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Inhibition of respiratory syncytial virus replication by antisense oligodeoxyribonucleotides

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Abstract

Oligodeoxyribonucleotides targeted against respiratory syncytial virus (RSV) genomic RNA inhibited RSV replication in cell culture by an apparent antisense mechanism. HEp-2 cells were infected with RSV strain A2 and incubated in the presence of oligonucleotides. Virus replication was measured by enzyme-linked immunosorbent assay (ELISA), virus yield assay, or production of specific RSV mRNAs. Using ELISA, 50% effective concentration (EC_{50}) values were about 0.5–1 μM for an antisense oligonucleotide targeted to the start of the NS2 gene. All oligonucleotides inhibited virus antigen production as measured by ELISA. In all assays, this antisense oligonucleotide was more potent than: (1) a control oligonucleotide containing the reverse sequence; (2) oligonucleotides targeted at RSV mRNA; (3) a random sequence oligonucleotide; and (4) ribavirin. Reverse transcriptase polymerase chain reaction (RT-PCR) showed sequence-specific depletion of the genomic RNA target following treatment of cells with the antisense oligonucleotide. Specific cleavage of the genomic target RNA has been detected at the antisense oligonucleotide binding site, suggesting that cellular RNase H participates in the reaction. These results indicate that antisense oligonucleotides targeted against RSV genomic RNA can effectively inhibit RSV replication and may have therapeutic value.

Keywords: Respiratory syncytial virus; Phosphorothioate oligonucleotides; Antisense inhibition

1. Introduction

Respiratory syncytial virus (RSV) causes severe lower respiratory tract disease in infants, young children and immunocompromised adults (Collins et al., 1996). RSV, a member of the family Paramyxoviridae, is an enveloped virus, containing an unsegmented, negative-stranded RNA genome of approximately 15 000 nucleotides (Collins et al., 1996). The genome encodes 10 viral proteins, which are translated from individual messenger RNAs (mRNAs). These RNAs are transcribed from the negative-stranded genome by the viral polymerase complex (N, P and L proteins). In addition, this complex replicates the negative-stranded genome from a positive-

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stranded intermediate for insertion into progeny virions. Replication occurs in the cytoplasm of the infected cells.

Currently, ribavirin is used therapeutically for RSV disease (Groothuis, 1994; Levin, 1994). Ribavirin is believed to inhibit RSV replication by several mechanisms: (1) inhibition of viral polymerase; (2) inhibition of 5′ cap formation of mRNAs; and (3) inhibition of IMP dehydrogenase which decreases intracellular GTP levels (Gilbert and Knight, 1986). Unfortunately, clinical benefits from ribavirin are small and only occur in a portion of RSV-infected individuals (Levin, 1994). There remains a need for development of therapeutics for RSV disease.

Phosphorothioate oligonucleotides are potent inhibitors of a wide range of viruses. These oligonucleotides have been targeted to inhibit expression of essential viral genes, thereby preventing virus replication. Oligonucleotides have been targeted to inhibit expression of structural genes of human immunodeficiency virus (HIV) (Lisziewicz et al., 1993, 1994; Anazodo et al., 1995), regulatory proteins of HIV (Matsukura et al., 1987), cytomegalovirus (CMV) (Azad et al., 1993; Pari et al., 1995), Epstein-Barr virus (EBV) (Roth et al., 1994; Daibata et al., 1996) and human papillomavirus (HPV) (Cowsert et al., 1993), and a variety of functions in hepatitis B virus (HBV) (Blum et al., 1991; Wu and Wu, 1992; Korba and Gerin, 1995). In two viruses, influenza virus and vesicular stomatitis virus, oligonucleotides were targeted at mRNA to inhibit protein translation and also to genomic RNA to inhibit RNA replication (Lemaitre et al., 1987; Leiter et al., 1990). In addition, phosphorothioate oligonucleotides have been shown to act by additional mechanisms, some sequence-specific, to inhibit replication of HSV, EBV and HIV (Gao et al., 1990; Yao et al., 1993; Wyatt et al., 1994; Buckheit et al., 1994; Ojwang et al., 1994, 1995). In antisense inhibition, the oligonucleotide binds to the complementary target RNA sequence specifically, resulting in reduced gene expression. In addition, the RNA/oligodeoxyribonucleotide complex may serve as a substrate for cellular RNase H cleavage, resulting in a decrease in intact target RNA, and the appearance of specific RNA cleavage products (Giles et al., 1995a,b).

In the present study, we have employed phosphorothioate oligonucleotides to inhibit RSV replication and to directly demonstrate the antisense mechanism of antiviral activity. We have targeted an oligonucleotide to the genomic strand of RSV in an effort to inhibit RNA replication and transcription of NS2 mRNA. This oligonucleotide inhibited virus replication and was a more potent inhibitor than control oligonucleotides or ribavirin. In addition, sequence-specific depletion of the genomic RNA target was observed following treatment of cells with the antisense oligonucleotide (and not with control oligonucleotides), and the expected RNA cleavage products were identified. This suggests that the oligonucleotide works, at least in part, by an antisense mechanism.

2. Materials and methods

2.1. Cells and virus

HEp-2 cells (ATCC CCL23; American Type Culture Collection (ATCC), Rockville, MD) were propagated in Dulbecco's modified Eagle medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) and penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C. RSV (strain A2, ATCC VR-1302) was grown in HEp-2 cells. Supernatants from infected cells were dispensed into microtubes and stored at -80°C. Virus titers were determined by limit dilution assays for virus cytopathic effects on HEp-2 cells in 96-well microtiter plates.

2.2. Oligonucleotide synthesis

Oligonucleotides (v590, v595 and NS2aug) have been targeted to a specific intergenic region/gene start region of the viral genome (at the start of the NS2 gene) or NS2 messenger RNA (Fig. 1). The v590 oligonucleotide is complementary to two RSV sites. The same 20-base sequence occurs at bases 590–609 at the start of the NS2 gene and bases 2323–2342 at the start of the P gene. The v1124 oligonucleotide was targeted to the gene start region of the N gene. A control oligonucleotide (v590s) contained the same bases as v590 with the sequence essentially reversed (Table 1).

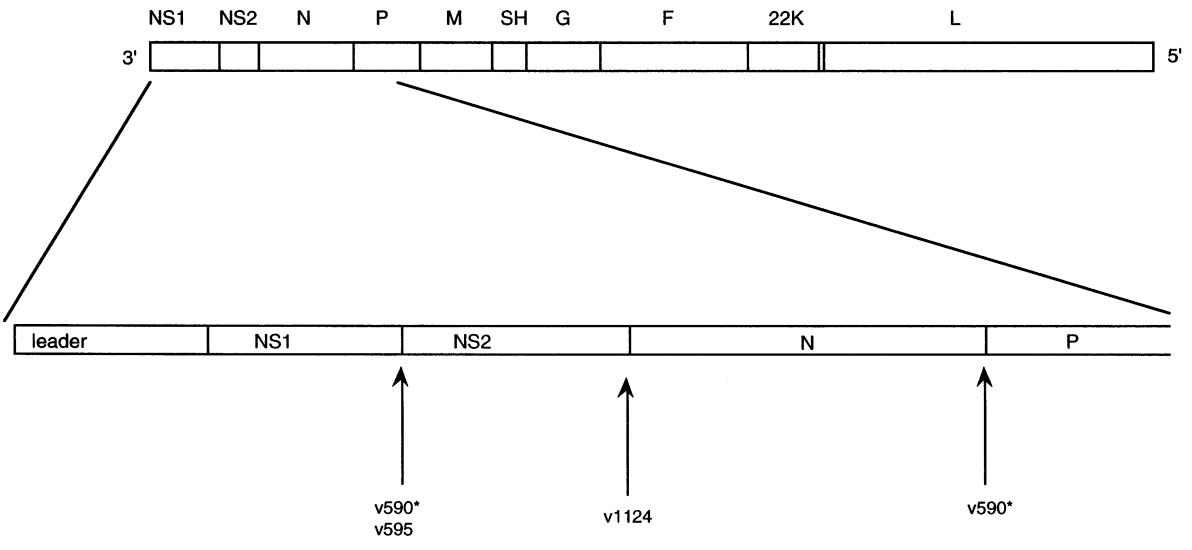

Fig. 1. Map of RSV genome and location of oligonucleotide target site.

Oligonucleotides were synthesized on a Pharmacia (Piscataway, NJ) Gene Assembler Plus using standard protocols as recommended by the manufacturers (Beaucage, 1993). For synthesis of random sequences (r20), the DNA phosphoramidites (Glen Research, Sterling, VA) were premixed in equimolar amounts in acetonitrile or mixed on the synthesizer using methods provided by the manufacturer. The thiosulfonating reagent used for all the phosphorothioate (PS) oligonucleotides was 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Iyer et al., 1990) (AIC, Natick, MA) as a 2% solution in low water acetonitrile (w/v). After deprotection, oligonucleotides were ethanol-precipitated twice.

Table 1
Anti-RSV oligonucleotides

v590	5′-AAAAATGGGGCAAATAAATC-3′
v595	5′-TGGGGCAAATAAATCAATTC-3′
v1124	5′-TGGGGCAAATACAAAGATGG-3′
NS2aug	5′-GTTGTGTCCATGGTTGGGTT-3′
v590s	5′-CTAAATAAACGGGGAAAAAT-3′
Randomer (r20)	All bases at all positions

2.3. Virus inhibition by oligonucleotides

HEp-2 cells were plated at a density of 10 000 cells/well in 96-well microtiter plates. Cells were treated, 6–7 h after plating, with oligonucleotides or ribavirin (Pharmatec, Alachua, FL) in triplicate at various concentrations in Opti-MEM (Gibco-BRL, Gaithersburg, MD) for 16–18 h at 37°C. Medium was removed and cells were washed to remove the residual oligonucleotides. Cells were then infected with RSV at an multiplicity of infection (MOI) of 0.01. After 1 h adsorption at 37°C, cells were washed and were then refed with fresh growth medium (Opti-MEM, 1% FBS) containing the same concentration of oligos as were used before infection. After 3 days of incubation at 37°C, supernatants were used for infectious virus yield assay and enzyme-linked immunosorbent assay (ELISA) was performed on the cells in the microtiter plate.

2.4. ELISA for RSV antigen

ELISA was conducted by modification of the procedure of Kang and Pai (1989). Cells were fixed in 0.05% glutaraldehyde at 4°C for 20 min, then blocked with phosphate-buffered saline

(PBS) containing 1% bovine serum albumin (BSA) for 30 min at room temperature. The primary antibody was a mouse anti-RSV antibody directed against the F protein of RSV (Chemicon International, Temecula, CA), diluted in PBS containing 1% BSA. After incubation at 37°C for 1 h, cells were treated with peroxidase-labeled goat anti-mouse IgG (Kirkegaard and Perry Labs, Gaithersburg, MD) diluted in PBS containing 1% BSA. After incubation for 1 h at 37°C, color was developed with 3,3′,5,5′-tetramethyl benzidine (TMB). Absorbance at 450 nm was recorded. Oligonucleotide- or ribavirin-treated cultures were compared to untreated, infected cultures to determine percent control. EC_{50} (50% effective concentration) was determined from graphs of the dose–response curves.

2.5. Infectious virus yield assay

The method used was a modification of the procedure of Prichard et al. (1990). Briefly, infected cell culture supernatants were titrated in 3-fold dilutions on HEp-2 cells in a microtiter assay. One well was used at each dilution. Virus titer was determined by ELISA. Virus yield from treated cultures was compared to untreated infected cultures to determine percent control yield reduction. EC_{99} (99% effective concentration) was determined from graphs of the dose–response curves. Statistical evaluation of ELISA and virus yield data was conducted using a Student's *t*-test.

2.6. Cytotoxicity

HEp-2 cells were plated at a density of 500 cells/well in 96 well-plates and incubated overnight. Cells were then treated with oligonucleotides or ribavirin diluted in Opti-MEM, 1% FBS and incubated for 96 h at 37°C. Cell growth was determined using the Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. Cells incubated in the presence of oligonucleotides or ribavirin were compared to untreated cells to determine percent control cell growth.

2.7. RT-PCR amplification of NS1 and NS1–NS2 regions of RSV

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using primers specific for the NS1 and NS2 regions of RSV. All primers were obtained from Oligo Therapeutics (Wilsonville, OR) or Gibco-BRL (Grand Island, NY). HEp-2 cells (1×10^5 cells/well in a 6-well dish) were treated with oligonucleotide (30 μM), washed, then infected with RSV (MOI = 0.2) for 1 h. Cells were then incubated in the presence of oligonucleotide for 24 h and total RNA was isolated using Trizol (Gibco-BRL) according to the manufacturer's instructions. RT-PCR was performed with the Gene Amp RNA PCR kit (Perkin-Elmer Cetus, Foster City, CA) according to the manufacturer's instructions with minor modifications. Reverse transcription was performed using primer BC1 (5′-GCAGCAATTCATTGAGTATG-3′) (Fig. 5A), which corresponds to 102–122 bp of RSV sequence (Stec et al., 1991; Mink et al., 1991). PCR was then conducted with 2 μl of the RT reaction mixture using three primers (OD1, BC2 and BC6) that are specific for the NS1 region of the viral genome (OD1 and BC2) and the NS1–NS2 region (OD1 and BC6). OD1 (5′-TGTTTGACAATGATGAAGTA-3′) corresponds to RSV bases 145–164; BC2 (5′-GTGACATTGATTTGCTAGTT-3′) corresponds to 537–557 bp of RSV sequence, and BC6 (5′-TAATTTTCAGGCTCCATCTG-3′) corresponds to 1065–1085 bp of RSV sequence (Stec et al., 1991; Mink et al., 1991). PCR was run using Ex-Taq polymerase (Pan Vera, Madison, WI) and Taq Start antibody (Clontech, Palo Alto, CA) for hot start PCR. PCR products were visualized after electrophoresis on 1% Seakem ME agarose (FMC, Rockland, ME) gels.

2.8. Quantitative competitive PCR

Competitive PCR was used to quantitate the amount of mRNA (Gilliland et al., 1990). The competitive template was generated from infected cells by RT-PCR (Perkin-Elmer Cetus) using primers BC1 and BC6. The 983-bp product was

cloned into pCR2.1 (Invitrogen, San Diego, CA) and then digested with *Acc*1 and *Hin*cII to delete a 408-bp fragment. After ligation, the plasmid contained NS1–NS2 sequences which were recognized by primers BC1 and BC6 to produce a 575-bp PCR product. Competitive PCR reactions contained oligonucleotide primers (BC1 and BC6, 0.75 μM each), deoxynucleoside triphosphates (dNTPs) (250 μM each), 10 μl of the RSV RT reaction mixture (see above) and dilutions of the competitive template (0.2–31 pg/ml). Ethidium bromide-stained gels were scanned (Umax, Hsinchu, Taiwan) to quantitate the amount of DNA in the PCR products. The amount of RSV cDNA was determined relative to the known amount of competitive plasmid according to published procedures (Gilliland et al., 1990). Control reactions with the RSV cDNA were conducted with primers BC1 and BC2 in the absence of the competitive template.

2.9. Evaluation of RSV RNA by 5′ RACE

Rapid amplification of cDNA ends (5′ RACE System, Gibco-BRL) was performed according to the manufacturer's instructions. Reverse transcription was performed with 2–4 μg total RNA; the reverse transcriptase primer was BC1, described above. Reverse transcription, RNA degradation, cDNA purification and terminal deoxynucleotidyl transferase tailing were all performed according to the manufacturer's instructions. PCR was conducted with the anchor primer (Gibco-BRL) and an RSV-specific primer, OD1. PCR was run using Ex-Taq polymerase (Pan Vera) and Taq Start antibody (Clontech, Palo Alto, CA) for hot start PCR. PCR products were then diluted 1:100 and a second PCR was conducted using the universal adapter primer (Gibco-BRL) and OD1. PCR products were visualized after electrophoresis on 2% 3:1 Nusieve:agarose (FMC, Rockland, ME) gels. PCR products from the reaction mixture were cloned into pCR2.1 (Invitrogen). Transformants were sequenced with Sequenase (US Biochemical, Cleveland, OH).

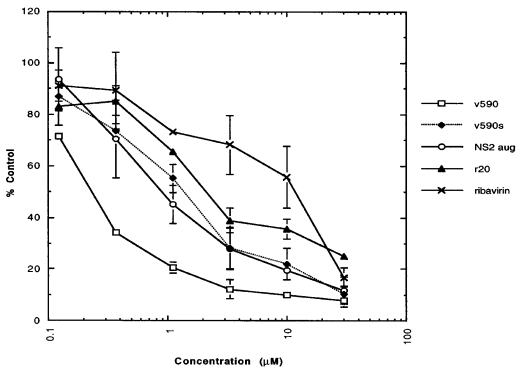

Fig. 2. Inhibition of RSV antigen production. RSV antigen was measured by ELISA in infected cells as described in Section 2. All treated cells were compared with untreated, infected cells to determine % control. Error bars indicate the standard deviation (S.D.) of triplicate values.

3. Results

3.1. RSV inhibition assays

Oligonucleotides inhibited RSV replication measured by ELISA and infectious virus yield. All oligonucleotides showed a dose-dependent reduction in RSV F antigen as demonstrated in Fig. 2. The most potent oligonucleotide was v590, inhibiting antigen production at 4–5-fold lower concentrations than control oligonucleotides, v590s and r20, or an oligonucleotide targeted at NS2 mRNA (Fig. 2, Table 2). The v590 oligonu-

Table 2
RSV inhibition by oligonucleotides

Compound	$EC_{50} \pm$ S.D. (μM, F antigen)	$EC_{99} \pm$ S.D. (μM, infectious virus)
v590	0.64 ± 0.70 (12)[a]	6.6 ± 3.7 (6)
vRNA590s	2.4 ± 2.1 (11)*	26 ± 8.9 (5)***
v595	1.15 ± 0.89 (7)	20 ± 11 (4)
v1124	9.9 ± 13 (4)	>30 (2)
NS2aug	8.0 ± 7.0 (3)	$\geqslant 30$ (2)
r20	17 ± 13 (10)	>30 (5)
Ribavirin	13 ± 5.7 (12)**	27 ± 6.2 (6)****

[a] Number of experiments.
* $P = 0.02$ vs. v590.
** $P = 0.001$ vs. v590.
*** $P = 0.006$ vs. v590.
**** $P = 0.0001$ vs. v590.

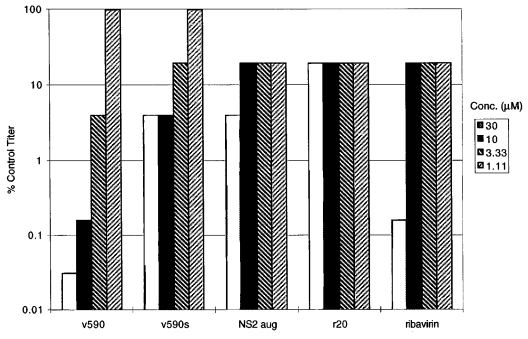

Fig. 3. Inhibition of infectious virus yield. Supernatant from infected cells was titrated in uninfected cells as described in Section 2 to determine infectious virus yield. Treated cultures were compared with untreated cultures to determine % control virus yield.

cleotide was also more than 10-fold more potent than ribavirin. EC_{50} values (Table 2) from multiple assays indicated that these differences were statistically significant ($P < 0.05$). Compared with other oligonucleotides targeted to gene start regions of the viral genome, v595 and v1124, v590 was 2–15-fold more potent in antigen inhibition (Table 2).

The v590 oligonucleotide (10 μM) and ribavirin (30 μM) decreased virus yield by over 99% (Fig. 3). EC_{99} values (Table 2) showed that v590 is 4–5-fold more active than control compounds (v590s and r20), and that these differences were significant ($P < 0.05$); v590 was also more active than v595 and v1124. Thus, the decrease in virus antigen detected in infected cells by ELISA reflected a decrease in the production of infectious virus.

3.2. Oligonucleotide toxicity

Cytotoxicity was measured using uninfected HEp-2 cells. Subconfluent cultures were incubated in the presence of oligonucleotides (or ribavirin) for 72 h. Under these conditions, cell growth did not plateau during the treatment period; cell doubling time was approximately 24 h (data not shown). Cell growth was determined and compared with untreated cultures. Fig. 4 shows that oligonucleotides did not inhibit cell growth at concentrations where antiviral activity occurred. Less than 50% growth inhibition was measured at concentrations as high as 100 μM. The selectivity index for RSV antigen inhibition for v590 was over 150.

3.3. Evaluation of RSV genomic RNA

To investigate the mechanism of v590 inhibition, genomic RSV RNA, the target for the v590 antisense oligonucleotide, was evaluated by RT-PCR. RNA, isolated 24 h post-infection, was reverse transcribed using BC1, a primer specific for the NS1 region. From this cDNA, PCR was then conducted using primers spanning the NS1–NS2 regions (OD1 and BC6, see Fig. 5A).In cells treated with v590, no PCR product was detected (Fig. 5B). In contrast, in cells treated with v590s or r20, a specific PCR product was present (Fig. 5B). Using the same cDNA, PCR was conducted using PCR primers for the NS1 region (OD1 and BC2, see Fig. 5A). In these reactions, a PCR product was detected in all samples, including v590.

In order to quantify the decrease in genomic RNA around the NS1–NS2 region, quantitative competitive PCR (QC-PCR) was conducted using PCR primers BC1 and BC6. PCR was conducted with cDNA prepared from oligonucleotide-treated, infected cells in the presence of a plasmid containing NS1–NS2 sequences. This plasmid

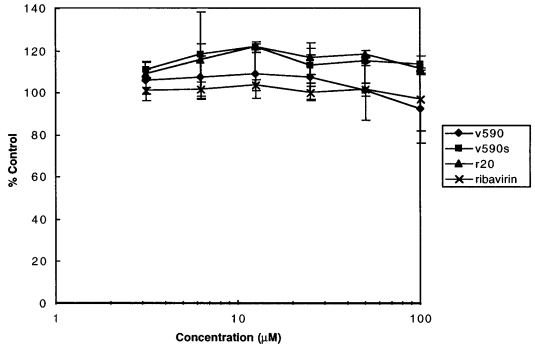

Fig. 4. Cytotoxicity: uninfected HEp-2 cell growth was determined as described in Section 2. All treated cells were compared with untreated, infected cells to determine % control. Error bars indicate the S.D. of triplicate values.

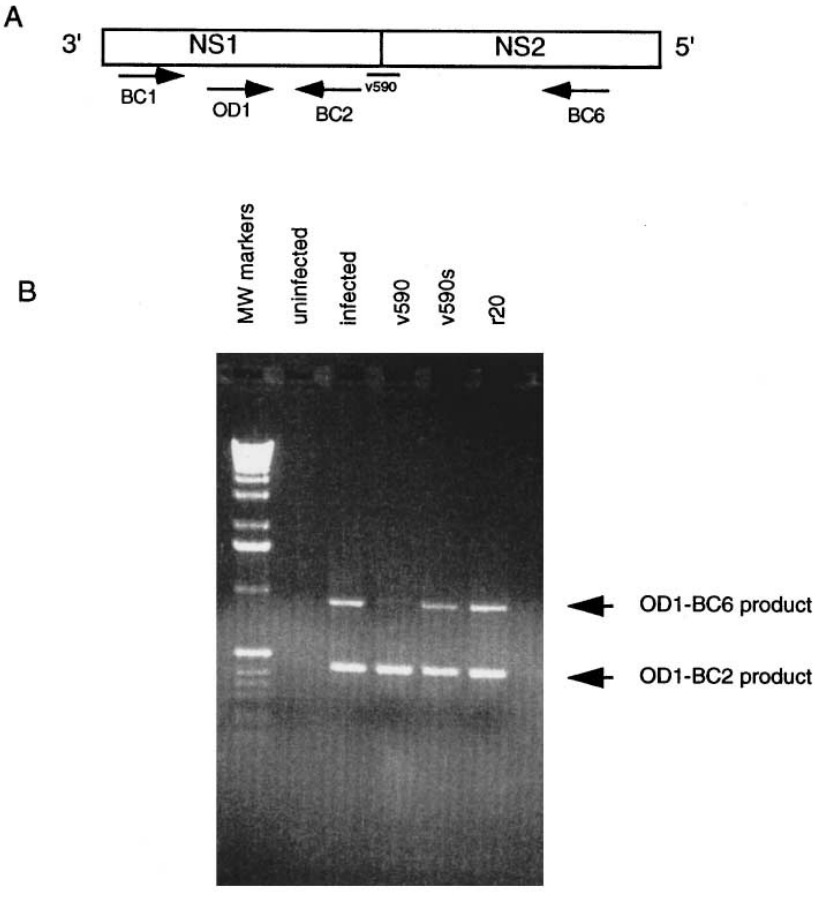

Fig. 5. Evaluation of genomic RSV RNA by RT-PCR. RT-PCR scheme (A) and results (B) are shown. RSV RNA was reverse transcribed using primer BC1. PCR was then conducted using OD1 and BC2 or OD1 and BC6 as described in Section 2. (B) shows the 410-bp product generated from OD1 and BC2 in all samples; the 940-bp product was generated from OD1 and BC6.

was prepared with a deletion in the NS1–NS2 region to provide a smaller PCR product (575 bp) than the genomic cDNA product (983 bp). Both products were clearly visible in cells treated with v590s (Fig. 6, right side). The amount of RSV cDNA in the v590s-treated cells was estimated to be 3 pg/ml. Similar amounts of RSV cDNA were measured in infected, untreated cells or cells treated with random oligonucleotide (data not shown). In cells treated with v590, RSV cDNA was not detected in competitive PCR reactions (Fig. 6, left side), indicating that the concentration was less than 0.2 pg/ml. This confirms that the genomic target was depleted after antisense oligonucleotide treatment.

4. Measurement of genomic RNA cleavage

RNase H cleavage of RNA in oligonucleotide/RNA duplexes has been proposed as a mechanism for antisense oligonucleotide inhibition of protein expression. The decrease in the amount of PCR product using NS1–NS2 primers suggested that RNA cleavage might have occurred. We looked for additional evidence of genomic RNA cleavage

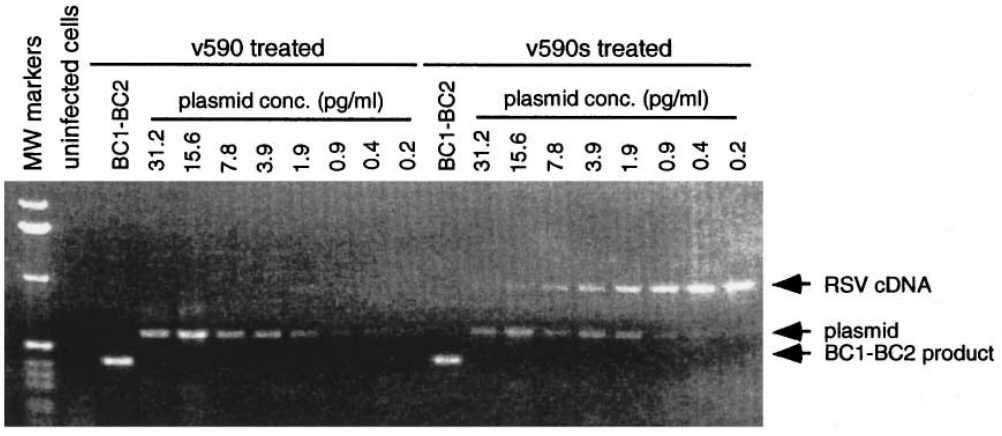

Fig. 6. Quantitation of genomic RSV RNA. RSV cDNA was prepared by reverse transcription. PCR was conducted with a constant amount of RSV cDNA in the presence of serial dilutions of a competitive plasmid template to determine the concentration of RSV RNA present after oligonucleotide treatment.

in the oligonucleotide target region by 5′ RACE. This technique was used to identify the 5′ end of genomic RNA in the oligonucleotide target region; such 5′ ends would only be amplified if RNA cleavage had occurred. This cleavage would result in the decrease of the RT-PCR product as demonstrated in Fig. 5.

5′ RACE was performed using RNA isolated from cells 24 h post-infection. RNA was reverse transcribed with an NS1-specific primer; cDNA was tailed with dCTP in the presence of terminal transferase. PCR was performed by using a primer specific for the oligo(dC) tail and a nested NS1-specific primer. In cells treated with v590, a PCR product was detected at approximately 510 bp (Fig. 7A); this is the size expected for a cDNA derived from RNA whose 5′ end is at the v590 target site. An additional product was detected at approximately 300 bp. Four clones from v590-treated cells which contained an insert corresponding to the 510-bp PCR product were sequenced. RSV RNA sequence was detected adjacent to a stretch of Gs introduced by terminal transferase (Fig. 7B). In v590-treated cells, this RSV sequence corresponds to the binding site for v590; all four clones indicated RNA cleavage in the v590 target site (Fig. 7C). Additional clones, corresponding to the 300-bp product, were sequenced and the 5′ end of the RNA appeared to be RSV base 295.

PCR products were also cloned from v590s- or r20-treated cells and untreated infected cells. A 300-bp product was also obtained from v590s- and r20-treated cells (data not shown). Sequencing indicated a 5′ end at base 295. None of the clones produced from RNA obtained from cells treated with control (v590s or r20) treated oligonucleotides, or infected, untreated cells produced a 5′ end at the v590 binding site.

5. Discussion

We have identified a phosphorothioate oligonucleotide as a potent inhibitor of RSV replication. The v590 oligonucleotide, targeted to genomic RNA, inhibited RSV antigen (measured by ELISA) and infectious virus yield 4–20-fold more effectively than ribavirin. Compared with a random mixture of 20-base oligonucleotides (r20), v590 was 26-fold more active in ELISA, and more than 5-fold more active in inhibiting infectious virus yield.

An additional control oligonucleotide (v590s) contained the same bases, with the sequence almost completely reversed (Table 1). This sequence

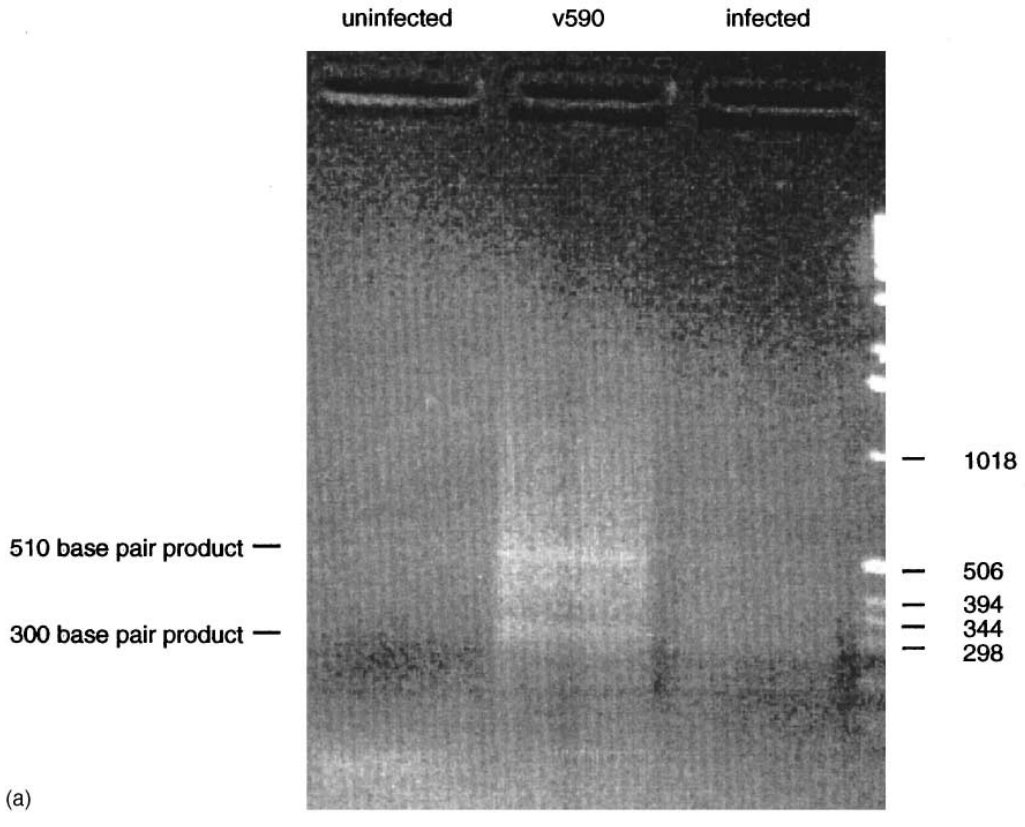

Fig. 7. Evaluation of genomic RSV RNA by 5′ RACE. (A) PCR products derived from 5′ RACE after treatment of cells with v590; (B) sequencing of cloned PCR products from RACE of RNA isolated from v590-treated cells. The location of the oligo(dG) tail, derived from the linker-specific primer, is indicated. The sequence adjacent to the tail, containing the 5′ end of the cDNA, is indicated. (C) Location of v590 oligonucleotide and v590-specific cleavage. Vertical arrows show location of 5′ end of RNA as detected by RACE. Additional products are also shown at base 295. The location of RT primer and RSV-specific PCR primers is also shown.

was chosen to maintain the same base composition as v590, and also to maintain a region containing four G residues. In other systems, four consecutive Gs contribute to non-antisense, sequence-specific inhibition of viruses or other cellular functions (Wyatt et al., 1994; Burgess et al., 1995; Ojwang et al., 1995). The v590 oligonucleotide was approximately 4-fold more active than v590s in ELISA and virus yield assays (Table 2). Another oligonucleotide containing four Gs and targeting the NS2 gene start region, v595, showed activity similar to v590. A four-G oligonucleotide targeting the N gene start region, v1124, was less active than v590. Other oligonucleotides targeted against the viral genome which did not contain four Gs were less active (data not shown). This suggests that the four Gs contribute significantly to the activity of the oligonucleotide, but are not the only important factor. An additional oligonucleotide (NS2aug), targeted against NS2 mRNA, was also less active than v590 (Fig. 2). Other oligonucleotides targeted against RSV mRNAs also gave results similar to those of the NS2aug oligonucleotide (data not shown).

The potency of v590 compared with all oligonucleotides evaluated, including three containing four contiguous Gs, suggested that this compound inhibited, at least in part, by a sequence-specific mechanism. However, control oligonucleotides were inhibitory in ELISA and

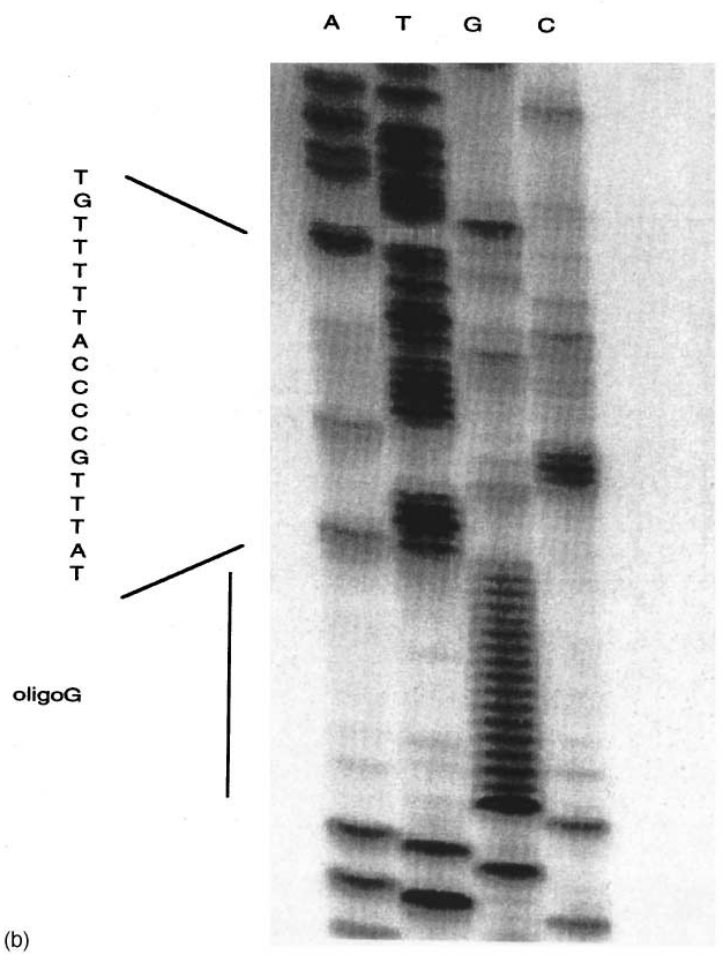

Fig. 7 continued.

virus yield assays. This suggests that these oligonucleotides may act by a non-antisense mechanism and inhibit the spread of virus infection in the 3-day assays.

The selectivity index for RSV antigen inhibition was determined to be more than 150 for v590, since v590 did not inhibit cell growth by as much as 50% at concentrations up to 100 μM. None of the oligonucleotides inhibited cell growth, suggesting that the inhibition was an antiviral effect and not non-specific cell growth inhibition.

The effect of v590 (and other oligonucleotides) on the genomic target sequence was evaluated to determine if sequence-specific inhibition involved an antisense mechanism. Previously, cellular RNase H has been implicated as a co-factor in antisense oligonucleotide inhibition of gene expression (Monia et al., 1993; Dean et al., 1994). This enzyme recognizes DNA/RNA hybrids, digesting the RNA strand. RNase H digestion of RSV RNA complexed with an antisense oligonucleotide would result in the depletion of genomic RNA. The v590 oligonucleotide specifically decreased the region of RSV RNA containing the v590 target sequence (Figs. 5 and 6), suggesting that RNA cleavage may have occurred. There was no effect on RNA 3′ to the target sequence (in the NS1 region), suggesting again that the interaction

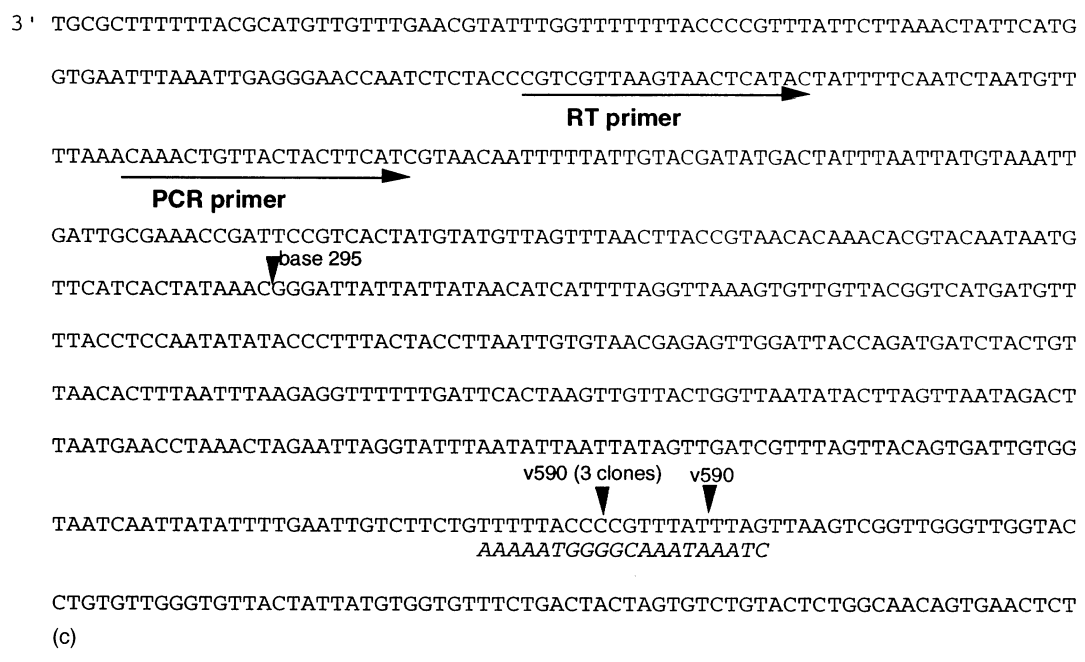

Fig. 7 continued.

was sequence-specific and that the RNA on the 3′ side of the cleavage was not digested by cellular nucleases.

Since RNA sequences 3′ to the putative RNase H cleavage were present, we used 5′ RACE to determine the 5′ end of the RNA cleavage. PCR products were isolated from RNA derived from v590-treated cells; sequence-specific RNA cleavage occurred within the oligonucleotide binding site. These experiments, using 5′ RACE, provide strong evidence that v590 interacted with its target RNA. A similar technique, RNA ligase-PCR, has been successfully used to establish that RNase H cleaved RNA/oligonucleotide duplexes in permeabilized cells or by oligonucleotides delivered with cationic lipid carriers (Giles et al., 1995b; Hanecak et al., 1996). In the present study, we observed that antisense inhibition can occur in the absence of cell permeabilization or an oligonucleotide delivery agent.

The apparent RNase-H-dependent cleavage was somewhat surprising since RNase H is believed to be a nuclear enzyme (Busen, 1980; Sawai et al., 1981) involved in DNA replication and RSV replicates in the cytoplasm. However, RNase H activity has been detected in cytoplasmic as well as nuclear fractions from cell lines (Rosolen et al., 1993), suggesting that this activity may be responsible for the sequence-specific cleavage we detected.

An additional PCR product from v590-treated cells or cells treated with other oligonucleotides showed apparent cleavage at base 295 (Fig. 7C). We believe that this apparent cleavage is a PCR artifact, since this site is adjacent to three consecutive Cs in the RSV sequence. The anchor primer (Gibco-BRL) which is used to amplify the oligo(dC)-tailed cDNA may have also recognized the three Cs in the RSV sequence, generating an apparent 5′ end. Since this product was also obtained after treatment with control oligonucleotides, it was not a sequence-specific product.

These results do not determine that antisense is the major mechanism for RSV inhibition by v590. Phosphorothioate oligonucleotides have been shown to inhibit virus replication by a variety of non-antisense mechanisms, including inhibition of virus binding, and inhibition of viral polymerases

(Gao et al., 1989, 1990; Wyatt et al., 1994; Ojwang et al., 1994, 1995). The v590 oligonucleotide (and other oligonucleotides) did not inhibit RSV binding to HEp-2 cells (data not shown). We have not performed additional experiments to determine what other mechanisms might be operating. Our experiments do show that sequence-specific interaction occurred between v590 and its target RNA, suggesting that v590 does act as an antisense oligonucleotide.

In summary, v590 is an antisense oligonucleotide which is a novel inhibitor of RSV replication. Inhibition was potent and sequence-specific. The v590 oligonucleotide was more active than ribavirin or control oligonucleotides, including a reverse sequence which contained four consecutive Gs. The four Gs alone did not account for the potency and specificity of inhibition. Additional in vivo studies will be required to determine whether v590 provides a valid alternative to ribavirin therapy of RSV disease. Since v590 inhibits RSV in part by a new, novel mechanism, it is possible that combination therapy with ribavirin may also prove to be beneficial. These studies are in progress.

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References

Anazodo, M.I., Wainberg, M.A., Friesen, A.D. and Wright, J.A. (1995) Sequence-specific inhibition of gene expression by a novel antisense oligodeoxynucleotide phosphorothioate directed against a nonregulatory region of the human immunodeficiency virus type 1 genome. J. Virol. 69, 1794–1801.

Azad, R.F., Driver, V.B., Tanaka, K., Crooke, R.M. and Anderson, K.P. (1993) Antiviral activity of a phosphorothioate oligonucleotide complementary to RNA of the human cytomegalovirus major immediate-early region. Antimicrob. Agents Chemother. 37, 1945–1954.

Beaucage, S. (1993) Oligodeoxyribonucleotide synthesis. Phosphoramidate approach. In: S. Agrawal (Ed), Methods in Molecular Biology, Vol. 20: Protocols for Oligonucleotides and Analogs, pp. 31–61. Humana Press, Totowa, NJ.

Blum, H.E., Galun, E., Weizsacker, F. and Wands, J.R. (1991) Inhibition of hepatitis B virus by antisense oligodeoxynucleotides (letter). Lancet 337, 1230.

Buckheit, Jr., R.W., Roberson, J.L., Lackman-Smith, C., Wyatt, J.R., Vickers, T.A. and Ecker, D.J. (1994) Potent and specific inhibition of HIV envelope-mediated cell fusion and virus binding by G quartet-forming oligonucleotide (ISIS 5320). AIDS Res. Hum. Retroviruses 10, 1497–1506.

Burgess, T.L., Fisher, E.F., Ross, S.L., Bready, J.V., Qian, Y., Bayewitch, L.A., Cohen, A.M., Herrera, C.J., Hu, S.S.-F., Kramer, T.B., Lott, F.D., Martin, F.H., Pierce, G.F., Simonet, L. and Farrell, C.L. (1995) The antiproliferative activity of c-*myb* and c-*myc* antisense oligonucleotides in smooth muscle cells is caused by a nonantisense mechanism. Proc. Natl. Acad. Sci. USA 92, 4051–4055.

Busen, W. (1980) Purification, subunit structure, and serological analysis of calf thymus ribonuclease H1. J. Biol. Chem. 255, 9434–9443.

Collins, P.L., McIntosh, K. and Chanock, R.M. (1996) Respiratory syncytial virus. In: B.N. Fields, D.M. Knipe and P.M. Howley (Eds), Virology, pp. 1313–1351. Lippincott-Raven, Philadelphia, PA.

Cowsert, L.M., Fox, M.C., Zon, G. and Mirabelli, C.K. (1993) In vitro evaluation of phosphorothioate oligonucleotides targeted to the E2 mRNA of papillomavirus: potential treatment for genital warts. Antimicrob. Agents Chemother. 37, 171–177.

Daibata, M., Enzinger, E.M., Monroe, J.E., Kilkuskie, R.E., Field, A.K. and Mulder, C. (1996) Antisense oligodeoxynucleotides against the BZLF1 transcript inhibit induction of productive Epstein-Barr virus replication. Antiviral Res. 29, 243–260.

Dean, N.M., McKay, R., Condon, T.P. and Bennett, C.F. (1994) Inhibition of protein kinase C-a expression in human A549 cells by antisense oligonucleotides inhibits induction of intercellular adhesion molecule 1 (ICAM-1) mRNA by phorbol esters. J. Biol. Chem. 269, 16 416–16 424.

Gao, W., Stein, C.A., Cohen, J.S., Dutschman, G.E. and Cheng, Y.-C. (1989) Effect of phosphorothioate homooligodeoxynucleotides on herpes simplex virus type 2-induced DNA polymerase. J. Biol. Chem. 264, 11 521–11 526.

Gao, W., Jaroszewski, J.W., Cohen, J.S. and Cheng, Y.-C. (1990) Mechanisms of inhibition of herpes virus type 2 growth by 28-mer phosphorothioate oligodeoxycytidine. J. Biol. Chem. 265, 20 172–20 178.

Gilbert, B.E. and Knight, V. (1986) Biochemistry and clinical applications of ribavirin. Antimicrob. Agents Chemother. 30, 201–205.

Giles, R.V., Ruddell, C.J., Spiller, D.G., Green, J.A. and Tidd, D.M. (1995a) Single base discrimination for ribonuclease H-dependent antisense effects within intact human leukaemia cells. Nucleic Acids Res. 23, 954–961.

Giles, R.V., Spiller, D.G. and Tidd, D.M. (1995b) Detection of ribonuclease H-generated mRNA fragments in human leukemia cells following reversible membrane permeabilization in the presence of antisense oligodeoxynucleotides. Antisense Res. Dev. 5, 23–31.

Gilliland, G., Perrin, S. and Bunn, H.F. (1990) Competitive PCR for quantitation of mRNA. In: M.A. Innis, D.H.

Gelfand, J.J. Sninsky and T.J. White (Eds), PCR Protocols. A Guide to Methods and Applications, pp. 60–69. Academic Press, San Diego, CA.

Groothuis, J.R. (1994) Role of antibody and use of respiratory syncytial virus (RSV) immune globulin to prevent severe RSV disease in high-risk children. J. Pediatr. 124 (Suppl.), S28–S32.

Hanecak, R., Brown-Driver, V., Fox, M.C., Azad, R.F., Furusako, S., Nozaki, C., Ford, C., Sasmor, H. and Anderson, K.P. (1996) Antisense oligonucleotide inhibition of hepatitis C virus gene expression in transformed hepatocytes. J. Virol. 70, 5203–5212.

Iyer, R., Phillips, L., Egan, W., Regan, J. and Beaucage, S. (1990) The automated synthesis of sulfur-containing oligodeoxyribonucleotides using 3*H*-1,2,-benzodithiol-3-one 1,1-dioxide as a sulfur-transfer reagent. J. Org. Chem. 55, 4693–4699.

Kang, J.-O. and Pai, C.H. (1989) In situ enzyme immunoassay for antiviral susceptibility testing of respiratory syncytial virus. Am. J. Clin. Pathol. 91, 323–326.

Korba, B.E. and Gerin, J.L. (1995) Antisense oligonucleotides are effective inhibitors of hepatitis B virus replication in vitro. Antiviral Res. 28, 225–242.

Leiter, J.M.E., Agrawal, S., Palese, P. and Zamecnik, P.C. (1990) Inhibition of influenza virus replication by phosphorothioate oligonucleotides. Proc. Natl. Acad. Sci. USA 87, 3430–3434.

Lemaitre, M., Bayard, B. and Lebleu, B. (1987) Specific antiviral activity of a poly(L-lysine)-conjugated oligodeoxyribonucleotide sequence complementary to vesicular stomatitis virus N protein mRNA initiation site. Proc. Natl. Acad. Sci. USA 84, 648–652.

Levin, M.J. (1994) Treatment and prevention options for respiratory syncytial virus infections. J. Pediatr. 124, S22–S27.

Lisziewicz, J., Sun, D., Metelev, V., Zamecnik, P., Gallo, R.C. and Agrawal, S. (1993) Long-term treatment of human immunodeficiency virus-infected cells with antisense oligonucleotide phosphorothioates. Proc. Natl. Acad. Sci. USA 90, 3860–3864.

Lisziewicz, J., Sun, D., Weichold, F.F., Thierry, A.R., Lusso, P., Tang, J., Gallo, R.C. and Agrawal, S. (1994) Antisense oligodeoxynucleotide phosphorothioate complementary to Gag mRNA blocks replication of human immunodeficiency virus type 1 in human peripheral blood cells. Proc. Natl. Acad. Sci. USA 91, 7942–7946.

Matsukura, M., Zon, G., Shinozuka, K., Mitsuya, H., Reitz, M., Cohen, J.S. and Broder, S. (1987) Phosphorothioate analogs of oligodeoxynucleotides: inhibitors of replication and cytopathic effects of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA 84, 7706–7710.

Mink, M.A., Stec, D.S. and Collins, P.L. (1991) Nucleotide sequences of the 3′ leader and 5′ trailer regions of human respiratory syncytial virus genomic RNA. Virology 185, 615–624.

Monia, B.P., Lesnik, E.A., Gonzalez, C., Lima, W.F., McGee, D., Guinosso, C.J., Kawasaki, A.M., Cook, P.D. and Freier, S.M. (1993) Evaluation of 2′-modified oligonucleotides containing 2′-deoxy gaps as antisense inhibitors of gene expression. J. Biol. Chem. 268, 14 514–14 522.

Ojwang, J., Elbaggari, A., Marshall, H.B., Jayaraman, K., McGrath, M.S. and Rando, R.F. (1994a) Inhibition of human immunodeficiency virus type 1 activity in vitro by oligonucleotides composed entirely of guanosine and thymidine. J. Acquir. Immune Defic. Syndr. 7, 560–570.

Ojwang, J.O., Buckheit, R.W., Pommier, Y., Mazumder, A., De Vreese, K., Esté, J.A., Reymen, D., Pallansch, L.A., Lackman-Smith, C., Wallace, T.L., De Clercq, E., McGrath, M.S. and Rando, R.F. (1995) T30177, an oligonucleotide stabilized by an intramolecular guanosine octet, is a potent inhibitor of laboratory strains and clinical isolates of human immunodeficiency virus type 1. Antimicrob. Agents Chemother. 39, 2426–2435.

Pari, G.S., Field, A.K. and Smith, J.A. (1995) Potent antiviral activity of an antisense oligonucleotide complementary to the intron–exon boundary of human cytomegalovirus genes UL36 and UL37. Antimicrob. Agents Chemother. 39, 1157–1161.

Prichard, M.N., Turk, S.R., Coleman, L.A., Engelhardt, S.L., Shipman, Jr., C. and Drach, J. (1990) A microtiter yield reduction assay for the evaluation of antiviral compounds against human cytomegalovirus and herpes simplex virus. J. Virol. Methods 28, 101–106.

Rosolen, A., Kyle, E., Chavany, C., Bergan, R., Kalman, E.T., Crouch, R. and Neckers, L. (1993) Effect of over-expression of bacterial ribonuclease H on the utility of antisense MYC oligodeoxynucleotides in the monocytic leukemia cell line U937. Biochimie 75, 79–87.

Roth, G., Curiel, T. and Lacy, J. (1994) Epstein-Barr viral nuclear antigen 1 antisense oligodeoxynucleotide inhibits proliferation of Epstein-Barr virus-immortalized B cells. Blood 84, 582–587.

Sawai, Y., Kitahara, N., Thung, W.L., Yanokura, M. and Tsukada, K. (1981) Nuclear location of ribonuclease H and increased level of magnesium-dependent ribonuclease H in rat liver of thioacetamide treatment. J. Biochem. (Tokyo) 90, 11–16.

Stec, D.S., Hill, M.G. and Collins, P.L. (1991) Sequence analysis of the polymerase L gene of human respiratory syncytial virus and predicted phylogeny of nonsegmented negative-strand viruses. Virology 183, 273–287.

Wu, G.Y. and Wu, C.H. (1992) Specific inhibition of hepatitis B viral gene expression in vitro by targeted antisense oligonucleotides. J. Biol. Chem. 267, 12 436–12 439.

Wyatt, J.R., Vickers, T.A., Roberson, J.L., Buckheit, Jr., R.W., Klimkait, T., DeBaets, E., Davis, P.W., Rayner, B., Imbach, J.L. and Ecker, D.J. (1994) Combinatorially selected guanosine-quartet structure is a potent inhibitor of human immunodeficiency virus envelope-mediated cell fusion. Proc. Natl. Acad. Sci. USA 91, 1356–1360.

Yao, G.-Q., Grill, S., Egan, W. and Cheng, Y.-C. (1993) Potent inhibition of Epstein-Barr virus by phosphorothioate oligodeoxynucleotides without sequence specification. Antimicrob. Agents Chemother. 37, 1420–1425.