Allele specific inhibition. A novel approach to cancer therapy

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CHAPTER 6

THE INVOLVEMENT OF HUMAN RIBONUCLEASES H1 AND H2 IN THE VARIATION OF RESPONSE OF CELLS TO ANTISENSE PHOSPHOROTHIOATE OLGONUCLEOTIDES

Anneloor L.M.A. ten Asbroek, Marjoni van Groenigen, Marleen Nooij, and Frank Baas

ABSTRACT

We have analyzed the response of a number of human cell lines to treatment with antisense oligodeoxynucleotides (ODNs) directed against RNA polymerase II, replication protein A, and Ha-ras. ODN-delivery to the cells was liposome-mediated or via electroporation, which resulted in different intracellular locations of the ODNs. The ODN-mediated target mRNA reduction varied considerably between the cell lines. In view of the essential role of RNase H activity in this response, RNase H was analyzed. The mRNA levels of RNase H1 and RNase H2 varied considerably in the cell lines examined in this study. The intracellular localization of the enzymes, assayed by green-fluorescent protein fusions, showed that RNase H1 was present throughout the whole cell for all cell types analyzed, whereas RNase H2 was restricted to the nucleus in all cells except the prostate cancer line 15PC3 that expressed the protein throughout the cell. Whole cell extracts of the cell lines yielded similar RNase H cleavage activity in an in vitro liquid assay, in contrast to the efficacy of the ODNs in vivo. Overexpression of RNase H2 did not affect the response to ODNs in vivo. Our data imply that in vivo RNase H activity is not only due to the activity assayed in vitro, but also to an intrinsic property of the cells. RNase H1 is not likely to be a major player in the antisense ODN-mediated degradation of target mRNAs. RNase H2 is involved in the activity assayed in vitro. The presence of cell-type specific factors affecting the activity and localization of RNase H2 is strongly suggested.
INTRODUCTION

Ribonucleases H (RNases H) are enzymes that specifically hydrolyze the RNA moiety in RNA-DNA duplexes \(^ {1,2}\). Proteins with RNase H activity are ubiquitous and have been isolated from a variety of organisms, ranging from viruses to prokaryotes and eukaryotes \(^ {3}\). The best characterized and functionally understood RNases H are the RNase H domains of retroviral reverse transcriptases, and the evolutionary related RNase HI of *Escherichia coli*. For both these enzymes crystal structures are available \(^ {4,5}\) and amino acid residues involved in substrate binding, metal binding, and catalysis have been identified and studied in detail by site-directed mutagenesis \(^ {6,7}\). Mammalian RNase H enzyme activities have been biochemically characterized in various tissues, including calf thymus \(^ {8}\), mouse cells \(^ {9}\), HeLa cells \(^ {10}\), human placenta \(^ {11}\) and human erythroleukemia cells \(^ {12}\). Based on differences in their biochemical characteristics and immunological cross-reactivity, RNase H activity in higher eukaryotes has been grouped into two classes \(^ {13,14}\). Class I enzymes have a native molecular weight of 68-90 kDa, are activated by both Mg\(^ {2+}\) and Mn\(^ {2+}\), and are active in the presence of sulfhydryl reagents. Class II enzymes have a lower molecular weight (30-45 kDa), are activated only by Mg\(^ {2+}\) and inhibited by additional Mn\(^ {2+}\), and are highly sensitive to sulfhydryl-blocking reagents.

Two different RNases H have been cloned and characterized in *Escherichia coli*: RNase HI \(^ {15}\) and RNase HII \(^ {16}\). The human sequence homologues of these bacterial enzymes have recently been identified and characterized \(^ {17-21}\). This has helped to link the biochemically characterized enzyme activities to the gene sequences. An overview of the two RNase H families, and their homologues identified in various species is given by Ohtani *et al.* \(^ {22}\). The human RNase H1 is a class I enzyme, and the sequence homologue of *E.coli* RNase HII, a prokaryotic minor enzyme which is not well characterized. Human RNase H2 is a class II enzyme, and the sequence homologue of *E.coli* RNase HI, the prokaryotic major enzyme that has been characterized in detail. RNase H enzymes are involved in removing RNA primers in prokaryotic and eukaryotic DNA synthesis reconstitution experiments *in vitro* \(^ {23,24}\). The physiological role of RNase HI in *E.coli*, however, is to prevent replication taking place from sites other than *oriC*. The RNA primer removal during replication *in vivo* is performed by the 5'-exonuclease activity of DNA polymerase I \(^ {25}\). Similarly, the removal of Okazaki RNA primers *in vivo* in eukaryotic cells does not necessarily involve RNase H; Dna2 helicase, helicase E, or Ku helicase, acting together with FEN1/RT H1 are also good and possible candidates \(^ {26}\). The physiological role of the eukaryotic RNases H remains as yet elusive.

The RNases H have gained renewed attention since the development of antisense drugs. Antisense oligodeoxynucleotides (ODNs) are widely used as a tool to down-regulate gene expression in a sequence-specific manner. The single-stranded DNA sequence binds to the complementary site in the target mRNA, upon which the RNA strand of the resulting DNA-
RNA hybrid is cleaved by RNase H\(^{(27)}\). Regular phosphodiester (PO) ODNs are rapidly degraded by cellular nucleases, and therefore modified ODNs have to be used. Phosphorothioate (PS) ODNs, in which a sulfur atom has replaced the non-bridging oxygen atom of the phosphate backbone, are most often used in practice. They are highly resistant to nucleases, able to recruit RNase H cleavage, and commercially available. Apart from their sequence-specific effects, however, these molecules also exhibit a number of sequence-independent artefacts, most of which are attributable to their ability to bind a number of heparin-binding proteins\(^{(28)}\).

In our search for allele-specific inhibitors based on single-nucleotide polymorphisms in target mRNA sequences using antisense PS-ODNs, which could provide a tumor cell specific anticancer therapy\(^{(29)}\), we encountered large differences in the responses of the various human cancer cell lines to the same ODN. We have examined this effect in detail and extended the analysis to different target sequences and ODN delivery methods. Furthermore, we investigated the role of RNase H2 in this process using \textit{in vitro} and \textit{in vivo} measurements.

**RESULTS**

Six human cell lines (embryonal kidney HEK293, prostate cancer 15PC3, pancreatic carcinoma MiaPacII, cervical carcinoma HeLa, bladder carcinoma T24, and rhabdomyosarcoma HTB82) were analyzed for their response on treatment with antisense ODNs. The initial experiments were performed using liposomal delivery of various antisense phosphorothioate ODNs. The response to ODN treatment varied considerably. 15PC3 and MiaPacII showed a good response, while HEK293 and HTB82 responded hardly at all, and HeLa and T24 showed an intermediate response. To investigate the nature of the differences in response to antisense ODNs we analyzed the RNase H levels in the cell lines, since RNase H is claimed to be a key component in the mechanism of inhibition of gene expression by antisense ODNs. The variation in RNase H mRNA levels is substantial (Figure 1). HEK293, HeLa and 15PC3 display a similar high level of RNase H1, whereas MiaPacII, T24 and HTB82 show a low level. The difference in intensity between the two groups, after normalization for 28S rRNA signal is about 10-fold. The RNase H2 mRNA level shows a 5- to 10-fold variation, but with a different distribution over the cell lines. 15PC3 and MiaPacII display the highest level of the 1.2 kb mRNA, and HEK293 the lowest. The 5.5 kb mRNA species detected by the RNase H2 probe (described by Wu \textit{et al.}\(^{(20)}\) to be a polyadenylated processing variant of the main 1.2 kb mRNA) shows a more or less consistent level in the various cell lines (variation is only 1- to 2-fold). Our subsequent analysis focused on the three cell lines that present the possible variation in mRNA levels: MiaPacII (low RNase H1, high
RNase H2), HEK293 (high RNase H1, low RNase H2) and 15PC3 (high RNase H1, high RNase H2).

Since mRNA levels do not necessarily reflect protein levels or activity, we measured the RNase H activity in an in vitro assay using whole cell extracts. An in vitro synthesized run-off RNA, corresponding to a part of the POLR2A mRNA sequence (GenBank accession X63564, position 2846 to 3306) was hybridized with a complementary phosphodiester (PO) ODN of 16 nucleotides (L5Cas16; position 3049 to 3064). Cellular extracts were used in a concentration series to assay the non-saturated part of the activity curve, and mixtures of two different cell extracts were compared to the separate extracts. A representative example of an RNase H assay is shown in Figure 2 (Figure 2A shows the experiment for HEK293 and 2B for MiaPacall). 10 μl or 20 μl extract yields the saturation level of substrate digestion by RNase H in the extracts. Roughly 10% of the input RNA remains uncut. In both cases the range from 0.5 μl to 2 μl extract is not yet saturating, indicating a similar level of activity in both cells. Perhaps we measure two distinct activities in these extracts, e.g. RNase H1 in HEK293 and RNase H2 in MiaPacall, which may be additive or for which one may be limiting. In order to exclude this possibility, equal amounts of both extracts were mixed and compared to the activity of one single extract. Figure 2A shows that 0.5 μl HEK293 extract plus 0.5 μl MiaPacall extract leads to 76% digestion of the input target RNA, whereas 1 μl extract of HEK293 gives 71% digestion. Similarly, 1 μl of both extracts combined versus 2 μl of single extract gives 81% versus 82% digestion respectively. The same is demonstrated in figure 2B, where the comparison of combined extracts to single MiaPacall extract is made. The difference in activity obtained with the combined extracts in panels A and B reflects the inter-experimental variation. The fact that the combined extracts are as active as the individual extracts demonstrates that both cell lines harbor similar RNase H enzyme activity.
Similar results were obtained with extracts of 15PC3 cells (not shown). Phosphorothioate (PS) ODNs behave similarly to PO-ODNs in the in vitro assay. They are slightly less efficient, yielding 50-60% cleavage of the target RNA with 1 μl extract, compared to 60-70% cleavage using the corresponding PO-ODN (unpublished results).

The in vivo performance of the cells to antisense ODN treatment was tested by transfection experiments. Antisense inhibition of gene expression is presumed to result in degradation of the target mRNA via RNase H activity. The efficacy of a particular ODN can therefore best be addressed by Northern blot analysis of the target mRNA, since the level of full-length mRNA can be assayed. To avoid scoring possible artefacts of the ODN delivery system and chemistry-related toxicity, we used liposomal delivery of PS-ODNs to the cells (PO-ODNs do not enter the cells via liposomes; unpublished observations) as well as delivery of PS- and PO-ODNs by electroporation. Figure 3A shows the effect of 20 hr treatment using liposomal transfection with 800 nM (i.e. 800 pmoles) PS-ODNs directed against RPA70 (replication protein A, 70 kDa subunit), oncogene Ha-ras, and POLR2A (RNA polymerase II, 220-kDa subunit) on the respective target mRNA levels. Figure 3B shows the result using electroporation of 800 nM of antisense ODN directed against RPA70. A PS- as well as a PO-version of the ODN was used in those experiments. Since PO-ODNs are quickly degraded by cellular nucleases, mRNA was assayed at 4 hr and 20 hr post-transfection. The anti-RPA70 PS-ODN yields maximum efficacy already within 4 hr post-transfection with liposomal delivery, at the same level as at 20 hr post-transfection (unpublished results).
A summary of the quantification of the intact target mRNA levels is presented in Table 1. With liposomal delivery the 15PC3 and MiaPacalII cells are the best responders, whereas HEK293 responds hardly at all. In 15PC3 cells the anti-RPA70 PS-ODN displays the same potency with electroporation as with liposomal transfection. The PO-ODN is also effective, although less than the PS-version and only when assayed at 4 hr, compatible with the intracellular instability of PO-ODN compared to PS-ODN. For MiaPacalII cells only the PS-ODN is effective, and the delivery method makes a big difference. HEK293 is a poor responder, although the anti-RPA70 PS-ODN performs better in electroporation than in

Figure 3: Northern blot analysis of the cell lines transfected with 800 nM ODNs directed against RPA70 and Ha-ras or POLR2A
Probes used are indicated on the left side. 28S rRNA and GAPDH hybridization were used for quantification of RNA loading. ODNs used are indicated on top of the lanes. A: Liposomal transfection of PS-ODNs: αRPA, ISIS12790 directed against RPA70; αRAS, ISIS2503 directed against Ha-ras; αPOL, L5Cas20 (for 15PC3 and HEK293) or L5Tas20 (for MiaPacalII) directed against POLR2A; 20mer, completely randomized control mixture of 20-mer PS-ODNs; mock, transfection without PS-ODN. RNA was isolated for analysis at 20 hr post-transfection B: Electroporation transfection of 800 nM PS-ODN ISIS 12790 (RPA-S) and the PO version of this ODN (RPA-O). RNA was isolated for analysis at 4 hr or 20 hr post-transfection as indicated on the right.
Table 1: Percentage of intact target mRNA after antisense ODN treatment

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>LIPOSOMAL 20 hr</th>
<th>ELECTROPORATION 20 hr</th>
<th>ELECTROPORATION 4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>19.7 ± 3.3 (n=6)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>MiaPacAll</td>
<td>26.0 ± 2.2 (n=3)</td>
<td>80.7 ± 9.0 (n=3)</td>
<td>72.3 ± 9.1 (n=3)</td>
</tr>
<tr>
<td>RPA-S</td>
<td>N.A</td>
<td>90.7 ± 8.4 (n=3)</td>
<td>70.0 ± 11.2 (n=3)</td>
</tr>
<tr>
<td>RPA-O</td>
<td>66.8 ± 4.6 (n=4)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>HEK293</td>
<td>93.3 ± 7.6 (n=3)</td>
<td>68.3 ± 6.9 (n=3)</td>
<td>69.0 ± 7.3 (n=3)</td>
</tr>
<tr>
<td>POL-S</td>
<td>N.A</td>
<td>108.3 ±1.7 (n=3)</td>
<td>72.7 ± 8.2 (n=3)</td>
</tr>
<tr>
<td>RPA-S</td>
<td>19.3 ± 1.2 (n=3)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>RPA-O</td>
<td>21.3 ± 0.5 (n=3)</td>
<td>28.0 ± 6.4 (n=3)</td>
<td>35.0 ± 7.3 (n=3)</td>
</tr>
<tr>
<td>15PC3</td>
<td>86.0 ± 9.3 (n=3)</td>
<td>46.7 ± 4.1 (n=3)</td>
<td></td>
</tr>
</tbody>
</table>

After treatment with 800 nM antisense ODNs, phosphorothioate (POL-S and RPA-S) or phosphodiester (RPA-O), cells were assayed for intact target mRNA at 20 hr or 4 hr post-transfection, using Northern blotting. Percentages, corrected for loading and normalized to the mock control transfections, are given as Average ± Standard Deviation for n independent experiments. n.d.: not done; N.A.: not available, since PO-ODNs do not enter cells when delivered by liposomal transfection reagents.

liposomal transfection of these cells. The delivery by electroporation is more prone to variation, because most cells are killed by the shock, and only the surviving cells are assayed that are attached to the culture plastic at time of analysis. This yields a larger deviation than the liposomal delivery, where cells are attached to the growth surface from start to finish. The cell internal fate of the ODNs was assayed with fluorescently labeled PS-ODNs using both delivery systems. With both methods at least 90% transfection efficiency was obtained, and the cells displayed little variation in staining intensity. All cell lines showed a similar uptake and distribution as shown in Figure 4 (the nucleus was identified by Hoechst staining of the DNA; not shown). Liposomal transfection results mostly in a bright nuclear fluorescence that is excluded from the nucleoli and appears as bright spherical structures in a diffuse nucleoplasmic staining, as well as some cytoplasmic staining in bright punctate structures. The electroporation transfection provides a completely different pattern, without nuclear fluorescence detectable, and with fine punctate perinuclear and cytoplasmic staining of other structures than appear following liposomal transfection. The corresponding PO-ODN shows a similar pattern and intensity as the PS-ODN in the fluorescent electroporation transfection (not shown).
A tritium-labeled PS-ODN (against RPA70) was used in both delivery systems to quantify the amount of ODN that is retained in the cells at time of mRNA analysis. The amount of ODN per cell was quantified as $^3$H dpm per μg protein and is shown in Table 2. The three cell lines assayed display similar cellular uptake. Thus, not only the intracellular distribution is similar for these cells (fluorescence), but also the intracellular concentration (tritium). Furthermore, the intracellular ODN concentration is a linear function of the ODN concentration administered at transfection (Table 2). Electroporation results in a roughly two-fold higher concentration than liposomal delivery. Overall only 2-3% of the $^3$H-labeled ODN that is put into the transfection is still detected at 20 hr post-transfection. The relative amount of tritium detected immediately after liposomal transfection is 2-fold higher for MiaPacalII and 15PC3.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>LIPOSOMAL dpm/μg protein</th>
<th>ELECTROPORATION dpm/μg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>MiaPacalII</td>
<td>4.4 ± 0.0</td>
<td>7.8 ± 2.0</td>
</tr>
<tr>
<td>400 nM</td>
<td>6.4 ± 0.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>600 nM</td>
<td>10.4 ± 0.9</td>
<td>24.2 ± 1.0</td>
</tr>
<tr>
<td>800 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEK293</td>
<td>4.2 ± 0.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>400 nM</td>
<td>6.8 ± 0.9</td>
<td>n.d.</td>
</tr>
<tr>
<td>600 nM</td>
<td>8.7 ± 0.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>800 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15PC3</td>
<td>3.1 ± 0.3</td>
<td>5.4 ± 0.6</td>
</tr>
<tr>
<td>400 nM</td>
<td>6.5 ± 0.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>600 nM</td>
<td>9.1 ± 1.4</td>
<td>13.4 ± 0.1</td>
</tr>
<tr>
<td>800 nM</td>
<td></td>
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</table>

$^3$H present in cells at 20 hr post-transfection of a concentration series of antisense RPA-S is given as Average ± Deviation for 2 independent experiments. n.d.: not done.
and 4-fold higher for HEK293 compared to the 20 hr data. This can largely be explained by cell division (as can be calculated from the total amount of protein measured at both time points).

The data obtained thusfar show that HEK293 cells have the lowest level of RNase H2 mRNA and display a very poor response to antisense ODN treatment. To test whether additional RNase H2 would lead to enhanced sensitivity to ODNs, we constitutively expressed RNase H2 in HEK293 cells. Clones expressing high levels of RNase H2 RNA were assayed in vitro and in vivo. The in vitro RNase H assay, using whole cell extracts of the transfectants, shows that the expressed RNase H2 RNA yields functional protein, whereas the vector alone (panel pcV) does not affect the RNase H activity of the cells (Figure 5A). The cell extracts of the RNase H2 transfectants (panels pcRH) have increased enzymatic activity. The lowest input (0.5 µl extract) already yields saturated enzyme activity levels. Activity could only be properly assayed using 10-fold diluted extracts (Figure 5B). The cells overexpressing RNase H2 are roughly 10-fold more active in this in vitro assay than the parental and vector control cells.

![Figure 5: In vitro RNase H assay with whole cell extracts of HEK293 transfectant cells](image)

Panel A shows the parental HEK293 cells (293 wt) and typical examples of a pcDNA3 vector-only control transfectant cell line (pcV) and a pcDNA3/RNase H2 transfectant cell line (pcRH8) using fresh extracts. Panel B shows a vector-only control (pcV) and three pcDNA3/RNase H2 transfectants (pcRH8, pcRH9, pcRH10) that showed the highest level of RNase H2 RNA on Northern blot analysis, using 10-fold diluted extracts. In comparison with figure 5A, a lower level of digestion is obtained in all cases, because frozen extracts were used for the dilution, and freezing the extract leads to loss of activity in our hands (unpublished observations). However, the relative differences in activity between the vector-only and RNase H2 transfectants are still retrieved.
<table>
<thead>
<tr>
<th>Probe</th>
<th>MiaPacall (MPII)</th>
<th>HEK293 (293)</th>
<th>pcV</th>
<th>pcRH8</th>
<th>pcRH9</th>
<th>pcRH10</th>
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<tr>
<td>aPOL</td>
<td></td>
<td></td>
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<td>RPA</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>28S</td>
<td></td>
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</tbody>
</table>

Figure 6: Northern blot analysis of 800 nM PS-ODN transfections of HEK293 cells overexpressing RNase H2

Cell lines shown are MiaPacall (MPII), HEK293 (293), pcDNA3 vector-only control transfectant of HEK293 (pcV), RNase H2 transfectant cell lines of HEK293 overexpressing RNase H2 (pcRH8, pcRH9, pcRH10). PS-ODNs used are indicated on top of the lanes. aPOL, L5Cas20 directed against POLR2A; aRPA, ISIS12790 directed against RPA70; 20mer, randomized control mixture. Probes (indicated on the left) are for POLR2A (top), RPA70 (middle) or 28S rRNA (bottom).

The RNase H2 overexpressing clones were tested in vivo using liposomal delivery of 800 nM PS-ODNs, directed against POLR2A and RPA70 (Figure 6). Assaying the RNase H2 transfectants using electroporation was not feasible due to extremely poor plating efficiency of the RNase H2 overexpressing lines following electroporation, even on poly-L-lysine coated plates. All six RNase H2 transfectants assayed (three of which are shown in figure 6) have the same low level of antisense inhibition as the parental and vector control cells (roughly 10% reduction of target mRNA). The high level of activity in vitro, and thus expression of functional protein, does not result in an increased response to antisense ODN treatment in vivo.

To rule out the possibility that different alleles of RNase H2 are expressed in MiaPacall, HEK293 and 15PC3, we sequenced the coding region in these cells. The coding regions were identical, except for one silent substitution of the wobble base of a triplet encoding a proline residue. Position 579 (GenBank accession AF039652) is an A in MiaPacall and 15PC3, but a G in HEK293.

The different response to antisense ODN treatment could also be attributed to a difference in enzyme localization within the various cell lines. To test this possibility the coding sequences of RNase H1 and RNase H2 were fused in frame to green-fluorescent protein (GFP). The six cell lines were analyzed by fluorescence microscopy following transient transfections (MiaPacall, Hek293 and 15PC3 are shown in Figure 7). Control experiments using the GFP vector alone showed a uniform distribution of fluorescence within the cells for all cell lines.
The expression of the GFP-RNase H1 protein results in fluorescence throughout the whole cell in all cases, although the expression in 15PC3 seems to be less uniform. The expression of RNase H2 is restricted to only the nucleus (identified by Hoechst staining; not shown) in all cases except 15PC3. In these cells RNase H2 displayed the same uniform expression pattern as RNase H1.

![Figure 7: Staining pattern of cells expressing green-fluorescent protein (GFP) and GFP fused to RNase H1 (GFP-H1) or RNase H2 (GFP-H2)](image)

**DISCUSSION**

In this study we showed that the reduction of target mRNA upon treatment with ODNs against the 220 kDa subunit of RNA polymerase II, the 70 kDa subunit of replication protein A, and the oncogene Harvey-ras varies considerably between human cell lines. Since the catalytic activity of an RNase H is essential for antisense-mediated RNA degradation we measured both mRNA and enzymatic activity. Large differences were observed in our cell lines in mRNA level of the two human RNase H enzymes. We focused on the comparison of the cell lines that displayed the major differences (Table 3). 15PC3 contains high levels of both RNases H1 and H2, MiaPacaII contains a low level of RNase H1 and a high level of RNase H2, whereas HEK293 contains a high level of RNase H1 and a low level of RNase H2 (respectively 10-fold more and 5-fold less than MiaPacaII cells as assayed by Northern analysis of total RNA). Despite these large differences in mRNA levels we detected a similar RNase H activity with the various cells when we used whole cell extracts in an *in vitro* RNase H assay. Single extracts displayed the same level of activity as mixed extracts,
indicating that similar enzymatic activities were measured in the various extracts. In vivo, however, the cell lines showed a different response with a number of target mRNAs, which depended in part upon the delivery method used (figure 3). 15PC3 cells performed well for all three targets, yielding on average 80% reduction of the target mRNA, whereas HEK293 always performed poorly (only 20-30% reduction was achieved). The response of MiaPaca II cells depended on the ODN delivery method, yielding 70-80% reduction of the target mRNA with liposomal delivery and only 20-30% with electroporation. The amount of cellular ODN, measured with $^3$H-labeled PS-ODN, was twice as big after electroporation than after liposome-mediated transfection. FITC-labeling disclosed a large difference in ODN localization, which depended on the method of transfection. In our hands, liposomal delivery of fluorescently labeled PS-ODNs resulted in a staining pattern that has been observed by others in various cell types, using different liposomes (37,38), or microinjection of PS-ODNs into the cytoplasm (38-41). This pattern was independent of the ODN sequence, length, or the fluorochrome used (38,40). The perinuclear and vesicular cytoplasmic staining resulted from accumulation of ODN in the endosomes and lysosomes (37,41). The bright nuclear ODN foci are the so-called PS-bodies, associated with the nuclear matrix; following mitosis they assemble de novo from diffuse PS-ODN pools in the daughter nuclei (38). While they retain their antisense capacity, PS-ODNs continuously shuttle between the nucleus and the cytoplasm (42). This nucleocytoplasmic shuttling is an active transport process, which probably involves binding to (unidentified) cellular proteins that exhibit shuttling. The nuclear localization of PS-ODNs seems to be an important prerequisite for their potential to exert antisense activity, despite their binding to nuclear matrix proteins (38).

The pattern of ODN localization after delivery with electroporation was completely different, displaying no fluorescence at all in the nucleus. The cytoplasmic structures had a different appearance than those following the liposomal delivery: many more and finer punctate structures. After electroporation, the staining patterns observed with PO-ODNs and PS-ODNs are similar. This makes it unlikely that backbone chemistry-related binding components are involved in the cytoplasmic delivery of ODNs by electroporation.

Since the fate of the ODNs within the different cell types was similar with respect to ODN accumulation and localization, a variation in response to ODN treatment must be an intrinsic property of the cells.

The mRNA data suggest that RNase H1 does not make a major contribution to the mRNA reduction of antisense treatment. Firstly, in vitro the three cell lines have similar RNase H activity, despite the big difference in RNase H1 mRNA levels, even when extracts are mixed. Secondly, in vivo the high level of RNase H1 in HEK293 compared to MiaPacalI does not result in an increased response to antisense ODN treatment, irrespective of the cellular ODN localization (liposomal delivery or electroporation of the ODNs). Finally, a GFP-RNase H1 fusion protein shows similar localization in all cell lines. This argues against a cell-specific restriction of RNase H1 to certain cellular compartments. Rather it suggests that RNase H1.
### Table 3: Summary of the results related to the involvement of RNase H1 and H2

<table>
<thead>
<tr>
<th>cell line</th>
<th>RH1 mRNA level</th>
<th>RH2 mRNA level</th>
<th>RH1 localization</th>
<th>RH2 localization</th>
<th>RH activity in vitro</th>
<th>target mRNA reduction in vivo *</th>
<th>liposomal</th>
<th>electroporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MiaPacall</td>
<td>+</td>
<td>++</td>
<td>whole cell</td>
<td>nucleus</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>15PC3</td>
<td>+++</td>
<td>+++</td>
<td>whole cell</td>
<td>whole cell</td>
<td>+</td>
<td>+++</td>
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<tr>
<td>HEK293</td>
<td>+++</td>
<td>+/-</td>
<td>whole cell</td>
<td>nucleus</td>
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<td>+</td>
<td></td>
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<tr>
<td>HEK293 pcRH</td>
<td>+++</td>
<td>++++</td>
<td>whole cell</td>
<td>nucleus</td>
<td>+++</td>
<td>+</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

*) +: 10-30% reduction of target mRNA level; +++: 70-90% reduction of target mRNA level. n.d.: not done; RH: RNase H.

which is the ortholog of the minor *E.coli* enzyme RNase HIII, with unknown function, is not a major player in the cell’s response to antisense ODN mediated cleavage of target mRNA.

The presence of two mRNA species, as well as a variation in the cellular localization complicates the interpretation of the role of RNase H2 (Table 3). The main 1.2 kb mRNA level varies substantially between the cell lines. In the *in vitro* RNase H assay, however, the three cell lines show similar cleavage activity. Thus, the activity measured in the *in vitro* assay does not correlate with the mRNA levels of either RNase H1 or H2. The discrepancy between the *in vivo* and *in vitro* measurements could be due to a compartmentalization of a component in the *in vivo* system. On the other hand we cannot exclude that the substantial amount of 5.5 kb mRNA present in all cells encodes a major contributor of the RNase H activity measured *in vitro*. There are several examples of apparent discrepancies between RNase H activity measurements in different assays in mammals and yeast.[36,43]. In mammalian cells the class I enzyme activity could only be measured in a liquid assay and was not detected with an in-gel assay; the class II activity measured in the liquid assay was of a monomeric enzyme, whereas the class II activity detected in-gel presented a multimeric enzyme form. In *Saccaromyces cerevisiae* the class I activity was detected only in in-gel assays, the class II activity of RNH(35) only in liquid assays, whereas the class II activity of RNH(70) was detected in neither assay.

In order to determine the contribution of the activity encoded by the 1.2 kb RNase H2 mRNA, we assayed six different transfectant clones of HEK293 (three of these are shown in figures 5 and 6) that expressed a spectrum of high levels of RNase H2, up to a 25-fold higher level than the endogenous 15PC3 RNase H2 mRNA. The increase in RNase H2 RNA in the transfectants resulted in increased enzymatic activity in the *in vitro* RNase H assay. This demonstrates that the overexpressed RNase H2 contributes substantially to the enzymatic activity assayed in whole cell extracts. However, these HEK293 transfectants overexpressing functional RNase H2 do not display an increased response to antisense ODN treatment *in
vivo. Due to an increased fragility of the transfectants, it was not possible to analyze the effects of ODNs delivered by electroporation. The data of the 15PC3 cells are compatible with the hypothesis that RNase H2 can play a role in the in vivo response of cells. They are the only cells that show a good response to antisense ODN treatment using electroporation of PS- and PO-ODNs. With this transfection method the ODNs (PS as well as PO) are only detected in the cytoplasm. 15PC3 cells are the only ones that have RNase H2 protein both in the cytoplasm and the nucleus, as opposed to a strict nuclear localization in the other cell lines tested. Thus the cytoplasmic localization of RNase H2 in 15PC3 might be responsible for the catalytic activity after electroporation of antisense ODNs. The cytoplasmic RNase H2 is not an absolute requirement for effective antisense inhibition, since MiaPacal1 cells that display nuclear fluorescence of GFP-RNase H2 show a similar reduction of the target mRNA as 15PC3 cells when PS-ODNs are transfected with liposomes. Nuclear location of RNase H2 is not sufficient for ODN-mediated mRNA degradation, however. HEK293 and MiaPacal1 cells display a similar localization of RNase H2, as well as similar ODN localization and accumulation. Nevertheless HEK293 cells do not respond to PS-ODN treatment, even when they express vast amounts of active enzyme.

Reviews discussing PS-ODN-mediated inhibition of gene expression warn against erroneous interpretation of results caused by the protein-binding capacity of PS-ODNs (27,28, and many more). The lack of reactivity of HEK293 cells in our study could therefore simply be explained by postulating a cell-specific factor that inactivates the PS-ODNs in these cells, which would imply that this factor is inactive in the in vitro RNase H assay, or that some other enzymatic activity is measured. The detection of increased activity in the transfectants overexpressing the coding region of the 1.2 kb RNase H2 mRNA suggests that at least the activity encoded by the 1.2 kb mRNA can be assayed in vitro. On the other hand, the fact that 15PC3 cells display RNase H2 not strictly in the nucleus as the other cells, but also in a large amount in the cytoplasm, clearly shows that cell-specific components exist that act on this RNase H enzyme. Since we deduce the cellular localization from the behavior of the GFP-RNase H2 fusion protein, the cellular factor must act with the RNase H2 enzyme. The previously mentioned nucleocytoplasmic shuttling of PS-ODNs with the help of shuttling cellular components (42) may play a role in the cell-specific variation in response to antisense ODN treatment.

A clear assignment of the role of RNase H2 in the PS-ODN mediated cleavage of target mRNA in vivo requires additional knowledge of at least two things. On the one hand, the components binding to this enzyme need to be identified to understand the cytoplasmic location of the enzyme in 15PC3 cells. This enzymatic location appears a necessity for activity towards ODNs that are restricted to the cytoplasm. On the other hand, the 5.5 kb mRNA species, whose sequence is unknown, awaits identification and characterization. We cannot exclude that it contributes to the activity essential for the antisense ODN-mediated inhibition of gene expression in vivo. This would be compatible with the finding that
antisense ODNs can be very effective in inhibiting gene expression in the brain\(^{44-46}\). Both in fetal and adult brain the main 1.2 kb RNase H2 mRNA can not (or hardly at all) be detected by Northern analysis\(^{47}\), whereas they do have a consistent amount of the 5.5 kb RNase H2 mRNA species.

Our findings are not compatible with a simple assignment of a single RNase H enzyme activity to the antisense ODN-mediated inhibition of gene expression in human cells \textit{in vivo}.

**MATERIALS AND METHODS**

**Cell culture**

Human cell lines HEK293 (embryonal kidney), 15PC3 (prostate cancer), MiaPacII (pancreatic carcinoma), T24 (bladder carcinoma), HeLa (cervical carcinoma) and HTB82 (rhabdomyosarcoma), were obtained from the American Type Culture Collection, or gifts from colleagues. Cells were maintained by serial passage in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin.

**Transfections**

Oligonucleotides were purchased from Isogen (The Netherlands). ODNs directed against \textit{POLR2A} have been described previously\(^{29}\). Basilion \textit{et al.}\(^{30}\) and Monia \textit{et al.}\(^{31}\) have described ODNs ISIS12790 and ISIS 2503 directed against RPA70 and Ha-ras respectively. ODN transfection with liposomal transfection reagent DAC-30 (Eurogentec) was as described previously and performed in a 6-well culture plate, with 1 ml serum-free medium containing DAC-30 and ODN\(^{29}\). ODN transfection by electroporation was done with a Bio-Rad Gene Pulser II with RF module. One day prior to transfection cells were plated such that at transfection roughly 70\% confluency was reached. Cells were harvested using trypsin followed by washing in PBS, and resuspended in HEPES-buffered media (2 mM HEPES, 15 mM K-phosphate buffer, 250 mM mannitol, 1 mM MgCl\(_2\), pH 7.2\(^{32}\)) at \(10^6\) cells per 100 \(\mu\)l. This was incubated with the ODN at ice for 10 min, transferred to an electroporation cuvet (0.2 cm; Bio-Rad) and shocked (280 V, 100\% modulation, 140 amplitude, 40 kHz RF, 1.5 msec burst duration, 15 bursts, 1.5 sec interval). The cuvet was placed on ice immediately after electroporation. Cells were washed out of the cuvet in complete culture medium and plated at appropriate density for recovery.

Plasmid transfections for transient expression of GFP-constructs were with 2 \(\mu\)g supercoiled plasmid on \(10^5\) cells.

For fluorescence microscopy cells were plated on glass coverslips in a 6-well culture plate, and transfected with FITC-labeled ODNs or GFP-expressing plasmids. For analysis cells
were fixed on the glass in PBS containing 4% paraformaldehyde and embedded in Vectashield Mounting Medium (Vector Laboratories Inc.). Fluorescence microscopy was done with a Vanox microscope and appropriate filters. For stable expression of RNase H2 in HEK293, cells were plated in 10-cm dishes at $10^7$ cells and transfected for 5 hr in 2.5 ml serum-free medium containing 12.5 µl transfection reagent DAC-30 (Eurogentec) and 2 µg linearized plasmid. Initial selection of transfected cells was with 1.5 mg G418 (Roche) per ml medium. Maintenance of selected clones was at 0.5 mg G418 per ml.

**Tritium ODN measurements**

Tritium labeling of the ODN was performed using the heat exchange method described by Graham *et al.* (33). Cells were transfected with $^3$H-labeled PS-ODN (specific activity 40260 dpm/µg ODN) using the liposomal or electroporation delivery described above and seeded in 6-well plates. At sampling cells were extensively washed with PBS (5x with 3 ml PBS per well) and lysed subsequently in 1 ml 1N NaOH per well. 500 µl was used for liquid scintillation counting. Protein was measured with BioRad DC reagent (Bio-Rad) using a BSA standard series for quantification.

**Plasmids**

C-terminal GFP fusion vector pEGFP-C1 was obtained from Clontech; pcDNA3 was obtained from Invitrogen. pcDNA3 derived constructs were linearized with restriction endonuclease PvuI (Roche) prior to transfection. Coding regions of RNase H1 (GenBank accession Z97029) and RNase H2 (GenBank accession AF039652) were cloned into pEGFP-C1 or pcDNA3 via RT-PCR with proofreading Taq polymerase (primer sequences available upon request). Constructs used for expression experiments were verified by DNA sequencing using Big-Dye terminator chemistry (Perkin-Elmer) and analyzed on an ABI377 sequencer.

**RNA analysis**

Northern blot analysis of RNA was as described (29). Hybridized probe was visualized and quantified on a PhospholImager (Molecular Dynamics). cDNA fragments to be used as probe were generated by RT-PCR and subsequent cloning into the pGEM-T Easy vector (Promega). Probes used were POLR2A (GenBank accession X63564, position 1608-2078), RPA70 (GenBank accession M63488, position 1066-1718), Ha-ras (GenBank accession J00277, position 1659-3485 exo sequences only), 28S rRNA (GenBank accession M11167, position 1635-1973), and GAPDH (GenBank accession M33197, position 245-536).

**In vitro RNase H assay**

The *in vitro* RNase H assay is a combination of two protocols described in literature (34,35). Whole cell extracts were prepared as follows: exponentially growing cells were harvested by scraping, washed once in PBS, and resuspended in 100 µl hypotonic lysis buffer (7 mM Tris-
Cl pH 7.5, 7 mM KCl, 1 mM MgCl₂, 1 mM β-mercaptoethanol) per 10⁶ cells. After 10 min incubation on ice, DNA was sheared by repeated passaging through a 27 Gauge needle. Then 0.1 volume of neutralization buffer (21 mM Tris-Cl pH 7.5, 116 mM KCl, 3.6 mM MgCl₂, 6 mM β-mercaptoethanol) was added. Cell debris was removed by centrifugation for 10 min at 4 °C. The supernatant was transferred to a fresh tube on ice and glycerol was added to a final concentration of 45%. The RNase H activity in these extracts is relatively labile and susceptible to freezing or diluting of the extracts. The extracts used in one experiment were always isolated at the same time and treated in the same way. So within one experiment the ratio of the extracts of different cell lines has to be compared. Absolute levels differ between the experiments. Template RNA was prepared by in vitro transcription of linearized target plasmid construct, using T7 RNA polymerase (Promega) and the manufacturer’s protocol. Run-off RNA and complementary ODN were denatured separately by boiling for 5 min in 100 mM KCl, 0.1 mM EDTA and slowly cooled to room temperature to allow folding of the template. 50 ng template RNA and 100 ng ODN were annealed at 37 °C for 15 min in 30 μl 100 mM KCl, 0.1 mM EDTA. Then RNase H mixture was added, comprised of 8.4 μl 5x buffer (250 mM Tris-Cl pH 7.5, 50 mM MgCl₂, 1 mM DTT, 2.5 mg/ml BSA), 1 μl RNasin (20 u/μl; Promega) and 5 μl cell extract, and incubated at 37 °C for 5 min. RNA was subsequently precipitated in the presence of glycogen, after removal of proteins by phenol extraction, and dissolved in gel loading buffer containing 95% formamide. Fragments were separated on a denaturing gel (6% acrylamide, 8 M urea), electroblotted onto Hybond-N+ membrane (Amersham), and visualized by hybridization with a probe derived from the insert of the plasmid used for run-off RNA synthesis.

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