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Analysis of the entire genomes of thirteen TT virus variants classifiable into the fourth and fifth genetic groups, isolated from viremic infants*

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Summary. TT virus (TTV) DNA in serum samples obtained from 24 TTV-infected infants was amplified by polymerase chain reaction (PCR) with inverse primers derived from the untranslated region. The amplified PCR products were molecularly cloned; six clones each were analyzed. Seventy-six (53%) of the 144 TTV clones were classified into group 4 (YONBAN isolates), and 22 (15%) into a novel genetic group (group 5). The TTV clones in group 4 were classified into 9 types, and those in group 5 into 4 types. The entire nucleotide sequence of one representative clone each from the 13 types were determined; they comprised 3570–3770 nucleotides, and had poor homology to TTVs of groups 1–3 (TA278, PMV and SANBAN isolates). A phylogenetic tree based on the entire nucleotide sequence of open reading frame 1 confirmed the presence of five distinct clusters separated by a bootstrap value of 100%. Analysis of 13 TTV variants demonstrated preservation of the genomic organization and transcription profile in all TTV groups. TTV group 4 was detected in 54% or 72% of 7-to-12-month-old infants in Japan and China, respectively, which is comparable with that among adults in the respective country, indicating early and frequent acquisition of this TTV group in infancy.

*The nucleotide sequence data in this paper have been deposited in the DDBJ, EMBL and GenBank databases under accession Nos. AB064595-AB064607 for the entire sequences of 13 TTV isolates, and AB064608-AB064657 for the sequences of the 5′-terminal and N22 region of 25 TTV clones.

Introduction

A novel, unenveloped human virus, named TT virus (TTV), was isolated from the serum of a patient with transfusion-related acute hepatitis of unknown etiology in 1997 [24]. The genome of TTV is a circular single-stranded DNA molecule of approximately 3.8 kilobases (kb) with negative polarity [17, 20, 27, 30]. TTV is most closely related to the virus family, *Circoviridae*, among the known animal viruses [22, 26, 43]; however, TTV differs considerably from members of the *Circoviridae* family in physicochemical properties and sequence at either the DNA and amino acid levels. TTV has an extremely wide range of sequence divergence for a DNA virus [15, 29], and at least 23 genotypes have been identified; the nucleotide sequence of the N22 region (central portion of open reading frame 1 [ORF1], corresponding to the area of the initial clone [N22 clone] recovered from the index patient) differs by more than 30% among each pair of genotypes [19, 29]. The sequence difference among some TTV isolates exceeds 40% at the entire genome level and 60% at the amino acid level, which reach the level of different viral species or even separate genera [11, 30, 41]. Therefore, at present, the 23 TTV genotypes are tentatively classified into four major genetic groups or phylogenetic clusters (groups 1–4) [19, 41]. Group 1 is represented by the prototype TTV (N22 clone and TA278 isolate) of genotype 1 [24, 27], and includes five additional genotypes (2–6) [29]. Group 2 comprises TTV isolates of genotypes 7, 8, 22 and 23 as well as 17, which is represented by the PMV isolate [10, 19]. Group 3 is composed of 11 genotypes (9–16 and 18–20) and includes the TUS01, SANBAN and TJN01 isolates [11, 30, 45], as well as eight genotypes of SEN virus (SENV) [21, 41]. Group 4 consists of a single genotype (genotype 21) of YONBAN isolates, with YONBAN meaning “the fourth” in Japanese [39].

Although TTV shows a great degree of genetic diversity beyond the genotypes described above, the nucleotide sequence of the untranslated region (UTR) of TTV is more conserved than that of the coding regions, and some blocks in the UTR are well conserved even among the four distinct genetic groups of TTV [11, 30, 39]. Hence, in the current study, taking advantage of the circular nature of the TTV genome, the entire genome was amplified by polymerase chain reaction (PCR) using inverse primers derived from the highly conserved area located just downstream of the TATA-box in the UTR of the prototype TTV (TA278) [31].

TTV is widely distributed throughout the world [1, 36]. Both acute resolving infection and chronic persistent infection have been recognized among TTV-infected humans [24]. Accumulating lines of evidence indicate that TTV is acquired in early childhood [7, 9, 14], and is prevalent in adults, who are frequently co-infected with TTVs of distinct genotypes and genetic groups [2, 23, 29, 40]. However, it remains unknown what kind of TTVs, in terms of genotype and genetic group, is transmitted to infants as a primary infection of TTV. Therefore, in the present study, six TTV DNA clones were isolated from each of 24 TTV-viremic infants born in Japan ($n = 17$) or the P. R. of China ($n = 7$), and

the entire nucleotide sequence of 13 isolates that were presumed to be classifiable into 9 types in group 4 and 4 types in the novel genetic group, provisionally designated as group 5, were determined, and their characteristics were analyzed molecularly and phylogenetically. In addition, using PCR with group 4-specific and group 5-specific primers, a preliminary study on the prevalence of TTVs of groups 4 and 5 was conducted in infants and voluntary blood donors living in Japan and China in an attempt to shed light on the molecular epidemiology of TTV infection.

Materials and methods

Serum samples

Serum samples were collected from 17 Japanese infants at 30–322 days (range) after birth, and 7 Chinese infants at 127–370 days after birth who were born in Jiujiang City of Jiangxi Province, P. R. of China (Table 1), who were positive for TTV DNA by UTR PCR as described below. In addition, for epidemiological study on TTV infection, serum samples were obtained from 93 Japanese infants at 200 ± 109 (mean $\pm$ standard deviation [S.D.]; range, 2–363) days after birth and 33 Chinese infants at 172 ± 126 (5–370) days after birth including the above-mentioned infants, 120 healthy individuals in Japan (40 ± 16 [16–64] years of age), and 29 healthy individuals (36 ± 10 [21–58] years of age) living in Jiujiang City ($n = 20$) or Beijing City ($n = 9$), China.

All of the serum samples were negative for hepatitis B surface antigen by a commercial kit (MyCell: Institute of Immunology Co. Ltd., Tokyo, Japan), antibodies to hepatitis C virus using a commercial kit (Abbott HCV PHA: Dainabot Co. Ltd., Tokyo, Japan), and antibodies to human immunodeficiency virus type 1 by a commercial kit (SERODIA-HIV: Fujirebio, Tokyo, Japan).

Detection of TTV DNA by PCR

Nucleic acids were extracted from 50 μl of serum using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) and dissolved in 50 μl of nuclease-free distilled water. A 10-μl aliquot of nucleic acids, equivalent to 10 μl of the sample, was tested for TTV DNA by UTR PCR which can detect essentially all TTV genotypes and genetic groups thus far identified [29, 32]. UTR PCR was carried out in the presence of Perkin-Elmer AmpliTaq Gold (Roche Molecular Systems, Inc., Branchburg, NJ) and nested primers by the method described previously [32], with a slight modification. In brief, primers NG472 (sense: 5′-GCG TCC CGW GGG CGG GTG CCG-3′ [W = A or T]) and NG352 (antisense: 5′-GAG CCT TGC CCA TRG CCC GGC CAG-3′ [R = A or G]) were used for the first-round PCR, and primers NG473 (sense: 5′-CGG GTG CCG DAG GTG AGT TTA CAC-3′ [D = G, A or T]) and NG351 (antisense: 5′-CCC ATR GCC CGG CCA GTC CCG AGC-3′) were used for the second-round PCR, which were derived from the same well-conserved area in the UTR of the TTV genome as in the original method [32]. The amplification product of the first-round PCR was 91 bp, and that of the second-round PCR was 71 bp. The genomic DNA of TTV-like mini virus (TLMV) which has a smaller genome (2.8–2.9 kb) [38] than TTV, is not amplifiable by this PCR method.

Three of the remaining four 10-μl aliquots of extracted nucleic acids were subjected to N22 PCR which can detect primarily TTVs of genotypes 1–6, or in other words, those

Table 1. Distribution of the TTV clones obtained from the serum samples from TTV-infected infants born in Japan and China among the five distinct genetic groups of TTV

Case ID (Name of TTV isolate)	Sex	Days after birth	TTV DNA (copies/ml)	No. of clones with the indicated TTV group[a]				
				1	2	3	4	5[b]
Japanese infants								
JT01	M	30	2.2×10^4	0	0	0	6	0
JT32	F	60	4.3×10^3	0	0	6	0	0
JT33	M	74	6.1×10^4	0	0	0	0	6[c]
JT34	M	77	4.6×10^2	0	0	0	0	6 (5+1)[d]
JT35	M	80	3.4×10^4	0	6	0	0	0
JT39	F	134	2.6×10^6	6	0	0	0	0
JT40	F	135	5.4×10^3	0	0	0	6	0
JT03	M	139	2.3×10^3	0	0	0	6	0
JT41	M	144	1.3×10^5	0	0	0	6	0
JT05	M	149	6.2×10^4	0	0	0	6 (4+2)	0
JT42	F	154	1.9×10^4	0	0	3	3	0
JT07	M	157	8.2×10^4	0	0	0	6	0
JT09	M	169	6.5×10^4	0	0	6	0	0
JT14	M	240	2.9×10^5	0	0	0	6 (5+1)	0
JT19	M	288	1.1×10^3	0	0	0	6	0
JT22	M	300	5.2×10^4	0	0	6 (4+2)	0	0
JT24	F	322	1.1×10^4	0	0	6	0	0
Subtotal				6 (6%)	6 (6%)	27 (26%)	51 (50%)	12 (12%)
Chinese infants								
CT39	M	127	3.8×10^2	0	0	0	5	1
CT23	F	177	9.3×10^4	2	0	0	1	3
CT25	M	226	6.7×10^4	0	0	0	6	0
CT27	M	294	9.7×10^4	0	0	3	3	0
CT44	F	315	5.9×10^5	0	0	0	0	6
CT30	F	327	6.9×10^4	0	0	2	4 (2+2)	0
CT43	F	370	2.3×10^5	0	0	0	6 (4+2)	0
Subtotal				2 (5%)	0	5 (12%)	25 (60%)	10 (24%)
Total				8 (6%)	6 (4%)	32 (22%)	76 (53%)	22 (15%)

[a]Six full-length TTV DNA clones of 3.6–3.8 kb were isolated from each infant. The number of clones with the indicated TTV genetic group, is shown. The genetic group numbers are in accordance with the previous report [19]

[b]A novel, fifth genetic group of TTV found in the present study was provisionally designated as group 5

[c]The entire nucleotide sequence of one of the isolated clones with underline was determined

[d]TTV clones in a genetic group that were classifiable into two distinct genotypes with the indicated number of clones, were obtained

of group 1 [27, 28], and to group 4- or group 5-specific PCR by the methods described below.

Quantitation of TTV DNA

For those samples that were positive for TTV DNA by UTR PCR and randomly selected for full-length inverse PCR (Table 1), TTV DNA was quantitated by real-time detection PCR using 5 μl of the nucleic acid solution as a template, primers NG473-NG352, a dual fluorophore-labeled probe [NG369-P: 5′-(Fam)-AGT CAA GGG GCA ATT CGG GCT CGG GA-(Tamra)-3′], and the LightCycler-FastStart DNA Master Hybridization Probes kit (Roche Diagnostics GmbH). PCR amplification was started with an initial denaturation at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 10 sec and annealing-extension at 62 °C for 30 sec. All reactions were performed in a LightCycler System (Roche Diagnostics GmbH). The quantification limit of the system was 3 to 5 copies per test capillary (20 μl of reaction mixture).

Molecular cloning and sequence analysis of TTV isolates

To amplify the entire TTV genome, nucleic acids extracted from the serum samples listed in Table 1 were subjected to full-length inverse PCR (3.6–3.8 kb) as described previously [31]. The amplification products were separated by electrophoresis on an agarose gel, ligated into pT7BlueT-Vector (Novagen Inc., Madison, WI), and used to transform *Escherichia coli*. The recombinant TTV DNA clones obtained from each infant were grouped according to the results of restriction fragment length polymorphism (RFLP) analysis using the restriction endonuclease, *Dra*I or the pair of *Eco*RI and *Pst*I (TaKaRa Shuzo, Shiga, Japan), and sequence analysis of the 5′ terminus (500 nt) of the cloned DNA corresponding to the region upstream of and within ORF2. To support this grouping, the sequence of the N22 region was determined for selected clones having a distinct RFLP pattern, as described previously [29]. Furthermore, the entire nucleotide sequence of selected 13 clones of 3.6–3.8 kb was determined, and their 5′ and 3′ terminal sequences corresponding to the inverted primers that were used, were confirmed by the sequences of the short PCR products (0.3–0.5 kb) covering the primer sequences. Both strands were sequenced by the BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA), as described previously [19].

Detection of TTV DNA of group 4 and group 5

Nested PCR was performed for the detection of TTV DNA of group 4 and TTV DNA of a novel genetic group which was provisionally designated as group 5 in the present study (see Results). The first round of PCR was carried out with group 4-specific primers, NG537 (sense: 5′-CTA CGT ACA CTT CCT GGG GYG TG-3′ [Y = T or C]) and NG538 (antisense: 5′-CCC GGC GGW CTC CAC GRC AT-3′), that had been derived from the UTR sequence of 9 distinct isolates belonging to group 4 identified in the present study, and Perkin-Elmer AmpliTaq Gold for 35 cycles [95 °C, 30 s (preheating at 95 °C for 9 min before the first cycle); 60 °C, 30 sec; 72 °C, 40 sec (additional 7 min for the last cycle)], and the second-round PCR was performed with NG539 (sense: 5′-CGA GAR CGC GAG CRA AGC GAG CG-3′) and NG538 for 25 cycles under the same conditions. The amplification product of the first-round PCR measured 269 bp, and that of the second-round PCR 182 bp. Similarly, for the detection of TTV DNA of group 5, the first round of PCR was performed with primers NG540 (sense: 5′-CGT AGC CAT GCT GCT GTT TG-3′) and NG541 (antisense: 5′-TCC ACC ATC CCC ATG CCA TG-3′), that had been deduced from the ORF2 sequence of four distinct isolates of group 5, for 35 cycles under the same condition as in the group 4-specific PCR except for

an annealing temperature of 58 °C, and the second-round PCR was performed with primers NG542 (sense: 5′-CTG TTT GTG GYT GTG GGG AT-3′) and NG543 (antisense: 5′-TCC CCA TGC CAT GGC AGG GC-3′) for 25 cycles. The amplification product of the first-round PCR was 222 bp, and that of the second-round PCR was 202 bp.

Analysis of nucleotide and amino acid sequences

Sequence analysis was performed using Genetyx-Mac version 10.1.4 (Software Development, Tokyo, Japan) and ODEN version 1.1.1 from the DNA Data Bank of Japan (DDBJ: National Institute of Genetics, Mishima, Japan) [12]. Sequence alignments were generated by CLUSTAL W (version 1.8) [42]. On the basis of the amino acid sequence alignment, the nucleotide sequence alignment was rearranged. A phylogenetic tree was constructed by the neighbor-joining method [37], based on the nucleotide sequence of the N22 region of 219–231 nt or the entire nucleotide sequence of ORF1. Bootstrap values were determined on 1000 resamplings of the data sets [8]. The final tree was obtained using the TreeView program (version 1.6.5) [35].

Results

Distribution of genetic groups of TTVs in viremic infants born in Japan and China

Six full-length TTV DNA clones of 3.6–3.8 kb were isolated from each of 17 Japanese infants and 7 Chinese infants, who were positive for TTV DNA with a titer of 4.6×10^2–2.6×10^6 (range, Japanese infants) and 3.8×10^2–5.9×10^5 (range, Chinese infants) copies/ml, respectively. The 144 clones obtained were subjected to analysis by RFLP and partial nucleotide sequencing. The results are summarized in Table 1. Thirty-nine clones obtained from 7 Japanese infants and 7 clones from 3 Chinese infants were the closest to TTVs of genetic group 1, 2 or 3, which are represented by the TA278, PMV or TUS01 isolate [10, 27, 30]. Of note, over 50% of the total clones obtained from the Japanese infants (63/102 [62%]) and Chinese infants (35/42 [83%]) were presumed to be classifiable into genetic group(s) other than groups 1–3. Then, one or two distinct clones in this group were randomly selected as representative clone(s) from each infant, and compared with the group 4 TTV which consists of a single genotype and is represented by the KC009 isolate [39]. Eleven representative clones from 9 Japanese infants and 8 representative clones from 6 Chinese infants showed the highest sequence similarity to the KC009 isolate, although the degree of similarity varied widely, ranging from 59% to 78% in the nucleotide sequence of the N22 region, suggesting the presence of additional novel genotypes in group 4. The phylogenetic tree based on the N22 region sequence of 219–231 nt, indicated that these 19 clones were classifiable into group 4, and furthermore into 9 types (one type was comprised of 9 clones, two types of 2 clones each, and six types of 1 clone each) (Fig. 1). Furthermore, 12 clones from 2 Japanese infants and 10 clones from 3 Chinese infants were not classifiable into any of the four known genetic groups; the representative clones from these infants had a nucleotide sequence similarity of $< 55\%$ in the N22 region to TTVs of groups 1–4, and were presumed to be

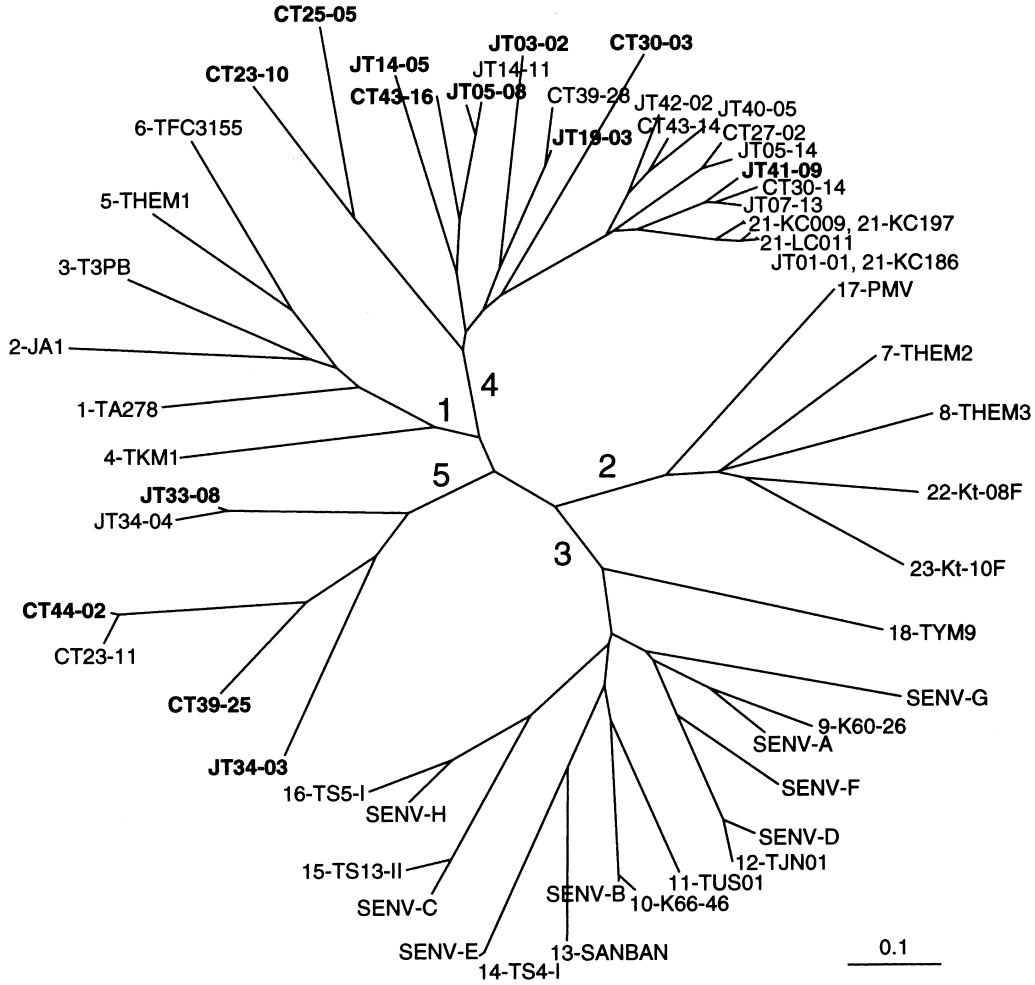

Fig. 1. Phylogenetic tree constructed by the neighbor-joining method of the nucleotide sequence of the N22 region of 57 TTV isolates. The N22 regions of 24 reported TTV isolates of genotypes 1–23 in groups 1–4 [10, 11, 19, 29, 30, 33, 39, 45] and SENV isolates of 8 genotypes A-H [41], as well as 19 clones (JT01-01, JT03-02, JT05-08, JT05-14, JT07-13, JT14-05, JT14-11, JT19-03, JT40-05, JT41-09, JT42-02, CT23-10, CT25-05, CT27-02, CT30-03, CT30-14, CT39-28, CT43-14, and CT43-16) in group 4 and 6 clones (JT33-08, JT34-03, JT34-04, CT23-11, CT39-25, and CT44-02) in a novel genetic group (tentatively designated as group 5) found in the present study, are compared. The 9 clones in group 4 and 4 clones in group 5 whose entire sequence were determined, are indicated in boldface type. Genotype numbers 1–23 and group numbers 1–4 are in accordance with our previous report [19, 29]

classified into novel genetic group(s). The phylogenetic tree in Fig. 1 shows that the 6 clones that could not be classified into any of the four genetic groups (1–4), belong to a novel, fifth genetic group, tentatively designated as group 5 in the present study, and were grouped into 4 types (two types comprised of 2 clones each, and two types of 1 clone each).

Full-length nucleotide sequences and genomic organizations of 13 TTV isolates

The entire nucleotide sequence of TTV was determined for 13 clones that were representative of each of the 9 types in group 4 (JT03-02, JT05-08, JT14-05, JT19-03, JT41-09, CT23-10, CT25-05, CT30-03, and CT43-16) and the 4 types in group 5 (JT33-08, JT34-03, CT39-25, and CT44-02). After determining the sequence of 0.3–0.5 kb covering the sequence of the inverse primers used for the full-length PCR, the entire genomic sequence was reorganized for the 13 isolates which were named JT03F, JT05F, JT14F, JT19F, JT41F, CT23F, CT25F, CT30F, and CT43F in group 4, and JT33F, JT34F, CT39F, and CT44F in group 5. The 9 isolates in group 4 and 4 isolates in group 5 possessed a circular genomic structure with a total genomic length of 3570–3729 nt (range, group 4) and 3701–3770 nt (range, group 5), respectively; the difference of the genomic length among the TTVs of group 4 (between CT23F [3729 nt] and CT30F [3570 nt]) was mainly ascribable to differences in the length of the hypervariable region [25] located in the middle of ORF1. The 13 TTV genomes each possessed the consensus sequence of donor and acceptor sites of the three splicings in mRNAs in accordance with the GT-AG splicing rule and the intron consensus rule [5, 18] (Fig. 2), similar to the VT416 and TYM9 isolates of TTV DNA whose three distinct mRNAs of 2.9–3.0 kb, 1.2 kb, and 1.0 kb in size have been observed in vitro and in vivo [13, 33]. The short splicing of 96–101 nt (range, Splice 1 in Fig. 2) was presumed to exist in the three mRNAs of all 13 isolates. The two longer splicings of 1473–1650 nt (range, Splice 2 in Fig. 2) and 1651–1891 nt (range, Splice 3 in Fig. 2) were deduced to be present in the 1.2-kb mRNA and the 1.0-kb mRNA, respectively.

The proposed genomic organizations of the CT23F and JT33F isolates, as the representative isolate for group 4 and group 5, respectively are illustrated in Fig. 3. A common internal promoter, i.e., the TATA-box (ATATAA), and a polyadenylation signal (AATAAA) were recognized in the locations shown in Fig. 3, in all 13 TTV genomes isolated in the present study. All of these genomes were presumed to possess the four major open reading frames (ORF1, ORF2, ORF3 and ORF4) that are present in the reported TTV isolates [13, 33]. The largest ORF (ORF1) and the shortest ORF (ORF2), which partially overlapped, encoded 680–746 amino acids (aa) (range) and 100–130 aa, respectively, in the 13 isolates. The ORF1 in all 9 isolates of group 4 lacked the ATG initiation codon, but instead, possessed a sequence matching Kozak's rule [16] of ACCACGG, GCCACGG, or ATCACGG (the possible initiation codon underlined) at the corresponding site, as that of the YONBAN isolates [39]. Hence, this ACG codon in ORF1 of the group 4 TTVs was assumed to work as an initiation codon; start codons other than ATG have been reported in other viruses such as the beak and feather disease virus of parrots [3] and Sendai virus [6]. ORF3 and ORF4 in the mRNAs of 1.2 kb or 1.0 kb, respectively, encode the putative joint proteins (271–302 aa and 266–297 aa, respectively, in the 13 isolates). The sequence between the end of ORF4 and the beginning of ORF2 was defined as the untranslated region (UTR),

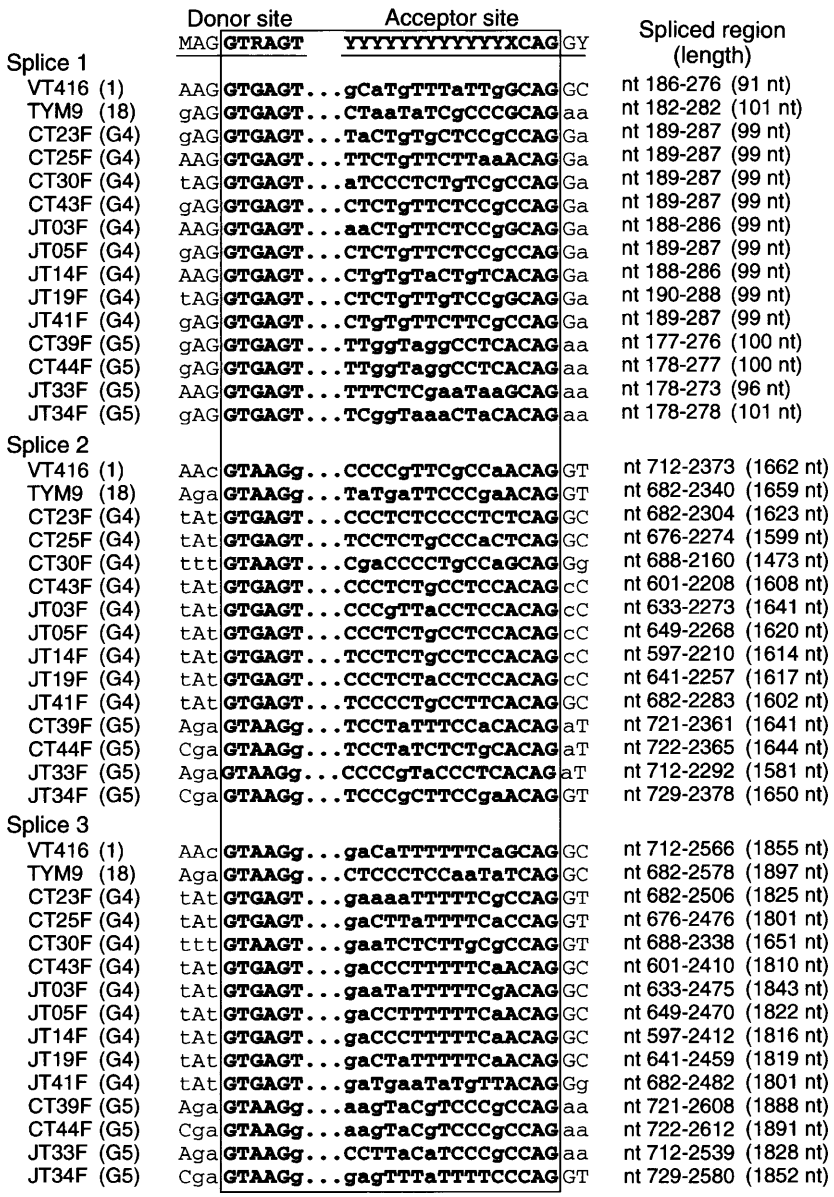

Fig. 2. Comparison of the nucleotide sequences of the donor and acceptor sites of the mRNAs of 9 TTV isolates in group 4 and 4 isolates in group 5. The consensus sequence of donor and acceptor sites in splicings [5, 18] is shown at the top. M denotes A or C, R denotes A or G, and Y denotes T or C. The sequence of the donor and acceptor sites in the first splicing (Splice 1) is shared by the 2.9- to 3.0-kb, 1.2-kb, and 1.0-kb mRNAs; that in the second splicing (Splice 2) is present in the 1.2-kb mRNA; and that in the third splicing (Splice 3) is present in the 1.0-kb mRNA. The mRNAs of the VT416 isolate of genotype 1 in group 1 [13] and TYM9 isolate of genotype 18 in group 3 [33] were previously analyzed, and their splicing sites are specified. On the right are shown the region of the intron and the number of nucleotides spliced out in parentheses

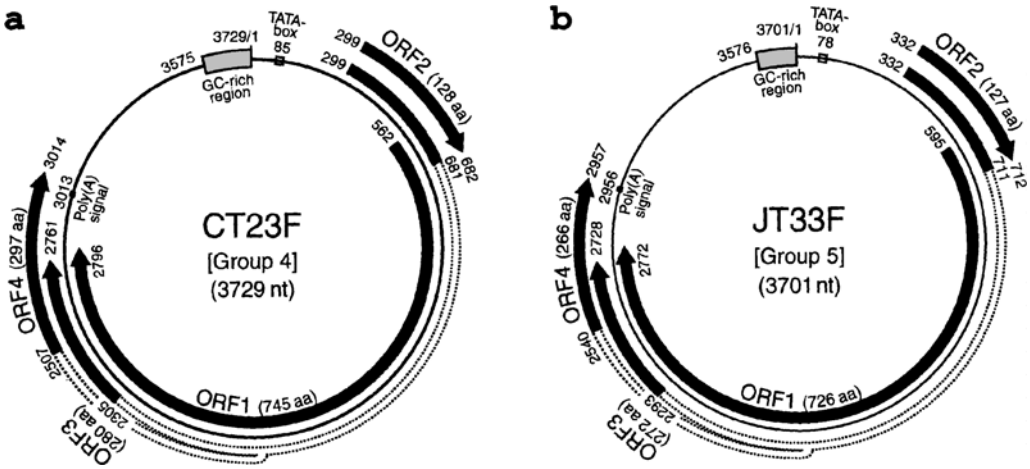

Fig. 3. Predicted genomic organization of **a** CT23F, a representative isolate of group 4 and **b** JT33F, a representative isolate of group 5. The closed box with arrow represent ORFs (ORF1-ORF4). The open box located between an upstream closed box and downstream closed arrow in ORF3 and ORF4, both of which encode joint proteins, indicates an area corresponding to an intron in the 1.2-kb and 1.0-kb mRNAs, respectively. The names of ORF3 and ORF4 are in accordance with previous reports [19, 33]. The poly(A) signal, GC-rich stretch and TATA-box are indicated by a small closed box, shaded box, and small open box, respectively

occupying 27.0–29.1% (989–1040 nt in the 9 isolates in group 4) or 28.5–29.0% (1070–1078 nt in the 4 isolates in group 5) of the TTV genome.

The GC-rich stretch of 130–160 nt in the 9 isolates of group 4 and 126–130 nt in the 4 isolates of group 5, was located in the middle of the UTR, and the GC-rich sequence was a little longer than that of the reported TTVs of groups 1–3 (108–122 nt) [17, 19, 20, 30]. Characteristic stem and loop structures were constructed by the GC-rich sequence and its upstream sequence (Fig. 4). The putative

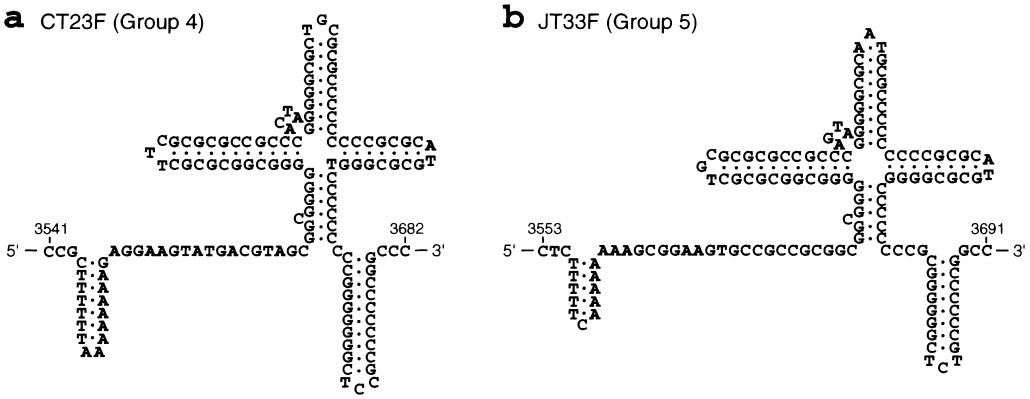

Fig. 4. Potential stem-loop structures in the GC-rich stretch and its upstream sequence. The sequence and predicted secondary structure of the **a** CT23F isolate and **b** JT33F isolate are shown. A bulge composed of a 4-nt sequence (ACTA or AGTA) was observed in a cross-like structure in CT23F and JT33F

secondary structures of the CT23F and JT33F genomes, forming a cross, resembled those of previously reported TTVs of groups 1–3. Interestingly, however, all 9 genomes of group 4 and 4 genomes of group 5 possessed in common a bulge in the cross-like structure, with a 4-nt sequence of ACCA or ACTA, and AGTA, respectively, which is not observed in any of the TTV genomes of groups 1–3 [19, 30, 45]. Its virological significance needs to be defined in future studies.

Comparison of the CT23F and JT33F genomes with the reported TTV genomes of groups 1–4 and other genomes in groups 4 and 5 identified in the present study

Figure 5 depicts a phylogenetic tree constructed based on the entire nucleotide sequence of ORF1 of 9 isolates in group 4 and 4 isolates in group 5, as well as 14 previously-reported TTV isolates (one representative isolate from each

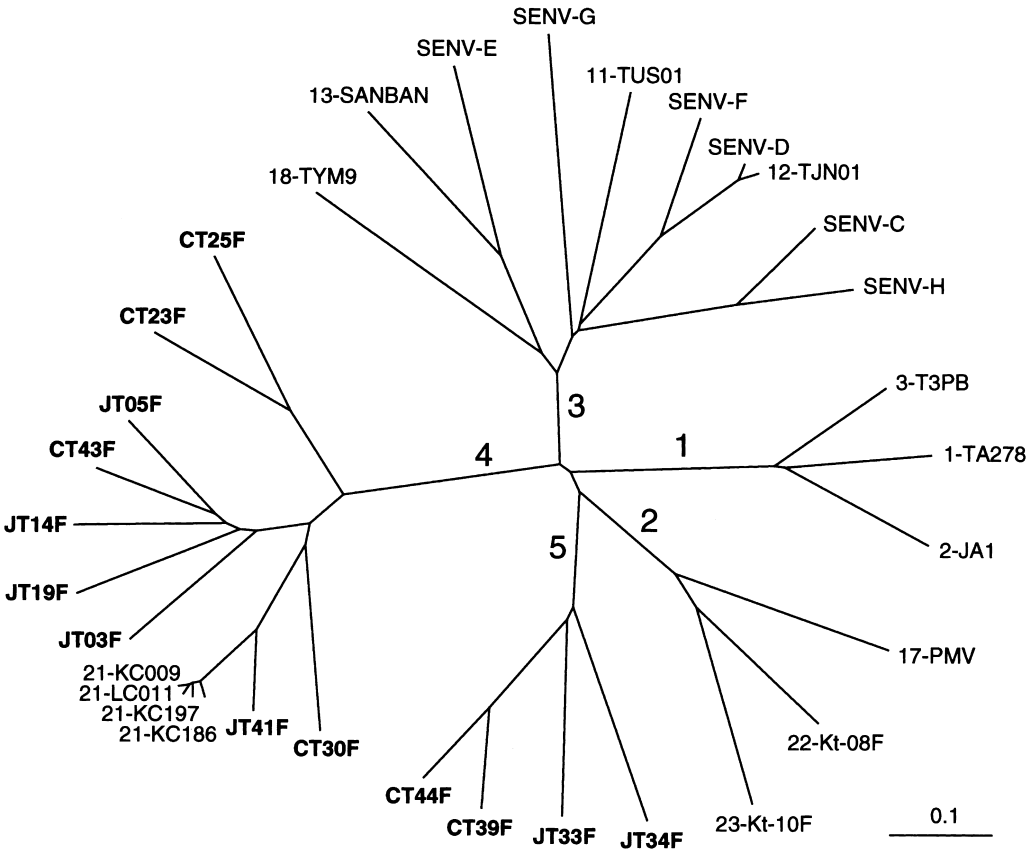

Fig. 5. Phylogenetic tree constructed by the neighbor-joining method of the nucleotide sequence of ORF1 in 33 TTV isolates. The nucleotide sequence of ORF1 of 20 TTV isolates in groups 1–4 (genotypes 1–3, 11–13, 17, 18 and 21–23, including 6 SENV genotypes), of which the entire sequence of ORF1 is known, as well as that of the 9 isolates in group 4 and 4 isolates in group 5 obtained in the present study and indicated in boldface type, are compared (see Introduction and the legend to Fig. 1 for isolate names and relevant references)

genotype in groups 1–3 [4, 10, 11, 19, 20, 30] and all 4 isolates in group 4 [39]) and 6 SENV isolates [41] whose entire ORF1 sequence has been reported. Nine isolates identified in the present study (JT03F, JT05F, JT14F, JT19F, JT41F, CT23F, CT25F, CT30F, and CT43F), were on the same branch as 4 previously-reported isolates (KC009, KC186, KC197, and LC011) [39] of genotype 21, making a group (group 4), but were separate from groups 1–3 which included the prototype TTV of group 1 (TA278), the PMV and Kt-08F isolates of group 2, and the TUS01 and SANBAN isolates as well as the SENV isolates of group 3. Furthermore, the 4 other isolates identified in the present study (JT33F, JT34F, CT39F, and CT44F) were clearly separate from groups 1–4, with a bootstrap value of 100%, confirming the presence of a novel genetic group, group 5.

The CT23F and JT33F genomes were compared with a representative TTV genome in each of the four groups, i.e., TA278 (group 1) [30], Kt-08F (group 2) [19], TUS01 (group 3) [30] and TTVyon-KC009 (group 4) [39] as well as 8 other isolates in group 4 or 3 other isolates in group 5, respectively, that were identified in the present study. Although the PMV isolate was the first TTV in group 2 whose entire genome sequence had been determined [10], another isolate in group 2 (Kt-08F) was used for comparison because ORF4 in the PMV genome is truncated due to premature termination. CT23F differed from TA278, Kt-08F, and TUS01 to a greater extent than from TTVyon-KC009 in the entire genome and regions thereof (Table 2). Remarkably, comparing the four ORFs, CT23F was only 28.8–36.0% similar to TA278, Kt-08F, and TSU01 at the amino acid level. Comparing the entire genomic sequence, CT23F was 65.0–75.9% similar to TTVyon-KC009 and 8 other isolates in group 4 identified in the present study, but was only 51.6–52.7% similar to TA278, Kt-08F, and TUS01, indicating that the 9 isolates represented by CT23F belong to the same group as the YONBAN isolates (group 4). The JT41F genome differed from TTVyon-KC009 by only 17.8%, but 8 other genomes in group 4 differed from KC009 by 35.5–39.6% at the nucleotide level of ORF1, indicating that JT41F is classifiable into the same genotype (genotype 21) as all 4 isolates in group 4 thus far reported. The 9 isolates in group 4 including JT41F differed from one another by 25.6–41.5% at the nucleotide level and 29.9–52.2% at the amino acid level of ORF1. These results suggest that 9 distinct isolates in group 4 identified in the present study are classifiable into 9 different genotypes including the one reported previously.

Comparing the entire genomic sequence, JT33F was merely 49.3–55.0% similar to TA278, Kt-08F, TUS01, and CT23F, but 68.4–69.2% similar to 3 other isolates in the same group (JT34F, CT39F, and CT44F). The amino acid sequences of ORF1-ORF4 of JT33F were 55.2–67.7% similar to those of 3 isolates in the same group, but only 29.4–42.4% similar to those of TTVs in groups 1–4, indicating that the 4 isolates represented by JT33F are distinct from known TTVs in groups 1–4, and are classifiable into a novel group (group 5). The CT39F and CT44F genomes were 85.9 similar in the entire genomic sequence and 80.3% similar in the amino acid sequence of ORF1, and therefore, they were considered to be within the same genotype. In contrast, the CT39F, JT33F, and JT34F

Table 2. Comparison of the CT23F and JT33F genomes with TTV genomes representative of the 4 distinct groups of TTV over the entire genome and in regions thereof

Region	Similarity (%) of CT23F with					Similarity (%) of JT33F with				
	TA278 (Group 1)	Kt-08F (Group 2)	TUS01 (Group 3)	KC009[a] (Group 4)	8 other isolates of group 4	TA278 (Group 1)	Kt-08F (Group 2)	TUS01 (Group 3)	CT23F (Group 4)	3 other isolates of group 5
Total	52.0	51.6	52.7	69.3	65.0–75.9[b]	50.2	51.5	55.0	49.3	68.4–69.2
Noncoding region[c]	60.7	61.5	64.9	86.0	86.9–90.6	73.1	73.2	76.0	63.4	82.6–83.3
GC-rich	82.2	81.0	81.7	84.7	92.3–95.6	80.2	83.0	77.7	90.6	93.1–94.5
Other	58.1	59.4	62.9	86.6	85.3–90.1	72.2	72.8	75.8	58.5	81.0–81.6
Coding region	50.1	51.6	48.6	64.5	60.1–71.4	50.7	53.3	50.1	48.4	64.0–65.2
ORF1	50.6	51.9	48.7	64.2	59.5–71.0	49.1	50.3	49.8	48.2	62.5–64.2
(amino acid)	35.1	32.7	34.8	54.0	47.8–68.0	39.9	42.4	38.9	37.0	55.2–57.6
ORF2	55.6	50.7	52.0	66.4	52.0–67.7	52.0	54.6	55.5	53.3	76.4–77.2
(amino acid)	36.0	32.8	33.6	59.7	34.0–57.6	34.4	29.7	37.7	31.7	63.8–67.7
ORF3	55.2	51.5	51.3	67.5	59.0–72.1	55.5	57.4	55.7	55.7	70.8–71.2
(amino acid)	31.4	29.1	31.8	53.2	37.0–55.9	37.5	34.2	34.8	32.5	56.2–59.3
ORF4	50.6	49.4	45.1	65.4	53.3–70.9	50.4	51.7	52.9	51.0	62.5–71.3
(amino acid)	29.9	31.1	28.8	52.9	39.2–59.2	29.8	29.4	34.7	31.1	56.1–59.1

[a]The sequence of the GC-rich stretch of the KC009 genome has not yet been completely determined [39]

[b]The range of similarities with the 8 other isolates in group 5 identified in the present study, is shown

[c]The GC-rich stretch resides in the middle of the noncoding region, and "Other" corresponds to the sequences 5′ to and 3′ to the GC-rich stretch

genomes differed from each other by 25.5–43.6% in the entire genomic sequence, but by 35.1–45.1% in the amino acid sequence of ORF1. Therefore, three distinct genotypes of TTVs were identified in a novel, fifth group in this study.

Of note, even in the UTR sequence where the nucleotide sequence is known to be well conserved, CT23F was similar to TA278 and Kt-08F by only 60.7% and 61.5%, respectively, and JT33F was similar to CT23F by merely 63.4%. Despite such a wide divergence, two specific regions downstream of the polyadenylation signal and just downstream of the TATA-box were well preserved among the TTVs of the five distinct groups (Fig. 6). The point mutations and deletion/insertion of nucleotide observed in the inverted repeat sequences downstream of the polyadenylation signal were covariant (Fig. 6b), suggesting the strict conservation of the putative stem-loop structures maintained by all TTV groups, as previously described for TTVs of groups 1 and 3 [11].

In the deduced amino acid sequences encoded by ORF1-ORF4, several motifs known to be characteristic of TTVs of groups 1–3 were also preserved in those of groups 4 and 5 identified in the present study. Namely, they all possessed an arginine-rich domain; motifs present in the putative replication-associated protein (Rep protein), which is involved in rolling-circle replication [20]; a glutamine/glutamic acid-rich domain in ORF1; the W-X_7-H-X_3-C-X_1-C-X_5-H motif in ORF2 common to TTV, TLMV and chicken anemia virus in ORF2 [11, 26, 38]; a serine-rich domain in the C-terminal region of ORF3; and a conserved motif in the C-terminal region of ORF4, E-X_8-R-X_2-R-$X_{4–6}$-P-$X_{5–11}$-P-$X_{1–8}$-V-X_1-F-X_1-L [34], that is common to TTVs that infect humans and non-human primates.

Detection of TTV DNAs of group 4 and group 5 in infants and adults in Japan and China

Using oligonucleotide primers specific for TTVs of group 4 or group 5, semi-nested or nested PCR was performed for the detection of TTV DNA of group 4 or that of group 5 in serum samples obtained from 93 infants and 120 healthy adults in Japan, as well as 33 infants and 29 healthy adults in the P. R. of China (Table 3). Among the 34 Japanese infants from whom blood samples had been obtained at 2–175 days after birth, group 4 TTV or group 5 TTV was detected in 5 (15%) and 3 (9%), infants, respectively, although TTV DNA was not detected in any infant by N22 PCR which can primarily detect TTV of group 1. Of interest, TTV DNAs of group 4 and group 5 were detected in 32 (54%) and 11 (19%), respectively, of the 59 Japanese infants from whom blood samples had been obtained at 181–363 days after birth, which were comparable with the frequencies among Japanese adults aged 16–64 years (49% and 23%, respectively). Similarly, infections of group 4 TTV and group 5 TTV were prevalent in China. TTVs of groups 4 and 5 were detected among the Chinese adults at frequencies significantly higher than among the Japanese adults (83% vs. 49% [$P<0.005$, χ^2-test] and 72% vs. 23% [$P<0.0001$, χ^2-test], respectively). TTV of group 4 was detected in 20% of the Chinese infants from whom blood samples had been obtained at 5–177 days after

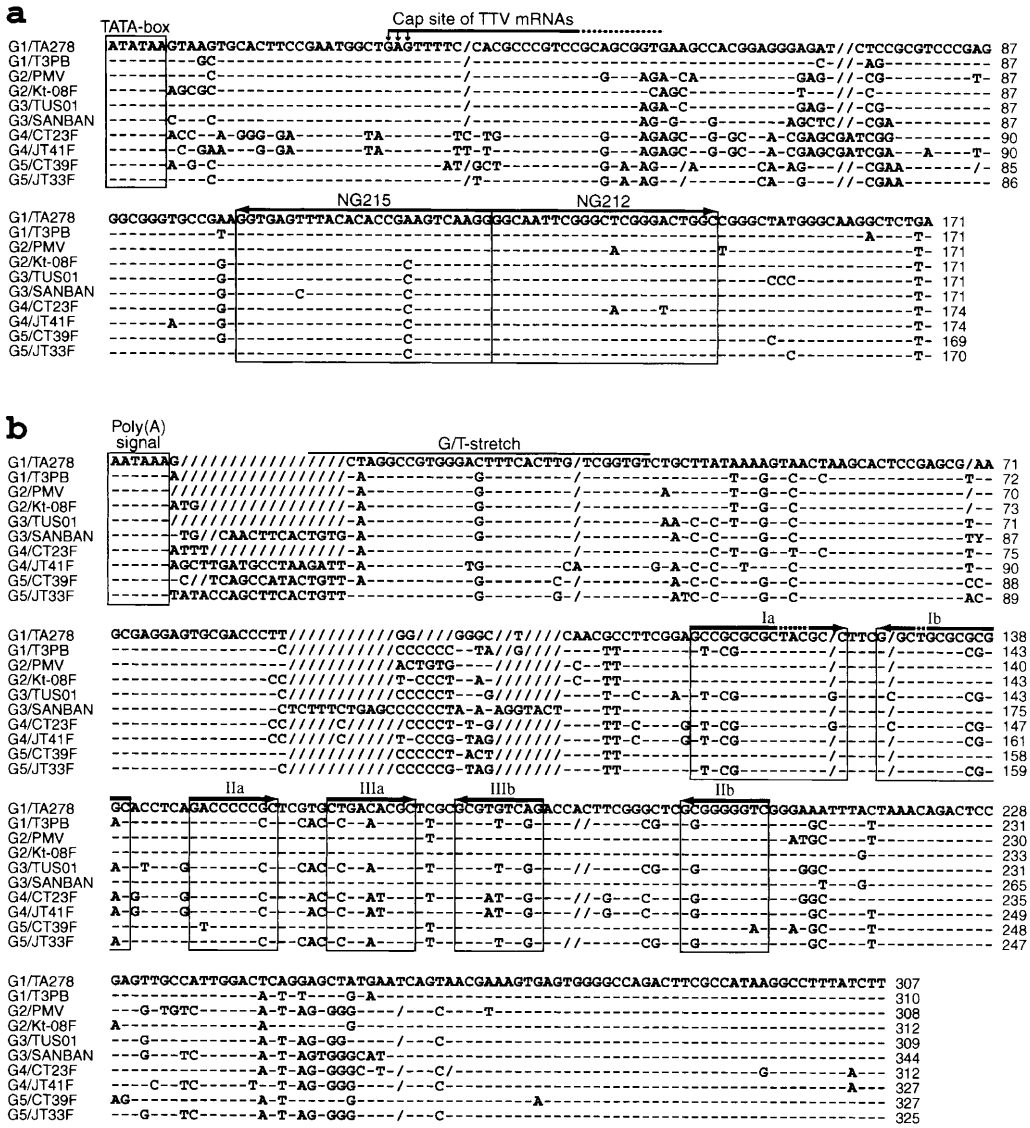

Fig. 6. Well-conserved sequences in the UTR among the TTV genomes in the five distinct genetic groups. **a** Nucleotide sequence of a span of 169–174 nt just downstream of the TATA-box in two representative isolates from each of the five genetic groups (1–5; G1–G5). The TATA-box of ATATAA is boxed. The putative cap-site of TTV mRNAs [33] is indicated by arrows with a horizontal bar. The inverse primers (NG212 [sense] and NG215 [antisense]) used for full-length PCR amplification of the TTV genome are shown by boxes with arrows. **b** Nucleotide sequence of a span of 307–344 nt just downstream of the polyadenylation signal in the same 10 isolates as in **a**. The G/T-stretch is overlined. The boxes with arrows represent three inverted repeats, which are conserved in all genetic groups and are possibly involved in the formation of stem structures (Ia and Ib, IIa and IIb, IIIa and IIIb)

birth, and in a much higher percentage of 72% among the Chinese infants from whom blood samples had been obtained at 188–370 days after birth, which was similar to that observed in adults in China (83%).

Table 3. TTV DNA of groups 4 and 5 in infants and adults living in Japan and China

Category	No. of cases	TTV DNA detectable by PCR with primers			
		UTR[a]	N22[b]	Group 4-specific	Group 5-specific
Japanese					
Infants					
2–175 days[c]	34	11 (32%)	0	5 (15%)	3 (9%)
181–363 days	59	55 (93%)	9 (15%)	32 (54%)	11 (19%)
Adults					
16–64 years	120	112 (93%)	18 (15%)[d]	59 (49%)[e]	28 (23%)[f]
Chinese					
Infants					
5–177 days	15	5 (33%)	1 (7%)	3 (20%)	3 (20%)
188–370 days	18	17 (94%)	6 (33%)	13 (72%)	7 (39%)
Adults					
21–58 years	29	29 (100%)	15 (52%)[d]	24 (83%)[e]	21 (72%)[f]

[a]UTR PCR can detect essentially all TTVs of the five distinct groups [29, 32]

[b]N22 PCR with primers derived from the well-conserved areas of the ORF1 sequence of the prototype TTV isolate (TA278), can detect primarily TTV of group 1 [27, 28]

[c]The range of the age of the infants at which the blood sample was obtained

[d]$P<0.0001$, [e]$P<0.005$, [f]$P<0.0001$ (χ^2-test) in comparison between the adults in Japan and China

Discussion

In the present study, we identified nine distinct TTV variants that belong to group 4, but which vary remarkably from each other. Based on pairwise comparison and phylogenetic analysis, 9 distinct genotypes were recognized among the TTV isolates of group 4 that were obtained from infants in Japan and China in the present study, one of which corresponded to the genotype of the YONBAN isolates [39]. In addition, four TTV variants which clearly differed from the previously described four genetic groups (groups 1–4) and which were classifiable into a novel, fifth genetic group tentatively designated as group 5, were identified in the present study. These four variants were classified into three distinct genotypes. Therefore, a total of 11 new TTV genotypes were identified in this study. As a result, more than 30 genotypes of TTVs have been identified up to the present. In this communication, however, we did not give a specific number to each genotype of TTV isolate(s) found in group 4 and group 5, because we thought it would be confusing and less informative to utilize such numbering system for a genotype consisting of a single isolate, one by one, at this preliminary stage.

As has been suggested by several investigators [11, 15, 21, 39, 41], the genetic variability among the TTV isolates of groups 1–5 seems to be far beyond the scope

of the usual virus in a single taxon. However, we did not try to call the various "TTV groups" as distinct virus species or separate genera due to the following reasons. First, the genomic DNAs of all TTV isolates identified thus far including those in groups 4 and 5, are amplifiable by PCR with primers derived from the UTR sequence of the prototype TTV (group 1), and have in common a strictly conserved genomic organization and putative transcription profile. Second, at this moment, we cannot deny the possibility of the presence of TTV variants that are intermediate between genotype and genetic group, similar to those that are located between TTV strains (or isolates) and TTV genotypes [15]; TTV might not have evolved in a two-tiered fashion, at least in a strict sense, as other viruses such as hepatitis C virus [44]. Whether homologous recombination among TTVs of distinct genetic groups plays a role in the wide diversification of this virus remains to be explored [46]. Third, based on the fact that one novel genetic group and 11 novel genotypes were identified from 24 viremic infants in the present study, it is likely that the number of TTV genotypes in each group and/or the number of genetic groups will increase with the accumulation of sequence data on TTV isolates from unexamined populations and countries in the world. Therefore, the validity of the classification into either genetic groups or virus species, has to be substantiated by extended studies in which virological, clinical, and epidemiological differences are attributed to these classifications.

It has recently been demonstrated that three distinct mRNAs of 2.9–3.0 kb, 1.2 kb and 1.0 kb in size, which have common 5′- and 3′-termini, are transcribed from the genomic DNA of TTV [13, 33]. All of these mRNAs arise from splicing, and the shorter mRNAs of 1.2 kb and 1.0 kb have additional splicing sites to link distant ORFs to create two new ORFs (ORF3 and ORF4). The 9 TTV variants in group 4 and 4 variants in group 5 obtained in the present study were presumed to have this transcription profile, based on the presence of consensus sequences of donor and acceptor sites of splicings (Fig. 2). This suggests that this unique transcription profile, which is not observed among known members (chicken anemia virus, porcine circovirus, and beak and feather disease virus of parrots) of the *Circoviridae* family [22, 26, 43], is common to members of the TTV family, irrespective of genotype and group. The complete preservation of coding capacity for two joint proteins (ORF3 and ORF4) and the previously predicted proteins (ORF1 and ORF2), as well as the presence and location of the GC-rich stretch, TATA-box, and polyadenylation signal in the TTVs of group 4 and group 5, as illustrated in Fig. 3, would indicate that the proposed genomic organization is characteristic to members of the TTV family, which now include five genetic groups of TTVs infecting humans. Two specific areas in the UTR, located just downstream of the TATA-box and just downstream of the polyadenylation signal, were highly conserved among the five distinct groups of TTVs. Interestingly, most of the mutations recognized between TTV isolates of groups 1–5 in the 100-nt sequence just downstream of the polyadenylation signal, were covariant and could serve to maintain the base-pairings required for the predicted stem-loop structures [11] (Fig. 6). It seems likely that the two specific areas in the UTR whose sequences were strictly preserved, are involved in pivotal

role(s) in the replication and protein expression of TTV, irrespective of genetic group. Furthermore, such conserved sequences are useful for the specific and sensitive detection of TTVs of all genetic groups, as was indicated in the present study.

TTV is widely distributed, with a high frequency of viremia in adults, who are frequently co-infected with TTVs of distinct genotypes and genetic groups [2, 23, 29, 39]. Of note, in the present study, the prevalence of TTV infection among infants less than 6 months of age was 32% in Japan and 33% in China, when TTV DNA was detected by UTR PCR which can detect TTVs of all five groups. However, the prevalence of TTV detectable by UTR PCR in infants of 7–12 months after birth was similar to that in adults in the respective country, indicating that most humans acquire TTV infection by 7–12 months postpartum at least in these Asian countries. In the present study, interestingly, TTV variants of group 4 were recovered from 9 (53%) out of the 17 viremic infants in Japan and 6 (86%) out of the 7 viremic infants in China, which suggests the early and frequent acquisition of TTV of this particular genetic group in infancy as a primary infection of TTV. Reflecting the high prevalence of group 4 TTV infection in early childhood, two infants each in Japan and China were co-infected with two distinct variants of group 4 TTV (Table 1). Surprisingly, a total of 9 different genotypes were identified among the 15 infants infected with group 4 TTV, further emphasizing the great degree of genetic diversity of TTV. Using the PCR method with group 4- or group 5-specific primers, group 4 TTV or group 5 TTV was detected at a frequency of 15% and 9%, respectively, among Japanese infants less than 6 months of age, despite the fact that TTV DNA was not detected in any of these infants by N22 PCR which primarily detects TTV of group 1. In contrast, TTV DNAs of group 4 and group 5 were detected in 54% and 19%, respectively, of the Japanese infants of 7–12 months of age, which were similar to those among the adults in Japan (49% and 23%, respectively). Similarly, group 4 TTV and group 5 TTV were detected at a high frequency of 72% and 39%, respectively, among the Chinese infants of 7–12 months of age, which were comparable to those among the adults in China (83% and 72%, respectively), suggesting that TTVs of groups 4 and 5 are frequently transmitted to humans in early childhood, at least in these Asian countries.

In conclusion, the results obtained in the present study indicate the presence of TTV variants classifiable into a fifth, novel genetic group (tentatively designated as group 5), that is clearly separate from the known 4 genetic groups (groups 1–4), and the early and frequent acquisition of TTVs of groups 4 and 5 (especially group 4) in infancy as a primary infection of TTV. Whether these TTV variants of groups 4 and 5 are more efficiently transmitted vertically from mother to baby or postnatally by breast-feeding, or through any other horizontal routes, than group 1 TTV represented by the prototype TTV, deserve analysis. Further extended virological and epidemiological studies deserve to be conducted in large cohorts not only in Asia but also in other countries in the world, in relation to TTV genotypes and genetic groups, to better understand the clinical significance and natural course of TTV infection.

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