Species-Specific TT Viruses in Humans and Nonhuman Primates and Their Phylogenetic Relatedness[1]

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By means of polymerase chain reaction with a primer pair (NG133-NG147) deduced from the untranslated region (UTR) of TT virus (TTV), TTVs with markedly distinct genomic lengths were recovered from sera of humans and nonhuman primates, and their entire nucleotide sequences were determined. A human TTV [TGP96 of 2908 nucleotides (nt)] was obtained that was about 900 nt shorter than heretofore reported TTVs (3787–3853 nt). Likewise, TTVs of chimpanzee occurred in two distinct genomic sizes [Pt-TTV6 (3690 nt) and Pt-TTV8-II (2785 nt)]. Two TTVs of Japanese macaque [Mf-TTV3 (3798 nt) and Mf-TTV9 (3763 nt)] were comparable in genomic length, but only 55% similar in sequence. These five human and nonhuman primate TTVs, along with TTVs of tamarin [So-TTV2 (3371 nt)] and douroucouli [At-TTV3 (3718 nt)], were compared over the entire nucleotide sequence. Although the seven TTVs were only ≤55% similar, they share a common genomic organization with two open reading frames (ORFs), designated ORF1 (654–735 amino acids) and ORF2 (91–152 amino acids). The N-terminal sequences of ORF1 proteins were rich in arginine, and sequence motifs necessary for transcription and replication were conserved among them all. Like the human prototype TTV (TA278), all seven TTVs from various animals possessed in common two 15-nt sequences (CGAATGGCTGAGTTT and AGGGGCAATTCGGGC) in the UTR that were covered by NG133 and NG147, respectively. These primers would be instrumental in research on TTVs in previously unexamined species for defining their virological characteristics and evolutionary relationships. © 2000 Academic Press

INTRODUCTION

TT virus (TTV) is a nonenveloped, single-stranded, and circular DNA virus of about 3.8 kb (Miyata *et al.,* 1999; Mushahwar *et al.,* 1999; Okamoto *et al.,* 1999a) that was discovered in a patient with posttransfusion hepatitis of unknown etiology (Nishizawa *et al.,* 1997; Okamoto *et al.,* 1998b). It is provisionally classified into the *Circoviridae* family (Miyata *et al.,* 1999; Okamoto *et al.,* 1999a), but differs considerably in the nucleotide sequence and genomic size from known animal circoviruses including porcine circovirus (PCV), beak and feather disease virus (BFDV) of parrots (Lukert *et al.,* 1995), and chicken anemia virus (CAV), now given a new genus, *Gyrovirus* (Pringle, 1999).

TTV has an extremely wide range of sequence divergence by which it is classified into at least 16 genotypes with sequence differences of >30% from one another (Mushahwar *et al.,* 1999; Okamoto *et al.,* 1998b, 1999b; Simmonds *et al.,* 1998; Takayama *et al.,* 1999; Tanaka *et al.,* 1998). The prevalence of TTV DNA, detectable by PCR with primers deduced from the untranslated region (UTR), is very high (up to 98%) in the general population (Itoh *et al.,* 1999; Takahashi *et al.,* 1998). TTV is transmitted parenterally by transfusions and intravenous drugs (Nishizawa *et al.,* 1997; Okamoto *et al.,* 1998b). It may also spread by a fecal–oral route, because TTV DNA is detected in the bile and feces (Okamoto *et al.,* 1998a; Ukita *et al.,* 1999), as well as saliva (Ross *et al.,* 1999), of infected individuals. Such a double mode of transmission presumed for TTV would enhance infection throughout a community.

There have been increasing lines of evidence for TTVs infecting nonhuman primates and other animals (Abe *et al.,* 2000; Leary *et al.,* 1999; Okamoto *et al.,* 2000a; Romeo *et al.,* 2000; Verschoor *et al.,* 1999). Furthermore, a circular single-stranded DNA virus resembling TTV, but with a smaller genomic size of approximately 2.9 kb, has been isolated from healthy individuals and tentatively designated TTV-like minivirus (TLMV) (Takahashi *et al.,* 2000). Hence, TTV would make a large "swarm" of circular single-stranded DNA viruses (Khudyakov *et al.,* 2000) that may infect animals frequently. In this study, complete nucleotide sequences of various genomic lengths were determined on seven TTVs from humans and nonhuman primates. Comparison of their sequence and genomic organization has disclosed marked variation as well as

TABLE 1

Positions and Nucleotide Sequences of Oligonucleotide Primers

Primer	Polarity	Nt position	Nucleotide sequence
Human (TGP96)			
NG353	Sense	230–254	5′-TGA ACT TGG GCG GGA GCC GAA GGT G-3′
NG354	Antisense	205–229	5′-GCC TGG AGT CAC TGA AGT GAT CCC G-3′
NG355	Sense	234–266	5′-GAG CCG AAG GTG AGT GAA ACC ACC-3′
NG356	Antisense	193–216	5′-GAA GTG ATC CCG TCT CCG TCT GGC-3′
Chimpanzee			
Pt-TTV2-II			
NG357	Sense	UTR[a]	5′-GGG AGC CGA AGG TGA GTG AAA CCA C-3′
NG358	Antisense	UTR	5′-GCC CTA GTC CAG CGA AGA TCA CCT TG-3′
NG359	Sense	UTR	5′-TGA GTG AAA CCA CCG TAG TCT AGG-3′
NG360	Antisense	UTR	5′-TGT GCT CTT CTC TCC GTC TAG CGG-3′
Pt-TTV8-II			
NG361	Sense	203–228	5′-GGC GGG TGC CGA AGG TGA GTG AAA CC-3′
NG362	Antisense	177–202	5′-CAG GGA CAG CGA AGT TAG TCT GCG AC-3′
NG363	Sense	218–241	5′-TGA GTG AAA CCA CCG AAG TCA AGG-3′
NG364	Antisense	156–179	5′-GAC TAT CTT CTC TCC GTC TAG CGG-3′
Japanese macaque (Mf-TTV9)			
NG345	Sense	166–189	5′-CCT GGT GAG TGA CCT CGG CCG CCC-3′
NG346	Antisense	142–165	5′-CTG CGC GGT TCG TCC TCG CCT GCG-3′
NG349	Sense	190–213	5′-GTG GGC GGG TGC CGA AGC GGG AGG-3′
NG350	Antisense	132–155	5′-CGT CCT CGC CTG CGT CTC GGG GGA-3′
Tamarin (So-TTV2)			
NG299	Sense	353–377	5′-AGC CCG TGG GCG GGA CCC GCA GAG C-3′
NG300	Antisense	329–352	5′-GCG GAT GCC TCT CCC TCC GGT CGC-3′
NG301	Sense	353–383	5′-AGC CCG TGG GCG GGA CCC GCA GAG CAT TCC G-3′
NG302	Antisense	323–352	5′-GCG GAT GCC TCT CCC TCC GGT CGC GTC TCC-3′

[a] Nt positions of Pt-TTV2-II primers were not specified, because the entire sequence of Pt-TTV2-II was not determined.

the evolutionary relationship of TTVs in primates for further characterization of this unusually prevalent and ubiquitous virus.

RESULTS

Two kinds of human TTVs with distinct UTR sequences

Of 574 sera from Japanese blood donors, 544 (94.8%) were positive for TTV DNA by UTR PCR (Itoh *et al.,* 1999). In 6 of the 544 (1.1%) positive sera, however, the products of 110 nt were not amplified in the second round of UTR PCR. When the amplification products by the first round of UTR PCR of the 6 sera were sequenced, their lengths (primer sequences at both ends were excluded) were 92 nt in 5 sera and 89 nt in the remaining serum. They were shorter than the 95 nt predicted for the 14 TTV isolates of various genotypes for which the entire genomic sequences are determined [TA278 (Accession No. AB017610), GH1 (AF122913), JA9 (AF122915), and JA20 (AF122914) of genotype 1; JA1 (AF122916), JA4 (AF122917), US32 (AF122921), and US35 (AF122920) of genotype 2; T3PB (AF247138) of genotype 3; TUS01 (AB017613) and TUPB (AF247137) of genotype 11; TJN01 (AB028668) of genotype 12; and TJN02 (AB028669) and SANBAN (AB025946) of genotype 13]. The products in

the first-round UTR PCR of the 6 TTV isolates (89–92 nt) were 75.3–96.7% similar to one another, but they were only 59.6–65.1% similar to the reported 14 TTVs. These results pointed to two kinds of human TTVs that possess distinct UTR sequences.

Entire nucleotide sequences of seven TTVs from humans and nonhuman primates

As listed in Table 1, nested and inverted primers were deduced from UTR sequences of 90–106 nt in TTVs of humans (TGP96, TA1817, and TM1251), chimpanzees (Pt-TTV2-II and Pt-TTV8-II), Japanese macaques (Mf-TTV7-II, Mf-TTV8-I, and Mf-TTV9), and cotton-top tamarin (So-TTV2). They were used as species-specific primer pairs for amplification of full-genomic sequences of nine TTVs by PCR. The migration patterns of the products of the second round of inverted full-length PCR are shown for TTVs in the nine sera from different species (Fig. 1).

Two kinds of TTVs of distinct genomic lengths were amplified in sera from both humans and chimpanzees. Three TTV DNAs from humans (lanes 1–3 in Fig. 1) and two from chimpanzees (Fig. 1, lanes 4 and 5) migrated to a position of 2.7–2.9 kb. They were much shorter than reported human TTV DNAs of 3.8 kb in size and the five chimpanzee TTV DNAs on a phylogenetic branch repre-
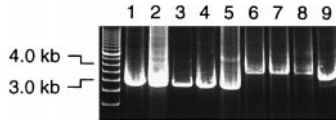
FIG. 1. Agarose gel electrophoresis of full-length TTV DNAs amplified by PCR with inverted nested primers. Migration positions of amplified full-length TTV genomes are shown for three human TTVs [lanes 1–3 (TGP96, TA1817 and TM1251)], two chimpanzee TTVs [lanes 4 and 5 (Pt-TTV2-II and Pt-TTV8-II)], three Japanese macaque TTVs [lanes 6–8 (Mf-TTV9, Mf-TTV8-I and Mf-TTV7-II)], and one cotton-top tamarin TTV [lane 9 (So-TTV2)]. The migration pattern of a molecular size marker [500-bp DNA ladder (TaKaRa Shuzo)] is shown on the left.

sented by Pt-TTV6 and with estimated sizes of 3.7 kb (Okamoto et al., 2000a). Three TTV DNAs from Japanese macaques (Fig. 1, lanes 6–8) migrated to a position of 3.7–3.8 kb, while the DNA from cotton-top tamarin (Fig. 1, lane 9) migrated to approximately 3.4 kb.

Seven representatives were selected from human and nonhuman TTVs of distinct sizes, and their entire genomic sequences were determined (Fig. 2). The representatives included one human TTV [TGP96 (2908 nt)],

two chimpanzee TTVs [Pt-TTV6 (3690 nt) and Pt-TTV8-II (2785 nt)], two Japanese macaque TTVs of similar sizes but with a marked sequence divergence [Mf-TTV3 (3798 nt) and Mf-TTV9 (3763 nt)], one cotton-top tamarin TTV [So-TTV2 (3371 nt)], and one douroucouli TTV [At-TTV3 (3718 nt)]. They all were deduced to be single-stranded from their behavior in digestion with S1 nuclease or mung bean nuclease, as was the genomic DNA of the human prototype TTV (TA278) (Okamoto et al., 1998b).

The seven TTVs were ≤55% similar over the entire nucleotide sequence of 2785–3798 nt. There were two chimpanzee TTVs of remarkably distinct genomic lengths. The longer one [Pt-TTV6 (3690 nt)] corresponded to the human prototype TTV (TA278) of 3853 nt (Okamoto et al., 1999a) and the shorter one [Pt-TTV8-II (2785 nt)] to a human TTV of a small genomic size [TGP96 (2908 nt)]; they were only 55.0 and 52.6% similar in sequence. Two TTVs of comparable genomic lengths from Japanese macaques [Mf-TTV3 (3798 nt) and Mf-TTV9 (3763 nt)] were only 54.8% similar. TGP96 and Pt-TTV8-II were 52.4–54.4% and 57.6–58.8% similar, respectively, to three human TTVs tentatively designated TLMV [CBD203
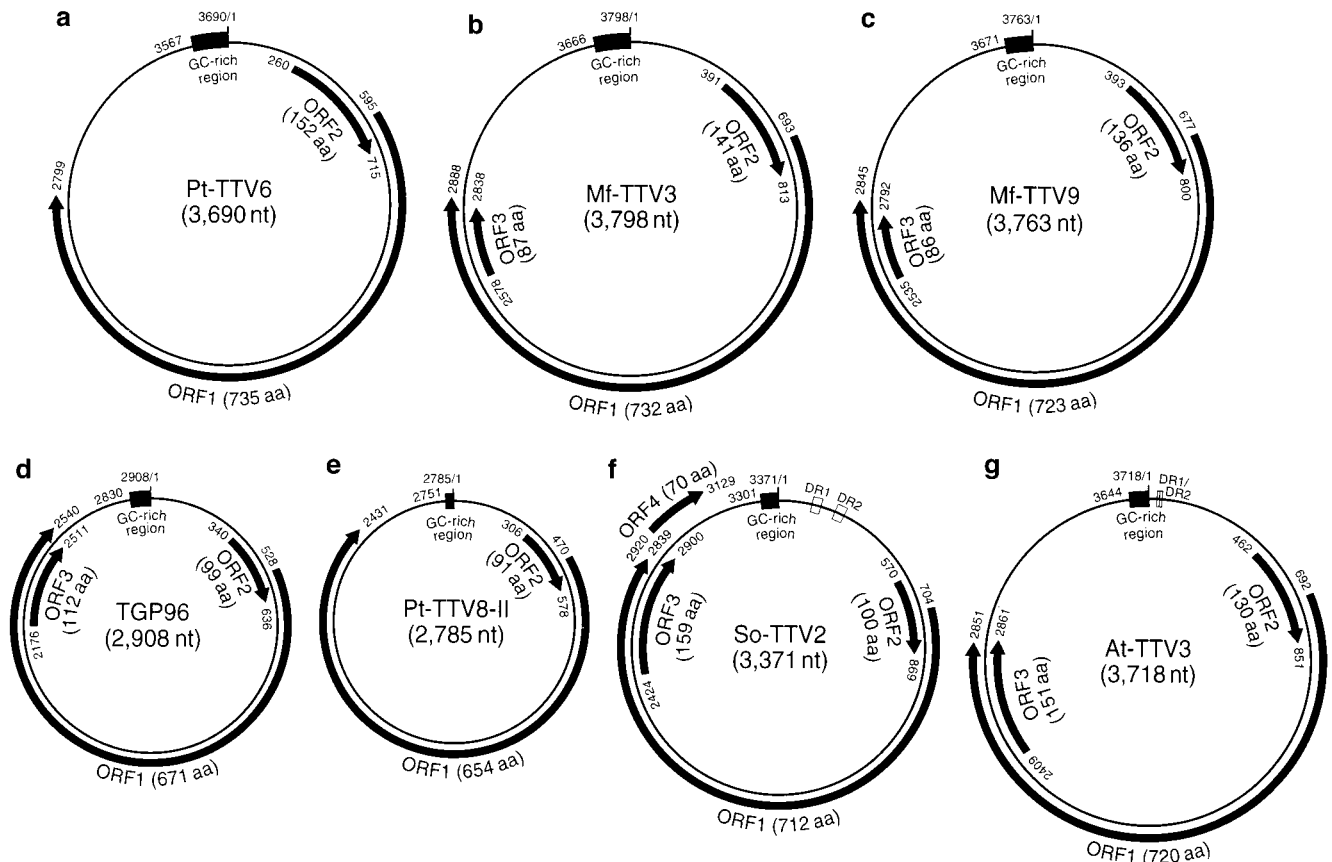
FIG. 2. Genomic organization of the seven TTVs from a human and nonhuman primates. The circumference of each circle represents the relative size of the genome. Arrows represent ORFs; ORF1 and ORF2 were shared by them all. ORF3, present in human TTVs except for those of genotype 1, was found in five of the TTVs. ORF4 was found only in cotton-top tamarin TTV (So-TTV2), which may correspond to the ORF encoding 56–58 aa in human TTVs, except for TJNO2 of genotype 13 (Erker et al., 1999; Ukita et al., 2000). A closed box indicates the GC-rich region, and open boxes indicate direct repeats with the nucleotide sequence specific for each TTV.

TABLE 2

Genomic Length and Key Elements of Species-Specific TT Viruses from Humans and Nonhuman Primates

Isolates	Genomic length (nt)	Nucleotide position (length) of					
		TATA box (ATATAA)	Sp1 site (GGGCGG)	Cap site (GGGGCAATT)	Coding region	Poly(A) signal (AATAAA)	GC-rich region
TA278[a]	3853	85–90	171–176	209–217	263–3074 (2812 nt)	3073–3078	3732–3853 (122 nt)
Pt-TTV6	3690	71–76	160–165	198–206	260–2799 (2540 nt)	2986–2991	3567–3690 (124 nt)
Mf-TTV3	3798	157–162	248–253	286–294	391–2888 (2498 nt)	3088–3093	3666–3798 (133 nt)
Mf-TTV9	3763	97–102	192–197	232–240	393–2845 (2453 nt)	3068–3073	3671–3763 (93 nt)
TGP96	2908	154–159	237–242	275–283	340–2540 (2201 nt)	2736–2741	2830–2908 (79 nt)
Pt-TTV8-II	2785	119–124	202–207	240–248	306–2431 (2126 nt)	2636–2641	2751–2785 (35 nt)
So-TTV2	3371	273–278	360–365	403–411	570–3129 (2560 nt)	3132–3137	3301–3371 (71 nt)
At-TTV3	3718	254–259	341–346	381–389	462–2861 (2400 nt)	3073–3078	3644–3718 (75 nt)

[a] The human prototype TTV (TA278) is shown as a reference (Okamoto et al., 1998b, 1999a).

(2897 nt), CBD231 (2860 nt), and CBD279 (2856 nt)] (Takahashi et al., 2000).

Major elements required for transcription, such as a TATA-box, Sp1 site, and poly(A) signal, were conserved in the seven TTVs, irrespective of distinct species and sizes, and the positions of their sequence motifs were well maintained with respect to coding regions (Table 2), as in the 14 human TTVs for which the entire sequence is known.

UTR sequences of the seven human and nonhuman primate TTVs

UTRs in the seven TTVs spanned 659–1318 nt and contained a GC-rich region of 35–133 nt in the middle (Fig. 2 and Table 2). Characteristic stem and loop structures were constructed by the GC-rich region and neighboring sequences (Fig. 3). Putative secondary structures of Pt-TTV6 and Mf-TTV3 genomes, forming a cross, resembled those of previously reported human TTVs (Okamoto et al., 1999a; Ukita et al., 2000). A similar cross-like structure was observed in the other TTV from Japanese macaque (Mf-TTV9). The predicted stem and loop structures of the other four TTVs were much different, however. TGP96, representing human TTVs of small genomic sizes, contained three successive direct repeats of 27 nt forming hairpins (DR1, DR2, and DR3). Pt-TTV8-II, representing chimpanzee TTVs of small genomic sizes, contained stem and loop structures giving rise to two GC-rich sequences that were different from each other. So-TTV2 also contained two stem-loop structures formed by

GC-rich sequences, with two direct repeats of 31 nt, downstream of the second stem–loop. At-TTV3 possessed two stem–loop structures composed of GC-rich sequences and a third large stem–loop structure, the loop of which contained a sequence [TAATTATGC (the same nt underlined)] similar to a nonamer motif (TANTAYTMS) conserved in plant and animal circoviruses (with the exception of CAV) that is required for replication (Bassami et al., 1998; Mankertz et al., 1998).

The common well-conserved sequence was identified in all seven TTVs 71–273 nt downstream of the end of GC-rich region (Fig. 4a). In addition to the above-mentioned TATA-box and Sp1 site, a sequence (GGGGCAATT) closely resembling the presumed cap site on the common sequence (GGGGCAAAT) in the 5′-ends of mRNAs of human respiratory syncytial virus genes (Collins et al., 1984), was found in all seven TTVs. A direct repeat sequence (GGGCAA) conserved in TTVs is identified in CAV also. Most remarkably, all seven TTVs from humans and nonhuman primates shared two conserved sequences of 15 nt (CGAATGGCTGAGTTT and AGGGGCAATTCGGGC), which exist in the 3′-terminal parts of the NG133 (sense) and NG147 (antisense) primer pair used in the first round of UTR PCR (Okamoto et al., 1999b, 2000a). These sequences are also maintained in all reported TTVs as well as TLMVs (Takahashi et al., 2000).

Figure 4b depicts a phylogenetic tree constructed using conserved sequences of the seven TTVs (Fig. 4a) along with those of human TTVs of distinct genotypes and two CAVs. Pt-TTV6 and Mf-TTV3 were close to hu-

a Pt-TTV6
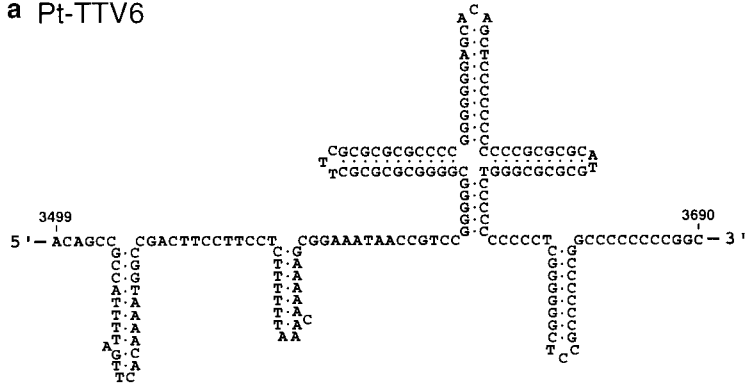
b Mf-TTV3

c Mf-TTV9

d TGP96
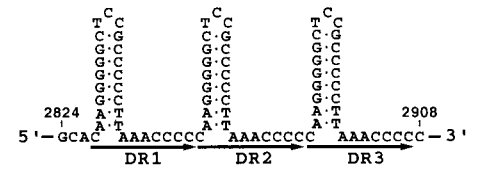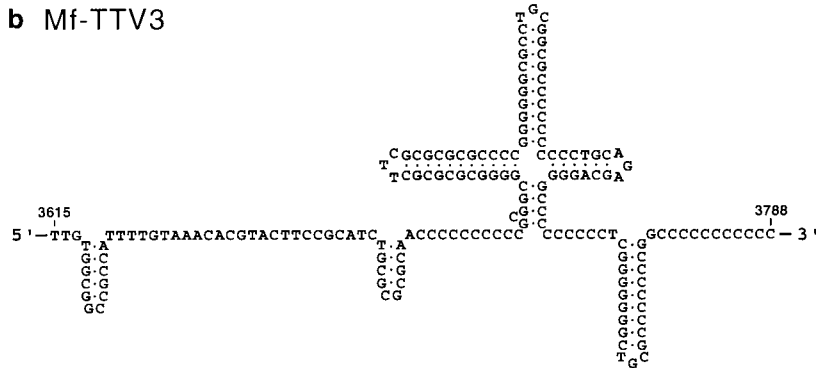
e Pt-TTV8-II
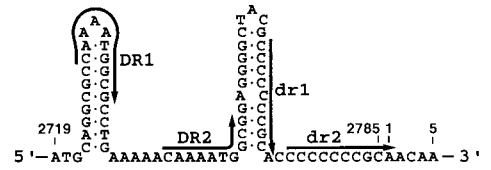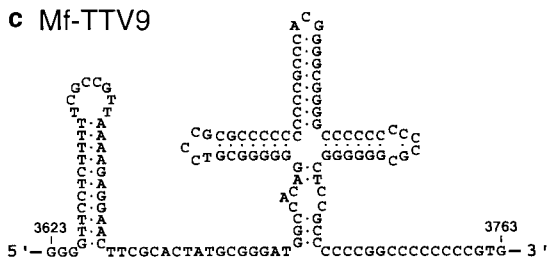
f So-TTV2
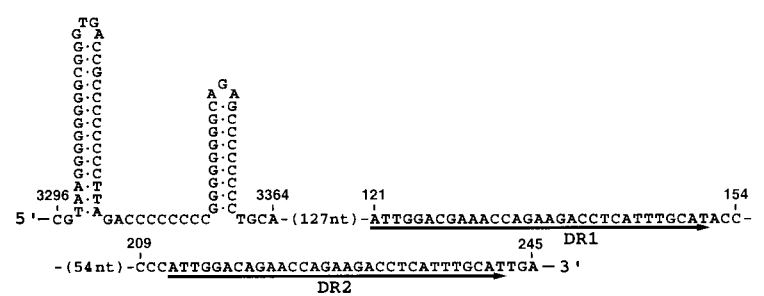
g At-TTV3
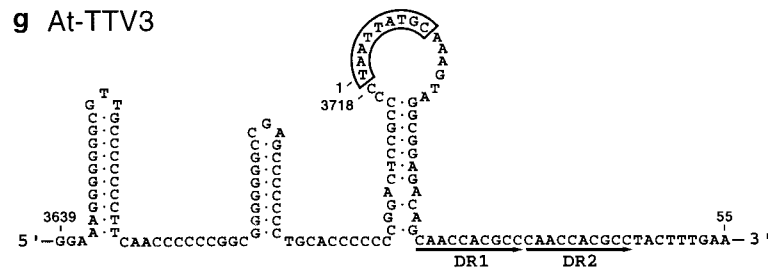
FIG. 3. Predicted secondary structures formed by a central portion of the UTR including the GC-rich region. The configurations are shown for seven TTVs from a human and nonhuman primates. Watson–Crick basepairings are marked by dots in between bases. Arrows represent direct repeats. A conserved sequence resembling the nonamer motif [TANTAYTMS (Bassami *et al.,* 1998; Mankertz *et al.,* 1998)] in a hairpin loop in At-TTV3 is boxed.

man TTVs of any genotype. Human and chimpanzee TTVs of smaller genomic sizes (TGP96 and Pt-TTV8-II, respectively), as well as TLMVs (CBD203 and CBD231), were clearly closer to human and nonhuman primate TTVs of larger genomic sizes than to CAVs.

Coding region sequences in the seven TTVs from humans and nonhuman primates

The seven TTVs possessed ORF1 and ORF2 like those in reported human TTVs of 3.8 kb (Fig. 2). ORF1s in the seven TTVs coded for 654–735 aa and were rich in Arg in

a

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            TATA-box        NG133
TA278(1)    ATATAAGTAAGTGCACTTCCGAATGGCTGAGTTTTCCACGCCCGTCCGCAGCGGTGAAGCCACGGAGGGAGATCTCCGCG/////////TCCCG
JA1(2)      ------|--GGCA-------------------------------------G-A-AG-G-----------TGATC-CGAA--//////////-----
T3PB(3)     ------|---GC-------------------------------------------------------C--AG----//////////-----
TUS01(11)   ------|-----------------------------------------AGA-C-----------GAG--CG----//////////-----
TJN01(12)   ------AG--CA---------------------------------------AG--GCAGC-A--------CG----//////////-----
SANBAN(13)  ------C---C---------------------------------------AG-G---G-------AGCTC-CGA--//////////-----
Pt-TTV6     ------C--GC--------------------------------AC-GAGGAGGCGCTGCC-CCGA-CG----TCG//////----
Mf-TTV3     ---A-|--------A-T-----------------------ATACT-T---G-----GTCG-TG-AGGA-CGACAGT-A-GA-C-CAGA///CCAG---/
Mf-TTV9     ---|--CCG-A---------------------------T-C-GA-A-GCAG---AG--C-AAC--CGCA-CCTGG-GA-T-ACCTCGGCCG----
TLMV-CBD231 ------C-----A---AGA---------------AT----A-A-G-AGA---CATCAGA--ACT-ACT-CCGG-T-ATC////////////T
TLMV-CBD203 ---A-|C---C--A-A-GA-----------------ATGC----TA-A-G-AGAGAAGAC--GTTACTGATCTTC//G-TAA////////////-TC
TGP96       ------CC---A------------------------ATGC----A-A-G-AGA---GATCA-TT-A-T-ACTCCAGG-T-AA////////////-T
Pt-TTV8-II  ------|TTC--C-----------------------ATGC----TA-A-G-AGAGAAGAT--T-G-A--CTA-CT--G-T-TC///////////-C
So-TTV2     ------|AG--CC-GGG-GA--------------------A-TC----G-GTGTAG-A-AC-CGA--GGA-G-A---GCA-------AG//////////----
At-TTV3     ------|C--------GA----------------------A--GT---G-GGA------TCTGCCGAG-----C--CGCGAC--AGCCGA////////C
CAV/M55918  -----TTG-GC/----A-A-CGG-C-GCAGTAGG-AT----AA-G-G-TCCG-----G-TG-----GAACG-CGGA-AA/////////////////
CAV/U65414  -----TTG-GC/----A-A-CGG-C-GCAGTAGG-AT----AA-G-G-TCCG-----G-TG-----GAACG-CGGA-AA/////////////////

            Sp1 site                                              NG147
TA278(1)    AGGGCGGGTGCCGAA//////GGTGAGTTTACACACCGAAGTCAAGGGGCAATTCGGGCTCGGGACTGGCCGGGC/TATGGGCAAG 164
JA1(2)      -------------G-//////------------------G---------|-------------------------/----------- 164
T3PB(3)     -------------T-//////----------------------------|-------------------------/----------- 164
TUS01(11)   -------------G-//////----------------C-----------|-------------------------/CCC------- 164
TJN01(12)   -------------G-//////----------------C-----------|-------------------------/CC--------- 164
SANBAN(13)  -------------G-//////-------C--------C-----------|-------------------------/----------- 164
Pt-TTV6     -----------A--CG-//////------------------G--T----|-------------------------/----------- 167
Mf-TTV3     T-------------G-//////----------------C-----------|------/A---/------------A-C--------- 168
Mf-TTV9     T-------------////GC--GAG------C-C-GCG------------|-------AA---CGGTCT---GA/CC------- 175
TLMV-CBD231 T--------A-------//////-------GA-AC------------T--|-------A-ATCAGTCTG-C-GA/-C------- 161
TLMV-CBD203 T--------A-------//////-------GA-AC------------T--|-------GA-TTAGTCTA-C-GA/CC------- 159
TGP96       T--------A-------//////-------GA-AC------T----T--|-------A-TTCAGTCTG-C-GA/CC------- 161
Pt-TTV8-II  T-------------//////-------GA-AC--------------|-------G--ACAGTCTA-C-GA/-C------- 161
So-TTV2     T------AC---C-GAGCATCC--AGG--TG-GG--C/--GT--|-------A----CTCCCGG-C-GA/CC------- 169
At-TTV3     T-------A---G/GAACAT/CC--AGG--TGTGG/AC/---T--|-------AG-A-CACCCG/-C--A/-C------- 166
CAV/M55918  CC---C-C--GG-GC//////A----A-CGG-G-TTA-CC-/AG--------CCT----/-CA-CGGA-----C--A//G------- 153
CAV/U65414  CC---C-C--GG-GC//////A----A-CGG-G-TTA-CC-/AG--------CCT----/-CA-CGGA-----C--A//G------- 153
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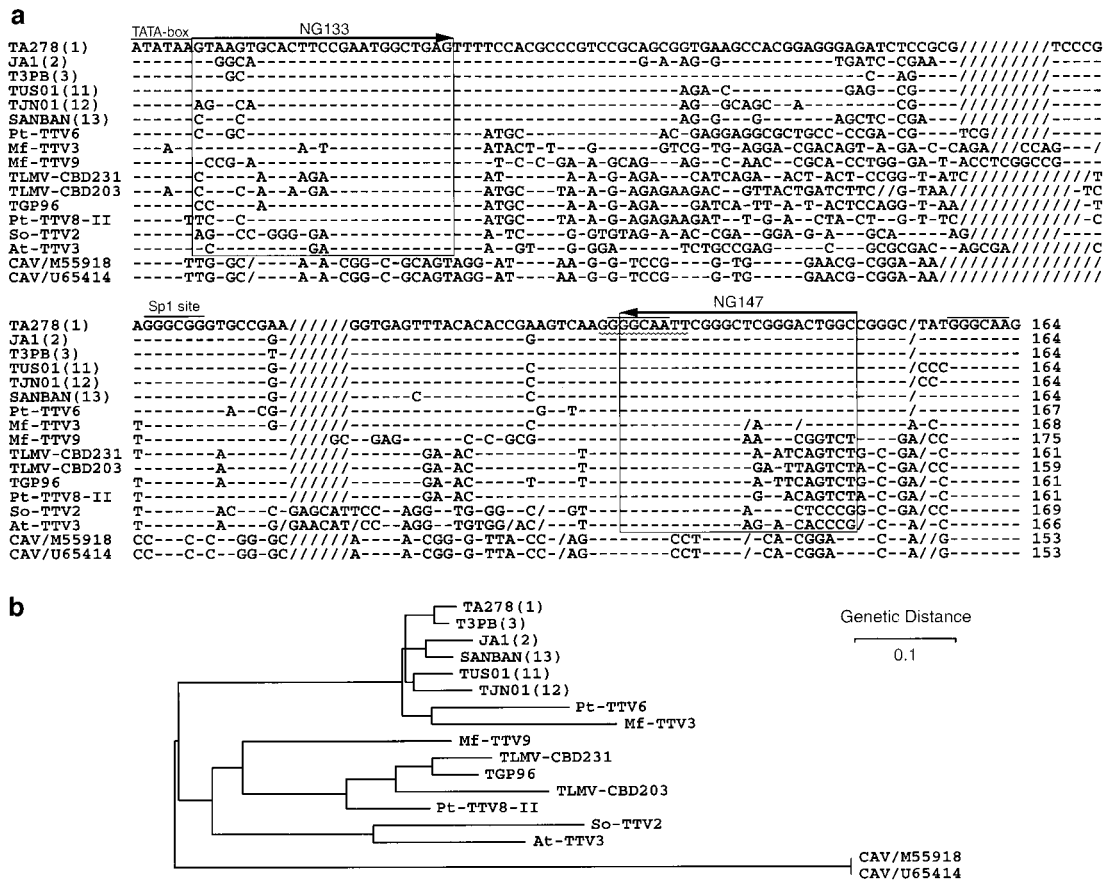
b

FIG. 4. Comparison of nucleotide sequences among human and nonhuman primate TTVs as well as CAV. (a) Nucleotide sequences of the conserved region in the UTR downstream of the GC-rich region are compared among TA278 of genotype 1 (Accession No. AB017610), JA1 of genotype 2 (AF122916), T3PB of genotype 3 (AF247138), TUS01 of genotype 11 (AB017613), TJN01 of genotype 12 (AB028668), SANBAN of genotype 13 (AB025946), TLMV-CBD231 (AB026930), and TLMV-CBD203 (AB026929), as well as CAV/M55918 and CAV/U65414. Overlined are the TATA box, the Sp1 site, and a direct repeat sequence. A putative cap site with the sequence GGGGCAATT is underlined with a wavy line. Sequences corresponding to primers NG133 (sense) and NG147 (antisense) are boxed. (b) A phylogenetic tree constructed using the sequences of 153–175 nt shown in (a) by the neighbor-joining method (Saitou and Nei, 1987). Bootstrap values were 100% for all nodes for 1000 resamplings of the data (Felsenstein, 1985).

their N-termini. As in reported human TTVs, nearly one-half (41–46 aa) of the N-terminal 100 aa were Arg in Pt-TTV6, Mf-TTV3, Mf-TTV9, So-TTV2, and At-TTV3. By contrast, human and chimpanzee TTVs of smaller genomic sizes (TGP96 and Pt-TTV8-II) had only 17 and 22 Arg residues in this region, respectively, comparable with 25 Arg residues in CAV. Likewise, the number of Phe residues was characteristic of the human and chimpanzee TTVs (Fig. 5a). Of the C-terminal 100 aa, on the other hand, CAV contained 3 Leu and 3 Gln residues, less than the seven TTVs of distinct genomic lengths that possessed 10–17 Leu and 7–17 Gln within this region.

Three of the four Rep protein motifs reported in animal circoviruses (Niagro et al., 1998) were conserved in all seven TTVs (Fig. 5b), except for motif 4 or the P-loop that represents a putative ATP/GTP-binding motif. In general, two His residues are conserved in motif 2, but one His in TTVs of humans and nonhuman primates is replaced by Arg, Lys, Gln, or Glu, which are physicochemically similar to His with respect to volume and polarity (Miyata et al.,

1979). Both His residues were conserved only in a TLMV (CBD231) (Takahashi et al., 2000).

Due to difficulties in constructing a phylogenetic tree using the full-genome sequence of TTVs, which markedly differ in sequence and size, a tree was constructed using the entire nucleotide sequence of 1962–2310 nt of ORF1 (Fig. 6). Human and chimpanzee TTVs of larger genomic sizes (TA278 and Pt-TTV6, respectively) were on one branch, while those of smaller genomic sizes (TPG96 and Pt-TTV8-II, respectively) were on another. The other nonhuman primate TTVs were separated from them all by a marked sequence divergence and resided on distinct branches. Although two TTVs from Japanese macaques (Mf-TTV3 and Mf-TTV9) bifurcated from the same branch, they were too divergent from each other to be classified in the same category. TTVs from the other two nonhuman primates in lower classes, tamarin (So-TTV2) and dourou-couli (At-TTV3), bifurcated from the branch harboring Japanese macaque TTVs, reflecting a close relationship of these three nonhuman primate TTVs.

a

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                                                                                                                    R    F
TA278(1)      MAYGWWRRRRRRWRRWRRRPWRRRWRTRRRRPARRRGRRRNVRRRRRGGRWRRRYRRWKRKGRRRKKAKIIIRQWQPNYRRRCNIVGYIPVLICGENTVS     44   0
TUS01(11)     MAYWFRRWGWRPRRRWRRWRRRRRLPRRRTRRAVRGLGRRRKPRVRRRRRTRRRTYRRGWRRRRYIRRGRRKKKLILTQWNPAIVKRCNIKGGLPIIIC      43   0
SANBAN(13)    MAWGWWRRWRRWPARRWRRRRRRRRPLRRRRAGRPARRYRRRRTVRTRRRRWGRRRYRRGWRRRTYVRKGRHRKKKKRLILRQWQPATRRRCTITGYLPIV     45   0
Pt-TTV6       MAWPWRRRRRWRRWRRRPRWRRRWRRRRTWRRRPRRAVRRRRVRRRGRGWRRLYRRYRRRRGRRTYKKKPVLTQWQPTVRRRCFIIGYMPLIICGENLTSKN     45   1
Mf-TTV3       MAWWWWRRWPRRRRRWRRWGRRRVGSWRRRGVAGRRRGRRVRRRRRRRVWRRGRRWRLRRRRRLRRYFRNTLILRQWQPTTIKSLRISGWFPGVVCSNRR      42   2
Mf-TTV9       MPWWPWRRRRRWRRRGNWRRRVAPRRYRRTARRAARRRKAVRRRRRRVRRRGYYRRYRRRRRYKRRLLILKQWQPTTNLRLTVKGLIPVVVMGKGKTQ       41   0
TLMV-CBD231   MPPFYYRRRWYPNRWRRTRLRFRRRRRFRRPIRKRFRRRRWVRRRRFFRFKRKLKSLNIKEWQPETIRKCHIKGNLCLLTCGRGNINHNYILTSESYVPTS    29   7
TLMV-CBD203   MPYFYRRWFPRRRRFRRSWRRRIRGPFRRKYWRRHWVRRYSRKRKLSKLNIKEWQPITIRKTKVKGLYPAFLCNDQRIGNNAIQYLDSIAPHHFPGGGGF     22   7
TGP96         MPYYYRRNYYPKWRRWRSRRNRFRRHFRRRTYRRRWVSNPFNKITLKQWQPPYKKTCYIKGQTCLIYYNNDRLGYNSTMYEKSIVPFHWPGGGSFSVSKY     17   5
Pt-TTV8-II    MPYYYRRNWRLPYRRRRWFWRRRPRPFIRRRHRRRRQPYFHKKLKKLKLQQYQPRTVRYCKIKGFQCLFEASKYRIGNNYTMYFNTFAPEHLPGGGG       22   8
So-TTV2       MAYGRRYWRRRRWRRRGGRWWRRRRPWKTRWRRRRRRWRRRYGRRARRFRRRHRVGRWRKLFRRVKSRVVRQWDPNVTRRCMINGYDHALFWGQQAQHR     42    3
At-TTV3       MVWRRRWWRRRRRPVWRRRYRRWRRRRRRWRRRPRRRRRPYRRRPYRRYGRRRKVRRRRRRTLVVRQFQPPYQRRCRVSGLEPLLYFGKKEEERHFYGNRQ     46   3
CAV/M55918    MARRARRPRGRFYSFRRGRWHHLKRLRRRYKFRHRRRQRYRRRAFRKAFHNPRPGTYSVRLPNPQSTMTIRFQGVIFLTEGLILPKNSTAGGYADHMYGA     25   7
CAV/U65414    MARRARRPRGRFYAFRRGRWHHLKRLRRRYKFRHRRRQRYRRRAFRKAFHNPRPGTYSVRLPNPQSTMTIRFQGIIFLTEGLILPKNSTAGGYADHLYGA     25   7
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b

```
                                   1                    2                3
                                futlt                 pHuHuuu          Yuxh
              consensus          uyp                   u    a                k

TA278(1)      266-FQVLQ-272      404-LEYHGGL-410       581-YRFK-584
TUS01(11)     273-FQVLR-277      416-YEYHCGW-422       579-YAYR-582
SANBAN(13)    275-FQVLK-279      399-YEYHLGM-405       445-YNTK-448
Pt-TTV6       264-FQVLG-268      385-LEYHAGI-391       562-YKFK-565
Mf-TTV3       163-FYRDP-167      234-FQAHFCN-240       378-YFDR-381
Mf-TTV9       259-FVNSP-263      332-NEIHTKI-338       423-YASQ-426
TLMV-CBD231   405-FLTLP-409      487-VHWHPKY-493       647-YRER-650
TLMV-CBD203   331-FGTSQ-335      514-CEAHFEY-520       590-YGTE-593
TGP96         219-FATAA-223      412-REGHGWD-418       505-YQTR-508
Pt-TTV8-II    101-FSIVK-105      307-RELHPTD-313       390-YLLK-393
So-TTV2       443-FCPYT-447      591-ARGHLGD-597       670-YDNH-673
At-TTV3       163-FWWTN-167      204-AKDHVTI-210       247-YIPR-250
CAV/M55918    313-FATLT-317      351-QRWHTLV-357       402-YALK-405
CAV/U65414    313-FATLT-317      351-QRWHTLV-357       402-YALK-405
BFDV/AF080560  14-F-TLN-17        50-PHLQGYF-56         89-YCSK-92
PCV/AF071879   16-F-TLN-19        53-PHLQGFA-59         93-YCSK-96
```

FIG. 5. Amino acid sequences of the ORF1 proteins of human and nonhuman primate TTVs. (a) N-terminal 100 amino acids in ORF1. Arg residues are indicated in boldface type. The number of amino acids is shown for Arg (R) and Phe (F) on the right. (b) Three of the four conserved amino acid sequence motifs of the Rep protein involved in rolling circle DNA replication. In the three consensus sequences, the amino acid residues conserved in almost all the proteins for rolling circle replication are in uppercase letters, and those conserved in at least half of them are in lowercase letters (Collins *et al.,* 1984). "u" denotes bulky hydrophobic residues [Ile (I), Leu (L), Val (V), Met (M), Phe (F), Tyr (Y), and Trp (W)] and "x" represents a lack of consensus at the indicated position.

ORF2 encoded 91–152 aa. A significant homology in the amino acid sequence was not observed in the translation products of ORF2s among human and nonhuman primate TTVs. The amino acid composition of ORF2 was comparable among them to some extent, however. For example, the TTV ORF2 contained less Ser and Gln than that of CAV. The conserved motif in ORF2 of reported human TTVs and CAVs (W-X$_7$-H-X$_3$-C-X$_1$-C-X$_5$-H) (Hijikata *et al.,* 1999; Noteborn *et al.,* 1991) was shared by TTVs of nonhuman primates.

An additional ORF, tentatively named ORF3 and encoding 86–159 aa, was found in the same frame as ORF2 only in Mf-TTV3, Mf-TTV9, TGP96, So-TTV2, and At-TTV3 among the seven TTVs. Like the corresponding ORFs in reported human TTVs of genotypes other than 1, ORF3s in the TTVs studied were rich in Ser (17–27 residues) and abundant in Arg and Lys (18–42 residues).

A fourth ORF (ORF4) was observed only in So-TTV2 encoding 70 aa in frame 3 bearing no other ORFs on it

and was positioned close to and downstream of the 3′-end of ORF1. Of 14 reported human TTVs with the entire sequence determined, the corresponding ORF is recognized in 13, except in TJN02 of genotype 13 (Ukita *et al.,* 2000), and encodes 56–58 aa. A conserved motif, E-X$_8$-R-X$_2$-R-X$_6$-P-X$_{12-19}$-F-X$_1$-L, was recognized in ORF4s in human TTVs and the cotton-top tamarin TTV (So-TTV2), but it was not identified in any of the three ORFs of CAV (M55918 and U65414), six ORFs of PCV (Y09921), or seven ORFs in BFDV (AF080560).

DISCUSSION

Since the discovery of TTV toward the end of 1997 (Nishizawa *et al.,* 1997), significant sequence heterogeneity of the TTV genome has been recognized within a remarkably short period of time. Initial studies determined TTV DNA of restricted genotypes by PCR with primers deduced from the coding region, and it was
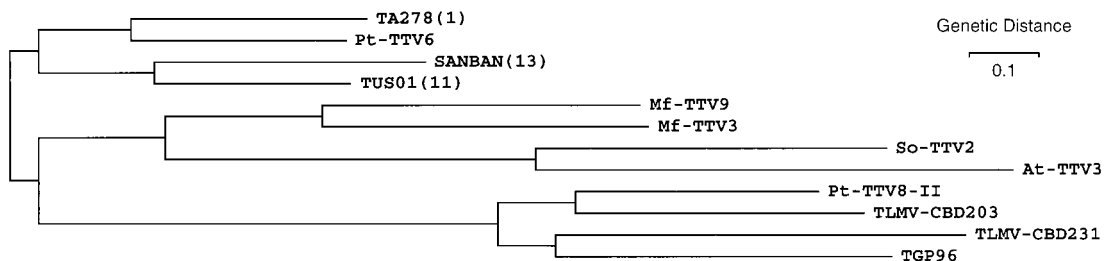

FIG. 6. A phylogenetic tree constructed using the entire nucleotide sequence of ORF1 by the neighbor-joining method (Saitou and Nei, 1987). Bootstrap values were 100% for all nodes for 1000 resamplings of the data (Felsenstein, 1985). Accession numbers are given in the legend to Fig. 4.

found in a few to 10% in the general population in the United States, England, and Japan (Charlton *et al.,* 1998; Okamoto *et al.,* 1998b; Simmonds *et al.,* 1998). As an extremely wide range of genomic divergence was disclosed, however, TTVs of at least 16 genotypes have been distinguished that differ by >30% over the entire or partial nucleotide sequence (Mushahwar *et al.,* 1999; Okamoto *et al.,* 1998b, 1999b; Simmonds *et al.,* 1998; Takayama *et al.,* 1999; Tanaka *et al.,* 1998). Despite outstanding sequence divergence, TTVs of any genotype have in common a conserved sequence in the UTR (Biagini *et al.,* 2000; Erker *et al.,* 1999; Hijikata *et al.,* 1999; Okamoto *et al.,* 1999a; Ukita *et al.,* 2000). This gave rise to universal UTR primers for use in PCR, which detect TTV DNA in the great majority of human beings, irrespective of their clinical status (Itoh *et al.,* 1999; Takahashi *et al.,* 1998).

Taking advantage of highly conserved UTR sequences, PCR with UTR primers was extended to sera from nonhuman primates (Abe *et al.,* 2000; Leary *et al.,* 1999; Okamoto *et al.,* 2000a). As a result, nonhuman primates including chimpanzees, Japanese macaques, tamarins, and douroucoulis have been found to carry species-specific TTVs at extremely high frequencies (Okamoto *et al.,* 2000a). Human TTV is particularly similar to chimpanzee TTV, so that chimpanzees are cross-infected with human TTV (Mushahwar *et al.,* 1999; Okamoto *et al.,* 2000a).

Two kinds of TTVs of distinct genomic sizes were recovered from humans and chimpanzees (Fig. 2). A human TTV of shorter genomic size (TGP96) corresponded to TLMV (Takahashi *et al.,* 2000). Human TTVs of larger and smaller genomic sizes, as well as chimpanzee TTVs of different genomic sizes, shared in common UTR sequences and genomic organization with two ORFs, although they differed in many other aspects. The question then is how to classify the two TTV species of distinct genomic sizes in humans and chimpanzees. In the interim, the two TTV species would better be regarded as members of TTV and distinguished by Roman numerals after dashes. Thus, human TTV with a larger genomic size is referred to as TTV-I and that with a smaller genomic size as TTV-II. Likewise, two species of chimpanzee TTVs may be called Pt-TTV-I and Pt-TTV-II.

A pair of universal primers (NG133–NG147) used in the first round of UTR PCR (Okamoto *et al.,* 1999b, 2000a) possess two 15-nt sequences (CGAATGGCTGAGTTT and AGGGGCAATTCGGGC) that helped detect TTVs in humans and nonhuman primates, because they were conserved in all human and nonhuman primate TTVs examined in the present study (Fig. 4). They might be useful for detecting TTVs in previously unexamined species; TTV-like sequences have been reported in cows, pigs, and sheep (Leary *et al.,* 1999).

In infected cells, viruses with a circular single-stranded DNA genome, such as TTVs, generate a dou-

ble-stranded DNA, which is needed for correct transcription and replication of the virus DNA (Noteborn *et al.,* 1991). Circular double-stranded TTV DNAs in replicative intermediate forms are detected in the liver and bone marrow cells (Okamoto *et al.,* 2000a,c), and TTV DNA is detected in hepatocytes from infected individuals by *in situ* hybridization (Rodriguez-Inigo *et al.,* 2000). It remains to be seen whether TTVs of smaller genomic sizes replicate in hepatocytes and hematopoietic cells like TTVs of larger sizes. This information would be required to determine whether two kinds of TTV genomes of distinct sizes exist in the same viral particle, as for some plant circoviruses (Boevink *et al.,* 1995; Burns *et al.,* 1995), or in separate viral particles and whether they depend on each other for replication. The detection of TTV-II in six individuals who were not infected with TTV-I lends support to the view that TTV-I and TTV-II would be in distinct viral particles and replicate independently.

Since TTV-IIs in six sera were recovered from human sera negative for TTV DNA in the second round of UTR PCR, it is not certain how many of the 538 sera testing positive in the second round, and for TTV-I by inference, would harbor TTV-II. The detection of species-specific TTV-I and TTV-II in two chimpanzees (Okamoto *et al.,* 2000a) reflects frequent coinfection with the two kinds of TTVs and suggests that it would be common in humans, also. In order to picture the epidemiology of TTV-II, PCR methods would need to be developed for specifically detecting DNA of TTV-II, in the presence of TTV-I, with primers from well-conserved regions of TTV-II. Like TTV-I, TTV-II may have many genotypes separated by a wide sequence divergence, which is predictable by comparison of only a few (Fig. 6).

Although two TTVs of Japanese macaques have comparable genomic lengths, they share only 55% of the nucleotide sequence. Whether the two belong to the same category of TTV or not needs to be determined by comparing their sequences against additional TTVs from Japanese macaques.

Despite a marked difference in genomic length and a wide divergence in sequence, TTVs from humans and nonhuman primates share conserved UTR sequences, which allows their detection by PCR with primers NG133 and NG147. They all possess a circular and single-stranded DNA genome with a similar genomic structure, expressing at least two major ORFs. They are suspected to replicate by a rolling circle mechanism as in known circoviruses (Niagro *et al.,* 1998; Noteborn *et al.,* 1991). Although sequence motifs for Rep protein, involved in rolling circle replication, are identified in the products of ORF1 of human TTV-Is, they seem to be only partially conserved (Erker *et al.,* 1999).

Rep protein motifs were identified in the product of ORF1 of all seven human or nonhuman primate TTVs examined in the present study (Fig. 5). One of the two His residues in motif 2 of most TTVs was replaced by Arg,

Lys, Gln, or Glu, which resemble His in polarity and molecular volume (Miyata *et al.,* 1979). A similar substitution of His for Arg is reported in CAV and that for Gln in PCV and BFDV (Ilyina and Koonin, 1992; Niagro *et al.,* 1998; Noteborn *et al.,* 1991), although Rep protein in PCV and BFDV is encoded by an ORF distinct from that for the capsid protein. In human TTV-I, motif 1 is immediately upstream of hypervariable region 1 (HVR1) and motif 2 is close to and downstream of HVR3; they are not in any of the three hypervariable regions (Nishizawa *et al.,* 1999) for excellent sequence conservation. Taken altogether, albeit Rep protein motifs of human and nonhuman primate TTVs were not typical (Fig. 5), they would be encoded by respective ORF1s as in CAV.

The data obtained in the present study suggest an outstandingly high degree of genetic complexity, as well as a common organization, within TTVs of humans and nonhuman primates. Whether homologous recombination among TTVs plays a role in the wide diversification of this virus remains to be explored (Worobey, 2000). Identification of a swarm of closely related but markedly different TTVs in humans and nonhuman primates would support their evolution over a long time. TTV deserves further study to determine its origin and to evaluate it for any disease-inducing capacity.

MATERIALS AND METHODS

Nonhuman primates

Two chimpanzees [*Pan troglodytes:* Nos. 201 (male, 5 years) and 258 (male, 9 years)] were bred in Kumamoto Primates Park (Sanwa Kagaku Kenkyusho Co. Ltd., Kumamoto, Japan). Pt-TTV6 was recovered from No. 201 and Pt-TTV8-II from No. 258; the chimpanzees had not received inoculation with human sera or received drugs at the time of blood sampling. Japanese macaque (*Macaca fuscata*), cotton-top tamarin (*Saguinus oedipus*), and douroucouli (*Aotes trivirgatus*) were caught in the wild and sera were obtained from the animals before they were used for medical experiments (Okamoto *et al.,* 2000a).

Extraction of nucleic acids and amplification by PCR

Nucleic acids were extracted from 50–100 μl of serum with a High Pure Viral Nucleic Acid Kit (Boehringer Mannheim, Mannheim, Germany) and dissolved in 50 μl of dH$_2$O. An amount equivalent to 10 μl of serum was subjected to PCR by the following two methods. UTR PCR was carried out with nested primers as described previously (Okamoto *et al.,* 1999b, 2000a). Long-distance PCR for the amplification of the full-length TTV genome was performed with nested inverted primers (Table 1) by the method described previously (Okamoto *et al.,* 2000a).

TTVs from humans and nonhuman primates

Full-length genomes were obtained by long-distance PCR with nested primers specific for nonhuman primates (Fig. 1 and Table 1). The genomes were TGP96, TA1817, and TM1251 from humans, Pt-TTV2-II and Pt-TTV8-II from chimpanzees, Mf-TTV7-II, Mf-TTV8-I, and Mf-TTV9 from Japanese macaques, and So-TTV2 from a cotton-top tamarin. Amplification products containing the full-length TTV genomes were separated by agarose gel electrophoresis, recovered from the gel, and ligated into pT7BlueT vector (Novagen Inc., Madison, WI). Thus, TGP96, Pt-TTV8-II, Mf-TTV9, and So-TTV2 clones were obtained. Similarly, Pt-TTV6, Mf-TTV3, and At-TTV3 were cloned from PCR products representing, respectively, the full-length TTV genomes of chimpanzee, Japanese macaque, and douroucouli reported previously (Okamoto *et al.,* 2000a).

Determination and computer analysis of TTV DNA sequences

Each of the full-length TTV DNA clones obtained was sequenced on both strands by the method described previously (Okamoto *et al.,* 2000a; Ukita *et al.,* 2000). Sequence analysis of TTV strains was performed with Genetyx-Mac version 10.1 (Software Development Co., Tokyo, Japan) and ODEN version 1.1.1 (Ina, 1994) of the DNA Data Bank of Japan (National Institute of Genetics, Mishima, Japan). Phylogenetic relatedness among TTV sequences was estimated by the neighbor-joining method (Saitou and Nei, 1987). The reliability of the phylogenetic results was assessed using 1000 bootstrap replicates (Felsenstein, 1985).

Strandedness of TTV genomes from humans and nonhuman primates

Extracted nucleic acids were treated with S1 nuclease or mung bean nuclease by the method described previously (Okamoto *et al.,* 1998b, 2000c). The genomes were subjected to PCR methods with several pairs of primer sets specific for each of the seven TTV isolates for determination of their strandedness.

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