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# Characterization of an Antisense Transcript Spanning the UL81-82 Locus of Human Cytomegalovirus

Mariana Bego,[1] J. Maciejewski,[2] S. Khaiboullina,[1] G. Pari,[1] and S. St. Jeor[1]*

*Department of Microbiology and Graduate Program in Cell and Molecular Biology, University of Nevada, Reno, Nevada,[1] and Cleveland Clinic, Cleveland, Ohio[2]*

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**In this study we present the characterization of a novel transcript, *UL81-82ast*, UL81-82 antisense transcript, and its protein product. The transcript was initially found in a cDNA library of monocytes from a seropositive donor. mRNA was obtained from monocytes isolated from a healthy donor with a high antibody titer against human cytomegalovirus (HCMV). The mRNAs were cloned into a lambda phage-derived vector to create the cDNA library. Using PCR, *UL81-82ast* was amplified from the library. The library was tested for the presence of numerous HCMV genes. Neither structural genes nor immediate-early genes were found. *UL81-82ast* was detected in five bone marrow samples from healthy antibody-positive donors. This same transcript was also found in in vitro-infected human fibroblasts early after infection but disappears at the same time that UL82 transcription begins. Not only was the transcript amplified using reverse transcription-PCR and sequenced but its protein product (UL82as protein) was detected by both Western blot and immunofluorescence. Phylogenetic studies using UL82as protein were conducted, showing a high degree of conservation in clinical isolates, laboratory strains of HCMV, and even in chimpanzee CMV. The transcript could be involved in the posttranscriptional regulation of the UL82 gene, affecting its mRNA stability or translation. Since the UL82 product, pp71, functions as an immediate-early transactivator, its posttranscriptional control could have some effect over latency reactivation and lytic replication.**

Human cytomegalovirus (HCMV) is a ubiquitous betaherpesvirus which occurs as a latent infection in the majority of adults (reviewed in reference 20). Infection in healthy immunocompetent individuals is typically asymptomatic followed by the development of a latent infection. Certain signaling events can induce the virus to enter into a productive cycle, resulting in recurrence of infectious virus and recrudescence of clinical symptoms (40). Over the past two decades, there has been a considerable rise in the number of patients undergoing immunosuppressive therapy following solid organ or bone marrow transplantation, as well as a dramatic increase in the incidence of AIDS. Consequently, HCMV, once considered a neonatal disease, has become an important pathogen in both immunocompromised and immunocompetent patients.

HCMV infects a variety of cells types including hematopoietic and stromal cells of the bone marrow (10, 24–26). Although an exact definition of CMV latency is unclear, it has been described operationally as the inability to detect infectious virus despite the presence of viral DNA. A functional viral genome is maintained in the host after clearance of productive primary infection (for a review, see references 9, 16, and 42).

The underlying mechanism regulating HCMV latency and reactivation remains unclear. The bone marrow is a probable site of HCMV latency (3, 24, 34, 37, 38), but the primary cellular reservoir harboring latent virus within the bone marrow is still unknown.

A number of cell types including monocytes, macrophages and endothelial cells are potential sites of latency (15, 24, 28, 35, 37, 40). Latent viral genomes are detected in $CD14^+$ monocytes and $CD33^+$ myeloid precursor cells (15, 44). HCMV DNA has also been detected in $CD34^+$ cells, which are the progenitors for cells of B, T, and monocyte/macrophage lineages, but not in mature B or T cells (15, 28, 36, 44). HCMV infection is influenced by the specific pathway of macrophage differentiation.

Differentiation of $CD14^+$ monocytes into macrophages produces a permissive environment for viral replication and infectious virus is produced (39, 45). It has been suggested that monocytes and macrophages are crucial for the maintenance of HCMV within the host.

In this study we sought to identify virus transcripts present in peripheral blood monocytes from seropositive donors. To this end, a cDNA library was generated in lambda phage with polyadenylated [poly$(A)^+$] RNAs isolated from monocytes of a healthy donor with a high antibody titer against HCMV. Phage DNA was extracted from the library and screened for putative latency-associated genes by PCR.

A unique transcript having an antisense orientation to the UL82 gene was found in the library. The presence of the identified transcript was also analyzed in bone marrow samples from antibody-positive donors. A unique conserved 133-amino-acid protein is expressed from the transcript, the UL82as protein, in HCMV-infected fibroblasts. The results suggest that this transcript may be associated with posttranscriptional regulation of the transactivator protein pp71.

* Corresponding author. Mailing address: Department of Microbiology, Graduate Program in Cell and Molecular Biology, University of Nevada, Reno, NV 89557. Phone: (775) 784-6161. Fax: (775) 327-2332. E-mail: stjeor@med.unr.edu.

## MATERIALS AND METHODS

**Cells, virus, and human samples.** HCMV strains were propagated in human foreskin fibroblasts (HFF) as previously described and maintained as frozen stocks (41). Human foreskin fibroblast cells were maintained in Dulbecco's

modified Eagle's medium supplemented with 10% fetal bovine serum. A low-passage clinical isolate was used (49) (stock titer $10^8$ PFU/ml). The extraction and purification of HCMV DNA have been described previously (41). The bone marrow samples were archived samples obtained from the Cleveland Clinic under an approved Institutional Review Board protocol and were sent to us as coded samples. The identity of the donor remains confidential. Blood from the donors used in preparation of the cDNA library was obtained under an approved University of Nevada at Reno Institutional Review Board protocol. The handling of human samples complied with federal guidelines and institutional policies.

**Monocyte isolation.** Fifteen milliliters of the blood was mixed with 15 ml HistoPaque-1077 cushion and centrifuged at 400 × *g* for 30 min at room temperature. The supernatant was then removed to reduce the number of contaminating platelets and the remainder was washed twice with 50 ml of Hanks' medium. The resulting white peripheral blood mononuclear cell pellet was resuspended by gentle mixing in Trizol reagent.

**Nucleic acid extraction.** Total RNA was isolated from fresh or frozen tissue by using guanidinium isothiocyanate-acid phenol (TRIZOL LS reagent, GIBCO BRL, Rockville, MD) according to the manufacturer's instructions and established methods (8). Cells were lysed in a 3.5-cm culture dish by adding 0.3 to 0.4 ml of TRIZOL LS reagent and passing the cell lysate through a pipette several times. After incubating the homogenized samples for 5 min at room temperature to obtain complete dissociation of nucleoprotein complexes, 0.2 ml of chloroform was added. Tubes were shaken vigorously by hand for 15 s, incubated at room temperature for 15 min, and centrifuged at 12,000 × *g* for 15 min at 4°C.

Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase containing RNA. After transferring the aqueous phase to a clean tube, RNA was precipitated by adding 0.5 ml isopropyl alcohol per 0.75 ml of TRIZOL LS reagent used for the initial homogenization. Samples were then incubated at room temperature for 10 min and centrifuged at 12,000 × *g* for 10 min at 5°C. After removing the supernatant, the RNA pellet was washed once by adding 1 ml of 75% ethanol per 0.75 ml of TRIZOL LS reagent used for the initial homogenization. Samples were mixed on a vortex and centrifuged at 7,500 × *g* for 5 min at 5°C. The RNA pellet was briefly dried and then reconstituted in RNase-free water.

**Poly(A)$^+$ RNA purification.** Poly(A)$^+$ RNA was purified using oligo(dT)-cellulose columns (Molecular Research Center, Inc.) according to the manufacturer's instructions. Total RNA was precipitated with isopropanol and dissolved in binding buffer (0.5 M LiCl, 50 mM sodium citrate, 0.1% sodium dodecyl sulfate) to a concentration of 4 mg RNA/ml and incubated at 70°C for 5 min. The RNA solution was applied to an oligo(dT)-cellulose column (Molecular Research Center, Inc.). The eluate was reintroduced in the column. The column was washed twice with 1 ml of binding buffer (1 mM sodium citrate, 0.1% sodium dodecyl sulfate) and eluted with 0.9 ml of elution buffer (1% sodium dodecyl sulfate, 75% ethanol, 1 M Tris, 5 M LiCl). The RNA concentration was measured spectrophotometrically. RNA was supplemented with LiCl and Polyacryl Carrier (Molecular Research Center, Inc.). The RNA-carrier was precipitated by centrifugation and dissolved in diethyl pyrocarbonate-treated water.

**Creation of cDNA library in lambda phage.** Poly(A)$^+$ RNA isolated from monocytes of a healthy donor with a high antibody titer against HCMV was used to construct a cDNA library and directionally cloned into lambda-ZAP (Lambda Zap Synthesis Kit, Stratagene) following the manufacturer's recommendations.

**First-strand synthesis.** Double-stranded cDNA was synthesized from 5 μg of poly(A)$^+$ RNA with 20 U of Moloney murine leukemia virus reverse transcriptase. Single-stranded DNA containing oligo(dT) and XhoI restriction endonuclease recognition sequences (ZAP cDNA Synthesis Kit; Stratagene) was used as a primer. The sequence of this primer is $(GA)_{10}$ACTAGT**CTCGAG**$(T)_{18}$. The GAGA sequence was added to protect the XhoI restriction site (bold). 5-methyl dCTP was used in the first strand synthesis. The end product of this reaction is a double-stranded 5-methyl cDNA-RNA molecule.

**Second-strand synthesis.** The sample was treated with RNase H to remove of the template RNA, and amplified with the same primer used for the first strand synthesis, single-stranded DNA containing oligo(dT) and XhoI restriction endonuclease recognition sequences. To measure the amount and size distribution of cDNA, an aliquot was amplified in the presence of [α-$^{32}$P]dATP (Amersham). The end products of this reaction are double-stranded DNA molecules where only one strand is methylated. The cDNA termini were blunt using blunting deoxynucleoside triphosphate mix and cloned *Pfu* DNA polymerase (ZAP cDNA Synthesis Kit; Stratagene).

**Ligation.** The hemimethylated, blunt-ended double-stranded cDNA was ligated to EcoRI linkers and digested with XhoI. Adapted cDNAs were separated from linker fragments by chromatography in an alkaline agarose gel. Column fractions 2 to 13 were collected, pooled, and extracted with phenol-chloroform and ethanol precipitation. After the size distribution was determined by agarose gel electrophoresis and autoradiography, the adapted cDNA was ligated to EcoRI- and XhoI-digested lambda-ZAP arms at 4°C for 48 hours. The ligated phage was packaged in vitro, and the resultant phage titer was determined by limiting dilution plaque forming assay *Escherichia coli* XL1-Blue MRF′ on NZY agar plates containing 5 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml and 3 μM of isopropylthiogalactopyranoside (IPTG). The stock library was prepared by plate lysate propagation of the phage in *E. coli* XL1-Blue MRF' on NZY agar plates.

TABLE 1. List of primers

| Primer | Sequence (5′→3′) |
|---|---|
| This study | |
| β-Actin R | TGGCTACAGCTTCACCACC |
| β-Actin F | ACTCCTGCTTGCTGAT CCAC |
| ZapR | GTAAAACGACGGCCAGT |
| ZapF | GGAAACAGCTATGAC CATG |
| AP | GGCACGCGTCGACTAG TACT (17T total) |
| UAP | CUAGGCCACGCGTCGACT AGTAC |
| IE-1 primers | |
| IEP 4BII | CAATACACTTCATCTCCTC GAAAGG |
| IEP 2AII | ATGGAGTCCTCTGCCAAGA GAAAGATGGAC |
| IEP 3A | GTGACCAAGGCCACGA CGTT |
| IEP 3B | TCTGCCAGGACATCTT TCTC |
| Previously described latency gene primers (18, 22) | |
| Anti-CLT F | ACTCATGGTCGCTCGGCAG CTCCTTGCTC |
| Anti-CLT R | GCACAAACCCCGACACGTA CCGTGGCA |
| CLT F1 | TAGTCTGCAGGAACGTCGT GGCCTTGGT |
| CLT F2 | TCGTCAGGATTATCAGGGT CCATCTTTCTCT |
| CLT F2a2 | GGAAGGGCCCTCGTCAGG ATTATCAGGGTC |
| CLT R1 | TACATCAATGGGCGTGGAT AGCGGTTTGAC |
| CLT R2 | AAATGGGCGGTAGGCGTG TACGGTGG |
| CLT R2-2 | GTATCATATGCCAAGTA CGCC |
| UL82as protein cloning primers | |
| TOPO-UL82as F | CACCATGACGAGCGTGAG AGCCC |
| UL82as R | TTTGGAACACCGACAGCCG |

**Phage DNA isolation.** The DNA from lambda phage was isolated as previously described (33). Briefly, DNase and RNase were added to a library aliquot to a final concentration of 100 μg/ml and incubated at 37°C for 30 min. Twenty microliters of 2 M $ZnCl_2$ was added per ml of lysate and incubated for 5 min at 37°C. The sample was pelleted by centrifugation and resuspended in 500 μl of TES (0.1 M Tris-HCl [pH 8], 0.1 M EDTA, 0.3% sodium dodecyl sulfate) and incubated at 65°C for 15 min. Then, 60 μl of 3 M potassium acetate was added, and incubated on ice for 20 min. The sample was pelleted again by centrifugation and the DNA present in the supernatant was ethanol precipitated.

**Oligonucleotide primers.** Synthetic oligonucleotide primers (Invitrogen, Inc.) for immediate-early 1 (IE1) HCMV genes (19), human β actin, and lambda ZAP (Stratagene, Inc.) were selected (Table 1). Previously described primers from the literature are also described in Table 1 (18, 22). Primers for several HCMV genes, within the UL80-UL82 region were designed (Table 2). The relative

TABLE 2. List of primers for area UL80-82[a]

| Primer | Sequence (5′→3′) |
|---|---|
| UL81 F1 | AGCACGCCGCACTACGACCTA |
| UL81 F2 | CCGCGGCAGACATCAGCA |
| UL81 R1 | GACGCTATATTTAGGGCTTCC |
| UL81 R2 | GGAAAAACACGCGGGGGA |
| UL81 R3 | GTGCTGGAAGTGGAAGCGG |
| UL82 F1 | ATGACCTCTCCTCCACACC |
| UL82 F2 | GAGCCTTGACGACTTGGTAC |
| UL80 F1 | AGCAGCAGCAACAACGTTAC |
| UL80 R1 | GGAAGAAGAACCTGCCGTCG |
| UL80 F2 | GCGGCAGAGTTCTACGTTAC |
| UL80 R2 | TTGGCACCTCCGGATAGAAG |
| UL82n F1 | AAGATGGTGTCGTGAGTCTG |
| UL82n R1 | TGACCGTAACGCCGAACTGG |
| UL82n F2 | CATTCTGGATCTGCACGATG |
| UL82n R2 | AACGGAGACGGACGAACGAG |

[a] For genes UL80 and UL82n, nested PCR was done in all cases with primers F1 and R1 in the first amplification and F2-R2 in the second.

orientation of primers in the UL81-UL82 region is shown in Fig. 1. The accuracy of every HCMV primer set was confirmed using HCMV DNA as a template.

**PCR amplification and cloning of HCMV-specific transcript cDNAs.** RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen). The program for the reverse transcription (RT)-PCR had an initial step of 1 h at 41°C instead of the initial denaturation. The PCR conditions were initial denaturation at 94°C, 30 cycles of 94°C for 30 seconds, 55 to 58°C (depending of the primers used) for 30 seconds, and 72°C for 1 min, followed by 10 cycles of 94°C for 30 seconds, 55 to 58°C for 30 seconds, and 72°C for 2 min. After the cycling process the products were exposed to final elongation of 72°C for 5 min. In case of the nested PCRs a 2-μl aliquot from the first round of amplification PCR was used to perform the second round of amplification.

Amplified products that did not have enough DNA for direct sequencing were cloned into plasmid vector by TA cloning (Invitrogen, Inc.). The unmodified PCR fragment was ligated to the pCR 2.1-TOPO vector for 5 min at room temperature and transformed in TOP10F' cells (Invitrogen, Inc.). Colonies were picked and grown in 2× YT medium overnight. An aliquot of the liquid culture (500 μl) was heat inactivated at 96°C for 10 min and spun down, and the supernatant was used for PCR using vector primers. Sequencing was done at the Nevada Genomic Center of University of Nevada Reno, using the ABI PRISM 3730 DNA analyzer. Purified PCR products were sequenced using the Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

**Southern blot analysis.** Southern blot analysis was performed with the following standard procedures. PCR products were separated by electrophoresis on a 1% agarose gel and ethidium bromide. The gel was treated with 0.2 N HCl (5 min), washed three times in di$H_2O$ (3 min each time) and treated with 0.4 N NaOH (5 min). The DNA was transferred overnight onto a nylon membrane by capillary blotting in 0.4N NaOH. The probe consisted of a small fragment of DNA corresponding to PCR fragment amplified with primers UL81F2 and UL81R3, starting with HCMV DNA as the template (Fig. 1). The PCR product was visualized in an agarose gel stained with ethidium bromide, cleaned and sequenced. The purified DNA fragment corresponding to fragment between primers UL81F2-UL81R3 was labeled with random primer using [α-$^{32}$P]dCTP (Amersham Pharmacia Biotech Inc.), and used as a probe. The nylon membrane was prehybridized for 1 h at 65°C and hybridized overnight with the described $^{32}$P-labeled probe at 65°C. The membrane was washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% sodium dodecyl sulfate (SDS) at 65°C for 15 min, and twice in 0.1× SSC with 0.1% SDS at 65°C for 30 min. Bands were detected by X-ray film at −80°C after different exposure times according to the experiment design.

**Rapid amplification of 3′ cDNA ends.** Total RNA, poly(A)$^+$ RNA and RNA extracted from HCMV-infected cells were used as a template. The RNAs were reverse transcribed using random primers, or specific UL81 primers and Superscript II reverse transcriptase. For completing the 3′ rapid amplification of cDNA ends (RACE), the cDNA was used as a template. Primers from UL81 region were used in combination with adaptor primer (AP, Table 1) to complete the first-round PCR. For the second-round PCR, the reaction medium (2 μl) from the first-round PCR was used as a template. Again, primers from the UL81 region were used but this time in combination with the universal adaptor primer (UAP, Table 1).

**Protein cloning and purification.** An open reading frame (ORF) corresponding to UL82as protein was amplified by PCR using primers TOPO-UL82as F and UL82as R (Table 1). To enable directional cloning, the forward primer was designed to contain CACC at the 5′ end, which pairs with the overhang sequence GTGG in the vector. The reverse primer was initiated with the codon preceding LUNA, the putative stop codon (TGA), so that the cloned PCR product produced an in-frame fusion with the C-terminal His tag. PCR constructs were verified by sequencing. The LUNA ORF PCR was cloned into pET102/D TOPO (Invitrogen). Recombinant LUNA was tagged with V5 and His epitopes. Recombinant protein was expressed in *Escherichia coli* BL21 start cells (Invitrogen) after induction with 1 mM isopropyl-β-D-thiogalactoside in LB for 24 h at 37°C. Cells were harvested by centrifugation. Recombinant LUNA protein was purified using HiTrap Chelating $Ni^{2+}$ affinity column (Amersham Biosciences) according to manufacturer's protocol.

**Sample preparation.** Bacterial cell pellets were resuspended in resuspension buffer (20 mM Tris-HCl, pH 8.0), using 4 ml of this buffer per 100 ml of initial culture used. Proteases inhibitor cocktail was added to this solution. Three cycles of freeze/thaw were conducted with dry ice and methanol mix and a 37°C water bath. Cells were further lysed using an 18-gauge syringe in between each cycle. Cells were centrifuged at high speed for 10 min at 4°C, and pellets were resuspended cold isolation buffer (2 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 2% Triton X-100, pH 8.0). A freeze and thaw procedure was repeated and followed by a high-speed centrifugation (10,000 × *g*) step for 10 min at 4°C. The pellet material was washed once in isolation buffer lacking urea, and resuspended in binding buffer (6 M guanidine hydrochloride, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0), using 5 ml of buffer for every 100 ml of initial culture. The samples were incubated at room temperature for 60 min with gentle rocking, followed by a high-speed centrifugation for 15 min at 4°C. Supernatant was filtrated using 0.45-μm filters in order to remove the remaining particles, before applying to the column.

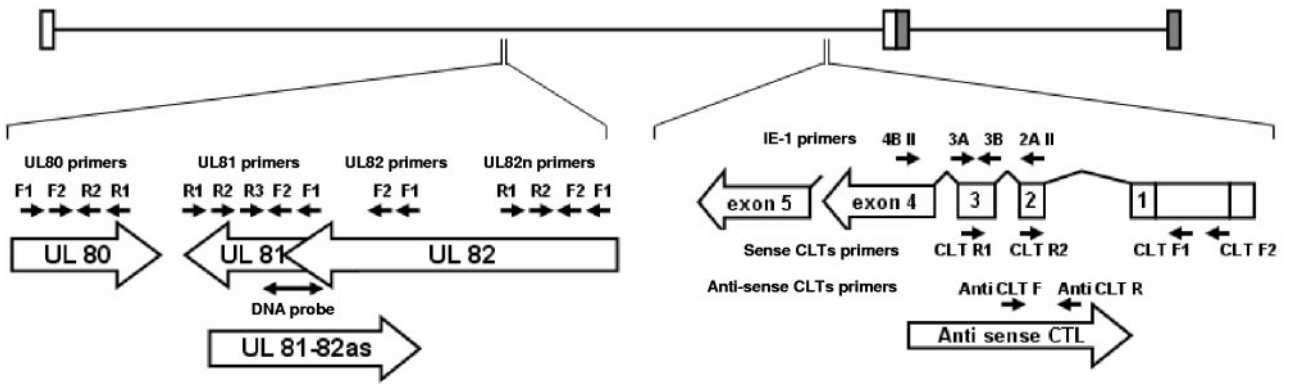


FIG. 1. Graphic representation of the HCMV genome, relative positions of genes analyzed in this study, and primers and probes used.

**Column preparation.** We washed 5 ml HiTrap Chelating columns with 5 ml of distilled water. Columns were loaded with 0.5 ml of 0.1 M $NiSO_4$, and excess salt was washed with 5 ml of distilled water. Approximately 5 to 10 ml of binding buffer (6 M guanidine hydrochloride, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0) was used to equilibrate the column before sample loading. After sample loading, the columns were washed with 10 ml of binding buffer, followed by 10 ml of wash buffer (6 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0). Proteins were refolded in the column using a linear 6 to 0 M urea gradient. The gradient started with the wash buffer described above and finished with refolding buffer (20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0). A gradient volume of 30 ml and a flow rate of 0.1–1 ml/min were used. Columns were washed with 5 ml of refolding buffer.

Refolded recombinant LUNA was eluted using a 0 to 1 mM 2-mercaptoethanol linear gradient starting with refolding buffer and ending with elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 0.5 M imidazole, 1 mM 2-mercaptoethanol, pH 8.0). A gradient volume of 20 ml and a flow rate of 0.1–1 ml/min were used. Aliquots were collected and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) gels. This procedure purified LUNA-$His_6$ to homogeneity based on Coomassie-stained SDS-PAGE (data not shown). For Coomassie staining, the gels were placed in a solution containing 0.25% Coomassie brilliant blue (Sigma) in 45% methanol, 10% acetic acid for 2 h to overnight and washed in the same solution with no dye. Western blot analysis also detected LUNA and the His tags.

**Polyclonal antibodies.** Polyclonal antibodies against purified recombinant LUNA were raised in rabbits. Two New Zealand female rabbits were used to obtain the polyclonal antibodies in sequential immunization every month, for 4 months. Before every immunization, blood samples were taken from the marginal vein of the rabbit ear, centrifuged and the sera were frozen. The immune sera were used in Western blot and immunoprecipitation experiments. Preimmune sera were used as negative controls.

**Serum purification (reabsorption).** The following is an adaptation of protocol described by Streit and Stern (42).

**Preparation of insoluble cell powder.** Human fibroblast cells were cultured in several T175 flasks to 100% confluence. Cells were washed with Hanks' balanced salt solution, trypsinized, and collected by centrifugation. Cell pellets were dissolved in four volumes of ice-cold acetone, and incubated on ice for 30 min. Cell solutions were spun down at 10,000 × *g* for 10 min, and cell pellets were washed once with 4 volumes of ice-cold acetone. Cell pellets were spread out on Whatman filter paper, and grinded to a fine powder using a pestle. Powder was air dried and stored at 4°C.

**Preabsorbing antibodies.** To preabsorbed serum, 3 mg of insoluble cell powder was weighed for every microliter of serum. One ml of TBST solution (Tris-buffered saline, 1% Tween, pH 7.4) was added to every 3 mg of insoluble cell powder, cells were vortexed and incubated for 30 min at 70°C. Cells were vortexed again and spun down at 10,000 × *g* for 1 min. Cell pellets were washed with TBST until supernatant came clear after centrifugation. After the final wash, cell pellets were mixed with the rabbit serum, and incubated for 2 to 3 h at room temperature with gentle shaking. The serum and insoluble cell powder mix was spun down at 10,000 × *g* for 3 to 5 min, and pellets were discarded.

**In vitro HCMV infections.** HFF cells were infected with an HCMV clinical isolate at different multiplicities of infection, from 0.5 to 5 PFU/ml. Cell monolayers were exposed to the virus for 1 hour, under normal growing conditions. After the incubation time, virus containing medium was removed, cells were washed with sterile phosphate-buffered saline, and medium was added.

**Western blot analysis.** Different time points of HCMV-infected cells were collected by centrifugation and pellets resuspended in 0.01% sodium dodecyl sulfate solution. Protein concentrations were determined using Coomasie Protein Assay reagent (Pierce). Samples (5 μg of protein) were mixed with 2x SDS-polyacrylamide gel electrophoresis loading buffer (125 mM Tris [pH 7.0], 20% glycerol, 10% β-mercaptoethanol, 6% SDS, and 0.2% bromophenol blue), boiled for 5 min, and were then separated by PAGE on 12% acrylamide gels. In some cases duplicate gels were analyzed by Coomassie staining and Western blot.

For Western blot analysis, after semidry electrophoretic transfer of proteins onto polyvinylidene difluoride membranes (Westran S 0.2-μm pore; Schleicher & Schuell BioScience), the membranes were incubated overnight with 5% powdered milk in Tris-buffered saline-Tween buffer (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], and 0.05% Tween 20) to block nonspecific protein interactions. The membranes were probed with one of the following primary antibodies: anti-LUNA preabsorbed rabbit serum (1:100 dilution), anti-IE1/2 monoclonal antibody (mAb810, Light Diagnostics), and anti-His coupled to horseradish peroxidase monoclonal antibodies (1:1,000; Invitrogen). Secondary antibodies used included goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated (Southern Biotechnology Associates, Inc.). Antibody-tagged protein bands on the probed membranes were detected using diaminobenzidine peroxidase substrate kit (Vector).

**Immunoflourescence microscopy.** Infected cells were plated on glass coverslips, and fixed at different times postinfection with 3:1 methanol:acetone and then permeabilized using a combination of glycine and Triton X-100. First coverslips were treated with 10 mM glycine solution for 30 min at room temperature, after a DPBS rinse 30 min incubation with 0.1% Triton X-100 solution in phosphate-buffered saline was done at room temperature. The coverslips were rinsed three times in PBT (phosphate-buffered saline plus 0.5% Tween), and then incubated with primary antibody solution in phosphate-buffered saline for 90 min at room temperature.

After incubation, the coverslips were washed in PBT three times for 5 min each and then incubated with a 1:1,000 dilution in phosphate-buffered saline of an Alexa Fluor conjugated secondary antibody (Molecular Probes, Inc.) for 30 min in the dark at room temperature, and rinsed again with PBT. All antibody solutions also contained 2% fetal bovine serum. The cells were again washed in PBT three times for 5 min each and then were incubated with Hoechst 33258 staining solution for 5 min at room temperature, in the dark. Prior to the addition of mounting solution, the cells were rinsed with PBT. Primary antibodies included Mab810 (Light Diagnostics), mouse monoclonal antibody against HCMV IE1-2 proteins, and rabbit polyclonal anti-LUNA. Secondary antibodies used were Alexa Fluor 555-conjugated (red) goat anti-mouse and Alexa Fluor 488-conjugated (green) goat anti-rabbit immunoglobulin antibody (Molecular Probes, Inc.) Images were captured with a Nikon E800 epifluorescence/confocal microscope using a 20× objective, or a 100× oil immersion objective.

**Accession numbers and alignments.** The following sequences were used for alignment analysis: gi 38093733 gb AC146999.1 AD169-BAC isolate (nucleotides 102936 to 102538), gi 59591 emb X17403.1 HEHCMVCG AD169 (nucleotides 117712 to 118110), gi 37777313 gb AC146904.1 PH-BAC isolate (nucleotides 109303 to 108905), gi 37654163 gb AC146851.1 Towne-BAC isolate (nucleotides 152184 to 152582), gi 38304373 gb AY315197.1 Towne (nucleotides 117157 to 117555), gi 39842016 gb AY446894.1 Merlin (nucleotides 119014 to 119412), gi 37777316 gb AC146907.1 FIX-BAC isolate (nucleotides 109215 to 108817), gi 37777315 gb AC146906.1 TR-BAC isolate (nucleotides 154705 to 155103), gi 37777314 gb AC146905.1 Toledo-BAC isolate (nucleotides 104619 to 104218), and gi 19881028 gb AF480884.1 chimpanzee cytomegalovirus (nucleotides 119078 to 119407). Protein alignment was done using MegaAlign, from Lasergene (DNAstar, Inc.), using Clustal W algorithms.

## RESULTS

**Isolation of monocytes and purification of poly(A)$^+$ RNA.** Because the number of infected monocytes from normal HCMV-positive donors as well as the number of HCMV transcript are likely to be extremely low it was decided to approach this problem by constructing a cDNA library to amplify any virus-specific transcripts.

Mononuclear cells were isolated from a donor with a high HCMV antibody titer. DNA was extracted from the monocytes and analyzed for the presence of HCMV DNA using IE1 gene primers in a nested-round PCR (Fig. 1). The resulting band was sequenced to further assess the presence of the intronic region of the area (Fig. 2a). Bands corresponding to DNA smaller than 100 bp present in water and donor DNA samples are indication of unused primers. Three samples of total RNA (samples numbered 1 to 3) were extracted from the same donor, and their integrity was verified in an agarose gel (data not shown). Poly(A)$^+$ RNA was purified from these total RNA samples.

Prior to the synthesis of the library the total RNA and poly(A)$^+$ RNA samples was tested for the presence of IE1 RNA. To test for the presence of a given transcript an aliquot of the total RNA or poly(A)$^+$ RNA was subjected to reverse transcription using random primers prior to the PCR step. As controls for the reactions, DNA was extracted from cells infected with an HCMV clinical isolate; in this case the RT step was omitted. To determine the quality of the purification the

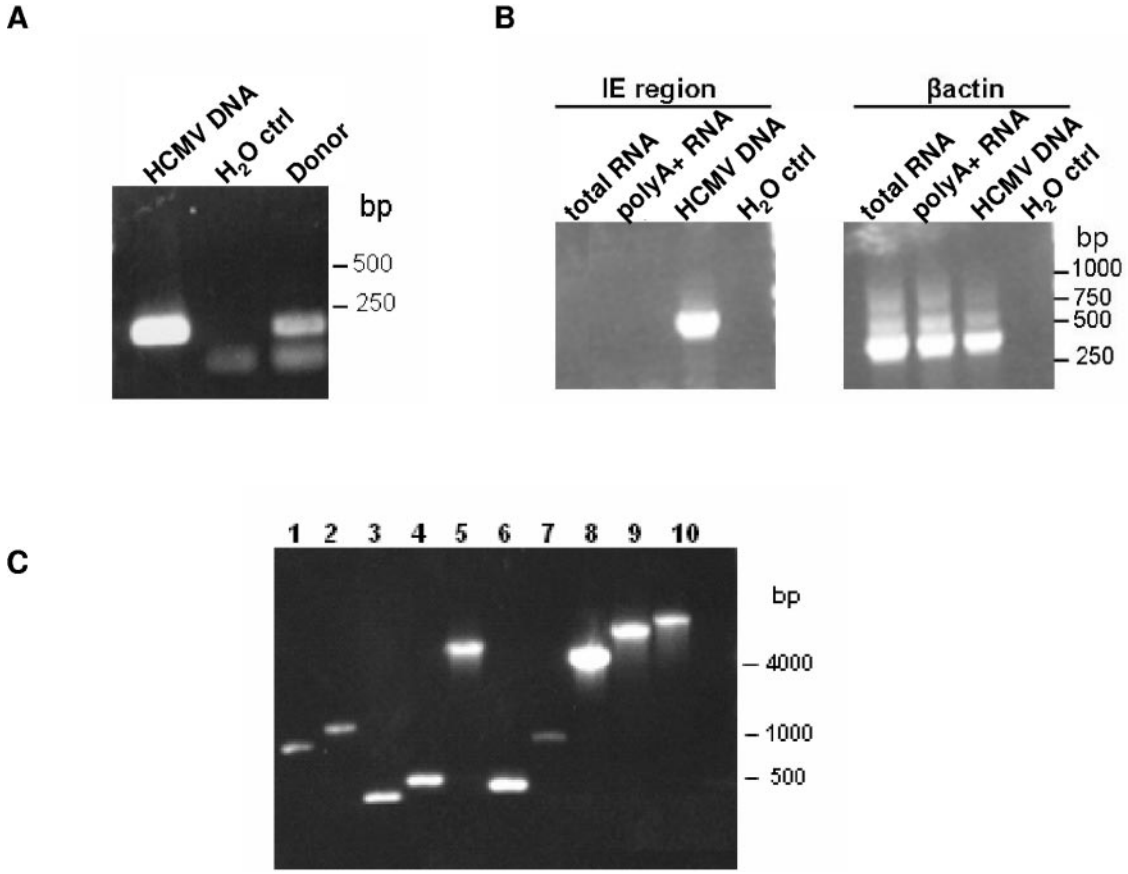


FIG. 2. cDNA library construction from monocyte mRNA. (A) Presence of HCMV DNA in the donor's monocytes. DNA was extracted from the isolated monocytes and tested for the presence of HCMV DNA by nested PCR, using primers IEP 4AII and 2BII, for the first round, and IEP 3A and 3B, for the second round. A positive (DNA from HCMV-infected cells) and a negative control are included. (B) Absence of IE-1 transcripts in monocyte RNA. Total RNA and poly(A)$^+$ RNA samples were subject to cDNA synthesis using random primers. As a positive control, DNA extracted from HCMV-infected HFF cells was used. All samples were subject to nested PCR using HCMV lytic genes primers IEP 4AII and 2BII, for the first round, and IEP 3A and 3B, for the second round. To test the quality of the cDNA/DNA samples, PCR was performed using β-actin primers (C) Size distribution of cDNA inserts in the phage DNA library. Phage DNA from 10 randomly selected clear plaques on NZY agar plates containing X-Gal and IPTG was amplified by using primers flanking the cDNA insert. PCR products representing cDNA inserts from approximately 500 bp to >3 kb were resolved by agarose gel electrophoresis and photographed after ethidium bromide staining. Bands from lanes 1 to 7 (marked with arrows) were too weak to be observed in the picture.

presence of β-actin transcripts was determined by one-round PCR using specific primers (Fig. 2b). No transcription from the IE1 gene was amplifiable from the RNA extracted from the purified monocytes using nested PCR (Fig. 2b). This result indicated not only the absence of detectable reactivation events, as indicated by the absence of IE1 transcripts, but also the absence of HCMV DNA contamination after the total RNA and poly(A)$^+$ RNA purifications.

**cDNA library construction.** RNA from latently infected monocytes was used to construct a cDNA library, by standard methods (Stratagene, Inc.). The primary library consisted of $2 \times 10^7$ recombinant phage/ml. Nearly all (>99.93%) phage contained inserts as determined by blue-white plaque screening on NZY agar plates containing X-Gal and IPTG. PCR amplification was performed on 10 randomly selected plaques from the library using vector-specific oligonucleotides primers flanking the cDNA insertion site. Most cDNA inserts ranged in size from approximately 500 bp to >3 kb (Fig. 2c).

To expand the in vitro-packaged recombinant phage, the primary library was propagated in *E. coli* XL1-Blue MRF' to yield a stock cDNA library containing approximately $10 \times 10^{12}$ phage/ml. Plaque lifting and hybridization were performed using a β-actin probe. This approach was not used for screening for HCMV-positive plaques due to the very low relative concentration of the viral transcript when compared to cellular transcripts instead a PCR-based approach was used.

**Identification of HCMV transcripts in the cDNA library.** Total DNA extracted from the cDNA library was tested by PCR. As a positive control β-actin was amplified by PCR (data not shown). Library DNA was also analyzed for the presence of the IE1 gene using previously described primers (19) in a nested-round PCR with negative results (Fig. 3a). A variety of previously described putative latency-associated transcripts (18, 22) were analyzed using the primers previously described (primers listed in Table 1). Transcripts analyzed included sense and antisense CMV latency-associated transcripts (18, 22) (Fig. 1).

To test the proper condition for the reactions, PCR was

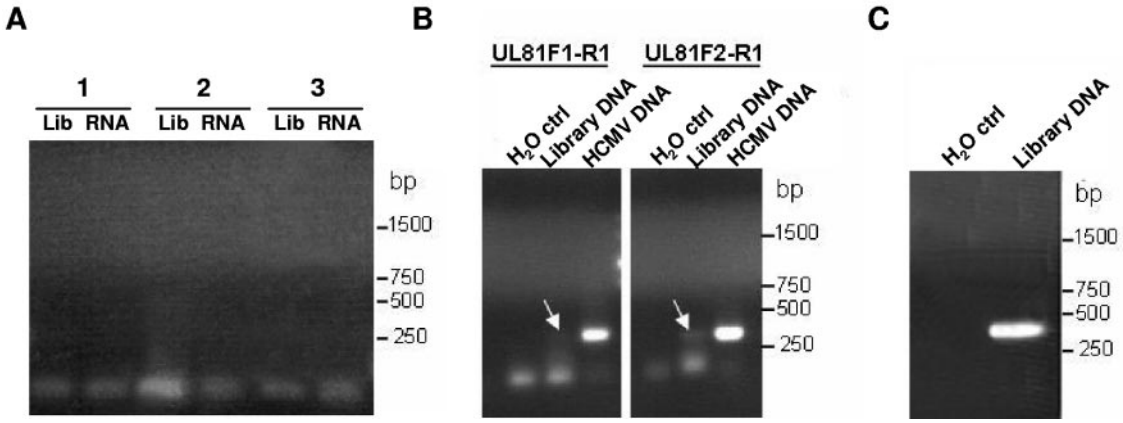


FIG. 3. Presence of HCMV transcripts in the phage DNA. (A) Presence of previously described CMV latency-associated transcripts. The presence of CMV latency-associated transcripts was studied in the library DNA as well as in the initial total RNA 3 extraction. DNA from the library and cDNA from total RNA 3 were tested by PCR using primer combinations antiCLT F1-R1, CLT F1-R2, and CLT F2-R2 (lanes 1 to 3, respectivley), as well as other combinations (data not shown). DNA from the library was tested by PCR using HCMV *UL81*-specific primers. As a positive control, DNA extracted from HCMV-infected HFF cells was used. (B) PCR using primers 81F1 and R1 or primers 81F2 and R1. The bands from the library sample are marked with an arrow. (C) Heminested PCR. To obtain higher amplification a heminested PCR was performed using primer 81F1-R1 in the first round and 81F2-R1 in the second round.

done using genomic HCMV DNA as a template. In order to examine these transcripts in our system, the primers were used in a PCR using DNA extracted from the cDNA library as a template. The transcripts were further analyzed by RT-PCR using RNA extracted from monocytes from the patient used to prepare our library; in this case cDNA was prepared first using random primers. In both experiments, using library DNA or monocyte RNA, we were unable to find CMV latency-associated transcripts, even when using nested PCR (Fig. 3a).

Previous work by Goodrum et al. (32) showed gene expression patterns in in vitro-infected $CD34^+$ cells (13). The HCMV gene array used (4) consisted of PCR products corresponding to the 203 known ORFs for the AD169 strain of HCMV, 19 additional ORFs identified in clinical isolates, negative controls, and 80 cellular cDNAs. For array experiments, $CD34^+$ cells were infected, and the level of gene expression was reported for 1, 5, and 8 days postinfection. Based on the result of this study we selected several genes that were upregulated at the latter time point, and designed primers based on published sequences in GenBank. Ongoing studies focus on the characterization of genes present in the library. Here we described a transcript corresponding to a region previously known as UL81.

Primers designed for regions UL81 amplified a product of the predicted molecular weight. Using several primers designed from the UL80-UL82 region (Table 2) of the HCMV genome, transcripts were amplified from the library by PCR (Fig. 3). Only weak bands were detected using one-round PCR, but the results were optimal using a heminested or nested PCR (Fig. 3b and 3c). The band size corresponded to that expected by comparison with the UL81 region that was used in the primer design. The band was sequenced and the results corresponded with the HCMV UL81 gene. Several combinations of primers were used to expand the amplified area. A total of 500 nucleotides corresponding to the region of UL81-82 of the HCMV genome was amplified. To verify the full-length and orientation of the HCMV transcript present in the library, we performed PCR using vector primers (lambda ZAP F and R; Fig. 4a). This PCR result allowed us to map both ends of our transcript. The results are shown in Fig. 4b and 4c.

The orientation was initially determined using several primer combinations and Southern blotting with a specific UL81 gene probe (Fig. 4b). The presence of several bands in the blot could suggest more than one transcript from that area. The bands (marked with white arrows in panel b, Fig. 4) appeared to be the main transcripts in the area, although only one was present in sufficient amount for sequencing analysis (band a from Fig. 4c). Only after long exposure could a signal be detected in the area of the gel that corresponded to amplification with forward primers and the lambda ZapR primer (black arrows in Fig. 4b). Because only one primer was specific for the template and the other primer binds to any template coming from the library (Zap primers bind to lambda phage backbone), many nonspecific bands are seen in the ethidium bromide-stained gel.

The HCMV-specific bands did not always coincide with the brighter PCR bands. Positive bands from amplifications with forward primers and the lambda ZapR primer did not have enough DNA for sequencing. Several PCR conditions were investigated without improving the final result. The combinations using primers UL82F1 were selected for sequencing analysis (Fig. 4d). All bands from Fig. 3c were sequenced, but only band a from PCR using UL82F1-ZapF was shown to be specific for HCMV by direct sequencing.

Sequencing using the ZapF primer allowed us to determine the 3′ end of the transcript. Interestingly, immediately after the stop codon there were 14 adenosines not coded for in the HCMV genome (tail sequence: gtgttccaaaTGAaaaaaaaaaaaa aa), followed by vector sequences from the multiple cloning site. The fact that we could achieve amplification using specific forward primers with the lambda ZapF primer suggested that the transcript present in the library corresponds primarily to the antisense orientation of UL81-82 (antisense transcript called UL81-82ast). As a control we designed primers that do not overlap UL81-82ast in order to amplify only gene *UL82*

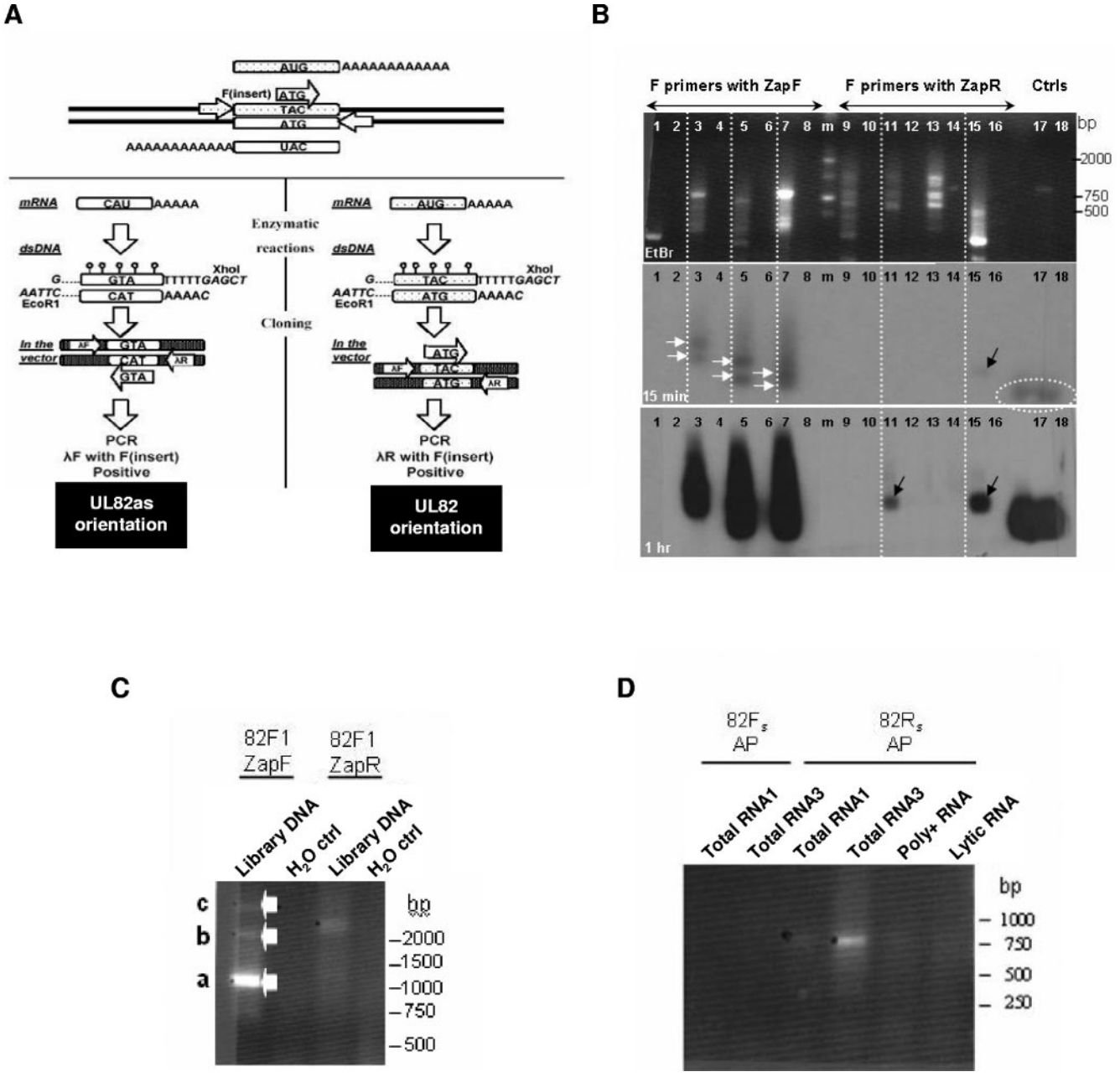


FIG. 4. Orientation of the UL81-82 transcript present in the library. (A) Directional cloning of cDNAs into phage library allows differentiating direction of the transcripts. (B) PCR using HCMV-specific primers from extracted library DNA. For lanes 1 to 16, odd numbers are library DNA, and even numbers are water controls. Positive controls in lanes 17 and 18, heminested amplification of library DNA with primers 81F2-81R2 (first round with 82F1-81R1). Primers used: lanes 1 and 2, 82Fi1-ZapF; lanes 3 and 4, 82F1-ZapF; lanes 5 and 6, 82F2-ZapF; lanes 7 and 8, 81F1-ZapF; lanes 9 and 10, 82Fi1-ZapR; lanes 11 and 12, 82F1-ZapR; lanes 13 and 14, 82F2-ZapR; lanes 15 and 16, 81F1-ZapR. Southern blotting and hybridization using PCR with primers UL81F2 and UL81R3 and HCMV DNA as a template (lanes 17 and 18) for the probe preparation. Developed for 15 minutes and 1 hour. (C) PCR using UL82 region and lambda vector primers from extracted library DNA. PCR using primer 82F1-ZapF or primer 82F1-ZapR was performed. The bands labeled a, b, and c were sequenced. (D) Orientation of the transcript present in monocyte RNA of latently infected patient using 3′RACE. Samples labeled 82Fs-AP underwent cDNA synthesis using primer UL81F1, and nested PCR using primers UL81F1-AP and UL81F2-UAP. Samples labeled 82Rs-AP underwent cDNA synthesis using primer UL81R1, and nested PCR using primers UL81R1-AP and UL81R2-UAP. mRNA corresponds to the poly(A) purification sample used for the library, and the sample lytic RNA is RNA extracted from HFF cells infected with Smith virus under lytic conditions. AP = adaptor primer, UAP = universal adaptor primer.

(Table 2, UL82n primers). Nested PCR for this transcript was negative. We also designed primers corresponding to gene *UL80* (Table 2), but we were not able to amplify this transcript in our library using nested PCR (data not shown).

**Identification of HCMV *UL81-82ast* in bone marrow and blood from donors with immunoglobulin G antibody against HCMV.** Monocytes from the bone marrow of patients with antibodies against HCMV were isolated. The same procedure

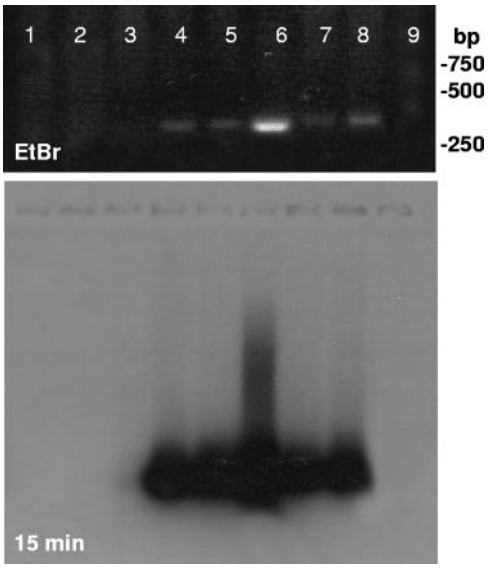


FIG. 5. Presence of *UL82ast* in bone marrow from latently infected patients. RNA was extracted by the Trizol method from latently HCMV-infected bone marrow samples. cDNA synthesis using random primers was performed using the extracted RNAs. Nested-PCR amplifications were performed using primers UL82F1 and UL81R1 in the first amplification and UL82F2 and UL81R2 in the second. Lanes 1 to 3 represent HCMV-negative donors 1051, 989, and 1134; lanes 4 to 8 represent the cDNAs from HCMV-positive donors 535, 553, 638, 234, and 376; lane 9 is the negative water control. Southern blotting and hybridization, developed for 15 min. Same probe as in Fig. 3B (PCR using primers UL81F2 and UL81R3 and HCMV DNA as a template).

was followed with bone marrow from uninfected individuals. Total RNA and poly(A)$^+$ RNA were purified as described previously. cDNA generated by random primers was used as templates for PCR. Every sample was tested for the presence of beta-actin transcripts as a control for the purification.

There was no amplification from the UL81-82 region using only one-round PCR in any sample (data not shown). Five out of five bone marrow samples from the seropositive donors were positive for the HCMV UL81-82 transcript by nested PCR and hybridization (Fig. 5). There was no amplification under these conditions in the HCMV-seronegative samples. There was no amplification from the IE1 region using one- or two-round nested PCR in any sample (data not shown). These data again indicated the absence of genomic HCMV DNA and lytic transcripts in our samples. As a control we used again the primers for nested PCR corresponding to regions of genes UL80 and UL82 (Table 2). We were not able to amplify these transcripts in bone marrow samples (data not shown).

To further assess the orientation of the transcript in vivo we used total RNA and poly(A)$^+$ purified fractions (designated the mRNA sample) obtained from the original donor used in construction of the library. cDNA was synthesized, using either UL81F1 or UL81R1 instead of random primers. RNA extracted late in infection from in vitro HCMV-infected fibroblasts was included as a control for a lytic virus infection. This RNA would be expected to contain only transcripts from lytic genes late in infection. Total RNAs 1 and 3 correspond to two RNA isolations from the same donor (total RNA 3 was used to make the library). A 3′ RACE reaction was performed using

TABLE 3. Expression of transcript *UL81-82ast* in in vitro-infected cells[a]

| Time of RNA sampling (h postinfection) | Sample/control | β-Actin | IE-1 | 81-82ast | UL82 |
|---|---|---|---|---|---|
| 0 (uninfected HFF) | Sample | Pos | Neg | Neg | Neg |
| | RNase/RT− | Neg | Neg | Neg | Neg |
| 2 | Sample | Pos | Pos | Neg | Neg |
| | RNase/RT− | Neg | Neg | Neg | Neg |
| 3 | Sample | Pos | Pos | Neg | Neg |
| | RNase/RT− | Neg | Neg | Neg | Neg |
| 4 | Sample | Pos | Pos | Neg | Neg |
| | RNase/RT− | Neg | Neg | Neg | Neg |
| 5 | Sample | Pos | Pos | Pos | Pos |
| | RNase/RT− | Neg | Neg | Neg | Neg |
| 6 | Sample | Pos | Pos | Pos | Pos |
| | RNase/RT− | Neg | Neg | Neg | Neg |
| 24 | Sample | Pos | Pos | Neg | Pos |
| | RNase/RT− | Neg | Neg | Neg | Neg |
| 48 | Sample | Pos | Pos | Neg | Pos |
| | RNase/RT− | Neg | Neg | Neg | Neg |
| 72 | Sample | Pos | Pos | Neg | Pos |
| | RNase/RT− | Neg | Neg | Neg | Neg |

[a] Human fibroblasts were infected with a clinical isolate of HCMV, and mRNA was collected at different time points as indicated. The sequences of the primers used for detection are shown in Tables 1 and 2. Nested PCR was performed in all cases except for β-actin.

combinations of UL81F1 or UL81R1 and AP or UAP. The absence of measurable amounts of the transcript in the RNA from in vitro-infected cells indicates either that the transcript is not present under these conditions, or that it is present at very low quantity. The positive result from the UL81R and AP primers for total RNA 3 was successfully cloned and sequenced (Fig. 4d). The sequence revealed a poly(A) tail as expected.

**Identification of HCMV *UL81-82ast* in in vitro-infected fibroblasts.** Human fibroblasts were infected with a clinical isolation of HCMV at a multiplicity of infection of 5. RNA was extracted at different time points ranging from 0 to 72 hours postinfection. Poly(A)$^+$ RNA was purified from these samples and cDNA was synthesized using random primers. Since HCMV DNA contamination is a common problem, two controls were used. First, all RNA samples were amplified without any cDNA synthesis step (RT- control), and aliquots of the RNA were treated with RNase prior to the cDNA step (RNase control). In all cases, the RNA samples were shown to be free of HCMV or cellular DNA (Table 3).

Transcripts in the IE1 region (primers Table 1) were amplified at all time points from 2 to 72 hours postinfection (Table 3). In infected cells transcripts from the UL81-82 region were amplified from a brief window of time, after 5 hours postinfection, but disappeared by 24 hours postinfection (Table 3). For this experiments heminested PCR for UL81-82ast was done using primers UL82F1 and UL81R1 for the first amplification and UL82F2 and UL81R1 for the second (Table 2). UL82 transcript (primers UL82n, Table 2) appeared after 6 hours postinfection and remained for the length of the experimental time (Table 3). The identity of the transcripts was confirmed by sequencing.

**Analysis of HCMV *UL81-82ast*.** The spatial localization of the *UL81-82* antisense transcript in the HCMV genome and

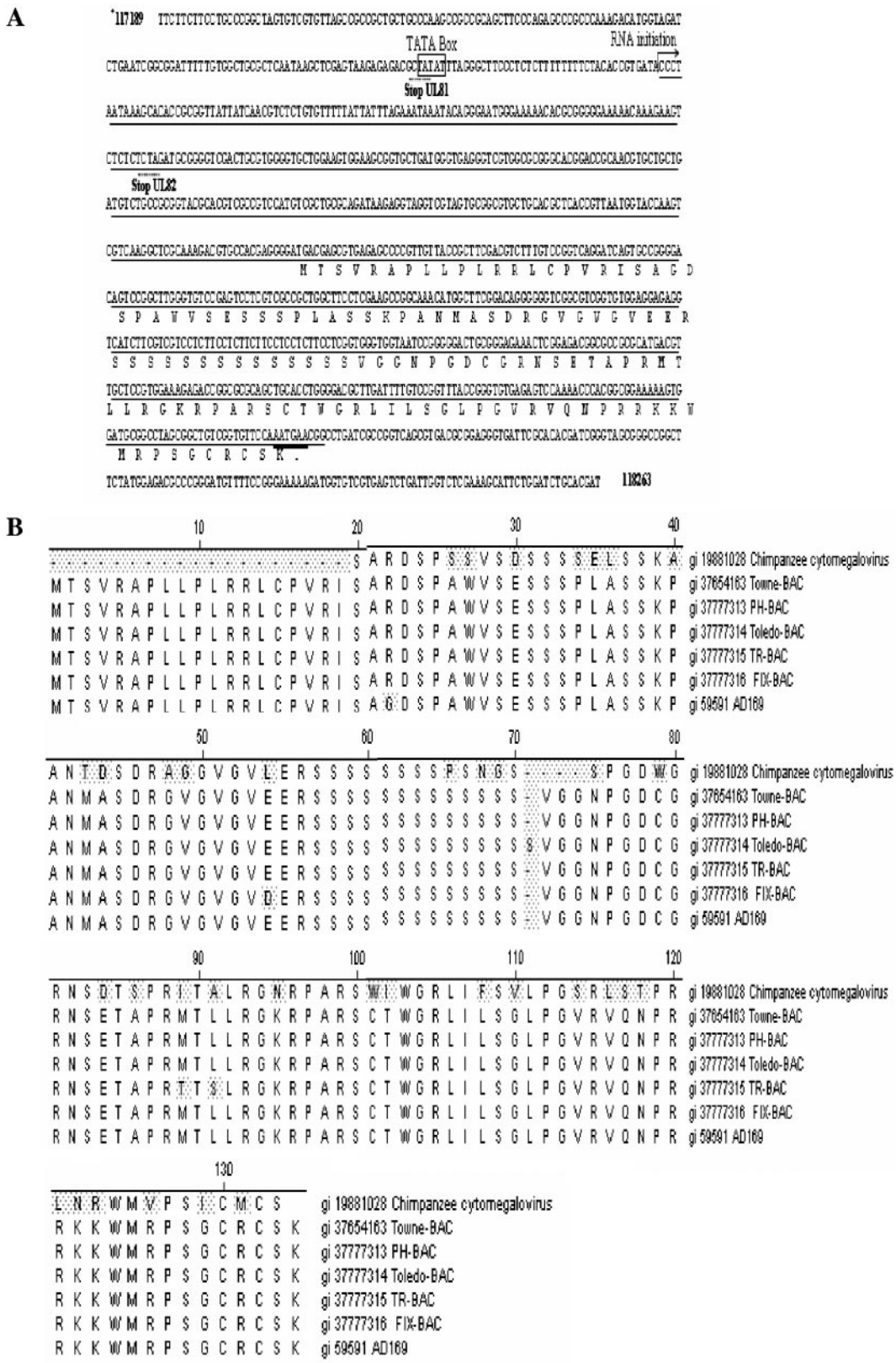


FIG. 6. Analysis of *UL81-82ast* and its protein product. (A) Map of the area showing the transcript (underlined) and the *UL81-82ast* ORF. *, Relative position compared to strain AD169 (accession number X17403). (B) Alignment of UL82as protein from several CMV genomes. Protein alignment was done using MegaAlign, from Lasergene (DNAstar, Inc.), and Clustal W algorithms. The compared genomes included human herpesvirus 5 strain AD169, Merlin strain, FIX-BAC isolate, TR-BAC isolate, PH-BAC isolate, Towne-BAC isolate, Toledo isolate, and chimpanzee CMV. See accession numbers in the Materials and Methods section.

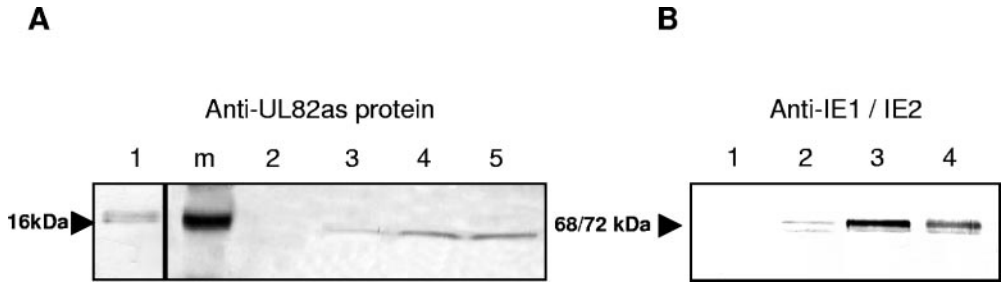


FIG. 7. Presence of UL82as protein in HCMV-infected cells. (A) Western blot of 12% acrylamide gel developed using rabbit polyclonal serum anti-UL82as as a primary antibody. Lane 1, *E. coli* purified recombinant UL82as protein. Lane 2, uninfected HFF cells. Lanes 3 to 5, HCMV clinical isolate-infected HFF cells, 7 hours postinfection, 24 hours postinfection, and 6 days postinfection, respectively. The molecular marker lane shows a 16-kDa band. (B) Western blot of 12% acrylamide gel developed using anti-IE1/IE2 monoclonal antibody mAb810 as a primary antibody. Lane 1, uninfected HFF cells. Lanes 2 to 4, HCMV clinical isolate-infected HFF cells, 7 hours postinfection, 24 hours postinfection, and 96 hours postinfection, respectively.

the primers used are shown in Fig. 1. The sequence of the area is detailed in Fig. 6a.

There is an open reading frame in the transcript, which codes for a 133-amino-acid serine-rich protein, UL82as protein (US82 antisense protein, predicted size, 14 kDa).

To study how conserved UL82as protein is among CMV clinical isolates and laboratory strains, a protein alignment was done using human herpesvirus 5 AD169 strain, Merlin strain, FIX-BAC isolate, TR-BAC isolate, PH-BAC isolate, Towne-BAC isolate, Toledo isolate, and chimpanzee CMV. As shown in Fig. 6b, only Toledo has an amino acid insertion (extra serine in the serine-rich domain) and only minor changes can be found in all others HCMV strains. Chimpanzee CMV also has a similar ORF in that same region, with the main differences located at the amino termini, and several deletions. Interestingly, 24 of the 27 serines are still conserved in the chimpanzee version of UL82as protein, possibly revealing their importance in protein's function. *UL81-82ast* ORF is not found in recent reanalyses of the HCMV genome (29). This is because in the analysis the authors required that any putative coding ORF did only overlap a known functional ORF by 396 bp. The cutoff was set based on UL76 and UL77 overlap, the longest previously known overlap among AD169 ORFs that were known to be functional. The *UL81-82ast* ORF was not found in their analysis because it overlaps the UL82 ORF by 399 bp, 3 bp above their cutoff value.

The presence of the protein was studied in infected HFF cells. The *UL81-82ast* ORF was cloned in a bacterial expression vector and purified (Fig. 7a, lane 1). Recombinant protein was tagged with a poly-His and a V5 tag. Purified protein was used as the antigen to raise rabbit polyclonal serum against the UL81-82as protein. The rabbit polyclonal serum was preabsorbed using uninfected HFF dry powder to eliminate unspecific binding, see the Materials and Methods section for details. HFF cells were infected with HCMV and different time points were studied by Western blot and indirect immunofluorescence microscopy.

Initially, the polyclonal serum was used to detect the presence of UL82as protein in HCMV-infected HFF cells (multiplicity of infection = 0.5) by Western blot. Figure 7a shows that UL82as protein can be detected as a faint band as early as 7 h postinfection, and its expression is noticeable up to 6 days postinfection (last time point analyzed). The identity of the band was confirmed by mass spectroscopy (data not shown). As a control, *E. coli* purified recombinant UL82as protein was used. The difference in molecular weight is due to the presence of fusion V5 and poly His tags in the recombinant protein. Also as a control, a Western blot using anti-IE1/IE2 mAb810 was done (Fig. 7b).

Further characterization was done by indirect immunofluorescence and confocal microscopy. HCMV-infected HFF cells (multiplicity of infection = 0.5) were stained for IE1/2 genes using mAb-810, and for UL82as protein using specific preabsorbed rabbit polyclonal serum (Fig. 8). Hoechst 33258 was used for nuclear staining; this dye shows a blue tint under fluorescent microscopy, but none when laser confocal microscopy is used. Figure 8, panel a, shows detection of UL82as protein from 8 to 96 hours postinfection using indirect immunofluorescence. As seen in the merge figures, UL82as protein is only present in a fraction of HCMV-infected cells (IE1/2-positive cells), confirming its specificity and absence of background or false-positive staining.

Figure 8, panel b, shows a 20× amplification of HCMV-infected HFF cells from 4 to 96 hours postinfection by confocal microscopy. The figure confirms that UL82as protein is not expressed in the earliest time points, such as 4 hours postinfection Also it is apparent that the number of HCMV-infected cells presenting UL82as protein in remarkably decreased at the latest time point, 96 hours postinfection (Fig. 8b). At 24 hours postinfection, 93% of IE1/2-positive cells are also positive for UL82as protein (average for three images, and approximately 50 infected cells per image). On the other hand, similar analyses showed only 46% of IE1/2-positive cells are also positive for UL82as protein at 96 hours postinfection At this point we are not able to determine whether the presence of UL82as protein is maintained for long periods of times or if its presence at later times is due to reinfections of previously negative cells.

Most of the images indicated a cytoplasmic localization of UL82as protein (Fig. 8). In some cases, heavy cytoplasmic staining seems to overlap the nucleus, but it is difficult to discriminate between nuclear and over the nucleus cytoplasmic staining. However in isolated events UL82as protein can be detected in Hoechst-negative nuclear spots, probably corresponding to nucleoli structures (Fig. 8, panel c).

## DISCUSSION

This manuscript describes the initial characterization of a novel HCMV transcript that is antisense to the UL81-82 re-

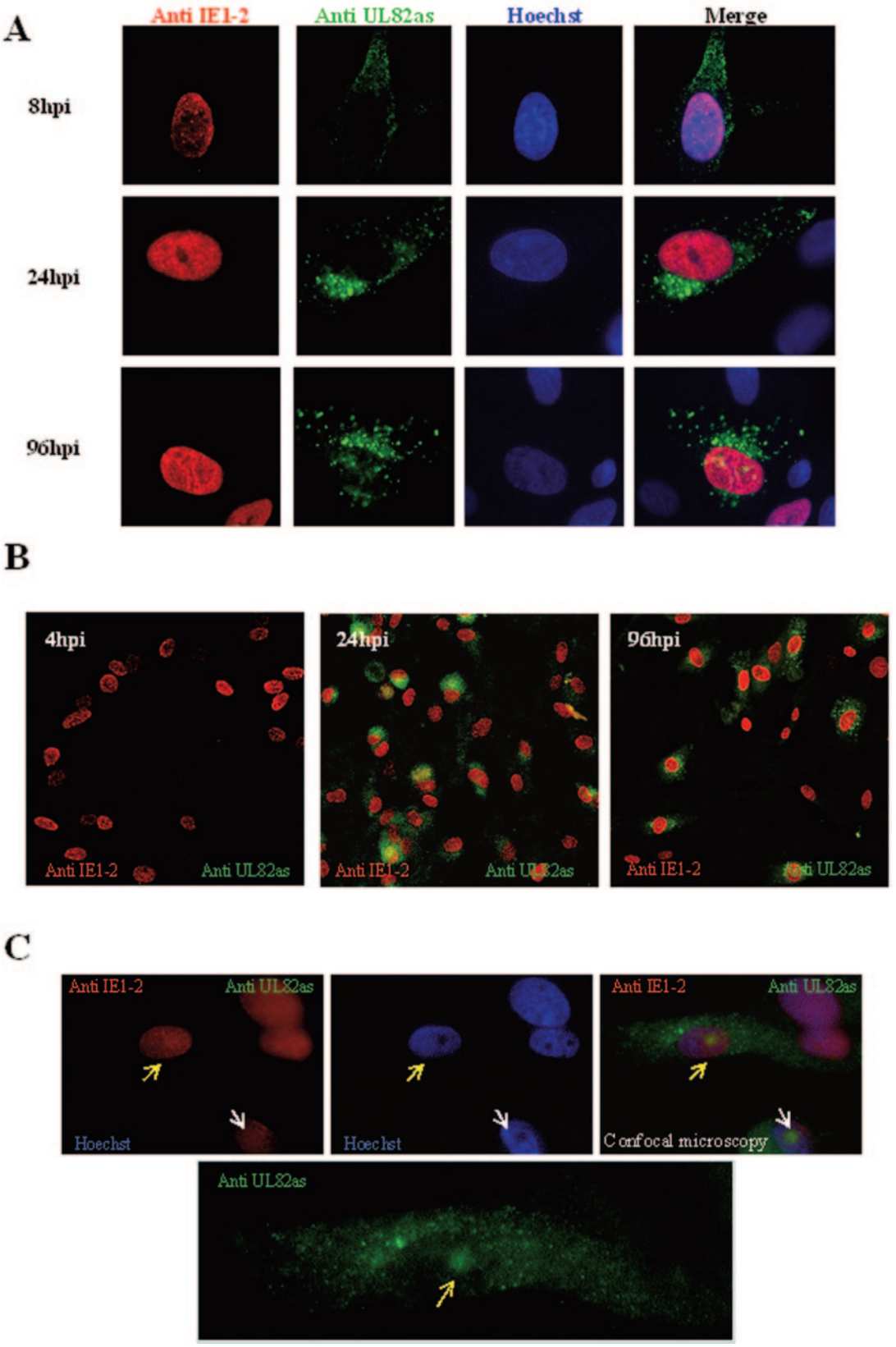


FIG. 8. Presence of UL82as protein in HCMV-infected HFF cells. Cells were stained using monoclonal anti-IE1/2 (Mab810), and rabbit polyclonal anti-UL82as as primary antibodies and anti-mouse Alexa Fluor 555-conjugated (imaged red) and anti-rabbit Alexa Fluor 488 (imaged green)-conjugated as the secondary antibody. Hoechst stain was used to identify cell nuclei, which are shown in blue. (A) Indirect immunofluorescence of HCMV-infected cells at different time points after infection (8, 24, and 96 hours postinfection, respectively). Magnification, 100×. (B) Confocal microscopy of HCMV-infected cells at different time points after infection (4, 24, and 96 hours postinfection, respectively). Magnification, 40×. (C) Apparent presence of UL82as in nucleoli. Visualized by indirect immunofluorescence and confocal microscopy after 24 hours postinfection in HCMV-infected HFF.

gion and overlaps over 700 nucleotides of the UL82 gene (UL81-82 antisense transcript or UL81-82ast) present in a cDNA library prepared from monocytes isolated from a seropositive donor. The library is restricted to polyadenylated messengers only because of the method used to create it. An additional 14 adenosine residues not coded for in the genome were found in the *UL81-82as* transcript, confirming that is indeed polyadenylated. There is a possibility that more than one transcript is expressed from the UL81-82 area in the antisense orientation. Differential initiation and termination sites and alternative splicing are under current investigation. UL81-82ast was present in monocytes isolated from a healthy donor with a high antibody titer against HCMV and in five bone marrow samples from antibody-positive donors, but not from antibody-negative donors. A sense orientation product of the UL81-82 region is transcribed, but because of its small amount, there was not enough material for sequencing.

There is a serine-rich peptide of 133 amino acids coded for by *UL81-82ast* (UL82as protein). Phylogenetic studies using UL82as protein were conducted, showing a high degree of conservation in clinical isolates, laboratory strains of HCMV and even in chimpanzee CMV. In HCMV-infected fibroblasts, UL82as protein is expressed in the cytoplasm, and in rare occasion in nuclear, nucleolus-like, inclusions.

The controlling region for *UL81-82ast* is likely to be a nonconventional TATA box (GC **TATA TTTA** GGGCT) that overlaps the *UL81* gene stop codon (underlined in Fig. 5). *UL81-82ast* may terminate following one of the two nonconsensus poly(A) sites. According to recent data (2, 14, 23), less than 70% of human 3′ expressed sequence tags contain one of the two optimal sequences (AAUAAA or AUUAAA). Moreover, use of alternative polyadenylation sites has been shown to be important for the regulation of gene expression (11). One of the nonconsensus poly(A) sites, AAUNAA, is present in approximately 6% of the pre-mRNA of human cells (48) There is a representative U-rich element (two GUs and a U or 2GU/U sequence) in the middle of the putative polyadenylation sites. The sequence maintains the consensus for the 2GU/U site (YGUGUUYY, where Y is C or T). It was emphasized that the precise sequence of the downstream element is, apparently, not crucial for the polyadenylation reaction (48). Furthermore, the downstream region is not described by any particular consensus.

*UL81-82ast* appears to be expressed very early after infection also (starting at 5 hours postinfection) in human fibroblast cells, and disappears by 24 hours postinfection. The mechanisms involving its regulation and its role in productive replication cycle are under investigation. Our data suggest that transcription of *UL81-82ast* is not detectable after the initiation of late gene transcription. However, UL82as protein is expressed in HCMV-infected HFF cells (multiplicity of infection = 0.5 PFU per cell) from 8 hours postinfection to at least 6 days postinfection There are two possible explanations for why the protein is present at late times and the transcript is only present at early times. First of all, because of its low abundance, the transcript was analyzed at a high multiplicity of infection (5 PFU per cell) whereas the protein which was more abundant was analyzed at a low multiplicity of infection (0.5 PFU per cell) to prevent lysis of cells at later times after infection. Thus, at a high multiplicity of infection all of the cells were initially infected, whereas at our low multiplicity of infection, the majority of cells were not infected from the initial virus inoculum. Either the protein has a long half-life, or what we are seeing is reinfection of HFF with newly generated virus. At this time we are unable to confirm that its expression at late times after infection is not due to reinfection.

Although UL82as protein was detected by Western blot up to 6 days postinfection, indirect immunofluorescence assays indicated that the ratio between IE1-2- and UL82as protein-positive cells decreases around 50% from 24 to 96 hours postinfection. In order to solve this problem, costaining for a late marker, such as pp65 or pp71, needs to be done. Costaining for pp71 would help visualize overall regulation involving orientation of the area in study. Such studies are ongoing. UL82as protein showed a clear cytoplasmic localization, although in isolated cases the protein was found in nuclear, possibly nucleoli, defined spots. It is possible that in cell types of hematopoietic origin that are involved in latent HCMV infections, expression patterns and localization of UL82as may be different than in HFF cells, which are permissive for HCMV infection. These studies are currently in progress.

Gene *UL82* encodes the tegument phosphoprotein pp71, which activates viral immediate-early transcription and thus has a role in initiating lytic infection. Expression of pp71 stimulates transcription from the HCMV major IE promoter and from heterologous promoters (1, 7, 21). Protein pp71 also has a general influence on gene expression since it increases the infectivity of transfected HCMV DNA independently from IE protein production (1). HCMV pp71 appears to be a functional counterpart of herpes simplex virus type 1 tegument protein VP16 and IE protein ICP0 (27). A latency-associated transcript, LAT RNA, present during herpes simplex virus type 1 latency is antisense to ICP0. LAT interferes with productive infection by inhibiting ICP0 expression (17). By analogy *UL81-82ast* might function similar to LAT and regulate UL82 expression.

Transcripts in the antisense orientation to lytic cycle genes during latency are present in an impressive collection of herpesviruses (5, 6, 12, 30–32, 46). It is possible that *UL82* and *UL81-82ast* transcripts are mutually exclusive. This could explain why *UL81-82ast* levels decreased during the in vitro fibroblast infections at the same time that transcription from the *UL82* gene begins. Analysis of the dynamics of the two promoters and their relationship to each other needs to be characterized further.

We were unable to amplify previously described latency transcripts from the IE region from either the library DNA or monocyte RNA. The absence of these putative latency-associated transcripts in our library could be explained by several mechanisms. It is possible that our donor does not express these transcripts that the abundance of these transcripts is below the limits of sensitivity of our PCR and RT-PCR assays. Another possibility is that these transcripts are not polyadenylated and therefore not represented in the library.

This is an initial report describing a new transcript present in monocytes from a seropositive donor and its protein product. The transcript could be involved in the regulation of gene *UL82*, but this needs to be investigated further. It is important to note that the transcript codes for a protein product that is present in infected cells. Although further work needs to be

done it is possible that this transcript and/or its protein product may play a role in HCMV latency. The presence of the transcript on monocytes and bone marrow of a healthy HCMV-seropositive donor and the absence of detectable mRNA for immediate-early or structural HCMV genes suggest that the transcript or its protein product may be involved in maintaining latent HCMV infection.

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