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Sequence-specific RNase H cleavage of *gag* mRNA from HIV-1 infected cells by an antisense oligonucleotide *in vitro*

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ABSTRACT

We have used a ribonuclease protection assay to investigate RNase H cleavage of HIV-1 mRNA mediated by phosphorothioate antisense oligonucleotides complementary to the *gag* region of the HIV-1 genome *in vitro*. Cell lysate experiments in H9 and U937 cells chronically infected with HIV-1 IIIB showed RNase H cleavage of unspliced *gag* message but no cleavage of spliced message which did not contain the target *gag* region. RNase H cleavage products were detected at oligonucleotide concentrations as low as 0.01 μM and the RNase H activity was seen to be concentration dependent. Similar experiments with 1-, 3- and 5-mismatch oligonucleotides demonstrated sequence specificity at low concentrations, with cleavage of *gag* mRNA correlating with the predicted activities of the parent and mismatch oligonucleotides based on their hybridization melting temperatures. Experiments in living cells suggested that RNase H-specific antisense activity was largely determined by the amount of oligonucleotide taken up by the different cell lines studied. RNase H cleavage products were detected in antisense oligonucleotide treated MT-4 cells acutely infected with HIV-1 IIIB, but not in infected H9 cells treated with oligonucleotide under the same conditions. The data presented demonstrate potent and specific RNase H cleavage of HIV-1 mRNA mediated by an antisense oligonucleotide targeted to HIV-1 *gag* mRNA, and are in agreement with previous reports that the major obstacle to demonstrating antisense activity in living cells remains the lack of penetration of these agents into the desired cellular compartment.

INTRODUCTION

During the past decade there have been numerous publications showing *in vitro* inhibition of HIV replication by antisense oligonucleotides through various suggested mechanisms of action (1–4). However, despite these initially promising data, the application of antisense oligonucleotide therapy for the potential treatment of HIV infection is an area of research that has received a number of setbacks. The non-specific effects of phosphorothiote oligonucleotides on virus adsorption make interpretation of many *in vitro* antiviral assays difficult (5–7). This has led to questions being raised as to whether these agents are actually working through a true antisense mechanism of action (8). Futhermore, initial *in vivo* studies have suggested problems with both the efficacy and toxicity of the oligonucleotides.

The phosphorothioate oligonucleotides have been shown to specifically modulate gene expression in a number of studies and numerous possible mechanisms of action have been suggested (1–4,6). Hybridization of antisense oligonucleotides to target mRNA molecules may interfere with RNA splicing or processing, may inhibit translation by either preventing the initiation of translation or blocking the progression of ribosomes along the mRNA, or may cause cleavage of RNA through an RNase H-mediated mechanism. Evidence for the involvement of RNase H in the antisense activity of the phosphorothioate oligonucleotides has been shown in relatively few studies and has only been shown in artificial sytems such as the microinjection of oligonucleotides into cells or treatment of cells with streptolysin O (9–12). A number of studies have demonstrated RNase H cleavage in cell lysate systems though these have used the addition of exogenous *Escherichia coli* RNase H, as opposed to endogenous cellular human RNase H (13–15). The majority of these studies have been with oligonucleotides directed to targets other than the HIV-1 genome.

We have shown previously a sequence-specific synergistic interaction between a 25mer phosphorothioate oligonucleotide complementary to the *gag* initiation site of HIV-1 and the nucleoside analog drugs in a cytoprotection-based assay in MT-4 cells (7). Although these results suggested an antisense component to the oligonucleotide anti-HIV activity, this study did not define the mechanism of action by which the oligonucleotides were exhibiting their antiviral effects.

In the current study we have established a ribonuclease protection assay to allow us to look for effects of oligonucleotides targeted to HIV-1 *gag* mRNA with respect to RNase H-mediated cleavage of the target sequence. Cell lysate experiments were carried out to look at the effects of oligonucleotide concentration and sequence specificity on RNase H-mediated cleavage and acute infection experiments with HIV-1 IIIB were performed to

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determine whether or not RNase H cleavage products could be detected in living cells.

MATERIALS AND METHODS

Oligodeoxynucleotides

Phosphorothioate oligonucleotides targeted to the *gag* region of HIV-1 IIIB and mismatch control oligonucleotides were synthesized by standard methods (4). The purity of the oligonucleotides was confirmed by polyacrylamide gel electrophoresis, hybridization melting temperature and ^{31}P-NMR analysis. For specific oligonucleotide HIV-1 IIIB *gag* complementary sequences and mismatch positions of control oligonucleotides see Table 1.

Cells and virus

The H9 T-lymphoblastoid cell line was originally obtained from R. Gallo (National Cancer Institute, Bethesda, MD). U937 cells, the human monocytic cell line obtained from a patient with histiocytic lymphoma, were acquired from the American Type Tissue Culture Collection (CRL 1593). CEM-SS and MT-4 cells were obtained from the AIDS Research and Reference Reagent Bank (Division of AIDS, NIAID, NIH), contributed by P. L. Nara and D. D. Richman, respectively.

Cell cultures were propogated in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% (U937, CEM-SS and MT-4 cells) or 20% (H9 cells) heat-inactivated fetal bovine serum (Sigma Chemical Co., St Louis, MO), 250 U/ml penicillin, 250 µg/ml streptomycin and 2 mM L-glutamine at 37°C in a 5% CO_2-gassed incubator.

HIV-1 IIIB was originally obtained from Dr Robert Gallo, National Cancer Institute. Virus stocks of HIV-1 were prepared from cell-free supernatant of chronically infected H9 cultures by the shaking method as described previously (16). H9 and U937 cells chronically infected by HIV-1 IIIB were maintained in continuous culture for at least 3 months after initial infection. The stability of the virus infection in the cultures was monitored by sequential p24 ELISA antigen measurements (NEN™ Life Science Products, Boston, MA).

Cell lysate assays

H9 and U937 cells, chronically infected with HIV-1 IIIB, were centrifuged at 300 *g* and washed in ice-cold buffer (100 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA and 50 mM tricine, pH 7.4). Washed cells (~5×10^6/sample) were resuspended in 50 µl of an 8:1:1 (by volume) mixture of buffer, Nonidet P-40 (NP-40) and RNase Block (40 U/µl; Stratagene, La Jolla, CA) containing antisense or mismatch control oligonucleotides (1 nM to 10 µM) and were incubated for 15 min at 37°C.

HIV-1 IIIB acute infection in H9 and MT-4 cells

H9 and MT-4 cells were incubated with HIV-1 IIIB under post-adsorption assay conditions as described previously (7). Briefly, MT-4 or H9 cells (1×10^7 cells/ml) were preincubated with a 500× $TCID_{CPE-90\%}$ concentration of HIV-1 for 4 h at 37°C, 5% CO_2 in the absence of oligonucleotides, washed to remove non-adsorbed virus, then added to 6-well plates (1×10^6 cells/well) containing antisense or mismatch control oligonucleotides (0.1–10 µM) and incubated for up to 4 days at 37°C, 5% CO_2. Anti-HIV activity was determined by supernatant p24 antigen measurement using HIV-1 p24 ELISA (NEN Life Science Products, Boston, MA) and cell viability was monitored by trypan blue dye exclusion and the MTT dye method (17).

Ribonuclease protection assay

The probe for the ribonuclease protection assay was designed to include the HIV-1 *gag* region and was obtained from the *Hin*dIII–*Acc*I fragment of the HXB2 clone (bases 531–959, with the numbering starting at the 5′ end of the LTR). This region was cloned into a vector containing an SP6 promoter and a biotin-labeled probe of 494 bp in length was produced by *in vitro* transcription using a T7 RNA polymerase (Ambion BrightStar BIOTINscript). The RNA probe was analysed and purified by electrophoresis on a 5% acrylamide, 8 M urea denaturing gel and then eluted by overnight incubation in elution buffer [0.5 M $NH_4(OAc)_2$, 1 mM EDTA, 0.2% SDS]. The elution buffer containing the probe was extracted once with an equal volume of water-saturated phenol and the probe was precipitated with ethanol and resuspended in nuclease-free water. Aliquots of the probe were stored at –80°C prior to use in the ribonuclease protection assay.

Cells treated with antisense or mismatch control oligonucleotides were collected by centrifugation at 300 *g* and lysed by the addition of cell lysis/denaturation solution (1×10^7 cells/ml) followed by vigorous vortexing. Cell lysates were stored at –20°C prior to analysis by ribonuclease protection assay.

HIV-1 *gag* probe (1000 pg) was mixed with 10 µl cell lysate (equivalent to total cellular RNA from 1×10^5 cells) and the ribonuclease protection assay was performed using the Direct Protect kit following the manufacturers' instructions (Ambion Inc., Austin, TX). The products of the ribonuclease protection assay were separated for analysis on a denaturing, polyacrylamide gel, transferred to a positively charged nylon membrane by electroblotting and the nucleic acid immobilized on the membrane by UV crosslinking. The biotinylated probe was then detected using a non-isotopic chemiluminescent secondary detection procedure (BioDetect kit, Ambion).

Densitometry

Ribonuclease protection assay data were analysed using an IS-2000 Digital Imaging System (Innotech Scientific Corp., San Leandro, CA).

RESULTS

The oligonucleotide concentration dependence and sequence specificity of RNase H cleavage of HIV-1 *gag* mRNA were defined with oligonucleotide 1 (GEM 91®). Figure 1 shows the HIV complementary region of the probe (531–959 nt) and the oligonucleotide complementary region (776–801 nt). As the protected region of the probe incorporates the major splice donor site at 743 nt, the probe is complementary to a 212 base region of the spliced message (543 nt to the splice donor site at 743 nt) as well as 428 bases of the unspliced *gag-pol* message. If GEM 91® hybridized to the unspliced message and mediated RNase H cleavage, generation of probe-complementary fragments of 245 nt (531–776 nt) and 158 nt (801–959 nt) would be expected. As the GEM 91® complementary region is located 3′ to the splice donor site, the oligonucleotide should not mediate RNase H cleavage of

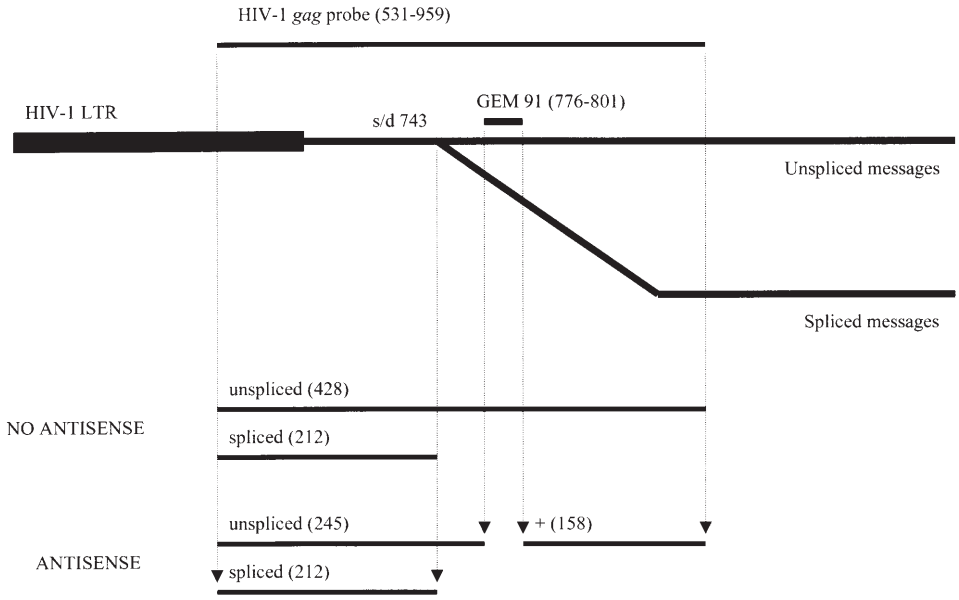

Figure 1. Design of probe for use in the ribonuclease protection assay detection of HIV-1 unspliced and spliced mRNA. Target sequence of oligonucleotide 1 (GEM 91®) is shown (776–801 nt) along with the expected mRNA cleavage products following antisense activity. Oligonucleotides 5 and 6 are complementary to sequences at 833–858 and 891–916 nt of the HIV-1 *gag* gene, respectively.

the spliced message. This unspliced message contains 212 bases complementary to the probe and serves as an internal control for all the ribonuclease protection assays carried out in these studies.

Cell lysate experiments were carried out to look at the concentration dependence of oligonucleotide 1 on RNase H cleavage of *gag* mRNA. The predicted RNase H cleavage products (245 and 158 nt) were detected at an oligonucleotide concentration as low as 0.01 µM in U937 cells chronically infected with HIV-1 IIIB (Fig. 2). With increasing concentrations of oligonucleotide, a gradual decrease in the unspliced *gag* message at 428 nt was observed. Complete cleavage of this unspliced message was seen at an oligonucleotide concentration of 10 µM. The spliced message, indicated by the band at 212 nt, is seen to be unaffected by oligonucleotide 1 and acts as an internal control for the assay. Similar RNase H cleavage patterns were observed in cells that were preincubated with oligonucleotide for 4 h and then washed to remove extracellular oligonucleotide prior to addition of NP-40 (data not shown). Comparable results were seen in T-lymphoblastoid H9 cells chronically infected with HIV-1 IIIB.

In order to look at the sequence-specificity of the RNase H-mediated cleavage of HIV-1 *gag* mRNA, cell lysate experiments were carried out with 1-, 3- and 5-mismatch oligonucleotides (oligonucleotides 2, 3 and 4, respectively). At an oligonucleotide concentration of 0.01 µM, i.e. the lowest concentration at which oligonucleotide 1 was shown to stimulate RNase H activity, no activity was seen in U937 cells chronically infected with HIV-1 IIIB with any of the mismatch oligonucleotides (Fig. 3). Further experiments were carried out to investigate the activity of a range of concentrations of oligonucleotides 2, 3 and 4 in comparison with oligonucleotide 1. Results showed that at higher concentrations, the mismatch oligonucleotides did exhibit RNase H cleavage of *gag* mRNA with the 1-mismatch oligonucleotide having a greater effect than the 3-mismatch, which in turn had a greater effect than the 5-mismatch oligonucleotide (Fig. 4). For example, at an oligonucleotide concentration of 10 µM, oligonucleotide 1 caused

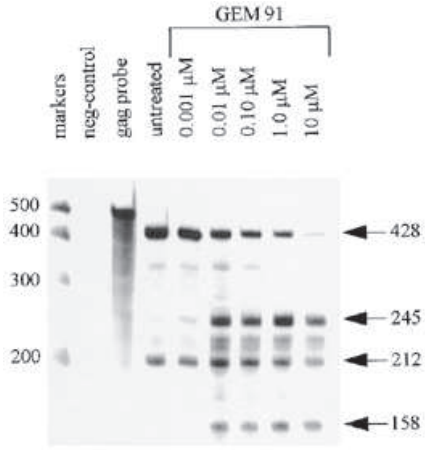

Figure 2. Ribonuclease protection assay analysis of U937 cells chronically infected with HIV-1 IIIB following treatment with NP-40 and incubation with increasing concentrations of oligonucleotide 1 (GEM 91®) for 15 min. The negative control lane represents uninfected U937 cell lysate, lane 3 shows undigested full length probe (494 nt) and lane 4 shows cell lysate with no oligonucleotide treatment.

total cleavage of unspliced *gag* messsage, the 1-mismatch oligonucleotide caused ~80% cleavage, the 3-mismatch 30% cleavage and the 5-mismatch <20% cleavage. This data is in agreement with the predicted activities of the four oligonucleotides based on their hybridization melting temperatures (Table 1).

Cell lysate experiments were also carried out with phosphorothioate oligonucleotides targeted to different regions of the HIV-1 *gag* gene. Oligonucleotides 5 and 6 (Table 1) showed comparable activity to oligonucleotide 1 whose target region included the *gag* translation initiation site. RNase H cleavage products observed following incubations of oligonucleotides 5 and 6 with HIV-1 IIIB chronically infected H9 cell lysates were as would be predicted from

Table 1. Antisense and control phosphorothioate oligonucleotide sequences, complementary HIV-1 HXB2 target regions and melting temperatures

No.	Oligonucleotide Sequence	HXB2 nt	T_m (°C)
1	d(CTC TCG CAC CCA TCT CTC TCC TTC T)	776-801	67.2
2	d(CTC TCG CAC CCA T**A**T CTC TCC TTC T)	-	58.9
3	d(CTC TCG C**T**C CCA T**A**T CTC **A**CC TTC T)	-	47.2
4	d(CT**A** TCG C**T**C CCA T**A**T CTC **A**CC T**G**C T)	-	38.6
5	d(CTG GCC TTA ACC GAA TTT TTT CCC A)	833-858	ND
6	d(CGT TCT AGC TCC CTG CTT GCC CAT A)	891-916	ND

Control oligonucleotide mismatches (oligonucleotides 2, 3 and 4) are shown underlined in bold. ND, not determined.

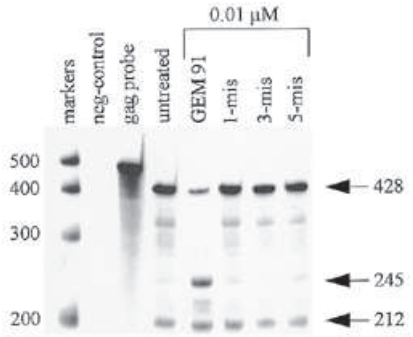

Figure 3. Ribonuclease protection assay analysis of U937 cells chronically infected with HIV-1 IIIB following treatment with NP-40 and incubation with oligonucleotide 1 (GEM 91®) or oligonucleotides 2, 3 and 4 (1-, 3- and 5-mismatch oligonucleotides, respectively) for 15 min at a concentration of 0.01 μM.

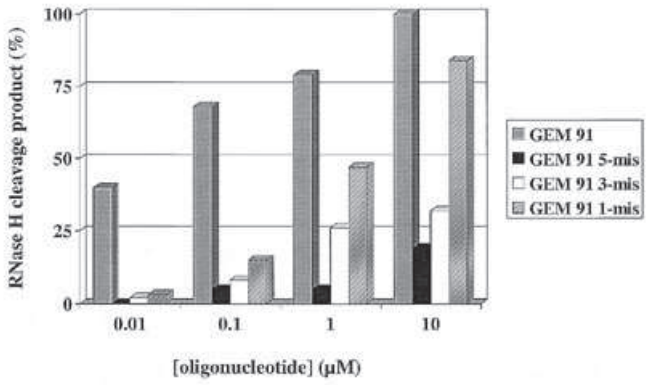

Figure 4. Densitometric analysis of RNase H cleavage of HIV-1 unspliced *gag* mRNA by oligonucleotide 1 (GEM 91®) and oligonucleotides 2, 3 and 4 (1-, 3- and 5-mismatch oligonucleotides, respectively) following treatment of U937 cells chronically infected with HIV-1 IIIB with NP-40. Data shown are from a single experiment.

their target regions (Table 1). A cleavage product of ~302 nt (complementary to region of *gag* probe from 531 to 833 nt) was observed with oligonucleotide 5 and a cleavage product of ~360 nt (531–891 nt) was generated with oligonucleotide 6 (Fig. 5).

Experiments carried out in H9 and MT-4 cells acutely infected with HIV-1 IIIB under post-adsorption conditions (Materials and Methods) gave contrasting results. The effect of oligonucleotide 1 on acutely infected H9 cells is shown in Figure 6. A delay in production of viral *gag* mRNA was seen as determined by ribonuclease protection assay over a period of 96 h under these assay conditions. Viral message was first detected at 48 h post-infection in untreated cells as compared with 72 h in cells treated with oligonucleotide 1 (10 μM). This was mirrored by a significant decrease in production of p24 antigen in this assay as measured by ELISA, with a 59% decrease in p24 levels seen at 72 h with 10 μM oligonucleotide and a 73% decrease after 96 h. The pattern of the bands in the ribonuclease protection assay did not give any indication of specific RNase H cleavage of HIV-1 mRNA in H9 cells under these assay conditions, only a reduction in the overall rate of infection.

Ribonuclease protection assay data from MT-4 cells acutely infected with HIV-1 IIIB and treated with oligonucleotide 1 are shown in Figure 7. Following a 48 h incubation with oligonucleotide 1 (10 μM), the RNase H cleavage product (245 nt) was detected and this correlated with a small decrease in unspliced *gag* message (428 nt); again the spliced message at 212 nt was unaffected by oligonucleotide 1 and acts as a control for the assay. In this case, specific RNase H-mediated cleavage of HIV-1 mRNA was observed within the infected cells. No cytotoxicity was observed under these assay conditions as monitored by trypan blue dye exclusion and MTT cell viability assays.

DISCUSSION

Many previous studies have shown anti-HIV activity of phosphorothioate antisense oligonucleotides by monitoring the production of p24 antigen, measuring viral mRNA levels or using cytoprotection-based assays (4,6,7,18,19). Although a number of these studies have exhibited differences in efficacy between antisense and control oligonucleotides, very few have shown any confirmation that an antisense mechanism of action was involved. We have used a ribonuclease protection assay to look at RNase

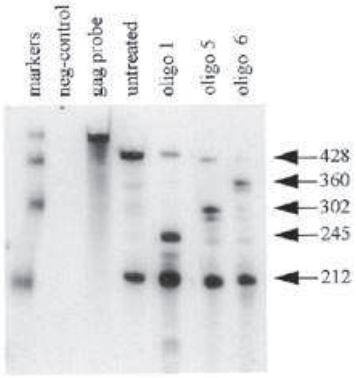

Figure 5. Ribonuclease protection assay analysis of H9 cells chronically infected with HIV-1 IIIB following treatment with NP-40 and incubation with oligonucleotide 1 (GEM 91®) or oligonucleotides 5 and 6 (HIV-1 HXB2 target regions 776–801, 833–858 and 891–916 nt, respectively) for 15 min at a concentration of 0.10 μM.

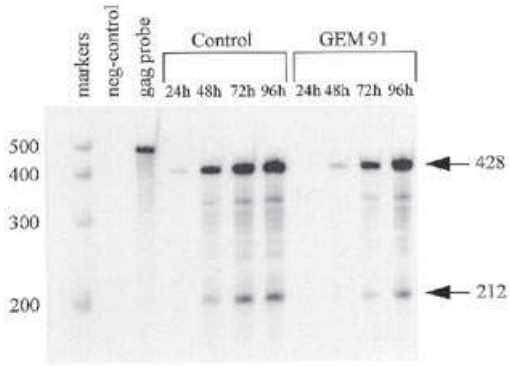

Figure 6. Ribonuclease protection assay analysis of H9 cells acutely infected with HIV-1 IIIB following incubation with or without oligonucleotide 1 (GEM 91®) for 24–96 h.

H-mediated cleavage by antisense phosphorothioate oligonucleotides targeted to the *gag* region of the HIV-1 genome, in both cell lysate assays and in living cells infected with virus.

Initial experiments were carried out using a cell lysate system to determine whether or not RNase H activity could be observed using RNA obtained from infected cells. This type of system established that HIV-1 mRNA was susceptible to cleavage and that adequate amounts of RNase H were available in the cell. This indicates that the *gag* initiation region is an appropriate target for antisense regulation and is not protected by RNA secondary structure or protein binding within the cell. Results indicated that RNase H-mediated cleavage of unspliced *gag* message could be seen at oligonucleotide concentrations as low as 0.01 μM, with complete cleavage occurring at 10 μM. Control oligonucleotides with 1, 3 and 5 base mismatches displayed decreasing activities in experiments carried out under identical conditions. It should be noted that no cleavage products were seen in control experiments where oligonucleotides were added after addition of cell lysis solution to the cells, this verifies that the oligonucleotide and RNase H are not active during the isolation process and that the cleavage product was generated under the assay conditions described.

The efficacy of the antisense oligonucleotides is thought to be correlated with the affinity with which it binds to its target mRNA, this can be characterized by the melting temperature (T_m)

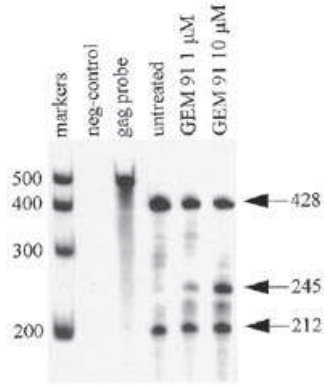

Figure 7. Ribonuclease protection assay analysis of MT-4 cells acutely infected with HIV-1 IIIB following incubation with or without oligonucleotide 1 (GEM 91®) for 48 h.

of the double-stranded nucleic acid that is formed (20,21). As the T_m value is largely determined by the concentration of the oligonucleotide, it is not surprising that some degree of RNase H cleavage is seen with the mismatch oligonucleotides. Indeed it is remarkable that at low concentrations a clear difference in antisense activity can be shown between two 25mer oligonucleotides differing in sequence merely by a single base change. Experiments with oligonucleotides targeted to different regions of the *gag* gene showed similar activity in mediating RNase H cleavage and cleavage products of the predicted sizes were produced. As well as providing further validation for the ribonuclease protection assay used in these experiments, these results also show that oligonucleotide targets that do not contain the translation initiation site of the gene are also suitable targets for antisense-mediated cleavage, this is in agreement with previous reports (22,23).

While the information obtained from these experiments clearly highlights the potential problems of oligonucleotide cellular uptake and availability of the oligonucleotide to the target RNA, the data suggest that if this barrier is removed (for instance by treating the cells with NP-40) then the drugs will indeed have their desired effect. These studies show that RNase H cleavage of unspliced *gag* message is observed without the need for addition of exogenous RNase H or the use of unrealistic oligonucleotide concentrations. RNase H cleavage was also observed in cells that were preincubated with oligonucleotide for 4 h and then washed to remove extracellular oligonucleotide prior to addition of NP-40 with no further addition of drug, i.e. the RNase H cleavage was brought about by oligonucleotide already taken up by the cells. This is in agreement with suggestions that phosphorothioate oligonucleotides are taken up into cells in sufficient amounts to have an antisense effect, but that the oligonucleotides may be bound in endosomes or vesicles and are therefore not able to interact with the target mRNA and/or cellular RNase H (24,25). However, although it is generally accepted that lipid carriers are required in the majority of cases to obtain antisense activity of oligonucleotides in cell culture assays, this may not be the case in *in vivo* studies (26–29).

Further experiments were carried out to determine whether or not RNase H cleavage could be observed in living cells acutely infected with virus. Two different T-lymphoblastoid cell types infected under post-adsorption conditions with the same strain of HIV-1 IIIB were studied and produced very different results.

Results from experiments with acutely infected H9 cells indicated a delay in replication of HIV-1 IIIB in H9 cells, with *gag* message being detected at 24–48 h post-infection in untreated cells but not until 48–72 h in cells treated with oligonucleotide 1 at a concentration of 10 μM. There was no indication of RNase H cleavage in this assay, suggesting that the effect of oligonucleotide 1 in inhibiting virus replication in acutely infected H9 cells was due to a non-antisense effect, or at least through a mechanism other than RNase H-mediated cleavage of viral message. This could be similar to sequence-independent oligonucleotide inhibition seen in other studies (6,7,30).

Experiments carried out with MT-4 cells, however, produced very different results. During the first 48 h following infection with virus, there was a small decrease in unspliced *gag* message and a corresponding production of the predicted RNase H cleavage product at 245 nt. This effect was not seen in untreated cells and was seen to be more marked at an oligonucleotide concentration of 10 μM than at 1 μM. We believe that this is the first evidence of RNase H activity mediated by antisense oligonucleotides in living cells without manipulation of cells to enable intracytoplasmic delivery of the oligonucleotides.

In light of our results showing RNase H activity at oligonucleotide concentrations as low as 0.01 μM in cell lysate assays, it is not surprising that some activity is seen in living cells despite the problems associated with cellular uptake of these agents. Previous reports have suggested that significant amounts of oligonucleotides can be taken up into cells but that they are compartmentalized in endosomes and antisense activity is not seen because the rate of efflux of oligonucleotide from the endosome to the cytoplasm is very slow (24). This would explain why we do not see RNase H cleavage in cells at concentrations <1–10 μM, i.e. a high extracellular concentration is required to enable sufficient oligonucleotide to enter the endosomes for efflux into the cytoplasm to occur. From our cell lysate data, we would estimate that <1% of the oligonucleotide added to the cells in the extracellular medium would need to be available freely inside the cells for RNase H cleavage to be seen. This may also explain why we do not see RNase H cleavage until 48 h after the oligonucleotide has been added, i.e. as the uptake of oligonucleotide increases with time, a point is reached between 24 and 48 h whereby the concentration of oligonucleotide inside the endosomes is sufficiently high for efflux to occur and for antisense activity to be observed. It is not clear why acutely infected H9 cells produced different results than the MT-4 cells. Experiments that we have carried out with fluorescein-labeled oligonucleotide have suggested that uptake is greater in MT-4 cells than H9 cells (unpublished data) and this may be one reason why differences were seen. However, as our experiments are only designed to investigate the possible role of RNase H in the observed antiviral activity, we cannot rule out the possibility that the oligonucleotide is having an antisense effect in the H9 cell assay mediated by a mechanism other than RNase H. Cytotoxicity studies showed no signs of oligonucleotide toxicity in either cell line within the concentration range used in these and previous studies (7). It is worth noting that within this field of research many previous studies have shown antisense oligonucleotide effects that are particular to one or two specific cell lines.

The evaluation of the potential of phosphorothioate antisense oligonucleotides as HIV therapeutics was difficult based on early studies where suitable control oligonucleotides were not always included and contributions from the significant non-specific artifacts generated in *in vitro* assays by the highly charged nature of these compounds were not appreciated (31). This led to suggestions that these drugs do not have an antisense mechanism of action. We have demonstrated in this report that oligonucleotides targeted to the HIV-1 *gag* gene are able to mediate RNase H cleavage of viral mRNA. RNase H cleavage was demonstrated in both cell lysate assays and in cells acutely infected with virus. Positive results in living cells were only observed in one particular cell line, however, and we have seen no consistent sequence specific reduction in *gag* mRNA or p24 production in living chronically infected cells. These results support the potential of antisense oligonucleotides as potent and specific agents to regulate gene expression. In spite of this potential, the uptake of these drugs, especially within the appropriate compartment of the cell, remains a major obstacle to their therapeutic application. It is hoped that such problems will be overcome in the near future with the design of new generation chemically modified antisense oligonucleotides and improved delivery systems.

REFERENCES

1 Matsukura,M., Shinozuka,K., Zon,G., Mitsuya,H., Reitz,M., Cohen,J.S. and Broder,S. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 7706–7710.
2 Agrawal,S., Goodchild,J., Civeira,M.P., Thornton,A.H., Sarin,P.S. and Zamecnik,P.C. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 7079–7083.
3 Agrawal,S. and Tang,J.Y. (1992) *Antisense Res. Dev.*, **2**, 261–266.
4 Lisziewicz,J., Sun,D., Weichold,F.F., Thierry,A.R., Lusso,P., Tang,J., Gallo,R.C. and Agrawal,S. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 7942–7946.
5 Akhtar,S. and Rossi,J.J. (1996) *J. Antimicrob. Chemother.*, **38**, 159–165.
6 Yamaguchi,K., Papp,B., Zhang,D., Ali,A., Agrawal,S. and Byrn,R. (1997) *AIDS Res. Hum. Retroviruses*, **13**, 545–554.
7 Veal,G.J., Agrawal,S. and Byrn,R.A. (1998) *Antiviral Res.*, **38**, 63–73.
8 Gura,T. (1995) *Science*, **270**, 575–577.
9 Shuttleworth,J. and Colman,A. (1988) *EMBO J.*, **7**, 427–434.
10 Shuttleworth,J., Matthews,G., Dale,L., Baker,C. and Colman,A. (1988) *Gene*, **72**, 267–275.
11 Giles,R.V., Spiller,D.G. and Tidd,D.M. (1995) *Antisense Res. Dev.*, **5**, 23–31.
12 Giles,R.V., Ruddell,C.J., Spiller,D.G., Green,J.A. and Tidd,D.M. (1995) *Nucleic Acids Res.*, **23**, 954–961.
13 Crooke,S.T., Lemonidis,K.M., Neilson,L., Griffey,R., Lesnik,E.A. and Monia,B.P. (1995) *Biochem. J.*, **312**, 599–608.
14 Agrawal,S., Jiang,Z., Zhao,Q., Shaw,D., Cai,Q., Roskey,A., Channavajjala,L., Saxinger,C. and Zhang,R. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 2620–2625.
15 Kandimalla,E.R., Manning,A., Zhang,Q., Shaw,D.R., Byrn,R.A., Sasisekharan,V. and Agrawal,S. (1997) *Nucleic Acids Res.*, **25**, 370–378.
16 Vujcic,L.K., Shepp,D.H., Klutch,M., Wells,M.A., Hendry,R.M., Wittek,A.E., Krilov,L. and Quinnan,G.,Jr (1988) *J. Infect. Dis.*, **157**, 1047–1050.
17 Mosmann,T. (1983) *J. Immunol. Methods*, **65**, 55–63.
18 Nakashima,H., Shoji,Y., Kim, S.-G., Shimada,J., Mizushima,Y., Ito,M., Yamamoto,N. and Takaku,H. (1994) *Nucleic Acids Res.*, **22**, 5004–5010.
19 Kuwasaki,T., Hosono,K., Takai,K., Ushijima,K., Nakashima,H., Saito,T., Yamamoto,N. and Takaku,H. (1996) *Biochem. Biophys. Res. Commun.*, **228**, 623–631.
20 Uhlman,E. and Peyman,A. (1990) *Chemical Rev.*, **90**, 543–584.
21 Szymkowski,D. (1996) *Drug Discovery Today*, **1**, 415–428.
22 Crooke,S.T. (1993) *FASEB J.*, **7**, 533–539.
23 Dean,N.M. and Griffey,R.H. (1997) *Antisense Nucleic Acids Drug Dev.*, **7**, 229–233.
24 Akhtar,S. and Juliano,R.L. (1992) *Trends Cell Biol.*, **2**, 139–144.
25 Beltinger,C., Saragovi,H.U., Smith,R.M., LeSauteur,L., Shah,N., DeDionisio,L., Christensen,L., Raible,A., Jarett,L. and Gewirtz,A.M. (1995) *J. Clin. Invest.*, **95**, 1814–1823.
26 Iverson,P.L., Crouse,D., Zon,G. and Perry,G. (1992) *Antisense Res. Dev.*, **2**, 223–233.
27 Pirruccello,S.J., Perry,G.A., Bock,P.J., Lang,M.S., Noel,S.M., Zon,G. and Iverson,P.L. (1994) *Antisense Res. Dev.*, **4**, 285–289.
28 Zhao,Q., Song,X., Waldschmidt,T., Fisher,E. and Krieg,A.M. (1996) *Blood*, **88**, 1788–1795.
29 Zhao,Q., Zhou,R., Temsamani,J., Zhang,Z., Roskey,A. and Agrawal,S. (1998) *Antisense Nucleic Acids Drug Dev.*, in press.
30 Matsukura,M. (1992) In Wickstrom,E. (ed.), *Antisense RNA and DNA*. Wiley-Liss, New York, pp. 285–304.
31 Agrawal,S. (1996) *Trends Biotechnol.*, **14**, 376–387.