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Expression of Human Cytomegalovirus UL36 and UL37 Genes Is Required for Viral DNA Replication

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It was previously reported that the region encoding human cytomegalovirus (HCMV) genes UL36 to UL38 was required for origin-dependent DNA replication. These genes encode transactivators that upregulate viral and cellular transcription. However, their requirement for viral DNA replication has not been demonstrated. We have now used an antisense phosphorothioate oligonucleotide complementary to the intron-exon boundary of the UL36 and UL37 unspliced RNA to show that these gene products are required for HCMV DNA replication. Southern analysis showed that this oligonucleotide almost completely inhibits HCMV DNA replication when used at concentrations as low as 0.08 μM. The ability of this oligonucleotide to inhibit DNA replication was not the result of an inhibition of virus adsorption. Southern blots showed no impairment of viral adsorption or internalization in the presence of either specific or nonspecific phosphorothioate oligonucleotides. In addition, Northern (RNA) blots confirm that this antisense compound specifically reduced UL36 mRNA in treated cells to undetectable levels while the steady-state levels of immediate-early transcripts IE1 and IE2 were unaffected. These results demonstrate that the UL36 and UL37 gene products provide an essential function in initiation of HCMV DNA replication.

Herpesvirus DNA replication is initiated at distinct *cis*-acting regions called origins of replication (2, 9, 18, 19, 37, 38). These origins have been defined with respect to sequences that are essential for viral replication, and in some systems, origin-binding proteins which recognize specific viral sequences within the origin have been identified (1, 9, 10, 18, 22, 28, 42). In most of these systems the viral factors required in *trans* to initiate and perform all the essential functions of replication have been elucidated by employing a cotransfection-replication assay (11, 29, 30, 43). This method most commonly involves cotransfection of subgenomic fragments along with a defined cloned origin of replication and assaying for *Dpn*I-resistant (replicated) plasmid. In some cases, for example with human cytomegalovirus (HCMV) and Epstein-Barr virus, these assays identified genes that may play an essential role other than an enzymatic function in DNA replication (11, 29, 30). These viral transactivators may be required for the interaction with or induction of host cell factors that aid in initiation of the replication process (17, 27). With herpes simplex virus type 1, the generation of *ts* mutants and insertional mutations have aided in the confirmation of knowledge relating not only to factors required for origin-dependent DNA replication but also to requirements for virus replication (4, 16, 23, 25, 32).

For HCMV, the cotransfection-replication assay has determined that 11 loci are required for origin-dependent DNA replication (29, 30). These loci contain the putative candidate HCMV homologs to herpes simplex virus type 1 replication genes (26, 29, 30, 43). These are UL54 (DNA polymerase), UL44 (polymerase accessory protein), UL57 (single-stranded DNA-binding protein), UL105 (helicase), UL70 (primase), and UL102 (primase-associated factor). In addition to these loci, other essential genes identified were UL84 (early protein), UL112 and UL113 (early proteins), IE1 and IE2 (immediate-early proteins), IRS1 (immediate-early protein), and UL36 to UL38 (immediate-early and early proteins). The proposed function, with respect to DNA replication, of some of these gene products is clear; however, others are still undefined. Direct evidence for the requirement of any of these genes, except polymerase (7), in virus replication has yet to be demonstrated. The lack of HCMV mutants or an efficient method to generate recombinants with mutations in essential genes makes it difficult to determine if genes required for origin-dependent replication are indeed required for virus replication. To this end, we used an antisense oligonucleotide directed against the UL36 and UL37 splice donor regions (39, 40) to show both the selective inhibition of UL36 mRNA and HCMV DNA replication.

Antisense phosphorothioate (PS) oligonucleotides and antisense RNA expression have been used to inhibit the expression of a number of viral genes, including those of HCMV (3, 13, 14, 21, 24, 33, 36, 44). Most of these applications of antisense were directed at genes known to be indispensable for replication in an effort to develop a potent antiviral agent. In this report we demonstrate that antisense technology can be used to show that a gene(s) required in the transient replication assay is necessary for virus replication.

Four RNAs are expressed from the UL36 to UL38 region; UL36, UL37, UL37X1, and UL38 (40). These proteins transactivate HCMV early gene and cellular promoters in transient assays (6). The transcripts encoded from these genes are the result of multiple spliced mRNAs (6, 40). Figure 1A illustrates the relative positions of these transcripts. A 100-bp intron is excised to produce the 1.68-kb UL36 mature transcript (40). UL36 mRNA is detected at immediate-early, early, and late times during infection (40). The UL37 3.2-kb mature transcript results from the excision of three introns, one of which is shared with UL36 mRNA (Fig. 1A) (40). The UL37 transcript is detected only under immediate-early conditions (40). UL36 and UL37 use the same polyadenylation signal, although the proposed protein-coding region of UL37 does not overlap with the coding region of UL36 (40). We targeted genes within this region because of their apparent requirement for origin-dependent DNA replication and to investigate whether they are also necessary for virus replication. This would be the initial

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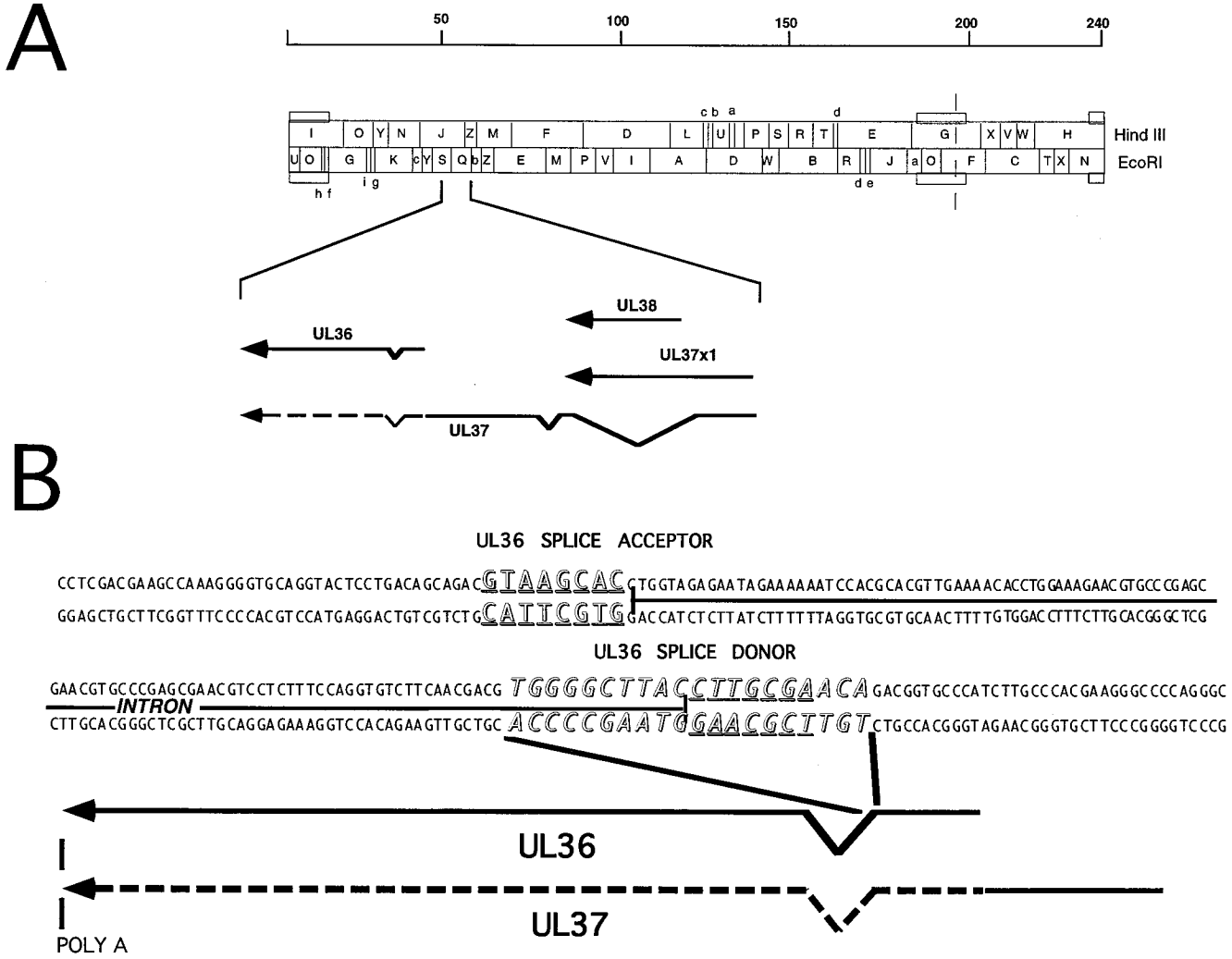

FIG. 1. UL36 to UL38 locus required for HCMV origin-dependent DNA replication. (A) Map of transcripts originating from UL36 to UL38 region. Arrows A and B denote the UL36ANTI antisense oligonucleotide complementary to both UL36 and UL37 splice donor regions. (B) Sequence of UL36-UL37 intron and partial sequence of exons 1 and 2. Underlined large letters indicate splice acceptor and donor regions that form the mature transcripts; the position and sequence of UL36ANTI antisense oligonucleotide 5′-TGGGCTTACCTTGCGAACA-3′ are also shown. The dashed lines in the UL37 transcript indicate the untranslated region of this RNA as predicted by nucleotide sequence analysis; UL36 and UL37 are presumed to use the same polyadenylation signal and therefore terminate at the same position.

step toward the goal of eventually determining their function in HCMV DNA replication within the context of the virus genome.

PS oligonucleotides were synthesized by using a Pharmacia gene assembler-special DNA synthesizer. Sulfur transfer was accomplished by using beta-cyanoethyl phosphoramidite chemistry and 3-H-1,2-benzodithiol-3-1,1,1-dioxide (Beaucage). Oligonucleotides used in cell culture assays were purified by either high performance liquid chromatography or ethanol precipitation and were determined to be 70 to 90% full-length products by gel electrophoreses. The PS oligonucleotides used in this report were 5′-TGGGGCTTACCTTGCGAACA-3′ (UL36 ANTI), complementary to the intron-exon boundary of UL36/37 and a control sense oligonucleotide, and 5′-TGTTCGCA AGGTAAGCCCCA-3′, referred to as SENSE and homologous to UL36 mRNA. These oligonucleotides correspond to HCMV genomic coordinates 49,565 to 49,584 (5). In addition, two other compounds were used as controls: 5′-TCTGGGTA ATTACAGCAAGC-3′, an unrelated nonspecific (NS) control oligonucleotide, and 5′-ACAAGCGTTCCATTCGGGGT-3′, a nonsense (NONSENSE) control oligonucleotide which is the same nucleotide sequence as UL36ANTI except in reverse order.

Initial screening for a UL36 to UL38 antisense oligonucleotide that could inhibit viral gene expression utilized an enzyme-linked immunosorbent assay (ELISA) to determine the production of HCMV UL44 (polymerase-associated factor). This protein was selected because it is an early protein that is required for HCMV DNA replication and therefore is a good indicator of the extent of virus replication. Figure 1B shows the position of the tested oligonucleotide. Human foreskin fibroblasts (HFF) 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 oligonucleotides at the various indicated concentrations in CCM5 medium (Hyclone, Logan, Utah) 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

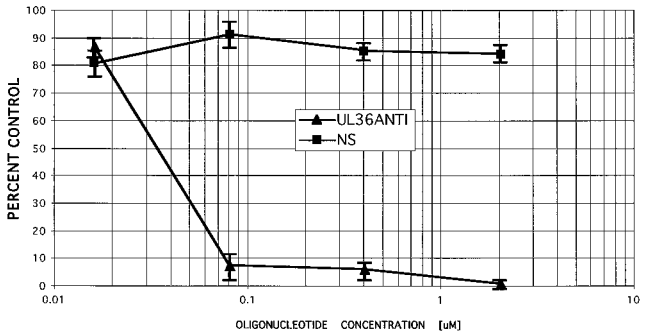

FIG. 2. UL36ANTI reduces the level of UL44. The results of an ELISA of antisense oligonucleotides complementary to mRNA from the UL36 to UL38 region are shown. HHF cells treated and infected as described in the text were screened for UL44 expression with a monoclonal antibody specific for the UL44 gene product. The UL36ANTI (complementary to UL36 intron-exon boundary of the unspliced RNA) antisense oligonucleotide reduced the expression of the UL44 gene product over 90% at 0.4 μM compared with the infected, untreated control. Error bars represent standard deviations calculated for datum points collected for each oligonucleotide concentration.

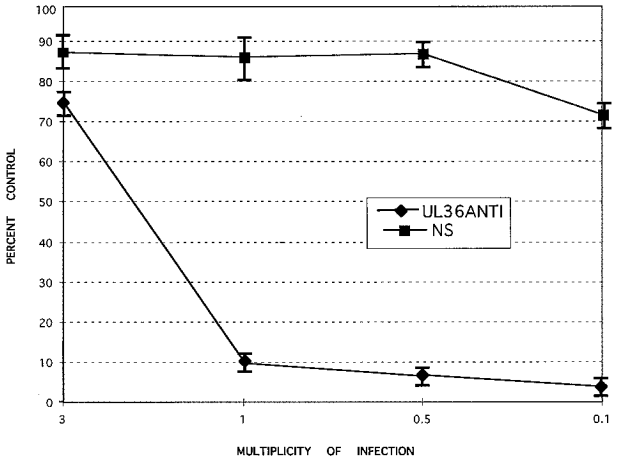

FIG. 3. Effect of different MOIs on antisense activity of UL36ANTI. The results of an ELISA show UL36ANTI inhibition of UL44 production at various MOIs. The oligonucleotide concentration used was 0.4 μM. The nonspecific (NS) oligonucleotide is described in the text.

any residual oligonucleotide. Cells were incubated with HCMV strain AD169 (ATCC VR-538) at a multiplicity of infection (MOI) of 0.1 for 1 h at 37°C. Cells were washed again and reefed in fresh growth medium containing serial dilutions of the antisense oligonucleotide at the same concentrations used in the preincubation. At 5 to 6 days postinfection (p.i.), cells were fixed (100% ethanol) and reacted with a primary antibody specific for the HCMV UL44 gene product (Advanced Biotechnologies, Rivers Park, Ill.). The cells were then reacted with anti-mouse immunoglobulin G conjugated to horseradish peroxidase-labeled (Kirkegaard and Perry, Gaithersburg, Md.) secondary antibody and developed with a tetramethylbenzidine substrate, and the optical density at 450 nm was determined by using a plate reader (Ceres 900, Biotek).

Figure 2 shows the result of a typical dose response ELISA experiment. The nonspecific oligonucleotide had no effect on antigen production even at a concentration of 2 μM; however, the antisense oligonucleotide complementary to the intron-exon boundary of UL36-UL37, UL36ANTI, inhibited UL44 expression almost completely at concentrations as low as 0.08 μM (Fig. 2). Antisense activity was lost however at an oligonucleotide concentration of 0.016 μM. Figure 1B shows the position and sequence of the antisense oligonucleotide 36ANTI, which contains a sequence complementary to 20 bp spanning the intron-exon boundary of the unspliced mRNA of both UL36 and UL37. Antisense oligonucleotides targeted against other splice donor or acceptor sites of UL37, UL37X1, or UL38 failed to reduce the level of the UL44 protein (34). We have also shown that the amount of virus yield is reduced to 99% of the amount of the control at a concentration of 0.08 μM (35).

The effect of different MOIs on antisense activity was also evaluated. Figure 3 is an ELISA experiment in which oligonucleotides were incubated with cells as described above except at a concentration of 0.4 μM. The cells were then infected at various MOIs. This experiment shows that significant inhibition of viral antigen expression was observed at all MOIs except 3.0.

To determine the effect of this oligonucleotide on HCMV DNA replication directly, we carried out Southern analysis on total infected-cell DNA treated with specific and nonspecific antisense oligonucleotides. HFF cells were seeded in 6-cm-diameter dishes at a density of 2×10^5 cells per dish and treated exactly as described above for the ELISA except that infection was carried out at an MOI of 0.4. At 5 to 6 days p.i., cells were lysed directly on the culture dish with 2% sodium dodecyl sulfate (SDS)–10 mM Tris-HCl (pH 7.5)–10 mM EDTA and transferred to a 1.5-ml microcentrifuge tube. Lysates were treated with 500 μg of proteinase K per ml for 2 h at 60°C, extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1), ethanol precipitated, and resuspended in Tris-EDTA. One microgram of DNA was cleaved with *Eco*RI, electrophoresed on 0.8% agarose gel, transferred to a Zeta probe nylon membrane (Bio-Rad, Hercules, Calif.), and hybridized with a random primer ^{32}P-labeled plasmid containing HCMV *Eco*RI M fragment according to the manufacturer's instructions. This fragment hybridizes to a 7.3-kb fragment of *Eco*RI-cleaved HCMV DNA. Blots were then exposed to X-ray film for 4 to 6 h at room temperature.

Figure 4 (top panel) is an autoradiogram of total infected cell DNA probed with HCMV *Eco*RI-M. At an oligonucleotide concentration of 0.08 μM, the amount of HCMV DNA was reduced about 50-fold compared with that of infected, untreated control samples. In addition, at 0.4 μM, HCMV DNA replication was almost completely inhibited. Oligonucleotides with unrelated sequences (NS) or oligonucleotides homologous to the mRNA of UL36 (36SENSE) and reverse-order sequences (NONSENSE) had no effect on HCMV DNA replication (Fig. 4). Figure 4 (bottom panel) shows that the reduction in HCMV DNA replication was not attributed to inhibition of virus internalization as measured by the presence of intracellular HCMV DNA. We performed Southern analysis on the relative amount of input intracellular virus present after adsorption. HFF cells were treated as previously described with an oligonucleotide concentration of 2 μM, infected at an MOI of 1, and allowed to incubate with virus for 2 h. Cells were then washed three times to remove residual virus, and total cellular DNA was harvested, cleaved with *Eco*RI, electrophoresed, blotted, and probed as previously described. Figure 4 (bottom panel) is an autoradiogram showing the relative abundance of intracellular virus. These results indicated that the oligonucleotide pretreatment with UL36ANTI had no significant effect on viral adsorption (Fig. 4, bottom panel).

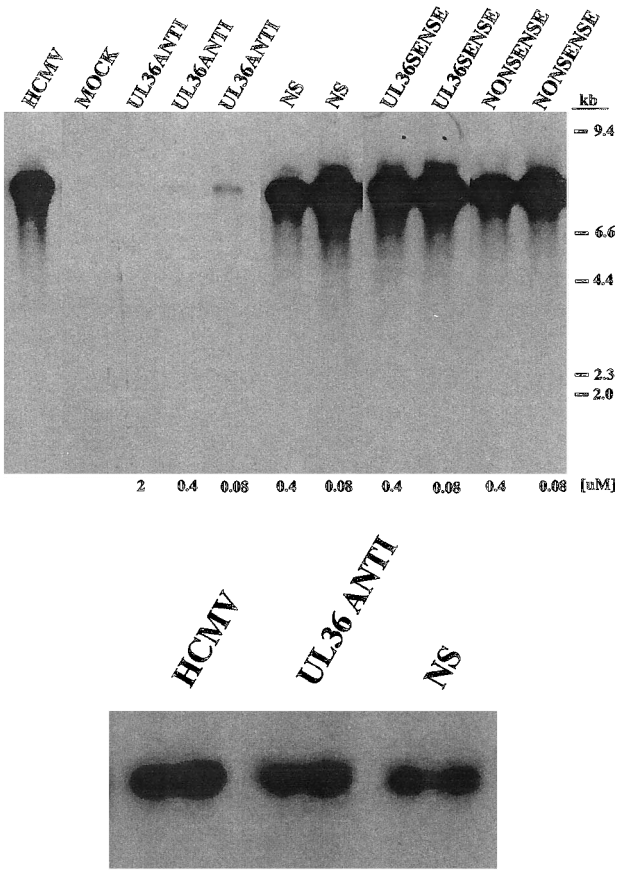

FIG. 4. (Top) Inhibition of HCMV DNA replication by UL36ANTI. Southern analysis of total infected cellular DNA hybridized with HCMV *Eco*RI M fragment. One microgram of total cell DNA from samples treated with the indicated oligonucleotides was cleaved with *Eco*RI and electrophoresed on a 0.8% agarose gel, blotted and probed. Antisense oligonucleotide complementary to UL36/37 (UL36ANTI), nonspecific (NS), an oligonucleotide complementary to the sense strand (UL36SENSE) or a nonsense sequence (NONSENSE) were used at the indicated concentrations in experiments evaluating their effect on HCMV DNA replication. (Bottom) Antisense oligonucleotides do not interfere with virus adsorption. Southern analysis of HCMV DNA 2 h p.i. showing that the presence of PS oligonucleotides incubated with HFF cells at a concentration of 2 μM prior to infection do not impair virus internalization. Cells were pretreated with UL36ANTI or nonspecific (NS) PS oligonucleotides 15 h prior to infection, washed three times, and infected (MOI of 1.0). Total cellular DNA was extracted 2 h p.i. and hybridized with HCMV *Eco*RI-M to determine the relative amounts of internalized HCMV DNA.

The lowest UL36ANTI concentration shown to inhibit UL44 production was 0.08 μM (Fig. 2). We also evaluated the lowest concentration that would inhibit HCMV DNA replication. Figure 5 is an autoradiogram of a Southern blot showing that at an oligonucleotide concentration of 0.016 μM UL36 ANTI no longer is capable of inhibiting DNA replication (Fig. 5, lane labeled UL36ANTI and 0.016 μM). This result is consistent with the ELISA result in Fig. 2; antisense activity is lost at a concentration of 0.016 μM.

The effect of the oligonucleotide UL36ANTI on viral cytopathic effect was also evaluated. Infected and treated HFF cells were photographed (magnification, ×100) prior to harvesting and extraction of total cellular DNA. Figure 6 shows that UL36ANTI significantly inhibits the development of cytopathic effect at 0.4 and 0.08 μM (panels B and C) compared with the development of cytopathic effect in untreated or nonspecific-oligonucleotide-treated, infected cells (panels A and D).

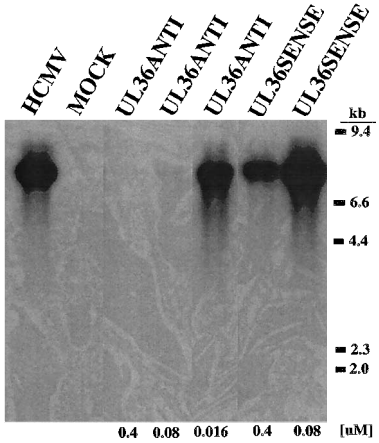

FIG. 5. UL36ANTI antisense activity is lost at an oligonucleotide concentration of 0.016 μM. A Southern blot shows that the ability of UL36ANTI to inhibit HCMV DNA replication was lost when an oligonucleotide concentration of 0.016 μM was used. Blots were hybridized as described in the text.

Once it was determined that the UL36 antisense oligonucleotide could inhibit viral DNA replication, we investigated its effect on the steady-state level of UL36 mRNA. HFF cells were plated in 6-cm-diameter dishes at a density of 10^6 cells per plate, pretreated with the UL36 antisense (UL36 ANTI) or nonspecific (NS) oligonucleotide as described above, and infected at an MOI of 0.8. At 6 h p.i., total cellular RNA was

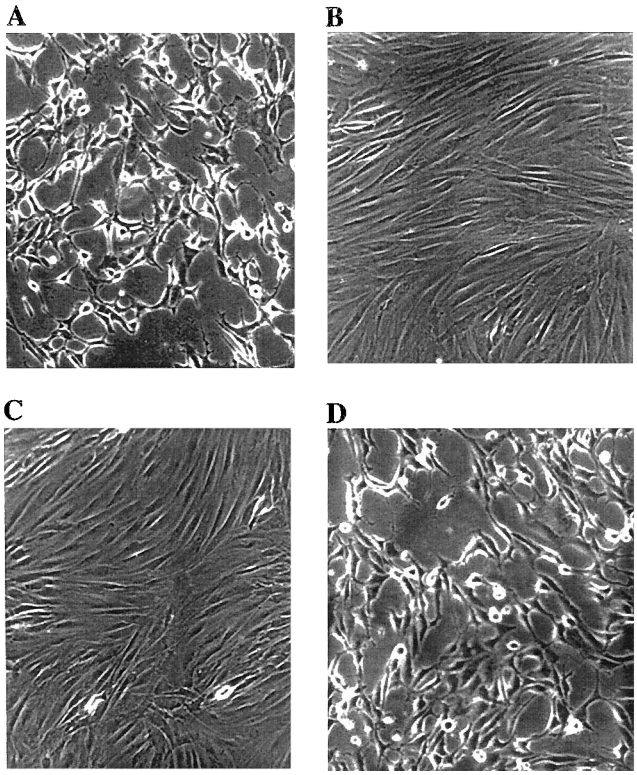

FIG. 6. UL36ANTI decreases viral cytopathic effect. HFF cells infected and treated with PS oligonucleotides were photographed at 6 days p.i. (MOI of 0.4). (A) HCMV-infected untreated cells; (B) HCMV-infected cells treated with 0.4 μM UL36ANTI; (C) HCMV-infected cells treated with 0.08 μM UL36ANTI; (D) HCMV-infected cells treated with 0.4 μM nonspecific oligonucleotide.

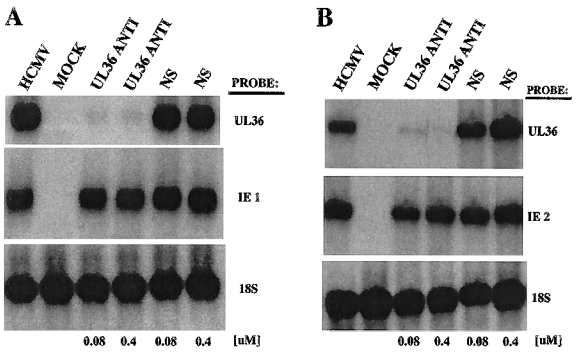

FIG. 7. Selective inhibition of UL36 mRNA. (A) Northern analysis of infected total cellular RNA harvested 6 h p.i. from samples treated at the indicated concentrations with oligonucleotides showing that UL36ANTI specifically decreases the steady-state level of UL36 RNA. The blot was hybridized with a probe specific for IE1 mRNA indicating that the steady-state level of IE1 RNA is unaffected by either UL36ANTI or nonspecific (NS) PS oligonucleotides. (B) Northern analysis of immediate-early RNA in the presence of specific and nonspecific oligonucleotides. An autoradiogram of a Northern blot hybridized with a probe specific for IE2 mRNA indicates that the steady-state level of IE2 is unaffected in the absence of UL36 mRNA and in the presence of UL36ANTI or nonspecific (NS) PS oligonucleotides.

prepared by the following procedure, as described previously (31). Infected treated and untreated, or mock-infected cells were lysed directly on a 6-cm-diameter dish with 500 μl of 2% SDS–200 mM Tris-HCl (pH 7.5)–1 mM EDTA, lysates were transferred to a 1.5-ml microcentrifuge tube, and 150 μl of ice-cold precipitation buffer (42.9 g of potassium acetate, 11.2 ml of acetic acid, water to 100 ml) was added. Tubes were vortexed and iced for 2 min and centrifuged for 5 min (room temperature), and supernatants were transferred to fresh tubes and extracted twice with 300 μl of chloroform-isoamyl alcohol (24:1). RNA was precipitated with 0.65 ml of ice-cold isopropanol and pelleted for 5 min at 14,000 × *g*. Pelleted RNA was resuspended in 50 μl of 100% formamide and stored at −80°C.

Ten micrograms (approximately 10 μl) of RNA was electrophoresed on a 1.0% agarose gel containing 6% formaldehyde and transferred to a Zeta probe nylon membrane. The filter was hybridized with ^{32}P-labeled single-stranded RNA probe (riboprobe) constructs complementary to either UL36 mRNA (genomic coordinates 48315 to 48959), IE1–1.95-kb mRNA (nucleotides 170887 to 173734), or IE2–2.2-kb RNA (nucleotides 169258 to 173734) in hybridization buffer (1.5× [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA; pH 7.7] SSPE, 1% SDS, 50% formamide, 0.5% nonfat dried milk, 100 μg of denatured salmon sperm DNA per ml) at 60°C for 16 h. Blots were then washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS (wt/vol) twice for 15 min each at room temperature and then with 0.1× SSC–0.1% SDS (wt/vol) twice for 45 min at 60°C. The blots were then exposed to X-Omat AR (Kodak) X-ray film at −80°C for 24 h.

Figure 7A shows the result of an autoradiogram of a typical Northern (RNA) blot. In samples in which the UL36 antisense oligonucleotide (lanes labeled UL36ANTI) was incubated with infected cells, no UL36-specific transcript could be detected at concentrations of 0.08 or 0.4 μM. The level of UL36 mRNA was unaffected by treatment with nonspecific oligonucleotide (lanes labeled NS). The blots were subsequently reprobed with probes specific to the immediate-early transcript IE1 (1.95-kb RNA). The level of IE1 mRNA in infected cells was unaffected by the presence of the UL36-specific antisense or nonspecific oligonucleotides or in the absence of UL36 mRNA (lanes labeled UL36ANTI and row labeled IE1). In addition, the level of 18S RNA was determined as a control for equivalent lane loading of the RNA sample. Similar results were obtained when UL36 ANTI-treated, infected cells were probed for the expression of the IE2 gene product (2.2-kb RNA). Figure 7B shows the result of an autoradiogram in which the IE2 mRNA level was unchanged when infected cells were treated with specific (lanes labeled UL36ANTI) or nonspecific (lane labeled NS) oligonucleotides. The UL36SENSE oligonucleotide also had no effect on the expression of UL36, IE1, or IE2 mRNA (34).

We have shown that the gene product(s) from UL36 and UL37 is required for HCMV replication. This was demonstrated by using a specific antisense PS oligonucleotide complementary to the intron-exon boundary of HCMV genes encoding UL36 and UL37. An HCMV subgenomic fragment encoding these genes was previously shown to be required for HCMV origin-dependent DNA replication (30).

The specificity of the UL36ANTI oligonucleotide was shown by Northern blot analysis in which the UL36 transcript was undetectable in infected cells when nanomolar amounts of oligonucleotide were used. RNA levels as well as HCMV DNA replication were unaffected by nonspecific, nonsense or sense control PS oligonucleotides (Fig. 4). It was also demonstrated that HCMV immediate-early transcripts IE1 and IE2 were unchanged in the absence of UL36 mRNA (Fig. 7). These experiments were done at times well before the onset of viral DNA replication (6 h p.i.). However, experiments done at later times p.i. (up to 120 h) revealed similar results (34), indicating that the UL36 gene product is essential for initiation of virus replication. We also demonstrated that adsorption of virus was unaffected and did not contribute to reduced levels of replication or transcription. The UL36ANTI oligonucleotide was also shown to be nontoxic to cells and did not affect the level of cellular transcription or metabolism when used at a level 50-fold in excess of HCMV inhibitory concentrations (34).

Antisense oligonucleotides have been used successfully to specifically inhibit cellular and viral gene expression, and in some of these cases a decrease in the steady-state level of targeted mRNA has been reported (8, 15, 33). Mechanisms of action of antisense oligonucleotides against various viral and nonviral targets have been previously described (41). Of these previously described mechanisms of action, RNase H degradation of target mRNA is most widely accepted. In this instance the oligonucleotide-mRNA hybrid becomes a substrate for cellular RNase H, and the target RNA is cleaved and subsequently degraded (12, 41). However, other mechanisms such as interference with transcription, splicing, or nuclear transportation have been postulated (33, 41). In the case of the HCMV UL36ANTI oligonucleotide described here, the lack of detection of UL36 mRNA as shown in Northern blots most likely indicates a nuclear localization and point of action for this oligonucleotide. This is based on the assumption that the UL36ANTI compound can hybridize only to the unspliced transcript present in the nucleus.

All experiments reported here involved pretreatment of cells with the antisense oligonucleotide. In the absence of pretreatment, inhibition of HCMV DNA replication was reduced. Subsequent experiments have shown that pretreatment can be shortened to 3 h prior to infection and the same degree of inhibition of HCMV DNA replication could be achieved (34). Pretreatment was probably necessary to achieve adequate intracellular concentrations of the oligonucleotide before infection. Since HCMV UL36 expression initially occurs before 4 h p.i. (40), accumulation levels of this message may be too high very early in infection. This may indicate that p.i. treatment

may not allow enough time for an effective intracellular build up of the antisense oligonucleotide.

The UL36 gene product has not been identified or characterized. However, regions of the predicted protein sequence have homology to known transcriptional activators (6). The UL37 gene product is predicted to be a membrane protein (6, 20). These gene products were shown to transcriptionally activate an HCMV early promoter in a synergistic manner along with IE1/IE2 (6). This observation along with the fact that these genes are required for both origin-dependent and, as demonstrated here, viral DNA replication emphasizes their importance in initiation of the HCMV replication process. Since our antisense oligonucleotide targets both UL36 and UL37 intron-exon boundaries we do not know whether one or both of these proteins are essential, although only the UL36 splice site is within the coding region of the proposed protein (40). The UL37 transcript can be detected only in the presence of a protein synthesis inhibitor (39). Northern blots have shown that neither of the transcripts is detected in the UL36ANTI oligonucleotide, cycloheximide-treated infected cells (34). Therefore, we have to assume that both RNAs are degraded and that neither gene product is expressed. Nevertheless, the application of antisense technology to demonstrate that these genes are required for HCMV viral DNA replication represents a true genetic approach to identifying genes essential for growth of this virus.

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