In Vivo and In Vitro Intragenomic Rearrangement of TT Viruses[▽]

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The in vitro replication of the Torque teno virus (TT virus) tth8 full-length genome and particle formation in a Hodgkin's lymphoma-derived cell line after transfection with cloned viral DNA were demonstrated. Analyses of the transcription patterns of tth8 and tth7 TT virus isolates in a number of lymphoma and T-cell leukemia cell lines indicated differential additional splicing events and intragenomic rearrangement generating open reading frames which could not be deducted from the genomic sequence. We also demonstrated the presence of rearranged TT virus genomes in vivo in sera taken from pregnant mothers whose children later developed childhood leukemia, as well as sera from control mothers. Control experiments using religated cloned genomic tth8 DNA mixed with cellular DNA did not result in such subviral molecules. These subviral isolates ranged from 172 bp to full-length TT virus genomes. Possible in vivo selection for specific rearranged molecules was indicated by the presence of one isolate (561 bp) in 11 serum samples. It remains to be clarified whether selected rearranged subviral components resulting from specific TT virus types may contribute to the initiation of disease. These data demonstrate new features of TT viruses suggesting possible similarities to plant viruses of the family *Geminiviridae*, as well as raise questions about the documented plurality and diversity of anelloviruses.

Several attempts have been made to link the ubiquitous Torque teno (TT) viruses to the etiology of a specific disease. These viruses were originally isolated from a patient suffering from posttransfusion hepatitis (32), but subsequent studies were unable to demonstrate a direct role of TT virus in the pathogenesis of hepatitis or hepatocellular carcinoma (14, 43).

TT viruses are circular, single-stranded DNA viruses of the genus *Anellovirus* that range in size from 2 to 3.8 kb. They have been isolated from humans and primates, as well as from pigs (2,878 bp), dogs (2,797 bp), and cats (2,064 bp) (15, 16, 37, 52, 53). Their genomes consist of two main open reading frames (ORFs) and additional smaller ORFs resulting from putative splicing events (15, 44). The putative noncoding region has a very high G+C content, with about 170 bp in this region being highly conserved among these viruses (30, 42). Their genome organization is similar to that of chicken anemia virus, which is vertically transmitted from infected parent chickens through the egg, causing anemia through lymphoid depletion in the progeny (29).

Peripheral blood mononuclear cells act as a reservoir for TT viruses (36, 63), whereas viral transcription and replication occur in bone marrow cells (19, 38, 39, 64). Investigations into a possible association between the presence of TT virus infections and leukemia or lymphoma have resulted in contradictory results (5, 10, 48). Epidemiological data on the distribution and prevalence of acute lymphoblastic leukemia led us to propose a possible role for TT virus-like viruses in the development of childhood leukemia and lymphomas (65). We pre-

viously isolated the full-length genomes of a spectrum of TT virus types from the spleen of a Hodgkin's disease patient (15). One group of six isolated complete genomes revealed the reorganization of the largest ORF, ORF1. Despite less than 1% overall nucleotide variation between these isolates, single-nucleotide differences resulted in the formation of two or several smaller ORFs within this region of the genome.

In the present study, we attempted to elucidate whether these rearranged TT virus genomes may be of significance in the pathogenesis of disease. We demonstrate in vitro TT virus DNA replication and particle formation after the transfection of a Hodgkin's lymphoma-derived cell line with the cloned full-length tth8 genome. Analyses of the transcription patterns of tth8 and tth7, which has an interruption in ORF1 (15), in a number of lymphoma and T-cell leukemia cell lines indicated differential additional splicing events and intragenomic rearrangement yielding ORFs which could not be deducted from the genomic sequence. The amplification of the replicating full-length viral genome after transfection in vitro led to additional off-sized viral DNA fragments, revealing intragenomic rearrangement events occurring in the viral genome. In addition, we demonstrated the presence of rearranged TT virus genomes ranging from 172 bp to full-length genomes in vivo in sera taken from pregnant mothers whose children later developed childhood leukemia, as well as sera from control mothers. Although a certain region of the TT virus genome seemed to be retained in these rearranged isolates, existing ORFs were sometimes completely deleted, and most were modified to induce new putative proteins. New ORFs were also introduced.

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MATERIALS AND METHODS

TT virus genomic DNA. Full-length genomes of the TT viruses tth8 (accession no. AJ620231) and tth7 (accession no. AJ620230) have been isolated previously from the spleen of a Hodgkin's disease patient (15). The viral sequences were

TABLE 1. Primers used in this study

Primer ^a	Nucleotide position ^b	Sequence ^c (5'–3')
Primers for RT-PCR and RACE		
ORF1s	598–618	TAT <u>TGA</u> <u>ATT</u> <u>CAT</u> GGC ATG GGG ATG GTG GAA
ORF1as	2827–2801	GCA GGC GGC CGC GAG CCG TAG GCA TGG GTT TAC ATG GAC CCC
h7g43s	568–588	CCT CAC AGG TTG ATT CGA GAC
h7g43as	1404–1384	ACC TGG AAG CTG ATG CAA GTG
5' GSPas	2660–2640	CCT CGG TCT CCC ACG GTC TCG
5'NGSPas	2592–2572	GGG ATG TCG ACC CGA GGC TTT
GAPDHs		TGG ATA TTG TTG CCA TCA ATG ACC
GAPDHas		GAT GGC ATG GAC TGT GGT CAT G
Primers for genomic PCR and long-PCR amplification		
Primer A	3211–3187	CGA AAC GCG CAA GCG TTT CGG GTG G
Primer B	3293–3317	GAA AGT GAG TGG GGC CAG ACT TCG C
tth25-1s	126–156	CCG CAG CGA GAA CGC CAC GGA GGG AGA TCC T
tth25-2as	125–95	ACG GGC GTG GAA AAC TCA GCC ATT CGG AAG T
tth25-7s	128–156	GCG GCG AGA ACG CCA CGG AGG GA
tth25-8as	127–101	GGA CGG GCG TGG AAA ACT CAG CCA TTC
jt34f-1s	223–247	GGC CGG GCC ATG GGC AAG GCT CTT A
jt34f-2as	220–195	TCC CGA GCC CGA ATT GCC CCT TGA CT
jt34f-3s	183–209	TTT ACA CAC CGC AGT CAA GGG GCA ATT
jt34f-4as	182–153	CTC ACC TCC GGC ACC CGC CCT CGG GTA CGT T
tth8-1s	133–164	AGA GGA GCC ACG GCA GGG GAT CCG AAC GTC CT
tth8-2as	132–102	CCG CGG ACG GGC AGC AAA AAC TCA GCC ATT C

^a s, forward; as, reverse.^b For RT-PCR and RACE primers, numbering corresponds to tth8; for genomic-PCR and long-PCR-amplification primers, numbering corresponds to TT virus.^c The EcoRI site in ORF1s and the NotI site in ORF1 as are underlined.

obtained after the cloning of the PCR-amplified genomes into the pUC18 vector (Invitrogen) by using the single cutter SalI. Cloned constructs were digested with the restriction enzyme SalI, and the linearized TT virus genomic inserts were separated from the plasmid DNA by gel purification prior to use for in vitro transfection assays (28, 56).

Cell culture and transfection. L428 (Hodgkin's lymphoma), Jurkat (human T-cell leukemia), HSB2 (T-cell leukemia), BJAB (Epstein-Barr virus-negative Burkitt's lymphoma), and Raji (Epstein-Barr virus-positive Burkitt's lymphoma) cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 0.01% penicillin-streptomycin in 5% CO₂ at 37°C. HepG2 (human undifferentiated hepatocellular carcinoma) and 293 (human embryo kidney) cell lines were maintained using Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and 0.01% streptomycin. Transfections were performed with the Nucleofector II transfection device and the nucleotransfection kit V using the respective protocol recommended for each cell type by the manufacturer (Amaxa Biosystems). Two micrograms of viral DNA was used to transfect 10⁶ cells of the 293 cell line and 5 × 10⁶ cells of each of the other cell lines. The plasmid pmaxGFP (Amaxa Biosystems) was used to measure the transfection efficiency in each transfection experiment.

Serum samples. The children of the 550,000 women who gave birth in Finland or Iceland from 1975 to 1997 formed a joint study cohort that was monitored for cancer before age 15 years through national cancer registries. First-trimester serum samples from the mothers of 342 acute lymphoblastic leukemia patients and 61 other leukemia patients, as well as samples from 1,216 matched control mothers, were retrieved from the Finnish and Icelandic Maternity Cohort Biobanks for a previous study on viral antibodies in maternal sera and childhood leukemia risk (22). For the present study, we selected serum samples from a subset of 30 Finnish mothers who gave birth to children who were diagnosed with childhood leukemia between the ages of 0 and 1.6 years, together with serum samples collected during the same time period from matched control mothers of similar ages whose children were monitored up through the ages at which the diagnoses of leukemia in the study children were made.

DNA isolation and PCR amplification. L428 cells transfected with full-length tth8 DNA were harvested 2 days after transfection, and total cellular DNA was extracted. Cell pellets were lysed in 0.5% sodium dodecyl sulfate–Tris-EDTA buffer and treated with 10 µg of RNase A (30 min at 37°C) and 200 µg of proteinase K (1 h at 50°C) before the DNA was extracted with phenol-chloroform and precipitated with ethanol. A second approach was also followed in order to separate smaller DNA molecules from the bulk of cellular DNA prior

to DNA extraction. In this case, transfected cells were initially centrifuged at 300 × g, followed by the centrifugation of the resulting supernatant at 2,750 × g. DNA was extracted from all pellets as described above. The isolated DNA was treated with DpnI (Fermentas) to remove the input plasmid DNA used for transfection. The replicating tth8 DNA was amplified from in vitro-transfected cells by long PCR with the primers A and B (Table 1). This touchdown long PCR was performed as follows: an initial denaturation at 95°C for 45 s was followed by five cycles of 1 min at 95°C and 5 min at 72°C; five cycles of denaturation for 1 min at 95°C, annealing for 1 min at 70°C, and elongation for 5 min at 72°C; 30 cycles of 1 min at 95°C, 1 min at 68°C, and 5 min at 72°C; and a final elongation step for 5 min at 72°C.

DNA was extracted from the serum samples by using the High Pure viral nucleic acid kit (Roche). These DNA samples were subsequently subjected to rolling-circle amplification with a TempliPhi kit (GE Healthcare). The presence of TT virus DNA in the serum samples was then demonstrated by PCR amplification using the primer combination NG133 and NG352 (resulting in an amplicon of 162 bp) followed by nested amplification with primers NG249 and NG351 (giving an amplicon of 134 bp) (36), as well as amplification with the primers NG472 and NG352 (resulting in an amplicon of 91 bp) followed by nested amplification with primers NG473 and NG351 (yielding an amplicon of 71 bp) (42). The resulting amplicons were separated by gel electrophoresis and eluted from the gel with a gel extraction spin kit (Genomed). Amplicons were each cloned into the vector pCR2.1 (TA cloning kit; Invitrogen) prior to sequencing. At least 10 clones from each amplicon were sequenced. Laboratory cross-contamination of these human samples with our previously described tth TT virus isolates (15) was highly unlikely as we did not include cloned TT virus DNA as positive controls in any of the PCR amplifications of DNA from the serum samples. The in vitro studies of two of our TT virus isolates, tth7 and tth8, were performed in separate locations.

Serum samples harboring TT virus DNA were subsequently subjected to long-PCR amplification using the TaKaRa LA *Taq* enzyme (TAKARA BIO INC., Japan) and at least one of the following primer combinations: tth25-1s and tth25-2as, tth25-7s and tth25-8as, jt34f-1s and jt34f-2as, and jt34f-3s and jt34f-4as (Table 1). Primers for this amplification were designed based on the respective 70-bp TT virus sequences obtained after amplification with the primers NG473 and NG351. The long-PCR amplification was performed using the touchdown stepwise reaction described above. All obtained amplicons were eluted and purified after gel electrophoresis, cloned, and sequenced. These reactions were controlled by the following. (i) A number of serum samples were

also analyzed by long PCR without prior rolling-circle amplification. (ii) An in vitro-religated, cloned, and purified full-length *tth8* genome mixed with 10 μ g of placenta or L428 cellular DNA served as a control for the long-PCR analysis of the serum samples. (iii) These control samples were analyzed by long PCR both with and without prior rolling-circle amplification. The primers (*tth8*-1 and *tth8*-2) used in this reaction were designed based on the *tth8* genomic sequence and corresponded to the back-to-back primers located in the control region of the TT virus genome in analogy to the primers used in the long-PCR analysis of the serum samples (Table 1).

Southern blot analysis. The in vitro replication of the viral DNA was monitored by transfecting L428 cells with the excised and linearized full-length *tth8* genome. These cells were harvested at days 1, 2, 3, and 7 after transfection. Total cellular DNA was isolated, and 10 μ g was digested with the restriction enzyme DpnI (Fermentas) to remove input viral DNA and the single cutter restriction enzyme AflII (New England Biolabs) to linearize replicating circular viral DNA. DNA samples were subjected to gel electrophoresis, after which the DNA was transferred onto a Hybond-N+ membrane (GE Healthcare). Hybridization, as well as labeling of the *tth8* ORF1 for use as a probe, was performed using the AlkPhos direct labeling kit (GE Healthcare). The blot was exposed for 2.5 h using Kodak MS Biomax film (Eastman Kodak).

RNA isolation and cDNA synthesis. Total RNA was isolated from transfected cells by using the mirVana kit according to the protocol of the manufacturer (Ambion). All RNA samples were treated with DNase I (New England Biolabs) to remove any residual DNA, followed by acid phenol (pH 4.8)-chloroform extraction and precipitation with ethanol. One microgram of RNA was reverse transcribed at 42°C for 1 h using 4 U of OmniScript reverse transcriptase from the OmniScript reverse transcription kit and 1 μ M oligo(dT)₁₂₋₁₈ primer according to the protocol of the manufacturer (QIAGEN). A second method for cDNA synthesis aimed at preventing secondary structure formation in RNA was performed for 1 h at 65°C using 15 U of ThermoScript reverse transcriptase (Invitrogen) and 1 μ M oligo(dT)₂₂ primer. The efficiencies of cDNA synthesis in all preparations were equal, as demonstrated using primers specific for the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene. A cDNA sample equivalent to 20 ng of total RNA was used as a template for each PCR amplification, which was performed with a final volume of 50 μ l by using specific *tth8* primers (Table 1). Ten microliters of the reaction mixture was analyzed by gel electrophoresis after 30, 33, and 36 cycles of amplification. PCR amplification was terminated after 39 cycles for samples in which no amplification was observed. All experiments were reproduced at least twice using different cDNA preparations.

The rapid amplification of 5' cDNA ends (5'-RACE) from *tth8* was carried out using the BD SMART RACE cDNA amplification kit according to the recommendations of the manufacturer (Clontech). The primers for 5'-RACE are listed in Table 1. Briefly, 400 ng of DNase-treated RNA was reverse transcribed with BD PowerScript reverse transcriptase for 1.5 h at 42°C. An aliquot of the reaction mixture was subjected to PCR amplification using UPM and GSP1 primers and a seminested reaction with UPM and NGSP1 primers and the Advantage 2 PCR kit reagents as recommended by the manufacturer (Clontech). The specificity of amplification was increased by using the following conditions: five cycles of 30 s at 94°C; annealing-extension for 3 min at 72°C followed by five cycles of 30 s at 94°C (denaturation), 30 s at 70°C (annealing), and 3 min at 72°C (extension); and finally, 25 cycles of 30 s at 94°C, 30 s at 69°C, and 3 min at 72°C. Amplicons were analyzed by gel electrophoresis in 2% agarose gels, eluted, and cloned into the vector pCR2.1 (Invitrogen).

Sequence analyses. All sequences were compared to sequences available in all relevant databases by using the HUSAR software package (9, 47).

Nucleotide sequence accession numbers. The sequences of the isolates obtained in this study have been submitted under the following accession numbers: sle1957, AM711976; sle2034, AM711977; sle2027, AM711978; sle2031, AM711979; sle2030, AM711980; sle2025, AM711981; sle2026, AM711982; sle2023, AM711983; sle2024, AM711984; sle1748, AM711985; sle1751, AM711986; sle1755, AM711987; sle1758, AM711988; sle1756, AM711989; sle1767, AM711990; sle1762, AM711991; sle1760, AM711992; sle1769, AM711993; sle1779, AM711994; sle1782, AM711995; sle1785, AM711996; sle1789, AM711997; sle1996, AM711998; sle1995, AM711999; sle1998, AM712000; sle2008, AM712001; sle2009, AM712002; sle1931, AM712003; sle1932, AM712004; sle2553, AM712005; sle2565, AM712006; sle2596, AM712007; sle2597, AM712008; sle2556, AM712009; sle2555, AM712010; sle2552, AM712011; sle2550, AM712012; sle2412, AM712013; sle2595, AM712014; sle2571, AM712015; sle2162, AM712016; sle2160, AM712017; sle2164, AM712018; sle2135, AM712019; sle2104, AM712020; sle2143, AM712021; sle2116, AM712022; sle2092, AM712023; sle2094, AM712024; sle2095, AM712025; sle1847, AM712026; sle1841, AM712027; sle1884, AM712028; sle1889, AM712029; sle2057, AM712030; sle2058, AM712031;

FIG. 1. In vitro replication and intragenomic rearrangement of *tth8* in the L428 cell line. (A) Southern blot analyses of total cellular DNA harvested at different time points after transfection. DNA was digested with DpnI to fragmentize input viral DNA (#). Hybridization was performed with labeled *tth8* DNA. The replication of genomic *tth8* DNA (AflII linearized, 3.7 kb; indicated by an arrow) increased from day 2 to day 7 following transfection. Lane 1, 1 day posttransfection; lane 2, 2 days posttransfection; lane 3, 3 days posttransfection; lane 4, 7 days posttransfection; lane 5, negative control with untransfected cells; lane 6, positive control for DpnI digestion of untransfected cellular DNA and religated *tth8* genome. M, DNA size marker. (B) Schematic presentation of the *tth8* genome after linearization with SalI. The DpnI digestion sites are indicated. ORF1as and ORF1s primers were used to amplify almost-full-length *tth8* DNA indicating the recircularization of the linearized *tth8* genome used for transfection (data not shown). (C, panel a) PCR amplification of full-length *tth8* DNA (3.7 kb), as well as shorter rearranged subviral fragments, 2 days after the transfection of L428 cells. Primers A and B were used for amplification. (C, panel b) Control long-PCR amplification of religated cloned genomic *tth8* DNA mixed with L428 cellular DNA. M, DNA size marker.

sle2072, AM712032; sle2061, AM712033; sle2065, AM712034; sle2018, AM712035; sle2016, AM712036; sle2019, AM712037; sle2022, AM712038; sle1999, AM712039; sle2015, AM712040; sle2002, AM712041; sle2001, AM712042; sle2004, AM712043; sle2003, AM712044; sle1988, AM712045; sle1983, AM712046; sle1963, AM712047; sle1981, AM712048; sle1975, AM712049; sle2052, AM712050; sle2053, AM712051.

RESULTS

In vitro replication of *tth8*. The release of TT virus particles into the supernatants of infected phytohemagglutinin-stimulated TT virus-negative peripheral blood mononuclear cell cultures and infected Raji cells has been described previously (7), but a cell culture system in which TT viruses will readily replicate is not yet available. Papillomaviruses cannot be readily propagated in cell culture. The in vitro transfection of cells with a cloned, linearized papillomavirus genome is frequently used in order to study the viral life cycle and to produce limited amounts of viral particles (28, 56). We therefore resorted to

FIG. 2. Electron micrograph of L428 cells 4 days after transfection with linearized full-length tth8 DNA. TT virus-like particles within a destructed and probably apoptotic cell, measuring approximately 30 to 40 nm in diameter, are visible.

this method to induce TT virus replication in vitro. We transfected L428 cells with SalI-linearized tth8 genomic DNA. Southern blot hybridization of the DpnI-AflII-digested total cellular DNA indicated the replication of the full-length tth8 genome already at 48 h after transfection. (Fig. 1A). The level of replicating tth8 genomic DNA increased up to day 7 after

transfection, at which point almost no input DNA was present as measured by DpnI digestion. As the restriction enzyme AflII is a single cutter of the tth8 genome, the hybridization of the tth8 DNA at ca. 4 kb indicates a recircularization of the SalI-linearized viral genome after transfection and during replication. (Fig. 1B). This recircularization was also indicated by the PCR amplification of cDNA covering the region of the genome harboring the SalI restriction site (data not shown). Electron micrographs of the transfected cells demonstrated cytoplasmic virus-like particles of the expected sizes of 30 to 40 nm (Fig. 2).

In vitro transcription of tth8. Our interest was to determine whether TT viruses may play a role in the etiology of hematopoietic diseases. Having demonstrated active tth8 virus replication after the transfection of cells with the full-length virus genome, we continued to analyze the viral transcripts in these cells in order to detect as-yet-uncharacterized viral mRNA species which may indicate additional translational events. The in vitro transcription of TT virus genomes has been reported previously (18, 44), confirming the three mRNA species of 2.9, 1.2, and 1.0 kb previously found in bone marrow cells (39). The alternative splicing events within the tth7 genome which we predicted previously (15) were recently confirmed for the TT virus genotype 6 (44). The tth8 and tth7 genomes differ by only 1% in their nucleotide sequences, and point mutations in the tth7 genome lead to the fractionation of ORF1 into two shorter ORFs (15). The L428 Hodgkin's lymphoma cells were transfected with tth7 and tth8 genomes, and the resulting transcripts were analyzed. We used a series of primer combinations to amplify specific regions within each of the cDNA clones. No

TABLE 2. tth7 and tth8 mRNA species with the respective splice donor and acceptor sites and their deviations from the consensus sequences^a

tth transcript(s)	Splice donor site		Splice acceptor site	
	Nucleotide position	Sequence	Nucleotide position	Sequence
Consensus		(A/C) <u>AGGT</u> (A/G) AGT		(C/T) ₁₅ <u>AG</u> (A/G)
<i>a</i> , <i>a1</i> ^{b,c}	181	<u>GAGGTGAGT</u>	276	<u>TTTTCGAATAAGCAGA</u>
	716	<u>GAAGAGTAAGG</u>	2565	<u>TTGTATTCTCAGG</u>
<i>b</i> , <i>b1</i> , <i>e</i> ^{b,c}	181	<u>GAGGTGAGT</u>	276	<u>TTTTCGAATAAGCAGA</u>
	716	<u>GAAGAGTAAGG</u>	2363	<u>TCATTGAACAGA</u>
<i>c</i> ^{b,c}	181	<u>GAGGTGAGT</u>	276	<u>TTTTCGAATAAGCAGA</u>
	389	<u>GTGGTATGA</u>	486	<u>CCATCCAACAGG</u>
	716	<u>GAAGAGTAAGG</u>	2565	<u>TTGTATTCTCAGG</u>
<i>d</i> ^b	181	<u>GAGGTGAGT</u>	276	<u>TTTTCGAATAAGCAGA</u>
	389	<u>GTGGTATGA</u>	2565	<u>TTGTATTCTCAGG</u>
<i>e1</i> ^{b,c}	181	<u>GAGGTGAGT</u>	276	<u>TTTTCGAATAAGCAGA</u>
	389	<u>GTGGTATGA</u>	486	<u>CCATCCAACAGG</u>
	716	<u>GAAGAGTAAGG</u>	2363	<u>TCATTGAACAGA</u>
<i>f</i> ^c	716	<u>GAAGAGTAAGG</u>	1281	<u>CTCTTTACAGA</u>
<i>g</i> ^c	831	ctGca <u>GTAAT</u>	1316	ACctcaCCCTTTT
<i>h</i> ^c	701	ctAGACgac	766	ActTACagac
	831	ctGca <u>GTAAT</u>	1316	ACctcaCCCTTTT
<i>i</i> ^c	716	<u>GAAGAGTAAGG</u>	2363	<u>TCATTGAACAGA</u>
	2387	ctTCcaGCca	2615	AAGAAGGAA <u>AG</u> ctca
<i>j</i> ^c	716	<u>GAAGAGTAAGG</u>	2583	CGGGTCGAC
<i>k</i> ^c	716	Gaaga <u>GTaa</u> GG	2590	ATCCCAaaCAGaa
<i>l</i> ^c	716	Gaaga <u>GTaa</u> gG	2594	CCaaAACaag
<i>m</i> ^c	716	Gaaga <u>GTaa</u> gG	2604	CaagaAACCcaaga
<i>n</i> ^c	716	Gaaga <u>GTaa</u> gG	2629	ACTCCAaag

^a Bold underlined letters represent key nucleotides in the consensus sequence; bold letters indicate nucleotides in transcript sequences that are identical to those in the consensus sequence. Lowercase letters represent possible repetitive sequences.

^b Detected by RACE.

^c Detected by RT-PCR.

FIG. 3. Schematic presentation of *tth7* and *tth8* transcripts. Solid lines indicate RNA sequences, and dashed lines represent introns or deleted regions. Nucleotide positions of the junction sites are indicated. Putative ORFs are represented by boxes, and the size of the predicted protein corresponding to each ORF is indicated. Primers used for amplification are shown by arrowheads. aa, amino acids. (A) ORFs as predicted from the full-length transcript of TT virus genomic DNA. (B) mRNA species (*a* to *e1*) obtained after 5'-RACE amplification using the 5'NGSP primer. Transcripts *a* and *a1* and transcripts *b*, *b1*, and *e* were identical in length, but sequence analyses resulted in putative proteins of various sizes. (C) mRNA transcripts (*f* to *h*) identified by RT-PCR amplification of ORF1 by using the h7g43s and h7g43as primers. (D) Transcripts (*i* to *n*) identified by RT-PCR using primers ORF1s and ORF1as.

obvious differences between *tth8* and *tth7* transcripts were noted. In order to map the *tth8* viral transcripts, we performed RACE. A total of 105 cDNA clones were sequenced and analyzed.

The *tth* mRNA species analyzed are listed in Table 2. The respective splice donor and splice acceptor sites, as well as deviations from the consensus sequences (1), are indicated. Figure 3 is a schematic presentation of the respective mRNA species. Transcripts *a*, *a1*, *b*, *b1*, and *e* correspond to the previously characterized TT virus mRNAs (18, 44), as well as previously predicted splicing patterns for *tth7* (15). Transcripts *c*, *d*, and *e1* have not been described before. Transcript *c* resembles transcript *a* except for additional splicing between nucleotides (nt) 389 and 486 (Table 2), and transcript *d* is spliced between nt 389 and 2565. Transcript *e1* also has the extra splicing site at nt 389 to 486. Both transcripts *e* and *e1* use

the splice acceptor site at nt 2363 and have a stop codon at nt 2589. The splicing donor and acceptor signals at the additional splicing site are well conserved. The translation of the ORFs in transcripts *e* and *e1* would result in putative proteins of 194 and 161 amino acids, respectively. Transcript *b* may code for the putative ORF3 protein, and *b1* may code for the ORF1m12 protein, whereas transcript *a* may code for the putative ORF4 protein and transcript *a1* may code for the ORF1m10 protein (15). Transcripts *f*, *g*, and *h* were identified by reverse transcription-PCR (RT-PCR) amplification of cDNA generated from *tth*-transfected cells by using the h7g43 primers, which amplify part of ORF1 (nt 568 to 1404) (15; Table 2). Transcripts *i* through *n* were also identified by RT-PCR but covered a larger region of ORF1, between nt 598 and 2827. For the most part, in transcripts *f* through *n*, either the splice donor and/or acceptor site was conserved or the junction covered possible repetitive sequences which may have been involved in these rearrangements. The junctions at nt 1316 and 2583, however, did not comply with either of these alternatives. The intragenomic rearrangement events at these sites were verified by repeating the cDNA synthesis with a reverse transcriptase which avoids the formation of RNA secondary structure artifacts.

Patterns of in vitro transcription of *tth8* and *tth7* in other hematopoietic cell lines. Hematopoietic cells act as the main reservoir for natural TT virus infections. We investigated whether the in vitro transcription of *tth7*, in comparison with that of *tth8*, would differ depending on cell type or the type of disease associated with the cells. Transcription in the T-cell leukemia cell lines Jurkat and HSB2, as well as that in the Burkitt's lymphoma cell lines BJAB and Raji, was analyzed, and transcripts were compared to the above-described transcripts from the L428 Hodgkin's lymphoma cell line. The human embryonal kidney cell line 293 was included based on the pathological changes reported in the kidneys of TT virus ORF1 transgenic mice (62). Double-stranded DNA as replicative forms of the TT virus genome was previously demonstrated to be present in liver cells of naturally infected subjects (40). We therefore included the HepG2 cell line in our experiments. Each of the cell lines was transfected with the full-length *tth7* and *tth8* genomes. Total RNA was isolated 48 h later, and oligo(dT) cDNA was synthesized. Transcription patterns were analyzed by PCR amplification with a series of primers located within ORF1 (Table 1). The patterns of transcription of *tth7* and *tth8* did not differ. Transcripts covering almost the complete sequence of ORF1 (obtained with primers ORF1s and ORF1as) were present in all cell lines except the HSB2 cell line (Fig. 4). We also did not detect any other *tth* transcripts in this cell line despite including positive controls for transfection and cDNA synthesis. We could not identify any obvious reason for this failure of transcription in HSB2 cells in comparison to the other cell lines tested, which included another T-cell leukemia cell line, Jurkat. A nonspliced transcript from the 293, Jurkat, L428, and BJAB cell lines was amplified with the h7g43 primers (Table 2 and Fig. 3), whereas transcripts *g* and *h* were present only in the L428 cells. These transcripts all contained ORFs for which the predicted proteins were partial ORF1 proteins varying in size. These results indicate a plurality of in vitro transcripts from one TT virus type probably arising from both alternative splicing events and intragenomic rearrange-

FIG. 4. Cell lines L428, BJAB, and HSB2 were transfected with *tth8* DNA, RNA was isolated 2 days after transfection, and oligo(dT) cDNA synthesis was performed. Regions within ORF1 were amplified. (A) RT-PCR amplification using primers ORF1s and ORF1as. Lanes 1, 9, and 13, negative controls; lane 2, *tth8* genomic DNA as a positive control for amplification; lanes 3 to 5, 6 to 8, and 10 to 12, L428, BJAB, and HSB2 samples subjected to 30, 33, and 36 cycles of RT-PCR amplification, respectively. Amplicons indicated the presence of transcripts *a* and *b* in L428 and BJAB samples, whereas no transcripts in HSB2 cells were detected. (B) RT-PCR amplification using primers *h7g43s* and *h7g43as* (Table 1). Amplicons indicated the presence of a nonspliced transcript, as well as the additional transcripts *g* and *h*. Lanes 3, 4, and 5, products from 30, 33, and 36 cycles of RT-PCR amplification, respectively; lane 1, negative control; lane 2, positive control for *tth8* genomic DNA amplification; M, DNA size marker.

ment but thereby opening the possibility for the expression of a variety of modified proteins.

In vitro intragenomic rearrangement of *tth7* and *tth8*. While verifying the in vitro replication of the full-length *tth8* genome in transfected cells by long-PCR amplification (performed with total DpnI-digested *tth8* DNA from transfected cells), resulting amplicon sizes did not indicate the presence of only the full-length viral genome but also additional smaller, off-sized viral DNA fragments (ranging from approximately 200 to 2,500 bp) (Fig. 1C). The primers used for the long amplification of the *tth8* genome were unable to amplify fragments of the DpnI-digested input DNA. The amplification of L428 cellular DNA mixed with religated *tth8* genomic DNA resulted in one amplicon corresponding in size to the full-length *tth8* genome (Fig. 1C). All amplicons were cloned and sequenced. Sequence data for the smaller amplicons revealed intragenomic rearrangement of the viral DNA occurring mainly at repetitive sequences present in the *tth8* genomic sequence. Repetitive sequences can lead to the formation of hairpin structures which then form the basis for subsequent recombinational events (59).

In vivo intragenomic rearrangement of TT virus genomes. Previous data on the prevalence of TT virus infections in blood samples from lymphoma and leukemia patients have been con-

FIG. 5. Examples of the long-PCR amplification of DNA from individual serum samples using *jt34f-1s* and *jt34f-2as* and *tth25-1s* and *tth25-2as*. Full-length TT virus genomes (3.5 to 4 kb) as well as off-sized rearranged subviral fragments were amplified. Serum sample numbers are indicated at the top. Long-PCR amplification was controlled by the amplification of religated and rolling-circle-amplified *tth8* genomic DNA mixed with placenta DNA (lane a, 10^2 copies, and lane b, 10^3 copies of the *tth8* genome). M, DNA size marker.

tradictory (5, 10, 48), and transplacental infection with TT virus has been described previously (12, 26, 31, 45). The highly conserved region (170 bp; amplicon from nested amplification, 135 bp) of the TT virus control region (36) was amplified in order to cover as broad a spectrum of TT virus types as possible. The amplification of the 3' half of this region (91 bp; amplicon from nested amplification, 71 bp), which exhibits an even higher level of conservation, allows for the detection of additional TT virus types (42). We amplified both these regions from all samples. All amplicons were cloned and sequenced. TT virus sequences were detected in 39 (65%) of 60 sera by amplifying the 170-bp region and 58 (97%) of 60 samples by amplifying the 71-bp fragment. No obvious differences in the frequency of TT virus DNA between study sample and control mothers were noted.

Because the high level of sequence homology between TT viruses in the 71-bp stretch prevents the unequivocal identification of the TT virus type, we continued to amplify complete TT virus genomes by using back-to-back primers (Table 1) designed based on the previously obtained TT virus sequence data. In addition, we argued that the simultaneous amplification of subviral genomes from in vivo samples would support our in vitro data. DNA samples from 36 sera were first subjected to rolling-circle amplification (TempliPhi) followed by long-PCR amplification. Amplicons corresponding to the sizes of complete genomes, as well as additional subgenomic amplicons, were obtained from a number of serum samples (Fig. 5).

FIG. 6. Breakpoint positions (arrows) in the intragenomic rearranged subviral isolates in relation to the *tth25* genome. Boxes indicate the positions of the respective TT virus ORFs.

Primers differing in single nucleotides and/or length were designed for use with the serum samples in order to optimize amplification conditions. The use of each primer combination with each individual serum sample resulted in amplicons differing in size. We processed tth8 genomic religated DNA mixed with placenta DNA and subjected the mixture to rolling-circle amplification under identical conditions to control for possible PCR artifacts (Fig. 5). Only full-length genomic DNA yielded a visible band in the tth8 control amplification. We also amplified DNA from a number of serum samples without prior rolling-circle amplification as an additional control (data not shown). Prior rolling-circle amplification of the serum DNA or tth8 DNA did not influence the outcome of the long-PCR amplification. The numbers and sizes of the subgenomic amplicons varied among serum samples. The presence of single subgenomic amplicons in a few samples (samples 71, 74, and 84) may reflect possible selection for such molecules. We isolated and cloned the full-length genomes, as well as the majority of the subgenomic amplicons, and analyzed 296 sequences attached to the respective 5' and 3' primer sequences. We took the presence of both primer sequences as an indication of a circular conformation of these isolates in the sera. The isolates ranged in size from 172 bp to full-length genomes. All sequences were compared to TT virus sequences available in the data banks by BLAST analyses. A very heterogeneous picture emerged from the sequence data. The rearrangement of the subviral isolates, as evidenced by homology to different fragmented regions of the TT virus genome, did not allow for the clear-cut identification of novel TT virus types. The degree of genomic rearrangement varied among serum samples. Examples of corresponding nucleotide positions (within the tth25 genome) at which breakpoints in the rearranged sequences were localized are depicted in Fig. 6. The breakpoint locations were randomly distributed throughout the genome, and distinct nucleotide patterns could not be determined, although the majority occurred in G+C-rich areas of the sequence.

Examples of the subgenomic viral molecules varying in size and genomic rearrangement are depicted in Fig. 7. All putative proteins encoded by ORFs present in the subviral DNA were analyzed for similarities to known proteins (Domainsweep) (9). Putative proteins showing similarity to TT virus proteins were present in the subviral isolates, and additional putative proteins (those larger than 50 amino acids are indicated in Fig. 7) which did not show homology to any protein listed in the data banks were identified. The overall observed genomic rearrangement among the multiple subgenomic molecules isolated from individual serum samples often varied from that observed among molecules from other serum samples. In groups in which, e.g., ORF2 remained largely intact (such as isolates from serum sample 82), ORF1 would vary in size (isolates from serum sample 52 encoded 52 to 224 amino acids)

or be absent (isolates from serum sample 53 were 172 bp and 737 bp). Interestingly, the ORF2 genes in the group of subviral isolates showing homology to TT virus saa-01 (accession no. ab060597) (35) (Fig. 7E) were either rearranged (seven isolates) or absent (two isolates).

Five serum samples (no. 49, 52, 74, 71, and 82) harbored TT virus sequences most closely related to TT virus tth25 (accession no. aj620222) (15) (Fig. 7A). The subviral isolates from serum sample 74 (13 isolates between 631 bp and 2,130 bp) largely retained the genome organization of TT viruses despite the decrease in total nucleotide length. ORF1 was located on the positive strand in eight isolates and on the negative strand in four other isolates and was absent in another. Similarly, ORFs in the two isolates from serum sample 52 which may encode putative proteins similar to the ORF3 protein were located on the negative strand. Serum sample 52, in addition, harbored rearranged isolates with the greatest degree of homology to the TT virus-like mini virus tlmv-nlc030 (accession no. ab038631) (52) (Fig. 7B). A putative ORF2 was absent from all these subviral isolates.

A third group of three serum samples (no. 45, 46, and 52) harbored TT virus sequences most closely related to the TT virus tchn-a (accession no. af345526) (23) (Fig. 7C).

Two subviral isolates (561 and 565 bp) showed homology to TT virus tjn01 (58) (Fig. 7D). The putative proteins from the only ORF present in both these clones showed similarity to the TT virus ORF2 protein. The fact that one subviral genome (sle2460, 561 bp) was isolated from 11 individual serum samples indicates possible positive selection for this specific TT virus sequence. These 11 serum samples originated from 7 study sample mothers and 4 control mothers.

DISCUSSION

The ubiquitous nature of TT viruses has led to the notion that infection with these viruses may occur without any obvious pathogenic effects. One report associated primary infection with respiratory disease in children (24), but this association still awaits confirmation by others.

Several previous studies have dealt with possible vertical transmission from mother to child (6, 20, 51). The presence of TT virus in cord blood has been described previously (11, 12, 26, 47, 61), whereas another study failed to confirm these results (34). In only a few cases can the corresponding TT virus type be detected in both mother and child immediately after birth; the majority of children acquire TT virus types other than those demonstrated to be present in their mothers (6, 34, 51). Our original investigation into a link between TT virus infection and disease led to the identification of TT virus-like sequences in tumors of the gastrointestinal tract and myelomas (8). We subsequently isolated a number of TT virus sequences

FIG. 7. Examples of the genomic organization of the rearranged subviral isolates from individual serum samples (numbers are indicated on the right side of each block). The genomic organization of the most closely related known TT virus is indicated at the top of each series. (A) Group most closely related to tth25 (accession no. aj620222); (B) group most closely related to tlmv-nlc030 (accession no. ab0038631); (C) group most closely related to tchn-a (accession no. af345526); (D) group most closely related to tjn01 (accession no. ab028668); and (E) group most closely related to saa-01 (accession no. ab060597). ORFs homologous to known TT virus ORFs are indicated by filled boxes, and those with no identified homology are indicated by unfilled boxes. Deviations from the sizes of known TT virus ORFs are indicated. aa, amino acids.

from the spleen of a patient with Hodgkin's disease (15). Based on published epidemiological data, as well as the findings of our previous studies and the fact that peripheral blood mononuclear cells act as a reservoir for these viruses (39, 40, 54, 64), we suggested a role for TT virus-like viruses in the pathogenesis of leukemia and lymphomas (65). These findings prompted us to analyze serum samples from mothers of children who had developed childhood leukemia in comparison with those from a group of mothers of healthy children for the presence of TT virus sequences. No difference in TT virus prevalence between the two groups of samples was noted. The majority of samples were found to contain TT virus sequences if the most-conserved 90 nucleotides in the noncoding region of the TT virus genome were targeted for amplification.

The high level of sequence homology in this region of the genomes of TT viruses did not permit distinction among TT virus types from the samples. As TT virus type specificity may be an indicator of the difference between the two groups of serum samples, we performed long-PCR amplification of the viral DNA in order to isolate complete viral genomes from the samples. The resulting amplicons represented not only complete TT virus genomes but also an additional spectrum of off-sized subviral genomes varying in size among individual samples, without, however, an association with the leukemia group. Analyses of these viral sequences revealed intragenomic rearrangement leading to rearrangements of ORFs, which in turn often resulted in putative proteins of undefined functions. The majority of rearranged subviral isolates had retained the noncoding region and the ORF2 gene of the TT virus genome. We also observed this phenomenon of rearrangement within the TT virus genome during *in vitro* replication studies of the tth8 TT virus genome. Subviral genomes, in addition to the replicating full-length genome, were detected as early as 2 days after transfection. This rearrangement was also mirrored in mRNA species isolated from these tth8-replicating cells. We partially confirmed the previously described transcription pattern for TT viruses (18, 39, 44), as well as identified a number of additional mRNA species. The latter included species from alternative splicing events, as well as species with junctions which may have resulted from the presence of repetitive sequences in the respective regions of the TT virus genome. An off-sized mRNA species probably resulting from recombination was detected during the replication of chicken anemia virus of the family *Circoviridae* (small, single-stranded DNA viruses) (17), and this mRNA species shows sequence homology to the noncoding regions of TT virus genomes (30).

The plurality of TT viruses has posed an additional problem in attempts to associate an infection with disease. This plurality became evident (3, 21, 35) soon after the first discovery of these viruses (32). Not only do the virus genomes vary in size (those of TT virus, TT virus-like mini virus, and small anellovirus), but the isolation of close to 100 individual TT virus types from human samples has been reported previously. Single-nucleotide differences which occur throughout the genomes of full-length isolates may lead to altered protein expression (3, 15), whereas sequence alteration in the defined hypervariable region of ORF1 of a TT virus type may play a role in immune modulation (15, 33). Intragenomic rearrangement involving larger fragments within one TT virus genome, however, has not been described previously. Homologous re-

combination between TT virus types has been discussed, and putative recombination breakpoints in the noncoding region have been identified (4, 60). The phylogenetic analysis of amplified sequences from a very short region within ORF1 also suggested recombinational events (25). The intragenomic rearrangement in our subviral isolates of various sizes, however, involves the rearrangement of genomic fragments resulting from breakpoints scattered throughout the genome (Fig. 6). No distinct sequence patterns or locations which could be causing the genetic instability (59) at these viral breakpoints were noted. Secondary structure formation within the viral genome involving repetitive sequences and G+C-rich regions is highly likely.

Among animal viruses, recombination in herpesviruses, where it occurs through either genomic segment inversion or recombination between highly related species, has been described previously. It is not known whether this recombination influences the virulence of these viruses (55). The high degree of heterogeneity in the human immunodeficiency virus genome results from slippage-mediated mutations involving single nucleotides or very small regions of the genome (13). This heterogeneity increases during the progression of disease (27). Hepatitis C virus, another RNA virus, continuously adapts to its host by cell defense evasion mechanisms and therefore represents a quasispecies (49). Examples in which recombination between viral and subviral components may aggravate disease symptoms can be found among the single-stranded DNA plant viruses of the family *Geminiviridae* (50). Recombination between DNA satellites plays a role in the evolution of these viruses (2), but deletions and recombination occurring at repetitive sequences within or between the DNA-A and DNA-B components of the begomoviruses lead to defective DNA components which in turn lead to a modification in the disease symptoms (41). The genomes of nanoviruses in plants consist of multiple circular single strands of DNA, the majority of which encode one gene each (57). Interfamilial recombination between geminiviruses and nanoviruses has been described previously (46). Recombinational events among single-stranded DNA viruses infecting humans, however, have not been described.

The isolation of subviral fragments of TT viruses described in the present study raises two questions. The first is whether these subviral isolates can, either alone or in combination with full-length TT virus genomes, induce pathogenic changes in infected cells. The presence of full-length TT virus genomes appears to be apathogenic, but the formation of accompanying subviral components may be necessary for the initiation of disease, as evident for the *Geminiviridae*. The possible selection for specific rearranged molecules *in vivo* as suggested by the presence of isolate sle2460 in 11 individual serum samples requires special attention. It remains to be clarified whether specific intragenomic rearrangements within specific TT virus types are required for the induction of disease. The second question arising from our study is whether the plurality of the known full-length TT viruses actually represents individual TT virus types or whether the shorter genomes may indicate previous intragenomic rearrangements. This question cannot presently be answered, as studies analyzing multiple isolates from serial samples from the same individual have not been reported. The genome structure of the recently isolated small

anellovirus type 2 (16) may, however, suggest such an event. More information on both these aspects, although addressing two completely different issues, will help to clarify the meaning of the ubiquitous nature, as well as the diversity and plurality, of the TT virus group.

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We declare that we have no conflict of interest.

REFERENCES

- Aebi, M., H. Hornig, R. A. Padgett, J. Reiser, and C. Weissmann. 1986. Sequence requirements for splicing of higher eukaryotic nuclear pre-mRNA. *Cell* 47:555–565.
- Amin, I., S. Mansoor, L. Amrao, M. Hussain, S. Irum, Y. Zafar, S. E. Bull, and R. W. Briddon. 2006. Mobilization into cotton and spread of a recombinant cotton leaf curl disease satellite. *Arch. Virol.* 151:2055–2065.
- Ball, J. K., R. Curran, S. Berridge, A. M. Grabowska, C. L. Jameson, B. J. Thomson, W. L. Irving, and P. M. Sharp. 1999. TT virus sequence heterogeneity in vivo: evidence for co-infection with multiple genetic types. *J. Gen. Virol.* 80:1759–1768.
- Biagini, P., P. Gallian, H. Attoui, M. Touinssi, J.-F. Cantaloube, P. de Micco, and X. de Lamballerie. 2001. Genetic analysis of full-length genomes and subgenomic sequences of TT virus-like mini virus human isolates. *J. Gen. Virol.* 82:379–383.
- Cacoub, P., E. Rosenthal, V. Gerolami, P. Hausfater, P. Ghillani, Y. Sterkers, V. Thibault, H. Khiri, J. C. Piette, and P. Halfon. 2003. Transfusion-associated TT virus co-infection in patients with hepatitis C virus is associated with type II mixed cryoglobulinemia but not with B-cell non-Hodgkin lymphoma. *Clin. Microbiol. Infect.* 9:39–44.
- Davidson, F., D. MacDonald, J. L. Mokili, L. E. Prescott, S. Graham, and P. Simmonds. 1999. Early acquisition of TT virus (TTV) in an area endemic for TT infection. *J. Infect. Dis.* 179:1070–1076.
- Desai, M., R. Pal, R. Deshmukh, and D. Banker. 2005. Replication of TT virus in hepatocyte and leukocyte cell lines. *J. Med. Virol.* 77:136–143.
- de Villiers, E. M., R. Schmidt, H. Delius, and H. zur Hausen. 2002. Heterogeneity of TT virus related sequences isolated from human tumor biopsy specimens. *J. Mol. Med.* 80:44–50.
- Ernst, P., K. H. Glatting, and S. Suhai. 2003. A task framework for the web interface W2H. *Bioinformatics* 19:278–282.
- Garbuglia, A. R., T. Iezzi, M. R. Capobianchi, P. Pignoloni, A. Pulsoni, J. Sourdis, E. Pescarmona, D. Vitolo, and F. Mandelli. 2003. Detection of TT virus in lymph node biopsies of B-cell lymphoma and Hodgkin's disease and its association with EBV infection. *Int. J. Immunopathol. Pharmacol.* 16:109–118.
- Gerner, P., R. Oettinger, W. Gerner, J. Falbrede, and S. Wirth. 2000. Mother-to-infant transmission of TT virus: prevalence, extent and mechanism of vertical transmission. *Pediatr. Infect. Dis. J.* 19:1074–1077.
- Goto, K., K. Sugiyama, T. Ando, F. Mizutani, K. Terabe, K. Tanaka, M. Nishihyama, and Y. Wada. 2000. Detection rates of TT virus DNA in serum of umbilical cord blood, breast milk and saliva. *Tohoku J. Exp. Med.* 191:203–207.
- Hamburgh, M. E., K. A. Curr, M. Monaghan, V. R. Rao, S. Tripathi, B. D. Preston, S. Sarafianos, E. Arnold, T. Darden, and V. R. Prasad. 2006. Structural determinants of slippage-mediated mutations by human immunodeficiency virus type 1 reverse transcriptase. *J. Biol. Chem.* 281:7421–7428.
- Hsu, H. Y., Y. H. Ni, H. L. Chen, J. H. Kao, and M. H. Chang. 2003. TT virus in healthy children, children after blood transfusion, and children with non-A to E hepatitis or other liver diseases in Taiwan. *J. Med. Virol.* 69:66–71.
- Jelcic, I., A. Hotz-Wagenblatt, A. Hunziker, H. zur Hausen, and E.-M. de Villiers. 2004. Isolation of multiple TT virus genotypes from spleen biopsy tissue from a Hodgkin's disease patient: genome reorganization and diversity in the hypervariable region. *J. Virol.* 78:7498–7507.
- Jones, M. S., A. Kapoor, V. V. Lukashov, P. Simmonds, F. Hecht, and E. Delwart. 2005. New DNA viruses identified in patients with acute viral infection syndrome. *J. Virol.* 79:8230–8236.
- Kamada, K., A. Kuroishi, T. Kamahora, P. Kabat, S. Yamaguchi, and S. Hino. 2006. Spliced mRNAs detected during the life cycle of *Chicken anemia virus*. *J. Gen. Virol.* 87:2227–2233.
- Kamahora, T., S. Hino, and H. Miyata. 2000. Three spliced mRNAs of TT virus transcribed from a plasmid containing the entire genome in COS1 cells. *J. Virol.* 74:9980–9986.
- Kanda, Y., Y. Tanaka, M. Kami, T. Saito, T. Asai, K. Izutsu, K. Yuji, S. Ogawa, H. Honda, K. Mitani, S. Ciba, Y. Yazaki, and H. Hirai. 1999. TT virus in bone marrow transplant recipients. *Blood* 93:2485–2490.
- Kazi, A., H. Miyata, K. Kurokawa, M. A. Khan, T. Kamahora, S. Katamine, and S. Hino. 2000. High frequency of postnatal transmission of TT virus in infancy. *Arch. Virol.* 145:535–540.
- Khudyakov, Y. E., M. Chong, B. Nichols, D. Reed, X. Dou, S. O. Viazov, J. Chang, M. W. Fried, I. Williams, W. Bower, S. Lambert, M. Purdy, M. Roggendorf, and H. A. Fields. 2000. Sequence heterogeneity of TT virus and closely related viruses. *J. Virol.* 74:2990–3000.
- Lehtinen, M., P. Koskela, H. M. Ögmundsdóttir, A. Bioigu, J. Dillner, M. Gudnadóttir, T. Hakulinen, A. Kjartansdóttir, M. Kvarnung, E. Pukkala, H. Tulinius, and T. Lehtinen. 2003. Maternal herpesvirus infections and risk of acute lymphoblastic leukaemia in the offspring. *Am. J. Epidemiol.* 158:207–213.
- Luo, K., H. He, Z. Liu, D. Liu, H. Xiao, X. Jiang, W. Liang, and L. Zhang. 2002. Novel variants related to TT virus distributed widely in China. *J. Med. Virol.* 67:118–126.
- Maggi, F., M. Pifferi, C. Fornai, E. Andreoli, E. Tempertini, M. Vatteroni, S. Presciutti, S. Marchi, A. Pietrobello, A. Boner, M. Pistello, and M. Bendinelli. 2003. TT virus in the nasal secretions of children with acute respiratory diseases: relations to viremia and disease severity. *J. Virol.* 77:2418–2425.
- Manni, F., A. Rotola, E. Caselli, G. Berorelle, and D. Di Luca. 2002. Detecting recombination in TT virus: a phylogenetic approach. *J. Mol. Evol.* 55:563–572.
- Matsubara, H., K. Michitaka, N. Horiike, T. Kihana, M. Yano, T. Mori, and M. Onji. 2001. Existence of TT virus DNA and TTV-like mini virus DNA in infant cord blood: mother-to-neonatal transmission. *Hepatol. Res.* 21:280–287.
- McNearney, T., Z. Hornickova, R. Markham, A. Birdwell, M. Arens, A. Saah, and L. Ratner. 1992. Relationship of human immunodeficiency virus type 1 sequence heterogeneity to stage of disease. *Proc. Natl. Acad. Sci. USA* 89:10247–10251.
- Meyers, C., T. J. Mayer, and M. A. Ozbun. 1997. Synthesis of infectious human papillomavirus type 18 in differentiating epithelium transfected with viral DNA. *J. Virol.* 71:7381–7386.
- Miller, M. M., K. A. Ealey, W. B. Oswald, and K. A. Schat. 2003. Detection of chicken anemia virus DNA in embryonal tissues and eggshell membranes. *Avian Dis.* 47:662–671.
- Miyata, H., H. Tsunoda, A. Kazi, A. Yamada, M. A. Khan, J. Murakami, T. Kamahora, K. Shiraki, and S. Hino. 1999. Identification of a novel GC-rich 113-nucleotide region to complete the circular, single-stranded DNA genome of TT virus, the first human circovirus. *J. Virol.* 73:3582–3586.
- Morricca, A., F. Maggi, M. L. Vatteroni, C. Fornai, M. Pistello, P. Ciccorossi, E. Grassi, A. Gennazzani, and M. Bendinelli. 2000. TT virus: evidence for transplacental transmission. *J. Infect. Dis.* 181:803–804.
- Nishizawa, T., H. Okamoto, K. Konishi, H. Yoshikawa, Y. Miyakawa, and M. Mayumi. 1997. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. *Biochem. Biophys. Res. Commun.* 241:92–97.
- Nishizawa, T., H. Okamoto, F. Tsuda, T. Aikawa, Y. Sugai, K. Konishi, Y. Akahane, M. Ukita, T. Tanaka, Y. Miyakawa, and M. Mayumi. 1999. Quasi-species of TT virus (TTV) with sequence divergence in hypervariable regions of the capsid protein in chronic TTV infection. *J. Virol.* 73:9604–9608.
- Ohto, H., N. Ujiie, C. Takeuchi, A. Sato, A. Hayashi, H. Ishiko, T. Nishizawa, H. Okamoto, et al. 2002. TT virus infection during childhood. *Transfusion* 42:892–898.
- Okamoto, H., T. Nishizawa, M. Takahashi, S. Asabe, F. Tsuda, and A. Yoshikawa. 2001. Heterogeneous distribution of TT virus of distinct genotypes in multiple tissues from infected humans. *Virology* 288:358–368.
- Okamoto, H., M. Takahashi, N. Kato, M. Fukuda, A. Tawara, S. Fukuda, T. Tanaka, Y. Miyakawa, and M. Mayumi. 2000. Sequestration of TT virus of restricted genotypes in peripheral blood mononuclear cells. *J. Virol.* 74:10236–10239.
- Okamoto, H., M. Takahashi, T. Nishizawa, A. Tawara, K. Fukai, U. Muramatsu, Y. Naito, and A. Yoshikawa. 2002. Genomic characterization of TT viruses (TTVs) in pigs, cats and dogs and their relatedness with species-specific TTVs in primates and tupaia. *J. Gen. Virol.* 83:1291–1297.
- Okamoto, H., M. Takahashi, T. Nishizawa, A. Tawara, Y. Sugai, T. Sai, T. Tanaka, and F. Tsuda. 2000. Replicative forms of TT virus DNA in bone marrow cells. *Biochem. Biophys. Res. Commun.* 270:657–662.
- Okamoto, H., A. Tawara, M. Takahashi, J. Kishimoto, T. Sai, and Y. Sugai. 2000. TT virus mRNAs detected bone marrow cells from an infected individual. *Biochem. Biophys. Res. Commun.* 279:700–707.
- Okamoto, H., M. Ukita, T. Nishizawa, J. Kishimoto, Y. Hoshi, H. Mizuo, T. Tanaka, Y. Miyakawa, and M. Mayumi. 2000. Circular double-stranded forms of TT virus DNA in the liver. *J. Virol.* 74:5161–5167.
- Patil, B. L., N. Dutt, R. W. Briddon, S. E. Bull, D. Rothenstein, B. K. Borah, I. Dasgupta, J. Stanley, and H. Jeske. 2007. Deletion and recombination events between the DNA-A and DNA-B components of Indian cassava-infect-

- ing geminiviruses generate defective molecules in *Nicotiana benthamiana*. *Virus Res.* **124**:59–67.
42. Peng, Y. H., T. Nishizawa, T. Takahashi, T. Ishikawa, A. Yoshikawa, and H. Okamoto. 2002. Analysis of the entire genomes of thirteen TT virus variants classifiable into the fourth and fifth genetic groups, isolated from viremic infants. *Arch. Virol.* **147**:21–41.
 43. Pineau, P., M. Meddeb, R. Raselli, L.-X. Qin, B. Terris, Z.-Y. Tang, P. Tiollais, V. Mazzaferro, and A. Dejean. 2000. Effect of TT virus infection on hepatocellular carcinoma development: results of a Euro-Asian survey. *J. Infect. Dis.* **181**:1138–1142.
 44. Qiu, J., L. Kakkola, F. Cheng, C. Ye, M. Soderlund-Venermo, K. Hedman, and D. J. Pintel. 2005. Human circovirus TT virus genotype 6 expresses six proteins following transfection of a full-length clone. *J. Virol.* **79**:6505–6510.
 45. Saback, F. L., S. A. Gomes, V. S. de Paula, R. R. da Silva, L. L. Lewis-Ximenez, and C. Niel. 1999. Age-specific prevalence and transmission of TT virus. *J. Med. Virol.* **59**:318–322.
 46. Saunders, K., and J. Stanley. 1999. A nanovirus-like DNA component associated with yellow vein disease of *Ageratum conyzoides*: evidence for interfamilial recombination between plant DNA viruses. *Virology* **263**:142–152.
 47. Senger, M., T. Flores, K. H. Glatting, P. Ernst, A. Hotz-Wagenblatt, and S. Suhai. 1998. W2H: WWW interface to the GCG sequence analysis package. *Bioinformatics* **14**:452–457.
 48. Shiramizu, B., Q. Yu, N. Hu, R. Yanagihara, and V. R. Nerurkar. 2002. Investigation of TT virus in the etiology of pediatric acute lymphoblastic leukaemia. *Pediatr. Hematol. Oncol.* **19**:543–551.
 49. Simmonds, P. 2004. Genetic diversity and evolution of hepatitis C virus: 15 years on. *J. Gen. Virol.* **85**:3173–3188.
 50. Stanley, J. 2004. Subviral DNAs associated with geminivirus disease complexes. *Vet. Microbiol.* **98**:121–129.
 51. Sugiyama, K., K. Goto, T. Ando, F. Mizutani, K. Terabe, Y. Kawabe, and Y. Wada. 1999. Route of TT virus infection in children. *J. Med. Virol.* **59**:204–207.
 52. Takahashi, K., M. Hijikata, E. I. Samokhvalov, and S. Mishiro. 2000. Full or near full length nucleotide sequences of TT virus variants (types SANBAN and YONBAN) and the TT virus-like mini virus. *Intervirology* **43**:119–123.
 53. Takahashi, K., Y. Iwasa, M. Hijikata, and S. Mishiro. 2000. Identification of a new human DNA virus (TTV-like mini virus, TLMV) intermediately related to TT virus and chicken anemia virus. *Arch. Virol.* **145**:979–993.
 54. Takahashi, M., S. Asabe, Y. Gotanda, J. Kishimoto, F. Tsuda, and H. Okamoto. 2002. TT virus is distributed in various leukocyte subpopulations at distinct levels, with the highest viral load in granulocytes. *Biomed. Biophys. Res. Commun.* **290**:242–248.
 55. Thiry, E., B. Muyllkens, F. Meurens, S. Gogev, J. Thiry, A. Vanderplasschen, and F. Schynts. 2006. Recombination in the alpha herpesvirus bovine herpesvirus 1. *Vet. Microbiol.* **113**:171–177.
 56. Thomas, J. T., S. T. Oh, S. S. Terhune, and L. A. Laimins. 2001. Cellular changes induced by low-risk human papillomavirus type 11 in keratinocytes that stably maintain viral episomes. *J. Virol.* **75**:7564–7571.
 57. Timchenko, T., L. Katul, Y. Sano, F. de Kouchkovsky, H. J. Vetten, and B. Gronenborn. 2000. The master rep concept in nanovirus replication: identification of missing genome components and potential for natural genetic reassortment. *Virology* **274**:189–195.
 58. Ukita, M., H. Okamoto, T. Nishizawa, A. Tawara, M. Takahashi, H. Iizuka, Y. Miyakawa, and M. Mayumi. 2000. The entire nucleotide sequences of two distinct TT virus (TTV) isolates (TJN01 and TJN02) remotely related to the original TTV isolates. *Arch. Virol.* **145**:1543–1559.
 59. Wells, R. D., R. Dere, M. L. Hebert, M. Napierala, and L. S. Son. 2005. Advances in mechanisms of genetic instability related to hereditary neurological diseases. *Nucleic Acids Res.* **33**:3785–3798.
 60. Worobey, M. 2000. Extensive homologous recombination among widely divergent TT viruses. *J. Virol.* **74**:7666–7670.
 61. Xiu, X., Z. Xiaoguang, Z. Ninghu, L. Youtong, X. Liumei, and Z. Boping. 2004. Mother-to-infant vertical transmission of transfusion transmitted virus in South China. *J. Perinat. Med.* **32**:404–406.
 62. Yokoyama, H., J. Yasuda, H. Okamoto, and Y. Iwakura. 2002. Pathological changes of renal epithelial cells in mice transgenic for the TT virus ORF1 gene. *J. Gen. Virol.* **83**:141–150.
 63. Yu, Q., B. Shiramizu, V. R. Nerurkar, N. Hu, C. M. Shikuma, M. E. Melish, K. Cascio, A. Imrie, Y. Lu, and R. Yanagihara. 2002. TT virus: preferential distribution in CD19(+) peripheral blood mononuclear cells and lack of viral integration. *J. Med. Virol.* **66**:276–284.
 64. Zhong, S., W. Yeo, M. Tang, C. Liu, X. R. Lin, W. M. Ho, P. Hui, and P. J. Johnson. 2002. Frequent detection of the replicative form of TT virus DNA in peripheral blood mononuclear cells and bone marrow cells in cancer patients. *J. Med. Virol.* **66**:428–434.
 65. zur Hausen, H., and E. M. de Villiers. 2005. Virus target cell conditioning model to explain some epidemiologic characteristics of childhood leukemias and lymphomas. *Int. J. Cancer* **115**:1–5.