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Variations in the Cytomegalovirus Major Immediate-Early Gene Found by Direct Genomic Sequencing

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An assay to detect and sequence DNA from human cytomegalovirus (HCMV) immediate-early gene region 1 has been developed; it involves in vitro amplification by the polymerase chain reaction and direct solid-phase sequencing of the amplified material. Urine samples from 16 patients tested positive for HCMV DNA in both a colorimetric assay for the detection of immobilized amplified nucleic acids and a standard polymerase chain reaction assay with agarose gel electrophoresis. Ten urine samples from healthy people tested negative in the same assays. Analysis of 106-bp fragments from seven patients and two laboratory HCMV strains (Ad 169 and Towne) demonstrated that the viral sequences were conserved in samples collected at different times from the same patient and in tissue-cultured samples. Two of the patient strains had variations in the amplified region, with a total of seven nucleotide substitutions yielding five amino acid alterations in the coding sequence.

Human cytomegalovirus (HCMV), a member of the *Herpesviridae* family, can cause serious illness in congenitally infected infants as well as in immunocompromised adults and patients suffering from AIDS (11, 12, 24, 31). HCMV has a large linear double-stranded DNA genome (240 kb) that is composed of two unique components (U_S and U_L) as well as terminal repeat sequences (13, 15, 25). HCMV undergoes temporally regulated viral gene expression, which can be broadly categorized into three sequential phases: immediate-early (IE), early, and late (39). The IE proteins are expressed 1 to 12 h after infection, even in the absence of protein synthesis (39). The IE products are proposed to switch the viral genome from restrictive to extensive expression in infected cells. HCMV strain Ad 169 has four different IE loci (21, 36). The loci IE-1, IE-2, IE-3, and IE-4 are encoded by the *Hin*dIII E fragment of strain Ad 169 (1). The IE-1 region, also referred to as the major IE gene, encodes the most abundant type of IE RNA, with a major size of 1.9 kb (36, 40). The major IE-1 product is a 491-amino-acid phosphoprotein (1, 34), which has a molecular mass of 72 kDa (36). The protein accumulates primarily in the nucleus of the infected cell (28). The proteins encoded by the IE-1 and IE-2 genes have been found to regulate transcriptional expression from the HCMV early- and late-gene promoters (5, 10, 32, 33) as well as the major IE promoter (6).

Numerous studies have dealt with the promoter and enhancer sequence upstream of the IE-1 gene (2, 16, 22, 26, 29, 35). Only two sequencing studies, however, have concentrated on the IE-1 gene: the IE-1 locus nucleotide sequences of Ad 169 and Towne were determined, and the transcripts were analyzed by nuclease mapping (1, 34). Restriction enzyme cleavage patterns of various laboratory and wild-type strains revealed distinct differences that may allow the identification of individual strains and lead to epidemiological conclusions (4, 17, 18). A study of HCMV strains has revealed an average of 80% DNA homology between them (19). The polymerase chain reaction (PCR) to amplify specific nucleotides has been used for the detection of HCMV with primers complementary to consensus regions (3, 9, 23, 30). It has also been possible to differentiate between HCMV patient strains by restriction enzyme analysis of a 2.6-kb fragment from the IE-1 locus after amplification by the PCR (8).

To acquire more knowledge of IE-1 gene variations, we here describe the development of a solid-phase approach (38) to detect immobilized amplified nucleic acids (DIANA assay) that permitted the detection of HCMV-specific DNA and direct genomic sequencing of a 146-bp amplified DNA fragment from patient samples. Samples were collected at different times from the same patient. To study variations of the IE-1 gene region, we compared the sequences obtained from clinical samples with those obtained from the corresponding cultured isolates.

MATERIALS AND METHODS

HCMV-infected cells and clinical specimens. Crude lysates of HL cells infected with HCMV Ad 169 or Towne were used as positive controls in the PCR. Uninfected cells as well as cells infected with other *Herpesviridae* family members (varicella-zoster virus, herpes simplex virus type 1, herpes simplex virus type 2, human herpesvirus 6, and Epstein-Barr virus) were used as controls. The cells were lysed in culture medium by being heated at 95°C for 10 min, and 10 μl of heat-treated cells (10^6 cells) was added to the PCR mixture.

Urine samples (n = 18) were collected from 16 patients with suspected HCMV infection; 10 patients were bone marrow or kidney recipients, 2 patients had leukemia, 3 patients were congenitally infected infants, and 1 patient was a tentative bone marrow donor. The urine samples were stored at −20°C until used in the PCR. Viruria was detected by virus isolation (14), and immunoglobulin G (IgG) and IgM antibodies to HCMV in sera were measured by an enzyme linked immunosorbent assay (37). No isolates were kept in

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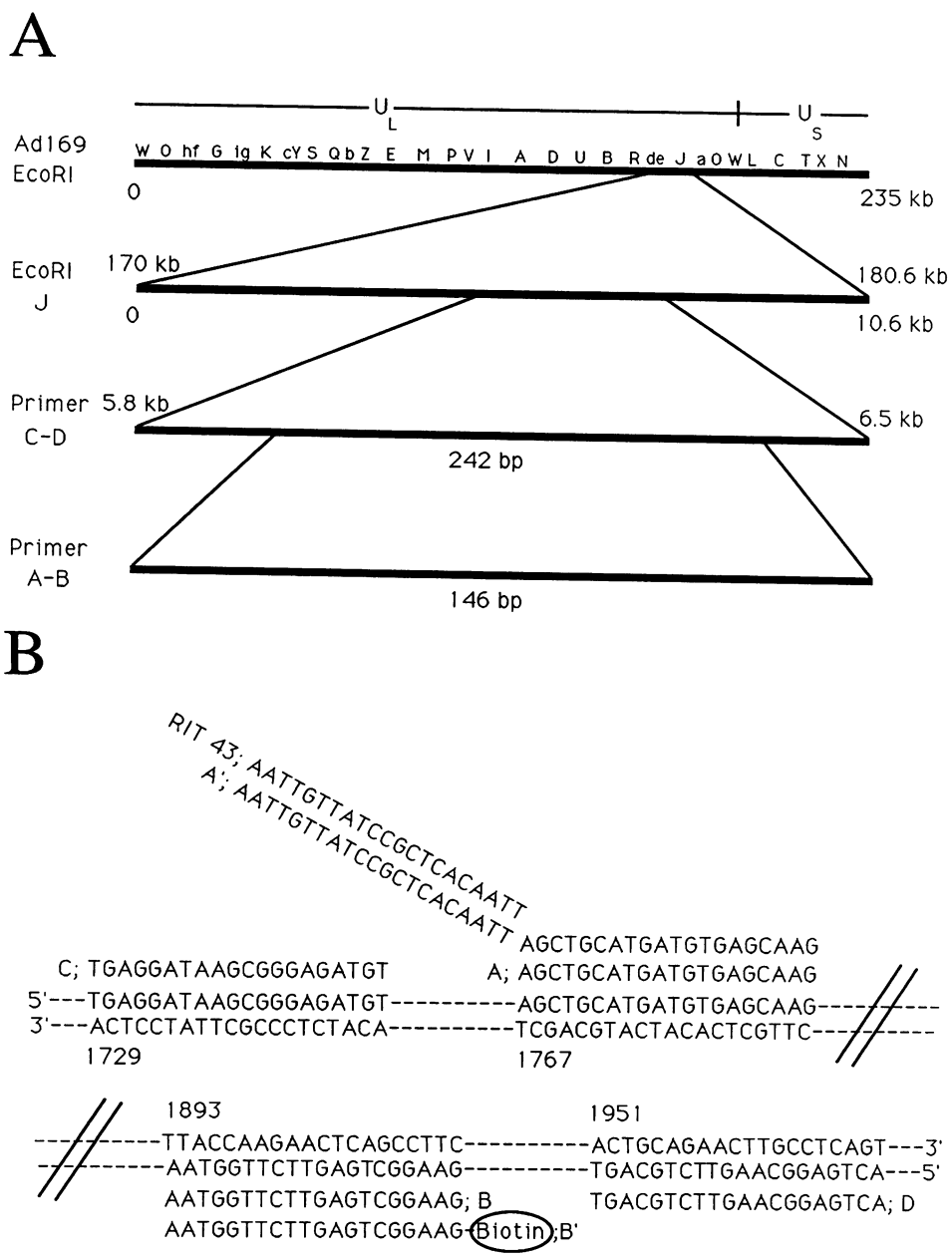

FIG. 1. (A) Primer locations in the HCMV Ad 169 genome. (B) Sequences and locations in the IE-1 gene (1) of the primer oligonucleotides used in the PCR (A, A', B, B', C, and D) and the sequencing oligonucleotide (RIT 43). Primers were selected for the HCMV Ad 169 sequence.

cultures for more than 8 weeks, and the isolates were stored at −20°C until used.

Oligonucleotide primers. The primers used in the HCMV-specific PCR were from the fourth exon of the IE-1 gene located in the *Eco*RI J fragment of strain Ad 169 (1) (Fig. 1). The primers were synthesized by phosphoramidite chemistry (Gene Assembler; Pharmacia LKB, Uppsala, Sweden) and purified as described by the manufacturer. The outer flanking primer pair consisted of primers C and D (3) (Fig. 1). The nested primer pair consisted of primers A and B (23) or A' and B' (Fig. 1). Primers A and B were used for PCR detection with agarose gel electrophoresis; with primers A' and B', a colorimetric analysis (DIANA assay) and direct sequencing were possible. Primer A' was obtained by adding 21 nucleotides of the *lac* operator (38) to the 5' end of primer A, and primer B' was obtained by 5' end biotinylation of primer B. The biotinylation was performed with biotin-X-NHS ester (Clontech, Palo Alto, Calif.). Sequencing primer RIT 43 (38) was labelled with fluorescein isothiocyanate (Pharmacia LKB).

PCR conditions. The PCR was performed as a double PCR with two sets of primers in two steps (3). In the first step, primers C and D were used, and in the second step, primers A and B or A' and B' were used.

All the reaction mixtures consisted of 10 mM Tris-HCl (pH 9.6), 10 mM $MgCl_2$, 50 mM NaCl, 0.2 μg of bovine serum albumin per μl, and 1 U of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin-Elmer Cetus, Emeryville, Calif.).

The variable parameters in the mixtures between the different PCR systems were the primers and the deoxynucleoside triphosphate concentrations. In the first step, 0.5 mM each deoxynucleoside triphosphate and 0.15 μM each primers C and D were used. In the second step, we used 1.0 mM each deoxynucleoside triphosphate and 0.60 μM each primers A and B or A′ and B′. The samples (2.5 μl of urine, 5 μl of culture medium from the virus isolation, or 10 μl of heat-lysed cells) were added to the C-D mixture, and water was added to a final volume of 50 μl. The tubes were covered with paraffin oil and preheated for 4 min at 92°C before the incubation cycles were started. Twenty incubation cycles consisting of 30 s at 92°C, 30 s at 53°C, and 30 s at 72°C were performed with an automated thermal cycler (DNA Thermal Cycler; Perkin-Elmer Cetus). The products from the first amplification were diluted 20 times with a new reaction mixture consisting of the A-B (50 μl) or the A′-B′ (100 μl) buffer system and reamplified by 40 incubation cycles with the temperatures and times described above. For excluding false-positives due to contamination, every fourth sample analyzed was a negative control.

PCR product detection. The PCR products amplified with primers A and B were detected by gel electrophoresis. The PCR products amplified with primers A′ and B′ were detected by the DIANA assay or gel electrophoresis.

In the gel electrophoresis assay, 10 μl of the amplimers were electrophoresed on 2% agarose gels (NA agarose; Pharmacia LKB) in 0.5× TBE (45 mM Tris-borate, 45 mM boric acid, 1 mM EDTA [pH 8.0]) containing 0.5 μg of ethidium bromide per ml. The gels were photographed with short-wave UV light (data not shown).

DIANA colorimetric detection (Fig. 2) was performed as described by Lundeberg et al. (27). Eighty microliters of the PCR mixtures was immobilized on 300 μg of magnetic beads covalently coupled to streptavidin (Dynabeads M-280 streptavidin; Dynal AS, Oslo, Norway). The beads with the immobilized DNA were washed three times to remove nonbound DNA and were then mixed with 100 μg of sonicated herring sperm DNA and 100 μl of fusion protein (LacI-LacZ; 0.2 mg/ml; purified as described previously [38]). The fusion protein consisted of the repressor protein (LacI), which binds to the introduced *lac* operator sequence, and of the enzyme β-galactosidase (LacZ). The β-galactosidase substrate, *o*-nitrophenyl β-D-galactoside, was added to the washed beads, and the change in the A_{405} per minute at room temperature was measured.

Solid-phase DNA sequencing. Only the amplimers made by the A′-B′ PCR system were sequenced. Samples which tested positive in the DIANA assay or gel electrophoresis were subsequently sequenced as described of Wahlberg et al. (38). Samples detected by gel electrophoresis were immobilized on magnetic beads before sequence reactions. The immobilized double strands of DNA were converted into single strands by alkali treatment. The beads with the immobilized single-stranded template DNA were transferred to a 96-well plate. All sequencing reactions were performed by use of 96-well plates, reagents from an Auto Read T7 sequencing kit (Pharmacia LKB), a Biomek-1000 work station (Beckman Instruments, Fullerton, Calif.), and a cooling-heating module (HCB-1000; Beckman) programmed as described by Hultman et al. (20). The annealing mixture consisted of beads, 60 nM RIT 43, and annealing buffer. The annealing mixture was heated to 65°C and allowed to cool to room temperature. The sequencing reaction mixture consisted of the annealing mixture, T7 DNA polymerase, and extension buffer. The mixture was dispensed into four wells and mixed with different dideoxynucleotides (containing A, C, G, and T). The four dideoxynucleotide mixtures were incubated for 5 min at 37°C, and stop solutions were then added. Each sequencing reaction mixture was heated just prior to loading of the gel. The fluorescing bands were read on a 6% polyacrylamide gel with an automated laser fluorescence (ALF) sequencing apparatus (Pharmacia LKB), and the nucleotide and amino acid sequences were deduced.

RESULTS

Basic concept. The primers used to detect and sequence the HCMV genome are shown in Fig. 1. A nested primer approach was used to minimize the amplification of nonspecific sequences (3, 38). In the second primer pair, one primer (B′) was biotinylated, while the other primer (A′) contained a 21-nucleotide *lac* operator. Thus, as outlined in Fig. 2, the specific amplimers can be captured on streptavidin-coated magnetic beads and colorimetrically detected with a fusion protein consisting of the LacI repressor and the enzyme β-galactosidase. This procedure enables the detection of the target genome and also subsequent direct DNA sequencing of fragments that are present in small amounts. The introduction of the *lac* operator into the target DNA allows solid-phase sequencing with a "general" primer corresponding to the *lac* operator sequence.

Specificity and sensitivity of the PCR and the DIANA assay. Since the DIANA assay can be automated, it may be of interest to use it for routine HCMV detection. The PCR with gel separation for detection and the DIANA assay were compared to evaluate the performance of the latter.

No PCR with uninfected cells or cells infected with the other five human herpesviruses was seen. The A-B PCR system has a sensitivity of 20 to 40 HCMV genomes (3). Urine samples from five healthy seropositive persons and five healthy seronegative persons were negative in the PCR. With this method, HCMV was detected in all 18 urine samples from patients with suspected HCMV infection, five virus isolation culture media, and HCMV Ad 169- and HCMV Towne-infected cell cultures. All the HCMV urine samples (18 of 18) were also positive in the DIANA assay. With virus isolation, 13 samples tested positive, 2 were infected with bacteria, and 3 tested negative. Three of the five patients whose urine samples were negative or contained bacteria had HCMV IgG and IgM antibodies. Two patients were HCMV IgG positive. One was a bone marrow recipient with pneumonia who died a few days later. The clinical diagnosis was suspected HCMV infection. No further samples were available for the PCR. The second patient was a tentative bone marrow donor with primary Epstein-Barr virus infection.

DNA sequencing. To determine the specific DNA sequence in the amplified region of the positive samples, we sequenced 14 samples (9 urine samples and 5 virus isolates) from seven patients and two laboratory HCMV strains (Ad 169 and Towne). We also tried to sequence cultured viral strain Towne that had been lysed by methanol after evaporation of the methanol by heat (3). The preparation of the sample may influence the subsequent sequencing, since the methanol-evaporated sample was amplified by the PCR, but the sequence could not be determined (data not shown).

Samples were taken at different times from two of the patients. Twelve days elapsed between patient samples 7a and 7b, and 52 days elapsed between patient samples 6a and 6b. The results of sequencing of the 106-bp region are shown in Table 1. No changes at the nucleotide level were found

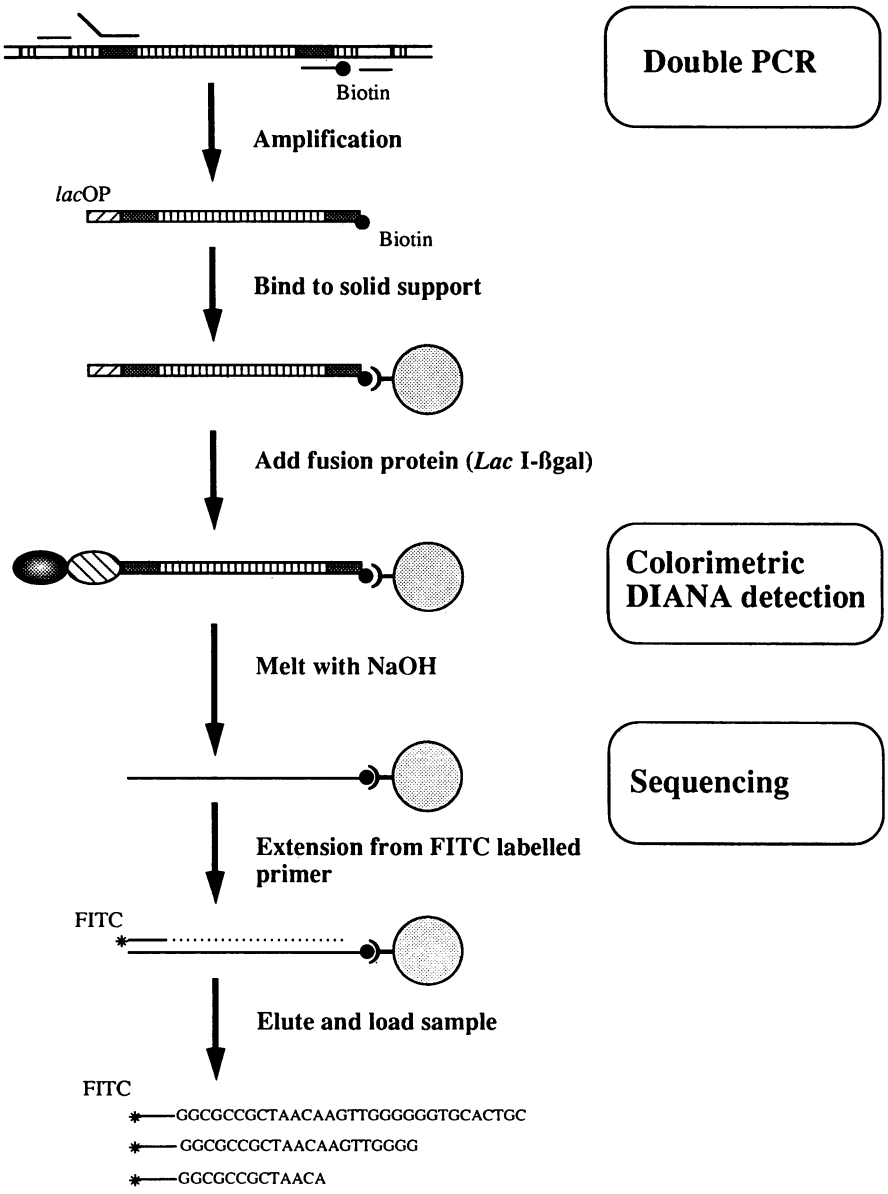

FIG. 2. Schematic drawing of the concept of solid-phase sequencing with PCR-amplified DNA. The primers were designed to amplify a region within the HCMV IE-1 gene. OP, operator; βgal, β-galactosidase; FITC, fluorescein isothiocyanate.

between the clinical samples and their respective cultured isolates. Sequences from samples collected from the same patient at different times were also conserved (Table 1).

Differences between the patient strains and the laboratory HCMV strains were found for two of the seven patients (patients 5 and 7) (Table 1). Substitutions were found in seven positions. The two patient strains had two silent nucleotide substitutions at nucleotide positions 1804 and 1837. Patient 5 had a nucleotide substitution at position 1851 that yielded an amino acid change from arginine (R) to lysine (K) at amino acid position 201 (Table 2). At nucleotide position 1890 in both samples 5a and 5b, we could not distinguish, from the automated laser fluorescence processed graph, whether the nucleotide was T or whether there had been a nucleotide substitution to C. Patient 5 seems to have had two strains, one of which had a nucleotide difference within the studied region. A C at nucleotide position 1890 will result in serine at amino acid position 214 instead of phenylalanine. This patient underwent bone marrow transplantation 12 weeks before sample collection. Viremia was detected by virus cultivation from a blood sample that was collected 5 weeks after the transplantation. It is possible that the virus strain had changed during these 7 weeks.

The virus from patient 7 had four nucleotide substitutions that resulted in three amino acid changes (Table 2). The nucleotide substitution at position 1817 caused an amino acid change from glutamine to lysine (at position 190), that at positions 1851 and 1852 caused an amino acid change from arginine to asparagine (at position 201), and that at position 1864 caused an amino acid change from methionine to isoleucine (at position 205). The sequence of the patient strain that had the majority of nucleotide substitutions had a

TABLE 1. Sequences of the IE-1 gene fragments and their nucleotide substitutions, compared with those of Ad 169 (1)

Sample[a]	Substitution at nucleotide[b]:						
	1804	1817	1837	1851	1852	1864	1890
Ad 169	—	—	—	—	—	—	—
Towne	—	—	—	—	—	—	—
Patient 1 (urine)	—	—	—	—	—	—	—
Patient 2 (urine)	—	—	—	—	—	—	—
Patient 3 (urine)	—	—	—	—	—	—	—
Patient 4, a (urine)	—	—	—	—	—	—	—
Patient 4, b (isolate from sample a)	—	—	—	—	—	—	—
Patient 5, a (urine)	G→A	—	A→G	G→A	—	—	T→Y
Patient 5, b (isolate from sample a)	G→A	—	A→G	G→A	—	—	T→Y
Patient 6, a (urine)	—	—	—	—	—	—	—
Patient 6, b (urine)	—	—	—	—	—	—	—
Patient 6, c (isolate from sample a)	—	—	—	—	—	—	—
Patient 7, a (urine)	G→A	C→A	A→G	G→A	G→C	G→T	—
Patient 7, b (urine)	G→A	C→A	A→G	G→A	G→C	G→T	—
Patient 7, c (isolate from sample a)	G→A	C→A	A→G	G→A	G→C	G→T	—
Patient 7, d (isolate from sample b)	G→A	C→A	A→G	G→A	G→C	G→T	—

[a] Letters following patient numbers indicate different samples from those patients.
[b] Y = T or C. Patient 5 seems to have had two HCMV strains, one with a C and the other with a T at nucleotide position 1890. Dashes indicate identity.

DNA homology, compared with the laboratory strain consensus sequence, of 94.4%.

DISCUSSION

We have shown that a solid-phase approach (38) can be used to detect and sequence HCMV DNA directly in clinical samples without a cumbersome cloning procedure.

Three urine samples were positive in the PCR and the DIANA assay, which had not yielded infectious virus, while the corresponding serological diagnoses indicated an HCMV infection. These assays do not require sterile, bacterium-free samples, like the virus isolation technique does. The fact that the DIANA assay allows both the detection of HCMV DNA and then direct sequencing of patient samples makes the method suitable for various epidemiological studies as well as studies of the structural-functional aspects of viral sequences. Such an approach will be still more valuable with a choice of primers flanking variable regions. Since positive samples can be directly sequenced, it is possible to confirm that HCMV-specific DNA has been amplified.

The fragment studied was from the fourth exon of the IE-1 locus encoding the major 72-kDa IE protein. Two of seven patient HCMV strains were found to have seven substitutions in the 106-bp fragment of the IE-1 gene, giving rise to five amino acid changes. In four of the five amino acid changes, the new amino acid was of an equal hydrophobic or hydrophilic character. One amino acid change, that at position 214, introduced a more hydrophilic amino acid compared with that in the reference strain (Table 2). The sequences of the strains from each patient were conserved, both in samples that were collected at different times from the same patient and in samples that were obtained from tissue cultures. This result is in accordance with those of several previous restriction enzyme cleavage studies, in which evidence for this type of conservation has been found (7, 8, 18). One patient may have excreted two HCMV strains differing by one nucleotide in this IE region.

Restriction enzyme cleavage patterns of various laboratory and wild-type HCMV strains revealed distinct differences that usually allowed monitoring and identification of individual HCMV strains (4, 17, 18). One study revealed an average of 80% homology between wild-type strains (19). Previous studies showed that the IE gene of strain Ad 169 was very similar to that of strain Towne. There were only three nucleotide differences in the coding sequence of 1,473 bp (1, 34). One of the mismatched nucleotides occurred in our primer D annealing region. We had no problems in amplifying target DNA containing one mismatch within the primer region. The other two mismatches did not occur in our sequence. Two of the three mismatched nucleotides gave rise to amino acid differences in Towne compared with Ad 169. A recent study showed that it was possible to distinguish different patient strains by use of a restriction enzyme-digested 2.6-kb PCR-amplified fragment from the IE-1 locus (8).

We found that the patient strain with the majority of nucleotide substitutions had a DNA homology, compared with strain Ad 169, of 94.4% and showed sequence conservation when compared with the tissue-cultured strain. Conservation is important in the use of the PCR and the DIANA assay to detect HCMV DNA and avoid false-negative results owing to strain sequence variations.

TABLE 2. Translated amino acid sequences of the samples from Table 1

Sample	Amino acid sequence[a]
Ad 169	GAANKLGGALQAKARAKKDELRRKMMYMCYRNIEF
Towne	----------
Patient 1	----------
Patient 2	----------
Patient 3	----------
Patient 4	----------
Patient 5	----------K----------*
Patient 6	----------
Patient 7	----------K----------N---I---------

[a] *, F or S. Patient 5 had two HCMV strains, with amino acid 214 being either phenylalanine or serine. Dashes indicate identity.

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