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RNase H-independent antisense activity of oligonucleotide N3′→P5′ phosphoramidates

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ABSTRACT

Oligonucleotide N3′→P5′ phosphoramidates are a new and promising class of antisense agents. Here we report biological properties of phosphoramidate oligonucleotides targeted against the human T cell leukemia virus type-I Tax protein, the major transcriptional transactivator of this human retrovirus. Isosequential phosphorothioate oligodeoxynucleotides and uniformly modified and chimeric phosphoramidate oligodeoxynucleotides containing six central phosphodiester linkages are all quite stable in cell nuclei. The uniformly modified anti-*tax* phosphoramidate oligodeoxynucleotide does not activate nuclear RNase H, as was shown by RNase protection assay. In contrast, the chimeric phosphoramidate–phosphodiester oligodeoxynucleotide is an efficient activator of RNase H. The presence of one or two mismatched nucleotides in the phosphodiester portion of oligonucleotides affected this activation only negligibly. When introduced into *tax*-transformed fibroblasts *ex vivo*, only the uniformly modified anti-*tax* phosphoramidate oligodeoxynucleotide caused a sequence-dependent reduction in the Tax protein level. Neither the chimeric phosphoramidate nor the phosphorothioate oligodeoxynucleotides significantly reduced *tax* expression under similar experimental conditions.

INTRODUCTION

Antisense oligonucleotides are currenly widely used to interfere in a sequence-specific manner with gene expression (1). Several types of backbone modifications have been developed to improve oligonucleotide stability against nucleases and to improve their affinity for RNA. However, the type of oligonucleotide chemical modification affects the potential mechanism by which it modulates gene expression. In contrast to modifications such as methylphosphonate, 2′-*O*-alkyl- or peptide oligonucleotides (PNAs), phosphorothioate oligodeoxynucleotides activate endogenous RNase H (2). For this reason, phosphorothioate oligodeoxynucleotides are often the most efficient type of antisense oligonucleotides (3,4). However, an increasing number of studies demonstrate non-specific effects of these compounds, presumably due to binding of cellular proteins or due to activation of RNase H and consequent cleavage of the mRNA sequences, which are only partially homologous (5–9). In addition, phosphorothioate oligonucleotides have relatively low affinity for RNA target sites (2).

Replacement of the 3′-oxygen by a nitrogen results in a N3′→P5′ phosphoramidate internucleoside linkage (10). Phosphoramidate oligodeoxynucleotides hybridize to complementary DNA or RNA in a sequence-specific manner with a much higher affinity when compared with the isosequential unmodified phosphodiester oligonucleotides (11). NMR studies show that despite the lack of a 2′-hydroxyl group, double helices formed between phosphoramidate oligodeoxynucleotides adopt the A-type conformation (12). However, very limited information is available about their biological properties, such as intracellular stability, potential activation of RNase H and antisense activity (13,14).

In the present study we examine the stability and gene inactivating potential of uniformly modified or chimeric phosphodiester–phosphoramidate oligodeoxynucleotides. We show that phosphoramidate internucleoside linkages confer a high degree of stability in the nuclear extract environment. In addition, we demonstrate that phosphoramidate oligonucleotides do not activate cellular RNase H, but chimeric oligomers do. Despite this, a uniformly modified phosphoramidate oligodeoxynucletide was the only compound able to specifically and selectively interfere with *tax* gene expression at low concentrations.

MATERIALS AND METHODS

Stability of oligonucleotides in nuclei suspension

Oligonucleotide phosphoramidates were prepared as described (15). Cell nuclei were isolated from *tax*-transformed mouse fibroblasts as described (16). The susceptibility of oligonucleotides to degradation by nuclear suspensions was examined by incubation in a reaction mixture (10 µl final volume) containing 300 nM 5′-^{32}P-labeled oligonucleotide, 25 mM Tris–HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$ and nuclei (8–12.5 µg protein/µl) at 37°C. Aliquots of 1 µl were withdrawn at the indicated time points, added to 2 µl stop mix (8 M urea, 50 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol) and analyzed by denaturing 20% polyacrylamide gel electrophoresis, followed by autoradiography.

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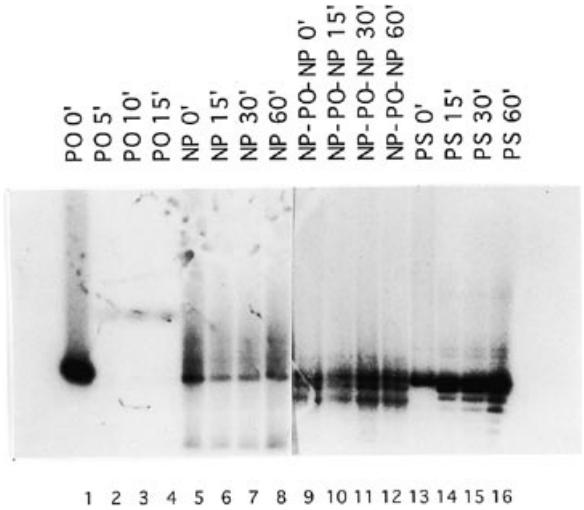

Figure 1. Stability of anti-*tax* oligodeoxynucleotides in cell nuclei suspension. Oligonucleotides (5′-^{32}P-labeled) were incubated with cell nuclei as described in Materials and Methods. Incubation times are indicated at the top of the figure. Control, oligodeoxynucleotide without incubation in cell nuclei suspension.

Induction of RNase H

Incubation of oligonucleotides with cell nuclei was performed in the presence of 25 mM Tris–HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, nuclei (8–12.5 µg protein/µl) and 1 µM oligonucleotide. After incubation for 1 h at 37°C, the RNA was isolated as described (16) and analyzed by the RNase protection assay followed by quantitation with a Pharmacia LKB Ultrascan XL laser scanner.

RNase protection assay

RNase protection assays were performed as described (16). The 227 nt run-off transcript obtained with pGEM3tax served as the probe. The L32 mRNA served as a loading control. Protected fragments were separated on a 5–10% polyacrylamide gel containing 8 M urea and analyzed by autoradiography.

Cell culture and transfections

The fibroblastic B cell line derived from LTRtax transgenic C57BL/6 mice (17) was grown in DMEM supplemented with 10% fetal calf serum at 37°C and 5% CO_2. B cells were trypsinized and adjusted to 10^5 cells/ml. They were seeded into 24-well plates at 500 µl/well and incubated overnight at 37°C, 5% CO_2 and 95% humidity. The following day the medium was aspirated, the cells were washed with serum-free DMEM (1 ml/well), followed by addition of 200 µl/well serum-free DMEM containing 30 µg/ml lipofectamine. After addition of 25 µl 20 µM oligonucleotide, the plate was incubated as above for 5 h, followed by addition of 500 µl complete medium. Protein was analyzed by immunoblotting after 16 h incubation.

RESULTS

Sugar–phosphate backbone-modified oligonucleotides in general and phosphorothioate compounds in particular exhibit superior resistance against nuclease degradation compared with unmodified phosphodiester oligomers (1). To examine the relative stabilities of the oligonucleotides in the nuclear compartment, 5′-^{32}P-labeled oligodeoxynucleotides were incubated in cell nuclei suspensions. For the unmodified oligonucleotide 1 (Table 1) incubated in nuclear extract, no full-length product could be detected after 5 min, the time point when the first aliquot was taken (Fig. 1, lane PO 5′). This is in accord with our previously published results (18) and demonstrates the extremely low stability of unmodified phosphodiester oligodeoxynucleotides under the test conditions. In contrast, compound 2, (Table 1), containing uniform phosphoramidate linkages, remained intact for at least 1 h incubation in nuclei suspension (Fig. 1, lanes NP). Similarly, the chimeric oligomer containing six central phosphodiester linkages flanked by 3′- and 5′-terminal phosphoramidate linkages was not degraded within 1 h incubation time (Fig. 1, lanes NP-PO-NP). The two shorter bands visible in these lanes are also present in the control lane, corresponding to starting material, and thus are not due to degradation during the incubation in nuclei suspension. This finding indicates that the main degrading activities in cell nuclei are exonucleases which are blocked by terminal phosphoramidate linkages. Interestingly, for an oligodeoxynucleotide containing exclusively phosphorothioate linkages (6, Table 1), a ladder of shorter bands appeared during the 1 h incubation in nuclei suspension. These shorter products have a relatively low intensity and suggest a slow degradation by at least 3′-exonucleases. This finding is not surprising, since several nucleases have a preference for one phosphorothioate P-diastereomer over the other and can cleave the corresponding phophorothioate linkage relatively efficiently (19). Since conventionally synthesized phosphorothioate oligonucleotides represent a racemic mixture, the bands may represent oligonucleotides which contained one or more phosphorothioate linkages in the cleavable configuration. Taken together, the results suggest an at least similar stability of the phosphoramidate and phosphorothioates oligonucleotides in cell nuclei extract. This does not represent differential susceptibility of the phosphodiester oligodeoxynucleotide to phosphatase, since we have demonstrated approximately equal susceptibility of phosphodiester, phosphoramidate and phosphorothioate oligonucleotides to phosphatase (13,18). Further, these stabilizing modifications are not present on the 5′-ends.

Table 1. Oligonucleotides used in this study

Number	Oligonucleotide	Type
1	5′-CCCTGGGAAGTGGGC-3′	TAXAS(PO)
2	5′-ccctgggaagtgggC-3′	TAXAS(NP)
3	5′-ccctGGGAAGtgggC-3′	TAXAS(NP-PO-NP)
4	5′-ccctGGGTAGtgggC-3′	TAXAS(NP-PO-NP)T
5	5′-ccctGGTTAGtgggC-3′	TAXAS(NP-PO-NP)TT
6	5′-CCCTGGGAAGTGGGC-3′	TAXAS(S)
7	5′-CCCTGGGTAGTGGGC-3′	TAXAS(S)T
8	5′-CCCTGGTTAGTGGGC-3′	TAXAS(S)TT
9	5′-atgggaaaatcccacA-3′	IL6κBAS(NP)
10	5′-tgtgggattttcccaT-3′	IL6κBS(NP)

Upper case letters indicate unmodified 3′-phosphodiester linkages, lower case letters 3′-phosphoramidate linkages. Phosphorothioate oligonucleotides are underlined. In each case, the corresponding modified linkage is on the 3′-side of the base. S, sense; AS, antisense. Composition is also indicated in parentheses: (PO), phosphodiester; (NP), phosphoramidate; (S), phosphhorothioate.

To investigate induction of endogenous RNase H activity by these different oligonucleotides, they were incubated in nuclei suspension and cleavage of endogenous *tax* mRNA was examined by RNase protection assay. Induction of RNase H-mediated RNA cleavage is indicated by additional protected fragments,

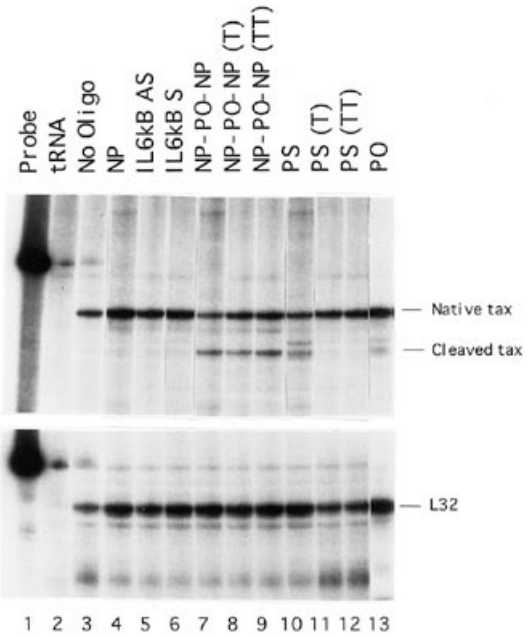

Figure 2. Activation of nuclear RNase H by anti-*tax* oligodeoxynucleotides. Oligodeoxynucleotides were incubated with cell nuclei for 1 h. The cleavage products were analyzed by RNase protection followed by denaturing polyacrylamide gel electrophoresis.

which are shorter than the fragment representing the uncleaved full-length *tax* RNA. Transcript of the ribosomal protein L32 served as a loading control. Neither the uniformly modified phosphoramidate 2 (Table 1) nor the two phosphoramidate control oligodeoxynucleotides (9 and 10, Table 1) activated RNase H (Fig. 2). The lack of induced cleavage by phosphoramidate 2 very likely reflects the different conformational form of this class of oligonucleotides, which form a double helix of the A-type with complementary RNA (11). As a result, RNase H may not recognize such hybrids. In contrast, the chimeric oligonucleotide 3 (Table 1) induces RNase H, as indicated by the distinct shorter band corresponding to the cleavage products (Fig. 2). In the case of both the phosphorothioate oligodeoxynucleotide 6 and of the unmodified 1 (Table 1), two more diffuse bands are visible, suggesting two preferential cleavage sites within the formed heteroduplexes. The second cleavage band suggests that the latter two oligonucleotides permit RNA cleavage by RNase H over their entire complementary sequence, whereas the chimeric NP-PO-NP oligomer restricts cleavage to the central six base pairs of the duplex. The second, longer fragment indicates cleavage close to the 3′-terminus of the oligonucleotide. The chimeric oligonucleotide does not support RNase H-mediated cleavage at this site because it contains phosphoramidate linkages in this region. The lower amount of cleavage products in the case of unmodified phosphodiester oligonucleotide 1 (Table 1) may be explained by its low hydrolytic stability in nuclei suspension (Fig. 1). To compare the sequence specificity of RNase H cleavage by the chimeric oligonucleotides with that of uniform phosphorothioate oligonucleotides, one or two central A→T or G→T mismatches were introduced. Phosphorothioate oligonucleotides bearing one or two central mismatches could no longer activate RNase H. In contrast, only minor reductions in RNA cleavage were observed for mismatched chimeric phosphoramidate oligonucleotides. Thus, chimeric phosphoramidate oligodeoxynucleotides containing at least six central unmodified phosphodiester linkages are able to support nuclear RNase H cleavage. However, they recruit this activity with lower sequence specificity than phosphorothioate oligodeoxynucleotides. This could result from the higher duplex T_m values conferred by the phosphoramidate flanking sequences. Such stabilization has been demonstrated to be ~1.9°C per linkage (10,11,13).

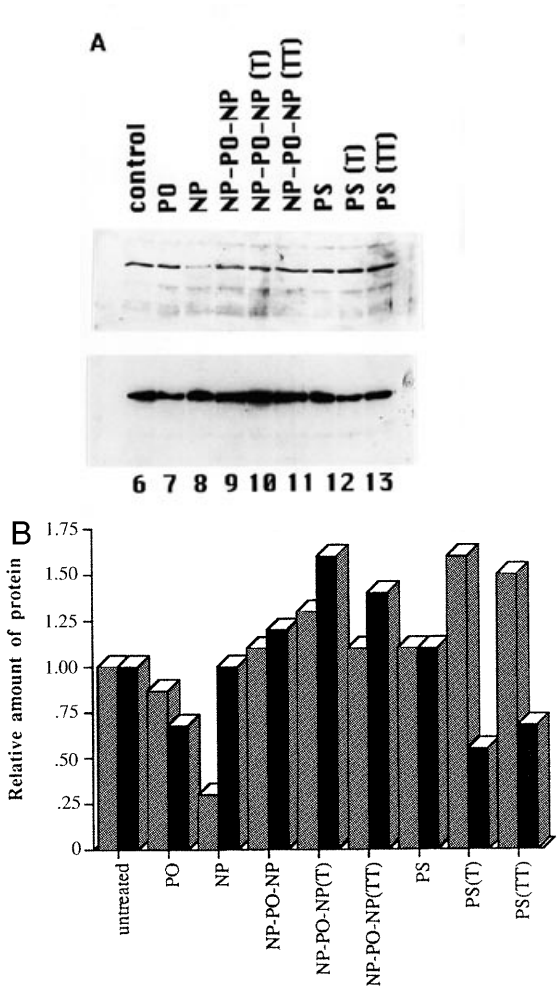

Figure 3. Inhibition of Tax expression by anti-*tax* oligodeoxynucleotides in cell culture. Cells were incubated with oligodeoxynucleotide–lipofectamine complexes as described in Material and Methods. After 16 h, cells were lysed and protein expression was analyzed by immunoblotting with anti-Tax antibody followed by reblotting with anti-RelA antibody. (**A**) Immunoblot with anti-Tax antibody or anti-RelA antibody. (**B**) Graphic representation of the protein band intensities. Gray columns, Tax protein; black columns, RelA protein.

To examine the inhibitory effects of the modified oligonucleotides in cell culture, oligomers were transfected with the cationic lipid lipofectamine into *tax*-transformed mouse fibroblasts. Results were reproduced three times. The half-life of HTLV Tax protein in this cell line is ~4 h (M.Nerenberg, unpublished results). Therefore, the amount of Tax protein was analyzed by immunoblotting 16 h after transfection. To control for protein loading and for specificity of inhibition, the blots were re-probed with RelA-specific antibodies. The intensities of the Tax bands were determined by laser densitometry and compared with the corresponding RelA intensities. Generally, the intensities of RelA bands varied within a factor of two. In a first experiment the ability of the different

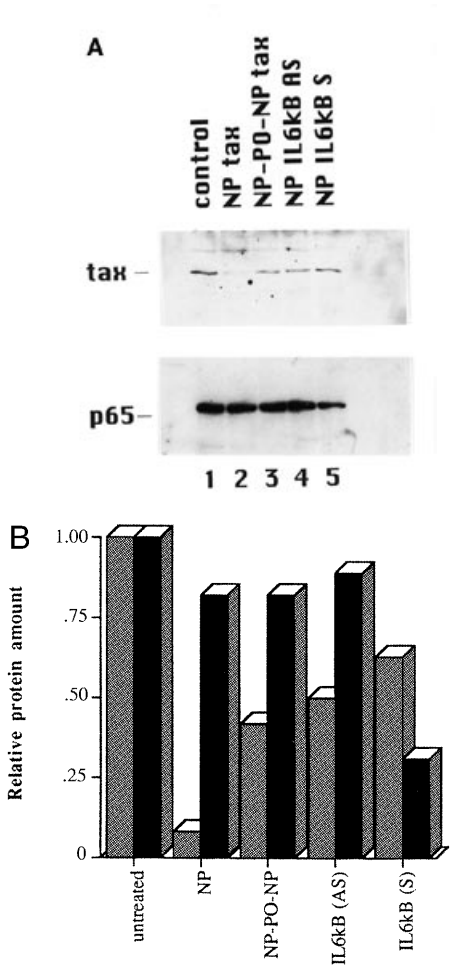

Figure 4. Sequence dependence of Tax inhibition by TAXAS(NP). Cells were treated as in Figure 3. (**A**) Immunoblot with anti-Tax antibody or anti-RelA antibody. (**B**) Graphic representation of the protein band intensities. Gray columns, Tax protein; black columns, RelA protein.

anti-*tax* oligonucleotides was examined. Neither the unmodified 1 nor any of the phosphorothioate or chimeric phosphoramidate oligodeoxynucleotides (3–8) caused any significant reduction in Tax protein (Fig. 3). In contrast, the antisense oligonucleotide containing exclusively phosphoramidate linkages (2) reduced the level of Tax protein to 30% when compared with untreated cells. The amount of RelA was exactly the same as in the control. Further, we were unable to detect any change in *tax* mRNA levels in phosphoramidate-treated cells. Thus, the inhibition of *tax* expression by this phosphoramidate antisense oligodeoxynucleotide was not RNase H dependent, as this oligonucleotide did not induce this activity in nuclei suspension.

In the second set of experiments, the sequence specificity of this observed Tax reduction was examined. This time, oligonucleotide 2 reduced Tax to 8% compared with the untreated control (Fig. 4). Again, the amount of RelA remained essentially unaffected at 82% of the control value. Transfection of *tax*-transformed cells with the chimeric oligomer 3 resulted in a 2-fold reduction in Tax. However, two control phosphoramidate oligonucleotides containing similar sequence motifs (3, 9 and 10) caused a similar decrease in the amount of Tax to 50 and 60% respectively. Therefore, this 2-fold reduction is very likely a sequence-non-specific effect or, alternatively, represents experimental variations between the different controls. However, even after a correction for this factor of two, inhibition by anti-*tax* phosphoramidate 2 was ~5-fold and agrees with the >3-fold reduction in the first experiment. Thus, the inhibition of *tax* expression by uniformly modified phosphoramidates appears to be sequence specific.

DISCUSSION

Phosphoramidate oligodeoxynucleotides represent a new class of potential antisense compounds with high affinity for complementary RNA and DNA oligomers (11). In the present study we compared the stability of oligonucleotides containing phosphoramidate linkages with those of unmodified or phosphorothioate oligodeoxynucleotides in intact cell nuclei. Under such conditions, the unmodified control oligodeoxynucleotide was degraded within 5 min incubation (Fig. 1). Similar to the phosphorothioate control, the corresponding phosphoramidate oligodeoxynucleotide remained completely intact after up to 1 h incubation. This shows that phosphoramidate internucleoside linkages confer high stability against nucleases present in cell nuclei. An oligonucleotide containing five consecutive phosphoramidate linkages at both the 5′- and the 3′-termini exhibited comparable stability, which points to exonucleases as being the major oligonucleotide degrading activities in cell nuclei. This conclusion is also supported by several other studies employing oligonucleotides bearing terminal modifications such as phosphorothioate or methylphosphonate linkages (18,20,24). Indeed, we have demonstrated 5′→3′ exonuclease to be important in nuclei (18). In contrast, data obtained with oligonucleotides injected into mice suggest that terminal phosphorothioate linkages are not sufficient to improve the stability or, consequently, bioavailability of oligonucleotides *in vivo* (22). Similar studies remain to be performed for phosphoramidate oligonucleotides.

Heteroduplexes formed by phosphoramidate and RNA strands are apparently not substrates for mouse nuclear RNase H. Similar results have been reported using a bacterial enzyme (14). In this respect, phosphoramidate oligodeoxynucleotides resemble compounds such as PNAs, methylphosphonates and 2′-*O*-alkyl oligonucleotides (2,23). However, an open question is whether the induction of RNase H by an antisense oligonucleotide is necessary for efficient inhibition of gene expression or whether binding of the oligonucleotide to its target sequence with high affinity is sufficient to achieve this goal. Several previous studies support the first assumption: oligonucleotides which could induce RNase H-mediated RNA degradation appeared much more potent inhibitors of gene expression than those not able to support RNase H (3,4). This could be complicated by differences in affinity. Further, this poses a dilemma for any exogenous antisense approach. On the one hand, from a statistical point of view, oligonucleotides of at least 15 nt in length may guarantee recognition of unique sites in the human mRNA pool but may have low T_m values. On the other hand, longer complementary sequences more easily tolerate single mismatches, thereby most likely reducing the specificity of the antisense oligonucleotide (24). Thus, an oligonucleotide which induces RNase H might cause degradation not only of its specific target RNA but also of other RNAs containing related sequence motifs (25,26).

One possible approach to solve this dilemma is the use of chimeric oligonucleotides. A central sequence containing 2′-deoxynucleoside moieties linked by phosphodiester or phosphorothioate linkages could be flanked by stretches of 2′-modified nucleoside moieties or, for example, methylphosphonate linkages. In this case only the

central part would induce RNase H, but not the flanking sequences. Studies by Monia *et al.* suggest that a 5 nt central stretch is sufficient to induce RNase H efficiently (3). Thus, one might expect that shortening the part relevant for RNase H induction to 5 nt might result in increased specificity of RNase H induction. Indeed, data obtained by Giles *et al.* (21) with chimeric anti-*bcr–abl* oligodeoxynucleotides containing five to nine central phosphodiester linkages flanked by methylphosphonate linkages support this.

In the present study, we compared phosphodiester, phosphorothioate and chimeric phosphoramidate oligodeoxynucleotides for their ability to elicit mammalian RNase H. Like the former two oligodeoxynucleotides, the chimeric phosphoramidate oligodeoxynucleotide containing six central phosphodiester linkages flanked by phosphoramidate linkages induced RNase H-mediated degradation of the endogenous *tax* transcript in cell nuclei suspension (Fig. 3). In addition, chimeric phosphoramidate oligodeoxynucleotides with central T-U or C-U plus T-U mismatches respectively still caused significant RNase H-mediated cleavage of *tax* RNA. In contrast, a completely phosphorothioate modified oligodeoxynucleotide of the same mismatched sequence could not induce degradation of *tax* RNA, despite its longer 'recognition surface' for RNase H. Whereas phosphorothioate linkages decrease the affinity of an antisense oligodeoxynucleotide for its complementary RNA, phosphoramidate linkages increase the T_m of the heteroduplex by ~2°C per residue (11). Thus, the lower specificity of RNase H-mediated RNA degradation in the chimeric molecules may be due to the superior stabilities of phosphoramidate–RNA duplexes compared with those of the phosphorothioate–RNA duplex. This implies that the amount of RNase H activation may be dependent on the overall stability of the heteroduplex and not directly on the number of RNase permissive Watson–Crick base pairs formed between oligonucleotide and target RNA. Similar results obtained with chimeric 2′-*O*-alkyl- and methylphosphonate oligonucleotides have recently been published by Larrouy *et al.* (26). Thus, activation of endogenous RNase H may be a poor choice for a mechanism for specific reduction of gene expression by antisense oligonucleotides, especially given its irreversible nature.

Finally, we compared the intracellular efficacy of RNase H-activating oligonucleotides with those of phosphoramidate oligodeoxynucleotides unable to activate RNase H. The oligonucleotides were introduced into *tax*-transformed fibroblasts with cationic lipids. At moderate concentrations neither the phosphodiester, the phosphorothioate nor the chimeric phosphodiester–phosphoramidate oligodeoxynucleotides caused a significant reduction in Tax protein (Fig. 4). It is not clear whether this lack of function is caused by a low intracellular stability and/or insufficient affinity of such oligonucleotides for their target RNAs. Alternatively, the intracellular concentration of phosphorothioate oligonucleotides able to bind to their target sequences may be reduced by non-specific interactions with cellular proteins (6,9). Only the completely modified phosphoramidate oligodeoxynucleotide 2 (Table 1) was shown to reduce the amount of Tax by 70–90% compared with cells treated with cationic lipids alone. Control phosphoramidate oligodeoxynucleotides containing similar purine-rich sequence motifs, such as GGGA or TGGG, had a <2-fold effect on Tax protein, suggesting a sequence-specific antisense effect by complementary phosphoramidate oligodeoxynucleotides (Fig. 4B). The mechanism of inhibition by phosphoramidates may be interference with RNA editing, processing, transport or steric blockage of the translational machinery (2).

The presented results show that antisense phosphoramidate oligodeoxynucleotides may mediate a potent RNase H-independent inhibition of gene expression. It also implies that RNase H-mediated RNA degradation may not be necessary for efficient inhibition of gene expression by antisense oligonucleotides. High RNA affinity conferred by a phosphoramidate oligonucleotide may be sufficient to effectively interfere with gene expression. However, other properties of phosphoramdiate oligonucleotides, such as decreased non-specific adsorption to proteins, may confer additional advantageous properties (13).

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