American Journal of Pathology, Vol. 145, No. 2, August 1994
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Cytomegalovirus-Related Sequence in an Atypical Cytopathic Virus Repeatedly Isolated from a Patient with Chronic Fatigue Syndrome

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An atypical virus, cytopathic for human and animal fibroblasts, was repeatedly cultured from a patient with chronic fatigue syndrome. Viral particles, suggestive of cytomegalovirus (CMV) were seen by electron microscopy. Infected cells did not, however, stain with antisera specific for CMV, herpes simplex virus, or human herpesvirus-6. Polymerase chain reaction (PCR) assays for these viruses were also negative. Two distinct products of approximately 1.5 kilobase pairs were amplified from virally infected cells using the human T lymphotropic virus-II tax gene reactive primer, SK44, in low stringency PCR. Sequencing of one of the amplified products showed a region of highly significant partial homology with the UL34 gene of CMV. The sequence of the other PCR product did not correspond with CMV or any other virus. DNA was extracted from the material pelleted by ultracentrifugation of filtered culture supernatants. It migrated in agarose gels as a single band of approximately 20 kbp. The banded DNA was digested with EcoRI and cloned. A 2.2 kbp plasmid containing the CMV-related sequence identified within the PCR product was recovered. Sequencing of this plasmid extended the region of partial sequence homology with CMV to include a portion of the UL35 gene of CMV. Initial sequencing of additional plasmids has confirmed the partial relatedness to CMV. The data indicate a novel type of CMV-related "stealth" virus that is able to establish a clinically persistent human infection. (Am J Pathol 1994, 145:440–451)

Chronic fatigue syndrome (CFS) refers to an illness characterized by unexplained severe prolonged fatigue often accompanied by a variety of neurocognitive symptoms.[1] Various reports have suggested that a viral infection may cause CFS. Postulated pathogens have included Epstein-Barr virus, human herpesvirus-6 (HHV-6), enteroviruses, and human T lymphotropic virus-II (HTLV-II)-related retrovirus.[2–5] We have used a combination of tissue culture and molecular techniques to probe a CFS patient for evidence of a persistent viral infection. This paper reports the isolation and initial molecular characterization of an atypical virus repeatedly isolated from this patient.

Patient: The 43-year-old health care provider (DW) was in her usual state of good health before an acute onset illness in August 1990. Her illness was characterized by intense headaches, generalized myalgia, and fever, developing 1 week after a sore throat. She was hospitalized with a diagnosis of possible encephalitis/meningitis. The cerebrospinal fluid (CSF) examination, however, was normal and the patient was discharged after 7 days' hospitalization. She continued to feel ill and was diagnosed as having CFS based on overwhelming fatigue necessitating elimination of virtually all social activities; a marked reduction in her capacity for even part-time work; impaired cognitive functions, including memory loss and difficulty in naming items (dysnomia); severe headaches; and nonrestorative sleep. She showed some improvement over the second and third year of her illness but continues to consciously avoid stressful situations for fear of exacerbating her persisting illness.

Materials and Methods

Viral Cultures

Heparinized blood of the patient was cultured on cell lines maintained in medium 199 plus 7% fetal calf

Accepted for publication May 10, 1994.

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serum (FCS). The inoculum consisted of Ficoll-Hypaque separated mononuclear cells admixed with the granulocytes from the buffy coat at the top of the erythrocyte layer. The cultures were rinsed free of contaminating erythrocytes at 1 hour and rinsed again at 24, 48, and 72 hours. Thereafter, the medium was replaced three times per week. The cells were observed for a cytopathic effect (CPE) at 2-day intervals. Routine passage of cultures was achieved by scrapping the cells into the culture medium and transferring 0.1 ml into a fresh culture tube. The fibroblastic/epitheloid cell lines used included human foreskin fibroblasts (MRHF), human lung fibroblasts (MRC-5), rhesus monkey kidney (RhMK), and primary kidney cultures of rabbit, duck, and chicken origin. The cells were obtained from BioWhittaker, Inc., (Walkersville, MD). HeLa (human), 3T3 (murine), H927 (feline), and CHO (hamster) fibroblastic cell cultures and Raji (B lymphoblastoid), HEp2 (liver epithelial), HTB152 (fibrosarcoma), and U118MG (glioblastoma) human cell lines were kindly provided by Dr. A. Epstein (USC School of Medicine).

Electron Microscopy

Cells were fixed in Karnovsky's glutaraldehyde/paraformaldehyde solution and postfixed in 1% osmium tetroxide. The samples were embedded in epon resin. Ultramicrotome-cut sections were stained with lead citrate/uranyl acetate. Pelleted material from ultracentrifuged culture supernatant was applied directly to grids and stained.

Immunofluorescence and ELISA Assays

Typing sera for cytomegalovirus (CMV), herpes simplex virus (HSV), adenoviruses, and enteroviruses were obtained from Bartel Laboratory (Sacramento, CA). The anti-CMV reagent (B1029-81) contains a mixture of three fluoresceinated monoclonal antibodies directed against the major immediate early (I-E) antigen, an undefined early nuclear antigen, and an undefined major late cytoplasmic component. Anti-CMV I-E antigen monoclonal antibody (code 500114) and anti-CMV pp65 lower matrix antigen monoclonal antibodies (C10 and C11) were obtained from Ortho Laboratories (Raritan, NJ) and Biotest Laboratories (Denville, NJ), respectively. The broadly reactive anti-HHV-6 monoclonal antibodies BO145 (against p41) and BO151 (against gp102)[6,7] were obtained from Universal Biotechnology Inc. (Rockville, MD). The sera and monoclonal were used as recommended by the suppliers. The testing for serum anti-HIV and anti-CMV antibodies were performed at an outside reference laboratory. Anti-HTLV (Abbott; ELISA) and anti-HHV-6 (Universal Biotechnology Inc.; immunofluorescence) testing were performed in our laboratory using undiluted and diluted antisera.

Viruses

HTLV-I-infected HUT cells were provided by Dr. Z. Salahuddin (USC School of Medicine). HTLV-II-infected Vero cells were provided by Dr. Irwin Chen (UCLA School of Medicine). The Z-29 strain of HHV-6 was obtained from the Centers for Disease Control and Prevention and was propagated in cord blood lymphocytes. CMV, HSV, and varicella zoster (VZV) isolates were obtained from the USC Infectious Disease Laboratory and propagated in MRC-5 cells. The patient's virus was maintained in MRC-5 and/or MRHF cells, with frozen stocks established at various intervals.

Polymerase Chain Reaction (PCR) Primers

Primers were synthesized on an Applied BioSystem model 380 synthesizer. The CMV primer set was designed to amplify a region within the I-E gene of CMV and has been described in an earlier study.[8] The primer set for the HTLV *tax* gene combined SK43′ (HTLV-I) and SK44″ (HTLV-II) primers and SK45′ probe.[9] Primers designed to amplify the region of HSV-1 between nucleotides 33,544 and 33,759 (GenBank accession number X14112), the region of VZV between nucleotides 110,034 and 110,359 (GenBank accession number X04370), and the region of the PZVH14 clone of HHV-6 between nucleotides 5,204 and 5,773 (GenBank accession number S57540) were selected from published reports. Based on the derived sequences of plasmids 15-5-2 and 15-5-4 (see Results), four sets of PCR primers with intervening detecting probes were also synthesized. For 15-5-2 the starting nucleotides on the positive and negative strands were 103 and 234 for set 1 and 631 and 1094 for set 2, respectively. The corresponding nucleotide numbers for the primers for plasmid 15-5-4 were 257 and 792 for set 3 and 728 and 849 for set 4, respectively. The primers were 20–22 nucleotides and the detecting probes 40 nucleotides.

DNA Sample Preparation

The cells scrapped from a single test tube were washed twice with phosphate-buffered saline (PBS)

incubated for 1 hour at 56 C with 100 μg proteinase K in 50 μl PCR buffer followed by heating to 100 C for 10 minutes. In some studies, purified DNA was obtained from blood or cultures using an Applied Bio-System DNA Extractor and the DNA resuspended at a concentration of 10 μg/ml. DNA was also extracted from the material pelleted by ultracentrifugation of either the culture supernatant or the lysate of infected MRHF cells. Seventy milliliters of cell-free supernatant was filtered through a 0.45-μ filter and spun at 29,000 rpm in an SW 41 Beckman rotor for 4 hours. Alternatively, $5 \times 10^7 - 10^8$ cells were twice frozen and thawed in 2 ml of medium. The lysate was diluted to 12 ml, clarified by low speed centrifugation and 0.45-μ filtration, and ultracentrifuged at 29,000 rpm in an SW 41 Beckman rotor for 12 hours. After ultracentrifugation, the medium was decanted and the bottom of the tubes rinsed in a total of 1 ml of Tris-buffered saline. The DNA in the pelleted material was extracted as described above and run in agarose gels. Control supernatants and cell lysates from uninfected cells were similarly processed.

PCR Assays

The PCR assays were performed in 100 μl containing 10 μl of the DNA template, 1 μmol/L of each primer, 2.5 U Taq polymerase, 200 μmol/L of each dNTP, 2 mmol/L Mg^{2+}, 50 mmol/L KCl, and 10 mmol/L Tris-HCl. The temperature and reaction times were chosen for either a low or a high stringency PCR. For low stringency PCR, annealing temperature of 42 C (30 seconds), extension temperature of 72 C (30 seconds), and denaturation temperature of 94 C (30 seconds) were used with rapid ramping. For high stringency PCR, annealing temperature of 60 C (30 seconds), extension temperature of 72 C (45 seconds), and denaturation temperature of 95 C (30 seconds) were used with rapid ramping. Forty cycles were run in both types of PCR assays. The PCR products were examined by agarose electrophoresis and by either dot blot (using 50 μl) or Southern blot (using 15 μl) hybridization with ^{32}P-labeled probes. For hybridization, membranes were ultraviolet cross-linked and baked in an oven at 80 C for 30 minutes. Hybridization was generally performed for 3 hours. Oligonucleotide probes were 5′ end-labeled, whereas PCR products were labeled by random priming incorporation of dCTP. Approximately 10^6 cpm of probe with a specific activity in the range of 10^8 cpm per μg were used in each assay. Membrane washing consisted of sequential incubations with 2× standard saline citrate (SSC) twice for 5-minute periods at room temperature, followed by 1× SSC for 30 minutes at 50 C and a fourth wash with 0.1× SSC for 30 minutes at 55 C. All wash solutions contained 0.2% sodium dodecyl sulfate. The washed membranes were monitored to check the negative and positive controls. If necessary, an additional wash was performed using 0.1× SSC at 60 C. The membranes were exposed to photographic film using two screens at −70 C for 12 to 18 hours.

Cloning and Sequencing of PCR Products and of DNA Obtained from Culture Supernatants

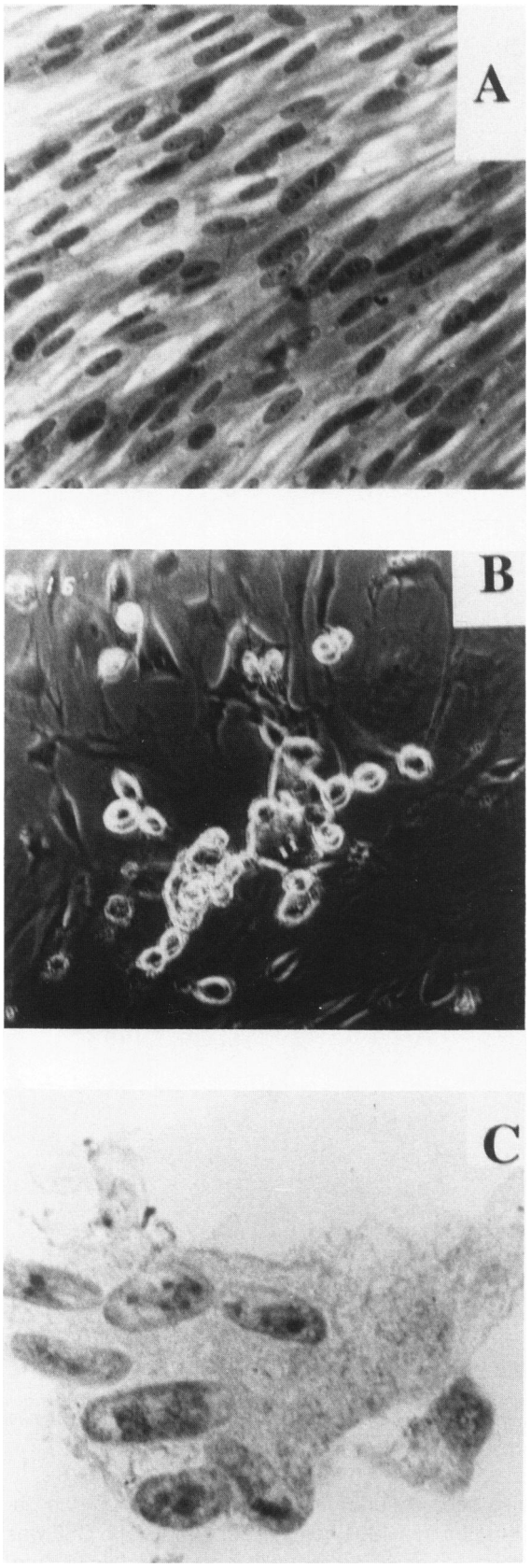
The PCR products were excised from sea plaque agarose gels, blunt ended using Klenow enzyme, phosphorylated, and cloned into pBluescript plasmids propogated in XL-1 bacteria. Sequencing of the plasmid inserts was performed by U.S. Biochemical Corp. (Cleveland, OH) with second strand verification. The inserts from these plasmids were excised using *Eco*RI and *Hin*dIII enzymes. Agarose-banded DNA derived from the material pelleted by ultracentrifugation of filtered culture supernatant was digested overnight with 10 U/μl of *Eco*RI enzyme. The resulting products were ligated into pBluescript.

Sequence Analysis

DNA and amino acid sequences were analyzed using the FastA and TFastA programs at the Genetics Computer Group (Madison, WI), the BlastN and BlastP programs of the National Center for Biotechnology Information, and the GeneWorks program from Intelligenetics, Inc.

Results

Blood obtained from the patient was initially cultured on MRHF human fibroblasts. A cytopathic effect (CPE) was observed after 8 weeks of culture. On repeat culturing of the patient's blood, CPE developed in MRHF, MRC-5, and RhMK cells. The CPE was transferable to secondary and subsequent cultures of these cells. CPE was also readily transferable to murine, feline, rabbit, hamster, duck, and chicken fibroblastic cell lines and to various long term human cell lines of epithelial, glial, and lymphoid origin. The distinctive CPE was, however, most readily observed in fibroblast cultures and in the fibroblastic component of primary kidney cultures. CPE development was promoted by regularly feeding the cultures at 2- to 3-day intervals and using a complete medium such as



medium 199 containing 7% FCS, as opposed to a basal medium with only 2% FCS. With these culture conditions, the CPE progressed to extensive cell destruction and vacuolated syncytial cell formation (Figure 1).

CPE was transmissible using cell-free supernatant passed through a 0.22-μ filter. Limiting dilution analyses have shown titers in the range of 10^{-2} to 10^{-3}. Infectivity is destroyed by boiling for 10 minutes and by exposure to chloroform/methanol. Infectious material could be stored at 4 C for over 1 year and could also be recovered from dessicated cultures retained at room temperature for 1 week.

Electron microscopic examination of infected cultures showed enveloped viruses approximately 180 to 200 nm in diameter. Viral particles were seen lining cytoplasmic vacuoles. Nonbudding viral capsids were readily apparent elsewhere within the cytoplasm and also in the nucleus. Prominent inclusions, similar to the dense body inclusions containing tegument proteins in CMV-infected cells,[10,11] were present in association with the cytoplasmic vacuoles containing viral particles (Figure 2).

The viral-infected cells did not stain by direct immunofluorescence with commercial typing antibodies for CMV, HSV, HHV-6, adenoviruses, or enteroviruses. The patient's sera gave negative reactions in ELISA assays for HIV and HTLV and in immunofluorescence assays for HHV-6. At the time of her initial hospital admission and on subsequent testing, she showed negative IgM and positive IgG antibody against CMV. She had positive but low titers of anti-Epstein-Barr Virus antibody. The patient's sera reacted in an immunofluorescence assay with cells infected with the virus derived from the patient to a dilution of 1:100. Quantitatively, similar reactions was seen, however, with sera from many normal individuals tested.

To exclude the possibility of laboratory contamination or other possible causes of a false positive culture, additional blood samples from the patient have been cultured on MRC-5, MRHF, and RhMK cells. On 15 of 18 separate occasions over a 3-year period, blood cultures have yielded strong CPE, transmissible to secondary and tertiary cultures. With frequent feeding of the cultures, the CPE from fresh inocula of the patient's blood was observable at approximately 2 to 3 weeks. In established cultures CPE can be seen

Figure 1. A: *Normal MRC-5 cells stained with hematoxylin and eosin (magnification ×200). Note the orderly formation of the elongated cells.* B: *Phase contrast of MRC-5 cells demonstrating the typical rounding and clumping of cells. The background cells show signs of toxicity that often accompanies the CPE.* C: *Hematoxylin and eosin stain of a large syncytial cell in a virus-infected MRC-5 culture. Note the multiple nuclei and vacuolated cytoplasm (×400).*

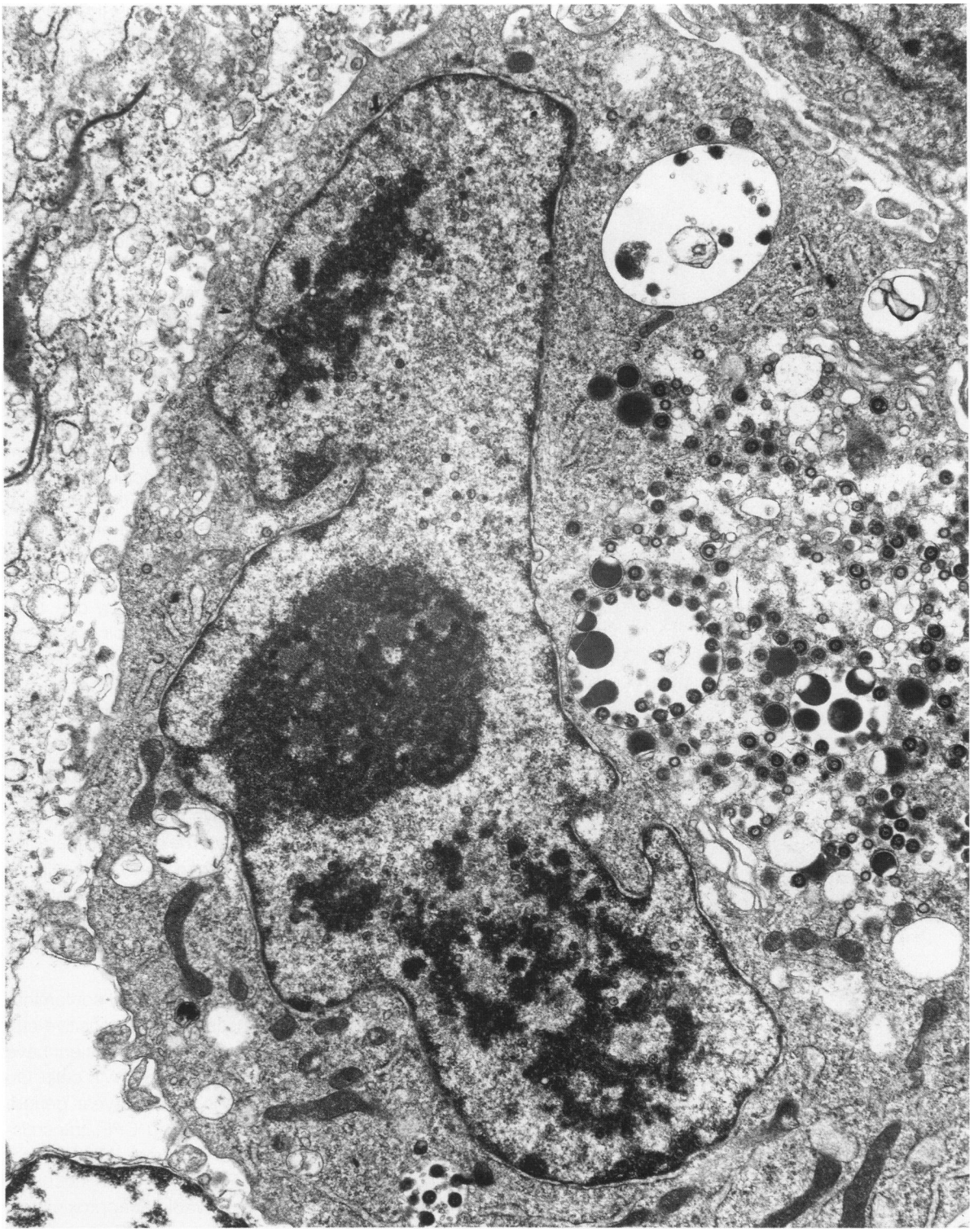


Figure 2. *Electron micrograph of MRC-5 cells infected with the virus isolated from the patient with CFS. Enveloped viral particles and inclusion bodies are conspicuously seen within cytoplasmic vacuoles. Viral particles are also present within the nucleus and elsewhere in the cytoplasm (×17,750).*

within several days of passage. Each of six positive cultures examined by electron microscopy have shown the same characteristic virus. The virus has also been cultured from CSF collected from the patient during a 1991 hospital admission. The CSF was otherwise unremarkable with normal protein (51 mg/

dl), glucose (49 mg/dl), and only a single white cell per cubic millimeter.

PCR primers sets reactive with an I-E gene of CMV gave negative results when tested on culture-derived DNA using low or high stringency PCR assays. Negative results were also seen with primer pairs derived from selected regions of HHV-6, HSV, and VZV genomes. In contrast to these results, low stringency PCR assays using HTLV *tax* primers consistently yielded PCR products easily observed in agarose gel electrophoresis. Most striking was an unexpectedly large product with an apparent size of 1.5 kbp. This band was clearly distinct from smaller products generated in the same PCR (Figure 3) and in several repeat PCR assays constituted the major product formed. In contrast to the 158-bp PCR product obtained using the *tax* primers on HTLV-I-infected cultures, none of the products generated from the patient's culture hybridized under stringent conditions with radiolabeled SK45′ probe. The 1.5 kbp product was excised from the gel then radiolabeled and used as a probe.

Pelleted material obtained by ultracentrifugation of either cell-free culture supernatant or the lysate from frozen-thawed cells was infectious for cell cultures and shown to contain viral particles by negative staining electron microscopy (data not shown). DNA was extracted from the material pelleted from filtered culture supernatant. On agarose gel, a faint band was observed that migrated slightly further into the gel than did uncut cellular DNA from either uninfected or infected cells (Figure 4, upper panel). The band was estimated to represent DNA of approximately 20 kbp. The material in the band, as well as residual material in the well, readily hybridized to the 1.5-kbp PCR product, as did cellular DNA from infected but not uninfected cells (Figure 4, lower panel). Hybridization did not occur if the material was pretreated with DNase I (data not shown). In a similar experiment, infected cells were lysed by freeze-thawing and after filtering through a 0.45-μ filter, pelletable material within the supernatant was obtained by ultracentrifugation. DNA/RNA was extracted and an aliquot was digested with *Eco*RI enzyme. As a control, DNA extracted from infected cells was similarly digested with *Eco*RI enzyme. DNA and RNA from the various samples were visualized on an ethidium-stained agarose gel. As shown in Figure 5, banded material of approximately 20 kbp was again present in the undigested aliquot obtained from the lysate of frozen-thawed infected

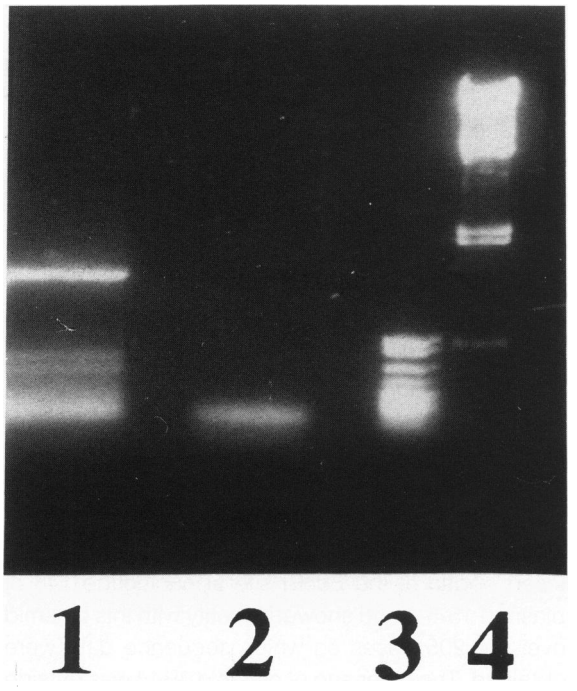


Figure 3. *Agarose electrophoresis of ethidium-stained PCR products obtained using the HTLV* tax *primers SK43′ and SK44″ on viral culture from patient DW (lane 1) or on HTLV-I-infected culture (lane 2).* Msp*I-digested pBR322 and* Hin*dIII-digested lambda are size markers in lanes 3 and 4, respectively. The sizes of the lower three* Hin*dIII bands shown are: 0.564, 2.027, and 2.322 kbp.*

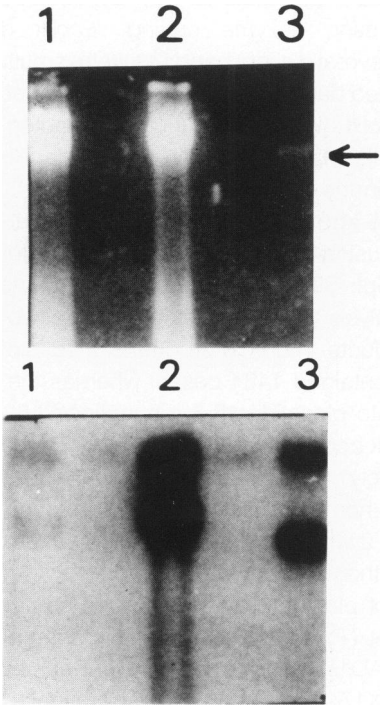


Figure 4. *Ethidium-stained agarose gel (top panel) and Southern blot hybridization pattern (bottom panel) of DNA isolated from uninfected (lane 1) and infected (lane 2) MRHF cells and from the material pelleted by ultracentrifugation of filtered supernatant from infected cultures (lane 3). On the agarose gel, a faint band could be seen in lane 3 at the region marked by the arrow. This band migrated further into the gel than did genomic DNA in lanes 1 and 2 and had an estimated size of approximately 20 kbp. The probe used was radiolabeled 1.5-kbp PCR product obtained using HTLV* tax *primer SK44″ on viral culture from patient DW.*

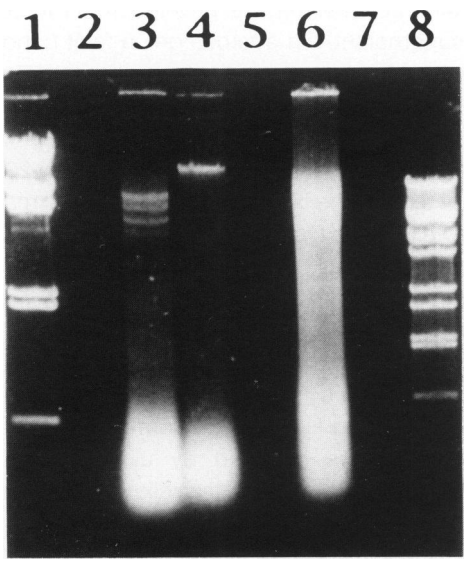


Figure 5. *Ethidium-stained agarose gel of* Eco*RI-digested (lane 3) and nondigested (lane 4) nucleic acids extracted from filtered, ultracentrifuged pelleted material derived from the lysate of frozen-thawed virus-infected MRHF cells.* Eco*RI digests of total DNA extracted from infected cells is present in lane 6. The size markers in lanes 1 and 8 are* Hin*dIII cut lambda phage and* Bst*E-II cut lambda phage, respectively. Lanes 2, 5, and 7 were blank.*

cells. The banded material was identified as dsDNA by restriction enzyme cutting. *Eco*RI digestion yielded several distinct bands as well as multiple, less well defined bands. Distinct bands could not be seen in the *Eco*RI digests of total DNA extracted from infected cells. Material migrating as cellular RNA could be seen in the lower regions of the gel.

The 1.5-kbp band generated from viral-infected cultures using the HTLV primers was cloned into pBluescript. Sequencing, as well as restriction enzyme analysis, indicated that there were two different PCR products. The PCR product cloned into plasmid 15-5-2 contained 1484 bases, whereas the product cloned into plasmids 15-5-4 contained 1539 bases (GenBank accession numbers U09213 and U09212, respectively). Computer-assisted analysis showed no apparent homology between the sequence in plasmid 15-5-2 with any viral or nonviral sequence contained in the GenBank data base. Analysis of the sequence of plasmid 15-5-4, however, showed highly significant ($P < 10^{-12}$ by BlastN) partial homology with the AD169 strain of CMV (GenBank accession number X17403). FastA analysis revealed a 58% identity over a 1201-bp overlap (Table 1). The overlapping regions identified by the FastA analysis extended from nucleotides 140 to 1,311 of the insert and nucleotides 44,705 to 45,891 of the CMV genome. This region of the CMV genome is contained within the transcripts of both the UL33 and UL34 genes and is part of the amino acid coding sequence of the UL34 protein, which extends from nucleotide 44,790 to 46,011.[13,14] The first 253 amino acids of the UL34 protein showed a highly significant 66% identity with a similar sized region in the predicted 285 amino acid sequence of an open reading frame (ORF) within the amplified PCR product (Table 2).

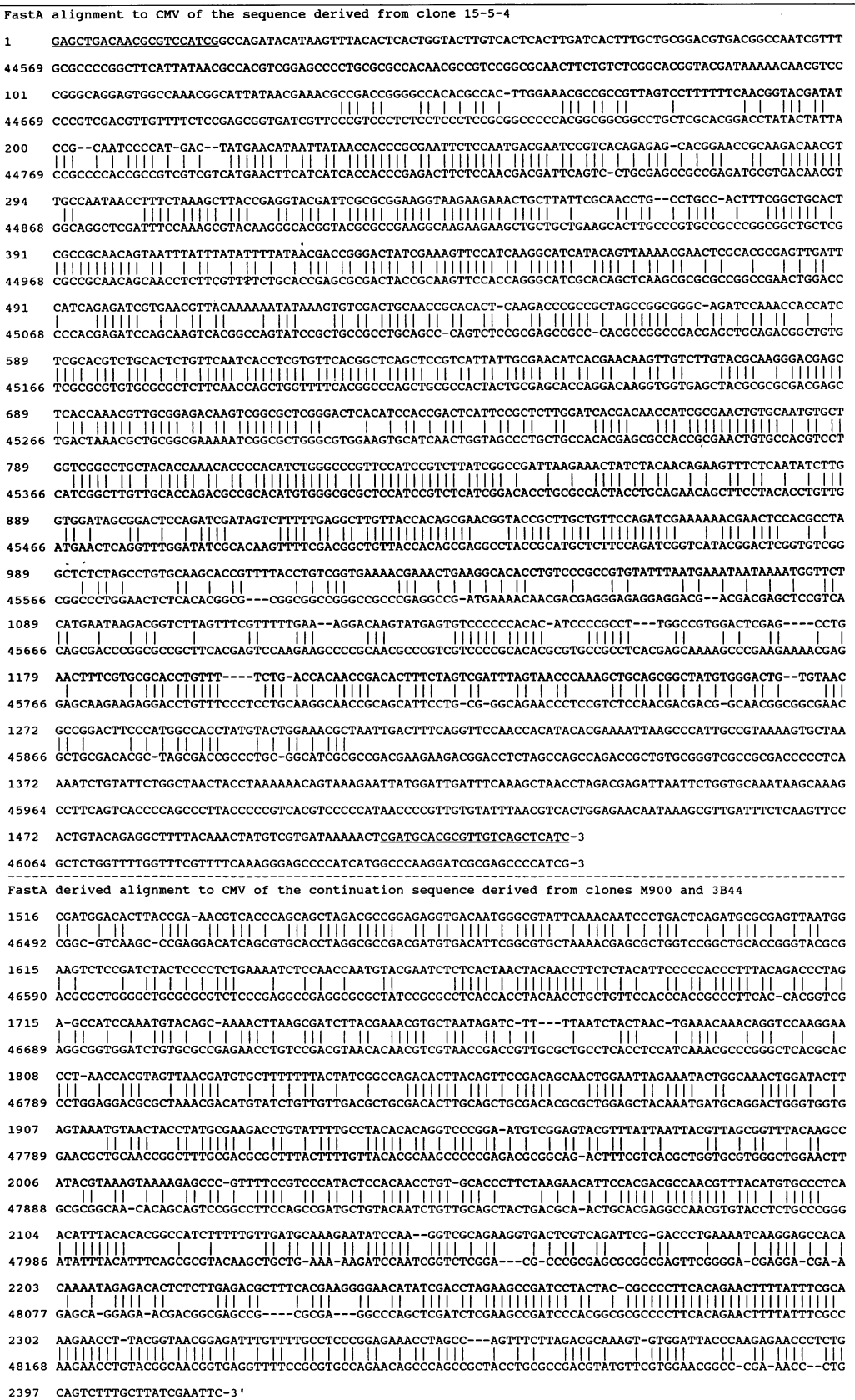
The inserts in plasmids 15-5-2 and 15-5-4 were excised and used as probes in Southern blots against *Eco*RI- and *Hin*dIII-digested DNA from infected and uninfected cells. Single hybridization bands were seen on DNA from infected cells with no hybridization with DNA from uninfected cells. The insert in plasmid 15-5-2 reacted with an *Eco*RI fragment estimated to be 8.5 kbp and with a *Hin*dIII fragment of 1.9 to 2.0 kbp. The insert in plasmid 15-5-4 reacted with an *Eco*RI fragment and a *Hin*dIII fragment estimated to be approximately 2.2 to 2.3 kbp (Figure 6).

The sequences in plasmids 15-5-2 and 15-5-4 were used to design four sets of virus reactive primers and detecting probes for use in high stringency PCR assays on the patient's cultures and blood. Figure 7 shows the positive results of Southern blot hybridization of PCR products generated on stored aliquots of eight separate cultures collected over a 2-year period. Figure 8 shows that a similar product was obtained by PCR testing of DNA extracted from the patient's blood.

Banded DNA extracted from the material pelleted by ultracentrifugation of culture supernatant was excised from the agarose gel. The DNA gave strong positive PCR assays using the primers based on the sequences of both plasmids 15-5-2 and 15-5-4 (data not shown). The extracted DNA was digested with *Eco*RI and ligated into pBluescript. After transfecting into bacteria, recombinant clones were screened with labeled inserts from plasmids 15-5-2 and 15-5-4. The 15-5-2 did not react with any of the currently available clones. The 15-5-4 insert reacted with a 2.2-kbp plasmid (3B44). The inserts in plasmid 3B44 hybridized as a single band of corresponding size in Southern blot analysis using *Eco*RI-digested DNA from infected cells (data not shown). No hybridization occurred with DNA from uninfected cells.

The terminal sequences of the insert in plasmid 3B44 were obtained. As expected, one end of the insert began at the *Eco*RI site at nucleotide 148 of plasmid 15-5-4 and showed identity with this plasmid over the 205 bases on which sequence data were obtained. The other end of plasmid 3B44 was outside the region covered by plasmid 15-5-4. A PCR product was obtained using primers based on this sequence and on the sequence from nucleotides 1465 to 1484 of plasmid 15-5-4. This product was cloned into plas-

Table 1. *Sequence Homology with CMV*



Demonstration of the FastA identified regions of nucleotide sequence homology between the clones derived from the patient's virus and CMV. Identical nucleotides within the homologous regions are indicated by the vertical lines. The nucleotide number is shown at the beginning of each row. The HTLV SK44″ primer is present at both ends of the sequence of clone 15–5–4 and is underlined. The sequence shown for clones M900 and 3B44 begins with the actual primer binding site corresponding to the 3′ end of plasmid 15–5–4. This region is complementary for 10 of 21 of the primer nucleotides, including the 5 nucleotides at the 3′ end of the primer.

Table 2. *Alignment of the Amino Acid Sequence Coded by a Predicted ORF in the Insert in Plasmid 15-5-4 with the Amino Acid Sequence of the UL34 Gene of CMV*

```
UL34      --MNFIITTR DFSNDDSVLR AAEMRDNVAG SISKAYKGTV RAEGKKKLLL   48
15-5-4    MTMNIIITTR EFSNDESVTE STEPQDNVAN NLSKAYRGTI RAEGKKKLLI   50

UL34      KHLPVPPGGC SRRNSNLFVF CTERDYRKFH QGIAQLKRAP AELDPHEIQQ   98
15-5-4    RNLP-ATFGC IRRNSNLFIF YNDRDYRKFH QGIIQLKRTR TRVDSSEIVN   99

UL34      VTASIRCRLQ PSLREPPTPA DELQTAVSRV CALFNQLVFT AQLRHYCEHQ  148
15-5-4    VTKNIKCRLQ PHTQDPPLAG GQIQTTISHV CILFNQLVFT AQLRHYCEHH  149

UL34      DKVVSYARDE LTKRCGEKSA LGVEVHQLMA LLEHERHREL CHVLIGLLHQ  198
15-5-4    EQVVLYARDE LTKRCGDKSA LGTHIHRLIA LLDHDNHREL CNVLMGLLHQ  199

UL34      TPHMWARSIR LIGHLRHYLQ NSFLHILMNS GLDIAQVFDG CYHSEAYRML  248
15-5-4    TPHMWARSIR LIGRLRNYLQ QKFLNILVDS GLQIDSLFEG CYHSERYRLL  249

UL34      FQIGHTDSVS AALELSHGAA AGPPEADENN DEGEEDDDEL RHSDPAFLHE  298
15-5-4    FQIEKTNS-- ---------- ---------- ---------- ---TPSSL--  262

UL34      SKKPRNARRP RTRVPPHEQK PEENEEEEEE LFPSCKATAA FLRAEPSVSN  348
15-5-4    ---------- ---------- ---------- ---ACASTV- -L---P----  270

UL34      DDGNGGERCD TLATALRHRA DEEDGPLASQ TAVRVAATPS PSVTPALTPV  398
15-5-4    ----VGE--- ---------- NETEG----- ---------- ----------  278

UL34      TSPITPLCI                                               407
15-5-4    -TPVPP-CI                                               285
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The boxed areas show identity between the two sequences.

mid M900 and fully sequenced with second strand verification. This has provided a composite sequence of 2410 bases extending from the 5′ end of plasmid 15-5-4 to the 3′ end of plasmid 3B44 (Table 1). Sequence alignment analysis indicated that the sequence extending beyond that of plasmid 15-5-4 has 52% nucleotide identity with CMV within the coding region of the UL35 gene. No ORF corresponding to the UL35 gene could, however, be identified. Furthermore, the homologous CMV region was not contiguous with the region of CMV homology identified from the sequence within plasmid 15-5-4 but required that a gap be placed within the CMV genome of 496 bases.

Discussion

This paper provides initial molecular characterization of an atypical cytopathic virus that has been repeatedly cultured from the blood of a patient diagnosed as having CFS. The virus induces a CPE in cells from multiple species. Although detailed description of the CPE will be provided elsewhere, it can best be summarized by the appearance of foamy cell syncytia. On electron microscopy, enveloped viral particles were seen that were consistent with a herpesvirus, most notably human CMV. Immunological and PCR-based assays for CMV detection were, however, negative. PCR products were generated from infected cultures

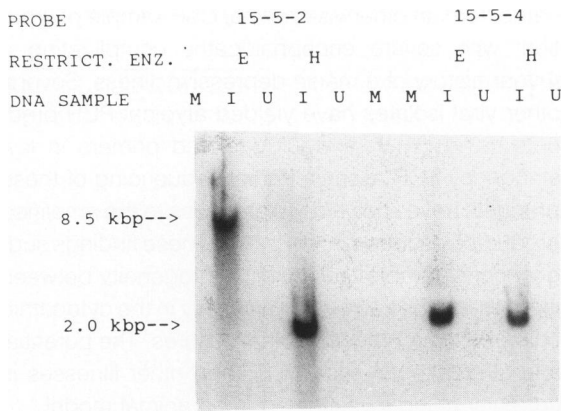

Figure 6. *Southern blot hybridization of* Eco*RI-(E) and* Hin*dIII-(H) digested DNA extracted from virus-infected (I) and uninfected (U) MRC-5 cells, or of enzyme-digested lambda DNA used as size markers (M). The probes were* ^{32}*P-labeled inserts obtained from plasmids 15-5-2 and 15-5-4. The insert was excised from plasmid 15-5-2 using* Eco*RI and* Hin*dIII. These enzymes cut slightly outside the* Eco*RV cloning site at the flanking ends of the insert. The insert in plasmid 15-5-4 was excised using* Hin*dIII, which cut within the plasmid at nucleotide 314 and within the distal pBluescript multiple cloning site.*

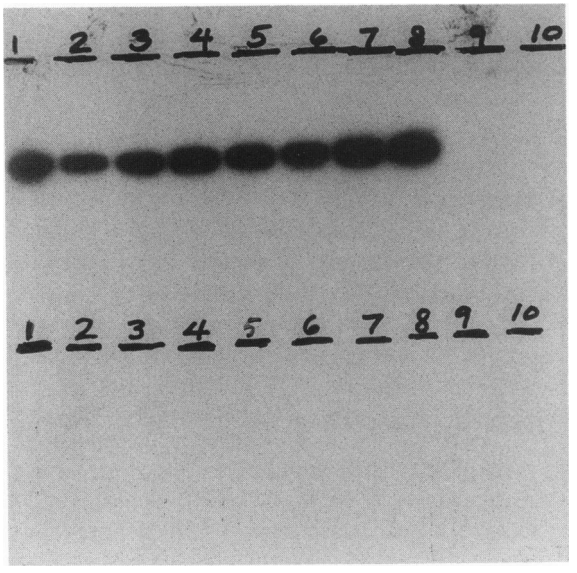

Figure 7. *Southern blot hybridization of PCR products generated from nonpurified DNA extracted from eight positive viral cultures independently obtained from patient DW over a 2-year period (lanes 1 to 8 in upper row). DNA from uninfected MRC-5, MRHF, and RbMK cultures and from various additional virus controls are shown in positions 9 and 10 in the upper row and 1 to 10 in the lower row. The primers and detecting probe used were based on the sequence of the insert in plasmid 15-5-4 (set 3 as described in the section on PCR primers in Materials and Methods). The hybridizing product was seen as a single band on agarose gel electrophoresis in the expected size range of 500 to 600 bp. Similar results were obtained using primers based on the sequences of plasmid 15-5-2. Positive PCR assays were also obtained in viral cultures repeatedly passaged in mouse and cat cell lines.*

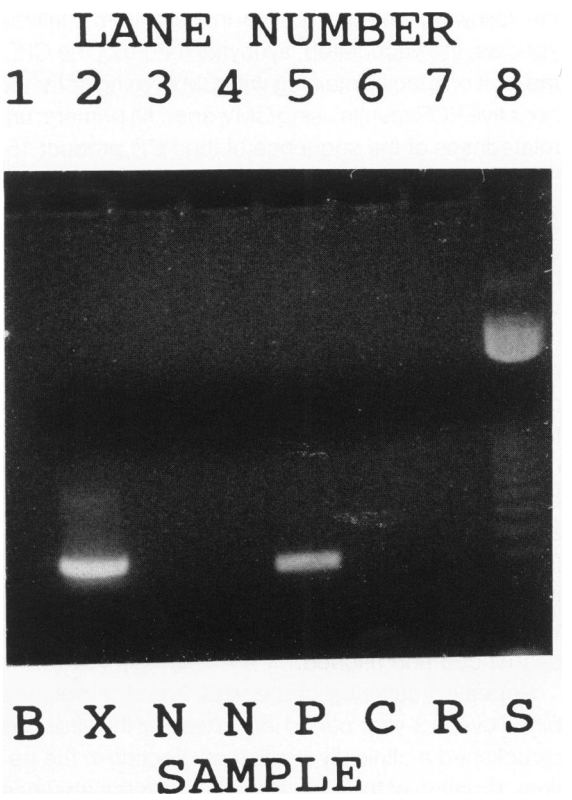

Figure 8. *Agarose gel electrophoresis of ethidium-stained PCR products obtained on purified DNA derived from the blood of the patient (P) collected in March 1993 (lane 5). Identically processed samples from two normal individuals (N) gave negative results (lanes 3 and 4). PCR-derived samples containing DNA from viral-infected (X, positive control) and uninfected (C, negative control) cultures are shown in lanes 2 and 6, respectively. Lane 1 was blank (B), lane 7 was the PCR reagent (R) control, and lane 8 contained size markers (S).*

using HTLV reactive primers in low stringency PCR assays. Although these products resulted from cross-priming (rather than from the presence of HTLV-specific sequences), they provided infected-cell-specific probes and material on which sequencing could be performed.

The banding pattern in agarose and the susceptibility to *Eco*RI enzyme digestion indicated that the pelleted material derived from infected cells contained dsDNA with one or more segments of approximately 20 kbp. Hybridization studies showed that the PCR products and an *Eco*RI fragment reacted with DNA from infected but not from uninfected cells. Sequencing studies on the 15-5-4 and M900 PCR products and the 3B44 *Eco*RI cloned fragment indicated that the genetic material contained regions of highly significant, partial homology to the UL34 and UL35 genes of CMV. The function of the UL34 gene is unknown and a corresponding gene has not been identified in other human herpesviruses.[13] The finding of partial homology at both the nucleotide and amino acid levels with the UL34 gene is, therefore, consistent with a CMV origin of at least part of the genetic material isolated from the infected cultures. The cultured virus is distinguishable from CMV, however, by

the following criteria: growth in cells from multiple species; the vacuolated, syncytial nature of the CPE; the lack of specific staining with CMV typing sera; the negative PCR results using CMV-specific primers; unrelatedness of the sequence of the PCR product 15-5-2 to that of CMV, and the apparent difference in the size of the genome.

Even within the CMV homologous regions of plasmids 15-5-4 and M900, the extent of sequence divergence from the AD169 prototype strain of CMV (GenBank accession number X17403) is far greater than the estimated 5% divergence observed between clinical CMV isolates.[15] This suggests that if derived from human CMV, this region of the viral genome has undergone multiple mutations. Preliminary partial sequencing of additional clones has strengthened this assessment with several clones showing a distant relatedness to various limited regions of the CMV genome. A clearer understanding of the structure of the virus should be forthcoming as these clones are fully sequenced and aligned.

Repeated culturing of the virus from the patient's blood over a 3-year period indicates that the virus has established a clinically persistent infection in the patient. Isolation of the virus from CSF and the absence of an accompanying inflammatory response suggest that the virus is neurotropic and yet noninflammatory. In addition to promoting viral growth by transactivating early viral genes, the proteins coded by the I-E genes of CMV are thought to provide important antigens for cellular immune reactions.[16,17] The major I-E gene of CMV was not detectable using either PCR or monoclonal antibody assays. It is conceivable, therefore, that the virus has arisen from CMV but that portions of its genetic machinery have been deleted or mutated as a mechanism of avoiding immune recognition. The absence of certain genes may also reduce susceptibility to cellular factors that restrict the interspecies growth of human CMV in animal cell lines. This could explain the broad *in vitro* host range of the virus.

Ongoing studies indicate that atypical viruses can be isolated from patients with a variety of neurological, psychiatric, and autoimmune diseases, as well as some asymptomatic individuals. The viruses are cytopathic for fibroblasts and other cell types of multiple species and do not give typical reactions in immunological and molecular probe assays for known human herpesviruses. Because of the absence of overt clinical signs of an inflammatory reaction in many of these patients, we have tentatively termed these agents stealth viruses. Of several isolates, one cross-reacts strongly with the PCR products generated against the virus described in this paper. This isolate came from an otherwise normal CSF sample of a patient with severe encephalopathy complicating a 4-year history of a manic depressive illness. Several other viral isolates have yielded atypical PCR products using HTLV- and CMV-related primers in low stringency PCR assays. Partial sequencing of these products have shown no relatedness to the amplified products described in this paper. These findings suggest considerable molecular heterogeneity between isolates, despite an overall similarity in the cytopathic changes induced in fibroblast cultures. The potential role of stealth viruses in CFS and other illnesses is currently being investigated in an animal model.

Acknowledgment

Financial support was gratefully received from the CFIDS Association of America, Charlotte, N. C. and from the Pegasus Medical Group, Los Angeles, CA. The work was performed within the USC Infectious Disease and Molecular Pathology Laboratories.

References

1. Holmes GP: Defining the chronic fatigue syndrome. Rev Infect Dis 1991, 13 (Suppl. I):S53–55
2. Schooley RT: Chronic fatigue syndrome: a manifestation of Epstein-Barr virus infection. Curr Topic Infect Dis 1988, 9:126–146
3. Buchwald D, Cheney PR, Peterson DL, Henry B, Wormsley SB, Geiger A, Ablashi DV, Salahuddin SZ, Saxinger C, Biddle R, Kikinis R, Jolesz FA, Folks T, Balachandran N, Peter JB, Gallo RC, Komaroff AL: A chronic illness characterized by fatigue, neurologic and immunoloogic disorders, and active human herpesvirus type-6 infection. Ann Intern Med 1992, 116: 103–113
4. Archard LC, Bowles NE, Behan PO, Bell EJ, Doyle D: Postviral fatigue syndrome: persistence of enterovirus RNA in muscle and elevated creatine kinase. J Roy Soc Med 1988, 81:326–329
5. De Freitas E, Hilliard B, Cheney PR, Bell DS, Kiggundu E, Sankey D, Wroblewska Z, Pallandio M, Woodward JP, Koprowski H: Retroviral sequences related to human T lymphocytotropic virus type II in patients with chronic fatigue immune dysfunction syndrome. Proc Natl Acad Sci USA 1991, 88:2922–2926
6. Ablashi DV, Balachandran N, Josephs SF, Hung CL, Krueger GRF, Kramarsky B, Salahuddin SZ, Gallo RC: Genomic polymorphism, growth properties and immunologic variations in herpesvirus-6 isolates. Virology 1991, 184:545–52

7. Schirmer EC, Wyatt LS, Yamanishi K, Rodriguez WJ, Frenkel N: Differentiation between two distinct classes of viruses now classified as human herpesvirus 6. Proc Natl Acad Sci USA 1991, 88:5922–26
8. Shibata D, Martin WJ, Appleman MA, Causey DM, Leedom JM, Arnheim N: Detection of cytomegaloviral DNA in peripheral blood of patients infected with human immunodeficiency virus. J Infect Dis 1988, 158: 1185–1192
9. Ehrlich GD, Greenberg S, Abbott MA: Detection of human T-cell lymphoma/leukemia viruses. In PCR Protocols: A Guide to Methods and Applications. Edited by Innis MI, Gelfand DH, Sninsky JJ, White TJ. New York, Academic Press, 1990, pp 325–336
10. Doane FW, Anderson N: Electron Microscopy in Diagnostic Virology: A Practical Guide and Atlas. Cambridge, MA, Cambridge University Press, 1987
11. Stinski MF: Human cytomegalovirus: glycoproteins associated with virions and dense bodies. J Virol 1976, 19:594–609
12. Wang WP, Myers RL, Chiu IM: Single primer-mediated polymerase chain reaction: application in cloning of two different 5′-untranslated sequences of acidic fibroblast growth factor mRNA. DNA Cell Biol 1991, 10: 771–777
13. Chee MS, Bankier AT, Beck AT, Bohni R, Brown CM, Cerny R, Horsnell T, Hutchison CA, Kouzarides T, Martignetti JA, Preddie E, Satchwell, SC, Tomlinson P, Weston KM, Barrell BG: Analysis of the protein coding content of the sequence of human cytomegalovirus strain AD169. Curr Top Microbiol Immunol 1990, 154: 126–169
14. Welch AR, McGregor LM, Gibson W: Cytomegalovirus homologs of cellular G protein-coupled receptor genes are transcribed. J Virol 1991, 65:3915–3918
15. Mocarski ES: Cytomegalovirus biology and replication. In The Human Herpesviruses. Edited by Roizman B, Whitley RJ, Lopez C. Raven Press, New York, 1993, pp 173–226
16. Alp NJ, Allport TD, Van-Zanten J, Rodgers B, Sissons JG, Borysiewicz LK: Fine specificity of cellular immune responses in humans to human cytomegalovirus immediate-early 1 protein. J Virol 1991, 65:4812–4820
17. Del-Val M, Schlicht HJ, Volkmer H, Messerle M, Reddehase MJ, Koszinowski UH: Protection against lethal cytomegalovirus infection by a recombinant vaccine containing a single nonameric T-cell epitope. J Virol 1991, 65:3641–3646