Effects of oligo sequence and chemistry on the efficiency of oligodeoxyribonucleotide-mediated mRNA cleavage

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Received March 13, 1990; Revised and Accepted May 18, 1990

ABSTRACT

Using the endogenous histone H4 mRNA of Xenopus oocytes as a target, we have previously shown that 20mer oligos complementary to different parts of this sequence vary in their effectiveness at causing mRNA cleavage in vivo, and that some of the RNA can never be cleaved. In this paper we show that the resistant RNA is not localised within one part of the oocyte, and that the relative resistance in vivo of endogenous or synthetic H4 mRNA to the different oligos is preserved in an in vitro assay system using deproteinised RNA. If an prior annealing step is included in vitro, all resistance is abolished. Chemical modification of one oligo by end substitution with methylphosphonate or phosphorothioate residues did not improve cleavage efficiency. Oligos with complete phosphorothioate substitution cause slower cleavage in vitro but persist for longer. Consequently phosphorothioate oligos are effective at lower doses than phosphodiester ones, provided that the incubation time is long enough (24 hours). Increasing oligo length from 20nt to 30nt increases phosphorothioate oligo efficiency over long reaction times in vitro, but decreases efficiency during short in vitro assays. Similar increases in length did not affect phosphodiester oligo performance in vivo, but caused a decrease in efficiency in vitro which was overcome by an annealing step.

INTRODUCTION

Oligodeoxyribonucleotides (henceforth referred to as oligos) are proving to be useful for inhibiting the expression of one selected gene without affecting others. Inhibition is specific because the oligo hybridises to the mRNA to which it is complementary. In some systems the RNA strand of the duplex is then cleaved at the oligo-binding site by an RNaseH-like activity, the RNA fragments being subsequently degraded. [1, 2, 3]. In addition, it is known that oligos bound to the 5' end of mRNAs in vitro can prevent the initiation (but not the translocation) of ribosomes, even in the absence of RNaseH [4]. It is not clear how important this mechanism is in vivo, but many authors have made oligos complementary to the area of the initiation codon to obtain any benefit arising from this method of inhibition.

The specificity of oligo-mediated cleavage has been exploited for hybrid arrest of translation in wheatgerm and rabbit reticulocyte lysates as a means of identifying the products of cloned genes [5, 3]. Oligos have also proved effective when applied externally to cultured cells or even to living organisms. Viral gene expression can be inhibited by incubation of infected cultured cells with oligos [6, 7] raising the hope of clinical use as an anti-viral agent. Cellular mRNAs, such as the c-myc proto-oncogene can also be affected by addition of oligos to the culture medium both of HL-60 cells (which over-express this gene) [8] and of T lymphocytes, where the gene is not constitutively expressed [9]. Addition of an oligo complementary to the common 5' end of trypanosome mRNAs to a culture of trypanosomes causes them to die, this effect not being seen with non-complementary oligos [10]. One of our own interests [11] and that of others [12, 13] is in using oligodeoxynucleotide injections as a method of producing Xenopus oocytes and embryos deficient in selected mRNAs as a substitute for conventional genetics.

One disadvantage with conventional phosphodiester oligos is that they are rapidly degraded by nucleases. There is currently much interest in the use of chemically novel oligos which are more resistant to enzymatic attack but still form stable complexes with RNA, and which are then good substrates for the RNaseH-like activity. It is also important that the oligos should be able to penetrate into the target cells easily, though design of oligos with this feature is handicapped by a lack of data on the method(s) by which any oligos enter the cell. A number of novel forms of oligo have been tried. These have included use of α-anomeric oligos [14, 15], methylphosphonates [16], phosphorothioates [17, 18], and oligos with various reagents covalently linked to one or both terminal residues [19, 20]. The resulting oligos have varied considerably in their utility. Moreover, it is apparent from several studies[2, 11, 19] that the primary sequence of an oligo can have a considerable influence on its effectiveness. Comparison of the different efficiencies of oligos of various types of modification (and of various sequences) at the molecular level is problematical in cultured cells. This is due to difficulties in studying the kinetics of the oligo-mediated cleavage of the RNA in vivo (if indeed it occurs), and due to ignorance of the route(s) and rates by which the oligos enter the cells. Xenopus oocytes are a good model system for the study of oligo-mediated cleavage because they can readily be microinjected, thereby circumventing the problem of different permeabilities of different oligos and
enabling a direct study of oligo-mediated cleavage to be made.

In previous papers [1, 11] we have described two oligos, H4-1 and H4-2, which are complementary to a major *Xenopus* histone H4 gene. We have shown that H4-1 injections cause the cleavage of 95%, but no more, of H4 RNA in the oocyte, whereas only 10% is cleaved after injections of H4-2. In this paper we further investigate the basis of this difference between the efficiency of different oligos, which we have reproduced in vitro. We also present some data on the effect on H4 mRNA of a new oligo, H4-3. Finally, in and attempt to reduce the doses of oligo to levels which are not toxic to embryos, but which still cause effective cleavage, we have prepared and tested a variety of modified oligos which are based on the H4-1 sequence. We find that an oligo with a complete phosphorothioate backbone works more slowly, but is active for longer than a phosphodiester oligo. Our results illustrate the difficulty of predicting oligo performance in vivo, and the consequent need for tests for oligo function, such as the simple in vitro assay which we describe.

**MATERIALS AND METHODS**

**Oocytes**

Oocytes were obtained and cultured as described by Colman [21]. Only Stage VI oocytes, as described by Dumont [22] were used in these studies. They were injected as described by Shuttleworth and Colman [1]. Oocytes injected with phosphodiester 20mers were incubated either for 3—5 hours or overnight before extraction of the RNA. We have previously shown [1, 11] that oligo-mediated cleavage is complete within 3 hours. In experiments with chemically modified oligos, or with oligos 25 or 30nt long, injected oocytes were incubated for 3—5 hours or for 24 hours. Incubation times for all experiments are specified in the text and figure legends. Unless otherwise stated, each oocyte received 50nl of 1mg/ml oligo solution. We prefer to state the amount of oligo injected rather than the final concentration in vivo. the amount of oligo injected rather than the final concentration in vivo of 0.5nl, the concentration of oligo in an oocyte injected with 50nl of 1mg/ml of a 20mer is 16μM. Variations in oligo sequence and backbone chemistry between the various 20mers alter this calculated concentration by less than 5%.

**RNA analysis**

Oocytes were frozen and RNA extracted by homogenising the oocytes in a buffer containing Proteinase K [23]. The RNA was recovered by phenol/chloroform extraction and ethanol precipitation. It was analysed by gel electrophoresis and Northern blotting as described by Shuttleworth and Colman [1]. Typically 2μg (0.5 oocyte equivalents) of RNA was loaded onto each track of 1.5% agarose, 2.2M formaldehyde gels, which were electrophoresed in MOPS buffer. After Northern transfer to nitrocellulose, filters were probed using antisense RNA transcripts of the plasmid pSP65XLH4, which contains a complete *Xenopus* H4 gene, transcribed from an SP6 promoter. Transcription reactions were carried out using the protocol of Melton et al [24], the radiolabel being [α-32P]UTP (Amersham, 800Ci/mm, 20mCi/ml). Hybridisation was by the method of Zinn et al [25].

**Oligodeoxynucleotides**

H4-2 and the phosphodiester H4-1 were as previously described [1]. Otherwise, oligodeoxynucleotides were synthesised on an Applied Biosystems 380A DNA synthesiser and purified by reverse phase hplc on a Waters μ-Bondpack C-18 column eluted with an acetonitrile gradient (14 to 45% in 30 min) in 0.1M triethylammonium acetate at pH 7.0. Phosphorothioate analogues were synthesised from nucleoside-3'-hydrogenphosphate monomers and isolated by trityl specific purification using OPC [26]. Oligodeoxynucleotides which contained both phosphodiester and phosphorothioate linkages were synthesised from cyanoethylphosphoramidites and sulphurised by the procedure of Stec et al [27]. Analogues with methylphosphonate linkages were prepared from deoxynucleoside methylphosphonamides with protocols supplied by Applied Biosystems, essentially as described by Agrawal and Goodchild [28]. Analogues containing up to four phosphorothioate or methylphosphonate linkages were purified on a Partisil 10-SAX column (Jones Chromatography) by the use of a phosphate gradient.

![Figure 2: Localisation of H4 mRNA](image-url)

**Figure 2: Localisation of H4 mRNA** Extracted RNA was analysed by Northern blotting. Tracks 1—3, nuclear/cytoplasmic location. Track 1, Total RNA from whole oocytes (T). Track 2, oocyte nucleus (Nu). Track 3, cytoplasm from enucleated oocyte (Cy). Tracks 4—9, Animal/Vegetal location. Track 4, total RNA from whole oocyte (T). Track 5, RNA from Animal hemisphere (An) of un.injected oocyte. Track 6, RNA from Vegetal hemisphere (Vg) of uninjected oocyte. Track 7, RNA from H4-1 treated whole oocyte. Track 8, RNA from Animal hemisphere of H4-1 treated oocyte. Track 9, RNA from Vegetal hemisphere of H4-1 treated oocyte. Injected oocytes were incubated overnight before assay.

Figure 1: The DNA sequence of the transcribed region of a *Xenopus laevis* histone H4 gene (from Ref. 30). The sequences of the oligos used in this study are shown under the regions of the H4 sequence to which they are complementary. Oligo sequences are underlined. The initiation codon of the H4 transcript is italicised (first line). The longer versions of the H4-1 oligo sequence have the same 5' end as the H4-1 20mer, and extend into the region indicated by the chevrons (<).
gradient (0.3M, 0–100% in 50min) in formamide (60%) followed by reverse phase hplc [29].

All oligos were fully complementary to known histone H4 sequences. Fig. 1 shows the DNA sequence of the transcribed region of a *Xenopus laevis* H4 gene (from Ref. 30). The sequences of the oligos used in these studies are shown in this figure, aligned with the regions of the H4 sequence with which they are complementary. Versions of the H4-1 oligo which were 25nt or 30nt in length had the same 5' end as the H4-1 20mer, but extended a further 5 or 10nt in the direction of the chevrons in Fig. 1.

In vitro cleavage reactions

Buffers:  
A1: 100mM KCl, 20mM Tris.HCl pH 7.4 (1x)
A2: 100mM KCl, 20mM Tris.HCl pH 7.4, 3mM MgCl₂, 2mM DTT, 0.1mg/ml BSA
B: 100mM KCl, 20mM Tris.HCl pH 7.4, 1.5mM MgCl₂, 1mM DTT, 0.05mg/ml BSA

For 'annealing' reactions 5µg of *Xenopus* total RNA (extracted as above) were dissolved in water. 2µg of oligo and then 2x A1 buffer were added to produce a final volume of 10µl in 1x A1 buffer. This was incubated at 60°C for 20 minutes, then briefly centrifuged to collect condensation from the sides of the tube. 10µl 1x A2 buffer was added and digestion started by addition of *E.coli* RNAseH (BRL) to 10u/ml. The enzyme was stored in 50% v/v glycerol, 1x buffer B at 20x working strength. Digestion was at 21°C for 1 hour. The reaction was then stopped by phenol extraction and the RNA recovered by ethanol precipitation.

For non-annealing reactions, 5µg RNA and 2µg oligo were mixed in a final volume of 20µl Buffer B. RNAseH was usually added to this immediately and digestion was as for annealed reactions. We have shown that this simplified protocol produces the same results as following the annealing protocol with the 20 minute incubation at 21°C instead of 60°C.

**RESULTS**

**H4-1 resistant RNA is present throughout the oocyte cytoplasm**

We have previously shown [1, 11] that 5–10% of the histone H4 RNA in the oocyte is not cleaved when oocytes are injected with the oligo H4-1, and that further injections of the oligo do not cleave this remaining RNA. We next investigated the possibility that the resistant RNA was localised in one part of the oocyte. Oocytes were injected with oligo H4-1, were incubated overnight, and were then bisected across the equator. Control oocytes, which had not been injected, were similarly dissected. In a separate experiment, oocytes were enucleated and RNA extracted from nucleus and cytoplasm. Fig 2 shows a Northern blot of the RNA recovered from the oocyte fragments in both experiments. Only the 5' cleavage fragment of H4 mRNA is visible in this figure, because the 3' fragment is unstable during overnight incubation [1]. All detectable H4 RNA from the enucleated oocytes was recovered from the cytoplasm (tracks 1–3 of Fig. 2). This is evidence that the H4-1 resistant RNA is not protected by virtue of being located in the nucleus. This is consistent with our previous demonstration that newly-synthesised or injected messages are susceptible to oligo-mediated cleavage in both the cytoplasm and the nucleus [1]. There is more H4 mRNA in the animal hemisphere of the oocyte than in the vegetal hemisphere (cf. tracks 5 and 6), and this is also the case with the H4-1 resistant fraction of the RNA (cf. tracks 8 and 9). We therefore have no evidence that the H4-1 resistant RNA is localised within one part of the oocyte cytoplasm, although this analysis would not have detected some forms of localisation (eg. in a circumferential layer).

Patterns of oligo-specific cleavage in oocytes can be simulated in vitro

Oligos H4-1 and H4-2, which are complementary to different regions of H4 mRNA, differ greatly in their effectiveness at cleaving this mRNA after injection into oocytes [1, 11]. This could either be an intrinsic feature of the RNA or oligos, or it could be the result of the environment of the RNA in the oocyte. To distinguish between these possibilities, phenol-extracted oocyte RNA was incubated with oligo and RNAseH as described in Materials and Methods. Three different anti-H4 oligos (H4-1, H4-2, H4-3).
H4-2 and H4-3) were used. In reactions with annealing (A+), the RNA and oligo were incubated together at 60°C before the addition of RNaseH. Reaction products were analysed by Northern blotting, as shown in Fig 3a. For comparison, the result of injecting oocytes with these oligos is shown in Fig 3b. Injected oocytes were incubated overnight before extraction of RNA. Consequently, only the 5' H4-1 cleavage fragment can be seen in Fig 3b, track 1. This fragment is the smaller of the two fragments visible in the corresponding in vitro reactions (Fig. 3a, tracks 1 and 2). Note that tracks 1 and 2 of Fig 3a contain half the amount of RNA present in the other samples.

In reactions in vitro without the annealing step, the three oligos differ in their effectiveness in cleaving the RNA, and this reflects the behaviour of the oligos in vivo. This suggests that the differences in effectiveness are not due to protection of some parts of the RNA by factors or structures in the oocyte. In either assay, oligo H4-1 is the most effective, oligo H4-2 is the least effective and H4-3 is intermediate (compare Fig 3a, tracks 2, 4 and 6 with Fig 3b, tracks 1, 2 and 3). If an annealing step is included in the in vitro assay however, these differences are abolished and all three of the oligos are fully effective (Fig 3a, tracks 1, 3 and 5). One possible explanation for these results is that different regions of the RNA adopt differing amounts of secondary structure, which inhibit oligo binding, but which are melted by the annealing treatment. This explanation, however, does not take into account the observation that the endogenous oocyte H4 mRNA pool contains molecules which vary slightly in sequence [1], due to transcription from several H4 genes. The results of our digestions in vitro with annealing suggest that all detectable H4 RNA molecules can hybridise to any of the oligos under some conditions. However, some H4 mRNA sequences might form hybrids less readily or stably, and the variation in oligo performance might reflect the proportion of the H4 RNA sequences with which each oligo can successfully hybridise. To exclude this possibility, we next performed experiments with a synthetic RNA preparation which was homogenous in sequence.

Oligo-mediated cleavage reactions were carried out with radiolabelled, capped sense-strand SP6 transcripts of an H4 clone, pSP64XLH4. All the sequences complementary to our oligos are fully conserved between this sequence and the one depicted in Fig.1. Synthetic H4 mRNA molecules were either injected into oocytes, which received injections of approximately 150pg of RNA and were then injected with H4-1, before incubation for 5 hours and extraction of RNA. For comparison, approximately 150pg of synthetic RNA was added to in vitro cleavage reactions containing one oocyte equivalent of total oocyte RNA. RNA products were extracted, electrophoresed, and detected by autoradiography of the dried gel (Fig 4). Only synthetic RNA molecules are detected by this analysis, since the endogenous Xenopus RNA is not radioactive. More of the synthetic H4 RNA is digested in the presence of H4-1 than H4-2, either in oocytes or in vitro (Fig 4, tracks 1 and 2, and 4 and 5). Each of the oligos shows a similar degree of effectiveness in the two systems. We conclude that polymorphism in the sequence of the target mRNA cannot explain the observed variation in the efficiency of different oligos. There are some differences between the patterns of fragments generated in vitro and in vivo. This is probably due to the presence of nucleases in vivo which attack the 3' cleavage fragment of the SP6 H4 RNA [1]. These differences are exaggerated in this figure, since larger RNA fragments will contain more radioactive nucleotides and will therefore register more strongly on the film.

Repeated digestion causes further cleavage in vitro

We have previously demonstrated that injection of a second dose of oligo H4-1 does not cause the cleavage of the RNA remaining after the first oligo injection [1, 11]. To determine whether this was also the case in vitro, phenol-extracted oocyte RNA was subjected to H4-1 mediated digestion in vitro without annealing, but with longer reaction times allowed (Fig 5). Oligo and RNA were either incubated together for 1 hour at 21°C before the addition of RNaseH (Fig 5, track 3), or digestion was allowed to proceed for 1 hour as in the standard assay, before the addition of a second aliquot of RNaseH and digestion for a further hour (track 4). Allowing a period for hybridisation before the addition of RNaseH did not affect the amount of cleavage (compare tracks 2 and 3). However, a longer RNaseH digestion does increase the amount of RNA which is cleaved (compare track 2 and track 4). Similarly, the H4 RNA in RNA extracted from oocytes which have been injected with H4-1 can be cleaved further by an in vitro reaction without annealing using the standard protocol (data not shown). The feature which confers resistance to H4-1 on a small fraction of the H4 mRNA would thus not appear to survive disruption of the oocyte and extraction of the RNA.

Modified H4-1 oligos in vivo and in vitro

We investigated the effectiveness of H4-1 oligos with chemically modified backbones at causing cleavage of H4 mRNA in oocytes and in the in vitro assay described above. We have previously observed that levels of phosphodiester-based oligos required to cause significant levels of cleavage were often toxic to eggs and embryos [11]. It was possible that modified oligos, expected to be more resistant to nucleases, might be effective at lower doses.

![Image](https://example.com/image.png)

**Figure 4:** Oligo-mediated digestion of 32p-labelled synthetic H4 mRNA. Autoradiograph of denaturing gel of extracted RNAs. Track 1, H4-1 digestion in vitro. Track 2, H4-2 digestion in vitro. Track 3, control; undigested RNA (C). Track 4, oocytes injected with SP6 H4 RNA only. Track 5, oocytes injected with SP6 H4 RNA then with H4-1. Track 6, oocytes injected with SP6 H4 RNA then with H4-2. Oocytes were incubated for 5 hours between injection and extraction of RNA.
Methylphosphonate (Nucleoside-OP(CH3)O2-Nucleoside) and phosphorothioate (Nucleoside-OP[S]O2-Nucleoside) versions of H4-1 were produced as described in Materials and Methods. At each terminus of some oligos, either one or two phosphate groups were modified, the rest of the residues having phosphodiester linkages. An H4-1 oligo with a complete phosphorothioate backbone was also produced. We call this oligo H4-1S. All these oligos were assayed by injection into oocytes (Fig 6), but at 0.1mg/ml, one-tenth of the concentration used previously. The phosphodiester H4-1 was also injected at 1mg/ml (Fig 6, track 2). Injected oocytes were incubated for 5 hours before extraction of RNA. When assayed by microinjection into oocytes, all the oligos have a similar effect to the injection of phosphodiester H4-1 at the same concentration (track 3). Note that the larger 3' H4 cleavage fragment, which is unstable in oocytes, is still present in the RNA from oocytes injected with H4-1S (track 8) but not in the other tracks. This implies that the phosphorothioate oligo was inactivated less rapidly than the others. However, as the amount of intact H4 mRNA remaining in all of the tracks 3 to 8 is similar, but is greater than the amount of RNA which is resistant to H4-1 at 1mg/ml (track 2), H4-1S must also act more slowly than the other oligos. We find that injection of 50nl 0.1mg/ml H4-1S does not inhibit oocyte protein synthesis as assayed by incorporation of 35S-methionine, or by analysis of the labeled proteins by electrophoresis (data not shown).

**Oligo effectiveness is affected by oligo length, and time of incubation**

It has been shown that phosphorothioate oligos are more resistant to nuclease than phosphodiester versions, but form less stable hybrids with RNA [16]. In an attempt to overcome the problem of reduced stability, we constructed phosphorothioate and phosphodiester oligos that were 25nt or 30nt long, reasoning that longer oligos would make the RNA-oligo hybrid more stable. The first 20nt from the 5' side of these oligos was the H4-1 sequence. H4-1, H4-1S and the longer versions of the oligos were injected into oocytes at 0.1mg/ml. RNA was then extracted either 3 or 24 hours later and analysed by Northern blotting (Fig 7). While there is little or no cleavage with the phosphodiester oligos after 3 hours, comparison of the amounts of intact RNA remaining after 3 and 24 hours in oocytes injected with phosphorothioate oligos shows that further reaction has occurred after the first time-point. Moreover, the phosphorothioate oligos become more effective with increasing size (this is more apparent in the 24 hour samples). There is no apparent difference between the effectiveness of different-sized phosphodiester oligos.

The same longer oligos were also assayed in vitro. The results from this 1 hour assay (Fig 6) contrast interestingly with the results of the longer incubations in vivo. In the in vitro assays with an annealing step, all the phosphodiester oligos are fully effective. However, the oligos become less effective in reactions without annealing as oligo size increases. All the phosphorothioate oligos are less effective than any of the phosphodiester ones, and the 25nt and 30nt oligos are not fully effective even in reactions with annealing. This apparent decrease in oligo effectiveness with increasing size is the opposite trend to that expected. It is not overcome when equal molar amounts of the different sized oligos are used, as opposed to equal amounts by mass as above (data not shown). Possibly the longer oligos are more likely to anneal to themselves. Among the phosphorothioate oligos, this feature seems to be compensated for by some advantage which longer oligos have during long incubations in vivo; possibly the longer oligos take longer to be inactivated by nuclease attack.

**DISCUSSION**

On the varying effectiveness of oligos of different sequences

In previous papers we have shown that two oligos complementary to different parts of the same H4 target RNA can vary in their effectiveness. A similar variation in the effectiveness of different oligos against calmodulin mRNA has been observed by Dash et al [2]. In this paper we have demonstrated that this variation in the effectiveness of different oligos can be preserved in vitro.
and that the three oligos we have used have the same order of effectiveness in vitro as in vivo. This in vitro assay may therefore be useful in the design and testing of future oligos; because of the considerable variation between the effects of different sequences, the most reliable strategy for oligo-mediated RNA cleavage must be to make several different oligos and then test them. Endogenous H4 mRNA contains molecules of several different sequences, and varying numbers of these may not be perfectly complementary to the different oligo sequences. However, this would not appear to cause the differences in effectiveness between oligos, which persist when a homogeneous population of cloned mRNA molecules is subjected to oligo-mediated cleavage either in vitro or in vivo.

Although we have not measured the dissociation temperatures of our different oligos, calculation according to equation (6) of Meinkoth and Wahl [31] suggests that the oligos should form hybrids of similar stability (TD_{H4,1}=64°C, TD_{H4,3}=62°C, TD_{H4,3}=68°C). Ranking the oligos in order of increasing calculated dissociation temperature would not lead us to predict their performance correctly. We suggest that the feature limiting the effectiveness of oligos in the in vitro assay is the secondary structure of the target RNA, which would prevent oligo binding to different extents depending upon the type and extent of secondary structure in the different regions of the message. The annealing treatment would be expected to denature the secondary structure, and this is consistent with the total effectiveness of all the oligos in assays with the annealing step, which one would not predict if the stability of the various oligo-RNA hybrids was the factor determining the performance of the oligos. We have previously commented upon the varying and inconsistent results which we have obtained by using computer programmes to predict the secondary structure of H4 mRNA [11]. A major source of difficulty is the lack of experimental data which can be used to assess the accuracy of rival computer predictions. We therefore have little faith in the value of our computer predictions in support of the role of secondary structure proposed above.

The implications of target message secondary structure for oligo-mediated RNA cleavage have also been considered by Shibahara et al.[32], who found that oligos complementary to both predicted single-stranded and double-stranded regions of the RNA WS-s(+1) were effective in vitro. However, their assay protocol contained a 60°C annealing step, and they do not appear to have tried protocols equivalent to our assays without annealing. Furthermore, some of the predicted regions of secondary structure were short and might be capable of local melting even at non-annealing temperatures.

We have previously shown that some of the H4 RNA in oocytes is resistant to cleavage mediated by oligo H4-1, in that repeated injection of the oligo does not cause further cleavage. This feature is not reproduced in the in vitro assay, in which RNA remaining from one round of oligo ablation either in vivo or in vitro can be digested further. Resistance to the oligo H4-1 would therefore seem to rely on a feature of the oocyte which does not survive from one round of oligo ablation either in vivo or in vitro. However, their assay protocol contained a 60°C annealing step, and they do not appear to have tried protocols equivalent to our assays without annealing.

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On the effectiveness of modified oligos

Modification of the terminal phosphodiester linkages of oligos to either methylphosphonate or phosphorothioate linkages has little effect on the performance of an oligo of a given sequence during short (5 hour) incubations in oocytes. A fully substituted phosphorothioate oligo is as effective as a phosphodiester version of the same sequence during a 3 or 5 hour incubation in oocytes, though the higher level of unstable RNA cleavage fragments in samples treated with phosphorothioate oligos suggest that the rate
of reaction is slower. This would also explain why a phosphorothioate oligo is less effective than a phosphodiester oligo of the same sequence in vitro, where the reaction time is only 1 hour.

During longer incubations in vivo (24 hours), further RNA cleavage occurs in oocytes injected with phosphorothioate oligos, though not in those injected with phosphodiester oligos. Presumably this reflects the greater stability of phosphorothioate oligos in oocytes. During 24 hour incubations, longer (25nt and 30nt) phosphorothioate oligos are more effective, a trend which is not noticeable with phosphodiester oligos. The increased effectiveness of the longer oligos could be due to more stable hybrid formation with the target RNA, or due to greater resistance to nucleases. We have previously shown that oligos as short as 10nt can mediate RNA cleavage [11]. Longer oligos will be reduced to this size more slowly by exonucleases, and endonuclease cleavage fragments of longer oligos are more likely to be large enough to continue to mediate RNA cleavage.

When assayed in vitro, the oligos show the unexpected trend of decreasing effectiveness with increasing size. We cannot explain this phenomenon, though possibly longer oligos are more likely to be capable of hybridising to themselves, a reaction which would compete with the formation of oligo-RNA hybrids. The phosphorothioate oligos appear to be less effective than phosphodiester versions in vitro; possibly this reflects a lower reaction rate. These effects are overcome by long (24 hour) incubations in vivo, the result being that phosphorothioate oligos are as effective as a ten-fold greater concentration of phosphodiester oligo of the same sequence.

Cazenave et al. [33] have examined the effectiveness of phosphorothioate and phosphodiester oligos complementary to globin mRNA on globin synthesis in cell-free translation systems and in globin-injected Xenopus oocytes. Their studies in some ways parallel our work on cleavage of endogenous, as opposed to injected, messages. They also observed that phosphorothioate oligos were effective at low concentrations (even at levels substoichiometric with the target RNA). This is fortunate, since large doses of phosphorothioate oligo (16µM, or approximately 40nl of 1mg/ml) were toxic to protein synthesis. We have not observed this effect at the lower doses (50nl of 0.1mg/ml) used in this study.

Of the several chemically modified oligo structures that have been used in oligo-mediated RNA cleavage experiments in the last few years, phosphorothioate oligos seem to be the most promising alternative to phosphodiester forms, offering the same advantages of specificity and suitability as a substrate for RNAseH, without the disadvantage of nuclease sensitivity. The literature shows that it is difficult to predict the performance of a given oligo in a living system. This is true whether comparing oligos which have different chemical modifications, or which are of the same chemical type, but differ in their sequence. We have described in this paper an in vitro assay which can be used to compare different oligo sequences, and have also presented evidence that properties of the target RNA other than its primary sequence can affect oligo-mediated cleavage. Such (currently) unpredictable effects are a hazard to the effective use of oligos only if the oligos are not tested for effectiveness before they are used in experiments where oligo performance can only be monitored indirectly (e.g. by the production of phenocopies). If suitable precautions are taken, oligo-mediated RNA cleavage is a powerful and specific method of temporarily disabling a gene, where this could not be done by other means.

ACKNOWLEDGEMENTS

We are grateful to Catherine Baker, who read the manuscript and made helpful suggestions. C.B. was supported by a Medical Research Council studentship, and A.C. acknowledges the support of Cancer Research Campaign, and the Medical Research Council.

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