Rational design of point mutation-selective antisense DNA targeted to codon 12 of Ha-ras mRNA in human cells

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ABSTRACT

Antisense oligodeoxynucleotides targeted to Ha-ras mRNA have been designed to discriminate between the codon 12-mutated oncogene and the normal proto-oncogene. An *in vitro* assay using two different sources of RNase H (rabbit reticulocyte lysates and nuclear extract from HeLa cells) was used to characterize oligonucleotide binding to normal and mutated Ha-ras mRNA. Short oligonucleotides (12- or 13mers) centered on the mutation had a very high discriminatory efficiency. Longer oligonucleotides (16mers) did not discriminate efficiently between the mutated and the normal mRNA. We have tested the efficacy of dodecanucleotides to induce RNase H cleavage of the full-length mRNA, moving the target sequence from the loop to the stem region which is formed in the vicinity of mutated codon 12. The most selective oligonucleotides were centered on the mutation which is located near the junction between the loop and stem regions even though they were less efficient at inducing RNase H cleavage than those targeted to the loop region. The 12mer antisense oligonucleotide with the highest discriminatory power was selected for cell culture studies. This oligonucleotide inhibited the proliferation of a human cell line which had been transformed with the mutated Ha-ras gene (HBL100ras1) but had no effect on the parental cell line which was transfected with the vector DNA (HBL 100neo) and expressed only the normal Ha-ras gene. Growth inhibition of HBL100ras1 cells was associated with specific ablation of targeted Ha-ras mRNA as shown by RT-PCR. These results show that '*in vitro*' evaluation using an RNase H assay allowed us to select an antisense oligonucleotide which elicited a selectivity towards point-mutated Ha-ras mRNA when added at 10 μ M concentration to the culture medium of cells expressing wild type and mutated Ha-ras mRNA.

INTRODUCTION

The three members of the *ras* gene family (Ha-ras, Ki-ras, N-ras) have been identified as active oncogenes in many different types of human tumors, including sarcomas, neuroblastomas, leukemias and lymphomas (1,2). Studies of *ras* oncogenes in different tumors established that point mutations leading to single amino acid substitutions were a universal feature of *ras* activation. Different amino acid substitutions at codon 12, 13 and 61 activate *ras* transforming potential (2). Furthermore, a point mutation in an intron of Ha-ras was found to affect splicing and elevate Ha-Ras protein levels, resulting in an increased transforming potential (3). Strategies aimed at selectively inhibiting expression of the mutated form of the Ha-ras oncogene could lead to new therapeutic approaches for the treatment of malignancies involving this gene. For this purpose, antisense oligonucleotides appear to offer considerable promise as specific inhibitors of oncogene expression (4,5).

We have previously reported that short modified antisense oligonucleotides directed against the Ha-ras codon 12 point mutation induced selective cleavage of the mRNA and inhibited the proliferation of T24 bladder carcinoma cells, which carry activated Ha-ras (6). Using a *ras*-luciferase fusion reporter gene assay, Monia *et al.* (7) have shown that a 5-fold selectivity was reached with a 17-nucleotide-long phosphorothioate oligonucleotide. Psoralen-oligomethylphosphonate conjugates have also been targeted to mutated Ha-ras mRNA (8). In the present study we have addressed the following question: is it possible to design an antisense oligonucleotide with a natural phosphodiester backbone, targeted to the region of the oncogene mRNA carrying the mutation (codon 12) that will be able to elicit a complete discrimination between oncogene and proto-oncogene and selectively inhibit the proliferation of cells carrying mutated as compared to normal Ha-ras?

Several factors such as stability, uptake and access to mRNA may potentially affect the ability of antisense oligonucleotides to inhibit oncogene expression in intact cells. However, all these important parameters depend on the nature of the antisense oligonucleotide, since any modification of the phosphodiester backbone or of the 3' or 5' ends of the oligonucleotide may affect

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stability, uptake and affinity of the oligonucleotide for its target. Data on hybridization efficiency and hybrid stability, as well as on the mechanism of action on target mRNA, should allow us to optimize the antisense sequence. In this report, unmodified oligonucleotides and substrate Ha-*ras* mRNA which contains secondary and tertiary structures were used to analyze RNase H cleavage in nuclear extracts from HeLa cells at 37°C. An increase in length of the oligomer is likely to increase rather than decrease the number of RNAs that can form hybrids stable enough to induce RNase H cleavage and lead to non-targeted degradation. Therefore attempts were made to keep the oligonucleotide length to a minimum. Here we show that high selectivity can be achieved with a dodecamer or a tridecamer, whereas oligonucleotides longer than pentadecamers induce RNase H cleavage of mismatched as well as fully matched targets.

In order to define the most selective and effective antisense oligomer we have used two RNase H assays with different 12mer oligonucleotides targeted to the region of the point mutation. We show that the 12mer which elicited the highest degree of selectivity towards the mutated mRNA was less efficient at inducing RNase H cleavage. These results are in agreement with the existence of a stable hairpin structure involving the target region (9). The 12mer targeted to the loop region induced efficient cleavage but did not discriminate between the mutant and wild-type mRNA. Finally, to link the *in vitro* assays with the activity in cultured cells (with respect to selectivity), a biological assay based on tumor cell proliferation was performed with the most selective dodecanucleotide.

MATERIALS AND METHODS

Oligonucleotide synthesis

Oligonucleotides were purchased either from Genset (France) or from Eurogentec (Belgium). Oligodeoxynucleotides were labeled at the 5' end using T4 polynucleotide kinase and [γ -³²P]ATP (Amersham) and were analyzed on a 20% polyacrylamide-7 M urea sequencing gel. Oligonucleotides used in cell culture assays were twice precipitated with 10-fold excess ethanol.

In vitro transcription and RNase H cleavage

Uniformly labeled capped SP6 transcripts were synthesized using an *Eco*RI linearized plasmid which contains the entire coding region of normal or activated Ha-*ras* as described (6). Rabbit reticulocyte lysate which contains active RNase H was obtained from Amersham. Nuclear extract from HeLa cells (HeLaScribe™ Nuclear Extract, transcription grade) was purchased from Promega. For RNase H assays performed in rabbit reticulocyte lysate, 15 µl of lysate was added to the reaction mixture (25 µl) supplemented with 25 mM methionine, oligonucleotide and transcript. For cleavage by RNase H contained in the nuclear extract from HeLa cells we added 1 µl of extract and 1 µg of carrier DNA, the mRNA and the oligonucleotide to the reaction mixture (25 µl) containing 40 mM Tris, pH 7.9, 100 mM KCl, 2 mM MgCl₂. To directly compare the efficacy of different RNase H preparations, we performed all cleavage reactions at 37°C for 30 min with 4 nM transcript and 2 µM oligonucleotide. In all cases, reactions were carried out without pre-mixing RNA with the oligomer. After phenol treatment and ethanol precipitation, RNase cleavage products were analyzed by electrophoresis on a 6% polyacrylamide-7 M urea sequencing gel. The gels were autoradiographed

and the amount of intact material and cleavage products was quantitated by densitometry.

RT-PCR

Total RNA was isolated from oligonucleotide-treated or untreated HBL100ras1 and HBL100neo cells. Lysis buffer (500 µl) containing 0.15 M NaCl, 10 mM Tris, pH 7.5, 1.5 mM MgCl₂, 0.65% NP-40 was added to a cell pellet containing 3–4 × 10⁶ cells. The mixture was incubated for 5 min at 4°C. After centrifugation, the supernatant was mixed with an equal volume of urea-SDS buffer (7 M urea, 10 mM Tris, pH 7.5, 10 mM EDTA, 1% SDS). Then protein precipitation was carried out in the presence of 2 M NaCl. The tubes were centrifuged and RNA was precipitated from the supernatant with 2 vol ethanol. Reverse transcription and PCR (2 µg RNA) were performed using a Gene Amp RNA PCR Kit according to manufacturer's instructions (Perkin Elmer Cetus). Reactions were performed on a Hybaid Programmable Thermable Reactor. As an internal standard, human β_2 -microglobulin cDNA was co-amplified. To ensure linear amplification the number of cycles was limited to 25 (94°C for 50 s, 57°C for 50 s, 72°C for 20 s). The primers used were as follows: *ras*: 5' amplimer: 5'-TGA GGA GCG ATG ACG GAA TA-3'; 3' amplimer: 5'-GTA TCC AGG ATG TCC AAC AG-3'; β_2 -microglobulin: 5' amplimer: 5'-AAG ATG AGT ATG CCT GCC GT-3'; 3' amplimer: 5'-ATG CTG CTT ACA TGT CTC GAT-3'. Reaction products were precipitated and separated on a 7% polyacrylamide gel and visualised by ethidium bromide staining.

Cell lines and proliferation assays

HBL100ras1 and HBL100neo cell lines, were a kind gift from Dr G. Goubin. These cell lines have been previously described in detail (10). Briefly, HBL100ras1 is a clone obtained from the human mammary cell line HBL100 transformed by pSV2 which carries the EJ/T24 human bladder carcinoma Ha-*ras* oncogene. This cell line therefore expresses normal Ha-*ras* as well as Ha-*ras* with a G → T point mutation at codon 12. HBL100neo is a clone transformed only with the pSV2 expression vector and it expresses only normal Ha-*ras*. Cells were grown in complete cell culture medium (Modified Eagle's Medium from Flow, Irvine, Scotland) supplemented with 7% heat-inactivated fetal calf serum (Seromed), antibiotics (50 U/ml penicillin and 50 U/ml streptomycin) and 4 mM glutamine (Flow). Cells were seeded in 96-well plates at a concentration of 4 × 10³ cells/well in 50 µl. After the cells were adherent to the culture dish (2–3 h) oligonucleotides were added at twice their final concentration in 50 µl of culture medium/well, resulting in a final volume of 100 µl/well. Following incubation at 37°C in humidified atmosphere with 5% CO₂, cell numbers were determined using an hemocytometer. Cell viability was examined at the same time by the trypan blue exclusion method and was found to be >95% in treated and untreated cells. Cells were also seeded in 6-well plates at a concentration of 10 × 10⁵ cells/well in 1 ml media. Oligonucleotides were added directly to the culture medium containing the cells before seeding in plates.

RESULTS

'Longer is not better' for discrimination between normal and mutated Ha-*ras* mRNAs

In a previous work we targeted a 9mer antisense oligodeoxynucleotide covalently linked to an acridine intercalating agent

and/or a hydrophobic tail to a region of Ha-ras mRNA including the point mutation G→U at the 12th codon (6). This mutation leads to a Gly → Val substitution in the Ras p21 protein. Using an *in vitro* RNase H assay we showed that under conditions where the activated ('VAL') mRNA was 70% cleaved in presence of the modified antisense 9mer, only 7% of the wild-type ('GLY') mRNA was cleaved. In order to achieve higher efficiency and better discrimination, we increased the length of the antisense oligonucleotide and centered it around the 12th codon as shown in Figure 1A. To determine whether eukaryotic RNase H could cleave perfect and mismatched duplexes, samples were incubated with nuclear extracts from HeLa cells known to contain high levels of RNase H activity. Capped mRNA of mutated and wild-type Ha-ras obtained by *in vitro* transcription (see Materials and Methods) were incubated with a nuclear extract from HeLa cells at 37°C for 30 min with antisense oligonucleotides of different lengths. The RNA was then analyzed on a 6% polyacrylamide sequencing gel. The mRNA was cleaved into two fragments of approximately 145 and 675 nucleotides (Fig. 1A) (for a complete analysis of cleavage products see Figure 3B). Uncleaved mRNA and the longer cleavage product, which represents the 3' part of the RNA transcript, are shown at the top of Figure 1B. The extent of cleavage was quantitated from densitomer tracing of the autoradiographs. For each oligonucleotide we used a range of concentrations (1–5 μ M) that led to nearly 100% cleavage on 'VAL' mRNA and, using these conditions, then determined the amount of cleavage induced on 'GLY' mRNA. Figure 1B shows that very good discrimination was achieved with the dodecamer and tridecamer while longer oligonucleotides induced RNase H cleavage of 'GLY' mRNA.

Dose-response studies were carried out with the 16mer and 12mer (dodecamer R5, see Fig. 3A for the sequence). Figure 2 shows that a 5-fold higher concentration of the 16mer was required to induce 50% cleavage of the mismatched (Gly) hybrid as compared with the matched (Val) hybrid. In contrast no cleavage was detected on the mismatched duplex with a concentration of 12mer 20 times higher than that required to induce 50% cleavage of the matched complex (Fig. 2). On the basis of the above results, we chose the 12mer oligonucleotide for further investigation.

Dodecamer efficiency and discrimination

One of the important factors that determines the efficiency of antisense oligonucleotides *in vivo* is the availability of the target sequence for hybridization. RNAs always contain secondary and tertiary structures. Binding affinity and rates of oligonucleotide hybridization are affected by target RNA structure (9,11,12a, b). RNase H assays have been used *in vitro* to test the efficacy of antisense oligonucleotides to direct mRNA cleavage (13). We used RNase H preparations from rabbit reticulocyte lysates (RRL) and nuclear extract from HeLa cells to compare the efficiency of RNase H-induced cleavage using eight different 12mers targeted to the region containing the point mutation (Fig. 3A). In addition, we addressed the question of discrimination in our assays by comparing cleavage of the messenger RNAs coding for wild-type and activated Ha-Ras protein.

Figure 3B shows the autoradiogram of the gel analyzing the cleavage products obtained after incubation of 'VAL' mRNA with eight different dodecamers in the presence of rabbit reticulocyte lysate. The cleavage induced by these oligomers was highly sequence-specific since only the predicted cleavage

Figure 1. (A) Oligonucleotides ranging in size between 12 and 16 bases and centered around the point mutation (G→U) are shown aligned with the complementary Ha-ras RNA sequence. The 12mer sequence corresponds to the R4- dodecamer shown in Figure 3A. The overall organization of the Ha-ras transcript is shown in the lower panel together with the location of the target sequence for antisense oligonucleotides. (a) indicates the full length mRNA, (b) and (c) the two RNA fragments which should be obtained after RNase H cleavage. (B) Cleavage by RNase H from nuclear extracts of HeLa cells of normal Ha-ras (GLY) and mutated Ha-ras (VAL) mRNA induced by oligodeoxynucleotides of different lengths (see A for oligonucleotide sequences). *In vitro* transcribed uniformly labeled RNA was incubated at 37°C with nuclear extract from HeLa cells in the presence of 5 μ M 12- and 13mer or 1 μ M 14, 15 or 16mers. The resulting RNA products were separated on a 6% polyacrylamide sequencing gel. Arrow (a) indicates the full length RNA (~820 nucleotides), (b) the position of the 3' fragment of the RNA transcript obtained after RNase H cleavage (~675 nucleotides). The percentage of cleavage of wild-type mRNA (filled bars) and mutated mRNA (hatched bars) was quantitated by densitometry.

Figure 2. Dose-response curves for the cleavage by RNase H from HeLa nuclear extracts of normal (Gly) and mutated (Val) Ha-*ras* mRNA hybridized to dodecamer and hexadecamer: 12mer with wild-type mRNA (□) and mutated mRNA (■), 16mer with wild-type mRNA (○) and mutated mRNA (●). Cleavage was quantitated as described in Figure 1.

products (arrows b and c) were obtained. Cleavage of the RNA always occurred in the region engaged in duplex formation with the antisense oligomer. The same concentration (2 μM) was used for all antisense oligonucleotides. Therefore we could directly compare the efficiency of the cleavage induced on 'VAL' mRNA to that of 'GLY' mRNA as shown in Figure 4 (top). These data were obtained after densitometric analysis of three different gels. RRL and nuclear extracts from HeLa cells were used as a source of eukaryotic RNase H and gave similar results (Fig. 4, top and bottom). In both cases R1 and R2 did not give any discrimination between normal and mutated Ha-*ras* mRNA, even though they were the most active at inducing RNase H cleavage. The best discrimination was achieved with oligonucleotides R4 and R5.

We selected oligonucleotide R5 for further studies as high discrimination between 'VAL' and 'GLY' mRNAs was achieved with this oligonucleotide at 2 μM concentration using both eukaryotic RNase H cleavage assays. The concentration of the oligonucleotide in nuclear extracts from HeLa cells was increased to 10 μM. At this concentration, R5 induced almost 100% cleavage on 'VAL' mRNA, while 'GLY' mRNA was not cleaved, in agreement with the results of Figure 2. These results show that it is possible to achieve nearly 100% discrimination using the appropriate dodecamer targeted to the region of Ha-*ras* mRNA carrying the point mutation. Therefore the R5 dodecamer was chosen for further cell culture studies.

***In vitro* assay and biological activity correlation**

The p21 Ras proteins are involved in growth regulation and differentiation in all eukaryotes. These proteins bind the guanine nucleotides GDP and GTP and can hydrolyze bound GTP. In many tumor cells, mutant oncogenic alleles of *ras* genes specify structurally altered forms of p21 ras that have a reduced ability to hydrolyze GTP when compared with the wild-type *ras* gene product. Because of their reduced intrinsic GTPase activity and

resistance to the ras GTPase activating protein (GAP), oncogenic forms of p21 ras are trapped in their activated GTP-bound state for extended periods of time, thereby flooding the cell with growth-stimulatory signals. The non-tumorigenic human mammary cell line HBL100 has been transformed with the EJ/T24 human bladder carcinoma Ha-*ras* gene (10). One of these cell lines (HBL100ras1) was shown to induce tumors in nude mice and has been used in a number of studies. In contrast HBL100 cells transfected with the vector which did not contain the Ha-*ras* gene (HBL100neo) were not tumorigenic (10).

As shown above, the R5 antisense dodecanucleotide was able to discriminate between wild-type and mutated Ha-*ras* mRNA, using *in vitro* assays. Therefore we addressed the question of whether R5 was able to induce selective cleavage of the mutated Ha-*ras* mRNA in cell cultures and whether this sequence selective effect would arrest the proliferation of HBL100 ras1 cells without affecting that of HBL100 neo cells. Figure 5A shows the dose-response curve for HBL100ras1 and HBL100neo growth inhibition. A single addition of 10 μM R5 dodecamer resulted in 75% growth inhibition of HBL100ras1 ras cells as measured 84 h after addition of the oligonucleotide to cell cultures. In contrast HBL100neo cells were inhibited by only 10%. Higher concentrations of R5 (20 μM) resulted in slightly higher inhibition but weaker discrimination. Figure 5B shows the time course of HBL100ras1 growth inhibition following a single addition of 20 μM R5 dodecamer (AS-Val), an inverted sequence of R5 (INV-Val) and the dodecamer targeted to the wild-type mRNA (AS-Gly). First, it was observed that the dodecamer targeted to the wild type mRNA (AS-Gly) did not inhibit HBL100ras1 proliferation nor did the inverted sequence. Secondly, there was no inhibition by the antisense oligonucleotide R5 (AS-Val) up to 48 h, then maximum inhibition was observed between 72 and 84 h and was lost after 210 h. This growth pattern is compatible with the reported 24 h cellular half-life of the Ras protein (14). Gray *et al.* (15) have shown that in NIH-3T3 cells transformed by the T24 human bladder carcinoma Ha-*ras* oncogene, three days of treatment with an antisense pentadecamer complementary to a target in the 5' untranslated region of the c-Ha-*ras* mRNA was necessary to reduce p21 levels by >90%. The half-life of the dodecamer in the culture medium when added at 20 μM concentration was ~4 h, whereas undegraded oligonucleotide was detected within cells at 48 h (data not shown). Cell viability examined at the same time was found to be >95% in both treated and untreated cells when examined using the trypan blue exclusion method.

Growth inhibition selectivity mirrors selective depletion of Ha-*ras* mRNA

In order to determine if growth inhibition of HBL100ras1 cells was associated with the specific inhibition of 'activated' Ha-*ras* mRNA we used RT-PCR to determine the level of Ha-*ras* mRNA in cells treated with oligonucleotides. Figure 6 shows that addition of 20 μM R5 (AS-Val) to HBL100ras1 cell culture medium led to an almost complete depletion (87%) of Ha-*ras* mRNA after treatment for 24 h when compared with β₂-microglobulin mRNA used as a control. Treatment with the inverted R5 sequence (INV-Val) dodecamer had no effect on Ha-*ras* mRNA. The dodecamer targeted to the wild-type mRNA (AS-Gly) had only a weak effect (19%) on Ha-*ras* mRNA, demonstrating the sequence and point mutation specificity of the R5 antisense oligonucleotide on HBL100ras1 cells. The mRNA depletion which occurred 24 h

Figure 3. (A) Sequences of the different dodecamers (R1–R8) targeted to the Ha-*ras* transcript (the box indicates the loop structure). The structure of a 120 nucleotide long Ha-*ras* transcript calculated according to Dessen *et al.* (28) is shown below the oligonucleotide sequences. (B) Cleavage of mutated Ha-*ras* (VAL) mRNA induced by different dodecamers (R1→R8). *In vitro* transcribed uniformly labelled RNA (4 nM) was incubated at 37°C for 30 min in rabbit reticulocyte lysate with 2 μM of oligomers. The resulting RNA products were separated on a 6 % polyacrylamide–7 M urea sequencing gel. Arrow (a) indicates the full length mRNA, (b) and (c) the position of the two RNA fragments obtained after RNase H cleavage. The mobility and the length of *Msp*I restriction fragments of pBR322 plasmid DNA are shown on the left-hand side of the figure.

after oligonucleotide treatment was maintained for up to 3 days (data not shown). In HBL100neo cells, R5 dodecamer treatment did not decrease wild-type mRNA levels in HBL100neo cells (3% depletion), while AS-Gly treatment abolished wild-type mRNA expression (Fig. 6).

DISCUSSION

Antisense oligonucleotides offer an interesting approach to inhibit cancer cell proliferation. Therefore it would be important to have rational rules for the design of efficient molecules. Beside the inherent vectorization problems, many of which still remain unsolved, a key point for a successful oligonucleotide therapy in cancer will be the discriminatory character of the molecule, as the target is often a mutated RNA which is not present in normal cells. Our work examined whether it was possible to draw inferences from an *in vitro* activity assay (RNase H cleavage) for use in cell experiments. RNase H activity has been identified as a key factor in the success of hybrid-arrested translation with oligodeoxynucleotides in *in vitro* translation assays (16–18).

RNase H-targeted cleavage of a mRNA by an oligodeoxynucleotide remains the most probable antisense mechanism when the target sequence is located within the coding region of a mRNA. Recently it was shown that antisense oligonucleotides can indeed direct RNase H cleavage at the antisense target site *in vivo* when introduced into cells by streptolysin O permeabilization (19). The antisense activity of oligonucleotides resulting from RNase H-induced cleavage should be dependent on (i) oligonucleotide (and target) sequence and structure (ii) oligonucleotide length, (iii) oligonucleotide concentration and (iv) enzyme activity.

Previous studies have used RNase H cleavage assays *in vitro* to determine the efficiency and the specificity of antisense oligonucleotides. However, different results are obtained depending on the source of RNase H and the temperature used for these experiments. In eukaryotes, at least two classes of RNase H activities have been described (see 20 for review). Experiments are usually carried out at 25, 30 and 37°C for wheat germ extract, rabbit reticulocyte lysate and HeLa cell extracts, respectively. Using *Xenopus* oocytes, experiments are usually performed at 22°C (21). In addition to the fact that different types of RNase H

Figure 4. RNase H-cleavage activity of 2 μ M dodecamers (R1→R8) targeted to wild-type (GLY) (filled bars) or mutant (VAL) (hatched bars) Ha-*ras* mRNA. Oligonucleotide sequences are shown in Figure 2. Following mRNA incubation with 2 μ M of oligonucleotide and Rabbit reticulocyte lysate (bottom) and nuclear extract from HeLa cells (top), cleavage products were separated on a 6% polyacrylamide–7 M urea gel and quantitated as described in Materials and Methods. Error bars represent the standard deviation from triplicate measurements.

do not share the same binding and catalytic properties, temperature has a very strong effect on oligonucleotide binding to the target mRNA, due to the high enthalpy change which accompanies duplex formation. To compare the selectivity of antisense molecules of different lengths targeted to the region of the point mutation, we used conditions leading to ~100% cleavage on perfectly matched hybrids ('VAL' mRNA). The cleavage was induced at 37°C without any prehybridization step to avoid denaturation of RNA structures. Under conditions where oligonucleotides ranging in size from 12 to 16 bases induced almost complete (>80%) cleavage of 'VAL' mRNA, only the 12- and

Figure 5. (A) Dose-response for growth inhibition of HBL100ras1 (hatched bars) and HBL100neo cells (filled bars) induced by antisense dodecamer R5. After addition of R5, (at the indicated concentration) cells were incubated for 84 h. (B) Kinetics of HBL100ras1 growth inhibition by 20 μ M dodecamer R5 (AS-Val), the dodecamer directed to wild-type mRNA (AS-Gly: 5'-CACCGCCGGCGC-3') and the inverted R5 dodecamer (INV-Val: 5'-CGCGGCAGCCAC-3'). Each point represents the means of triplicate cultures with error bars as indicated. Percentage of inhibition was calculated according to: $100 \times (N_n - N_0) / (N_n - N_0)$, where N_0 is the cell count at the beginning of the experiment, N_n is the number of untreated cells after n days of culture and N is the number of treated cells after n days.

13mer did not induce any cleavage on 'GLY' mRNA. Therefore, using unmodified oligonucleotides centered around the codon 12 point mutation, high selectivity was achieved with a dodecamer or a tridecamer. Longer oligomers lost the ability to discriminate between the two mRNAs. The free energy loss due to a mismatch within the double-helical region of an oligonucleotide-mRNA hybrid does not depend on the length of the oligonucleotide, provided the mismatch is not close to either end. When the oligonucleotide length increases, lower concentrations of oligonucleotide should be used to ensure a similar coverage of the target. As shown in Figure 2, there is a 5-fold difference in the concentrations of a 16mer leading to 50% RNase H cleavage of

Figure 6. Ha-*ras* and β_2 -microglobulin mRNA expression in HBL100ras1 and HBL100neo cells exposed to 20 μ M of R5 (AS-Val), AS-Gly, INV-Val (sequences given the legend of Fig. 5). Cells were treated with dodecamers for 24 h. Total RNA was extracted from cells and reverse transcribed. The resulting cDNA was amplified by PCR using primer pairs specific for Ha-*ras* and β_2 -microglobulin as described in Materials and Methods.

normal as compared with mutated Ha-*ras* mRNA. However the 12mer exhibited a much higher discrimination between normal and mutated Ha-*ras* mRNA than expected on the basis of differential binding to the two targets. The site of binding and cleavage of RNase H clearly should play an important role. The sites of cleavage by RNase H from HeLa cells are located in the vicinity of the mutation (data not shown). It is therefore expected that a higher discrimination may be achieved when the binding and/or cleavage sites of RNase H coincides with or overlaps the mutant site. We have recently investigated hexose-containing tridecamers centered on the mutation (22). The tridecamer with a pyranose nucleoside at the mutation site gave low cleavage (10%) of the mutated Ha-*ras* mRNA, while the same tridecamer with two hexoses positioned six and seven bases upstream induced strong cleavage (60%) of mutated Ha-*ras* mRNA, even though the stabilities of the duplexes formed by the two oligonucleotides with the mRNA were almost identical. These results suggest that RNase H plays an active role in the discrimination reported in our studies.

The most selective dodecamer (R5) specifically inhibited HBL100ras1-mediated cell proliferation. This inhibition was expected to be due to greatly decreased Ha-*ras* mRNA levels if RNase H-induced cleavage of the mRNA was involved in the antisense effect. Reverse transcription of total RNA from cells (HBL100ras1, HBL100neo) treated or untreated with R5 dodecamer and amplification of the cDNA, provided evidence that the point mutation-specificity observed *in vitro* was maintained within cells and that R5 induced sequence-selective depletion of mutant Ha-*ras* mRNA in HBL100ras1 cells.

Monia *et al.* (7) have used a thermodynamic model to predict the specificity that can be achieved by an antisense oligonucleotide targeted to a single base point mutation. Using a reporter gene assay they have shown that, as predicted by the thermodynamic model, a maximum selectivity of 5-fold was achieved with a 17mer phosphorothioate centered around the Ha-*ras* codon 12 point mutation. The biological assays were carried out using transient DNA transfections and the phosphorothioate oligonucleotides were introduced into the cells using lipofectin.

The results presented in our study demonstrate that an *in vitro* RNase H assay can be used to predict the selectivity of antisense oligonucleotides with respect to two mRNAs which differ by a single base. The selected dodecamer exhibited a much higher

discrimination between normal and mutated Ha-*ras* than predicted by a thermodynamic model. These results suggest that RNA structure as well as RNase H interactions with the duplex RNA-DNA play an important role in oligonucleotide discrimination. It is well documented that a central mismatch is more destabilizing than a terminal one. In the case of the dodecamer centered on the point mutation of Ha-*ras* mRNA the presence of a stem structure near the mismatch is likely to contribute to discrimination by destabilizing the antisense-mRNA hybrid. We have recently shown that chemically and photochemically activatable oligonucleotides targeted to the same region did not induce efficient reactions in the absence of a 'helper' oligonucleotide whereas they provided good substrates for RNase H cleavage. The important role played by RNase H in the antisense activity and selectivity of oligonucleotides has also been suggested by studies involving chimeric oligonucleotides composed of terminal modifications which do not induce RNase H cleavage surrounding a central phosphodiester region (24–26). Our study shows that high selectivity can be achieved using an unmodified oligonucleotide selected *in vitro* and added to culture medium in the absence of a delivery system. Experiments on cell cultures showed that the selective depletion of the targeted mutant mRNA was obtained after cell uptake of the antisense dodecamer. We have shown recently that the R5 antisense dodecanucleotide adsorbed to polyalkylcyanoacrylate nanoparticles inhibited Ha-*ras*-dependent tumor growth in nude mice after subcutaneous injection (27). The development of antisense oligonucleotides targeted to mutated regions of mRNAs in tumor cells represents an interesting approach to selectively inhibit tumor growth, with little effect expected on normal cells which carry the unmutated gene.

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REFERENCES

- 1 Bos, J.L. (1989) *Cancer Res.* **49**, 4682–4689.
- 2 Barbacid, M. (1990) *Eur. J. Clin. Invest.* **20**, 225–235.
- 3 Cohen, J.B., Broz, S.D. and Levinson, A.D. (1989) *Cell* **58**, 461–472.
- 4 Neckers, L., Whitesell, L., Rosolen, A. and Geselowitz, D.A. (1992) *Crit. Rev. Oncogenesis*, **3**, 175–231.
- 5 Saison-Behmoaras, E. and Hélène, C. (1992) *Cancer Biology and Medicine: The search for new anticancer drugs*, M.J. Waring and B.A.J. Ponder (eds), Kluwer Academic Publishers Dordrecht/Boston/London, Ch. 10, pp. 259–277.
- 6 Saison-Behmoaras, T., Tocqué, B., Rey, I., Chassignol, M., Thuong, N.T. and Hélène C. (1991) *The EMBO J.*, **10**, 1111–1118.
- 7 Monia, B.P., Johnston, J.F., Ecker, D.J., Zounes, M.A., Lima W.F. and Freier, S.M. (1992) *J. Biol. Chem.*, **267**, 19954–19962.
- 8 Chang, E.H., Miller, P.S., Cushman, C., Devades, K., Pirolo, K.F., Ts'o, P.O.P. and Yu, Z.P. (1991) *Biochemistry* **30**, 8283–8286.
- 9 Lima W.F., Monia, B.P., Ecker, D.J. and Freier S.M. (1992) *Biochemistry*, **31**, 12055–12061.
- 10 Lebeau, J., Le Chalony, C., Prosperi, M.T. and Goubin, G. (1991) *Oncogene*, **6**, 1125–1132.
- 11 Fedor, M.J. and Uhlenbeck, O.C. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 1668–1672.
- 12 (a) Herschlag, D. and Cech, T.R. (1990) *Biochemistry*, **29**, 10159–10171.
(b) Herschlag, D. and Cech, T.R. (1990) *Biochemistry*, **29**, 10172–10180.
- 13 Hélène, C. and Toulmé, J.J. (1990) *Biochim. Biophys. Acta*, **1049**, 99–125.

- 14 Magee, A.I., Gutierrez, L., McKay, I.A., Marshall, C.J. and Hall, A. (1987) *EMBO J.*, **6**, 3353–3357.
- 15 Gray, G.D., Hernandez, O.M., Hebel D., Root, M., Pow-Sang, J.M. and Wickstrom, E. (1993) *Cancer Res.* **53**, 577–580.
- 16 Minshull, J. and Hunt, T. (1986) *Nucleic Acids Res.* **14**, 6433–6451.
- 17 Cazenave, C., Loreau, N., Thuong N.T., Toulmé, J. J and Hélène, C. (1987) *Nucleic Acids Res.* **15**, 4717–4736.
- 18 Walder, R.Y and Walder, J. A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5011–5015.
- 19 Giles, R.V., Ruddell, C.J., Spiller, D.G., Green, J.A and Tidd, D.M. (1995) *Nucleic Acids Res.* **23**, 954–961.
- 20 Crouch, R.J and Dirksen, M. L. (1982) In *Nucleases*. S.M Linn and R.J. Roberts (eds), pp. 221–244, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- 21 Woolf, T.M., Melton, D.A. and Jennings C.G.B. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 7305–7309.
- 22 Augustyns, K., Godard, G., Hendrix, C., Van Aerschot A., Rozenski, J., Saison-Behmoaras T. and Herdewijn, P. (1993) *Nucleic Acids Res.* **21**, 4670–4676.
- 23 Godard, G., François, J-C., Duroux, I., Asseline, U., Chassignol, M., Thuong, N., Hélène, C. and Saison-Behmoaras, T. (1994) *Nucleic Acids Res.* **22**, 4789–4795.
- 24 Giles, R.V. and Tidd, D.M. (1992) *Nucleic Acids Res.* **20**, 763–770.
- 25 Larrouy, B., Blonski, C., Boiziau, C., Stuer, M., Moreau, S., Shire, D. and Toulmé, J.J. (1992) *Gene*, **121**, 189–194.
- 26 Monia, B.P., Lesnik, E.A., Gonzalez, C., Lima, W.F., McGee, D., Guinosso, C.J., Kawasaki, A.M., Cook, P.D and Freier, S.M (1993) *J. Biol Chem*, **268**, 14514–14522.
- 27 Schwab, G., Chavany, C., Duroux, I., Goubin, G., Lebeau, J., Hélène, C. and Saison-Behmoaras, T. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 10460–10464.
- 28 Dessen, P., Fondrat, C., Valencien, C. and Mugnier, C. (1990) *CABIOS* **6**, 355–356.