Comparative Analysis of Human Cytomegalovirus *a*-Sequence in Multiple Clinical Isolates by Using Polymerase Chain Reaction and Restriction Fragment Length Polymorphism Assays

JOHN A. ZAIA,^{1*} GHISLAINE GALLEZ-HAWKINS,¹ MARGARET A. CHURCHILL,¹
ANGELA MORTON-BLACKSHERE,¹ HEMA PANDE,² STUART P. ADLER,³
GERHARD M. SCHMIDT,⁴ AND STEPHEN J. FORMAN⁴

Division of Pediatrics and Department of Hematology and Bone Marrow Transplantation,⁴ City of Hope National Medical Center,¹ and Division of Immunology, Beckman Research Institute of the City of Hope,² Duarte, California 91010, and Department of Pediatrics and Medicine, Medical College of Virginia, Richmond, Virginia 23284³

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The human cytomegalovirus (HCMV) *a*-sequence (*a*-seq) is located in the joining region between the long (L) and short (S) unique sequences of the virus (L-S junction), and this hypervariable junction has been used to differentiate HCMV strains. The purpose of this study was to investigate whether there are differences among strains of human cytomegalovirus which could be characterized by polymerase chain reaction (PCR) amplification of the *a*-seq of HCMV DNA and to compare a PCR method of strain differentiation with conventional restriction fragment length polymorphism (RFLP) methodology by using HCMV junction probes. Laboratory strains of HCMV and viral isolates from individuals with HCMV infection were characterized by using both RFLPs and PCR. The PCR assay amplified regions in the major immediate-early gene (IE-1), the 64/65-kDa matrix phosphoprotein (pp65), and the *a*-seq of the L-S junction region. HCMV laboratory strains Towne, AD169, and Davis were distinguishable, in terms of size of the amplified product, when analyzed by PCR with primers specific for the *a*-seq but were indistinguishable by using PCR targeted to IE-1 and pp65 sequences. When this technique was applied to a characterization of isolates from individuals with HCMV infection, selected isolates could be readily distinguished. In addition, when the *a*-seq PCR product was analyzed with restriction enzyme digestion for the presence of specific sequences, these DNA differences were confirmed. PCR analysis across the variable *a*-seq of HCMV demonstrated differences among strains which were confirmed by RFLP in 38 of 40 isolates analyzed. The most informative restriction enzyme sites in the *a*-seq for distinguishing HCMV isolates were those of *MnII* and *BssHII*. This indicates that the *a*-seq of HCMV is heterogeneous among wild strains, and PCR of the *a*-seq of HCMV is a practical way to characterize differences in strains of HCMV.

The human cytomegalovirus (HCMV) genome is composed of unique long (U_L) and short (U_S) sequences containing terminal segments with inverted repeating elements (Fig. 1). Each U_L and U_S component, because of the reiteration of the terminal direct repeat sequences (ab . . . b'a' and a'c' . . . ca), can exist in the mature virion DNA in one of four possible orientations relative to each other (17). The region where these two components meet is termed the L-S junction, and in HCMV DNA (strain Towne), the repeated sequences in this junction region consist of 11-kbp repeats in U_L and 2-kbp repeats in U_S (12, 18, 28). These regions vary in size between clonal derivatives of otherwise identical HCMV strains because of heterogeneity in the types and numbers of repeat elements present in the L-S junction (28). In addition to the variability in the repeat elements, the structure of the L-S junction region has been found to contain a region homologous to the *a*-sequence (*a*-seq) of herpes simplex virus (HSV) types 1 and 2 (33). The *a*-seq of HSV is a 0.25- to 0.5-kbp sequence, bounded by direct repeats, that is thought to serve as a signal for cleavage of DNA concatemers formed during viral replication (20). The homologous *a*-seq of HCMV has been shown to substitute

for the HSV cleavage and packaging function of defective HSV (27).

The presence of hypervariable regions within the joining region of HCMV U_L and U_S has been used to characterize efficiently the differences between strains by using restriction fragment length polymorphism (RFLP) (31). This methodology has permitted epidemiologic evaluation of HCMV infection in a variety of important clinical settings, including organ transplantation centers (7, 8), neonatal units (14, 29, 35), and day-care centers (1, 2) and in individuals with the acquired immune deficiency syndrome (9, 11, 30). RFLP analyses have contributed useful information regarding the spread of single strains of HCMV and the potential presence of multiple strains of HCMV in individuals with active infections. However, because of the time requirement for wild virus replication in vitro, RFLP is an inefficient procedure for analyzing large numbers of viral isolates. It has been shown that the polymerase chain reaction (PCR) can be used for detection of HCMV infection in urine (10) and blood (4, 13, 15, 24). For this reason, we characterized the differences between strains using PCR. In this report, we show that PCR amplification of the *a*-seq from clinical isolates of HCMV can be used to characterize differences between strains. This technology has the advantage of being rapid and does not require the growth of virus in tissue culture, as is needed by RFLP.

* Corresponding author.

FIG. 1. Localization of HCMV AD169 PCR amplification sites. (A) Schematic description of HCMV genome structure showing U_L and U_S sequences containing the PCR target sites for HCMV-pp65 (PCR-pp65) and HCMV-IE-1 (PCR-IE1). The joining sequences between U_L and U_S are indicated by ab...b'a' and a'c'...ca and are homologous to the cosmid probe pCM1035. The *a*-seq of this joint region is shown as an *XhoI* fragment and contains degenerate direct repeat sequences and the PCR site of amplification (PCR-*a*-seq). (B) Sequence of the amplified *a*-seq for AD169. Primers are underlined, and the herpesvirus *pac*-1 and *pac*-2 homologies and the restriction enzyme sites are indicated with brackets.

MATERIALS AND METHODS

Source of viruses. The laboratory strains of HCMV used in this investigation included Towne, which was originally provided by S. Starr (Philadelphia, Pa.); AD169, which was originally provided by J. Waner (Oklahoma City, Okla.); and Davis, which was obtained from the American Type Culture Collection (Rockville, Md.). Simian CMV strain Colburn was provided by W. Gibson (Baltimore, Md.), HSV type 2 strain MS was provided by R. Eberle (Stillwater, Okla.), and varicella-zoster virus was obtained from a clinical isolate. In addition, clinical HCMV isolates were obtained from a repository of virus isolates prepared in this laboratory from bone marrow transplant recipients at the City of Hope National Medical Center by using cultures of leukocyte, urine, or bronchoalveolar lavage (BAL) specimens by previously published virus isolation techniques (23, 36). Isolates of HCMV were chosen based on the availability of at least two isolates from a patient, one from a leukocyte specimen and the other from either a urine or a BAL specimen. In addition, isolates were obtained from children and staff of three day-care centers in Richmond, Va.

Isolation and growth of HCMV. For cultures of clinical specimens, we used a primary cell line of human foreskin fibroblasts maintained in Dulbecco modified Eagle medium (GIBCO BRL, Grand Island, N.Y.) containing 1,000 mg of glucose per liter and supplemented with 10% fetal bovine serum (Irving Scientific, Santa Ana, Calif.). The cultures were maintained for 4 weeks and then were passed once into fresh human foreskin fibroblast cultures when a cytopathic effect was observed. HCMV was confirmed by using a monoclonal antibody stain for HCMV immediate-early gene (IE-1) antigen (NEN-Dupont, Boston, Mass.). Isolates were frozen in 10% dimethyl sulfoxide when a 4+ cytopathic effect was reached and were stored at -70°C until use in this study. HCMV isolates were selected from the repository, thawed, and passaged three or four times in human foreskin fibroblasts to obtain a high-titer stock in two 75-cm² flasks. The infected cells were harvested by trypsinization and then frozen at -70°C as a cell pellet until DNA extraction.

DNA extraction. The cells were thawed and extracted by

the method of Chandler and co-workers (5). Briefly, cell pellets were resuspended in 1 ml of extracting buffer (10 mM Tris, 10 mM EDTA, 150 mM NaCl, 0.4% sodium dodecyl sulfate [SDS] [pH 7.4]), with proteinase K added to a final concentration of 1 mg/ml. The mixture was incubated at 65°C for 30 min and at 37°C for 18 h. The DNA was purified by two extractions with equal volumes of phenol-chloroform and then precipitated overnight in 0.3 M sodium acetate and 2.5 volumes of 100% ethanol, recovered and resuspended in 200 μl of water.

RFLP analyses. RFLP analyses were performed by using an *EcoRI* digestion of 2 μg of DNA per 10 μl of buffer which was then electrophoretically resolved in components on a 0.7% agarose gel in $1\times$ TAE (5) for 16 h at 30 V. Before blotting, the gel was soaked in 0.25 M HCl, denatured in 0.4 M NaOH, and transferred by alkaline blotting onto a Zeta-probe membrane (Bio-Rad, Richmond, Calif.). After neutralization in $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), the membrane was hybridized with pCM1035 (a gift from G. Jahn, Erlangen, Federal Republic of Germany), which was previously labeled by nick translation with [α -³²P]dATP (BRL, Gaithersburg, Md.), and then incubated at 42°C in 50% formamide–0.25 M Na₂HPO₄ (pH 7.2)–0.25 M NaCl–7% (wt/vol) SDS–1 mM EDTA. After hybridization, the membrane was washed for 15 min at room temperature with $2\times$ SSC–0.1% SDS, $0.5\times$ SSC–0.1% SDS, and $0.1\times$ SSC–0.1% SDS. The filters were exposed to Kodak X-Omat AR-5 film in the presence of intensifying screens at -70°C for 1 to 5 days.

PCR assay. PCR was performed by using a commercial kit (GeneAmp DNA Amplification Reagent Kit; United States Biochemical Corp., Cleveland, Ohio); and selected regions, IE-1, 64/65-kDa matrix phosphoprotein (pp65), and *a*-seq, of the HCMV genome were chosen for amplification (Fig. 1A). Amplification involved 25 cycles in which primers (Genosys Biotechnologies Inc., The Woodlands, Tex.) for each such region were annealed at 55°C , extended at 72°C , and denatured at 94°C . HCMV IE-1 was amplified between nucleotides 1154 and 1330 (3) by using 5'-₁₁₅₄CGA GAC ACC CGT GAC CAA GG₁₁₇₃-3' as the first primer and 3'-₁₃₁₁CTC TTT

FIG. 2. RFLP of herpesviruses by using Southern blot analyses of DNAs from various herpesviruses digested with *Eco*RI and probed with pCM1035 by using laboratory strains (A) and clinical isolates of HCMV (B). (A) Lanes: L, 1-kb ladder; 1, pCM1035; 2, HCMV Towne; 3, HCMV AD169; 4, HCMV Davis; 5, simian CMV Colburn; 6, HSV MS; 7, vervet CMV; 8, varicella-zoster virus. (B) HCMV clinical isolates. Lanes (sources are indicated in parentheses): 1, 155G (leukocytes); 2, 183G (BAL); 3, 408G (leukocytes); 4, 412G (urine); 5, 345G (leukocytes); 6, 331G (leukocytes); 7, 407G (leukocytes); 8, 428G (BAL); 9, 379G (BAL); 10, 374G (leukocytes).

CTA CAG GAC CGT CT₁₃₃₀-5' as the second primer. HCMV pp65 was amplified between nucleotides 866 and 1025 (22) by using 5'-₈₆₆AAA GAG CCC GAC GTC TAC TAC ACGT₈₉₀-3' as the first primer and 3'-₁₀₀₁CTG GTC ATG CAG TTC CAC ATG GACC₁₀₂₅-5' as the second primer. The L-S junction region was amplified in the conserved region of the *a*-seq between nucleotides 751 and 889 (19) by using 5'-₇₅₁TTC CCC GGG GAA TCA ^ΔAC AG₇₇₁-3' as the first primer and 3'-₈₆₉AAA GTG GGG GGG CGA TTT TT₈₈₉-5' as the second primer.

The amplified PCR products were run on a 1.8% agarose gel for 1 h with a 123-bp sizing ladder (BRL), and were transferred onto a Zeta-probe membrane in 0.4 M sodium hydroxide for Southern blotting. The probes for these amplified products were as follows: IE-1, 3'-₁₁₈₂AAG GAC GTC TGA TAC AAC TCC TT₁₂₀₄-5'; pp65, 3'-₉₄₁CGC GTG CTC GAC CAA ACG AGG TAC CTC TTG₉₇₀-5'; *a*-seq, pCM1035 DNA. The oligonucleotide probes were labeled at the 3' ends with [α -³²S]dATP by terminal deoxynucleotidyl transferase by using a commercial kit (NEN-DuPont), and the pCM1035 DNA was labeled with [α -³²P]dATP by nick-translation by using a commercial kit (BRL). The restriction enzyme sites for HCMV *a*-seq are shown in Fig. 1B. In the digestion of the *a*-seq PCR product, 5 μ l of amplified DNA was mixed with 3.5 μ l of water, 0.5 μ l of the appropriate 10 $\times$ buffer, and 1 μ l of enzyme in a final volume of 10 μ l. This was incubated according to enzyme specifications, electrophoresed on a 1.8% agarose gel, and analyzed by Southern blotting, as described above for RFLP analysis.

RESULTS

Several herpesviruses, including HCMV (strains Davis, Towne, and AD169), simian CMV (strain Colburn), vervet CMV, HSV type 2 (strain MS), and varicella-zoster virus, were compared by RFLP (Fig. 2A). The HCMV laboratory strains were markedly different by RFLP analysis, as expected, and except for HSV, none of the other herpesviruses hybridized to the pCM1035 probe for the L-S junction of HCMV. In addition, 30 clinical isolates were analyzed in a similar manner, and these RFLP-characterized viruses

FIG. 3. PCR of HCMV *a*-seq, IE-1, and pp65 for laboratory strains of HCMV. (A) PCR amplification products are shown for HCMV strains Towne, AD169, and Davis by using primers specific for *a*-seq (lanes 1, 2, and 3, respectively), IE-1 (lanes 4, 5, and 6, respectively), and pp65 (lanes 7, 8, and 9, respectively). (B) Lanes 1', 2', and 3' are Southern blot analyses of the *a*-seq amplification products from HCMV Towne, AD169, and Davis, respectively, hybridized with ³²P-labeled pCM1035. Lane L, 123-bp ladder.

formed the basis for further PCR comparisons. Representative RFLP analyses of 10 such isolates are shown in Fig. 2B.

The HCMV laboratory strains Towne, AD169, and Davis had DNA amplification products of different lengths when *a*-seq PCR was used but had amplification products of similar lengths when IE-1 and pp65 PCR amplifications were used (Fig. 3). None of the other herpesviruses used in RFLP analysis showed amplification by the *a*-seq PCR (data not shown). Southern blot analysis of the amplified products confirmed that the *a*-seq DNA hybridized to the L-S junction probe (Fig. 3, lanes 1', 2', and 3') and that the IE-1 and pp65 DNAs hybridized to their specific probes (data not shown). This type of analysis was then applied to 30 wild isolates to

FIG. 4. PCR of HCMV clinical isolates: PCR amplification products by using primers specific for HCMV IE-1 (A), HCMV pp65 (B), and HCMV *a*-seq (C). Isolates in lanes 1 to 10 are the same as those shown in Fig. 2B (see Fig. 2B legend).

TABLE 1. Enzyme digestion of HCMV *a*-seq PCR product^a

HCMV strain (source)	<i>Sma</i> I	<i>Mlu</i> I	<i>Mae</i> I	<i>Bss</i> HII	<i>Fnu</i> 4HI	<i>Mn</i> II	<i>Spe</i> I
pCM1035	+	+	+	+	+	+	+
AD169	+	+	+	+	+	+	+
Towne	+	+	-	+	+	-	-
Davis	-	-	-	-	+	-	-
155G (leukocytes)	+	+	-	+	+	-	-
408G (leukocytes)	+	+	-	-	+	+	-
412G (urine)	+	+	-	+	+	-	-
407G (leukocytes)	+	+	-	+	+	-	-
374G (leukocytes)	+	+	-	-	+	+	-

^a +, Enzyme cleavage of DNA; -, no enzyme cleavage of DNA.

determine whether the *a*-seq variability extended to clinical isolates of HCMV. The PCR results for 10 HCMV isolates are shown in Fig. 4 and are matched with the isolates characterized in Fig. 2B. The IE-1 and pp65 PCR products were indistinguishable (Fig. 4A and B), but the *a*-seq PCR products had variable DNA product lengths. Of these 10 strains, 5 (lanes 1, 2, 4, 7, and 8 in Fig. 2B and 4C) were identical by RFLP analysis and generated identical PCR products in all PCR assays. With rare exceptions, isolates with different RFLP patterns generated different *a*-seq PCR products. Two such RFLP-distinct HCMV strains, which were isolated from different specimens from the same individual, were also distinct by *a*-seq PCR (compare lanes 3 and 4 in Fig. 2B and 4C). Even though DNA from all isolates studied by PCR supported amplification when the priming site was within conserved regions such as IE-1 and pp65, 4 of 40 isolates were not amplified when they were primed from the variable *a*-seq. The explanation for the failure of amplification of certain samples is most likely due to differences in primer site sequences in the hypervariable *a*-seq, but we have no sequence data to confirm this.

To investigate the degree of differences among the sequences of *a*-seq for distinct isolates of HCMV, a restriction enzyme analysis of the *a*-seq PCR product was performed. The presence of restriction sites for *Sma*I, *Mlu*I, *Mae*I, *Bss*HII, *Fnu*4HI, *Mn*II, and *Spe*I was variable in these HCMV strains (Table 1). The *Fnu*4HI site was present in all HCMV isolates studied, and *Sma*I and *Mlu*I were present in all strains except Davis. The overlapping sites, *Spe*I and *Mae*I, were present only in AD169. The *Bss*HII and *Mn*II enzyme digestions were the most informative because they permitted easy distinction between isolates. DNA from *a*-seq PCR analysis of five HCMV isolates was analyzed following digestion with either *Mae*I or *Bss*HII (Fig. 5). Note that after the initial PCR (Fig. 5, lanes 1 to 5), *Mae*I failed to cut any of these strains (Fig. 5, lanes 1', 2', 3', and 4'), but *Bss*HII cut three of them (Fig. 5, lanes 1'', 2'', and 4''), thereby confirming the initial RFLP similarities.

We analyzed multiple HCMV isolates obtained from 14 different bone marrow transplant recipients using this same method. Of these, 11 patients had two strains of HCMV from different sites which were identical by RFLP analysis. PCR analysis of these strains showed them to be identical by product size and enzyme digestion analysis. Three pairs of isolates were not amplified, even though their IE-1 and pp65 gene sequences were amplified. In the three instances of dissimilar HCMV isolates, two pairs of isolates were different by both RFLP and PCR analyses, and one pair of isolates that was different by RFLP was identical by PCR.

The utility of this assay was further evaluated by testing 10 HCMV DNA specimens prepared from HCMV obtained

FIG. 5. Restriction enzyme digestion of *a*-seq PCR product. Five HCMV isolates from Fig. 2B (lanes 1 to 5) were analyzed by HCMV *a*-seq PCR, subjected to Southern blot analysis, and stained with ethidium bromide (A) or subjected to pCM1035 hybridization (B). The PCR product was uncut (lanes 1 to 5) or was digested with *Mae*I (lanes 1' to 5') or with *Bss*HII (lanes 1'' to 5''). Lane L, 123-bp ladder.

from individuals in day-care settings. Isolates were obtained and evaluated by RFLP, and then the identity of the specimen was masked prior to PCR analysis. Among 10 isolates of HCMV from urine specimens (Fig. 6B), there were three PCR patterns: (i) a predominant pattern found in eight identical isolates (see lanes 1, 2, 3, 5, 6, 8, 9, and 10), (ii) one amplification product which was shorter than the others (lane 7), and (iii) one reaction which was not amplified by using *a*-seq PCR (lane 4). When the specimens were decoded and compared with the RFLP results, there were also three distinct RFLP patterns in isolates obtained from individuals at three different day-care centers (Fig. 6A). Pattern A included specimens 1, 2, 3, 5, 6, 9, and 10; pattern B included specimen 7; and pattern C included specimen 4. Specimen 8 appeared to have RFLP patterns A and C combined and most likely represented a dual infection with isolate 4 which was from an individual at the same day-care center. This was confirmed by additional RFLP analyses by using *Bam*HI (data not shown). DNA specimen 8 repeatedly produced an *a*-seq PCR identical to those of isolates with RFLP pattern A, further suggesting that the contaminating strain was similar to isolate 4 and was not amplified by *a*-seq PCR. Thus, the isolates which were distinct by RFLP were distinct by PCR. These HCMV strains yielded *a*-seq PCR product DNA which contained *Mn*II and *Bss*HII sites and therefore could not be distinguished further by our restriction enzyme analyses.

DISCUSSION

We demonstrated that a selective region near the packaging signal sites of the L-S junction can serve as an amplification site for comparison of HCMV strains. In selective

FIG. 6. RFLP and *a*-seq PCR of HCMV isolates from individuals at three day-care centers. DNAs from 10 HCMV clinical isolates from individuals at three day-care centers were digested with *Eco*RI and analyzed by Southern blot (A) by using 32 P-labeled CMV DNA labeled in vivo (1, 2). This DNA was coded and subjected to PCR analysis (B) by using primers specific for HCMV *a*-seq. The code numbers 1 to 10 are indicated; specimens 1, 2, 3, 5, 6, 9, and 10 came from day-care center 1; specimens 4 and 8 came from day-care center 2; and specimen 7 came from day-care center 3. Lane L, 123-bp ladder. The individual specimens are indicated as lane numbers.

situations, PCR combined with restriction enzyme analysis of the amplified DNA can distinguish isolates obtained from the same individual. Conventional RFLP methods for differentiating strains of HCMV are tedious. RFLP analysis requires the isolation of virus and growth of sufficient amounts of DNA for enzyme digestion. Use of *a*-seq PCR analysis permits rapid identification of strains in a fraction of the time usually required for RFLP. For example, RFLP requires 1 to 4 weeks for virus isolation plus another 4 weeks for tissue culture passage and production of sufficient viral DNA for analysis, whereas PCR results can be obtained on the initial unpassaged isolate and analyzed in 3 days. Furthermore, although we did not attempt to demonstrate this, we speculate that *a*-seq PCR-based strain differentiation of HCMV could become even more efficient if used directly on the original clinical specimen, without first obtaining a virus isolate.

The optimal site(s) for amplification of HCMV deserves further investigation. In this study, we were able to amplify across only a small, relatively conserved region of the HCMV *a*-seq. Attempts to extend this PCR amplification across the more variable regions of the L-S junction, which contains the regions of degenerate direct repeat sequences, were unsuccessful. Even the use of the reiterated repeat TTGGGTGTG as a primer was not reliable for extended amplification. In addition, HCMV Davis and several clinical isolates were not amplified by using the *a*-seq PCR. This indicates that primer site base pair mismatches were present in these virus strains and resulted in poor or absent amplification under the relatively stringent annealing condition of

the assay. It is possible that additional primer sites which could be used to distinguish viruses can be found in the L-S junction region. In addition, it has been shown that highly conserved regions such as IE-1 and other gene-coding regions contain variable sites (6). These regions might be exploited for selective differences which would permit differentiation of viral strains.

It has been shown that, for HSV, the *a*-seq of the L-S junction region is important as a terminal direct repeat region which serves as a *cis* packaging signal (25–27, 32, 34). In this way, multiple-length copies of the viral DNA can be cleaved into a single genome length for packaging. In addition, it is postulated that these inverted terminal direct repeats present in *a*-seq play a role in the inverted orientation of the L-S junction region during viral DNA replication (21). The *a*-seq of HCMV contains two regions of homology (*pac*-1 and *pac*-2), which is similar to the case in HSV (16, 19) (Fig. 1). Within the *pac*-2 motif, there is a DNA recognition sequence, for which a nuclear protein has been isolated (16). The biological relevance of this is not yet clear, but it should be possible to use *a*-seq amplification to determine natural variability in this region. The data from this report, demonstrating discernible variability in the *a*-seq of clinical HCMV isolates, suggest the possibility that differences in this functionally important region can be systematically analyzed and possibly correlated with biologic characteristics of HCMV strains.

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ADDENDUM IN PROOF

It has recently been shown that HCMV strain differentiation can be successfully performed on clinical specimens prior to virus isolation using PCR (S. Chou, *J. Infect. Dis.* 162:738–742, 1990).

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