Intragenic Variability of Human Cytomegalovirus Glycoprotein B in Clinical Strains

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Human cytomegalovirus (HCMV) strains can be classified into four glycoprotein B (gB) genotypes, and there has been evidence of differences in viral virulence. In this study, intragenic variability of HCMV gB strains was analyzed. The gB gene was amplified by nested polymerase chain reaction using samples from immunosuppressed patients. The genotype of fragments corresponding to the cleavage site of gB was determined by restriction fragment analysis; fragments corresponding to the N- and C-termini (gBn and gBc) were sequenced and compared with published sequences. At the cleavage site, the four known genotypes were found. Typing revealed four major genotypes at the N-terminus and two at the C-terminus. In 22 of 44 strains, the gB type determined at the cleavage site was different from the gBn or gBc type (or either), indicating that intragenic variability within the gB gene occurs frequently.

Glycoprotein B (gB) of the human cytomegalovirus (HCMV) plays an important role in virus infectivity. This protein is a major component of the virion envelope and is transported to the plasma membrane of infected cells. It has been shown that monoclonal antibodies to gB inhibit virus penetration into cells and block transmission of infectious virus from cell to cell [1]. Furthermore, anti-gB antibodies constitute up to 70% of the total serum neutralizing activity found in persons with past HCMV infection, indicating that gB is a major target for neutralizing antibodies [2, 3]. Common and strain-specific epitopes have been described [4, 5]. In addition, it has been shown that gB protein induces cytotoxic T cell responses [6–9].

The gB precursor protein of 906 amino acids undergoes glycosylation and proteolytic cleavage between residues 460 and 461 during transport through the exocytotic pathway. The amino- and carboxy-terminal fragments remain disulfide-bonded. The gene coding for gB is highly variable in regions corresponding to the N-terminus and the cleavage site and less variable in regions corresponding to the C-terminus of the protein [10, 11]. Several studies on clinical HCMV isolates showed that sequence variation was restricted to a limited number of gB types [10–12]. Chou and Dennison [10, 11] classified HCMV strains into one of four variant groups, each with a characteristic nucleotide and peptide sequence. They proposed a genotyping scheme that employs restriction fragment length polymorphism (RFLP) after amplifying a fragment that corresponds to the cleavage site of the gB protein.

There is increasing evidence that strains with different gB genotypes may vary in virulence. Infections with HCMV gB type 1 were previously correlated with a more favorable outcome than infections with gB types 2–4 when bone marrow transplant recipients and human immunodeficiency virus (HIV)–infected patients were analyzed [13–15]. Therefore, genotyping of gB may be helpful in predicting the clinical outcome of HCMV infection.

It has been argued that the four gB genotypes determined in samples from geographically restricted areas are not representative and that additional gB genotypes may be found in samples from other geographic areas. Therefore, we sequenced variable regions within the N- and C-terminal parts of gB from a vast number of clinical isolates from German patients and compared the sequences obtained with published gB sequences from other geographic areas.

Furthermore, evidence for intragenic variability within the gB gene has previously been reported. Chou [11] described 2 HCMV strains that showed the typical sequence of one gB genotype crossing then over to the typical sequence of another type [11]. To analyze the frequency of intragenic variability, we determined the genotype at the region corresponding to the cleavage site of gB using RFLP from 44 different clinical samples. The genotype was compared with sequence data from the N- and C-terminal regions.

Materials and Methods

Cells and viruses. Blood, urine, and bronchoalveolar lavage samples were obtained during routine screening from 44 immunosuppressed patients with active HCMV infection (16 bone marrow, 16 kidney, 1 heart, and 3 liver transplant recipients; 5 connately infected infants; 2 HIV-infected patients; and 1 patient with auto-

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immune disease). Blood was separated to obtain peripheral blood leukocytes (PBL) as described [16]. HCMV strains were isolated from urine and PBL. Laboratory strain AD169 and clinical isolates were propagated in human foreskin fibroblasts (HFF) cultured in Eagle MEM (Gibco, Glasgow, UK) supplemented with 5% fetal calf serum (ccPro, Karlsruhe, Germany). Cells were harvested after 8–10 passages, when cytopathic effect was visible in >80% of HFF. A total of 30 clinical isolates and 14 PBL samples was further analyzed by RFLP and DNA sequencing.

DNA preparation. HFF infected with clinical isolates and native PBL obtained from viremic patients were counted, and 2×10^6 cells were pelleted by centrifugation at 400 *g* for 15 min. Cells were resuspended in 250 μL of a polymerase chain reaction (PCR)–compatible lysis buffer (10 m*M* Tris at pH 8.3, 2.5 m*M* MgCl2, 50 m*M* KCl, 0.5% Tween 20, and 0.5% NP-40) supplemented with 0.2 mg/mL proteinase K. After incubation (56°C, 120 min), proteinase K was inactivated (95°C, 10 min). DNA was purified by ethanol precipitation and dissolved in 100 μL of distilled water. A 1-μL aliquot of this extract was used for amplification by PCR.

Amplification of the gB gene by nested PCR. The oligonucleotide primers used for PCR amplification were commercially synthesized (TibMolbiol, Berlin) and are listed in table 1. The entire gB gene (3029 bp) was amplified in a first-round PCR (gBallout5′/gBallout3′). Fragments around the cleavage site (gBcls), the N-terminal region (gBn), and the C-terminal region (gBc) were amplified in the second-round PCR. The fragments around the cleavage site (293–296 bp, size varies by strain) were amplified by primer set gB1319/gB1604, the gBn fragments (1676 bp) by primer set gBallin5′/gB1604, and the gBc fragments (1400 bp) by primer set gB1319/gBallin3′.

The first round of amplification was performed in a total volume of 100 μL using 1 μL of DNA extract and 99 μL of PCR mix (10 m*M* Tris at pH 8.3, 2.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.01% gelatin, the four dNTPs at 200 μM each, and 1.25 units of Taq polymerase) supplemented with 20 pmol of each outer primer. Second-round amplification was performed in a total volume of 50 μL, adding 1 μL of first-round amplification product to 49 μL of PCR mix containing 15 pmol of each inner primer. Samples of both rounds were denatured (95°C, 360 s) and amplified within 25 cycles. Annealing time was 15 s for all PCRs; annealing temperature was 61°C for amplification of the entire gB gene and the gBc fragment and 56°C for the gBn and gBcls fragments. Elongation time was 120 s for amplification of the entire gB gene and 40 s for all other PCRs; elongation temperature was 72°C for all PCRs. Amplification was performed on a thermal cycler (model 9600; Perkin Elmer, Norwalk, CT). Aliquots (10 μL) of the amplification products were analyzed on 1.5% agarose gels stained with ethidium bromide.

RFLP. Fragments around the cleavage site amplified by primer set gB1319/gB1604 (codon 439–534) were digested with restriction enzymes *Rsa*I and *Hin*fI (New England BioLabs, Beverly, MA) and analyzed on a 3.5% agarose gel (Metaphor; FMC, Risingevej, Denmark). The four types of gB were distinguished by different pattern lengths of fragments as described [10].

DNA sequencing. PCR-amplified gBn- and gBc-terminal fragments were purified using a PCR purification kit (Qiaquick; Qiagen, Hilden, Germany). Sequencing was performed directly, without subcloning, and was based on the dideoxy chain-terminator technique using nucleotides labeled with four different fluorescent dyes (prism sequenase terminator single-stranded DNA sequencing kit; Applied Biosystems, Weiterstadt, Germany). The sequencing reactions were performed according to the manufacturers instructions on a thermal cycler (model 9600, Perkin Elmer) using each of the sequencing primers listed in table 1 at 10 pmol/μL (gBNi5′/gBNi3′ for N-terminal fragments, and gBCi5′/gBCi3′ for C-terminal fragments). Sequencing reaction products were purified by gel filtration using spin columns (S-200; Pharmacia, Freiburg, Germany) and analyzed on a sequencer (ABI 373A; Applied Biosystems).

As a control, laboratory strain Ad169 was sequenced repeatedly, and sequence data were compared with published sequences. No aberrant point mutations could be observed (data not shown).

Computer analysis and genotyping of sequences. DNA sequence editing, comparison, and alignment were performed on a Macintosh PowerPC using the DNAStar sequence analysis software. All alignments were performed with the algorithm described by Hein and Stovlbaek [17]. A standard identity residue weight table was used for phylogenetic analysis. The numbering of all base pairs refers to the AD169 gB gene, with 1 being the first bp of the gB start codon.

Results

Typing of fragments corresponding to the gB cleavage site. The gB genotype of 30 clinical HCMV isolates and of 14 PBL

Table 1. gB primer sequences.

Primer	Primer sequences	Position* on AD169 genome
gBallout5′	5′-GGGAGCCGCACCGACCTT-3′	−132 to −115
gBallout3′	5′GGTCACGCCGCCGCTCAG-3′	2880 to 2897
gBallin5′	5′-CGACACGCAAGAGACCACGAC-3′	−57 to −37
gBallin3′	5′-TGCAGCACCTAGATATCCAGTTTA-3′	2783 to 2806
gB1319	5′-TGGAACTGGAACGTTTGGC-3′	1319 to 1337
gB1604	5′-GAAACGCGCGGCAATCGG-3′	1603 to 1620
gBNi5′	5′-ACGAACATGGAATCCAGGATCTGG-3′	−6 to 18
gBNi3′	5′-CGTCAAAACATTTTGGTAGACCCG-3′	421 to 444
gBCi5′	5′-GACCTCGAAGAGATCATGC-3′	2032 to 2050
gBCi3′	5′-TAAGCCTGCTCGTTGGTGTA-3′	2515 to 2534

NOTE. All sequences are based on the AD169 sequence (GenBank accession no. X04606).
* 1 = first bp of gB start codon.

samples from viremic patients was determined using RFLP, as previously suggested by Chou and Dennison [10]. The DNA was extracted, and a fragment corresponding to the cleavage site of gB was amplified by PCR. The gB type of the amplification products was determined by RFLP, which gave characteristic patterns of fragment length (data not shown). All HCMV strains adopted one of four gB types, and no aberrant patterns suggesting the presence of additional genotypes were observed. In summary, in 44 samples, gB type 1 was found in 16, gB type 2 in 14, gB type 3 in 7, and gB type 4 in 7.

Definition of prototypes and typing of fragments corresponding to the N-terminus of gB. The part corresponding to the N-terminus of gB, previously found to be highly variable, was genotyped by sequence analysis and alignment with published sequences [10, 11]. For comparison, a representative strain of each of the four groups previously identified by RFLP was defined as a prototype strain (table 2). Although prototypes 2 and 3 were clearly distinguishable at the cleavage site of gB, Chou [11] found that these strains and 5 of 11 clinical isolates were indistinguishable between bp 81–201 in the major variable N-terminal region and therefore belonged to a 2/3 genotype [11].

In the present study, a longer fragment (bp 47–387) was analyzed from a higher number of clinical samples (N1–N44). Sequences N1–N44 were aligned and compared with sequences of the prototype strains. Figure 1 shows the alignment of the N-terminal sequences from all clinical and prototype strains that indicated the gBn genotype. Only the variant triplets from bp 47 on (triplet 16) are shown. The triplet numbering refers to the AD169 gB gene, with 1 being the first bp of the gB start codon. Although genotypes 2 and 3 were clearly related, they differed by several point mutations strongly conserved within one genotype (triplets 16, 56, 113, and 116), leading to 4 amino acid exchanges. In contrast, genotypes 1 and 4 were distinct from the other genotypes and showed characteristic deletions and insertions (e.g., triplets 25, 28, 39, 56, 87, and 88).

Sequences of 15 samples were similar to that of prototype 1 and therefore were classified as gBn 1 genotype. Four sequences were similar to the sequence of prototype 2 (gBn 2 genotype), 13 were similar to the sequence of prototype 3 (gBn 3 genotype), and 10 were similar to the sequence of prototype 4 (gBn 4 genotypes) (figure 1). Two sequences (N29 and N30), however, were similar to the sequence of prototype 1 and changed, downstream of bp 167, to a genotype similar to prototype 3. Therefore, the majority of HCMV strains could be classified into one of four genotypes at the N-terminus of gB, and only a minority was not groupable.

Within one gB genotype, the sequence similarities ranged from 97% to 100% and between different gB genotypes, from 86% to 95% (table 2). The similarity of strains N29 and N30 (not groupable) was 99%.

Next, we compared these sequences (N1–N44) with sequences obtained from other geographic areas. Sequences M64956, M64938, M64949, M64937, and M64939 (from a German study by Lehner et al. [12]), sequences X76145–X76159 (from a Chinese study by Shiu et al. [18]), sequences M60924–M60927, M60929, M60931–M60934, M85228, and M85229 (from the Portland, Oregon, study by Chou et al. [10, 11]), and the sequence of strain Towne (M22343) were analyzed. A fragment between bp 47 and bp 250 of all sequences, together with prototype sequences, were displayed in a phylogenetic tree (figure 2). The N-terminal sequences clustered with one of the four known prototype sequences, with the exception of four sequences (N29, N30, X76152, and M60927). As described above, sequences N29 and N30 showed homology to those of prototypes 1 and 3. Sequences X76152 and M60927 were similar to those of prototypes 2 and 3 and changed, downstream of bp 211, to a genotype similar to prototype 1.

Typing of fragments corresponding to the C-terminus of gB. The part corresponding to the C-terminus of gB was found previously to be more conserved than the N-terminus and the cleavage site [11]. To analyze the variability of this part of gB

Table 2. Percent similarity of N-terminal sequences compared with prototype strains of gB.

	Prototype sequences			
N-terminal sequences	gBn 1	gBn 2	gBn 3	gBn 4
gBn 1	99%–100%*	86%	87%	86%–87%
gBn 2	86%	99%–100%*	93%–94%	92%–93%
gBn 3	87%–88%	94%–95%	97%–99%*	92%–93%
gBn 4	86%–87%	92%–93%	92%	99%–100%*
Not groupable	93%	90%	90%	90%

NOTE. N-terminal sequences obtained in this study were aligned with N-terminal sequences of prototype strains (prototype sequences gBn 1–4). Strain C327A (GenBank M60929) was defined as gB prototype 1, strain AD196 (X04606) as gB prototype 2, strain CO76A (M85228) as gB prototype 3, and strain C194A (M60926) as gB prototype 4.

* % similarity within 1 gB group.

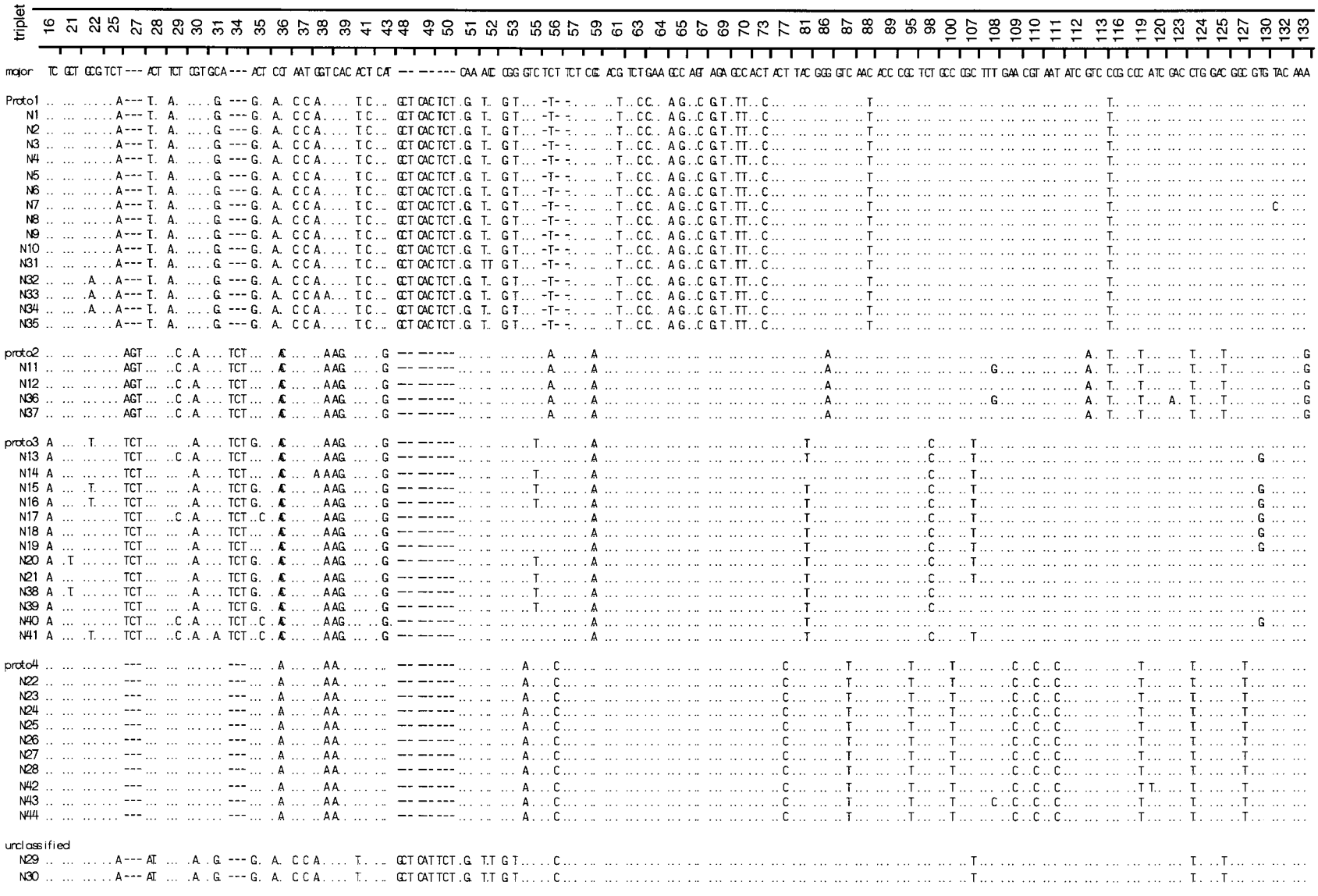

Figure 1. Nucleotide sequence of fragment corresponding to N-terminus of gB. Sequences of clinical strains (clinical isolates N1–N30, peripheral blood lymphocyte samples N31–N44) were compared with those of prototype strains (proto 1–4). Only variant triplets from bp 47 (on triplet 16) are shown. Triplet numbering refers to AD169 gB gene, with 1 being first bp of gB start codon. Majority of all sequences (major) and all variant triplets at each location are shown. Dots indicate nonaberrant sequences; dashes indicate deletions compared with majority.

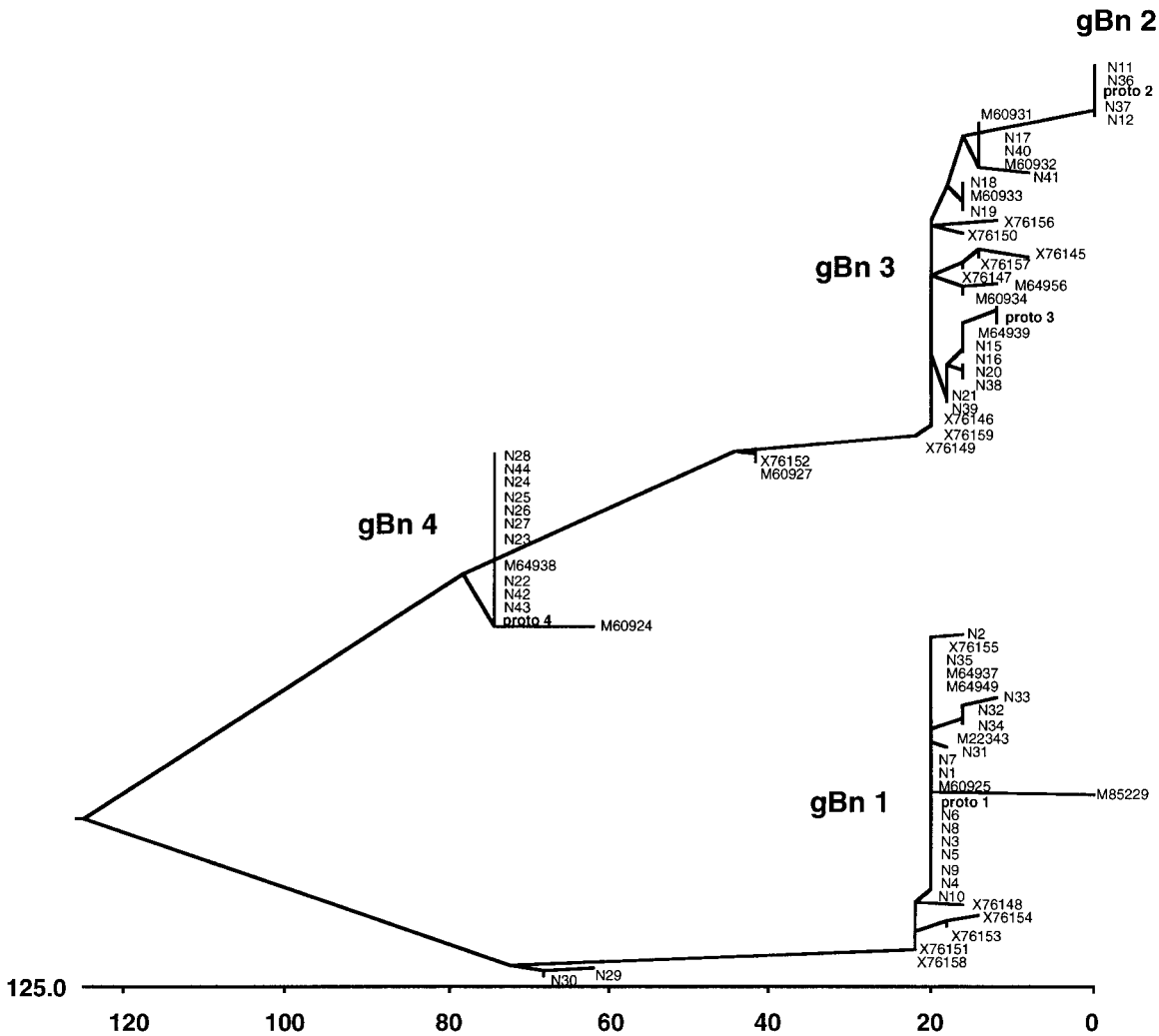

Figure 2. Phylogenetic tree analysis of N-terminal gB sequences. Sequences of strains N1–N30 were obtained from clinical isolates and of strains N31–N44 from peripheral blood lymphocyte samples. Prototype strains (proto 1–4) and strains M64956, M64938, M64949, M64937, M64939, X76145-X76159, M60924-M60927, M60929, M60931-M60934, M85228, M85229, and M22343 were published as described in Results. Relations between N-terminal gB genotypes (bp 47–250) analyzed by sequence analysis software are given. Scale measures distance between sequences. Units indicate no. of substitution events.

in our study population, we sequenced a 315-bp fragment (bp 2075–2390) of gB from all 44 clinical strains (C1–C44). The sequences were aligned and compared with sequences of the prototype strains. In agreement with the previous study, the C-terminal sequences were more conserved than the N-terminal sequences, and similarities ranged from 93% to 100%. The sequences were displayed in a phylogenetic tree together with sequences of prototype strains, strain Towne (GenBank accession no. M22343), and two additional clinical isolates published by Chou (M60931, M60927) [11]. As shown in figure 3, the C-terminal sequences clustered into two different groups. Prototype sequences 1 and 2 clustered in one group, and prototype sequences 3 and 4 clustered in a second group. Thus, only two gBc genotypes could be distinguished.

Comparison of gBn and gBc genotypes with the gB type at the cleavage site. Previously it was found that some HCMV strains cross over from one gB genotype to another. One of 11 clinical HCMV strains and the Towne strain changed the gB genotype within the gene, and it was hypothesized that this was due to recombination [11]. Therefore, in the present study, the frequency of intragenic variation was analyzed. The genotypes of all HCMV strains determined at fragments corresponding to the N terminus, the C terminus, and the cleavage site of gB were compared. In 22 of 44 strains, the gBn or gBc (or both) genotypes were different from the gB type determined at the cleavage site (figure 4). Therefore, 50% of the strains showed intragenic variation. This clearly shows that changes of gB genotype occur frequently.

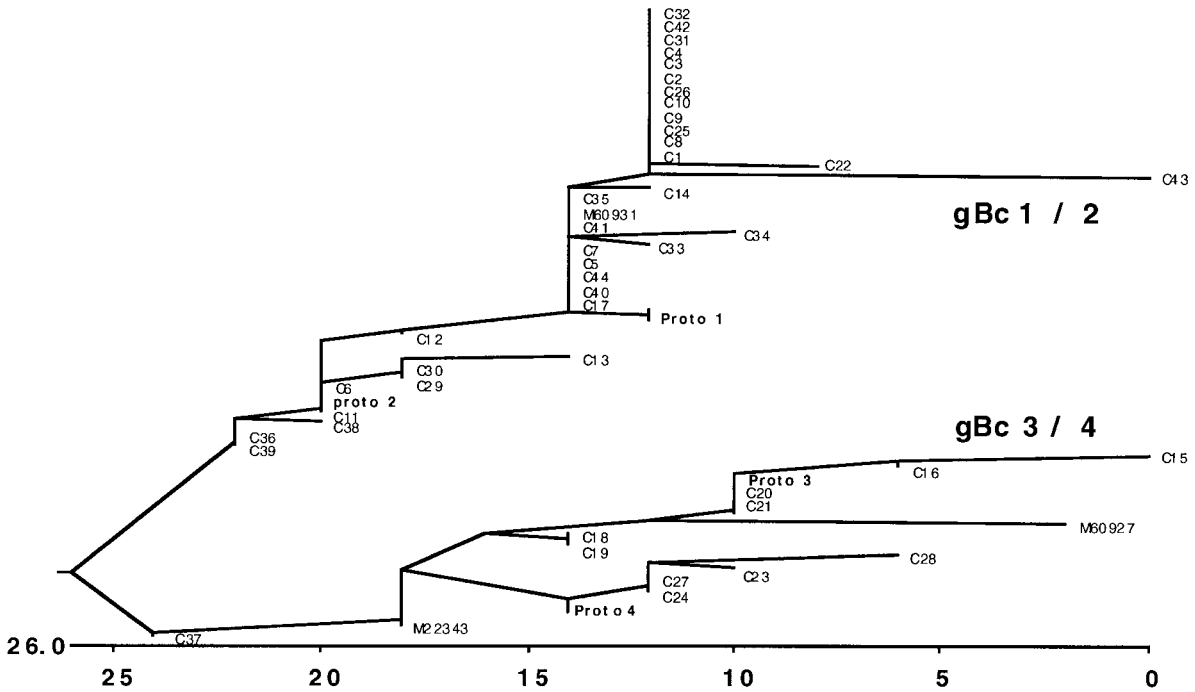

Figure 3. Phylogenetic tree analysis of C-terminal gB sequences. Strains C1–C30 were obtained from clinical isolates and strains C31–44 from peripheral blood lymphocyte samples. Prototype strains (proto 1–4) and strains M60927, M60931, and M22343 were published as described in Results. Relations between C-terminal gB genotypes (bp 2075–2390) analyzed by sequence analysis software are given. Scale measures distance between sequences. Units indicate no. of substitution events.

Furthermore, there was evidence that the frequency of intragenic variation is associated with the gB type at the cleavage site. gB type 1 was significantly less variable than gB type 2 (2-test, $P = .0094$). As shown in figure 4, only 3 (19%) of 16 strains with type 1 at the cleavage site changed genotype. In contrast, 12 (86%) of 14 strains with type 2 at the cleavage site changed genotype. Within the group of strains with types 3 and 4 at the cleavage site, 4 (57%) of 7 and 3 (43%) of 7 strains, respectively, changed genotype.

Discussion

Here we show that HCMV strains can be classified into four genotypes by RFLP of a fragment corresponding to the gB cleavage site, as previously proposed by Chou and Dennison [10, 11]. No additional gB types were found. Furthermore, we show that a fragment (bp 47–387) corresponding to the N-terminus of gB can also be classified into four genotypes and a fragment (bp 2075–2390) corresponding to the C-terminus of gB can be classified into two genotypes. Finally, we present evidence for frequent intragenic variability.

It has been reported that gB genotypes 2 and 3 are indistinguishable at the N-terminal region. Chou et al. [10, 11] classified virus strains with <6 amino acid exchanges in a major variable N-terminal region (bp 81–201) as one gB genotype. Since gB types 2 and 3 show only 2 amino acid exchanges in this major variable region and only 5 clinical strains were analyzed, they were indistinguishable. We analyzed a longer fragment (bp 47–387) of the N-terminal region, in which 4 amino acid exchanges occurred. This allowed us to discriminate the clearly related gB types 2 and 3 in a higher number of clinical samples.

In accordance with the Portland study, a higher degree of homology was found at the C-terminus of gB (bp 2075–2392). However, the high number of clinical isolates analyzed in the present study allowed us to distinguish two genotypes in this region (gBc type 1/2 and gBc type 3/4). In clinical isolates, both genotypes could be detected, whereas the sequences obtained directly from PBL belonged to gBc type 1/2 only. This may be a distorted picture due to the lower number of PBL samples ($n = 14$) than virus isolates ($n = 30$). Furthermore, this may be due to a different tissue tropism, since only 5 of 30 virus isolates were obtained from PBL, all showing the gBc 1/2 configuration.

Several studies from different geographic areas (western Europe, China, USA) have determined sequences of the gB gene from clinical isolates [10–12, 18]. These sequences, however, have so far never been compared. We aligned the published sequences with sequences we obtained from the N- and C-terminal parts of gB. We could show that the presence of a limited number of gB genotypes is not due to local epidemiology but is observed in different geographic areas.

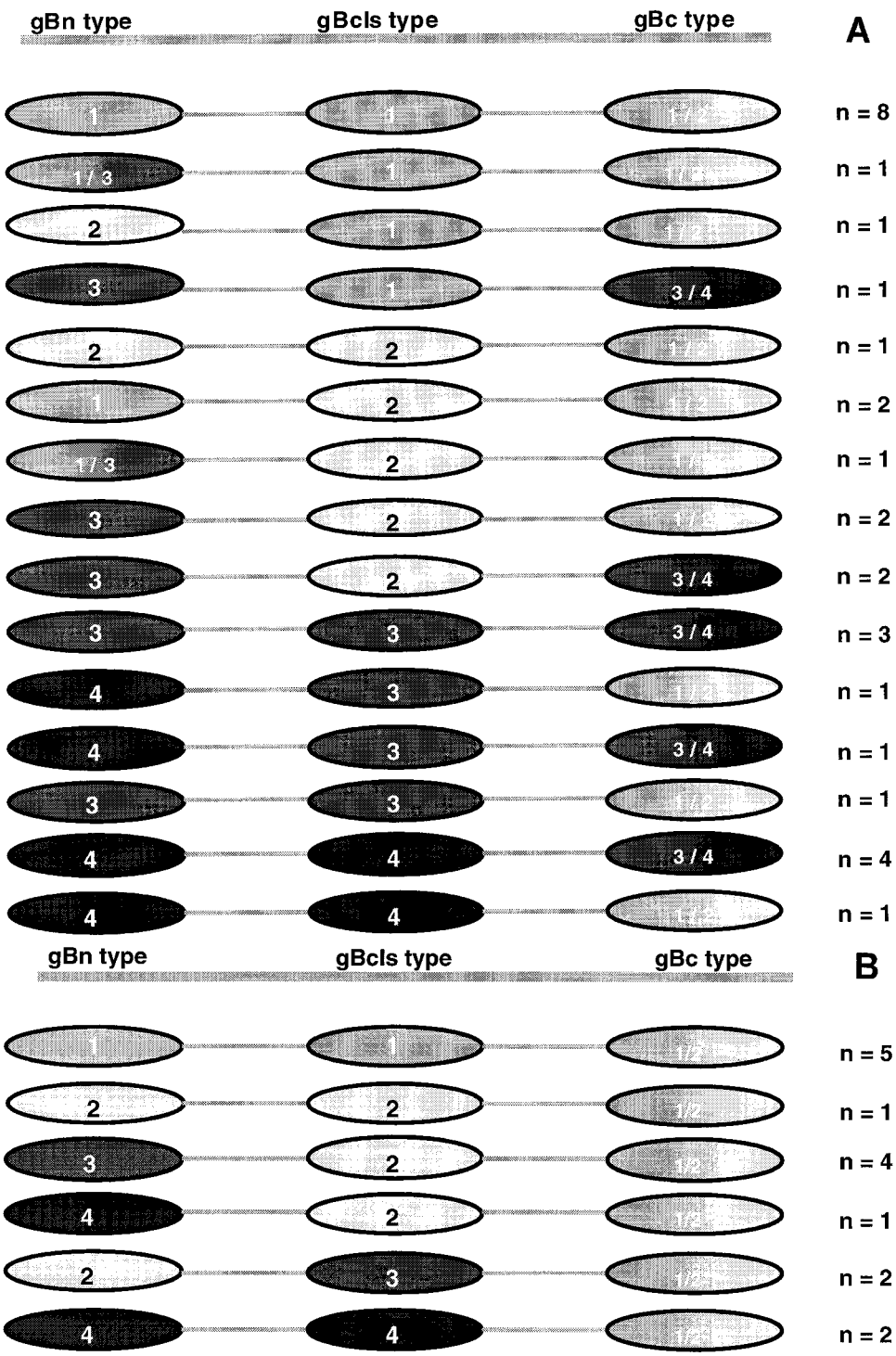

Figure 4. Comparisons of gB genotypes determined in clinical isolates (**A**) and in peripheral blood lymphocyte samples (**B**). gB genotypes were determined in fragment corresponding to N-terminus of gB (gBn type), cleavage site (gBcls type), and C terminus (gBc type). n = no. of samples investigated.

At the N-terminus of gB, not all HCMV strains could be classified according to the genotyping scheme. Two of 44 clinical HCMV strains showed high similarity to a gBn 1 genotype and changed, downstream of bp 146, to a genotype similar to gBn 3.

Chou [11] and Shiu et al. [18] also describe strains (M60927, M22343, and X76152) with the typical picture of one genotype, then crossing over to a different genotype [11, 18]. Therefore, deviations from the typical four genotypes at the N-terminus are rare, but a low number of aberrant gB variants circulate in the population.

Furthermore, it was suspected that these changes in gB genotype were due to recombination [10, 11]. We therefore tested

the frequency of intragenic variability in our samples. The gB types determined at the N- and C-terminal fragments of the gB gene (gBn and gBc types) were compared with the gB types determined at the cleavage site. In 22 (50%) of 44 strains, the genotype was not consistent in these three regions. We excluded the existence of an artifact due to genetic recombination during culture of strains derived from doubly infected patients because changing gB types were also found in native PBL samples without prior virus isolation. We could also show that the frequency of variation was associated with the gB type at the cleavage site. HCMV strains of gB type 1 at the cleavage site were significantly less variable than those of gB type 2 ($P = .0094$). This phenomenon cannot be explained at present because the mechanism for gB variation is not known. It can only be speculated that the immunologic pressure on HCMV strains differs and that this may lead to a different frequency of variation.

Taken together, our data show that variation within the gB gene of HCMV occurs frequently. Further in vitro studies will be necessary to clarify whether gB variation is due to recombination.

In previous studies, a high frequency of gB variation was not observed. Those studies sequenced HCMV strains obtained in the years 1985 to 1990 [10, 12]. One may speculate that with progression of the HIV epidemic, HCMV infections with multiple strains may have occurred more frequently and may have led to the occurrence of variant strains circulating in the population. The number of patients we studied here, however, was too small to investigate whether virus variants were detectable more frequently in patients with primary infections than in patients with reactivation of infection.

In summary, our study clearly shows that it is not sufficient to characterize gB genes at only the cleavage site. At least two hypervariable regions (N-terminus and cleavage site) have to be analyzed. These findings should be taken into account in studies that correlate the gB genotype with the clinical outcome of HCMV infection and might be important for vaccine development.

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