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NOTES

Potent Antiviral Activity of an Antisense Oligonucleotide Complementary to the Intron-Exon Boundary of Human Cytomegalovirus Genes UL36 and UL37

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An antisense phosphorothioate oligonucleotide complementary to the intron-exon boundary of human cytomegalovirus genes UL36 and UL37 (UL36ANTI) reduced the yield of infectious virus by 99% and inhibited human cytomegalovirus DNA replication at a concentration of 0.08 μM. In addition, oligonucleotides with base substitutions which resulted in base pair mismatches showed lesser degrees of activity, indicating a sequence-specific antisense mechanism. UL36ANTI was also shown to inhibit DNA replication of ganciclovir-resistant strains and human cytomegalovirus clinical isolates.

The use of antisense phosphorothioate (PS) synthetic oligonucleotides to selectively inhibit cellular and viral gene expression has been demonstrated in a variety of systems (1, 2, 5, 11, 19). Inhibition of virus replication by using antisense oligonucleotides complementary to the RNA of essential genes may be an effective antiviral therapy because of the specificities and low levels of toxicity of these compounds (25). Antisense PS oligonucleotides bind to mRNA in a sequence-specific manner, and as a result they are substrates for endogenous cellular RNase H, which cleaves the mRNA, and subsequently the message is degraded (25). Although, in theory, this interaction between antisense oligonucleotide and mRNA is specific, it must be recognized that nonspecific interactions can also result in the inhibition of virus replication (8, 9, 13, 25).

Recently, the genes required for human cytomegalovirus (HCMV) origin-dependent DNA replication were elucidated by a cotransfection replication assay (16, 17). The loci identified in those reports encode genes that were previously identified on the basis of nucleotide sequence homology to be candidate homologs to herpes simplex virus type 1 replication genes (3, 15, 26). However, other loci contained genes not previously implicated in HCMV DNA replication (16, 17). These include HCMV immediate-early and early kinetic class proteins, one of which is the region encoding HCMV genes UL36 to UL38. In a previous report we described the use of an antisense oligonucleotide, UL36ANTI, to demonstrate that the UL36 and UL37 gene products are required for HCMV DNA replication (21). The selective inhibition of UL36 mRNA and not other immediate-early genes also strongly suggests an antisense mechanism of action (21). In this report we describe the use of this antisense oligonucleotide to show that this compound can potently inhibit virus replication and is a potential antiviral agent.

Cells, viruses, and oligonucleotides. Human foreskin fibroblast (HFF) cells were propagated in CCM2 (Hyclone, Logan, Utah) supplemented with kanamycin.

HCMV strain AD169 was purchased from the American Type Culture Collection (ATCC VR-538). Ganciclovir (GCV)-resistant HCMV recombinant viruses were a generous gift from S. Stanat and K. Biron (Burroughs Wellcome); strains and their characterizations are as follows: (i) 8805 contains a mutation that leads to a Met→Val amino acid change at position 460 of the protein sequence in the UL97 gene; (ii) 8704 contains a mutation that corresponds to a Leu→Ser amino acid change at position 595 of the protein sequence of the UL97 gene. Both mutated genes were obtained from clinical isolates and were recombined into AD169, replacing the native UL97 gene (7, 22). All GCV-resistant strains were shown in our laboratory to grow in the presence of 100 μM GCV (20). HCMV clinical isolates were a gift from S. St. Jeor (University of Nevada-Reno); their characterization is as follows: CI1 was isolated from monocytes of a heart transplant patient with an active CMV infection. CI2 was isolated from the kidney of a 2-month-old male with an active CMV infection.

HCMV AD169 nucleotide sequence coordinates are from Chee et al. (3).

PS oligonucleotides were synthesized at Hybridon Inc. on a Millipore model 8800 DNA synthesizer. Sulfur transfer was accomplished by using β-cyanoethyl phosphoramidite chemistry and 3-*H*-1,2-benzodithiol-3-1,1,1-dioxide (Beaucage). The oligonucleotides used in cell culture assays were purified by either high-pressure liquid chromatography or ethanol precipitation (full-length purity, 70 to 90%).

Screening for inhibition of HCMV UL44 production by ELISA. HFF cells were plated at a density of 5,000 cells per well in a 96-well microtiter plate (Falcon, Franklin Lakes, N.J.) 24 h prior to treatment. Cells were pretreated with antisense or control oligonucleotides at the various indicated concentrations in CCM2 medium (Hyclone) for up to 15 h. The growth medium was then removed, and the cells were washed three times with Dulbecco's phosphate-buffered saline to remove any residual oligonucleotide. Cells were incubated with HCMV strain AD169 at a multiplicity of infection (MOI) of 0.05 for 1 h at 37°C. Cells were washed again and were refed fresh growth medium containing serial dilutions of antisense oligonucleotide at the same concentrations as those used during preincubation. At 5 to 6 days postinfection (p.i.) cells were fixed (100% ethanol) and reacted with primary antibody specific for the HCMV UL44 gene product (Advanced Biotech-

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TABLE 1. Oligonucleotide screening for anti-HCMV activity

Oligonucleotide name	Oligonucleotide sequence (5′–3′)	HCMV gene target	EC_{50} (μM)[a]
IRS1ANTI-A	CCG TTG CGC TGG GCC ATG GG	IRS1 ATG	0.3 (1)
IRS1ANTI-B	CAT GGG CGC CGG ACA CCT GC	IRS1 ATG	0.8 (1)
UL101ANTI-A	GGC TGA GCG GTC ATC CTC GGA	UL101 ATG	0.2 (2)
UL102ANTI-A	GCG AAA CGA CAT GGC CAA ATC	UL102 ATG	0.35 (2)
UL102ANTI-B	GCG CGT GGG TGC CAT ACT CTT	UL102 ATG	0.5 (2)
UL36ANTI-A	GAC GTG GGG CTT ACC TTG CG	UL36 splice donor	0.2 (2)
UL36ANTI-B	CAA CGA CGT GGG GCT TAC CT	UL36 splice donor	0.27 (2)
UL36ANTI-C	TCT TCA ACG ACG TGG GGC TT	UL36 splice donor	0.08 (3)
UL36ANTI-D	CTT GCG AAC AGA CGG TGC CC	UL36 splice donor	0.27 (1)
UL36ANTI	TGG GGC TTA CCT TGC GAA CA	UL36 splice donor	0.06 (6)
UL36ANTI-2	ACA GCA GAC GTA AGC ACC TG	UL36 splice acceptor	0.15 (1)
UL37ANTI-A	CGC GGC ATG GTG AGA CTG CT	UL37 spliced transcript	1.4 (1)
UL37ANTI-B	ACC CCT GCT TAC TGG TGA GA	UL37 splice donor	1.4 (1)
UL37ANTI-C	GTT GTT TTT ACC TGA AAC CC	UL37 splice acceptor	3 (1)
UL37ANTI-D	CCG AAC GGC GGT TTC TCC AC	UL37 splice acceptor	0.5 (1)
UL44ANTI-A	CTT GCG ATC CAT CCC GGA CAG	UL44 ATG	0.85 (1)
UL44ANTI-B	CTC CGA GAG GCG CGT CTT GC	UL44 ATG	1.1 (1)
UL84ANTI-A	GAC GCG TGG CAT GCT TGG TGT	UL84 ATG	0.08 (2)
UL84ANTI-B	AGG TTG GGG TCG ACG CGT GGC	UL84 ATG	0.48 (1)
Unrelated oligonucleotide (NS)	TCT GGG TAA TTA CAG CAA GC		2.1 (6)
PFA			67 (6)
UL36SENSE	TGT TCG CAA GGT AAG CCC CA	UL36 sense strand	
UL36NONSENSE	ACA AGC GTT CCA TTC GGG GT		
ISIS 2922	GCG TTT GCT CTT CTT CTT GCG	IE2 coding region	

[a] Number of experiments is given in parentheses. EC_{50}, 50% effective concentration. The 50% effective concentrations of UL36ANTI, NS, and PFA were determined from six experiments each, as discussed in the text.

nologies, Columbia, Md.). Cells were then reacted with anti-mouse immunoglobulin G conjugated to secondary antibody labeled with horseradish peroxidase (Kirkegaard & Perry, Gaithersburg, Md.). The secondary antibody was developed with tetramethylbenzidine substrate, and the optical density at 450 nm was determined with a plate reader (Ceres 900; Biotek). The 50 and 90% effective concentrations (EC_{50}s and EC_{90}s, respectively) for UL36ANTI were calculated by using the results of at least three separate experiments.

UL44 encodes the HCMV polymerase processivity factor and is present in infected cells at early times in infection (10, 12). The level of this antigen was used as an indicator of HCMV DNA replication and antiviral efficacy since this protein is essential for the replicative process (17). HFF cells plated in a 96-well format were pretreated with various PS antisense oligonucleotides complementary to HCMV replication gene transcripts as described above. Initial screening of the oligonucleotides was done at 10, 2, and 0.4 μM. Table 1 shows the results of the enzyme-linked immunosorbent assay (ELISA) screening. The antisense oligonucleotide UL36ANTI was the most active with respect to inhibition of UL44 production, having a highly reproducible EC_{50} of 0.06 ± 0.02 μM (standard deviation; $n = 6$). At oligonucleotide concentrations of 0.08 μM, antigen production was reduced by more than 90%. EC_{50}s for UL36ANTI were unchanged when higher MOIs were used. At an MOI of 0.8, EC_{90}s were calculated to be 0.15 ± 0.13 μM (standard deviation; $n = 4$). The UL36 to UL38 region encodes four transcripts, and UL36ANTI is complementary to 10 nucleotides of the intron and 10 nucleotides of the exon in the splice donor junction of these unspliced transcripts (21).

Evaluation of other UL36 sequences and base pair mismatches of UL36ANTI. It was determined previously that UL36ANTI could inhibit viral DNA replication almost completely at an oligonucleotide concentration of 0.08 μM (21). In order to further define the sequence specificity of UL36ANTI we introduced various base changes into this oligonucleotide. This would result in base pair mismatches that would affect the ability of UL36ANTI to bind to a target. We then evaluated the abilities of these altered compounds to inhibit HCMV DNA replication.

Cells were incubated with oligonucleotide and were infected as described above. The level of HCMV DNA replication was determined by Southern blot analysis (21). The base pair mismatches created by the substitution of any one base essentially eliminated the abilities of these oligonucleotides to inhibit DNA replication at 0.08 μM (Fig. 1). However, some substitutions retained good inhibition at 0.4 μM (see lanes MM1 and MM4). The oligonucleotide MM1 contains a G-to-C substitution in the center of the oligonucleotide. This substitution resulted in a loss of activity at 0.08 μM (Fig. 1, lane MM1). These mismatches result in melting temperatures (T_ms) of 55.6°C for the oligonucleotide MM1 and 60.8°C for the oligonucleotide MM4. Other base pair mismatches introduced in the middle of the oligonucleotide, for example, in lane MM2, appeared to have a greater effect on activity than those changes introduced toward the 3′ end of the molecule (Fig. 1, lanes MM2 and MM4). UL36ANTI-A and UL36ANTI-B are two antisense oligonucleotides whose sequences shift 4 and 8 bases, respectively, downstream (in the 3′ direction) compared with the sequence of UL36ANTI (Table 1). These oligonucleotides were active at 0.4 μM; however, at 0.08 μM they were significantly less active than UL36ANTI (compare the lanes marked UL36ANTI-A and UL36ANTI-B with the lane marked UL36ANTI in Fig. 1). Base pair mismatches introduced at the 5′ end also had a marked effect on activity (Fig. 1, lane MM3). T_ms for oligonucleotides MM2 and MM3 were determined to be 48.8 and 51.6°C, respectively. There is a correlation between the relative decrease in T_ms resulting in different binding affinities and the activity of UL36ANTI (Fig. 1). UL36-D is an antisense oligonucleotide that is complementary to a sequence that is completely within the intron of

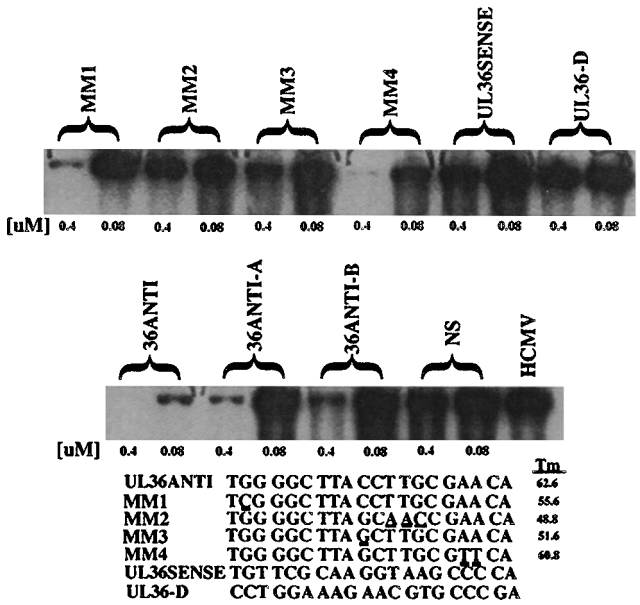

FIG. 1. UL36ANTI base pair mismatches and their effects on inhibition of HCMV DNA replication. Southern analysis was performed as described previously (21). Relative band intensities for Southern blots were calculated by using Scan Analysis software (Biosoft, Ferguson, Mo.). Positive control samples are reported as having a value of 1; all other band intensities are reported as some decimal fraction of this amount. UL36ANTI and various other oligonucleotides are shown below; underlined bases indicate substitutions. Relative differences in T_ms, given at the right of the oligonucleotide sequences, were determined by incubating oligonucleotide with target DNA that was 5 bases longer in the 3′ and 5′ directions in a buffer of 100 mM NaCl, 10 mM MgCl, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.0) and generating A_{260}-temperature curves.

UL36-UL37 (21). This oligonucleotide was completely inactive at 0.08 and 0.4 μM. In addition, an oligonucleotide complementary to UL36ANTI, UL36SENSE, had no effect on the level of HCMV DNA replication (Fig. 1, lane UL36SENSE).

Infectious virus yield reduction assay. HFF cells were treated with oligonucleotide as described previously by using an MOI of 0.4 (21). At 7 days p.i. supernatants were harvested, diluted serially from 10^{-3} to 10^{-7}, and used to infect fresh fibroblasts seeded in six-well plates at a density of 10^5 cells per well. After a 1-h incubation, virus was removed and cells were overlaid with 0.4% agarose containing Dulbecco's modified Eagle's medium plus 10% fetal calf serum. At 14 days p.i. the cells were fixed with 10% formalin and stained with 0.2% methlyene blue, and virus plaques were counted. Figure 2 shows a dose-response that indicates that UL36ANTI treatment at 0.08 μM was sufficient to reduce infectious virus yield by 99% when compared with that in infected, untreated control samples. Similar results were achieved with ISIS 2922 when it was used at a concentration of 2 μM, which is in agreement with published results (2). GCV required a concentration of greater than 20 μM to reduce virus yield by 99% in this assay. UL36SENSE oligonucleotide failed to reduce the level of infectious virus at any concentration tested.

UL36ANTI is active against GCV-resistant strains and clinical isolates. For GCV-resistant strains 8805 and 8704, complete inhibition of viral DNA replication was apparent at 0.4 μM, and a significant inhibition occurred at 0.08 μM (Fig. 3A, lanes UL36ANTI). Some nonspecific effects on replication were observed when virus 8704 was used (Fig. 3A, lanes NS [nonspecific oligonucleotide] 2 and 0.4 μM). In other experiments, AD169 recombinant virus strains that were either foscarnet (PFA) resistant or that contained a deletion in the UL97 region were also similarly inhibited by UL36ANTI when it was used at a concentration of 0.08 μM (20). UL36ANTI was also active against HCMV clinical viral isolates CI1 and CI2 (Fig. 3B).

Comparison of UL36ANTI with current anti-HCMV agents. Figure 4 is an autoradiogram of a Southern blot showing the activities of UL36ANTI, ISIS 2922 (see Table 1), PFA, and GCV with respect to inhibition of HCMV DNA replication. By Southern analysis, UL36ANTI was more than 6,000 times more active than PFA and more than 1,000 times more active than GCV (Fig. 4, lanes UL36ANTI [0.08 μM], PFA [500 μM], and GCV [100 μM]). In addition, ISIS 2922, an antisense

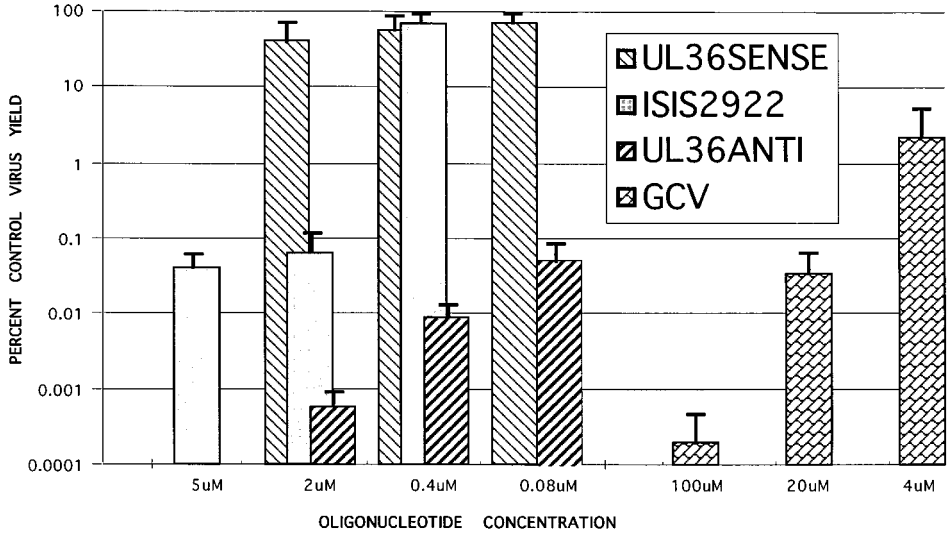

FIG. 2. Infectious virus yield reduction assay. Samples were treated as described in text. The dose-responses of oligonucleotides listed were generated by using a plaque assay to evaluate infectious virus in the supernatants from cells at 7 days p.i. Virus titers of untreated samples were approximately 10^6 PFU/ml. Titers of UL36ANTI-treated samples were 1×10^2 and 6.5×10^2 PFU/ml at 0.4 and 0.08 μM, respectively. NS-treated titers were approximately 5×10^5 PFU/ml at an oligonucleotide concentration of 0.08 μM. Results are plotted as a percentage of the yield of infected, untreated cultures.

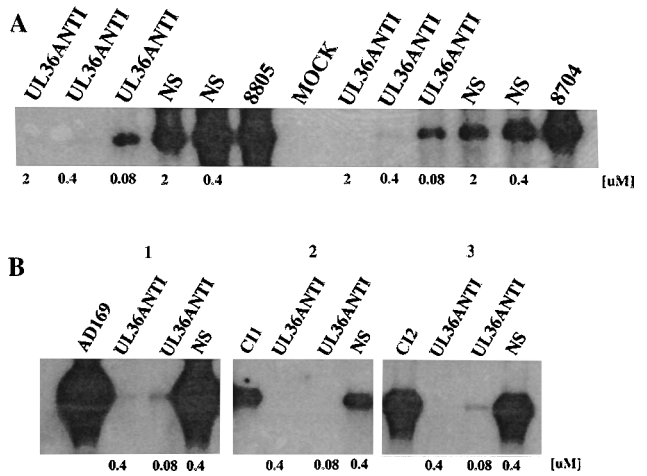

FIG. 3. UL36ANTI is active against HCMV recombinant GCV-resistant viruses and clinical isolates. Autoradiogram of a Southern transfer of total cell DNA infected with either recombinant viruses 8805 or 8704 (A) or HCMV clinical isolates Cl1 or Cl2 (B). Oligonucleotide concentrations are given below the autoradiogram. See text for descriptions of viruses.

oligonucleotide that is complementary to the immediate-early RNA IE2 of HCMV (2) is approximately 60 times less active than UL36ANTI (Fig. 4, lanes UL36ANTI [0.08 μM] and ISIS2922 [5 μM]). These results indicate that UL36ANTI is a potent inhibitor of HCMV DNA replication compared with conventional anti-HCMV drugs and other antisense drugs with anti-HCMV activity.

Evaluation of cell growth and cytotoxicity by the MTT assay. Antisense oligonucleotides UL36ANTI or NS were incubated with HFF cells in 200 μl of CCM5 medium. The cells were seeded in 96-well plates at a density of 1,000 cells per well. Control cells were treated in the same manner as the test samples, except that no oligonucleotide was added. Cells were allowed to incubate with oligonucleotide for 5 days. After this incubation 50 μl of MTT (2 mg/ml) was added and the mixture was allowed to incubate for 2 h at 37°C. The medium was then removed, and 100 μl of dimethyl sulfoxide was added to each well. The optical density at 570 nm was determined with a Biotex Ceres 900 plate reader. The average for triplicate samples was determined, and values are reported as a percentage of the value for the control.

The results show that with the use of oligonucleotide concentrations of 12.5 μM or less, no apparent effect on viable cell growth was observed compared with the effect on growth of untreated control cells. At oligonucleotide concentrations of 100 μM, viable cell growth was decreased by 40%, indicating that the effective antiviral concentration of 0.08 μM for UL36ANTI is more than 1,200 times less than the concentration necessary to significantly affect cell growth (20).

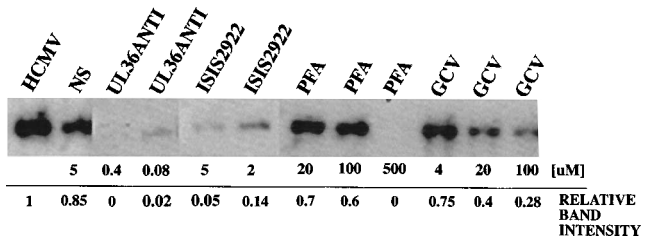

FIG. 4. Comparison of anti-HCMV activity of UL36ANTI with those of other anti-HCMV therapeutic agents. An autoradiogram of Southern transfer performed as described previously shows the concentration required to inhibit HCMV DNA replication. The relative band intensity is shown below each band.

Previously, it was shown that UL36ANTI, complementary to the intron-exon boundary of HCMV genes UL36 and UL37, can selectively inhibit these mRNAs, resulting in a significant decrease in HCMV DNA replication (21). These genes are required for HCMV origin-dependent DNA replication and therefore make logical targets for antiviral intervention (17). The exact roles of the UL36 to UL38 gene products in HCMV DNA replication have not been determined, although they are transactivators, upregulating promoters of both cellular and viral origins (4). Although the functions of UL36 and UL37 with respect to HCMV DNA replication have not been determined, their requirement for viral growth has now been demonstrated (21).

The antiviral activity of UL36ANTI was demonstrated by inhibition of viral DNA synthesis, inhibition of viral antigen production, and inhibition of infectious virus yield. Several lines of evidence indicate that these effects are due to a sequence-specific antisense activity. First, the introduction of base substitutions that result in base pair mismatches decrease and in some cases abolish the ability of the oligonucleotide to inhibit HCMV DNA replication. Second, oligonucleotide sequences complementary to sequences just upstream or downstream of UL36ANTI show slight decreases in potency, indicating that UL36ANTI (complementary to exactly 10 bp within the intron and 10 bp within the exon of UL36-UL37) is the most active sequence. Third, no antiviral activity was observed when infected cells were treated with an oligonucleotide complementary to the sense strand of UL36-UL37. In addition, negative results were also observed when either nonspecific or reverse sequence UL36ANTI oligonucleotides were used. Finally, UL36ANTI selectively inhibited UL36-UL37 mRNA expression, while the steady-state levels of HCMV immediate-early RNA IE2 were unaffected (21). In addition, oligonucleotides did not inhibit the attachment and adsorption of virus, as measured by the amount of input viral DNA present within cells incubated with oligonucleotide (21).

Northern analysis of RNA at 6 h p.i. indicated that the level of UL36 mRNA originating from input virus was undetectable in treated cells (21). RNA and DNA harvested at later time points showed that viral replication was inhibited and that UL36 mRNA was still at undetectable levels (21). This indicates that the expression of UL36-UL37 is essential for virus replication (21). It is likely that the UL36 and UL37 gene products encode proteins that transactivate other factors essential to HCMV. UL36 mRNA is detectable as early as 4 h p.i. and is present throughout the infection cycle (23, 24). RNA harvested at 6 h p.i. is transcribed before the onset of viral DNA replication which initiates at approximately 36 h p.i. (14). The level of transcription measured at later times showed the extent of viral infection and confirmed that the spread of infection was efficiently halted (20).

HCMV genes that control transcription or that transactivate other viral genes can be excellent targets for antiviral agents. These genes are primarily immediate-early genes that are initially expressed 0 to 4 h p.i. (14). In theory, inhibition of expression of these gene products should result in an overall decrease in HCMV gene expression, including the expression of genes required for HCMV DNA replication. Indeed this seems to be the case for inhibition of UL36-UL37 gene expression as presented in this report. UL36 and UL37 are immediate-early genes expressed in the absence of de novo protein synthesis (24).

The UL36 to UL38 region is required for HCMV origin-

dependent DNA replication and hence provides necessary factors that participate either directly or indirectly in replication (17). The protein products of these transcripts were shown to transactivate both cellular and viral promoters (4). The UL36 mRNA is present at immediate-early, early, and late times during infection and encodes a protein with an unknown function (24). UL37 encodes an immediate-early protein that is predicted to be a membrane protein (24). The UL37 transcript is 3′ coterminal with UL36; however, nucleotide sequence analysis indicates that the protein-coding regions do not overlap and that the UL37 protein terminates upstream of the putative ATG codon for UL36 (23, 24).

All experiments reported here involved pretreatment of cells with the antisense oligonucleotide. In the absence of pretreatment, inhibition of HCMV DNA replication was reduced about 5- to 10-fold, but it was determined that the oligonucleotide pretreatment time can be as short as 3 h with no loss of activity with respect to inhibition of HCMV DNA replication (20). Pretreatment is probably necessary to achieve adequate intracellular concentrations of oligonucleotide before infection, because UL36-UL37 RNA is very abundant as early as 4 h p.i. (24). This may indicate that postinfection treatment may not allow enough time for an effective intracellular buildup of antisense oligonucleotide at these time points. However, this limitation of cell culture may not be a significant consideration with regard to treatment in an infected host, since uninfected cells could potentially build up amounts of oligonucleotide sufficient to halt the spread of infection.

Antisense PS oligonucleotides may act through a mechanism in which the oligonucleotide-RNA complex is a substrate for cellular RNase H degradation of the target RNA (25). This may be one possible mode of action for the UL36ANTI oligonucleotide. However, since this oligonucleotide is targeted against an intron-exon boundary, inhibition of splicing or transportation into the cytoplasm and subsequent degradation of the UL36 transcript may also be other possible mechanisms. UL36ANTI, with its potent anti-HCMV activity, represents an attractive antisense candidate for further drug development.

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