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Increased specificity for antisense oligodeoxynucleotide targeting of RNA cleavage by RNase H using chimeric methylphosphonodiester/phosphodiester structures

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ABSTRACT

One of the inherent problems in the use of antisense oligodeoxynucleotides to ablate gene expression in cell cultures is that the stringency of hybridization in vivo is not subject to control and may be sub-optimal. Consequently, phosphodiester or phosphorothioate antisense effectors and non-targeted cellular RNA may form partial hybrids which are substrates for RNase H. Such processes could promote the sequence dependent inappropriate effects recently reported in the literature. We have attempted to resolve this problem by using chimeric methylphosphonodiester/phosphodiester oligodeoxynucleotides. In contrast to the extensive RNA degradation observed with all-phosphodiester oligodeoxynucleotides, highly modified chimeric antisense effectors displayed negligible, or undetectable, cleavage at non-target sites without significantly impaired activity at the target site. We also note that all of the all-phosphodiester oligodeoxynucleotides tested demonstrated inappropriate effects, and that such undesirable activity could vary widely between different sequences.

INTRODUCTION

Antisense oligodeoxynucleotides and oligodeoxynucleotide analogues, have been used to inhibit gene expression in a variety of systems (reviewed in Tidd [1], and Uhlmann and Peyman [2]). The antisense effects observed with phosphodiester oligodeoxynucleotides in Wheat Germ Extracts [3], Xenopus oocytes [4] and Xenopus embryos [5] are mediated by ribonuclease-H (RNase H), an enzyme which degrades RNA at the site of hybridization with the complementary oligodeoxynucleotides. In addition, it is widely believed that the antisense effects observed in cell cultures may be mediated, or at least enhanced, by RNase H–like activity. However, oligodeoxynucleotides which can direct RNase H have recently been reported to show undesirable sequence dependent activity in Xenopus embryos [5,6] and in cell cultures [7,8]. Since the stringency of hybridization in vivo may not be optimal, it is not inconceivable that such inappropriate effects were due to the cleavage, by RNase H, of non-target RNA at sites of partial complementarity to the antisense effectors. This significant problem has recently led one group to question the potential development of antisense oligodeoxynucleotides as future cancer and anti-viral chemotherapeutic agents [8]. Undesirable sequence dependent effects of this nature may be a potential drawback with all oligodeoxynucleotides and analogue structures which can direct RNase H. Indeed, phosphorothioate oligodeoxynucleotide analogues may be particularly susceptible to such inappropriate activity as they have been reported to direct RNase H to cleave hybridized RNA with greater efficiency than equivalent phosphodiester oligodeoxynucleotides [9,10].

On the other hand, non-ionic methylphosphonodiester oligodeoxynucleotide analogues, developed by Miller and Ts'o [11], have been shown to be incapable of directing RNase H [12], which may account for their poor overall performance as antisense effectors. It would appear that methylphosphonates may only induce antisense effects through physical obstruction of splicing reactions and ribosome binding upon hybridizing to the RNA at these specific sites. Additionally, methylphosphonodiester/RNA heteroduplexes display low melting temperatures (Tm's) [1,13] indicating a low ability of methylphosphonates to hybridize to RNA, and this may further limit their potential as antisense effectors.

However, the apparent deficiencies of methylphosphonodiester oligodeoxynucleotides in terms of their inability to direct RNase H and poor hybridization to RNA may be exploited to resolve the problem of undesirable sequence dependent RNase H effects. Chimeric antisense effectors, composed of terminal methylphosphonodiester sections separated by a central phosphodiester region, could significantly reduce RNase H cleavage at non-target sequences. Firstly, any partial complementarity solely between the methylphosphonodiester sections of a chimeric antisense effector and RNA would not result in RNase H activation. Secondly, a certain minimum level of heteroduplex stability may be required to activate RNase H. Because the methylphosphonodiester sections in a chimeric molecule hybridize poorly to RNA, longer regions of partial complementarity between RNA and chimeric oligodeoxynucleotides, than between RNA and phosphodiester oligodeoxynucleotides, would be required for the same level of heteroduplex formation at 37°C. Therefore, RNA sequences with sufficient partial complementarity to direct RNase H should, statistically, become more scarce upon reducing the size of the

phosphodiester region within a chimeric oligodeoxynucleotide analogue of constant length, or upon reducing the hybridization strength of that phosphodiester region by, for example, increasing the A+T to G+C ratio.

We have investigated the sequence dependent inappropriate activity of oligodeoxynucleotides in an *in vitro* system with a variety of phosphodiester and chimeric methylphosphonodiester/phosphodiester analogue structures of the type previously described [13]. Our results demonstrate that chimeric methylphosphonodiester/phosphodiester oligodeoxynucleotide analogues, with small phosphodiester regions containing high A+T to G+C ratios, may eliminate undesirable activity and may be particularly suitable for use as antisense agents in situations where RNase H can mediate the antisense effects. In addition to their low promiscuous substrate activity with RNase H, demonstrated in this report, we have previously shown that they still activate the enzyme to cleave perfectly complementary sequences with high efficiency [13]. Furthermore, the terminal methylphosphonodiester sections protect the oligodeoxynucleotide analogues from degradation by serum [14] and cellular [15] nucleases.

METHODS

Oligodeoxynucleotide synthesis

Phosphodiester, chimeric methylphosphonodiester/phosphodiester and methylphosphonodiester oligodeoxynucleotides were synthesized on an Applied Biosystems 381A DNA Synthesizer and purified by solid phase extraction on C-18 Sep Pak cartridges (Walters Chromatography Division, Millipore (U.K.) Ltd), as previously described [14,16].

Polymerase chain reactions (PCR's)

DNA for *in vitro* transcription was amplified by PCR on an IHB2024 Thermal Cycler (Cambio) situated in a +4°C cold room, with a thermal profile of: 1 cycle of 95°C for 4 minutes, 60°C for 1 minute, 72°C for 3 minutes; 29 cycles of 95°C for 1 minute, 60°C for 1 minute, 72°C for 3 minutes; 1 cycle of 72°C for 12 minutes. The reactions contained 200μM each dNTP, 0.5μM each primer, 50pg to 500pg template DNA and 2U Taq polymerase (Cambio) in 100μl of 1×PARR buffer (unspecified composition, Cambio), and were overlaid with 100μl of liquid paraffin (BDH). A 1780 base-pair c-*myc* DNA fragment containing an SP6 RNA polymerase promoter and the region between −270 and 1434 of the c-*myc* cDNA sequence [17] was amplified from 50pg of pSP65 plasmid [18] containing a c-*myc* cDNA (Cambio), using the 5′ primer, 1, 5′ GTACATATTGTCGTTAGAACGCGGCTACAA 3′ and the 3′ primer, 2, 5′ GTCTCAAGACTCAGCCAAGGTTGTGAGGTT 3′. A 1304 base-pair p53 DNA fragment was amplified from 500pg of CMV expression vector containing a cDNA of the p53 Harlow sequence [19] (a generous gift from Dr. J. Jenkins) using the 5′ primer, 3, 5′ GGTACCTAATACGACTCACTATAGGGAGAAGCTTAACGGTGACACGCTTCCCTGGATT 3′ and the 3′ primer, 4, 5′ AGAGAGGGATCCAAGGGAACAAGAAGTGGAGAAT 3′. The amplified p53 DNA contained the region −52 to 1203 of the p53 cDNA sequence, with the T7 RNA polymerase promoter being introduced by primer 3. The amplified fragments were purified by electrophoresis in 1% low melting temperature agarose gel/TAE buffer, followed by band excision and phenol extraction [20] prior to transcription.

In vitro transcription

1μg of DNA was incubated with 50U of bacteriophage T7 or SP6 RNA polymerase (Cambio or Boehringer), 20U Human placental RNase inhibitor (Amersham) in 40mM Tris-HCl pH 7.9, 6mM $MgCl_2$, 10mM NaCl, 10mM dithiothreitol, 2mM spermidine in a total volume of 40μl. Unlabelled RNA was obtained with each rNTP present at 1mM, digoxigenin labelled RNA was obtained with 1mM ATP, CTP and GTP, 0.9mM UTP, and 0.1mM digoxigenin-11-UTP (Boehringer). Incubation was at 37°C (T7) or 40°C (SP6) for 2 hours.

RNase H assays

2μl of unpurified transcription reactions (ca. 500ng digoxigenin labelled RNA) were incubated with 0.5U RNase H (Amersham),

Human c-myc gene sequence

Met Pro Leu Asn Val Ser

5'...ACG ATG CCC CTC AAC GTT AGC...3'

Codon No. 1 2 3 4 5 6

c-myc oligonucleotides	Code 5'-3'
Antisense oligodeoxynucleotides	
3'...T-A-C - G-G-G - G-A-G - T-T-G - C-A-A...5'	M14pAS
3'...T/A/C / G-G-G - G-A-G - T-T-G / C/A/A...5'	M383AS
3'...T/A/C / G/G/G - G-A-G - T/T/G / C/A/A...5'	M545AS
3'...T/A/C / G/G/G / G/A-G - T-T-G / C/A/A...5'	M347AS
3'...T/A/C / G/G/G / G/A/G - T-T-G - C/A/A...5'	M248AS
3'...T/A/C / G/G/G / G/A/G / T/T/G / C/A/A...5'	M14mAS
Sense oligodeoxynucleotides	
5'...A-T-G - C-C-C - C-T-C - A-A-C - G-T-T...3'	M14pS
5'...A/T/G / C-C-C - C-T-C - A-A-C / G/T/T...3'	M383S
5'...A/T/G / C/C/C - C-T-C - A/A/C / G/T/T...3'	M545S
5'...A/T/G / C/C/C / C/T/C / A/A/C / G/T/T...3'	M14mS
Nonsense oligodeoxynucleotide	
5'...G-T-A - C-G-G - T-A-A - C-G-G - G-A-T...3'	M14pNS

Human c-Ha-ras gene sequence

Met Thr Glu Tyr Lys Leu Val

5'...ATG ACG GAA TAT AAG CTG GTG...3'

Codon No. 1 2 3 4 5 6 7

c-Ha-ras oligonucleotides	Code 5'-3'
Antisense oligodeoxynucleotides	
3'...C-T-G - C-C-T - T-A-T - A-T-T - C-G-A...5'	R14pAS
3'...C/T/G / C/C/T - T-A-T - A/T/T / C/G/A...5'	R545AS
Sense oligodeoxynucleotides	
5'...G-A-C - G-G-A - A-T-A - T-A-A - G-C-T...3'	R14pS
5'...G/A/C / G/G/A - A-T-A - T/A/A / G/C/T...3'	R545S

Figure 1. Human c-*myc* and c-Ha-*ras* oligodeoxynucleotide structures and sequences. A dash (−) between bases indicates a normal phosphodiester internucleoside linkage, whereas a slash (/) indicates a methylphosphonodiester linkage. The name of each oligodeoxynucleotide is shown to the right of the sequence and is derived from the structure and sequence as below. The series is indicated by the first letter, 'M' or 'R', indicating c-*myc* and c-Ha-*ras* respectively. The next three alphanumerics indicates structure in the conventional 5′ to 3′ direction. For example, '14p' has all internucleoside linkages of the normal phosphodiester type, '347' has three and seven methylphosphonodiester linkages at the 5′ and 3′ termini respectively, separated by four contiguous phosphodiester linkages, '14m' has all internucleoside linkages of the methylphosphonodiester type. The remaining character(s) indicate actual sequence, 'AS' being antisense, 'S' being sense and 'NS' nonsense (randomized antisense sequence). The antisense sequences are shown 3′ to 5′ to demonstrate complementarity to the sense sequence.

20U Human placental RNase inhibitor (Amersham) in 20μl of 40mM Tris-HCl (pH 7.6 at 37°C), 4mM $MgCl_2$, 1mM dithiothreitol, 0.003% bovine serum albumen [13] at 37°C for 0 to 60 minutes. Oligodeoxynucleotides or analogues, where added, were present at 1μM (a roughly 20 fold molar excess over RNA). Samples (3μl) were removed at intervals, mixed with 47μl ice cold 100ng/μl E.coli rRNA in 1mM EDTA pH 8.0, and extracted with phenol/chloroform/isoamyl-alcohol (25:24:1 v:v:v). 2μl of the extracted solution was removed for 2.2M formaldehyde−1.5% agarose gel separation as previously described [20] except for the addition of ethidium bromide to the loading buffer [21] and formaldehyde to the running buffer (final concentration 2.2M). The gel was blotted onto nylon membrane (Hybond N, Amersham) and the digoxigenin labelled RNA fragments detected directly using the proprietary immunological system (Boehringer). Densitometric measurements were made across the blots at a wavelength of 555nm with a Shimadzu Model CS9000 instrument. The ordinate scale of the densitometers output was varied to permit observation of all the relevant peaks.

Northern blotting

Unlabelled RNA transferred to nylon membranes was detected using the probing and detection procedure recommended by Boehringer. Briefly, UV exposed membranes were probed at 42°C overnight with digoxigenin labelled c-*myc* DNA (produced by random priming of Klenow fragment) in the manufacturer's recommended hybridization solution (50% formamide, 5×SSC, 5% blocking reagent 0.1% N-lauroylsarcosine (Na salt), 0.02% SDS, 100μg/ml sonicated herring sperm DNA). The probed membranes were washed to high stringency (65°C, 0.1% SDS, 0.1×SSC) and bound digoxigenin labelled probe detected using the immunological detection system, as above.

RESULTS

The specificity of targeting cleavage of RNA by RNase H was investigated by exposing *in vitro* transcribed RNA to ca. 20 fold molar excesses of various oligodeoxynucleotides in the presence of the enzyme (0.025U/μl). The oligodeoxynucleotide structures and sequences synthesized and used in this study are shown in Figure 1. The c-*myc* antisense oligodeoxynucleotide sequence was taken from the literature where it has been reported to be a specific antisense effector [22−25]. The c-*myc* sequences may be seen to have a slight preponderance of G and C residues, whereas the c-Ha-*ras* oligodeoxynucleotides have an excess of A and T residues.

Figure 2 presents the results obtained from the RNase H digestion of digoxigenin labelled, *in vitro* transcribed, 1700 nucleotide (nt) c-*myc* RNA when directed by all-phosphodiester c-*myc* antisense, sense and nonsense 15-mer oligodeoxynucleotides. The sense and nonsense oligodeoxynucleotide controls should not, in principle, direct RNase H cleavage at all. The antisense oligodeoxynucleotide should, theoretically, direct RNase H to cleave the 1700nt RNA once, at the targeted translation initiation region, to produce fragments of 270nt and 1430nt. Figure 2B shows that by 30 minutes very little of the expected 1430nt fragment remained in the antisense reaction, due to extensive digestion at a number of non-target sites. Such undesired cleavage by RNase H was also observed with both the sense and nonsense oligodeoxynucleotides, although at fewer sites, and to a lesser extent at those sites, than was observed with

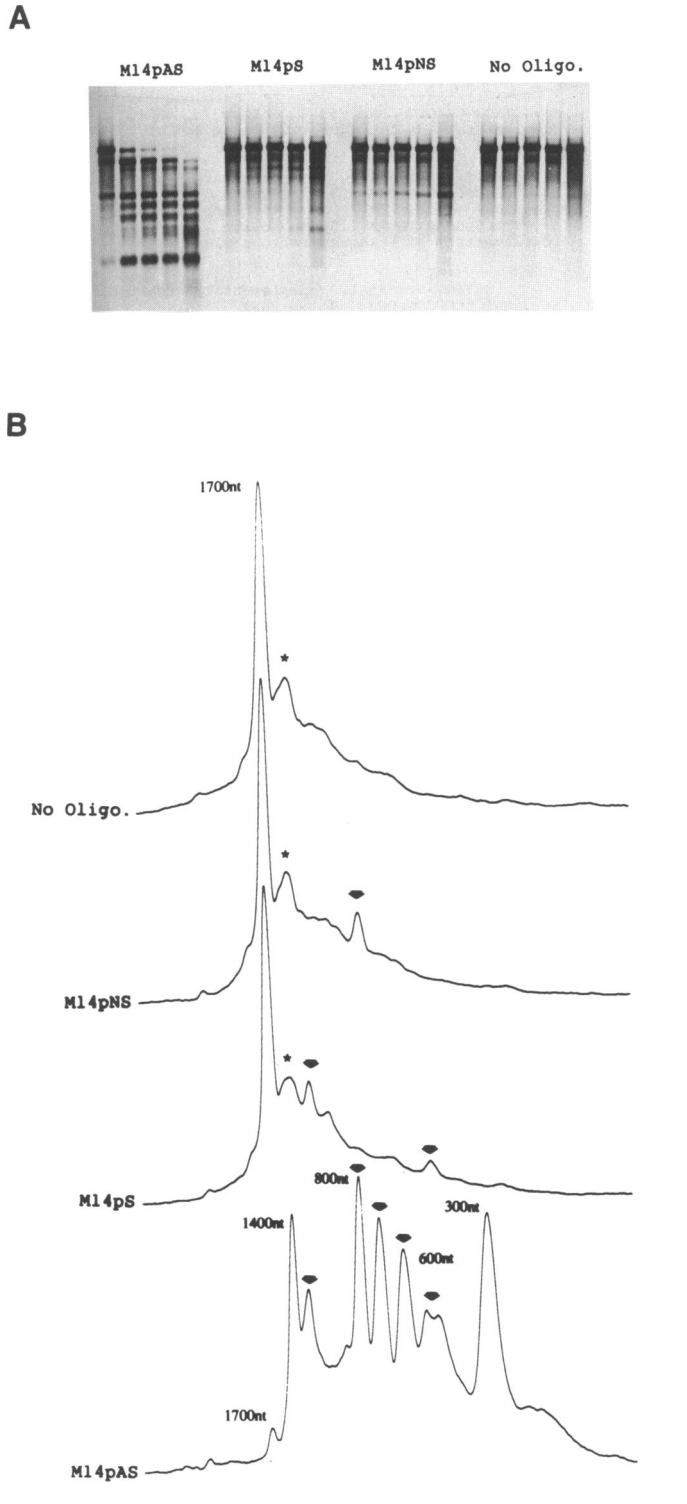

Figure 2. Incubation of digoxigenin labelled, 1700nt, *in vitro* transcribed c-*myc* RNA (500ng) with RNase H (0.025U/μl) in the absence of oligodeoxynucleotide or in the presence of the c-*myc* all-phosphodiester oligodeoxynucleotides M14pAS, M14pS or M14pNS (1μM). The structure, sequence and abbreviation of the oligodeoxynucleotides is shown in Figure 1. RNA bands produced by premature termination during *in vitro* transcription are indicated with an asterisk (*). RNA fragments generated through undesired cleavage by RNase H are indicated with an arrow (→). **A.** RNA immunologically detected on a blot which shows samples removed from reactions containing the indicated oligodeoxynucleotide after (from left to right within each block) 0, 5, 15, 30 and 60 minute incubations. **B.** Densitometric traces (555nm) of the thirty minute time points on the blot shown in **A** above.

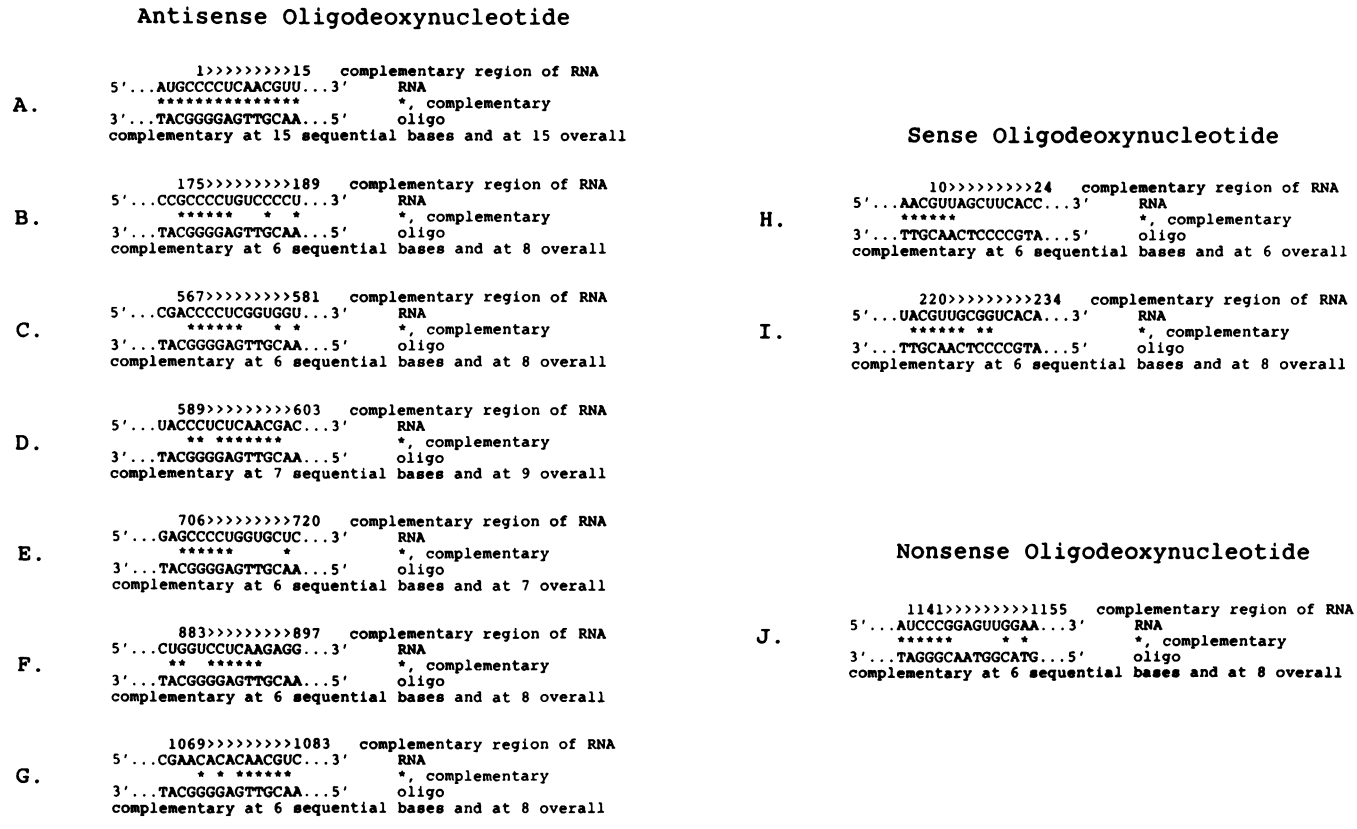

Figure 3. Regions of partial complementarity which may form at least 6 contiguous base pairs between the 1700nt, *in vitro* transcribed c-*myc* RNA and the indicated c-*myc* oligodeoxynucleotide sequences. The c-*myc* antisense sequence is fully complementary to 1 region, the target site (A), and partially complementary to 6 other regions (B to G). The c-*myc* sense sequence is partially complementary to 2 regions (H and I) and the c-*myc* nonsense oligodeoxynucleotide sequence is partially complementary to 1 region (J).

the antisense oligodeoxynucleotide. No non-specific activity was observed in the absence of oligodeoxynucleotide. The same results were obtained with *in vitro* transcribed RNA which was not itself labelled with digoxigenin but which was detected on the blot by probing with digoxigenin labelled c-*myc* DNA (data not shown). This indicated that the digoxigenin label in the RNA did not promote these effects as artefacts of the assay system. Additionally, the low salt buffer used in these experiments, which supplies Mg^{2+} ions essential for RNase H activity, did not significantly alter the melting temperatures of heteroduplexes from the values observed in an Mg^{2+}-free 'physiological' buffer [13].

It was apparent from these results that the oligodeoxynucleotides were able to form hybrids at regions of partial complementarity within the RNA, which had sufficient stability or lifetime at 37°C to permit cleavage of the RNA at such sites by RNase H. The sites of potential hybridization between the 1700nt c-*myc* RNA and the c-*myc* oligodeoxynucleotides were identified by computer searching of the RNA sequence [17] for regions of partial complementarity to the oligodeoxynucleotides, which extended over at least six contiguous bases. The results of these computer scans are presented in Figure 3. The c-*myc* antisense sequence, in addition to its target site (A), showed significant sequence complementarity to 6 other regions along the 1700nt c-*myc* RNA sequence (B to G). Partial complementarities to the c-*myc* RNA were also observed with the sense (2 regions, H and I) and nonsense (1 region, J) sequence oligodeoxynucleotides, although fewer in number and of lower G + C content than observed with the antisense sequence. It may be that the c-*myc* antisense sequence was an unfortunate choice for an antisense effector molecule in that it appears to contain the complement of commonly occurring mRNA sequence motifs and, additionally, is capable of forming a predominance of stabilizing GC base pairs in partial sequence hybrids. A partial complementarity of six contiguous base pairs appears to be sufficient for the undesired activation of RNase H. For example the 700nt fragment produced on directing RNase H to cleave the c-*myc* RNA by M14pAS (Figure 2) was most probably derived from further cleavage (non-target site E, Figure 3, position 706 to 720 on the RNA) of the expected 1430nt fragment, obtained initially from strand scission at the target site A.

Since, in contrast to the sense and nonsense controls, the c-*myc* antisense all-phosphodiester sequence was able to direct efficient RNase H cleavage of the single c-*myc* RNA at several non-targeted sites, in addition to the targeted region at codons 1 to 5, it seemed likely that it would also promote the degradation of other, non-targeted, RNA molecules. If this were true, then apparent sequence-specific antisense effects achieved, in some cases, with this oligodeoxynucleotide might be spurious, resulting from the generalized degradation of RNA in the presence of RNase H-like activity. To address this possibility, RNA of the tumour suppressor gene, p53 was selected as an irrelevant, non-target, control RNA molecule, and the experiments repeated. Figure 4 presents the results obtained from incubating RNase H at 37°C with *in vitro* transcribed, digoxigenin labelled, 1250nt p53 RNA in the presence of c-*myc* antisense, sense and nonsense

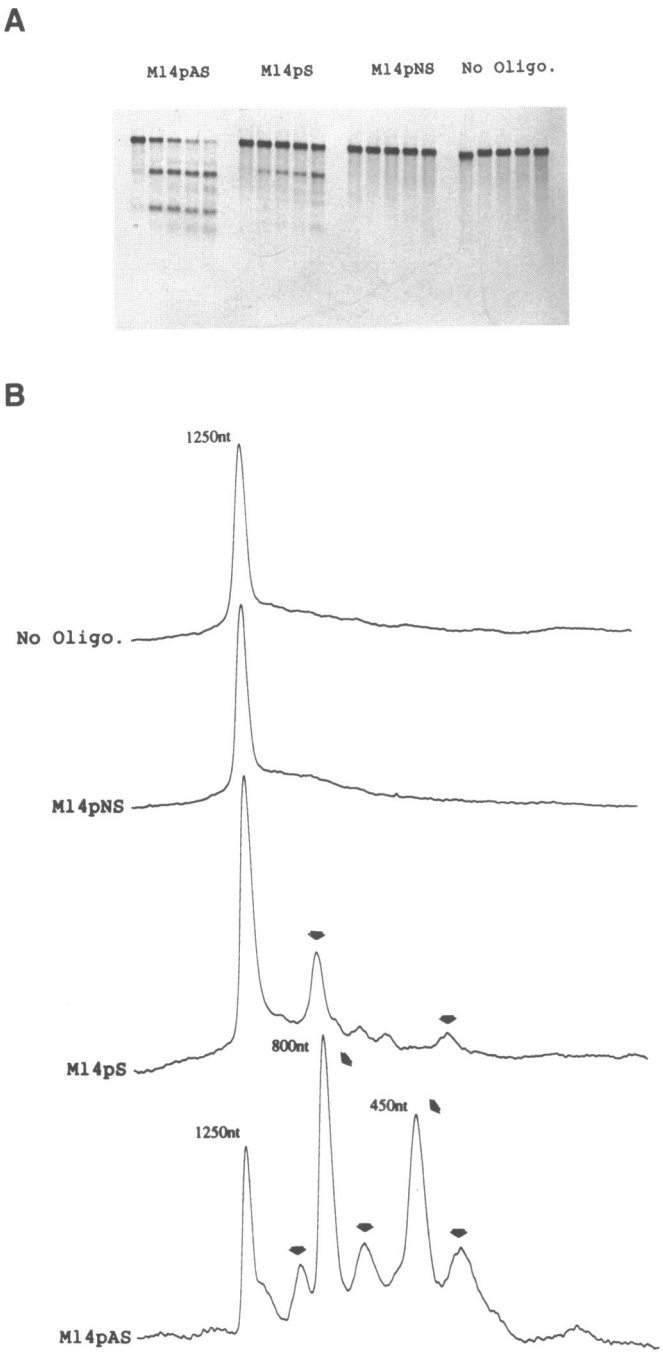

Figure 4. Incubation of digoxigenin labelled, 1250nt, *in vitro* transcribed p53 RNA (500ng) with RNase H (0.025U/μl) in the absence of oligodeoxynucleotide or in the presence of the c-*myc* all-phosphodiester oligodeoxynucleotides M14pAS, M14pS or M14pNS (1μM). The structure, sequence and abbreviation of the oligodeoxynucleotides is shown in Figure 1. RNA fragments generated through undesired cleavage by RNase H are indicated with an arrow (→). **A.** RNA immunologically detected on a blot which shows samples removed from reactions containing the indicated oligodeoxynucleotide after (from left to right within each block) 0, 5, 15, 30 and 60 minute incubations. **B.** Densitometric traces (555nm) of the thirty minute time points on the blot shown in **A** above.

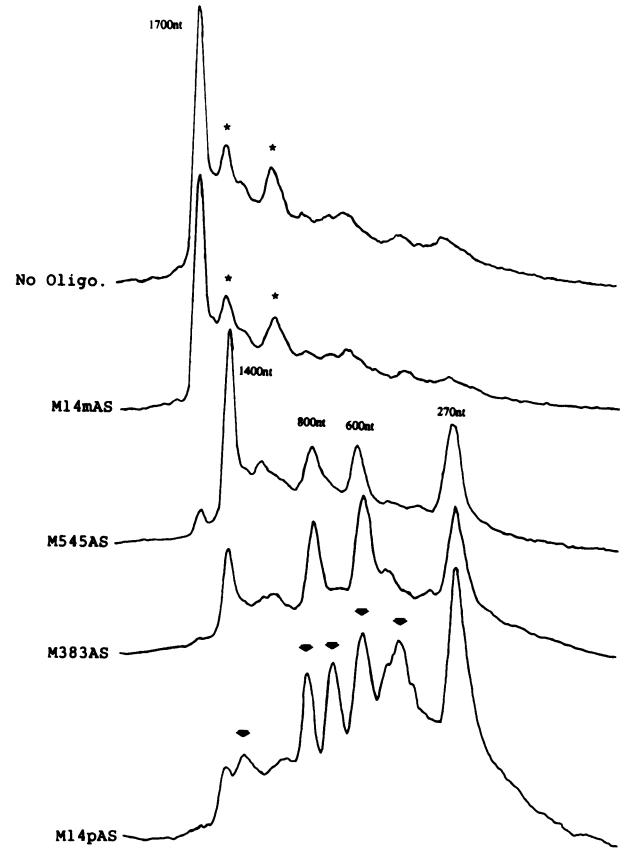

Figure 5. Densitometric traces (555nm) of RNA blot band intensities after 30 minute incubation of digoxigenin labelled, 1700nt, *in vitro* transcribed c-*myc* RNA (500ng) with RNase H (0.025U/μl) in the absence of oligodeoxynucleotide or in the presence of the c-*myc* oligodeoxynucleotides M14pAS, M383AS, M545AS or M14mAS (1μM). The structure, sequence and abbreviation of the oligodeoxynucleotides is shown in Figure 1. RNA bands produced by premature termination during *in vitro* transcription are indicated with an asterisk (*). RNA fragments generated through undesired cleavage by RNase H are indicated with an arrow (→).

phosphodiester oligodeoxynucleotides. The p53 control RNA should not have been digested by RNase H at all under these conditions, in the absence of any undesired activity. In fact, it may be seen, from Figure 4B, that by 30 minutes M14pAS, the c-*myc* antisense oligodeoxynucleotide, had directed RNase H to almost completely degrade the p53 RNA. In the same time significant, but much lower undesired RNase H activity was observed with M14pS, the c-*myc* sense oligodeoxynucleotide, whereas no cleavage whatsoever occurred with the nonsense oligodeoxynucleotide, M14pNS, or in the absence of oligodeoxynucleotide.

The regions of partial complementarity between the 1250nt p53 RNA sequence and the c-*myc* oligodeoxynucleotide sequences were identified (data not shown). Again, to score, the minimum partial complementarity required between RNA and oligodeoxynucleotide sequences was six contiguous bases. The c-*myc* antisense oligodeoxynucleotide sequence was found to be partially complementary to a remarkable 11 regions of the 1250nt p53 RNA, while the sense oligodeoxynucleotide sequence was partially complementary to just 2 regions. No region within the p53 RNA sequence was partially complementary to the nonsense oligodeoxynucleotide sequence over at least six consecutive bases. Such data, along with the empirical results presented in Figure 4, would appear to confirm that the c-*myc* antisense oligodeoxynucleotide was an unfortunate choice of sequence for specifically targeting c-*myc* mRNA.

Because methylphosphonates are unable to activate RNase H, we investigated the potential for reducing undesirable cleavage

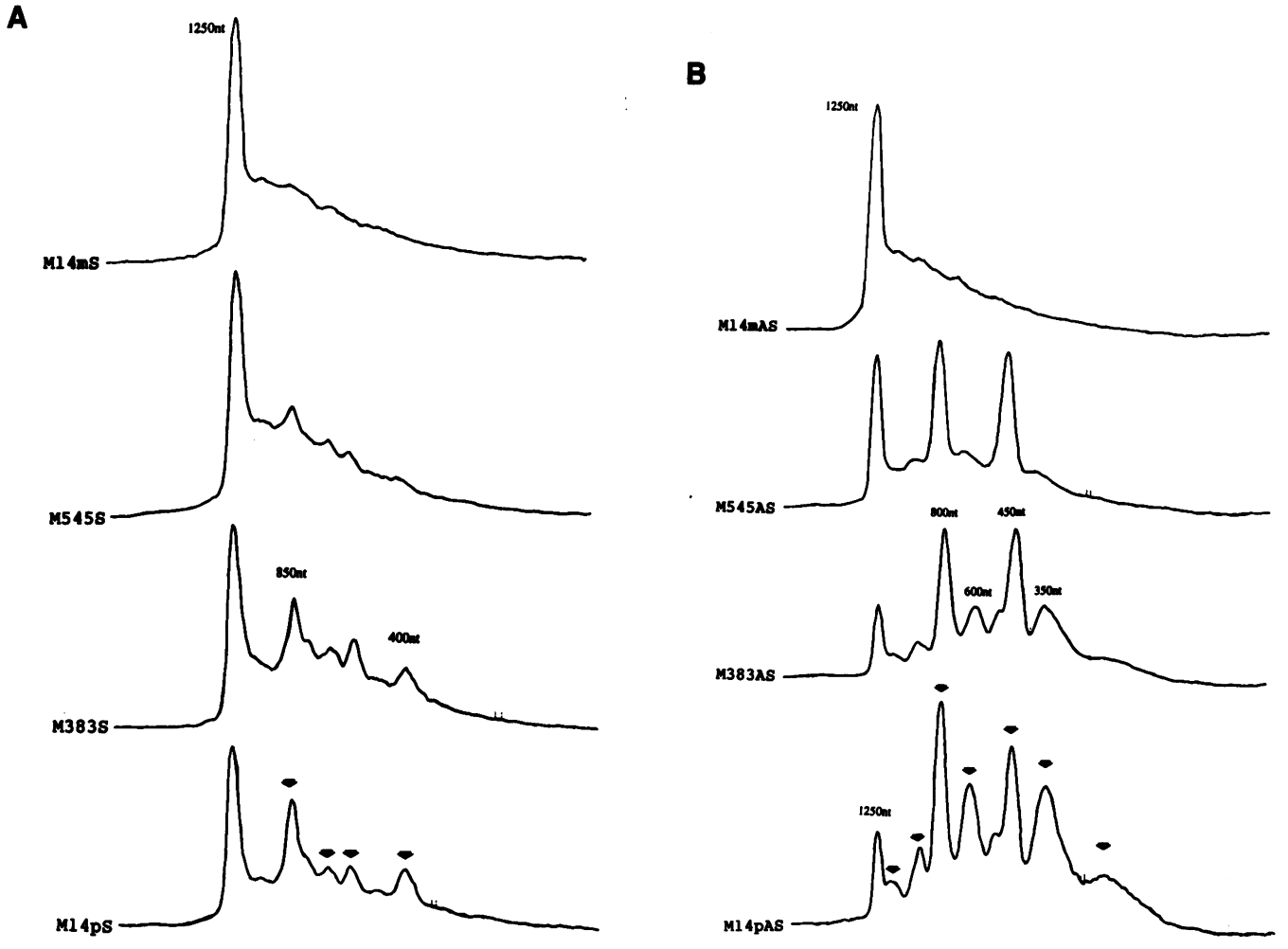

Figure 6. Densitometric traces (555nm) of RNA blot band intensities after 30 minute incubation of digoxigenin labelled, *in vitro* transcribed, 1250nt p53 RNA (500ng) with RNase H (0.025U/μl) and c-*myc* oligodeoxynucleotides (1μM) of the indicated sequence and structure (as described in Figure 1). RNA fragments generated through undesired RNase H activity are indicated with an arrow (→). **A.** Sense c-*myc* oligodeoxynucleotides M14pS, M383S, M545S and M14mS. **B.** Antisense c-*myc* oligodeoxynucleotides M14pAS, M383AS, M545AS and M14mAS.

of RNA by increasing methylphosphonodiester replacement of terminal phosphodiester internucleoside linkages within the oligodeoxynucleotides. Figure 5 presents the results obtained when the 1700nt digoxigenin labelled c-*myc* RNA was exposed to RNase H for 30 minutes in the presence of c-*myc* antisense oligodeoxynucleotides with 14p (all-phosphodiester), 383 (three methylphosphonodiester internucleoside linkages at each end separated by eight contiguous phosphodiester internucleoside linkages), 545 (five methylphosphonodiester linkages at each end separated by four contiguous phosphodiester internucleoside linkages) and 14m (all-methylphosphonodiester) structures. As expected, no degradation of RNA occurred during incubation with the antisense all-methylphosphonodiester oligodeoxynucleotide, M14mAS. The all-phosphodiester oligodeoxynucleotide, M14pAS, as before, promoted significant undesired RNase H activity such that by 30 minutes very little of the expected 1430nt product remained. On the other hand, increasing methylphosphonodiester substitution of terminal phosphodiester internucleoside linkages reduced RNase H activation at non-target sites while cleavage efficiency at the target site, codons 1 to 5, was retained. M383AS promoted RNase H activity at fewer inappropriate sites than M14pAS, and M545AS directed RNase H less efficiently than M383AS at the one remaining non-target site (Figure 3, D). M545AS, therefore, directed undesirable RNase H activity both at fewer non-target sites, and with lower efficiency than the all phosphodiester oligodeoxynucleotide of the same sequence (M14pAS), while the ability to produce the desired cleavage of the RNA at the antisense target site was not significantly affected.

Having established that undesired RNase H activity against c-*myc* RNA was reduced by incorporating c-*myc* oligodeoxynucleotide sequences into chimeric methylphosphonodiester/phosphodiester structures, it was of interest to determine if the same manipulations also eliminated inappropriate activity with c-*myc* oligodeoxynucleotides against the non-target p53 RNA control. Figure 6 presents the results obtained when the control digoxigenin labelled 1250nt p53 RNA, was incubated for 30 minutes in the presence of RNase H with c-*myc* sense, Figure 6A, and antisense, Figure 6B, oligodeoxynucleotides of 14p, 383, 545 and 14m structures. As expected the all-methylphosphonodiester, 14m, structures did not direct RNase H whereas the all-phosphodiester, 14p, structures promoted substantial undesired RNase H activity, as before. However, the chimeric 383 and 545 oligodeoxynucleotide structures displayed reduced inappropriate

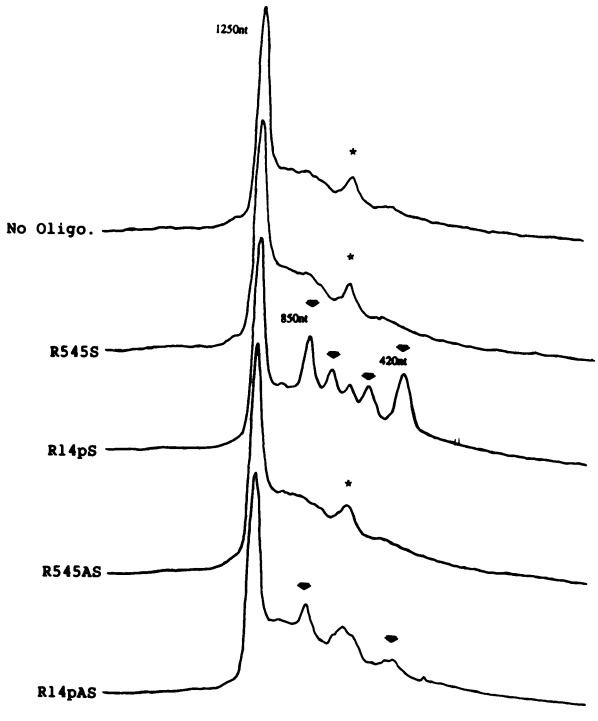

Figure 7. Densitometric traces (555nm) of RNA blot band intensities after 30 minute incubation of digoxigenin labelled, *in vitro* transcribed, 1250nt p53 RNA (500ng) with RNase H (0.025U/μl) and the c-Ha-*ras* oligodeoxynucleotides R14pAS, R545AS, R14pS and R545S (1μM), and without added oligodeoxynucleotide. The structure, sequence and abbreviation of the oligodeoxynucleotides is shown in Figure 1. RNA bands produced by premature termination during *in vitro* transcription are indicated with an asterisk (*). RNA fragments generated through undesired RNase H activity are indicated with an arrow (→).

activation of RNase H, with the 545 structures of each sequence displaying the lowest undesired activity. Indeed with the chimeric c-*myc* sense M545S oligodeoxynucleotide only negligible undesired RNase H activity remained.

An attempt was made to further reduce the undesirable activity of c-*myc* antisense sequence oligodeoxynucleotides by moving the phosphodiester region to a position with lower G + C content, M347AS, or to a position adjacent to the poorly hybridizing methylphosphonate A residues, M248AS. These 'off centre' effectors were tested against both c-*myc* and p53 digoxigenin labelled *in vitro* transcribed RNA, in the presence of RNase H. A substantial improvement was observed compared to M545AS (data not shown) although it was not found possible to completely abolish the inappropriate effects in this way.

The c-Ha-*ras* sequence oligodeoxynucleotides (Figure 1) were used in further tests of the ability of the chimeric methylphosphonodiester/phosphodiester structure to reduce non-targeted RNase H effects. Figure 7 presents the results obtained when the digoxigenin labelled 1250nt p53 RNA was exposed to RNase H for 30 minutes in the presence of the c-Ha-*ras* sense and antisense, phosphodiester and chimeric 545 structure oligodeoxynucleotides. Ideally, the irrelevant control c-Ha-*ras* phosphodiester oligodeoxynucleotides should not direct RNase H dependent cleavage of p53 RNA. The 545 structure c-Ha-*ras* oligodeoxynucleotides would be expected to direct only very low levels of undesired RNase H activity because, in addition to the previously described advantages which chimeric methylphosphonodiester/phosphodiester nucleic acid analogues appear to generally possess, the phosphodiester region contains only A + T residues and so should have very low hybridization potentials to partially complementary RNA. In agreement with these predictions it can be seen from Figure 7 that although both all-phosphodiester oligodeoxynucleotides promoted undesired RNase H activity, neither of the chimeric c-Ha-*ras* oligodeoxynucleotides (R545S, R545AS) induced inappropriate effects.

DISCUSSION

The target specificity of antisense oligodeoxynucleotides is a matter of some concern when considering the goal of inhibiting expression of single genes amidst the general milieu of genetic information transfer within living cells. Given certain basic assumptions, Helene and Toulme [26] have calculated that the oligodeoxynucleotide length required to specify a unique sequence of human mRNA is 11nt if the oligodeoxynucleotide contains only G and C residues and 15nt if it contains only A and T residues. Therefore, many investigators have employed 15 to 20-mer oligodeoxynucleotides which contain all four bases, because such antisense effectors should specify unique mRNAs with a significant margin of safety. However, since the stringency of hybridization within cells is beyond control, this theoretical level of specificity could be severely compromised if hybrids formed between other, non-targeted mRNAs and partially complementary oligodeoxynucleotides, and these hybrids were then subject to attack by RNase H. For example, one 15-mer may also be viewed as containing eight overlapping 8-mers and the perfect complement of each of these 8-mers would be expected once in every 65500 bases of random sequence. Therefore, the frequency of potential hybridization sites which may direct RNase H may be increased from the theoretical 1 in 10^9 bases of random sequence, for the entire 15-mer, to 1 in 8192 bases for the sum of the eight constituent 8-mers. Inappropriate effects due to partial sequence hybrids is, potentially, a problem for all oligodeoxynucleotides that are capable of directing RNase H. Phosphorothioate antisense effectors may be particularly prone to directing RNase H at non-target sites due to the nuclease-resistant [9] phosphorothiodiester internucleoside linkage extending the active life of these molecules in biological media [27], and also as a consequence of the high intrinsic activity of the enzyme with hybrids of the analogue oligodeoxynucleotide and RNA [9,10], although this may be mitigated somewhat by the reduced Tm of RNA/phosphorothioate heteroduplexes.

The results presented in this paper demonstrate that RNase H may, indeed, cleave non-target RNA sites where partial hybrids are formed between the RNA and oligodeoxynucleotides. By comparison of observed and predicted fragment sizes we have found that regions of partial complementarity as small as six contiguous base pairs, with a high G + C content, appear sufficient to direct undesired RNase H activity at 37°C (Figures 2 and 3). All of the all-phosphodiester oligodeoxynucleotides tested directed undesired RNase H activity to some degree (Figures 2 and 7), although large differences were observed between the extent of the inappropriate effects caused by different base sequences. The c-*myc* antisense oligodeoxynucleotide [22–25], M14pAS, was found to direct RNase H with particular promiscuity (Figures 2 and 4) and to exhibit an unexpectedly large number of partial complementarities to both c-*myc* RNA (Figure 3) and the control p53 RNA. This sequence may, therefore, contain the complement of mRNA motifs which occur

frequently, due to the non-random nature of mRNA base composition, and so would be an unfortunate choice of antisense effector in situations of high RNase H activity. The possibility that different oligodeoxynucleotides may differ significantly in their frequency of partial complementarity to non-targeted RNA casts some doubt over the popular use [28,29] of sense and nonsense sequence phosphodiester oligodeoxynucleotides, in isolation, as negative controls for phosphodiester antisense effectors.

Methylphosphonate oligodeoxynucleotide analogues, formed by replacement of the phosphodiester internucleoside linkages with the nuclease-resistant methylphosphonodiester groups, are incapable of directing RNase H [12], and methylphosphonodiester/RNA heteroduplexes display low melting temperatures [1,13]. These characteristics may explain why methylphosphonates have been widely described as inefficient antisense effectors, requiring high concentrations for activity [30–32]. However, their inactivity with RNase H and low RNA heteroduplex melting temperatures may be exploited in designing chimeric methylphosphonodiester/phosphodiester antisense effectors which eliminate undesired RNase H effects without significantly impairing activity at the target site. The terminal methylphosphonate sections limit RNase H attack to hybrids which include a significant proportion of the central phosphodiester region, while the reduced melting temperature, conferred on the hybrid by the methylphosphonate sections of the chimeric oligodeoxynucleotide, requires that there be extended partial complementarity between RNA and oligodeoxynucleotide to produce heteroduplexes with sufficient stability or lifetime to direct RNase H.

The superiority of chimeric methylphosphonodiester/phosphodiester oligodeoxynucleotides, in terms of reduced inappropriate RNase H effects, in an *in vitro* system, has been demonstrated by the experimental results presented in this report. Increasing methylphosphonodiester substitution of terminal phosphodiester internucleoside linkages produced chimeric oligodeoxynucleotides with substantially reduced undesired activity (Figures 5 and 6) without, at the same time, significantly affecting cleavage of RNA at the antisense target site (Figure 5). Further reductions in undesired activity with the c-*myc* antisense sequence were achieved with chimeric oligodeoxynucleotides containing 'off-centre' phosphodiester regions, M347AS and M248AS (data not shown) probably as a result of additional destabilization of partial sequence heteroduplexes. Again, these improvements were secured without compromising targeted cleavage of the RNA. However it did not prove possible to totally abolish inappropriate effects with the c-*myc* antisense oligodeoxynucleotides and, therefore, this may be a particularly difficult sequence to work with.

In conclusion, unwanted sequence dependent side effects resulting from non-targeted cleavage of mRNA by RNase H may be substantially reduced by designing antisense oligodeoxynucleotides as chimeric structures with terminal methylphosphonodiester sections separated by a phosphodiester region. To minimize undesirable activity the short phosphodiester region should be rich in A and T residues, with the G and C residues restricted, as far as possible, to the distal parts of the methylphosphonate regions. Under these circumstances, RNase H cleavage of target sequence RNA is maintained [13], whereas non-targeted RNAs which exhibit partial complementarity extending across the phosphodiester section of the oligodeoxynucleotides will have such low hybridization affinity at 37°C, that insignificant RNase H activity will result.

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