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Enzymatic Amplification of Human Cytomegalovirus Sequences by Polymerase Chain Reaction

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Polymerase chain reaction (PCR) amplification was used to detect human cytomegalovirus (HCMV) sequences. The fragments selected for amplification were fragments of 130 and 152 base pairs (bp) located at two opposite ends of HCMV strain AD169 *Eco*RI fragment D. Amplification of the 152-bp DNA was consistently greater than that of the 130-bp DNA. At the optimal Mg^{2+} concentration of 5 mM, specific PCR amplification of 152-bp DNA with Taq polymerase was sensitive; only one AD169-infected fibroblast cell or 0.01 pg of AD169 fragment D DNA was needed for detection. This specific amplification was also found with various clinical HCMV isolates and peripheral blood cells and urine from patients. In 37 urine samples analyzed simultaneously by PCR and by virus cultivation, identical results were found in 35 samples, while 2 scored positive only by PCR. This suggests that specific amplification of 152-bp DNA is sensitive and can be used for rapid detection of HCMV infections.

Human cytomegalovirus (HCMV) infections play an important role in human disease. The virus is a major pathogen in immunosuppressed individuals and a common infectious cause of congenital abnormalities. Conventional methods of HCMV serodiagnosis are based on poorly defined viral antigens and only give indirect evidence of HCMV infection. For the diagnosis of an active HCMV infection, virus isolation is usually required; however, HCMV grows slowly in culture and tissue culture is work intensive and expensive.

To facilitate the understanding of HCMV infection, we sought to improve on current methods of detection. We chose to adapt the new in vitro gene amplification technology, the polymerase chain reaction (PCR), for this purpose (7, 12). In the PCR, target DNA sequences are selectively amplified through repeated cycles of denaturation, annealing with oligomer primers complementary to flanking regions of the target sequence, and primer extension with DNA polymerase. The amount of target DNA increases exponentially as a function of the number of cycles (2^n), facilitating subsequent detection with a labeled complementary oligomer probe.

In this study, the fragments selected for amplification were fragments of 130 and 152 base pairs (bp) lying within HCMV strain AD169 *Eco*RI fragment D DNA, which is transcribed early and in abundance late in infection. Using the primer pairs flanking these fragments, we showed that these regions can be amplified exponentially on target DNAs containing these sequences, such as subgenomic AD169 DNA fragments and human fibroblast DNAs infected with HCMV genomes, including two laboratory-adapted HCMV strains (AD169 and Towne) and a number of clinical HCMV isolates. In addition, we showed that enzymatic amplification of DNA by PCR can be performed directly with crude cell lysates from a small number of cells, without going through a tedious DNA extraction procedure. Furthermore, we carried out this PCR amplification procedure with clinical urine samples, which results in faster detection of HCMV genomes with higher sensitivity than conventional virological culture methods, suggesting its potential application for clinical detection of HCMV.

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MATERIALS AND METHODS

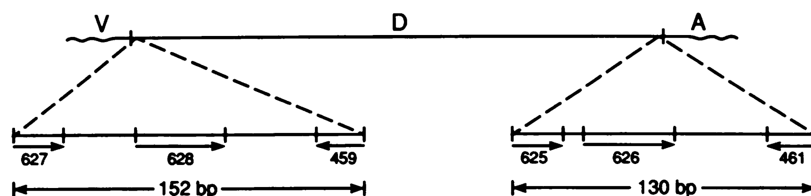
Virus and cells. HCMV strain AD169 was obtained from the American Type Culture Collection (Rockville, Md.) and passaged in human foreskin fibroblast (FF) cells. Clinical specimens were cultured for HCMV as previously described (17).

Oligomer primers and probes. Four synthetic primers (461, 625, 459, and 627) and two synthetic probes (626 and 628) were used in this study. Their sequences and locations in AD169 *Eco*RI fragment D DNA are diagramed in Fig. 1. Each primer is a 20-mer, and each probe is a 40-mer. These oligomers were synthesized on a DNA synthesizer (model 380A; Applied Biosystems, Inc.) and purified by high-pressure liquid chromatography.

Preparation of target DNAs for PCR. The sources of target DNAs used for PCR in these experiments were from four categories.

(i) AD169 *Eco*RI fragment D DNA. Purified AD169 DNA was digested with *Eco*RI, and the 17-kilobase fragment D DNA was subsequently purified on an agarose gel as previously described (16).

(ii) Purified cellular DNA. Cells infected (or mock infected) with HCMV were initially lysed with a lysis buffer (10 mM Tris hydrochloride [pH 7.4], 10 mM disodium EDTA, 0.6% sodium dodecyl sulfate) (3) containing 50 μg of proteinase K (Sigma Chemical Co., St. Louis, Mo.) per ml. Cellular DNAs were successively extracted from cell lysates with equal volumes of phenol, phenol-$CHCl_3$-isoamyl alcohol (25:24:1), and finally with $CHCl_3$-isoamyl alcohol (24:1). The aqueous phase was ethanol precipitated, and DNAs were suspended in $TE_{7.5}$ buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM disodium EDTA). After digestion with 0.1 mg of RNase A (pancreatic; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml, cellular DNAs were extracted again with phenol and the $CHCl_3$-isoamyl alcohol mixture as above. The final aqueous phase was then ethanol precipitated and suspended in sterile water. Usually, 1 μg of



Primers:

Probes:

FIG. 1. Sequences of the synthetic oligonucleotide primers and probes and their locations in HCMV AD169 *Eco*RI fragment D DNA.

purified cellular DNA was used for each 100 μl of gene amplification reaction mixture.

(iii) Crude cell lysates. Tissue culture cells were washed with phosphate-buffered saline, and cell numbers were counted. Cell fractions containing 10, 100, and 1,000 cells in microcentrifuge tubes were brought up to 50 to 70 μl with H_2O and were freeze-thawed three times, followed by heating at 95°C for 5 to 10 min. Complete cell disruption was verified by light microscopy. For the gene amplification reaction, substrates for PCR were added directly into these crude cell lysates.

(iv) Urine. Urine samples (10 ml) were first clarified by centrifugation at 900 × *g* for 20 min, and virus was pelleted by ultracentrifugation at 35,000 rpm (90,000 × *g*, Beckman ultracentrifuge, 60 Ti rotor) for 1 h. Pellets were washed once with cold STE buffer (50 mM NaCl, 10 mM Tris hydrochloride [pH 7.5], 1 mM disodium EDTA) and were suspended in 50 μl of sterile H_2O. Virus particles in the suspension were then disrupted by freeze-thawing three times, followed by heating at 95°C for 5 to 10 min prior to PCR.

PCR. The PCR was done by the method of Saiki et al. (12), with modifications depending on the experiment. In PCR experiments involving the use of the Klenow fragment of *Escherichia coli* polymerase I, each 100-μl reaction mixture contained, in addition to the target DNA and primers, 1.5 mM each deoxynucleotide (dATP, dCTP, dGTP, and dTTP) (Pharmacia, Inc., Piscataway, N.J.), 10 mM Tris hydrochloride (pH 7.5), 50 mM sodium acetate, and 10 mM $MgCl_2$. DNAs were initially denatured at 95°C for 5 min. After annealing with primers for 2 min at 30°C, 1 U (1 μl) of Klenow polymerase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was added and the primers were allowed to extend for 2 min at 30°C. Subsequent cycles of PCR were done in the following order: denaturation at 95°C for 2 min, primer annealing at 30°C for 2 min, addition of 1 U (1 μl) of Klenow polymerase, and final extension at 30°C for 2 min.

The PCR with Taq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) was performed according to the instructions of the manufacturer. In addition to the target DNA, each 100-μl reaction mixture in these experiments consisted of 100 to 150 pmol of each primer, 1 mM each deoxynucleotide (dATP, dCTP, dGTP, and dTTP), 5 mM $MgCl_2$, 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, and 0.01% gelatin. Normally, PCR was run for 20 to 25 cycles. The first cycle involved an initial DNA denaturation at 94°C for 1.5 to 2 min, followed by primer annealing at 37°C for 2 min. After the addition of Taq polymerase (2 U), nascent DNA chains were allowed to extend for 3 min at 72°C. The subsequent cycles were: DNA denaturation at 94°C for 2 min, primer annealing at 37°C for 2 min, and primer extension at 72°C for 3 min. For the last cycle of PCR, DNAs were allowed to extend for 6 min at 72°C.

At the end of PCR, 20 μl of each amplification mixture was added to 4 μl of 6× loading buffer containing 60% (vol/vol) glycerol and 0.15% each of bromophenol blue and xylene cyanole in 0.3× TAE buffer (12 mM Tris base, 0.3 mM disodium EDTA [pH 8.0], 0.03% [vol/vol] glacial acetic acid). This was kept at 4°C for gel electrophoresis. The remaining amplification mixture (approximately 80 μl) was stored at −20°C for slot blot analysis.

Labeling of oligomer probes. The 626 and 628 oligomers were each end labeled with T4 polynucleotide kinase in a 50-μl reaction mixture containing 1× kinase buffer (50 mM Tris hydrochloride [pH 7.6], 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM disodium EDTA [pH 8.0]), 100 pmol of the oligomer, and 100 μCi of [γ-^{32}P]ATP (10 mCi/ml, 3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). The labeling reaction was started with the addition of 10 U of T4 polynucleotide kinase (Bethesda Research Laboratories, Inc.). After incubation at 37°C for 30 min, the reaction was stopped with the addition of 2 μl of 0.5 M disodium EDTA (pH 8.0). The labeled probes were purified on DE52 (Whatman, Inc., Clifton, N.J.) columns.

After the columns were washed sequentially with TE_8 buffer (10 mM Tris hydrochloride [pH 8.0], 1 mM disodium EDTA [pH 8.0]) and with TE_8 plus 0.2 M NaCl, the labeled probes were eluted with TE_8 plus 0.5 M NaCl and were then ethanol precipitated and suspended in H_2O. The average efficiency of incorporation of [γ-^{32}P]ATP into oligomer probes is approximately 10^8 cpm/100 pmol of the probe.

Gel electrophoresis and Southern and slot blot analyses. A portion of the gene amplification reaction mixtures was analyzed by electrophoresis through 4% Nusieve GTG agarose (FMC Corp., Philadelphia, Pa.) gel (containing 1 μg of ethidium bromide per ml) in 1× TAE buffer (40 mM Tris base, 1 mM disodium EDTA [pH 8.0], 0.1% [vol/vol] glacial acetic acid). For Southern blot analysis (15), gels were briefly rinsed with H_2O and were denatured at room temperature for 30 min (i.e., twice for 15 min each time) in a denaturing solution containing 1 M NaCl and 0.5 N NaOH. Denatured gels were again rinsed with H_2O and were neutralized at room temperature for 30 min (twice for 15 min each time) with a neutralizing buffer containing 0.5 M Tris hydrochloride (pH 7.4) and 1.5 M NaCl. Neutralized gels were blotted onto Nytran 0.2-μm membrane (Schleicher & Schuell, Inc., Keene, N.H.) with 5× SSPE (50 mM phosphate buffer [pH 7.4], 0.75 M NaCl, 5 mM disodium EDTA). After being rinsed briefly with 5× SSPE, blots were air dried and baked at 75 to 80°C (in a vacuum oven) for 30 to 60 min. This was followed by a prehybridization of the blots at 40°C for 4 h in a buffer containing 3× SSPE, 30% formamide (Bethesda Research Laboratories, Inc.), 0.5% sodium dodecyl sulfate, and 5× DET (5× Denhardt solution, 1 mM Tris hydrochloride [pH 8.0], 1 mM disodium EDTA [pH 8.0]). Hybridization was done overnight at the same temperature after the addition of the ^{32}P-kinase-labeled probe (usually 0.5×10^8 to 1.0×10^8 cpm of the probe were used for each small blot of 4.5 by 6 cm). After hybridization, the blots were washed at room temperature with 2× SSPE (plus 0.1% sodium dodecyl sulfate) three times for 30 min each time. Damp blots were wrapped with Saran Wrap and were exposed to XAR films (Eastman Kodak Co., Rochester, N.Y.) at −70°C in the presence of an intensifying screen (Cronex Lightning-Plus).

For slot blot analysis, a portion of the gene amplification reaction mixtures was heated at 95°C for 5 min to denature DNA, followed by rapid cooling on ice. Denatured DNAs were applied immediately onto 0.2-μm Nytran membrane (prewetted with 5× SSPE) in a preassembled slot blot apparatus (Schleicher & Schuell). Blots were dried and hybridized under the same conditions as described for Southern blot analysis.

RESULTS

Specific amplification of HCMV DNA fragments by PCR. Two regions lying within AD169 *Eco*RI fragment D DNA were selected for amplification with PCR (Fig. 1). Region 1 is 101 bp from the *Eco*RI fragment D-A junction. Using the primer pair (461 and 625) flanking this region and the AD169 *Eco*RI fragment D DNA (17 kbp) as the target, a DNA fragment of 130 bp was detected on ethidium bromide-stained agarose gel after 15 cycles of PCR with the addition of Klenow fragment of *E. coli* DNA polymerase I at each cycle (Fig. 2A). Amplification of this 130-bp DNA by PCR is sequence specific, since in the Southern blot, the 130-bp band was hybridized to the labeled 40-mer probe (626) having a sequence located in the middle of this fragment (Fig. 1 and 2B). In fact, with the use of the ^{32}P-kinase-labeled

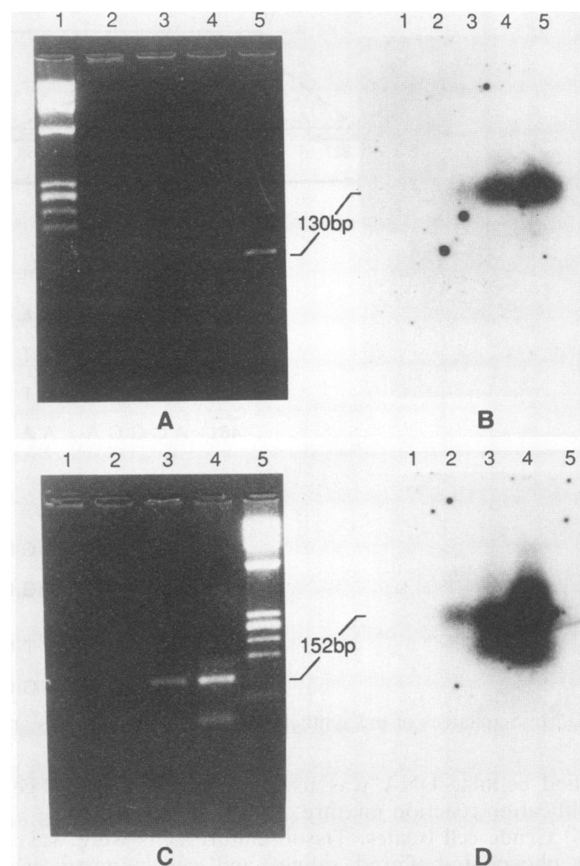


FIG. 2. Amplification by PCR of the 130- and 152-bp HCMV DNA fragments from AD169 *Eco*RI fragment D DNA. PCR was done in 100-μl reaction mixtures containing 0.5 nM AD169 *Eco*RI fragment D DNA as the target and two separate primer pairs (3 μM each), 461 and 625 (A and B) and 459 and 627 (C and D), for 20 cycles with the addition of 1 μl of Klenow polymerase (1 U/μl) at each cycle. At the end of 5, 10, 15, and 20 cycles, 10 μl of each PCR mixture was withdrawn and subjected to agarose (4% Nusieve GTG) gel electrophoresis. (A and C) Ethidium bromide-stained gels. (B and D) Autoradiographs of gels A and C, respectively, after blotting to Nytran membrane and hybridization with ^{32}P-labeled 626 (B) and 628 (D) probes. Lanes 2 through 5 in panels A and B and lanes 1 through 4 in panels C and D correspond to the reaction mixtures collected after 5, 10, 15, and 20 cycles of PCR, respectively. Lanes A1 and C5 each contain 2 μg of *Hae*III-digested ϕX174RF DNA marker fragments.

626 probe, amplification of the 130-bp DNA could be detected after 10 cycles of PCR.

Similarly, with the primer pair (459 and 627) situated 160 bp from the *Eco*RI fragment D-V junction, a DNA fragment of 152 bp was found to be amplified after 10 cycles of PCR with Klenow polymerase, as shown by ethidium bromide staining (Fig. 2C). Again, much greater sensitivity was revealed by hybridizing the Southern blot with the labeled 628 DNA probe, which corresponds to a sequence located in the middle of this 152-bp DNA fragment (Fig. 1 and 2D). In this experiment, amplification of 152-bp DNA by PCR, however, appeared to be spurious, because there was concomitant synthesis of two other DNA fragments having sequences related to probe 628 (Fig. 2C and D). Spurious amplification of DNA fragments by PCR has been described by others and can at times be eliminated or minimized by modification of the PCR system, such as the use of a

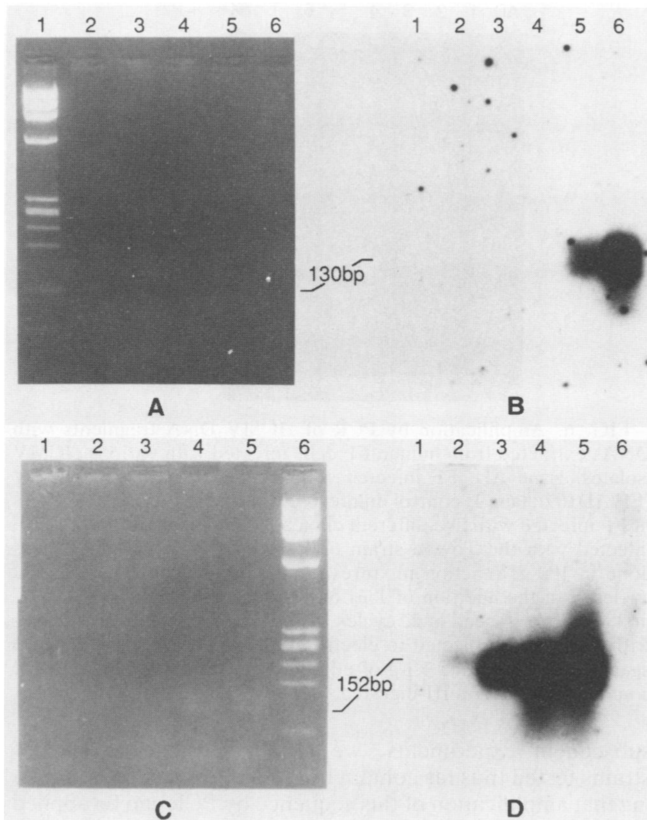


FIG. 3. Amplification by PCR of the 130- and 152-bp HCMV DNA fragments from FF/AD169 DNA. PCR was done in 100-μl reaction mixtures containing 1 μg of FF/AD169 DNA and 125 pmol each of the primer oligomers: 461 and 625 (A and B) and 459 and 627 (C and D). The reactions were for 25 cycles, with the addition of 1 μl of Klenow polymerase (1 U/μl) at each cycle. At the end of 5, 10, 15, 20, and 25 cycles, 10 μl of each PCR mixture was withdrawn and analyzed as described in the legend to Fig. 2. (A and C) Ethidium bromide-stained gel. (B and D) Southern blot analyses of panels A and C with the ^{32}P-labeled 626 and 628 probes, respectively. Lanes 2 through 6 in panels A and B and lanes 1 through 5 in panels C and D correspond to the reaction mixtures collected after 5, 10, 15, 20, and 25 cycles of PCR, respectively. Lanes A1 and C6 each contain 2 μg of *Hae*III-digested φX174RF DNA marker fragments.

thermostable DNA polymerase instead of heat-sensitive Klenow fragment DNA polymerase (2, 8). This issue will be addressed below.

Amplification of HCMV sequences in infected human fibroblast DNA. Our next set of experiments were designed to amplify the same HCMV sequences present in infected human cells. Initially, AD169-infected human FF (FF/AD169) DNA (1 μg) was used as the target and was amplified with 25 cycles of PCR with the addition of Klenow polymerase at each cycle (7). Figure 3 shows the agarose gel and Southern blot analyses of the reaction products with 10% of PCR mix collected after 5, 10, 15, 20, and 25 cycles with the two primer pairs. In these experiments, it appeared that amplification of the 130-bp HCMV DNA was consistently less efficient than that of the 152-bp HCMV DNA under the same PCR conditions. For example, we were unable to detect the 130-bp DNA on ethidium bromide-stained agarose gel after 25 cycles of PCR (Fig. 3A), whereas synthesis of the 152-bp DNA was readily visible after 20 cycles (Fig. 3C). Synthesis of the 130-bp DNA was detected, however, after Southern blot analysis with the ^{32}P-labeled 626 probe after 20 cycles of PCR (Fig. 3B). Similarly, with greater sensitivity, the ^{32}P-labeled 628 probe could detect the 152-bp HCMV DNA after only 10 cycles of PCR with Klenow fragment (Fig. 3D). The efficiency of amplification of specific HCMV DNA sequences by PCR can be estimated by comparing the intensity of ethidium bromide-stained amplified HCMV DNA with that of the marker DNA fragments (e.g., *Hae*III-digested φX174RF DNA). In this experiment, assuming that there is only one copy of HCMV DNA per haploid cell genome, approximately 100 ng of the 152-bp DNA was synthesized after 25 cycles of PCR from 1 μg of FF/AD169 DNA, giving rise to approximately 2×10^6-fold amplification of this fragment ($100 \text{ ng}/[(1{,}000 \text{ ng} \times 152 \text{ bp})/3 \times 10^9 \text{ bp}] = 2 \times 10^6$). However since approximately 100 copies of the AD169 strain of HCMV may be present per infected cell, a more realistic estimate is that the 152-bp DNA was amplified in this experiment approximately 2×10^4-fold after 25 cycles of PCR.

During these studies, a thermostable DNA polymerase extracted from *Thermus aquaticus* (i.e., Taq polymerase) became available which has two major advantages over the Klenow fragment of *E. coli* DNA polymerase I in the PCR amplification procedure: (i) convenience, since the Taq polymerase only needs to be applied once for the entire 25 cycles of PCR in contrast to the repeated addition of Klenow enzyme at each cycle; and (ii) specificity, since polymerization of DNA at high temperature (e.g., 72°C) with Taq polymerase minimizes nonspecific DNA synthesis observed at lower temperature (e.g., 30°C) with Klenow polymerase (2, 11).

Using the Taq polymerase with our primer pairs, we found a very stringent requirement for optimal Mg^{2+} concentration in PCR. After 22 cycles of PCR at four different Mg^{2+} concentrations (0.5, 1.5, 10, and 50 mM) with FF/AD169 DNA as the target and the 459 and 627 20-mers as the primer pair, the 152-bp DNA was detected on ethidium bromide-stained agarose gel only when 10 mM Mg^{2+} was used in the PCR with Taq polymerase (Fig. 4A). Although DNA fragments larger than 152 bp (primarily a band of about 700 bp) were also found when PCR was done with 10 mM Mg^{2+}, virtually no DNA was detected on the agarose gel after staining with ethidium bromide after 22 cycles of PCR performed at other Mg^{2+} concentrations (0.5, 1.5, and 50 mM). Subsequently, we found that synthesis of this larger DNA fragment (~700 bp) could be eliminated or minimized with PCR done at 5 mM Mg^{2+}, a condition at which the synthesis of the 152-bp DNA was found to be the most specific and optimal among different Mg^{2+} concentrations tested (Fig. 4B). At this Mg^{2+} concentration, the 152-bp DNA was amplified 10^5- to 10^6-fold from 1 μg of FF/AD169 DNA after 20 cycles of PCR, assuming 100 copies of AD169 viral DNA were present per infected cell. A similar degree of amplification was also reached with purified AD169 *Eco*RI fragment D DNA (17 kbp). After PCR with Taq polymerase at 5 mM Mg^{2+}, the specific synthesis of the 152-bp DNA fragment D DNA could be detected with 0.01 pg of AD169 fragment D DNA, a signal which was otherwise detected with 1 ng of the same DNA prior to PCR (Fig. 5B). The efficiency of amplification of HCMV DNA by PCR thus appeared to be enhanced at least 10-fold when Taq polymerase was used to replace the Klenow fragment of *E. coli* DNA polymerase I.

Because clinical isolates of HCMV are known to be heterogeneic, our next series of experiments were designed to examine whether the 152-bp HCMV DNA sequence could

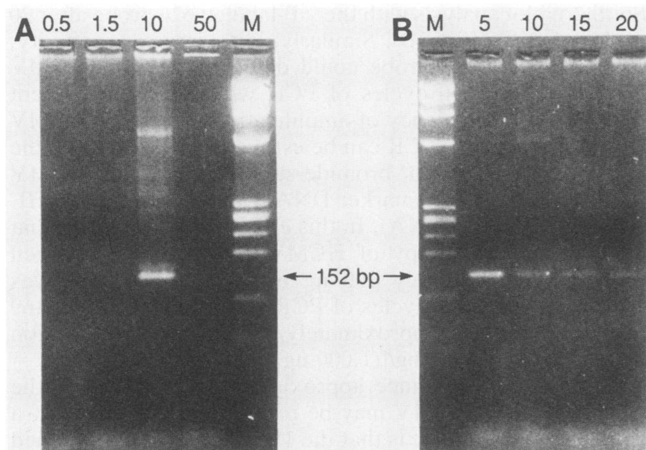


FIG. 4. Determination of the optimal Mg^{2+} concentration required for amplification of 152-bp HCMV DNA fragments by PCR with Taq polymerase. PCR was done in 100-μl reaction mixtures containing 2.5 μg (A) or 1.0 μg (B) of FF/AD169 DNA as the target and 100 pmol each of 459 and 627 oligomers as the primers. After 22 (A) and 20 (B) cycles of PCR with the addition of 1 μl of Taq polymerase (2 U/μl) at the first cycle, 10 μl of the reaction mixtures was electrophoresed through a 4% Nusieve GTG agarose gel containing 1 μg of ethidium bromide per ml. (A) Amplification of the 152-bp HCMV DNA in the presence of 0.5, 1.5, 10, and 50 mM Mg^{2+}; (B) amplification of the 152-bp HCMV DNA in the presence of 5, 10, 15, and 20 mM Mg^{2+}. Lane M contains 2 μg of *Hae*III-digested φX174RF DNA.

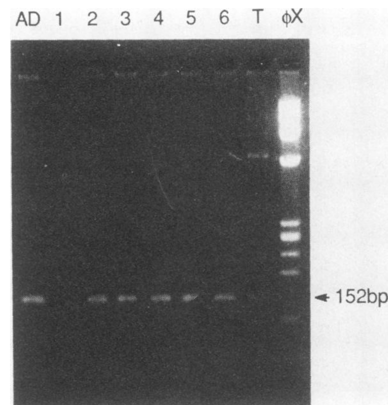


FIG. 6. Amplification by PCR of HCMV DNA fragments with DNAs extracted from human FF cells infected with various HCMV isolates. Lane AD, FF infected with the AD169 strain of HCMV (FF/AD169); lane 1, control uninfected FF; lanes 2 through 6, DNAs of FF infected with five different clinical HCMV isolates; lane T, FF infected with the Towne strain of HCMV (FF/Towne). PCR was done in 100-μl reaction mixtures containing 1 μg of DNA for 22 cycles with the addition of 1 μl of Taq polymerase (2 U/μl) at the first cycle. At the end of 22 cycles, 5 μl of each reaction mixture was withdrawn and subjected to electrophoresis in a 4% Nusieve GTG agarose gel containing 1 μg of ethidium bromide per ml. Lane φX contains 1 μg of *Hae*III-digested φX174RF DNA.

be amplified from different HCMV clinical isolates to the same degree as from the AD169 strain. DNAs were extracted from cultured human FF cells infected with five clinical HCMV isolates, as well as from those infected with two laboratory-adapted HCMV strains, namely, AD169 and Towne. PCR was done with 1 μg of each DNA with Taq polymerase at a Mg^{2+} concentration of 5 mM. After 22 cycles of PCR, DNAs from all five HCMV clinical isolates exhibited the same degree of amplification of the 152-bp sequence as that from AD169 DNA (Fig. 6). No amplification was seen when uninfected FF DNA was used (Fig. 6). These results indicated that this 152-bp sequence is specific and is conserved among various HCMV isolates. In fact, in subsequent experiments, we found that all the HCMV strains tested thus far contain this 152-bp sequence, suggesting that amplification of this sequence by PCR can be applied for clinical detection of HCMV genomes.

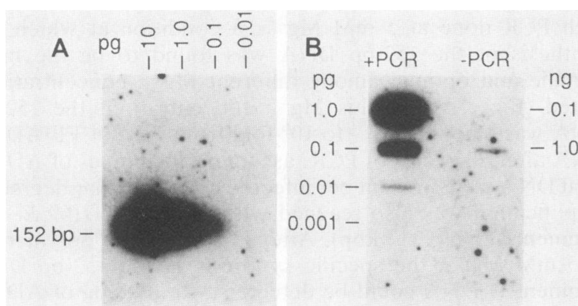


FIG. 5. Slot blot and Southern blot analyses of PCR-amplified AD169 *Eco*RI fragment D DNA. PCR was done with five different amounts of purified AD169 *Eco*RI fragment D DNA (100, 10, 1, 0.1, and 0.01 pg). After 25 cycles of PCR with 2 U of Taq polymerase (and 5 mM Mg^{2+}), 10% of the reaction mixtures with 100, 10, 1, and 0.1 pg of fragment D DNA was subjected to Southern blot analysis (A) and 10% of the reaction mixtures with 10, 1, 0.1, and 0.01 pg of fragment D DNA was used for slot blot analysis (B), with the ^{32}P-labeled 628 probe.

PCR with crude cell lysates including peripheral blood leukocytes. To avoid the tedious procedure of extracting cellular DNA, we applied PCR directly to disrupted tissue culture cells. In this experiment, 10, 100, and 1,000 cultured cells corresponding to uninfected FF, FF/AD169, and FF infected with two clinical isolates were freeze-thawed three times, followed by heating at 95°C for 5 to 10 min to ensure complete disruption of cells. After 20 cycles of PCR of these crude cell lysates with the primer pair used for amplification of 152-bp HCMV DNA, 20% of each 100-μl PCR mix was taken for slot blot analysis and 10% of the mix (i.e., 10 μl) was used for Southern blot analysis as described in Materials and Methods. Amplification of HCMV DNA by PCR with unfractionated crude cell lysates appeared to be quite sensitive (Fig. 7A). While ^{32}P-kinase-labeled 628 probe could detect amplified HCMV sequence from 20 FF cells infected with clinical HCMV isolates, only 2 FF/AD169 cells were needed for detection. In fact, in subsequent experiments, we repeatedly detected the HCMV sequence amplified from only 1 FF/AD169 cell and from less than 10 cells infected with clinical isolates using the [^{32}P]ATP kinase-labeled 628 probe (data not shown). The higher sensitivity for detection of amplified HCMV sequence in FF/AD169 cells than that in FF infected with clinical isolates in these experiments may be attributed to the higher copy number of HCMV genome present in the FF/AD169 cells.

The specificity of amplified HCMV DNA by PCR with crude cell lysates was also examined by Southern blot analysis with ^{32}P-labeled 628 probe. The single DNA band (152 bp) observed after PCR with all three HCMV-infected cell cultures (FF/AD169 and two FF cultures infected with clinical isolates) but not with the uninfected FF cells (Fig. 7B) indicated that amplification of 152-bp DNA by PCR with crude cell lysates is very specific.

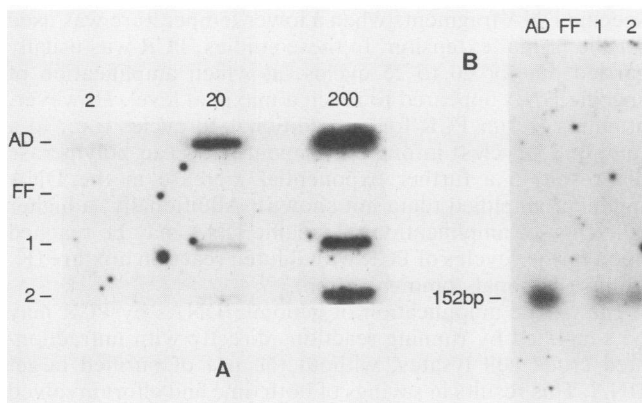


FIG. 7. Slot blot and Southern blot analyses of PCR-amplified HCMV DNA fragment from unfractionated crude cell lysates. PCR was done directly with crude cell lysates prepared from 10, 100, and 1,000 cells each of FF/AD169 (AD), of uninfected FF (FF), and of FF infected with two different clinical isolates (1 and 2). After 20 cycles of PCR with 2 U of Taq polymerase, 20% of each of the 100-μl reaction mixtures (corresponding to 2, 20, and 200 cells, respectively) was used for slot blot analysis (A) and 10% of each reaction mixture from 1,000 disrupted cells (corresponding to 100 cells each) was subjected to Southern blot analysis (B), with the ^{32}P-labeled 628 probe.

In addition to HCMV-infected tissue culture cells, PCR amplification of 152-bp DNA was also found with crude cell lysates prepared directly from peripheral leukocytes from patients. After separation of blood from a patient into mononuclear and polymorphonuclear leukocyte fractions, PCR amplification of HCMV DNA was clearly detectable with 200 cells of both fractions (Fig. 8). In fact, equivalent amplification of HCMV DNA was found with mononuclear cells after their further separation into lymphocyte and monocytes-macrophage fractions (data not shown).

The rapidity plus the qualitative and quantitative nature of this PCR experiment with unfractionated crude cell lysates thus suggests that prompt detection of clinical HCMV isolates can be obtained through this in vitro DNA amplification procedure.

PCR of urine samples. Since HCMV can be isolated from urine at the time of active HCMV infection, one way to determine whether the virus is present in the urine is to perform PCR of urine samples. Urine samples were collected from 10 patients with acquired immunodeficiency syndrome and from 2 healthy individuals. A portion of the

FIG. 8. PCR of peripheral leukocytes. Heparinized blood from a patient with acquired immunodeficiency syndrome was fractionated by Ficoll-Hypaque gradient centrifugation into mononuclear and polymorphonuclear leukocyte fractions. Crude cell lysates prepared from 10, 100, and 1,000 cells of each of these fractions were subjected to 20 cycles of PCR, followed by slot blot analysis with ^{32}P-labeled 628 probe. A, Mononuclear leukocytes; B, polymorphonuclear leukocytes. Lanes 1, 2, and 3 of rows A and B represent crude cell lysates of 2, 20, and 200 cells, respectively. Lanes 1 and 2 of row C represent a no PCR control of A3 and B3, respectively.

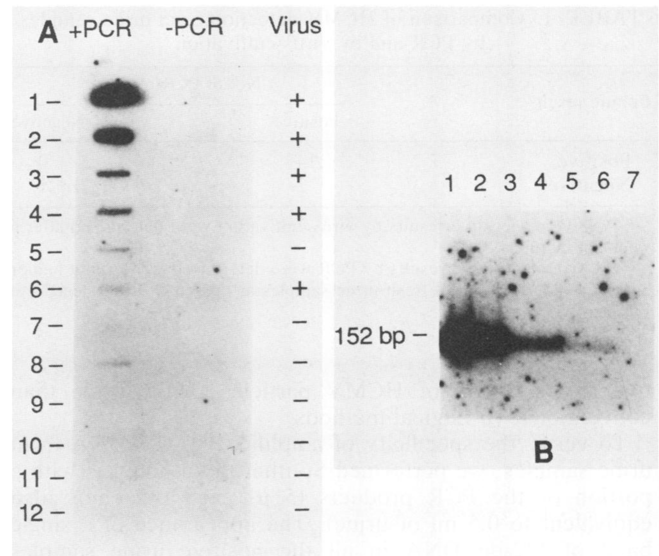


FIG. 9. PCR of urine samples. Urine samples were clarified and supernatant virus fractions were precipitated as described in Materials and Methods. Virus suspensions were freeze-thawed and boiled and subjected to 25 cycles of PCR with the use of the primer pair (150 pmol each) flanking the 152-bp HCMV DNA. (A) Slot blot analysis with the ^{32}P-labeled 628 probe with 10% of the reaction mixture (i.e., equivalent to 1 ml of urine) and the results of standard virus cultivation of the same specimen. −PCR represents the control PCR mixtures that did not go through PCR cycles. (B) Southern blot analysis of 5% of selected PCR reaction mixtures (i.e., equivalent to 0.5 ml of urine) with the ^{32}P-labeled 628 probe after electrophoresis through a 4% Nusieve GTG agarose gel. 1 through 10, Urine samples collected from 10 patients with acquired immunodeficiency syndrome; 11 and 12, urine samples from two healthy individuals.

same urine samples was taken for routine virus culture. Portions of 10 ml of the remaining urine samples were clarified, and virus particles were precipitated and disrupted as described in Materials and Methods. Virus suspensions were subjected to 25 cycles of PCR with the primer pair flanking the 152-bp HCMV DNA. A 10% portion of each PCR product (i.e., 10 μl, which is also equivalent to 1 ml of urine) was analyzed by slot blot hybridization with the ^{32}P-labeled 628 probe. Amplified HCMV sequence could be detected from 1 ml of urine from 7 (of 10) patients after 25 cycles of PCR (Fig. 9A); little or no HCMV DNA could be detected in any of these samples prior to PCR reaction. Correlation of these data with the results obtained from routine virological culture showed that two of these seven positive samples after PCR were virus negative by the routine virological assay (Fig. 9A). In fact, in a total of 37 clinical urine samples analyzed simultaneously by PCR and by virus cultivation, identical results were found in 35 samples (21 were negative and 14 were positive); while 2 samples scored positive only by the PCR procedure (Table 1). In each of the two cases in which a urine sample was positive by PCR but negative by culture, urine specimens obtained from these patients were subsequently found to be positive for HCMV as assayed by tissue culture. (Titers of culture-positive urine specimens ranged from 10 to 10^4 50% tissue culture infective doses per ml.) To date, we have not obtained negative PCR results on urine samples which were positive by virological culture methods. This suggests that the amplification procedure for HCMV DNA is more sensi-

TABLE 1. Comparison of HCMV detection from urine samples by PCR and by virus cultivation

Culture result[a]	No. of PCR results[b]	
	Positive	Negative
Positive	14	0
Negative	2	21

[a] Positive or negative results by virus cultivation were determined after 6 weeks of culture.

[b] Positive or negative results by PCR were determined by slot blot and/or Southern blot analyses of fresh urine samples as described in the legend to Fig. 9.

tive for detection of HCMV particles in the urine than conventional virological methods.

To verify the specificity of amplified HCMV DNA from urine samples, we performed Southern blot analysis with a portion of the PCR products (5 μl each, which is also equivalent to 0.5 ml of urine). The appearance of a single band of 152-bp DNA in all the positive urine samples analyzed suggests that amplification of the 152-bp HCMV DNA by PCR is specific (Fig. 9B). In addition, the relative intensities of these 152-bp DNA bands seen in the Southern blot analysis (Fig. 9B) appeared to correlate well with those observed by slot blot analysis (Fig. 9A). Thus, amplification of the 152-bp DNA by PCR may provide a sensitive qualitative and quantitative diagnostic tool for detection of HCMV particles in the urine.

DISCUSSION

We described methods of in vitro amplification by PCR of small fragments of HCMV DNA using samples of subgenomic viral DNA from tissue culture cells infected with the virus and from urine. In these studies, we performed PCR with two different primer pairs located at opposite ends of *Eco*RI fragment D of the AD169 genome. Enzymatic amplification by PCR with primer pairs located at the 5′ end of fragment D gave rise to accumulation of an authentic HCMV 152-bp sequence, whereas amplification of a 130-bp DNA was found with primer pairs located at the 3′ end of this D fragment. Amplification of the 152-bp DNA by PCR was consistently greater than that of the 130-bp DNA. Additionally, amplification of the 152-bp HCMV DNA by PCR was specific and sensitive among different HCMV strains, suggesting that it can be used as a diagnostic tool for clinical detection of HCMV genomes.

In approximately 3 years since the first published report, in vitro enzymatic amplification of DNA fragments by PCR has already been applied to DNA cloning and sequencing (6, 13, 18, 19), to the diagnosis of genetic disorders (1, 4, 12), and to the detection of infectious disease pathogens, including human immunodeficiency virus (5, 9) and human papillomavirus (14). In this report, we presented evidence to show that subgenomic HCMV can be selectively amplified between 10^5- and 10^6-fold after 25 cycles of PCR with Taq polymerase (Fig. 4B, 5, and 6). Although a theoretical value of 10^6-fold amplification after 20 cycles of PCR ($2^{20} = \sim 10^6$) has not been reached, similar efficiency of in vitro DNA amplification has nevertheless been obtained in other PCR systems (12). The efficiency of amplification of the same HCMV DNA was, however, shown to be approximately 10-fold lower with the use of the Klenow fragment of *E. coli* DNA polymerase I (Fig. 3C), presumably owing to competition for factors needed for DNA synthesis by other non-specific DNA fragments when a lower temperature was used for the primer extension. In these studies, PCR was usually carried out for 20 to 25 cycles, at which amplification of specific DNA appeared to reach a maximal level. However, attempts to run PCR for an additional 10 cycles (i.e., to a total of 35 cycles) in one experiment with Taq polymerase did result in a further exponential increase in the DNA fragment amplified (data not shown). Additionally, a higher efficiency of amplification of specific DNA may be reached upon further cycles of PCR with diluted reaction mixture (K. Mullis, personal communication).

Enzymatic amplification of genomic DNAs by PCR may be simplified by running reactions directly with unfractionated crude cell lysates, without the use of purified target DNA. This results in savings of both time and effort involved in the process. Saiki et al. (10) previously detected specific DNA amplification from crude cell lysates prepared from 75 MOLT-4 and SC-1 cells. Results obtained in our laboratory showed that enzymatic amplification of a specific HCMV DNA fragment could be repeatedly observed with cell lysates prepared from approximately one FF/AD169 cell (Fig. 7; unpublished data). Since there may be 100 copies of HCMV genome per infected FF cell, our results suggest that at least equivalent efficiency of amplification of HCMV DNA can be reached by PCR with crude cell lysates of AD169-infected cells. Using unfractionated crude cell lysates, PCR amplification of HCMV DNA was also demonstrated with peripheral leukocytes from patients with acquired immunodeficiency syndrome (Fig. 8). The lower efficiency of amplification observed in these cells (20 to 200 cells) compared with that of the fully infected tissue culture cells (2 to 20 cells) (Fig. 7A) likely indicates a lower percentage of cells infected with HCMV in leukocytes from these patients.

Utilizing the enzymatic amplification procedure, we successfully detected the HCMV genome in urine samples. Correlation of these data with those obtained from conventional virological culture methods (Fig. 8; Table 1) showed that while 2 of 37 clinical urine samples were shown to be false-negative by virological culture assay, no false-negative or false-positive results were obtained with the PCR method. This suggests that detection of the HCMV genome by PCR amplification is specific and sensitive and can serve as a powerful tool for clinical detection of virus particles. Since in individuals infected with HCMV virus may be isolated from sources other than urine, such as saliva, bronchial alveolar lavage fluid, buffy coat, etc., we are interested in applying this PCR procedure to these other clinical specimens. In preliminary experiments, we have successfully applied PCR to detect HCMV DNA from lung and buffy coat specimens of viremic patients, and we are currently examining the use of nonisotopically labeled oligonucleotide probes for the detection of DNAs amplified by PCR. Although this work is still in the early phases of development, we believe that the data presented in this report indicate that PCR will have broad application for the rapid detection of HCMV in clinical specimens for diagnosis and studies of HCMV pathogenesis.

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