengths from the 5′ terminus of the oligonucleotide primer used to generate both the probes and the sequencing ladders are indicated at left in *A* and *B*. The predicted lengths (in nucleotides, nt) of the protected cDNA fragments are indicated at right. Two different autoradiographic exposures of the S1 products are shown in order to identify the less abundant products. (*A*) Sequencing ladder of oligonucleotide 2-primed α-globin cDNA alongside protected fragments of the same cDNA when hybridized to RNA extracted from oocytes injected as follows: no injection (lane 1); globin mRNA (lane 2); globin mRNA plus oligonucleotide 1 (lane 3); globin mRNA plus oligonucleotide 3 (lane 4). Longer exposures of the same lanes are shown as lanes 1′, 2′, 3′, and 4′. (*B*) S1 protection as in *A* for the calmodulin cDNA primed with oligonucleotide 9. RNAs used for hybridization were from yeast (control; lane 1), uninjected oocytes (lane 2), oocytes injected with oligonucleotide 8 (lane 3), or oocytes injected with oligonucleotide 7 (lane 4). Longer exposures of the same lanes are shown as lanes 1′, 2′, 3′, and 4′.

oligonucleotide 3 (Fig. 3*A*, lane 4) revealed an additional series of fragments whose lengths again approximated the predicted fragments of 295 nucleotides and 135 nucleotides (see Fig. 2).

The specificity of these cleavage reactions supports the idea that RNase H activity is present in the oocyte. Moreover, since more than 50% of the total injected RNA can be detected as specific cleavage products within only 30 min after injection, the RNase H activity must account for at least a significant amount of the total degradation detected in the blots (Fig. 1 *A* and *B*).

Similar S1 mapping experiments were used to analyze the degradation of the endogenous *Xenopus* calmodulin mRNA. RNA from control oocytes protected the full length *Xenopus* calmodulin cDNA probe of 323 nucleotides (Fig. 3*B*, lane 2). RNA from oocytes injected with oligonucleotide 8 (lane 3) protected new complementary DNA fragments whose lengths approximated the predicted 3′-terminal probe fragment of 184 nucleotides (Fig. 2) in addition to the full-length probe of 323 nucleotides. In some experiments, a further set of faint bands could be observed that were specific to RNA extracted from oocytes injected with oligonucleotide 8 and whose lengths corresponded to the predicted 5′-terminal probe fragment of 109 nucleotides (Fig. 2). These extreme differences in cDNA fragment detectability may reflect differential stabilities of the RNase H-like products in the oocyte. As predicted, RNA from oocytes injected with oligonucleotide 7 protected two new sets of cDNA products whose lengths approximate the predicted 5′- and 3′-terminal cDNA fragments (234 nucleotides and 59 nucleotides long) of S1 nuclease digestion.

DISCUSSION

Confirming previous work (11, 14, 30), we find that complementary DNA sequences effectively promote degradation of target mRNA molecules. We have now demonstrated that complementary DNA sequences are also capable of degrad-

ing mRNA sequences *in vivo* and that they do so by a mechanism that involves an endogenous RNase H-like activity. Our results suggest three further points: (*i*) that destruction of the target sequences is rapid and complete when appropriate concentrations of complementary DNA molecules are employed; (*ii*) that any portion of the mRNA may serve as a target; and (*iii*) that only relatively small regions of perfect homology (as few as 15 nucleotides) are necessary for cleavage to occur.

The inactivation of target mRNA by this mechanism should be irreversible. Indeed, we found it difficult to detect all the fragments generated by the RNase H-like activity presumably due to rapid mRNA degradation following cleavage. Although the destruction of target mRNA molecules is sequence-specific, we found that, at high concentrations, the β-globin complementary 30-mer (oligonucleotide 5) but not the 15-mer (oligonucleotide 6) caused some degradation of α-globin mRNA (data not shown). A careful sequence analysis revealed that both of these oligonucleotides are ≈60% complementary to α-globin mRNA. Although we have not characterized this further, it is probable that a relatively small degree of complementarity can cause mRNA degradation if the complementary region is sufficiently long. Where a high degree of specificity is required, it would therefore be advantageous to utilize smaller oligonucleotides, since they seem to form equally good substrates for the RNase H-like activity.

It is attractive to suggest that complementary DNA-mediated mRNA destruction could be employed in other cell types that lack highly developed genetics. We emphasize, however, that the technique as described here could have certain limitations. First, we have relied on an endogenous RNase H-like activity present in *Xenopus* oocytes to degrade the mRNA. It is not known whether this RNase H activity is a property of all eukaryotic cells. Second, we have introduced complementary DNA molecules by injection, a technique limited in application to moderately large cells. The development of other types of oligonucleotide derivatives might overcome this limitation. Third, the short half-life of injected oligonucleotides in the *Xenopus* oocyte (8) could limit the utility of this approach to the study of those phenotypes that are a function either of long-lived mRNAs with short-lived protein products or of mRNAs that are rapidly and transiently induced. It may be possible to prevent rapid DNA degradation, however, through the use of modified or circular DNA molecules.

Nonetheless, we have successfully used a modification of this method based on *in vitro* hybridization of oligonucleotides to mRNAs to block assayable nerve-cell functions such as inducible currents attributable to specific ion channels (32). This modification may be useful for cloning genes that can only be identified by means of bioassay.

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