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Single base discrimination for ribonuclease H-dependent antisense effects within intact human leukaemia cells

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ABSTRACT

We have previously demonstrated, *in vitro*, that phosphodiester and phosphorothioate antisense oligodeoxynucleotides could direct ribonuclease H to cleave non-target RNA sites and that chimeric methylphosphonodiester/phosphodiester analogue structures were substantially more specific. In this report we show that such chimeric molecules can promote point mutation-specific scission of target mRNA by both *Escherichia coli* and human RNases H *in vitro*. Intact human leukaemia cells 'biochemically microinjected' with antisense effectors demonstrated efficient suppression of target mRNA expression. It was noted that the chimeric methylphosphonodiester/phosphodiester structures showed single base discrimination, whereas neither the phosphodiester nor phosphorothioate compounds were as stringent. Finally, we show that the antisense effects obtained in intact cells were due to endogenous RNase H activity.

INTRODUCTION

Antisense oligodeoxynucleotides, and analogue structures, have been intensively examined for activity as inhibitors of gene expression (for reviews see 1–5) owing to their potential utility as therapeutic agents to combat HIV and cancer. Many clinically relevant target mRNAs result from the transcription of point mutated genes (for example *p53*, the *ras* family, c-*src* and *neu*/c-*erb*B-2/HER-2). Therefore, any antisense compound used to inhibit expression of such genes would need to demonstrate point mutation specificity, so that levels of the unmutated proto-oncogene protein, which may have essential function(s), remain unperturbed.

It has been reported that both phosphodiester and the relatively nuclease-resistant phosphorothioate structures exhibit sequence-dependent undesired activity (6–8). We have shown that, *in vitro*, phosphodiester and phosphorothioate effectors can hybridize to non-target RNAs over partial complementarities as short as six contiguous bases and that these inappropriately formed heteroduplexes may then act as substrates for ribonuclease H (RNase H). Clearly, this level of non-targeted antisense function could severely limit the ability of these compounds to selectively target point mutated mRNAs. On the other hand, we have also demonstrated that chimeric antisense structures, composed of terminal methylphosphonodiester sections surrounding a central phosphodiester region (9), elicited increased specificity of RNase H-dependent RNA cleavage (10,11). Homologous antisense structures were also found to be comparatively selective in *in vitro* translation systems (12), as were methoxyethylphosphoramidate/phosphodiester chimeric effectors in *Xenopus* and tissue culture systems (13,14).

Here we report that chimeric methylphosphonodiester/phosphodiester antisense oligodeoxynucleotides could direct both *Escherichia coli* and human cell extract RNases H with point mutation specificity *in vitro*. Our previous observations with *E.coli* RNase H (10,11) were extended to show that both phosphodiester and phosphorothioate compounds were less selective than their chimeric congeners at directing the activity of human cell extract RNase H. Most importantly, we demonstrate that efficient single base discriminating antisense effects may be obtained within living human leukaemia cells which were loaded with chimeric antisense effectors using streptolysin O 'biochemical microinjection' (15). Using the reverse ligation-mediated PCR (RLPCR) procedure (16), previously used to demonstrate *in vivo* ribozyme function (17) and RNase H-mediated antisense oligodeoxynucleotide effects in a human chronic myelogenous leukaemia cell line (18), we note that the single base-specific antisense effects observed in the human acute lymphoblastic leukaemia MOLT4 cell line were also mediated by RNase H.

MATERIALS AND METHODS

Oligodeoxynucleotide synthesis

Phosphodiester, phosphorothioate and chimeric methylphosphonodiester/phosphodiester oligodeoxynucleotides were synthesized and purified as previously described (15,18,19). The structures and sequences of the *p53* antisense oligodeoxynucleotides used in this study are shown in Figure 1.

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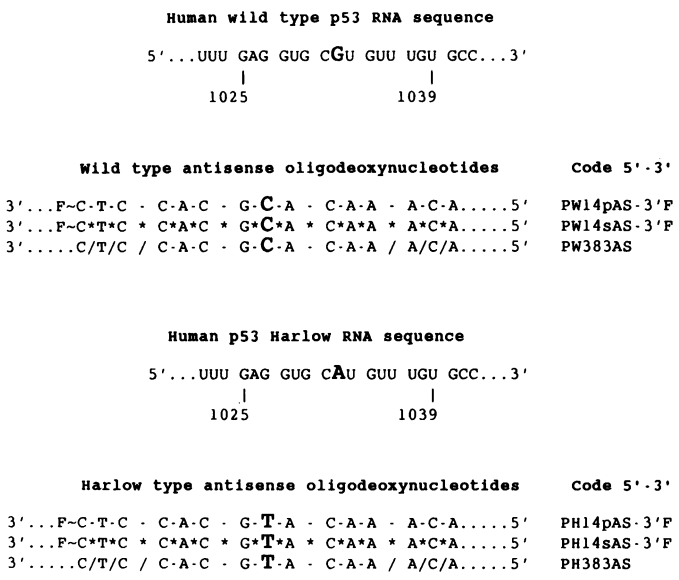

Figure 1. Human *p53* antisense oligodeoxynucleotide structures and sequences. The position targeted in the human *p53* cDNA sequence (hump53t, GenBank accession no. K03199) is indicated. A dash (-) between bases indicates the presence of a normal phosphodiester linkage, whereas a slash (/) indicates a methylphosphonodiester internucleoside linkage and an asterisk (*) indicates a phosphorothioate linkage. Fluorescein attachment to the 3'-termini, via a 6-amino-1-hexanol linker, is indicated by F~. The name of each oligodeoxynucleotide is shown to the right of the sequence and is derived from the structure and sequence as follows. The series is shown by the first two alphanumerics, PW or PH indicating wild-type and Harlow mutation sequence targets respectively; the next three alphanumerics indicate structure in the conventional 5' to 3' direction, 14p has all internucleoside bonds of the normal phosphodiester type, 383 has three methylphosphonates at each end and eight central phosphodiesters, 14s has all internucleoside linkages of the phosphorothioate type; the next two characters indicate actual sequence, AS being antisense. Finally, -3'F denotes fluorescein attachment to the 3' termini.

Cell culture

The acute lymphoblastic leukaemia MOLT4 cell line (European Collection of Animal Cell Cultures, Porton Down, UK) was maintained in exponential growth in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% heat inactivated (65°C, 1 h) fetal calf serum (Sera-Lab, Crawley Down, UK). All procedures were performed upon cultures assayed to be >95% viable by Trypan blue exclusion.

Subcloning of *p53* cDNAs into pGEM4Z

Eukaryotic expression vectors containing the point mutant *p53* Harlow sequence (20) and, separately, a 'rationalised' (rational) pseudo-wild-type *p53* sequence (restriction endonuclease sites introduced into the DNA sequence by mutations which are silent at the protein level) were generously supplied by Dr John Jenkins (Marie Curie Research Institute, Oxted, UK). The *p53* portions of these plasmids were amplified by PCR as previously described (10), using the upstream primer 5'-GGT ACC TAA TAC GAC TCA CTA TAG GGA GAA GCT TAA CGG TGA CAC GCT TCC CTG GAT T-3' and the downstream primer 5'-AGA GAG GGA TCC AAG GGA ACA AGA AGT GGA GAA T-3'. The 1304 bp PCR products were digested with *Bam*HI and *Hin*dIII (Boehringer Mannheim, Lewes, UK), ligated into *Bam*HI/*Hin*dIII double-digested pGEM4Z (Promega, Southampton, UK) to produce 1270 bp inserts. DNA present in the ligation reactions was transformed into *E.coli* JM109 (Promega) and colonies containing recombinant plasmids were selected using standard ampicillin antibiotic and IPTG/X-GAL blue–white screening. The identity of the inserts was confirmed by analysing for the presence of the *p53* rational *Sma*I site and by non-radioactive *f*mol sequencing (Promega) using the digoxigenin-labelled *p53* primer 5'-digoxigenin-TTG CGG AGA TTC TCT TCC TCC TCT-3'.

Transcription/degradation *in vitro*

Site-specific cleavage of *in vitro*-transcribed RNA was obtained by doping standard T7 RNA polymerase *in vitro* transcription reactions (10) with 1 μM antisense oligodeoxynucleotide and 0.02 U/μl *E.coli* RNase H (Epicenter, UK distributors Cambio). Reactions were stopped, concentrated by ethanol precipitation and resuspended in formaldehyde gel loading buffer (10). Reaction substrate and products were fractionated through formaldehyde–agarose gels and visualized by ethidium bromide staining and UV-induced fluorescence.

Preparation of human cell extract

Whole cell extract was obtained from the MOLT4 human cell line using the ammonium sulphate fractionation technique described by Manley (21). Small aliquots (100 μl) of the dialyzed final solution were stored in liquid nitrogen and could be thawed on ice/snap frozen at least three times before significant reduction of RNase H activity was evident.

Cell extract assays

Assays were conducted with 50–100 ng digoxigenin-labelled *in vitro* transcript (synthesized and purified as previously described; 10) per 10 μl reaction at 37°C in an intracellular buffer (11 mM potassium phosphate, pH 7.4, 108 mM KCl, 22 mM NaCl, 1 mM dithiothreitol, 3 mM $MgCl_2$, 1 mM ATP) with 1 μM oligodeoxynucleotide, 1 U/μl human placental ribonuclease inhibitor (Stratagene, Cambridge, UK) and 1 μg/μl yeast tRNA. Whole cell extract was added in a volume equal to 10% of the final reaction volume. Reactions were stopped after 15 min by addition of 400 μl modified RNA extraction buffer (22) and, following extraction with 50 μl chloroform, the RNA was recovered from the aqueous phase by ethanol precipitation. RNA precipitates were resuspended in 90% formamide, 0.03% Xylene cyanol, 0.03% Orange G, heated to 90°C and quenched on ice prior to loading on the gel. Electrophoretic separation of substrate and product RNA species was obtained using a 0.75 mm × 20 cm 4% polyacrylamide (19:1 acrylamide:bis-acrylamide)–7 M urea gel thermostatically maintained at 50°C. Nucleic acids contained in the gel were electroblotted (TransBlot SemiDry, BioRad, Hemel Hempstead, UK) onto Nytran NY12N membrane (Schleicher & Schuell, UK distributors Anderman and Co. Ltd, Kingston-Upon-Thames, UK). Transfer was routinely assessed by examining both gel and membrane for the UV shadows of yeast tRNA. Digoxigenin-labelled RNAs, UV cross-linked to the membrane

(2 min, 254 nm), were detected chromogenically as previously described (10).

Reversible cell permeabilization with streptolysin O

Cells were treated with streptolysin O essentially according to the protocol of Barry *et al.* (23) as modified by Spiller and Tidd (15). Briefly, 10^6 cells were washed in permeabilization buffer (137 mM NaCl, 100 mM PIPES, pH 7.4, 5.6 mM glucose, 2.7 mM KCl, 2.7 mM EGTA, 1 mM ATP) and resuspended in 100 µl pre-activated (5 mM dithiothreitol, 1 mM ATP, overnight) streptolysin O solution (40 U/ml; Sigma Chemical Co., Poole, UK) in permeabilization buffer containing 0 or 20 µM antisense oligodeoxynucleotide and incubated at 37°C for 30 min. Resealing was achieved by addition of 1 ml RPMI 1640 medium containing 10% fetal calf serum and incubation was continued at 37°C for a further 210 min before RNA extraction. One hour prior to isolation of total RNA, 100 µl (~10^5 cells) culture samples were removed and dead cells counter-stained with propidium iodide (Sigma; final concentration 10 µg/ml). These samples were analysed for cell viability and extent of permeabilization by two colour flow cytometry as previously described (15). Post-streptolysin O treatment cell viabilities in these experiments were 76.3 ± 9.2%.

RNA isolation

Total RNA was isolated from experimental cell cultures using a guanidinium thiocyanate/acid phenol method (22). RNA precipitates were resuspended in water and their concentration estimated by gel electrophoresis and ethidium bromide staining.

Northern blots

Denaturing agarose gel electrophoresis, transfer of RNA to Hybond N (Amersham International plc, Little Chalfont, UK), probing with digoxigenin-labelled nucleic acids and subsequent chromogenic and chemiluminescent visualization procedures were all performed as previously described (11). Gels were evenly loaded with RNA as assessed by routine staining with ethidium bromide and analysis of UV-induced fluorescence (data not shown).

Reverse ligation-mediated PCR

p53 mRNA 3′ fragments generated by RNase H activity within MOLT4 cells were detected by RLPCR (see Figure 5) as previously described (18). Briefly, total RNAs extracted from experimental cells were subjected to ligation reaction conditions with an *in vitro*-transcribed 25mer RNA linker and T4 RNA ligase (Boehringer Mannheim), such that the linker would be covalently bonded to any RNA molecules present bearing a 5′-phosphate group. The RNA was then re-isolated from the reaction mixture as above. Reverse transcription reactions were carried out as described (16) and were stopped by heating to 95°C for 5 min, followed by treatment with 1 µg RNase A (type III-A; Sigma) for 20 min at 37°C prior to PCR amplification. Reverse transcription and PCR primers: (i) RNA linker-specific 5′ PCR primer, 5′-GGG CAT AGG CTG ACC CTC GCT GAA-3′; (ii) *p53* reverse transcription primer, 5′-CGA AGC GCT CAC GCC CAC GGA T-3′; (iii) *p53* PCR1 primer, 5′-TGG GCA GTG CTC GCT TAG TGC-3′; (iv) nested labelled *p53* PCR2 primer, 5′-digoxigenin-TTG CGG AGA TTC TCT TCC TCT-3′. Final analysis of RLPCR products was accomplished by electrophoresis through 0.75 mm 6% polyacrylamide–7 M urea sequencing gels, semi-dry electrotransfer onto Nytran NY12N membrane and detection by anti-digoxigenin–alkaline phosphatase Fab fragments (Boehringer Mannheim) with a chromogenic procedure (10).

Direct sequencing of RLPCR products

One µl of a 1 in 10 dilution of PCR1 reactions was used as the template for 30 cycle *f*mol sequencing reactions (Promega) using the digoxigenin-labelled primer 5′-digoxigenin-TTG CGG AGA TTC TCT TCC TCT-3′ (*vide supra*). Products were electrophoresed through 0.4 mm 6% polyacrylamide–7 M urea sequencing gels, diffusion blotted onto Nytran NY12N membrane and the sequence developed as above.

Densitometry

A Shimadzu CS9000 instrument was used as previously described (10).

RESULTS

It has previously been reported that chimeric methylphosphonodiester/phosphodiester antisense effectors demonstrate greater selectivity for the target RNA site, when directing *E.coli* ribonuclease H (RNase H) activity, than phosphodiester and phosphorothioate congeners (10,11). We decided, therefore, to investigate whether single base mismatch discrimination could be obtained with such antisense analogues and *E.coli* RNase H. A novel assay system was developed, called 'transcription/degradation', in which DNAs with a suitable promoter were incubated with bacteriophage RNA polymerase in the presence of antisense oligodeoxynucleotides and RNase H. The RNA run-off transcripts produced by the RNA polymerase can act as substrates for oligodeoxynucleotide-directed RNase H activity. Oligodeoxynucleotide activity and specificity can then be evaluated simply by fractionating the DNA template, RNA substrate and RNA reaction products in an agarose gel and staining with ethidium bromide.

Plasmid DNA was purified from 15 independent colonies harbouring pGEM4Z-*p53* cDNA recombinants (see Materials and Methods) and linearized downstream of the *p53* insert. Figure 2 presents the results obtained when these DNAs were subjected to transcription/degradation in the presence of oligodeoxynucleotides whose sequences differ at only one base (see Fig. 1). In Figure 2A it may clearly be seen that the point mutant Harlow sequence, ~1270 nt *in vitro*-transcribed RNA (lanes 7–15) was efficiently cleaved by RNase H in the presence of the Harlow sequence chimeric oligodeoxynucleotide, PH383AS (three methylphosphonates at each terminus and eight central phosphodiester internucleoside linkages) into the expected fragments of ~880 and 390 nt. The rational sequence RNA (lanes 1–6) was subject to only very low levels of RNase H-dependent cleavage in the presence of PH383AS, presumably due to the single base pair mismatch between the rational RNA and the Harlow oligodeoxynucleotide. Conversely, it may be seen in Figure 2B that the wild-type rational *in vitro*-transcribed ~1270 nt RNA (lanes 1–6) was efficiently digested to ~880 and 390 nt fragments by RNase H in the presence of the wild-type chimeric antisense

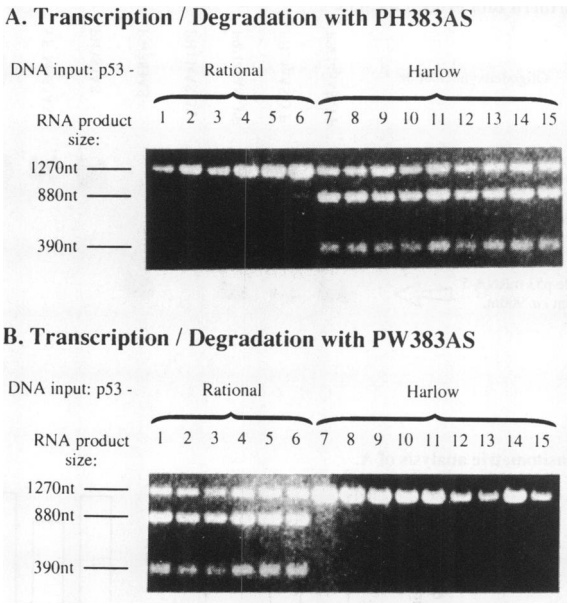

Figure 2. *In vitro* transcription/degradation. Restriction endonuclease linearized pGEM4Z-*p53* recombinant plasmids were transcribed by T7 RNA polymerase in the presence of 0.02 U/μl *E.coli* RNase H and (**A**) 1 μM PH383AS or (**B**) 1 μM PW383AS for 1 h at 37°C. Reactions were stopped, concentrated by ethanol precipitation, analysed by formaldehyde–agarose gel electrophoresis and visualized by ethidium bromide staining and UV-induced fluorescence. Lanes 1–6, plasmid DNA prepared from six independent colonies resulting from pGEM4Z-*p53* rational recombination. Lanes 7–15, plasmid DNA prepared from nine independent colonies from pGEM4Z-*p53* Harlow recombination. The structure and sequence of the *p53* oligodeoxynucleotides are described in Figure 1.

effector PW383AS and that the Harlow sequence *p53* RNA (lanes 7–15) was essentially uncleaved, presumably because of the single base pair mismatch between the Harlow RNA and the wild-type antisense effector. We have found that phosphorothioate oligodeoxynucleotides may not be used in transcription/degradation reactions because they inhibit the bacteriophage RNA polymerase (data not shown). Phosphodiester effectors have been found to be less selective than congenic chimeric methylphosphonate/phosphodiester analogues in this system (data not shown).

The above results show that a chimeric oligodeoxynucleotide composed of terminal methylphosphonate sections and a central phosphodiester section could efficiently discriminate between its target RNA sequence and one which differed by only one residue in 15 when directing *E.coli* RNase H *in vitro*. The next logical step was to investigate whether such point mutation specificity could be maintained when using a partially purified human cell extract (21) as the source of RNase H. It was also desirable to directly compare chimeric methylphosphonate/phosphodiester effectors with the more commonly used all-phosphodiester and all-phosphorothioate analogues.

The results presented in Figure 3 were obtained when digoxigenin-labelled 1270 nt *p53 in vitro* transcripts were incubated for 15 min at 37°C with the range of *p53* Harlow and *p53* wild-type antisense effectors (see Fig. 1) and human MOLT4 cell extract in an intracellular buffer. The histogram describes the extent of RNA cleavage (rational *p53* transcript, filled bars; Harlow *p53* transcript, open bars) by oligodeoxynucleotide, thus facilitating the identification of antisense effector cross-reactivity. Both the wild-type and Harlow sequence all-phosphodiester oligodeoxynucleotides (PW14pAS-3′F and PH14pAS-3′F respectively) may be seen to have induced extensive human RNase H-dependent cleavage of both the rational and Harlow *p53* RNAs. The wild-type PW14pAS-3′F oligodeoxynucleotide induced scission of >80% of its rational target RNA, but also ~45% of non-target, point mutated Harlow RNA. Similarly, the Harlow sequence PH14pAS-3′F phosphodiester effector directed cleavage of >80% its target Harlow mutation RNA, but also a substantial 70% of non-mutant rational RNA. The phosphorothioate *p53* antisense analogues appeared to be somewhat more specific than their phosphodiester congeners in this assay system (perhaps due to the reduced RNA–heteroduplex melting temperatures for phosphorothioates relative to equivalent phosphodiester molecules; 24). The wild-type phosphorothioate analogue, PW14sAS-3′F, induced human RNase H-mediated cleavage of ~70% of its target RNA, whereas only a low level of non-target Harlow RNA cleavage was observed. Similarly, the Harlow antisense phosphorothioate compound, PH14sAS-3′F, induced RNase H-dependent scission of ~70% of its target Harlow RNA transcript, but also 40% of non-target rational transcript. In contrast, neither the wild-type nor the Harlow chimeric '383' antisense effectors induced significant RNase H scission of non-target RNA transcript and both efficiently directed cleavage of their target RNAs. The wild-type chimeric structure, PW383AS, directed human RNase H to cleave ~60% of the rational RNA transcript, while no scission of the non-target Harlow transcript was detected. Similarly, the Harlow antisense chimeric effector, PH383AS, induced ~60% cleavage of the Harlow RNA, although a very low level of human RNase H-dependent cleavage of non-target rational RNA was also observed.

Future therapeutic use of antisense oligodeoxynucleotides would require reliable, sequence-specific antisense effects within intact cells. An efficient method for 'biochemical microinjection' of oligodeoxynucleotides into live cells has recently been described (23) and confirmed (15), thus overcoming the major problem of cell membrane impermeability to antisense compounds. This procedure was used to introduce the *p53* antisense effectors into living human cells so that the activity and selectivity of these compounds could be tested *in vivo*.

Human acute lymphoblastic leukaemia MOLT4 cells (which possess the wild-type sequence at the *p53* Harlow mutation position) were reversibly permeabilized with streptolysin O in the presence (20 μM external concentration) of the range of *p53* antisense effectors, one control oligodeoxynucleotide, 5′F-M383AS (antisense to proto-oncogene c-*myc*: 5′...F~A/A/C/G-T-T–G-A-G–G-G-G/C/A/T...3′, chimeric with three methylphosphonates at each end, eight central phosphodiesters and fluorescein-tagged at the 5′-end) and in the absence of any antisense effector. Cell viabilities were analysed by two colour flow cytometry (15) 3 h after treatment with streptolysin O + oligodeoxynucleotide and were found to be 76.3 ± 9.2%. At this time point, at least, there was no differential decrease in cell viability associated with treatment with streptolysin O + oligodeoxynucleotide, as compared with treatment with streptolysin O alone. RNA purified from the experimental cultures after a 4 h incubation at 37°C was analysed by Northern hybridization for level of intact 2500 nt *p53* mRNA and these results are presented in Figure 4A. The relative levels of intact *p53* mRNA remaining

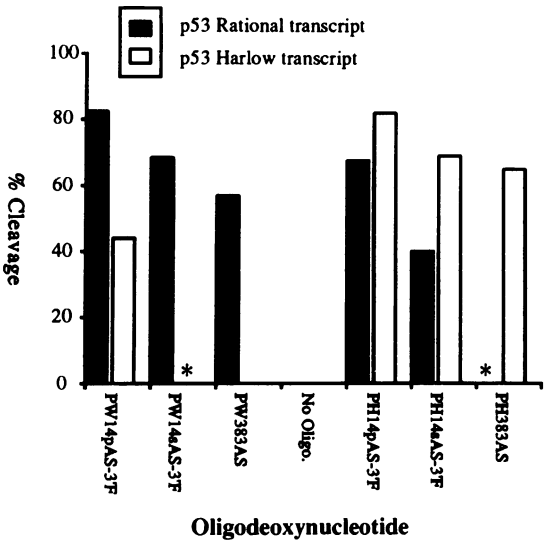

Figure 3. Human cell extract RNase H cleavage of *in vitro*-transcribed *p53* RNA (~1270 nt, rational sequence filled bars and Harlow sequence open bars) directed by *p53* wild-type and Harlow chimeric methylphosphonodiester/phosphodiester, phosphodiester and phosphorothioate antisense oligodeoxynucleotides. Digoxigenin-labelled RNA was incubated at 37°C for 15 min with MOLT4 cell extract (10% by volume) and the indicated oligodeoxynucleotide in an 'intracellular' buffer solution. Extent of RNA cleavage was determined by densitometric analysis of the chromogenically developed blots. *Not determined; although a low level of non-targeted cleavage of Harlow RNA in the presence of PW14sAS-3'F oligodeoxynucleotide and rational RNA in the presence of PH383AS oligodeoxynucleotide was apparent upon visual examination of the blot, uncertainty in the baseline assignment under the fragment peak on the densitometric trace prevented accurate determination of the extent of cleavage. The structure and sequence of the *p53* oligodeoxynucleotides are described in Figure 1.

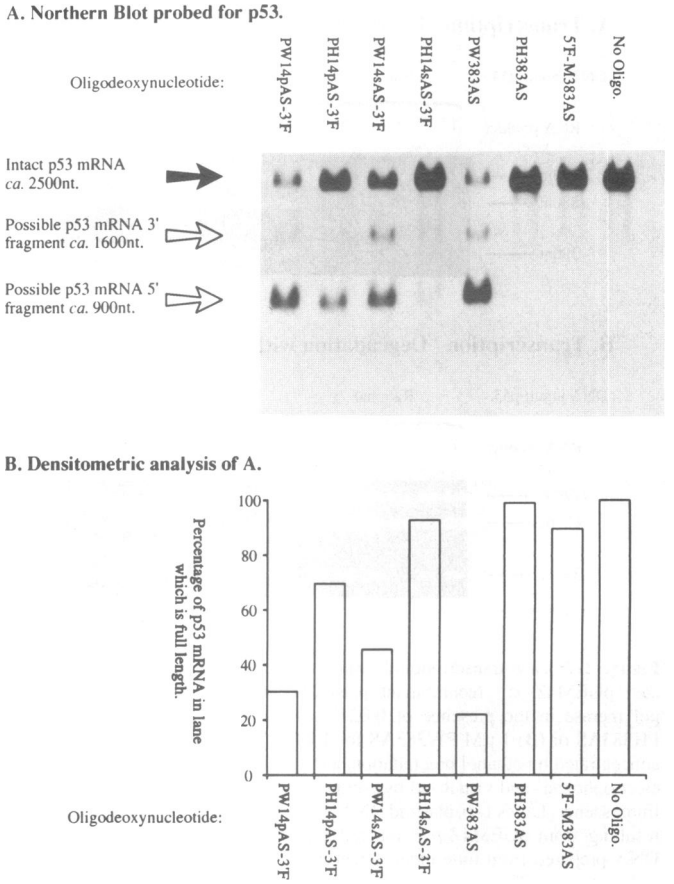

Figure 4. Antisense oligodeoxynucleotide-mediated reduction of *p53* mRNA level in living MOLT4 cells biochemically microinjected with antisense effectors. MOLT4 cells were reversibly permeabilized with streptolysin O in the presence of the indicated oligodeoxynucleotides (20 μM). Total RNA was extracted after an incubation period of 4 h and analysed for *p53* mRNA level. (**A**) Northern hybridization with a *p53* probe to assess level of intact 2500 nt *p53* mRNA (indicated with the filled arrow). The open arrows at ~900 and 1600 nt indicate putative RNase H fragments of *p53* mRNA. (**B**) Densitometric quantitation of the intact *p53* mRNA present in (A). Percentage full-length *p53* RNA was calculated as [densitometric area of full-length *p53* band/(densitometric area of full-length + shorter fragments)] × 100%. The structure and sequence of the *p53* oligodeoxynucleotides are described in Figure 1. The c-*myc* antisense control oligodeoxynucleotide 5'F-M383AS is described in the text.

were quantitated by scanning densitometry of the chemiluminescent exposure. The histogram of Figure 4B presents the percentage amount of *p53* hybridizing material per lane, which is full-length *p53* mRNA. It can be seen from Figure 4A and B that extensive reduction in intact *p53* mRNA level (filled arrow) was achieved *in vivo* with both the fully complementary PW14pAS-3'F and the single base pair mismatch PH14pAS-3'F all-phosphodiester oligodeoxynucleotides. Values of 30 and 70% intact mRNA respectively were recorded. The phosphorothioate compounds were substantially more specific, although less efficient, than the phosphodiester structures *in vivo*. Expression of full-length *p53* mRNA was reduced to 45% by the fully complementary PW14sAS-3'F, while only a minor reduction in expression (to 93%) was observed with the single base mismatch PH14sAS-3'F. The chimeric methylphosphonate/phosphodiester antisense effectors showed efficient single base discrimination. The fully complementary PW383AS caused the greatest reduction in full-length *p53* mRNA (to 22%), whereas no significant reduction in *p53* mRNA was observed in cells treated with the Harlow sequence chimeric compound PH383AS (99%). Only a minor reduction in *p53* mRNA expression was found in cells treated with the chimeric c-*myc* control oligodeoxynucleotide (90%), despite previous observations that oligodeoxynucleotides with this *myc* sequence could be quite non-selective *in vitro* (10). An identical ranking of antisense activity was obtained when the densitometric area of the full-length *p53* signal was normalized to the no oligodeoxynucleotide control (PW383AS > PW14pAS-3'F > PW14sAS-3'F > PH14pAS-3'F > PH14sAS-3'F > PH383AS), further confirming the comparative efficacy and selectivity of these *p53* chimeric methylphosphonodiester/phosphodiester structures.

The short RNAs which were detected by the *p53* probe used in the blot of Figure 4A (open arrows) were of suitable lengths [observed ~900 and 1600 nt, expected ~950 and 1560 + poly(A) tail] to be *p53* mRNA fragments resulting from cleavage of the *p53* mRNA at the site of oligodeoxynucleotide hybridization by RNase H *in vivo*. We have previously identified a role for RNase H in the mediation of antisense effects observed in a human chronic myelogenous leukaemia cell line (KY01) using RLPCR (18). RLPCR permits unequivocal identification of mRNA 3' fragments generated by the activity of RNase H. An experimental procedure flow diagram for RLPCR is presented in Figure 5.

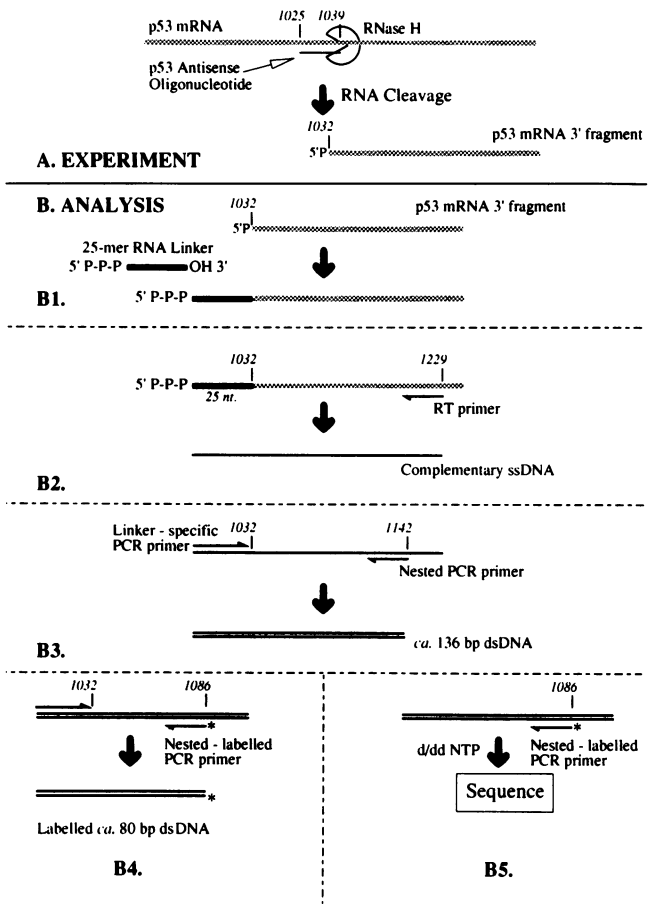

Figure 5. Experimental procedure flow diagram for detection by RLPCR of 3′ fragments produced by *in vivo* RNase H-mediated scission of *p53* mRNA. The numbers in italics above the lines representing *p53* sequence nucleic acids indicate position along the *p53* sequence (hump53t, GenBank accession no. K03199). (**A**) A *p53* oligodeoxynucleotide introduced into human cells hybridizes with *p53* mRNA to form a substrate for RNase H, which cleaves the mRNA. One of the products of this reaction is a 3′ mRNA fragment which has a 5′-phosphate group. In this example only the 3′ mRNA fragment produced by scission of the phosphodiester bond 5′ to base 1032 of the mRNA is considered. Standard procedures are used to isolate total RNA from the treated cells. (**B**) RLPCR analysis proceeds by: (B1) ligation of a 25mer *in vitro* transcript linker to all available 5′-phosphates in the sample of isolated RNA using T4 RNA ligase; (B2) reverse transcription using a *p53*-specific primer; (B3) PCR amplification of the cDNA using a linker-specific upstream primer and a *p53*-specific downstream primer to produce an ~136 bp product; (B4) subsequent amplification of the 136 bp product with the same linker-specific upstream primer and a digoxigenin-labelled downstream primer produces an ~80 bp digoxigenin-labelled product which is fractionated by denaturing gel electrophoresis and visualized by blotting (see Materials and Methods); the 136 bp product is also positively identified by direct cycle DNA sequencing (B5) using the same *p53*-specific primer as used in (B4).

Oligodeoxynucleotides which obtain a cytoplasmic or nuclear location may hybridize to their target mRNA and thereby generate substrates for RNase H. RNase H activities cleave the mRNA within the region of the heteroduplex, to produce 5′ products with a 3′-OH group and, significantly, 3′ fragments with a 5′-PO_4 group (25) (Fig. 5A). Analysis proceeds by ligating a short RNA linker onto all available 5′-phosphates present in a sample of extracted RNA (Fig. 5B1) using T4 RNA ligase. Products of the reverse ligation reaction are reverse transcribed using a gene-specific primer (Fig. 5B2) and then amplified (Fig. 5B3) using one primer with the same sequence as the RNA linker molecule and a gene-specific primer complementary to a region upstream of the reverse transcription primer (nested). The first PCR product is then analysed in two ways. Further exponential amplification using the RNA linker-specific primer and a digoxigenin-labelled primer, nested inside the first PCR primer (Fig. 5B4), yields a number of products with lengths dependent upon the precise internucleoside linkage within the heteroduplex originally cleaved by RNase H. In the case of a 15mer phosphodiester or phosphorothioate antisense oligodeoxynucleotide targeted to positions 1025–1039 of the *p53* mRNA sequence, denaturing gel electrophoresis resolves a spread of products centred around 80 nt, but ranging from 73 to ~85 nt. The first PCR product is also directly sequenced (Figure 5B5), which permits positive identification of the *p53* sequence and the exact point of transition to the RNA linker sequence where the mRNA was cleaved by RNase H *in vivo*. This provides unequivocal proof that RNase H-mediated cleavage of the mRNA *in vivo* occurred at the site targeted by the antisense oligodeoxynucleotide.

Portions of the RNA extracted from an identical *in vivo* experiment to that analysed for intact *p53* level by Northern hybridization (Fig. 4A and B) were analysed for RNase H-dependent cleavage fragments of *p53* mRNA using RLPCR. The results are presented in Figure 6. It can be seen that no RLPCR signal was generated from the streptolysin O without oligodeoxynucleotide control, but that RNase H-dependent cleavage fragments could be amplified from the RNA extracted from cells treated with streptolysin O and both the fully complementary and the single base mismatch phosphodiester and phosphorothioate oligodeoxynucleotides (PW14pAS-3′F, PH14pAS-3′F, PW14sAS-3′F and PH14sAS-3′F respectively). This indicated that both the targeted and non-targeted antisense effects obtained within living human acute lymphoblastic leukaemia MOLT4 cells were RNase H-dependent. The single base mismatch-specific effects of chimeric methylphosphonodiester/phosphodiester structures *in vivo* (Fig. 4A and B) may also be seen to have been mediated by endogenous RNase H over a shorter range of cleavage sites than for the all-phosphodiester and all-phosphorothioate oligodeoxynucleotides (Fig. 6), consistent with the reduced length of the enzyme-directing phosphodiester section present in the chimeric molecules. The RLPCR products were sequenced to confirm that in each case the *p53* mRNA was cleaved *in vivo* at the antisense target site within the RNase H substrate region of the heteroduplexes formed between the *p53* mRNA and the antisense oligodeoxynucleotides (data not shown).

DISCUSSION

Chimeric methylphosphonodiester/phosphodiester oligodeoxynucleotides have previously been shown to possess advantageous characteristics for antisense effectors, namely reduced sensitivity to exonuclease degradation (9) whilst retaining the ability to activate RNase H efficiently (26) and with enhanced selectivity for the target RNA (10–12). In this report we have extended our previous observations to show that such chimeric effectors targeted to a *p53* mutation site can direct both *E.coli* and human RNases H *in vitro* with point mutation selectivity (Figs 2 and 3). Furthermore, we have demonstrated that when directing human RNase H activity *in vitro*, phosphorothioate analogues do not

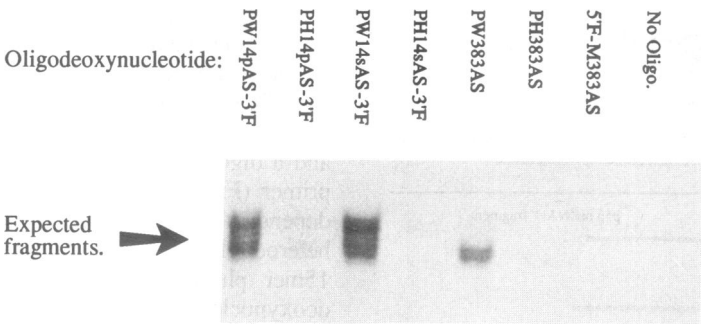

Figure 6. Detection of RNase H-generated 3′ fragments of *p53* mRNA present in total RNA extracted from MOLT4 cells 4 h after biochemical microinjection of *p53* antisense oligodeoxynucleotides. The RNA isolated from an identical experiment to that described in Figure 4 was analysed by *p53*-specific RLPCR according to the protocol presented in Figure 5. The arrow indicates the position to which a 78 nt product would run in the electrophoretic gel. The multiple bands in each lane identify products which differ in length successively by 1 nt and thus provide a visual representation of the range of RNase H cleavage sites within the parent *p53* mRNA/antisense oligodeoxynucleotide heteroduplex *in vivo*. The structure and sequence of the *p53* oligodeoxynucleotides are described in Figure 1. The c-*myc* antisense control oligodeoxynucleotide 5′F-M383AS is described in the text.

reliably provide point mutation specificity and that phosphodiester effectors present a substantial deficiency in selectivity (Fig. 3). Attempts have been made to obtain point mutation specificity with phosphodiester oligodeoxynucleotides (27). High selectivity was obtained when the size of the antisense effector was reduced to 12 bases, although this oligodeoxynucleotide was found to have reduced potency against long RNA species (>100 nt), presumably as a result of a limited ability to invade stable RNA secondary structures. In addition, the strategy of using small antisense effectors (~12 bases) may not be general, for it has been calculated that around 15 bases are required in order to uniquely specify a single mRNA species (28).

The transcription/degradation system, presented in Figure 2, can provide almost point mutation specificity when used in combination with chimeric antisense effectors. We have used this system for other targets (data not shown) and have found that significant non-targeted cleavage may occur when using phosphodiester oligodeoxynucleotides, while phosphorothioate antisense effectors appear to inhibit the activity of the bacteriophage RNA polymerases. On a technological note, we have found that the very high specificity of transcription/degradation allows its use as a rapid and reliable procedure to positively identify plasmid preparations and PCR products.

The high selectivity of chimeric compounds observed *in vitro* is also evident when they are used *in vivo*. When introduced into living MOLT4 cells using streptolysin O biochemical microinjection (15,23), chimeric methylphosphonate/phosphodiester analogues demonstrated both the highest targeted activity at reducing *p53* mRNA levels among the fully complementary (PW-, wild-type) oligodeoxynucleotide series and the lowest non-target activity amongst the single base mismatch (PH-, Harlow) effectors, as shown in Figure 4. The all-phosphodiester structures showed good targeted activity *in vivo*, but very poor selectivity, whereas the sulphur-modified phosphorothioate analogues produced the poorest targeted activity, but reasonable selectivity.

All three *p53* antisense oligodeoxynucleotide structure types (phosphodiester, phosphorothioate and chimeric methylphosphonate/phosphodiester) were unequivocally shown to direct RNase H at the antisense target site in *p53* mRNA *in vivo* (Fig. 6) when introduced into the cell by streptolysin O permeabilization. It would thus appear that endogenous RNase H is important for mediating the antisense effects obtained, at least with these compounds and at least in human acute lymphoblastic (this report) and chronic myelogenous (18) leukaemia cell lines. However, it is essential to note that we have never obtained an antisense effect in living cells, either RNase H-dependent or -independent, unless artificial mechanisms were used to introduce antisense effectors into the cells. Previous studies have targeted *p53* for antisense inhibition (29), although not at a point mutation site. Furthermore, no attempt was made to ensure that the oligonucleotide reached the appropriate cellular compartment and no evidence for an antisense mechanism was presented.

This report presents evidence that chimeric methylphosphonodiester/phosphodiester antisense effectors may be highly effective and selective *in vivo* and that they function via an RNase H mechanism. We believe that this structure type represents a good base for future chemical modifications intended to increase the intracytoplasmic delivery of oligodeoxynucleotides. Phosphodiester compounds, in addition to their poor selectivity *in vivo* (presented above), are highly susceptible to nucleases, which appear to be ubiquitous in biological fluids. On the other hand, phosphorothioate oligodeoxynucleotides, in addition to their poor activity *in vivo* (described above), have been widely reported to induce many non-antisense toxic effects via interaction with cellular proteins (see 5,30,31, and references therein).

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