Human Torque teno virus:
epidemiology, cell biology and immunology

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ACADEMIC DISSERTATION

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“Two little mice fell in a bucket of cream. The first mouse quickly gave up and drowned. The second mouse, wouldn't quit. He struggled so hard that eventually he churned that cream into butter and crawled out. Gentlemen, as of this moment, I am that second mouse.”

Frank Abagnale Sr. in the movie Catch Me If You Can

To my siblings: Sanna, Riikka-Eliina, Helmi-Maria and Heikki-Pekka

CONTENTS

ABSTRACT

Torque teno virus (TTV) was discovered in 1997 in the serum of a Japanese patient with post-transfusion hepatitis of unknown etiology. TTV is a non-enveloped small virus containing a circular single-stranded negative-sense DNA genome. TTV resembles in genome organization the animal pathogen, chicken anemia virus (CAV), of the *Circoviridae* family. Thus, TTV represents the first circovirus-like virus found in humans. Within a few years after its discovery, TTV was noted to have several genotypes. To date, these viruses are classified into a new floating genus, Anellovirus. TTV was soon shown to be globally highly prevalent among healthy individuals; up to 94% of subjects are viremic. Persistent infections and co-infections with several genotypes occur frequently. However, the pathogenicity of TTV is at present unclear.

To determine the prevalence of TTV in the Finnish population, we set up PCR methods and examined the sera of nonsymptomatic subjects for the presence of TTV DNA and for genotype-6 DNA. We showed that TTV is also very common in Finland: TTV DNA was detected in 85% of the subjects, and genotype-6 DNA in 4%.

We studied the prevalence of TTV DNA and genotype-6 DNA in human tissues. TTV DNA was detected in peripheral blood mononuclear cells (PBMC), adenoids, tonsils, synovia, skin, bile and liver. Genotype 6 TTV-DNA was detected in the latter three, with no tissue-type or symptom specificity.

The cell biology events during TTV infections are at present unknown. Replicating TTV DNA has been detected in liver, in PBMC, and in bone marrow; and alternative splicing has been shown to generate three mRNAs. In order to characterize TTV cell biology in more detail, we cloned and sequenced the full-length genome of TTV genotype 6. We showed that in clone-transfected human kidney-derived cells TTV produces, by alternative splicing and by alternative translation initiation, altogether six proteins with distinct subcellular localizations. The mRNA transcription was detected in the seven cell lines, and TTV DNA replication in several of them, including cell lines of erythroid and of hepatic origin. We demonstrated that the replication can be blocked by the drug aphidicolin, indicating that TTV utilizes for replication the cellular DNA polymerases.

Diagnoses of TTV infections have been based almost solely on PCR. However, serological tests, measuring antibody responses, would give more information on TTV infections. In addition, the mechanism for the sustained occurence of the virus in the circulation is at the moment unknown. To investigate the immunology behind the TTV infections in more detail, we expressed the six TTV proteins in bacteria and in insect cells for use as antigens in immunoblotting. We detected in human sera IgM and IgG antibodies to occur simultaneously with TTV DNA, appearance of TTV DNA regardless of pre-existing antibodies, and disappearance of TTV DNA after the appearance of antibodies. The genotype-6 nucleotide sequence remained stable for years within the infected

individuals, suggesting that some mechanism other than mutations is used by TTV to evade our immune system and to establish chronic infections in healthy individuals.

In this thesis work we have set up a wide array of methods (e.g. molecular biology and serology), with which we have determined TTV prevalence in Finland, studied TTV-related cell biological events (i.e. transcription, replication, and translation), and expressed TTV proteins for immunological studies. The methods and results presented will be valuable in TTV research and diagnosis, and can be used to reveal the clinical significance of this new virus genus/family, Anellovirus/*Anelloviridae*.

ORIGINAL PUBLICATIONS

This thesis is based on the following original articles that are referred to in the text by their Roman numerals.

I Kakkola L, Hedman K, Vanrobaeys H, Hedman L, Soderlund-Venermo M. (2002). Cloning and sequencing of TT virus genotype 6 and expression of antigenic open reading frame 2 proteins. Journal of General Virology, 83:979-90.

II Kakkola L, Kaipio N, Hokynar K, Puolakkainen P, Mattila PS, Kokkola A, Partio EK, Eis-Hubinger AM, Soderlund-Venermo M, Hedman K. (2004). Genoprevalence in human tissues of TT-virus genotype 6. Archives of Virology, 149:1095-106.

III Qiu J, Kakkola L, Cheng F, Ye C, Soderlund-Venermo M, Hedman K, Pintel DJ. (2005). Human circovirus TT virus genotype 6 expresses six proteins following transfection of a full-length clone. Journal of Virology, 79:6505-10.

IV Kakkola L, Tommiska J, Boele LCL, Miettinen S, Blom T, Kekarainen T, Qiu J, Pintel D, Hoeben RC, Hedman K, Söderlund-Venermo M. (2007). Construction and biological activity of a full-length molecular clone of human Torque teno virus (TTV) genotype 6. FEBS Journal, 274:4719-30.

V Kakkola L, Bondén H, Hedman L, Kivi N, Moisala S, Julin J, Miettinen S, Ylä-Liedenpohja J, Kantola K, Hedman K, Söderlund-Venermo M. Human Torque teno virus (TTV) of genotype 6: expression of proteins in bacteria and in insect cells, and analysis of IgG prevalences. Manuscript in submission.

ABBREVIATIONS

aa	amino acid
ALT	alanine aminotransferase
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BFDV	beak and feather disease virus
bp	base pair
CAV	chicken anemia virus
CPV	canine parvovirus
DAB	3,3'-diaminobenzidine
DIG	digoxigenin
D-MEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GFP	green fluorescent protein
GST	glutathione S-transferase
HA	hemagglutinin
HAART	highly active antiretroviral therapy
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HPV	human papillomavirus
HRP	horseradish peroxidase
HVR	hypervariable region
ICTV	International Committee on Taxonomy of Viruses
IgG-ETS EIA	IgG epitope type specificity enzyme immuno assay
IPTG	isopropyl β-D-1-thiogalactopyranoside
MEM	modified Eagle medium
mRNA	messenger-RNA
NBT	4-nitro blue tetrazolium chloride
nt	nucleotide
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV	porcine circovirus
PMWS	post weaning multisystemic wasting syndrome
RDA	representational difference analysis
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SAV	small anellovirus
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SISPA	sequence-independent single primer amplification
SLE	systemic lupus erythematosus
ssDNA	single-stranded DNA
SV40	simian virus 40
TAIP	TTV-derived apoptosis-inducing protein
TBE	tick borne encephalitis
TTLMV	TTV-like minivirus
TTMDV	Torgue teno midivirus
TTMV	Torgue teno minivirus
TTV	TT-virus, Torque teno virus
UTR	untranslated region

REVIEW OF THE LITERATURE

1 INTRODUCTION

Viruses have been infecting cells since the origin of organisms, maybe even since the first cells. Viruses infecting humans today have either co-evolved with us for thousands of years, or have entered human populations at various time points. Viruses and viral diseases have had major impacts on humans throughout history. Nevertheless, it was only until the 19th century that viruses were actually found and described. The first virus to be discovered was tobacco mosaic virus of a plant. The initial finding, showing that the disease was caused by something smaller than bacteria or fungi, came in 1892 from a Russian scientist Dimitrii Ivanovsky. Six years later Martinus Beijerinck from Holland confirmed that this agent must be smaller and different from any bacteria known: the agent passed through filters, and multiplied only in a live host. Beijerinck named this filtrate *contagium vivum fluidum* ("soluble living germ"), and the agents were named "ultrafilterable viruses" (virus = "poison" in Latin) (Flint et al., 2004).

2 PROPERTIES OF VIRUSES

Since the discovery of the tobacco mosaic virus, it has become evident that viruses are well suited for their task: to reproduce themselves. Characteristic for viruses is that they do not reproduce by division, i.e. viruses do not "grow"; instead, they replicate within cells. Viruses are very small, they contain nucleic acid (RNA or DNA) that is protected by a capsid of protein(s). Some viruses have also a lipid bilayer surrounding the capsid (i.e. enveloped viruses), or inside the capsid (e.g. some bacteriphages). Viruses exist in many forms (spherichal, helical, with spikes etc.) and sizes. All viruses are obligate cellular parasites, meaning that viruses totally depend on living host cells for reproduction. Viruses do not encode ribosomes or mitochondria, and thus can not synthesize proteins or produce energy. Instead, these life sustaining processes are provided by the host cell. Viruses are passively moved (by air, water, vectors etc.) into contact with their host cells, into which they invade, i.e. hijack for their own purposes. Even though viruses are considered "simple" forms of life, they are far beyond that. They may be small in size, but are able to perform gigantic tasks, and do have major impacts on their hosts.

Viruses on the whole are cabable of infecting all living organisms: bacteria, fungi, plants, and animals. In humans, viruses can either invade our body, or infect the cells on our surfaces (i.e. skin or mucosa). Depending on the infecting virus, and other factors (e.g. the

subject's physiological state, environmental factors, etc.), the infection can lead to disease or remain sub-clinical. When the virus encounters its host cell, the viral life cycle begins: the virus attaches to the cell, enters it, releases nucleic acid into the cell (uncoating), replicates genomic material, produces viral proteins (for particles, for genomic synthesis, and for interactions with the environment), assembles (and matures) into virions, and finally is released from the cell either with or without cell lysis. Different viruses have various ways to accomplish this life cycle (Flint et al., 2004).

3 DISCOVERY OF HUMAN VIRUSES AND OF TORQUE TENO VIRUS

3.1 Overview of history and methods in discovery of human viruses

Scientists have always tried to find the underlying cause for various diseases, and to date viruses have been identified as causal agents for several of those. Interestingly, although the first vaccine against a virus-caused disease (smallpox) was developed already in 1798, it was until in 1901 that the first human virus (yellow fever virus) was discovered. By experimentally infecting humans with mosquitoes and with serum, scientists proved that mosquitoes were transmitting the disease-causing filterable agent, a virus. The discovery of human viruses was very slow due to dangers and difficulties in handling of the disease-causing (and often fatal) agents introduced into human volunteers. The next important human virus to be discovered was the influenza virus in 1933. Scientists infected ferrets with human throat washings and were able to isolate the virus. Later, mice and chicken embryos were shown to be infectable with influenza virus, eggs being used still today in influenza research. These findings led to the use of animal models, which still are invaluable tools in virus research. The new era on virus research began along with the invention of the electron microscope in the 1930s. This allowed the visualization of viruses that had earlier not been possible with light microscopes. In the 1950s cell and tissue culture methods were developed, leading to isolation of many human viruses, including adeno, measles and rubella. These systems allowed for detailed study of the virus life cycle, providing information not possible to obtain with other methods (Flint et al., 2004).

In the 1980s a dramatic improvement in methods came with the invention of the polymerase chain reaction, PCR, used for the amplification of DNA (or RNA). With this method it became possible to amplify genomes of unculturable viruses, and to detect very small amounts of viral nucleic acids. The post-PCR era has had a major impact on discovery and characterization of viruses. To date, several methods have been developed that utilize some form of amplification of viral sequences. In PCR, the restriction for amplification of unknown viruses is that the sequence has to be known. Therefore, PCR-based methods for which prior knowledge of the target sequence was not needed were developed, e.g. use of degenerate primers or random PCR. In addition, several sophisticated

amplification techniques have been developed. For example, sequence-independent single primer amplification (SISPA) has been used to detect unknown sequences that exist in low amounts. In SISPA, the genetic material is first converted to double-stranded DNA (dsDNA), then adapters are linked to restriction enzyme digested DNA, and primers specific for the adapter are used for amplification of the target sequence in between the adapters. In addition, the differential display technique is used for investigation of differences in messenger-RNA (mRNA) expression; whole genome amplification techniques are used for amplification of viral genomes etc. The products are usually cloned and sequenced, or analyzed by microarray techniques, or by restriction enzyme analyses combined with gel electrophoresis techniques (Ambrose et al., 2006).

One technique also used in TTV research (see below), is representational difference analysis (RDA), which reveals the differences in nucleic acid composition between sequential samples (e.g. during and after disease). In this method, restriction enzyme digested DNAs from two samples are mixed, then melted, and allowed to anneal. Nucleic acids in one of the samples are, prior to mixing, linked to a primer. The primer is used subsequently in PCR to amplify sequences in which the primers are present, while the unlinked nucleic acids remain non-amplified. Thus, exponential amplification of nucleic acids present only in the primer-labeled sample (i.e. primers at both ends of DNA fragments) takes place (Ambrose et al., 2006; Lisitsyn et al., 1993).

3.2 Emerging viruses

Emerging viruses are generated from existing ones by mutation (e.g. influenza virus), by exposure of naïve populations to the existing virus (e.g. due to movement of the infected hosts), and by changing the microbial host-range (e.g. HIV and CPV). New possibilities for emergence are often provided by human activity: high density populations, water regulation, drug abuse, sexual behaviour, increased speed and distance in travelling, and invasion into new environments (e.g. jungle, sea).

The Webster's Encyclopedic Unabridged Dictionary of the English Language defines the verb emerge as follows: 1) to rise or come forth from or as if from water or other liquid; 2) to come forth into view or notice, as from concealment or obscurity; 3) to come up or arise, as a question or difficulty; 4) to come into existence, develop; 5) to rise, as from an inferior or unfortunate state or condition. In this regard, the above explained concept of emerging viruses should be widened to include viruses that emerge (i.e. are found or detected and thereby come into existence) by human research. During the post-PCR era, several viruses that have existed with us for millennia, but unnoticed, have "emerged". Anelloviruses described in this thesis are an excellent example; not merely one virus, but a large virus family living with us.

3.3 Emergence of TTV

Hepatitis, i.e. inflammation of the liver, affects millions of people worldwide. Severity and outcome of the disease depend on the etiological agent. Most of the hepatitis cases are caused by infection with one of the hepatitis viruses; hepatitis A, B, C, D or E (http://www.who.int/csr/disease/hepatitis/en/). However, there are still numerous hepatitis cases in which the causal agent is not known. In the search for yet unidentified hepatitis viruses, TT-virus (TTV) was found in 1997 in Japan from a patient with post-transfusion hepatitis of unknown etiology. The patient was 58 years old, and had received 35 units of blood for heart surgery. TTV was detected with the RDA in the serum sample taken during hepatitis. A clone of approximately 500 bp was obtained, which showed no similarities with any sequences deposited in databases. TTV DNA was detected in the blood transiently, during a peak of a liver enzyme, alanine aminotransferase (ALT), elevation (Nishizawa et al., 1997).

3.4 Evidence for the viral nature of TTV

Nishizawa and coworkers showed by PCR that the TTV genome was of DNA. In addition, as this DNA was not amplified from the tissues of humans (presumably) free of TTV infection, it was concluded that it did not originate from human genome. Furthermore, since it fractionated in sucrose gradient at 1.26 g/cm^3, and was resistant to DNaseI, it was concluded to be encapsidated, and thereby a putative virus. This newly found virus was named TT-virus according to the initials of the patient (Nishizawa et al., 1997). Later, TTV was named as Torgue teno virus (latin “torques”, necklace; “tenuis”, thin) (Biagini et al., 2004). Further studies showed that serum-derived TTV is sensitive to Mung Bean Nuclease, but resistant to RNaseA and restriction enzymes. Thus TTV was concluded to be a DNA virus that had a single stranded genome (ssDNA) (Mushahwar et al., 1999; Okamoto et al., 1998b). Detergent treatment destroys the viral envelope altering the density of the virus. Since the density of the Tween-80 treated TTV remained unchanged in sucrose gradient, TTV was concluded to be a non-enveloped virus (Okamoto et al., 1998b). Furthermore, Mushahwar and coworkers have estimated by filtration that the TTV virion is 30-50 nm in diameter (Mushahwar et al., 1999). In conclusion, a new, small, non-enveloped, ssDNA virus was found in humans.

4 CHARACTERISTICS OF TORQUE TENO VIRUS

4.1 Genome

As the DNA of TTV was concluded to be of viral origin, it was studied further for genome organization and for transcription properties. The TTV genome was soon shown to be of circular ssDNA, and to contain a GC-rich (89%-90.6%) region of 117 nucleotides (Fig. 1). Similarities, in genome organization and in transcription regulation sites, were noticed with chicken anemia virus (CAV), an animal pathogen of the *Circoviridae* family (Miyata et al., 1999; Mushahwar et al., 1999). In addition, Mushahwar and coworkers showed with hybridization and nuclease protection that the TTV genome is negative sense DNA, i.e. mRNAs are produced from its complementary strand (Mushahwar et al., 1999).

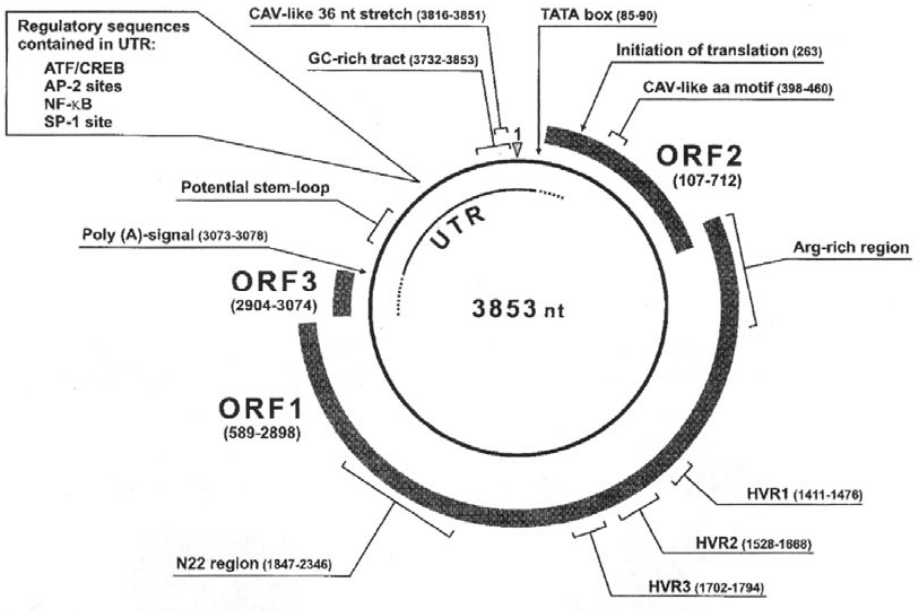

Figure 1. Genome organization of TTV (reproduced from Bendinelli et al., 2001, with permission).

The lengths of the genomes vary among TTV isolates, the average being around 3.8 kb. (Erker et al., 1999; Hallett et al., 2000; Heller et al., 2001; Hijikata et al., 1999b; Miyata et al., 1999; Peng et al., 2002). The genome contains a coding region of approximately 2.6 kb and an untranslated region (UTR) of 1.2 kb. The sequence variation at the nucleotide level is quite extensive (see below); however, some areas are conserved. For example the GC-rich region, although varying in length (108-160 nts), is present in all the isolates (Hallett et al., 2000; Heller et al., 2001; Peng et al., 2002). Also, a poly-A sequence downstream and a TATA-box upstream of the coding region are conserved (Erker et al., 1999; Hallett et al.,

2000; Heller et al., 2001; Hijikata et al., 1999b). At the nucleotide level the UTR is more conserved than the coding area, and contains elements for transcription regulation (Kamada et al., 2004; Miyata et al., 1999; Suzuki et al., 2004).

4.2 Transcription capacity

When the full-length sequences of TTV isolates were analyzed, 2-3 open reading frames (ORF) were identified (Erker et al., 1999; Miyata et al., 1999; Mushahwar et al., 1999; Okamoto et al., 1998b) (Fig. 1). However, it was not known whether the ORFs were transcribed. Kamahora and coworkers cloned a genotype-1 isolate in full length, transfected it into the monkey kidney cell line Cos-1, and identified three clone-derived mRNA species: 3.0, 1.2 and 1.0 kb in length (Fig. 2). The transcripts used the same poly-A at the end of the coding region, and began from the same area near the TATA box. All the transcripts showed the same splicing at the beginning of the mRNAs. The 3.0-kb mRNA contained the complete ORF1, the 1.2-kb mRNA had a second splicing that joined ORF2 to another ORF in the same reading frame, and the 1.0-kb mRNA also had a second splicing that joined ORF2 to another ORF in a different reading frame (Kamahora et al., 2000). The splice sites were found to be conserved among different genotypes (Kamahora et al., 2000; Peng et al., 2002), and the same mRNAs were also identified *in vivo* in bone marrow cells from a TTV infected subject (Okamoto et al., 2000c). Thus, it was proved that TTV uses alternative splicing to create mRNAs for various putative proteins.

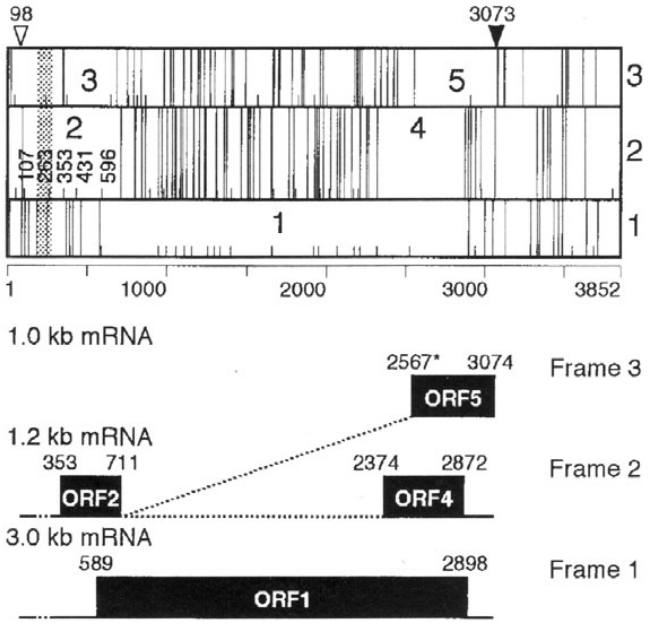

Figure 2. The three mRNA species identified in the genotype-1 transfected cells. The dashed line represents the spliced areas (reproduced from Kamahora et al., 2000, with permission).

The UTR is used for transcription control; transcription factor binding sites are located in the UTR (Miyata et al., 1999), and the promoter and enhancer elements have been mapped within the UTR (Kamada et al., 2004; Suzuki et al., 2004). Interestingly, not all the enhancer motifs at the UTR are conserved among the various isolates. This has been suggested to result in different transcriptional regulation depending on host cell and/or on TTV isolate (Kamada et al., 2004). In addition, the presence of additional splice variants has been suggested (Leppik et al., 2007), the implications of which in TTV biology remain to be elucidated.

4.3 Proteins

The open reading frames encoding TTV proteins are located in the complementary DNA strand, the plus strand. ORFs are found in all three reading frames, and they overlap with each other (Miyata et al., 1999). Despite the sequence variability among TTV isolates, the locations of the ORFs are conserved (Erker et al., 1999; Heller et al., 2001; Hijikata et al., 1999b). Initially, it was thought that two to three ORFs are present in TTV (Erker et al., 1999; Miyata et al., 1999; Mushahwar et al., 1999; Okamoto et al., 1998b), however, splicing of the mRNAs yields additional transcript variants (Kamahora et al., 2000).

ORF1: The longest ORF, named ORF1, is found in all TTV isolates, and it has been suggested to encode a capsid protein (Takahashi et al., 1998). The predicted length of the ORF1-encoded protein varies, ranging from 719-770 amino acids (Erker et al., 1999; Heller et al., 2001; Tanaka et al., 2000b; Ukita et al., 2000). In some isolates, stop codons are found in the middle of ORF1, shortening or interrupting the putative protein (Erker et al., 1999; Jelcic et al., 2004; Luo et al., 2002). It has been suggested that the interrupted ORF1 could form defective viruses (Pollicino et al., 2003). The ORF1 sequence varies at the amino acid level among TTV isolates, showing amino acid differences of the order of 50-70% (Luo et al., 2002; Ukita et al., 2000). However, these differences are not uniformly distributed since ORF1 contains hypervariable regions (HVRs) in which mutations/nucleotide changes leading to amino acid changes occur more frequently than in the remaining part of the protein (Jelcic et al., 2004; Luo et al., 2002; Mushahwar et al., 1999; Nishizawa et al., 1999; Takahashi et al., 1998). It has been suggested that TTV could escape immunological responses by mutating these HVRs (see below). The ORF1-encoded protein is also assumed to be involved in replication, since ORF1 has two rolling circle replication motifs, so called Rep-motifs, of which at least one seems to be conserved among the majority of TTV isolates (Erker et al., 1999; Luo et al., 2002; Mushahwar et al., 1999; Tanaka et al., 2001) In addition, the ORF1 encoded protein has an arginine-rich N-terminus (Hijikata et al., 1999b; Mushahwar et al., 1999; Okamoto et al., 1998b; Takahashi et al., 1998; Tanaka et al., 2001) that might have DNA-binding activity and function in packaging of viral DNA into capsids (Erker et al., 1999). The capsid protein, VP1, of CAV

has Rep-motifs and an arginine-rich N-terminus similar to the ORF1 encoded protein of TTV, and is assumed to function as a replication associated protein (Niagro et al., 1998). The TTV ORF1-encoded protein contains also potential glycosylation sites (Tanaka et al., 2000b) that vary between isolates, and could have some effect on protein function and/or antigenicity (Bendinelli et al., 2001; Hijikata et al., 1999b).

ORF2: The small ORF2 encodes a putative protein of approximately 200 amino acids. However, in several genotypes a stop codon divides ORF2 into two smaller protein coding areas, ORF2a and ORF2b. The ORF2b is less conserved in amino acid sequence than ORF2a (Tanaka et al., 2000b; Ukita et al., 2000), however, the ORF2 (or ORF2b) has a conserved amino acid motif, $WX_7HX_3CX_1CX_5H$, that is also found in the CAV protein (Hijikata et al., 1999b; Peng et al., 2002; Tanaka et al., 2001). This conserved motif corresponds to the protein-tyrosine phosphatase (PTPase) signature motif, and the protein might be involved in the regulation of cellular and/or viral proteins during infection (Peters et al., 2002).

Other ORFs: The nomenclature of the remaining other TTV proteins/open reading frames varies to some extent among publications. The ORF3-encoded protein, i.e. ORF2/2 in this thesis and ORF2-4 on the work of Kamahora and coworkers (Kamahora et al., 2000), of approximately 280 amino acids, has a serine-rich domain preceded by basic amino acids at the C-terminus (Peng et al., 2002; Tanaka et al., 2001). ORF3 encoded protein of genotype-1a has been predicted to have similarities with the hepatitis C- virus (HCV) non-structural protein 5A (NS5A), which is involved in cell cycle regulation and in suppression of interferon-induced anti-viral resistance. In Cos-1 cells, two forms of the TTV ORF3 encoded protein were produced. It was shown that the slower-migrating form was phosphorylated more at serine residues in the C-terminal region. However, the function of this protein during the viral life cycle is not yet known. Phosphoproteins of other viruses are usually involved in virus replication or in the regulation of cellular proteins, suggesting similar functions for the TTV protein (Asabe et al., 2001).

The ORF2-5 (i.e. ORF2/3 in this thesis) -encoded protein, of approximately 280 amino acids, has been shown to have similarities with proteins possessing transcription-factor activity and could thus have some role in the regulation of TTV transcription (Kamahora et al., 2000). A conserved amino acid motif, $E\text{-}X_8\text{-}R\text{-}X_2\text{-}R\text{-}X_6\text{-}P\text{-}X_{12\text{-}19}\text{-}F\text{-}X_1\text{-}L$, is found in the C-terminus of the protein (Okamoto et al., 2000b; Peng et al., 2002), with an unknown function.

One putative protein of 105 amino acids, named TTV-derived apoptosis-inducing protein (TAIP), has been shown to induce apoptosis in hepatocellular carcinoma cells, resembeling

thus the apoptin-protein of CAV which causes apoptosis in cancer cells (Kooistra et al., 2004). However, whether this TAIP is expressed by all TTV isolates, or has some cell/tissue-type specificity, remains to be studied.

In addition to the proteins listed, more ORFs have been suggested to arise by intragenomic rearrangements and by alternative splicing (Leppik et al., 2007), thus potentially further expanding the coding capacity and the variability of TTV isolates.

4.4 Structure

By filtration studies TTV has been estimated to be 30-50 nm in diameter (Mushahwar et al., 1999). In three studies potential TTV particles have been identified. Itoh and coworkers showed that TTV particles in sera, but not in feces, of HIV positive individuals are complexed with IgG. Virus-like particles were detected with electron microscopy (Fig. 3) in fecal and serum samples of patients with high viral copy numbers (10^8/ml) (Itoh et al., 2000).

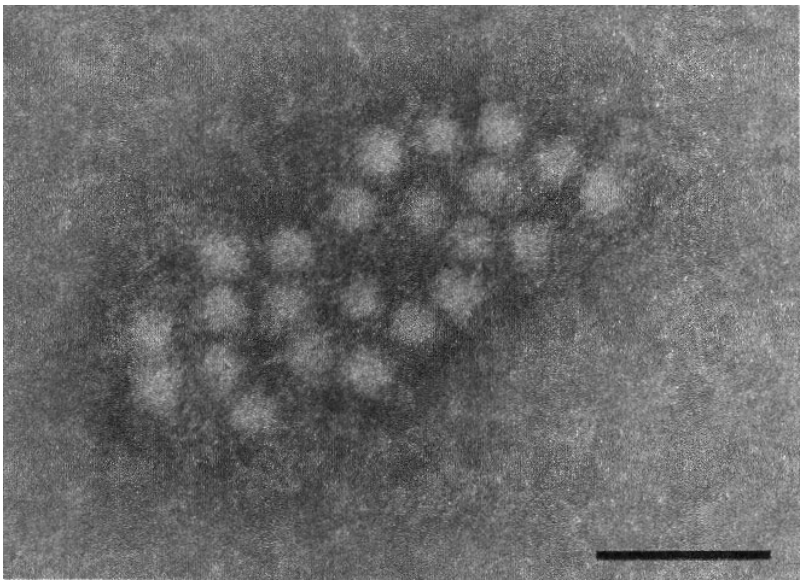

Figure 3. Electron microscopy of TTV aggregates in a serum sample with a TTV level of 10^8 copies/ml. Scale bar, 100 nm. (Reproduced from Itoh et al., 2000, with permission.)

Zur Hausen and coworkers have identified TTV-like particles in lymphoma- and leukemia-derived cell lines (zur Hausen et al., 2005). In addition, Leppik and coworkers have detected virus-like particles in the Hodking's lymphoma-derived cells transfected with a full-length clone of TTV (Leppik et al., 2007).

However, due to the lack of TTV-specific and well-characterized antibodies, immuno-electron microscopy has not been performed, and therefore the TTV specificity of the particles has not been confirmed. In addition, there is no cell system that would produce high amounts of capsid proteins which could form virus-like particles. When these applications are available, the appearance and fine-structure of TTV capsid can eventually be revealed.

4.5 Replication mechanism

The exact mechanism of TTV replication is not known. It is assumed, based on similarities with other circular ssDNA viruses, that TTV uses the rolling circle mechanism (Mushahwar et al., 1999). To this end, other circoviruses encode special proteins with Rep-motifs which bind to the initiation site of replication (Mankertz et al., 1998; Niagro et al., 1998). Based on the amino acid sequence, TTV ORF1 has been shown to contain such Rep-motifs (Erker et al., 1999; Luo et al., 2002; Mushahwar et al., 1999; Tanaka et al., 2001). In addition to replication-associated proteins, animal circoviruses have conserved nonanucleotide sequences, repeptitive sequences, and stem-loop structures that are involved in replication (Mankertz et al., 2004; Niagro et al., 1998; Todd et al., 2004). The UTR of TTV also contains potential stem-loop structures (Hijikata et al., 1999b; Mushahwar et al., 1999; Peng et al., 2002). Whether these structures and proteins of TTV are used in viral replication, is currently not known.

4.6 Sequence variation

Quite soon after the discovery of TTV it was shown that extensive divergence occurs among TTV isolates, both at the nucleotide and at the amino acid level (Okamoto et al., 1998b): divergences of e.g. 47-70% at the amino acid level have been reported (Biagini et al., 1999; Luo et al., 2002). Interestingly, the divergence in the coding area is higher at the amino acid than at the nucleotide level (Tanaka et al., 2000b), a situation that often is the opposite. In phylogenetic analyses, TTVs have been found to form a large phylogenetic tree with five major clusters (Biagini et al., 2004) (Fig. 4).

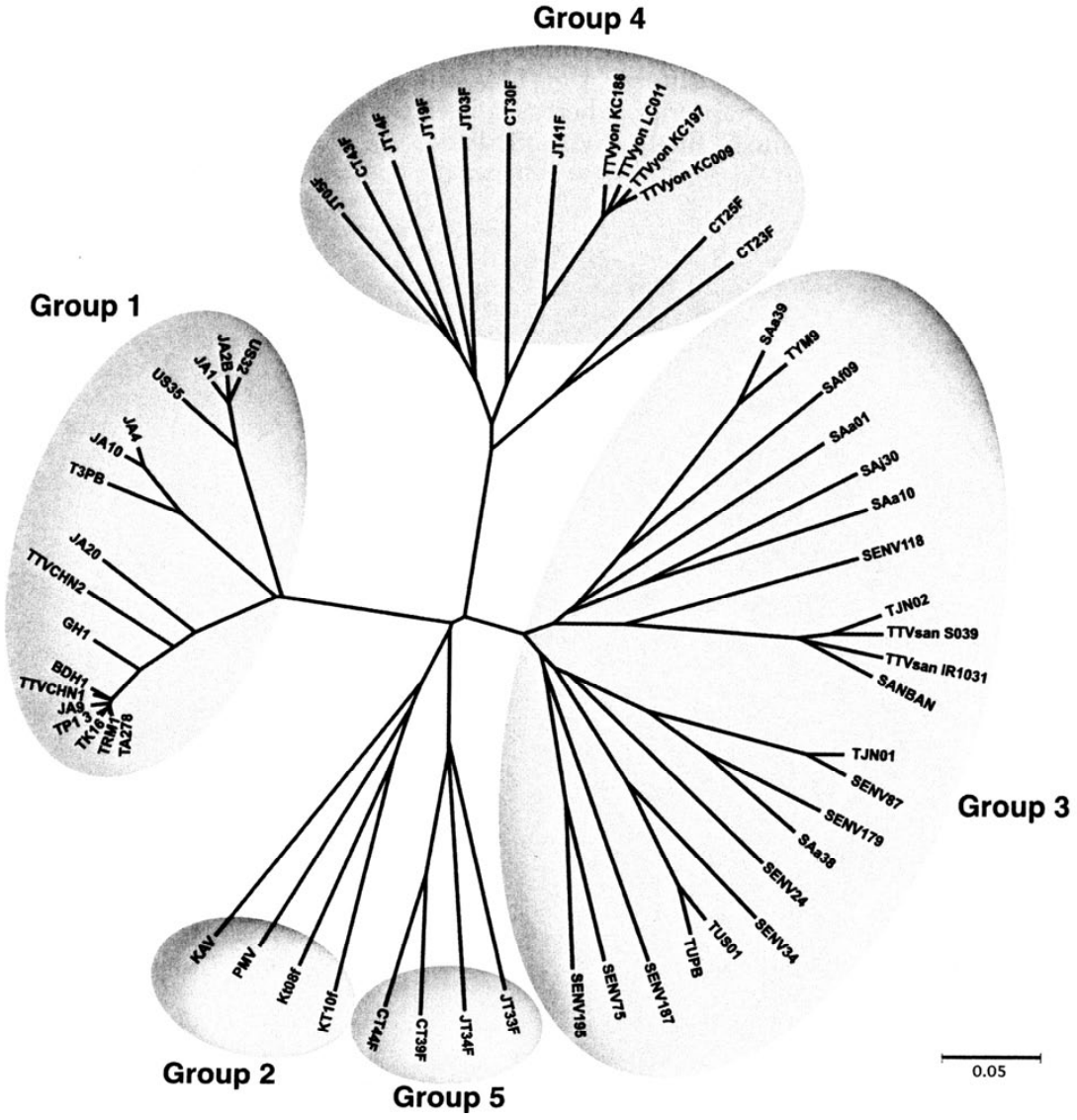

Figure 4. Phylogenetic tree of 61 TTV isolates. The scale bar represents a genetic distance of 5% (reproduced from Biagini et al., 2004, with permission).

The reason for and the mechanism of this extensive genomic variation are not known, and several possibilities have been suggested. One is that TTV mutates with high frequency. There are studies in which the nucleotide sequences from sequential samples have been compared, showing either persistence of the same sequence for years, or alterations and evolution of sequences within individuals (Ball et al., 1999; Biagini et al., 1999; Gallian et al., 1999; Irving et al., 1999). An extensive mutation rate is usually not assumed to be characteristic for DNA viruses since they often use cellular DNA polymerase(s) which, unlike the reverse transcriptase encoded and used by RNA viruses, has strong proofreading capacity. However, recently it was shown that ssDNA containing parvoviruses evolve with a rate comparable to RNA viruses. It was suggested that this high mutation rate could be

associated with the single-stranded nature of the genome and/or the coding capacity for proteins involved in replication (Shackelton et al., 2006). It is thus possible that TTV has a similar mechanism that allows it to mutate at a high rate. The majority of amino acid changes in closely related TTV sequences have been located in the HVRs of ORF1, and it is thus suggested that TTV circulates within individuals as quasispecies. By mutating HVRs, TTV could evade immune responses and establish persistent infections (Jelcic et al., 2004). It has also been suggested that only a minor fraction of the TT-viruses has all the components required for successful infection, or that co-infection of isolates brings the necessary components together (Khudyakov et al., 2000; Luo et al., 2002). Such phenomena are known with other viruses: e.g. HIV is known to produce large amounts of viruses of which some, but not all, are capable for productive infection, and plant-infecting nanoviruses require several different virus particles to co-infect the same cell until the production of progeny virions occurs.

One common mechanism for viruses to create new variants is recombination, and with TTV such events have been estimated to occur quite frequently (Manni et al., 2002; Worobey, 2000). However, Jelcic and coworkers question whether the observed recombinations could be due to PCR artefacts (Jelcic et al., 2004), while Worobey (2000) reports that the observed recombinations were not PCR-derived (Worobey, 2000). Recently, also intragenomic rearrangements have been suggested to contribute to the genomic variation of TTVs (Leppik et al., 2007).

In general, viruses evolve by producing large numbers of descedants and by generating large numbers of mutants during replication. In addition, recombinations and reassortments (of segmented genomes) contribute to viral evolution. Evolution, however, can not proceed without limitations, e.g. capsid size limits the amount of packaged genetic material. In addition, extensive mutations can lead to non-viable viruses with lost protein functions, some of which the virus needs to conserve in order to replicate within its host cells. Furthermore, an excessively virulent virus kills its host and thus itself. The ultimate goal for a virus would be to achieve such a balance with its host that it can replicate and reproduce itself without causing the host to die (too soon).

As for TTV, the virus has been suggested to have co-evolved with us for millions of years, resulting in extremely wide variation (Ball et al., 1999; Biagini et al., 1999; Gallian et al., 2000; Hijikata et al., 1999b; Prescott et al., 1999). The heterogeneity of TTVs at the nucleotide level could be due to a low replication rate, generation of mutations, a long history of human infections, low genetic fitness, and/or lack of competition between isolates (Khudyakov et al., 2000). In addition, frequent recombination events could smoothen the differences between virus populations (Manni et al., 2002). Whatever the

mechanism behind the heterogeneity of TTV is, it is evident that humans are not infected by a signle TTV species, but by a large family of TT viruses.

5 PREVALENCE OF TORQUE TENO VIRUS

5.1 TTV DNA prevalence in blood

After identification of the new virus infecting humans, it became crucial to determine its prevalence among healthy individuals, and especially in various patient groups for possible disease associations. TTV DNA prevalence was estimated to be low initially at a time when its wide sequence variation was not known. The PCR conditions, and especially the primers used in research, differed significantly, some detecting fewer genotypes than others, resulting in discrepancy (Ali et al., 2002; Irving et al., 1999; Leary et al., 1999; Lopez-Alcorocho et al., 2000; Niel et al., 1999; Nishiguchi et al., 2000; Okamoto et al., 1999b; Pollicino et al., 2003; Ukita et al., 2000). To date, with knowledge of the genomic variation, various primer combinations have been tested to develop a PCR method which would detect, quantitate, and/or differentiate all the TTV genotypes and TTV-like viruses (Biagini et al., 2001a; Devalle et al., 2004; Hu et al., 2005; Maggi et al., 2003b; Moen et al., 2002b). In addition, new sequence-independent methods, like rolling circle amplification, have been used for amplification of divergent, and new, TTV isolates (Biagini et al., 2007; Niel et al., 2005).

Regardless of the technical issues, it became soon apparent that TTV occurs worldwide in the general population (Abe et al., 1999; Okamoto et al., 1999b; Prescott et al., 1998; Viazov et al., 1998a) and that its prevalence increases with age (Hsieh et al., 1999; Saback et al., 1999; Umemura et al., 2001a; Zhong et al., 2001a). The reported DNA prevalences vary, depending on the PCR methods used, but in general are very high: for example TTV DNA positivity in sera of blood donors is 46-62% in Brazil (Devalle et al., 2004; Niel et al., 1999) 51.6-82.7% in Turkey (Erensoy et al., 2002; Yazici et al., 2002), 90% in Norway (Huang et al., 2001), and 53.3% in China (Zhong et al., 2001a). Variation in geographical prevalence of some genotypes, or evolution of genotypes within isolated ethnic groups has been suggested to occur (Prescott et al., 1999; Umemura et al., 2003; Vasconcelos et al., 2003. However, results showing no geographical differences in TTV DNA prevalence or genotype/genogroup prevalence have also been presented (Gallian et al., 2000; Huang et al., 2001; Manni et al., 2002; Mushahwar et al., 1999; Pisani et al., 1999). Some genogroups are suggested to be more common than others (Maggi et al., 2005a; Maggi et al., 2006), but this might reflect the use of suboptimal primers or some viral characteristics of these genogroups. In addition, the fluctuating and/or low virus loads could account for variation in results under slightly suboptimal PCR conditions (Huang et al., 2001).

The knowledge of TTV prevalence changes all the time. New variants are discovered and new methods are developed (Biagini et al., 2007; Niel et al., 2005). What is the true representation of TTV in humans is an important topic of forthcoming studies. Nevertheless, it is obvious that this virus (or these viruses) is frequently (or constantly) present in blood of the majority of healthy individuals.

5.2 TTV DNA prevalence in tissues and body fluids

By PCR methods, TTV DNA has been detected in several organs, cells and body fluids, including peripheral blood mononuclear cells (PBMC), saliva, urine, feces, throat swabs, liver, bile, cervical swabs, semen, hair, skin, bone marrow, lymph node, muscle, thyroid gland, lung, spleen, pancreas, kidney, cerebrospinal fluid, gastrointestinal tract, and brain (Chan et al., 2001a; Deng et al., 2000; Fornai et al., 2001; Gallian et al., 2000; Inami et al., 2000a; Ishikawa et al., 1999; Jiang et al., 2000; Lopez-Alcorocho et al., 2000; Maggi et al., 2001a; Maggi et al., 2003a; Martinez et al., 2000; Matsubara et al., 2000; Okamoto et al., 1998b; Okamoto et al., 1999a; Okamoto et al., 2001a; Okamura et al., 1999; Osiowy et al., 2000; Pollicino et al., 2003; Rodriguez-Inigo et al., 2000; Ross et al., 1999; Sospedra et al., 2005; Suzuki et al., 2001; Ukita et al., 1999; Yamamoto et al., 1998; Yu et al., 2002; Zhong et al., 2002). It seems that there is no preference for a given genotype to exist in certain tissues (Nakagawa et al., 2000; Pollicino et al., 2003). The PBMC contains several subsets of different cells, and the TTV DNA in PBMC seems to show no restriction to a specific cell type: it is found in B-lymphocytes, T-lymphocytes, monocytes, polymorphonuclear leukocytes, granulocytes, and in natural killer cells, but not in red blood cells or platelets (Maggi et al., 2001b; Takahashi et al., 2002). There are also contradictory results on the presence of TTV DNA in B-lymphocytes (Garbuglia et al., 2003) and in T-lymphocytes (Yu et al., 2002). Besides TTV DNA, TTV mRNA has been detected at least in PBMC, cerebrospinal fluid, breast, colon, omentum, gallbladder, liver, bone marrow and thyroid (Mariscal et al., 2002; Okamoto et al., 2000c; Pollicino et al., 2003).

In addition to normal tissues, TTV DNA has been detected in tissues of several cancer types (de Villiers et al., 2002), including brain tumors (Sospedra et al., 2005). However, as noted by Takahashi and coworkers, since TTV is present in the cells of the immune system that invade multiple tissues with inflammation, it should be carefully determined whether TTV really harbours the cells inherent in particular tissue (Takahashi et al., 2002).

6 TORQUE TENO VIRUS-LIKE VIRUSES

6.1 Torque teno minivirus

During the ten years since the discovery of TTV, TTV-like viruses have also been identified in humans. TTV-like minivirus, TLMV (later termed Torque teno minivirus, TTMV), was found in a TTV-viremic serum by serendipity; the primers and PCR conditions that were used favored amplification of TTMV instead of TTV (Takahashi et al., 2000). The genome of TTMV consists also of circular negative sense ssDNA of approximately 2800-2900 nucleotides, i.e. is shorter than that of TTV. The particle size of TTMV has been estimated to be less than 30 nm, going below TTV also in diameter (Takahashi et al., 2000).

TTMV resembles TTV in genomic structure, possessing the GC-rich region and the coding area. TTMV also contains a CAV-like motif in the ORF2 region, and an ariginine-rich N-terminus as well as Rep-motifs in the ORF1 (Biagini et al., 2001b; Takahashi et al., 2000). The ORF2 protein has been shown to act as a dual-specificity phosphatase (Peters et al., 2002). ORF3 was shown to have a serine-rich C-terminus, exhibiting some homology with a topoisomerase I protein and thus potentially being involved in the replication process (Takahashi et al., 2000).

TTMV is also highly divergent; the first three TTMV sequences discovered differed from each other by 42% at the nucleotide level, and by 67% at the amino acid level (Takahashi et al., 2000). Phylogenetically TTMV, as well as TTV, forms a large family or cluster of isolates (Niel et al., 2001) that diverge from each other as much as 47% within the ORF1 area (Biagini et al., 2006b; Biagini et al., 2007) (Fig. 5). The mechanism for this divergence is not known, but similar recombination-prone sites as in TTV have been postulated to exist (Biagini et al., 2001b).

TTMV is distributed worldwide among healthy individuals without (apparent) geographical clustering (Biagini et al., 2001b; Niel et al., 2001). For example, in sera of blood donors the TTMV DNA prevalence is 62% in France (Biagini et al., 2006b), 67-72% in Brazil (Devalle et al., 2004; Niel et al., 2001), and 48% in Norway (Moen et al., 2002a). TTMV isolates have been shown to occur in various tissues, such as plasma/serum, PBMC, feces, saliva, bone marrow, spleen and cervical swabs, in varying viral loads ($1.3x10^3$-$1.7x10^8$ copies per μg of DNA) (Biagini et al., 2001b; Fornai et al., 2001; Thom et al., 2003; Vasconcelos et al., 2002).

6.2 Small anellovirus and Torque teno midivirus

Small anelloviruses (SAV), were found by the DNase-SISPA method in patients with an acute viral infection syndrome (Jones et al., 2005). SAVs also share the genomic structure of other TTVs; a GC-rich region and a coding region are found in their genomes that are of 2249-2635 nt in length (Jones et al., 2005). The SAV ORF2 area was shown to possess a similar CAV-like motif as in the other TTVs (Andreoli et al., 2006). Similar to TTVs, SAV isolates seem to form a large phylogenetic tree (Fig. 5), showing genomic variation of up to 41%. SAV isolates have also been found in various tissues, including saliva and PBMC (Biagini et al., 2006a) as well as in nasopharyngeal aspirates (Chung et al., 2007). As other TTVs, SAVs are common among healthy inividuals; they occur e.g. in 20% of French blood donors (Biagini et al., 2006a) and in 34.5% of Korean children (Chung et al., 2007). In addition, isolates related to SAV, but with even shorter genomes (2002 nt and 2454 nt) have been identified. They differed from SAVs by approximately 40%, forming a cluster of their own within the SAV sequences (Biagini et al., 2007).

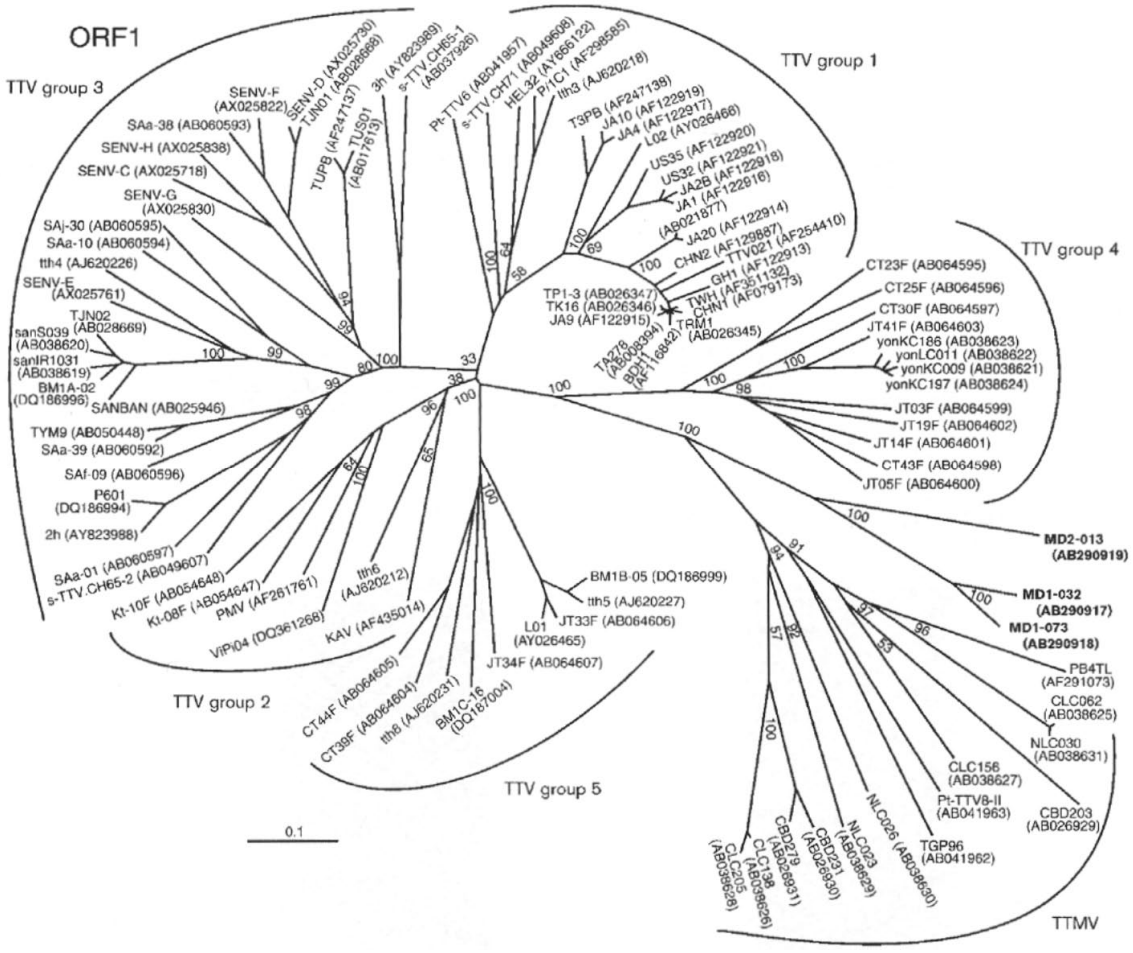

Figure 5. Phylogenetic tree constructed from ORF1 sequences of TTVs, TTMVs and TTMDVs (marked as MDx-xxx) (Adapted from Ninomiya et al., 2007, with permission).

Recently Ninomiya and coworkers reported, while attempting to amplify SAV sequences in sera, amplicons longer than expected. The full-length clones were of 3242-3253 nt, with all the characteristics of TTV-like viruses. Most importantly, the previously described SAVs were reported to be deletion mutants or artefacts generated during amplification of these longer isolates. These newly identified isolates were termed Torgue teno midiviruses (TTMDV) (Ninomiya et al., 2007a). Upon analyzing more TTMDV sequences, the authors noticed that these formed a large swarm of isolates differing in length (3175-3230 nt) and in sequence (33% divergence at nucleotide level and 60.7% at amino acid level of ORF1) (Fig. 5). In addition to other TTV-like characteristics, also three Rep-motifs were identified in the ORF1 region, as well as putative stem-loop structures in the GC-rich region. Based on this study, TTMDVs occur in at least 40% of nonsymptomatic individuals (Ninomiya et al., 2007b), i.e. apparently ubiquitously worldwide.

It has been speculated that these smaller TTVs could be the result of intragenomic rearrangements of full-length TTVs (Leppik et al., 2007). However, in the original investigation TTMV was clearly demonstrated not to be a satellite genome of TTV, but a complete virus (Takahashi et al., 2000). It is highly probable that with new amplification methods, additional variants of TTV will be discovered, thus continuing the expansion of the phylogenetic tree (Fig. 5).

6.3 Torque teno viruses of animals

TT-viruses are not restricted to humans, and various animal species have been shown to carry their own TTVs. Non-human primates are infected with simian TTV, showing a high DNA prevalence (Abe et al., 2000; Cong et al., 2000; Okamoto et al., 2000a; Romeo et al., 2000; Thom et al., 2003). The genomic organization of simian TTVs is similar to that of human TTVs (Inami et al., 2000b; Okamoto et al., 2000b), and co-evolution of TTVs with their hosts has been suggested (Thom et al., 2003).

Pets also have been shown to have TTVs. For example several isolates have been found in cats and dogs (Biagini et al., 2007; Okamoto et al., 2002). In addition, other mammals, such as tupaias (Okamoto et al., 2001b) and pigs have species-specific TT viruses (McKeown et al., 2004; Niel et al., 2005; Okamoto et al., 2002). The swine TTV has been shown to form at least two genogroups (Niel et al., 2005) that are found worldwide in pigs (Kekarainen et al., 2006; McKeown et al., 2004). The high prevalence of swine TTV raises a possible risk in xenotransplantation for immunosuppressed organ recipients (McKeown et al., 2004). Interestingly, genogroup 2 was found more often than genogroup 1 in pigs with post-weaning multisystemic wasting syndrome, the meaning of which remains to be studied (Kekarainen et al., 2006). Whether all animals have species-specific TT viruses, remains

investigated. However, based on the current information on this virus family, it would not be surprising to find TT viruses in all animal species.

7 TAXONOMY OF VIRUSES

The International Committee on Taxonomy of Viruses (ICTV) maintains the universal taxonomy, i.e. classification, of viruses. The classification of viruses varies depending on our knowledge of the particular virus: the demarcation for species within the same genus can be based on structure, genome, host organism, mode of transmission, or other properties common to all the viruses within that species. The definition for a virus species is "a polythetic class of viruses that constitute a replicating lineage and occupy a particular ecological niche". The virus species form genera, which constitute families that are universal classes, the members of which have to share key properties (e.g. circular ssDNA genome and infect vertebrates) (Ball, 2004)
(http://www.virustaxonomyonline.com/).

7.1 Taxonomy of TTVs

Before TTV was known to have a circular genome, it was thought to represent a new parvovirus, i.e. a ssDNA virus with a linear genome (Okamoto et al., 1998b). When the circular nature of TTV genome was revealed, TTV was concluded to closely resemble CAV, a member of the *Circoviridae* family, in genome organization. Thus it was proposed that TTV could be the first circovirus found in humans (Miyata et al., 1999; Takahashi et al., 1998), or that TTV could form a new virus family (Mushahwar et al., 1999). When the TTMV was discovered, it was proposed to be an intermediate between CAV and TTV, and it was suggested that all these three viruses could be evolutionarily linked and classified into a new virus family (Takahashi et al., 2000).

It now seems that the TTV genotypes form a phylogenetic tree with five major clusters (Figs. 4 and 5) (Biagini et al., 2004; Peng et al., 2002). Jelcic and coworkers have suggested that the TTV isolates could form a new virus family with a subdivision into genera, species, types, subtypes and variants based on the sequence divergence (Jelcic et al., 2004). In addition to TTV and TTMV, the recently identified SAVs/TTMDVs shall also be included in the classification. These three virus species consist each of a wide repertoire of isolates with sequence divergences within the whole genome of at least 50%, 40% and 33%, respectively (Ninomiya et al., 2007b). In addition, when the role of the smaller, rearranged TTVs (Leppik et al., 2007) is fully revealed, the genotyping of TTVs might further change.

To date, TTV and TTV-related isolates are officially classified as a floating genus Anellovirus (Biagini et al., 2004), yet the nomenclature of isolates and genotypes still varies within publications. Based on a collaborative work, it has been proposed by *Circoviridae-Anellovirus* Study Groups of ICTV that all the anellovirus members (TTV, TTMV, SAV/TTMDV, and highly divergent animal isolates) could be classified into a new viral family, *Anelloviridae*. By phylogenetic analysis of the entire ORF1 of anellovirus isolates, the family would have several genera with species and subspecies (http://www.ictvonline.org/index.asp?bhcp=1) (Biagini et al., taxonomic proposal ICTV Report, unpublished data). It has also been foresightedly suggested that since CAV has several similarities to TTVs (i.e. mRNA splicing, genomic organization etc.), CAV and anelloviruses could be included into the same virus family, but within their own subfamilies (Biagini et al., unpublished data).

7.2 Family *Circoviridae*

The known vertebrate infecting circular ssDNA viruses are found only in the family *Circoviridae*. Other viruses with circular ssDNA genomes include bacteria-infecting *Microviridae* and *Inoviridae*, and plant-infecting *Geminiviridae* and *Nanoviridae* (ICTVdB - The Universal Virus Database, version 4.) (Fauquet et al., 2004). It has been suggested that circoviruses have evolved from plant nanoviruses, which switched host from plant to vertebrate, subsequently recombining probably with calicivirus, establishing a new virus lineage, circoviruses (Gibbs et al., 1999).

Within the family *Circoviridae* (Fig. 6) chicken anemia virus is classified into the genus *Gyrovirus*, and porcine circovirus (PCV) and beak and feather disease virus (BFDV) into the genus *Circovirus* (Pringle, 1999). All the viruses within the family *Circoviridae* have a circular ssDNA genome, but they differ in structure (Crowther et al., 2003) (Fig 7.) and in genomic organization (ambisense vs. antisense). Circoviruses replicate via double-stranded DNA intermediate that is used as a template for the generation of viral ssDNA by the rolling circle mechanism. For replication, viruses use the host cell DNA polymerase in the S phase of cell division. The infections are highly prevalent worldwide, and are transmitted feco-orally and vertically. Circoviruses cause subclinical infections in their animal hosts, but are also associated with various diseases, caused by infection-related lymphoid depletion and immunosuppression (Todd et al., 2004).

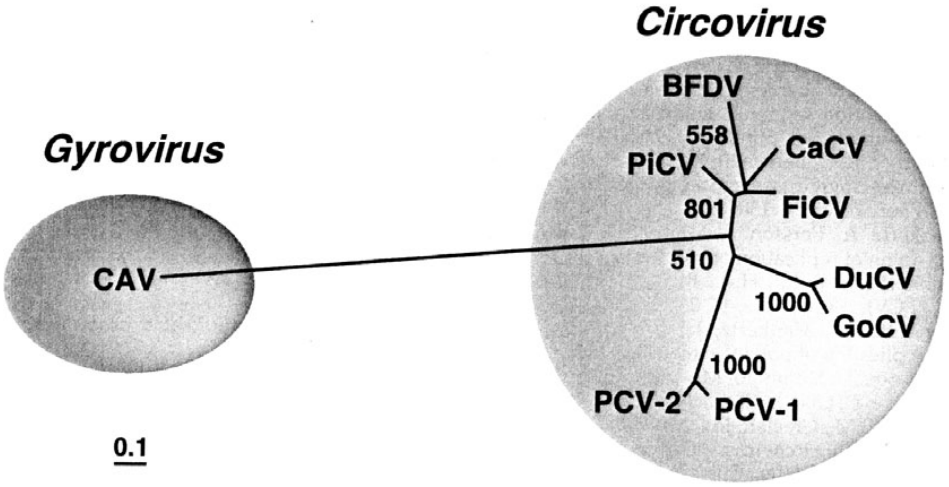

Figure 6. Phylogenetic tree based on Rep protein sequences of the family *Circoviridae*. (Reproduced from Todd et al., 2004, with permission.)

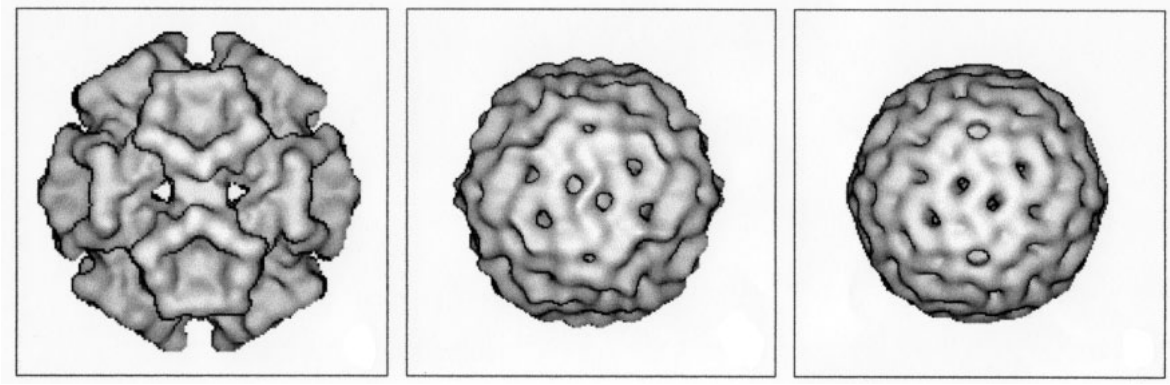

Figure 7. Three-dimensional drawings of circoviruses, based on computational analysis of electron micrographs: (from left to right) CAV, PCV-2, and BFDV. (Adapted from Crowther et al., 2003, with permission).

Genus Circovirus

Viruses in the genus *Circovirus* have ambisense genomes, i.e. the viral proteins are encoded both in sense and anti-sense orientation, resulting in multiple mRNAs (Bassami et al., 1998; Cheung, 2003a; Cheung, 2003b; Niagro et al., 1998).

Porcine circovirus (PCV) has two strains (PCV1 and PCV2) with divergence of approximately 20%, of which PCV2 is pathogenic and causes post weaning multisystemic wasting syndrome (PMWS) in pigs (Hamel et al., 1998; Meehan et al., 1998). The PCV

genome is approximately 1760 nt long and contains a conserved nonanucleotide motif in the potential stem-loop structure that is used for initiation of replication (Todd et al., 2004). The ORF2-encoded virus protein is the only protein forming the capsid (Nawagitgul et al., 2000). ORF1-encoded replication associated protein, Rep (Mankertz et al., 1998), is produced in two forms by alternative splicing (Mankertz et al., 2001; Mankertz et al., 2004).

Beak and feather disease virus (BFDV) infects young psittacines and causes persisting lymhoid depletion predisposing the birds to secondary infections with potentially lethal pathogens (Todd et al., 2004). In addition to BFDV, various avian circoviruses are included in the genus [ICTVdB - The Universal Virus Database, version 4.; (Todd et al., 2004)]. The avian-infecting circoviruses have genomes of approximately 1800-2000 nt, and are distributed widely in various tissues, frequently causing sub-clinical infections. Viral loads have been suggested as a correlate to disease severity (Niagro et al., 1998; Todd et al., 2004)

Genus Gyrovirus

Chicken anemia virus (CAV) is the sole member of the genus *Gyrovirus* (Todd et al., 2004). CAV has a genome of 2300 nt in anti-sense organization, i.e. the proteins are encoded from one direction on the complementary DNA-strand (Noteborn et al., 1991; Noteborn et al., 1992; Todd et al., 1990). Until recently it was thought that CAV encodes one long polycistronic mRNA (Noteborn et al., 1992; Phenix et al., 1994), however, Kamada and coworkers showed CAV to generate by alternative splicing at least three mRNA species, similar to TTV (Fig. 8). The previously described unspliced mRNA is also produced, and the meaning for the CAV life cycle of the three spliced mRNA species remains to be elucidated (Kamada et al., 2006).

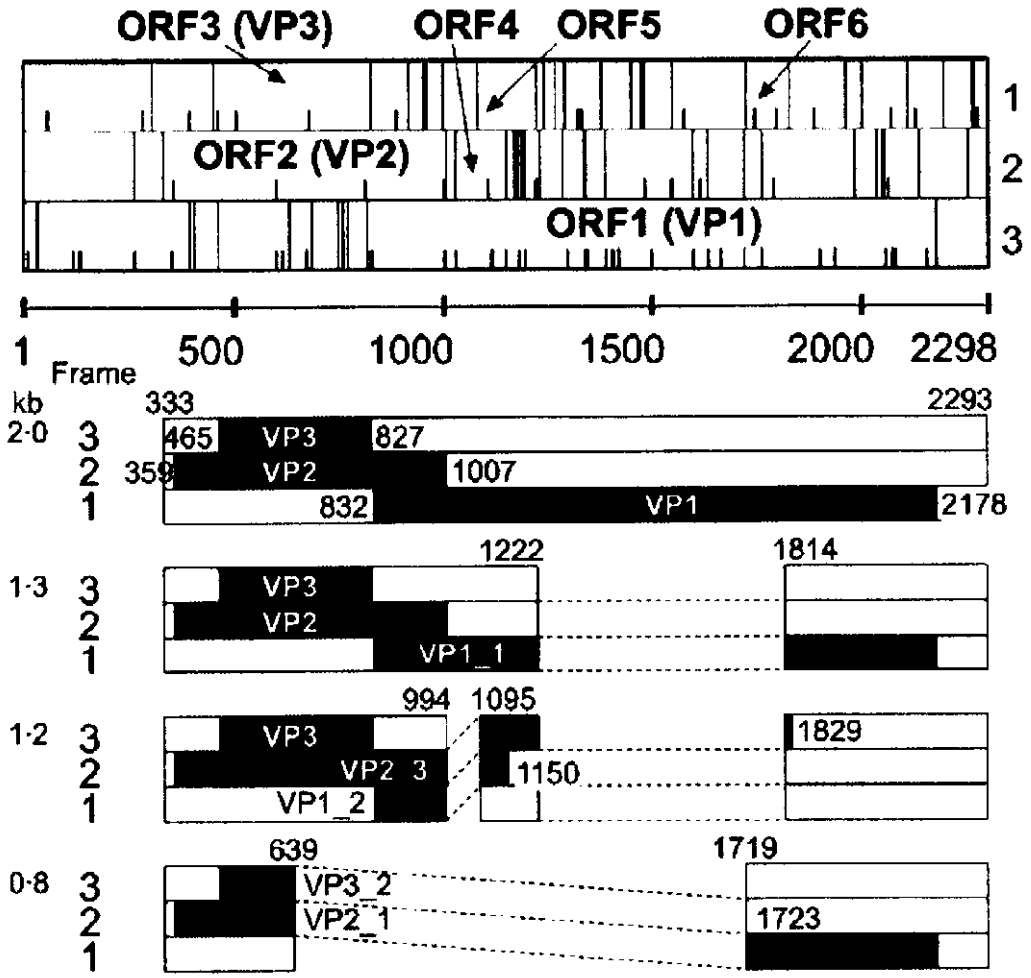

Figure 8. The transcription map of CAV. (Reproduced from Kamada et al., 2006, with permission).

VP1 is the only protein forming the capsid of CAV, however, VP2 co-expression is required for generation of neutralizing antibodies, VP2 probably acting as a scaffold protein and providing proper folding to VP1 (Noteborn et al., 1998; Todd et al., 1990). VP1 possibly also acts in replication since the Rep-motifs occur within (Todd et al., 2004). VP3, called Apoptin, causes apoptosis in infected cells (Noteborn et al., 1994; Noteborn, 2004). This Apoptin-induced apoptosis occurs selectively also in human cancer cells, and Apoptin has been investigated for therapeutic potential (Danen-Van Oorschot et al., 1997; Noteborn, 2004). VP2 acts as a dual-specificity protein phosphatase, as does ORF2 protein of TTMV (Peters et al., 2002), the activity of which is required for replication, cytopathogenicity, and MHC-I down regulation in infected cells (Peters et al., 2005; Peters et al., 2006). CAV seems to have two strains (ICTVdB - The Universal Virus Database, version 4.), with a divergence of ~4% (Natesan et al., 2006).

CAV induces immunosupression in chickens by depleting hemocytoblasts and thymocytes (Miller et al., 2004) by apoptosis (Jeurissen et al., 1992). In addition, CAV infection causes impairment in development of pathogen specific cytotoxic T-cells, and leads to more severe diseases in co-infection with other pathogens (Markowski-Grimsrud et al., 2003). The clinical symptoms depend on the age of the infected chicken, on the presence of antibodies, and on the presence of secondary infections. Chickens of all ages are prone to infection, but clinical disease develops only in young or in immunosuppressed chickens. In addition, CAV can establish latent infection in reproductive tissues from which it is activated at the time of reproductive maturation (Miller et al., 2004).

8 TRANSMISSION, INFECTION AND HOST CELLS OF TORQUE TENO VIRUS

Viruses have various ways of transmission. For example HIV is transmitted via blood, influenza virus via aerosols, caliciviruses often via water, and TBE via ticks. These forms of passive physical transport or live vectors eventually bring the virus into contact with its target cell, and the viral life cycle can begin. The virus is said to have infected its host when it has entered the target cell. Viruses can cause acute, latent, or persistent infections. Acute infections, and acute phases of chronic infections, are usually short-lived: virus hits the target and escapes before the host dies or elicits a strong immune response. Latent infections often last for life: the virus hides inside the cells, either as an episome or as integrated into the host genome, and avoids the immune response. Under certain circumstances the virus is rescued and starts to produce progeny virions. Persistent infections (including chronic infections) last for a long time, either due to low virus production, or due to viral infection of confined body compartments, or due to dysfunction of the host's immune system.

8.1 Transmission of TTV

Since TTV has been found in a myriad of tissues and body fluids (see section "Prevalence of Torque teno virus") several transmission routes are possible. Due to the emergence of TTV in post-transfusion hepatitis, blood and blood products have been thoroughly investigated for the source of TTV infection. Indeed, TTV DNA prevalence has been shown to be higher in patients having received several blood transfusions or blood products (Forns et al., 1999; Maeda et al., 2000; Prati et al., 1999; Simmonds et al., 1998; Viazov et al., 1998b), although there are also results that do not support this (Rubinstein et al., 2000; Schröter et al., 1998). In addition, TTV has been found in various blood products, including first-generation recombinant factor VIII concentrates, factor IX concentrates (Azzi et al.,

2001; Simmonds et al., 1998; Yokozaki et al., 1999), and intramuscular immunoglobulin batches (Pisani et al., 1999). In addition to TTV, TTMV could potentially be transmitted via blood transfusions (Takahashi et al., 2000). Blood borne viruses, such as HIV, are transmitted among drug users via shared needles. However, it has been shown that this is not the main route for TTV transmission (MacDonald et al., 1999; Yazici et al., 2002). Due to the transmission via blood, some research groups have called TTV a "transfusion transmitted virus". However, this nickname is misleading because TTV most probably is transmitted via other routes as well.

Transmission via blood does not explain the prevalence of TTV worldwide. Most of the other viruses showing high prevalence at the population level are usually efficiently transmitted via the feco-oral route. This most probably is also the case with TTV, since the virus is found in feces (Okamoto et al., 1998a; Ross et al., 1999; Ukita et al., 1999), even among 7-12 month-old healthy children (Lin et al., 2000).

Viruses are transmitted via food and water. As is typical for a highly prevalent virus, TTV is found in various aquatic environments. TTV was detected in 5% of surface water samples from a river in Japan (Haramoto et al., 2005a), as well as in waste water (Haramoto et al., 2005b; Vaidya et al., 2002). In addition, TTV and TTMV were detected in 8% of Norwegian shellfish (Myrmel et al., 2004), indicating that TTV is present in water.

Viruses are also effectively spread by droplets. Indeed, higher TTV loads detected in saliva than in serum (Deng et al., 2000; Gallian et al., 2000), presence of TTV in throat swabs (Ishikawa et al., 1999), and detection of SAVs in nasopharyngeal aspirates (Chung et al., 2007) suggest that spreading via the respiratory tract and droplets could be one transmission route for TTV.

Sexual transmission is another important way for a virus to spread within the adult population. TTV has been found in cervical swabs (Calcaterra et al., 2001; Chan et al., 2001c; Fornai et al., 2001) and in semen (Inami et al., 2000a; Martinez et al., 2000) suggesting sexual transmission. However, this mode of transmission has been proposed to be of minor importance (MacDonald et al., 1999; Salakova et al., 2004; Yazici et al., 2002).

It seems that humans acquire TTV infection(s) very early in life. TTV DNA has been detected in blood of newborns, in cord blood, and in amniotic fluid, suggesting transmission already *in utero* (Bagaglio et al., 2002; Gerner et al., 2000; Matsubara et al., 2001; Morrica et al., 2000; Saback et al., 1999). In addition, newborns acquire TTV from the environment within a few months (Davidson et al., 1999; Kazi et al., 2000). This

environmentally acquired infection could be transmitted from the mother, since TTV has been found in breast milk (Gerner et al., 2000; Iso et al., 2001; Matsubara et al., 2001; Ohto et al., 2002; Schröter et al., 2000). Also, results contradictory to *in utero* transmission have been presented, showing TTV DNA-negative cord blood samples, and TTV DNA appearance from a few months of age upwards (Davidson et al., 1999; Hsieh et al., 1999; Iso et al., 2001; Kazi et al., 2000; Ohto et al., 2002; Salakova et al., 2004; Simmonds et al., 1999; Sugiyama et al., 1999). However, the absence of TTV DNA could be due to the PCR methods used (Matsubara et al., 2001), and due to possibly low virus load in infants. When TTV sequences from children and from mothers have been compared, they show both similar and divergent sequences, indicating both mother-child transmission and environmental transmission (Bagaglio et al., 2002; Davidson et al., 1999; Lin et al., 2002; Ohto et al., 2002; Sugiyama et al., 1999). Also, TTMV has been detected in cord blood, amniotic fluid, breast milk and newborn sera, with sequence similarity to those of the mothers (Matsubara et al., 2001). For HBV it has been shown, that in hyper-endemic areas perinatal and early-life horizontal transmission of the virus results in high prevalence and a chronic carrier stage (Custer et al., 2004). The mother-to-child transmission route could in part explain the high prevalence and persistent infectious characteristics for TTV.

It is quite obvious that such a prevalent virus group as anelloviruses may have several modes of transmission; feco-oral and droplet spread being at the moment the most probable candidate routes.

8.2 TTV infections in animals

Animal models are still very important in the research of virus transmission and infections. Rhesus monkeys and chimpanzees can be infected orally or intravenously with human TTV originating from human feces and/or serum, indicating presence of infectious TTV and transmission to animals (Luo et al., 2000; Mushahwar et al., 1999; Okamoto et al., 2000a; Romeo et al., 2000; Tawara et al., 2000). In Rhesus monkeys, a few days post-infection, the virus was detected in liver (in the nuclei and/or cytoplasm of hepatocytes), and was excreted in bile, feces, and blood. Chronic infection was sustained for at least six months. The infection could be passaged to other monkeys, with no significant nucleotide changes, thus indicating stability of the replicating TTV sequence (Luo et al., 2000). In chimpanzees, TTV was detected transiently in the sera, with no signs of hepatitis (Mushahwar et al., 1999). In another study, both transient and persistent infections in chimpanzees were observed, with mild biochemical and histological changes in liver (Tawara et al., 2000). The difference between those two experiments could be due to the TTV loads in the inoculums or due to the amount of immunocomplexes (inoculum from persistent infection with more immunocomplexes in the former study, and from acute infection with less immunocomplexes in the latter) (Tawara et al., 2000). To further support the

transmissibility of human TTVs to animals, human TTV sequences have been identified in samples obtained from captive chimpanzees and from animals used for transmission studies of human viruses (Cong et al., 2000; Okamoto et al., 2000b; Romeo et al., 2000). TTV sequences identified in gibbons bred in captivity were found to be closely related to human sequences, and the virus was hypothesized to have been transmitted to gibbons from the animal handlers (Noppornpanth et al., 2001).

Interestingly, simian-TTV infections occur also in humans, whereby TTV has been postulated to be of zoonotic origin (Iwaki et al., 2003). However, it is also possible that some TTVs found in apes and classified as simian-TTVs are actually human, as transmitted during capture, transportation or handling of the animals, as has been speculated for human TTV sequences detected in gibbons (Noppornpanth et al., 2001). Furthermore, zoonotic origin alone would not fulfill the worldwide occurence of TTV in humans. Nevertheless, it is possible that animals could, in part, serve as reservoirs of human TTV. This scenario is supported by the results showing human TTVs in farm animals such as chickens, pigs, cows and sheep (Leary et al., 1999) and in camels in the United Arab Emirates (Al-Moslih et al., 2007), but others have not been able to verify the results on farm animals, leaving this scenario unresolved (Thom et al., 2003).

8.3 TTV infections in humans

In the first TTV publication by Nishizawa and coworkers, TTV infections in humans were shown to be either transient or persistent (Nishizawa et al., 1997). The time course (i.e. kinetics) of TTV infections has been studied in various patient groups. TTV viremia has been shown to persist for several years (Azzi et al., 2001; Ball et al., 1999; Biagini et al., 1999; Irving et al., 1999; Lefrere et al., 2000; Sugiyama et al., 1999), but also clearance of the virus has been detected (Ohto et al., 2002; Prati et al., 1999; Simmonds et al., 1998; Wilson et al., 2001). One reason for the variable results in the acute vs. persistent infection could be fluctuation of virus levels. In persisting infections, the DNA sequence has been shown to either remain unchanged, or change with time (Ball et al., 1999; Biagini et al., 1999; Irving et al., 1999) indicating true persistence, superinfections, and/or evolution of quasispecies. TTV infection has been suggested to be a very dynamic process: it has been estimated that over 90% of the virions are cleared every day, with generation of 3.8×10^{10} virions to replace the cleared ones (Maggi et al., 2001c).

In general, the levels of TTV viremia in healthy subjects are assumed to be low, and in comparison with cancer patients, the viral levels in PBMC were shown to be two logs lower (Zhong et al., 2001b). In addition, it has been shown that among the study subjects the levels of TTV DNA either remain steady or fluctuate, in the range of 10^2 to 10^8 copies/ml (Ball et al., 1999; Maggi et al., 2005b; Pistello et al., 2001; Zhong et al., 2001a).

However, all these results have been obtained in variable study populations with PCR methods which are affected by the presence of several isolates in fluctuating levels, and thus the interpretation of results is difficult.

Co-infections with multiple TTV genotypes are common, even in healthy individuals (Ball et al., 1999; Biagini et al., 1999; Chan et al., 2001a; Forns et al., 1999; Gallian et al., 1999; Jelcic et al., 2004; Mushahwar et al., 1999; Niel et al., 2000; Okamoto et al., 1999b; Prescott et al., 1999; Takayama et al., 1999b). The presence of mixed infections could 1) infer that TTV is not cleared from the system, 2) that re-infections and super-infections occur frequently, 3) that the virus is spread via several routes, 4) that the mutation rate is high, and/or 5) that a previous infection does not protect against a re-infection (White et al., 2000). Of these possibilities, at least infections with new genotypes have been documented to occur during follow-up (Gallian et al., 1999; Maggi et al., 2006). In addition to mixed infections with various TTV genotypes, mixed infections with several TTMV isolates (Vasconcelos et al., 2002), as well as co-infections with TTMV and TTV have been reported to occur in healthy individuals (Biagini et al., 2006b; Moen et al., 2002a; Niel et al., 2001; Vasconcelos et al., 2003). Also co-infections with the three anelloviruses (TTV, TTMV and SAV/TTMDV) have been reported (Biagini et al., 2006a; Ninomiya et al., 2007a).

Whether the recently identified rearranged sub-genomic fragments (Leppik et al., 2007) will further make the picture of anellovirus infections more complex remains to be seen. In any case, data of sewage waters suggest that there are no seasonal changes in TTV infections (Haramoto et al., 2005b; Vaidya et al., 2002), i.e. it seems that we acquire TTV throughout the year and throughout our lives.

8.4 TTV host-cell tropism

The presence of TTV DNA in several organs and tissues suggests a wide host cell tropism. A search for the host cell for TTV has been attempted by showing the location of TTV DNA (*in situ* methods or DNA quantification) in certain cells, or by detecting replicating TTV DNA in tissues.

Cells circulating in blood and originating from the hematopoietic compartment have been the strongest candidates for TTV host cells, and the presence of TTV in PBMC has been confirmed by fluorescent *in situ* hybridization (Lopez-Alcorocho et al., 2000; Mariscal et al., 2002; Zhong et al., 2002). Since the TTV sequences found in serum were different from those in PBMC, it has been suggested that some genotypes could have preference for PBMC (Chan et al., 2001a; Okamoto et al., 1999a; Okamoto et al., 2000d). In addition, since the sera of some PBMC-positive patients were TTV DNA negative, it has been

suggested that PBMC could serve as a reservoir for TTV (Garbuglia et al., 2003; Maggi et al., 2001c). Furthermore, PBMC can be infected with TTV (Zhong et al., 2002), and when stimulated, produce infectious TTV (Maggi et al., 2001b; Mariscal et al., 2002). Bone marrow contains precursors for hematopoietic cells. The notion of decreasing levels of TTV-DNA in bone marrow transplant recipients during myelosuppression led to a suggestion that hematopoietic cells could sustain TTV replication (Kanda et al., 1999). Further evidence, showing replication of TTV in bone marrow cells (Yu et al., 2002; Zhong et al., 2002) and clearance of TTV after bone marrow transplantation (Chan et al., 2001b), strengthen the possibility that the hematological compartment could be the site of replication and/or persistence of TTV.

The presence of high TTV-DNA levels in saliva (Lopez-Alcorocho et al., 2000; Okamoto et al., 1998b; Rodriguez-Inigo et al., 2000; Suzuki et al., 2001) has led to investigations of oropharyngeal tissue and/or salivary glands as a putative target/host tissue for TTV infection (Deng et al., 2000). Indeed, TTV has been detected by *in situ* hybridization in the cytoplasm of oral epithelial cells (Rodriguez-Inigo et al., 2001), however, the meaning of this finding is not clear. Maggi and coworkers have shown that TTV DNA loads in the nasal cavity exceed those in the sera and thus the nasal cavity could be the primary site of TTV infection (Maggi et al., 2003b) further supporting the droplet spread hypothesis.

Due to the history of TTV as a potential hepatitis virus, liver as a main target organ for TTV infection has also been studied extensively. TTV is found by *in situ* methods in the nucleus and/or the cytoplasm of liver cells in patients with liver damage (Cheng et al., 2000; Jiang et al., 2000), but without any cytopathological changes (Comar et al., 2002; Ohbayashi et al., 2001).

Replication of TTV in tissues has been mostly studied by gel separation, as well as with strand-specific primer extension, and full-length PCRs. With these methods (although not ideal for detection of replication), double-stranded, circular and potentially replicating forms of TTV have been detected in a variety of tissues: lung, stimulated PBMC, bone marrow cells, liver, lymph node, thyroid gland, spleen, pancreas, and kidney (Bando et al., 2001; Mariscal et al., 2002; Okamoto et al., 2000e; Okamoto et al., 2000f; Okamoto et al., 2001a). In Rhesus monkeys experimentally infected with TTV, the viral DNA was detected in various organs (Luo et al., 2000), whereas replicative forms were detected only in liver, bone marrow and the small intestine (Xiao et al., 2002).

Several viruses are known to integrate into host genomes, however, TTV seems not to integrate, at least not in hepatocytes, PBMCs or hematopoietic tumor cells (Nishizawa et al., 1997; Tanaka et al., 2000a; Yamamoto et al., 1998; Yu et al., 2002). In addition to the

presence of TTV DNA in certain cell types, the enhancer activity of the TTV promoter has been studied in various cell lines. The activity was highest in K562 human erythroleukemia cells, and also in HepG2 human hepatocellular carcinoma cells (Kamada et al., 2004), further supporting that these two cell types are the targets of TTV infection.

Only two *in vitro* studies on TTV infection in cell lines have been published. Desai and coworkers infected with TTV genotype 1-positive pooled sera a Chang liver cell line, the B lymphoblast cell line Raji, and stimulated PBMC. Chang liver cells produced constant, low amounts of TTV upon cell passage, whereas in PBMC and in the Raji cell line the virus release was transient (Desai et al., 2005). Leppik and coworkers transfected Hodgkin's lymphoma-derived cell line L428 with a full-length TTV clone, and demonstrated TTV DNA recirculation and replication in the cells (Leppik et al., 2007).

The best candidates at the moment for TTV host cells are cells of hepatic and those of erythroid origin. However, the lack of efficient virus culture systems and well-characterized animal models, leave our current knowledge on anelloviruses' life cycle, infection mechanisms, and target cells/organs inadequate.

9 IMMUNOLOGY OF VIRUS INFECTIONS

9.1 Immunological battle between viruses and hosts

When a virus infects its host, specific defence mechanisms are usually initiated by the host to clear the infection: for example plants (and higher organisms) use micro-RNAs, bacteria use restriction enzymes and animals have an immunological system. The human immune system is divided into innate and adaptive immunity. Innate immunity is the second-line defence, while skin and mucosa are the first line, against invading microbes. As players of the innate system, phagocytic cells (macrophages and neutrophils) ingest and destroy invaders, whereas natural killer cells recognize and destroy the infected cells by sensing viral proteins and MHC-I reduction. Complement protein C3b binds to invaders opsonising them for phagocytes and also directly lysing enveloped viruses by activating the lysis complex. Infected cells and cells of the innate system secrete cytokines [e.g. interferons (INF) α, β and γ, and TNF-α], compounds that activate and coordinate other innate immunity cells and initiate the adaptive immunity reaction. In addition, IFNs-α and β produced by infected cells, induce the cells to die, and predispose the neighbouring cells also to die upon infection. Complement-coated viruses are efficiently targeted to B cells and follicular dendritic cells, and large natural IgM/antigen complexes are efficiently filtered by the spleen, thus increasing the concentration of the antigen in lymphoid organs, thereby linking innate and adaptive immunity (Flint et al., 2004; Hilleman, 2004; Ochsenbein et al., 2000; Zinkernagel, 1996).

The cytokines produced by the cells of the innate system activate dendritic cells in tissues. Dendritic cells engulf viruses, migrate to lymph nodes and present antigens to helper T cells, and thus activate the adaptive immune system. The helper T cells orchestrate the adaptive immunity by activating antigen-specific B cells, and by involvement in activation of cytotoxic T cells. The activated B cells mature into plasma cells that produce large amounts of antibodies (IgM, IgG and IgA), and also form memory B cells. Activated cytotoxic T cells migrate to the infected tissue and destroy infected cells. The cell-free virions are bound by antibodies which block the infectivity of viruses and/or opsonise them to be engulfed by phagocytes. In addition, antibodies also promote cellular cytotoxicity. The two branches of the immune system interact: the innate immune system signals to the adaptive immunity system which agent has invaded the body. Innate immunity is fast, non-specific (recognizes patterns) and has no memory, while adaptive immunity is slow, specific (recognizes specific antigens), and can protect against re-infection. The adaptive immunity can not be fully launched unless the innate immunity is triggered by the invader (Flint et al., 2004; Hilleman, 2004; Ochsenbein et al., 2000; Zinkernagel, 1996).

Viruses have generated numerous ways to overcome the immune responses in order to survive within the host. Large viruses have several genes encoding proteins for these purposes. Viruses try to avoid innate immunity by releasing progeny virions by budding (i.e. without cell lysis), keeping dsRNA levels low or absent (i.e. no cytokine production from infected cells), and by producing proteins that interfere with interferon responses. Furthermore, viruses can block the effects of cytokines by interfering with cytokine or receptor production, and with their functions. Viruses can mislead NK cells by expressing MHC protein analogs on the surface of the infected cells. Adaptive immunity is fought by the production of a large number of defective virions that exhaust virus-specific antibodies, by reduction of MHC-I expression (i.e. less cytotoxic T-cell activity), and by mutations (i.e. antibodies do not recognize the mutated protein). Viruses can further evade immune responses by producing proteins that restrict, modify, redirect or ablate the expression or function of cells of the host immune system. Proteins can also act as ligands or receptors that antagonize/agonize host cell functions in favor of the virus. In addition, a high mutation rate, especially among RNA viruses, and antigenic shift and drift help to escape the immune control. In persistent infections, viruses can infect non-permissive or semi-permissive cells, remaining latent and non-replicating, and thus avoiding cell destruction. The virus may also be stored in various body compartments that immunological responses do not reach, for example in the brain. Viruses have also developed several ways to prevent cells from presenting antigens. The cell cycle and apoptosis are also controlled in various ways, and several mechanisms are used to evade antibody and complement actions. Moreover, some viruses, like Aleutian mink disease virus of the family *Parvoviridae* (Best

et al., 2005), even take advantage of antibodies in order to infect their target cells; antibody-dependent enhancement of infection (Hilleman, 2004; Ochsenbein et al., 2000; Takada et al., 2003; Zinkernagel, 1996).

9.2 Immunology of TTV infections

TTV, harboring a small genome, has restricted capacity to encode proteins for immune evasion. For TTV the studies on this topic are limited. Antibodies against TTV have been determined with various methods. Tsuda and coworkers mixed patient serum with fecal-derived TTV, followed by immunoprecipitation. TTV PCR was performed on the supernatant and from the pellet: a higher intensity of PCR-amplicon signal from the pellet than from the supernatant represented immunoprecipitation of TTV particles, and thus the presence of TTV-specific antibodies in the serum. With this crude method the presence of IgG antibodies in patients with and without TTV DNA, as well as after clearance of TTV DNA, was demonstrated (Tsuda et al., 1999). For detection of IgM antibodies a method using IgM capture, followed by incubation with TTV genotype-1 positive serum, was developed. The IgM-bound TTVs were detected by PCR. With this method, IgM antibodies for TTV were not detected in TTV DNA-negative healthy individuals, but were found in patients with hemophilia or with HCV infection. In three patients, it was shown that after TTV-DNA clearance, short-lived IgM antibodies appeared, followed by long-lasting IgG antibodies (detected by the immunoprecipitation method) (Tsuda et al., 2001). Both of the above studies were carried out on TTV genotype-1, however, it is not yet known whether the TTV genotypes also represent serotypes, i.e. whether the antibodies cross-react between genotypes.

Two research groups have reported the expression of the putative capsid protein, ORF1, in two parts: the N-terminus (amino acids 1-411) (Handa et al., 2000) and the C-terminus (amino acids 504-752) (Ott et al., 2000). Both proteins were expressed as His-tagged in *E. coli*, and used as antigens in immunoblots for detection of TTV-specific antibodies. Handa and coworkers detected antibodies against the N-terminus in 38% of US blood donors. The majority of the antibody-positives were also TTV-DNA positive (one for the same genotype as the protein), indicating that the antibodies could be non-neutralizing and non-protective against re-infection (Handa et al., 2000). Ott and coworkers detected TTV-specific antibodies (IgG+IgA+IgM) in 98.6% of the tested sera, and TTV DNA in 76.1%. When IgM was tested alone, no responses were detected (Ott et al., 2000). In addition, one group has reported the expression of the N22 region within ORF1 (120 amino acids) but found no antibodies in immunoblotting (Lo et al., 1999) suggesting that the area expressed was non-immunogenic (or was not immunogenic under denaturating conditions).

TTV particles can form immunecomplexes in the sera of infected subjects (Itoh et al., 2000; Maggi et al., 2006; Tsuda et al., 1999), and over 90% of the virions are cleared every day (Maggi et al., 2001c). Interestingly, it has been suggested that TTVs are immunecomplexed in persistent infection, but not in acute infections (Nishizawa et al., 1999). Super-infection with a new genotype was shown to result in an increase of viral load, and in a reduction of immunecomplexed virions. After the super-infecting genotype was resolved, the immunecomplexes and viral loads reverted. It is puzzling why the underlying genotypes were not resolved, however, as suggested by the authors, one explanation could be that the preformed antibodies are more effective in clearing acute than established infection (Maggi et al., 2006).

It has been shown that the viral loads and/or TTV DNA prevalence increase in immunosuppressed subjects, such as transplant recipients (Moen et al., 2003) and HIV-infected patients (Sagir et al., 2005a; Touinssi et al., 2001). Higher TTV levels have been observed in patients with lower $CD4^+$ cell counts (Christensen et al., 2000; Sagir et al., 2005b; Thom et al., 2007), suggesting that the viral load could reflect the patient's immune status (Madsen et al., 2002; Sagir et al., 2005a; Shibayama et al., 2001). On the other hand, TTV viremia does not appear to correlate with a decreased $CD4^+$ cell count, suggesting that HIV-related immunedeficiency alone is not a sufficient cause for elevated TTV or TTMV viremia. In addition, results indicating that HAART (highly active antiretroviral therapy) reduces TTV levels even without increase in $CD4^+$ cell count, suggest that some immunological mechanism other than $CD4^+$ cell-related acts in reduction of TTV viremia (Madsen et al., 2002; Moen et al., 2002c).

Interferons, the mediators of the immune system, are widely used for the treatment of chronic hepatitis B and C virus infections. The effect of interferons has been studied also in TTV infections. Several studies have shown TTV and TTMV levels to decrease during treatment, but no permanent outcome was achieved. This indicated suppression of TTV replication but not eradication of the virus (Akahane et al., 1999; Ali et al., 2002; Chayama et al., 1999; Kao et al., 2003; Maggi et al., 2001c; Tokita et al., 2001; Umemura et al., 2002a; Watanabe et al., 2000). Whether this re-occurence is due to re-infection or re-activation of TTV is not known. Moen and coworkers have shown that in the interferon-treated patients the TTV and TTMV viruses re-appeared with the same viral sequences dominating, thus supporting the view of a re-activation. In one patient they observed appearance of a novel strain, indicating re-infection (Moen et al., 2003). It has also been suggested that some genotypes could be more resistant than others to interferon treatment (Chayama et al., 1999), but these results have not been fully supported (Watanabe et al., 2000).

In order to establish persistent infections, like anelloviruses do, the virus must somehow evade the immune response. It has been speculated that the HVRs of ORF1, the putative capsid protein, are frequently mutated due to immune pressure (Jelcic et al., 2004; Umemura et al., 2002b) and that TTV could escape immune surveillance by circulating as quasispecies (Nishizawa et al., 1999; Umemura et al., 2002b). However, there are also publications indicating that HVRs do not mutate during follow up (Ukita et al., 2000). Interestingly, it has been suggested that in chronic and persistent TTV infection, the HVRs mutate and quasispecies exist, while in acute and in resolving TTV infection, the HVRs remain stable (Nishizawa et al., 1999).

Since animal circoviruses exert immunomodulation in their hosts, similar immunomodulatory effects of TTV infection have been searched for. It seems that TTV infection in hemodialysis patients has no effect on the activation or distribution of lymphocytes (Fodor et al., 2002). However, in children with acute respiratory disease, the TTV loads showed a correlation with the B-cell percentage, and an inverse correlation with the T cell percentage, possibly suggesting that TTV replication could cause lymphocyte imbalance and be immunosuppressive (Maggi et al., 2003c). It is not known what the mechanism and consequences of this phenomenon are; neither is it known are the viral loads a cause or an outcome of lymphocyte imbalance. A recent publication by Zheng and coworkers showed - intriguingly - that the ORF2 protein could suppress the NF-κB activity and thereby inhibit transcription of downstream genes involved in virus-related inflammations. This could be one mechanism for TTV to modulate the immune response and to establish persistent infection (Zheng et al., 2007).

How TTV can subvert the antibody responses and why the antibodies produced are not able to eradicate the virus, is a mystery. In addition, the entire field of TTV-specific T-cell immunity in healthy individuals remains to be explored.

10 DISEASE ASSOCIATIONS

Viruses can cause diseases by a number of different mechanisms. Replication may lead to cell lysis upon release of progeny virions, viral infection may induce the cell to kill itself (apoptosis), the immune reaction may eliminate the infected cells, or may lead to immunopathogenic events, or viral infection may cause uncontrolled cell proliferation. Also infection of an unnatural host can lead to disease, e.g. tick-borne encephalitis virus (TBE) causes no disease in rodents but encephalitis in humans.

Approximately 800 articles have been published on anelloviruses (January 2008; www.pubmed.gov), of which the majority attempt to link anelloviruses with various

diseases. Since the outcome of a given study is highly dependent on the PCR method used, considerable discrepancies have arisen. Taken together; it is possible that some isolates/genotypes could cause some diseases, either alone or co-infecting with other TTV strains or other pathogens. It is likewise possible that TTVs per se do not cause any disease, but that they somehow have an effect on the outcome or the progression of some disease(s) (either good or bad). Also the levels of TTV in tissue and/or in blood could affect any of the conditions mentioned above. And finally, it is possible that TTV does not have any effect on our well-being. Taking into consideration the wide array of different isolates/genotypes, and bearing in mind that the only methods in current use for detection of TTV infections (only acute and persisting, not past infections) are PCR-based, it is obvious that the results on disease associations of TTVs are inconsistent. Two excellent review articles exist on this (Bendinelli et al., 2001; Hino et al., 2007), in addition to two noteworthy editorials (Naoumov, 2000; Simmonds, 2002), and the following section simplifies and briefly summarizes what has been studied and found.

10.1 Diseases potentially related to TTV infection

Liver diseases: Due to the discovery of TTV from a patient with hepatitis (Nishizawa et al., 1997), many studies have addressed the connection (if any) between TTV and hepatic disorders including non-A to -E viral hepatitis, transfusion associated hepatitis, cryptogenic chronic liver disease, liver chirrosis, hepatitis-associated aplastic anemia, fulminant hepatitis, hepatocellular carcinoma and alcohol related liver diseases.

The initial studies did show an association between TTV prevalence and/or loads and various hepatic disorders (Charlton et al., 1998; Ikeda et al., 1999; Kanda et al., 1999; Nishizawa et al., 1997; Okamura et al., 2000; Tanaka et al., 1998a). It has also been suggested that persistent TTV infections in the patients could contribute to hepatic failure (Takayama et al., 1999a). However, contradictory results were presented indicating that TTV is not associated with ALT levels or with hepatic disorders (Hijikata et al., 1999a; Hsieh et al., 1999; Nakano et al., 1999; Naoumov et al., 1998; Niel et al., 1999; Prati et al., 1999; Viazov et al., 1998b; Yamamoto et al., 1998). It has been noted that the levels of TTV viremia can be higher in patients with hepatitis, which, in consideration of methodological sensitivity, could be interpreted as a “higher prevalence” of TTV in those patients (Pistello et al., 2001). Whether TTV replication would be a cause or a consequence of the liver damage is not clear (Nobili et al., 2005).

According to one hypothesis certain genotypes/genogroups could be associated with hepatic disorders (Okamura et al., 2000; Tokita et al., 2001; Tuveri et al., 2000). Especially SENV isolates have been extensively studied in this regard. Even though, based on some studies, the SENV-D and SENV-H isolates are more common in patients with hepatitis (He

et al., 2003; Umemura et al., 2001b), it seems that infection is not related to hepatitis or other liver diseases (Akiba et al., 2005; Kao et al., 2002; Momosaki et al., 2005; Schröter et al., 2003; Shibata et al., 2001; Umemura et al., 2001a; Yoshida et al., 2002).

Co-infections of TTV with other hepatitis viruses (mainly HBV and HCV) have been widely studied. Although high TTV loads have been associated with the occurrence of hepatocellular carcinoma in patients with HCV-related chronic liver disease, it is not clear if TTV is a cause, a co-factor or a marker of the disease (Tokita et al., 2002). In any case, based on several studies, co-infections with TTV seem not to have any effect on the severity of hepatitis or other hepatic disorders due to HBV or HCV (Campo et al., 2000; He et al., 2003; Hsu et al., 2003; Kato et al., 2000; Schröter et al., 2003; Tuveri et al., 2000; Umemura et al., 2001a).

In summary, TTV does not fulfill the criteria for being a hepatitis virus: it does not appear to cause any damage to the liver cells (Shimizu et al., 2000), transmission to chimpanzees does not cause elevated ALT levels or hepatitis (Mushahwar et al., 2001), and according to several reports in addition to those cited, no statistically important difference in comparison to controls exists in TTV prevalence, loads, sequence variation, genotype distribution, or co-infection among liver disease patients (Ali et al., 2002; Azzi et al., 2001; Chan et al., 2001a; Erensoy et al., 2002; Hsu et al., 2003; Huang et al., 2000; Ikeuchi et al., 2001; Kato et al., 2000; Kurihara et al., 2001; Masia et al., 2001; Mikuni et al., 2002; Nishiguchi et al., 2000; Tanaka et al., 1999; Watanabe et al., 2000; Wong et al., 2002).

Respiratory tract disorders: TTV infection has been suggested to have a role (either active or opportunistic) in children with acute respiratory disease. TTV has been found in higher loads in nasal swabs especially in patients with a more severe respiratory tract disease, like bronchopneumonia (Maggi et al., 2003b). Also the prevalence of SAV was higher in children with acute respiratory syndrome (Chung et al., 2007). In addition, children with high TTV loads in nasal specimens were shown to have worse spirometric values, and TTV was suggested to contribute to the pathogenesis of asthma. It was suggested that TTV replication could twist the immunobalance towards the Th2 response that is known to have a role in the pathogenesis of asthma (Pifferi et al., 2005). In idiopathic pulmonary fibrosis patients a poorer survival rate was suggested to correlate with TTV infection. Interestingly, TTV replication has been shown to occur in lung tissue (Bando et al., 2001). Furthermore, high TTV loads were associated with severity of bronchiectasis (Pifferi et al., 2006). However, as with all the other disorders mentioned, it is not known if TTV is the cause or the result of (or a bystander in) the disease. In one study, a newborn and the parents were followed for TTV loads in saliva. The newborn acquired a TTV strain from the mother

within a few days after delivery, and showed high viral loads in saliva associated with benign rhinitis (Biagini et al., 2003).

Co-infections with other pathogens: Co-infection of cervical cells with TTV and human papillomavirus (HPV) has been shown to occur. Even though women with multiple HPV infections had a higher prevalence of TTV, there was no correlation to high-risk or low-risk HPV strains (Calcaterra et al., 2001). Higher TTV loads have been detected in gastrict tissue of patients with gastritis and with *Helicobacter pylori* possibly suggesting some role for TTV infection (Maggi et al., 2003a).

Immunocompromised patients: It has been suggested that some genotypes could be more common in HIV-infected patients than in healthy individuals (Shibayama et al., 2001). In addition, TTV viral loads have been shown to increase in HIV patients who are progressing towards AIDS, and to correlate with a low $CD4^+$ cell count (Christensen et al., 2000; Sagir et al., 2005a; Shibayama et al., 2001; Thom et al., 2007; Touinssi et al., 2001). However, this most probably reflects the overall immune status of the patient and has no consequence on the underlying disease.

Cancer: TTV DNA has been found in various lymphoma types as often as in healthy individuals, and the virus has been shown to localize in non-neoplastic cells. However, it was postulated that TTV could somehow modulate the T cells it infects and thus have some role in the pathogenesis of lymphomas (Garbuglia et al., 2003). Zur Hausen and coworkers have presented an interesting hypothesis in which they suggest that persistent TTV infections increase the risk for specific translocations, having thus an effect on the development of childhood leukemias and lymphomas (zur Hausen et al., 2005). Co-infection with HPV and TTV genotype-1 have been related to poor outcome of laryngeal carcinoma, however, it is not known if TTV has any effect on the cancer progression (Szladek et al., 2005). TTV DNA has been detected in a wide variety of neoplastic tissues (de Villiers et al., 2002), however, this could be due to tissue inflammation, or to the rapidly dividing cancer cells supporting TTV replication. Presence of TTV in colorectal-cancer specimens has been suggested to have some pathogenetic role, however, the same TTV isolates were found also in non-cancerous tissue (de Villiers et al., 2007). TTV DNA was detected more often in the sera of patients with classical Kaposi's sarcoma than in sera of healthy subjects. In addition, TTV DNA has been detected in lesional skin, as well as in healthy skin of patients, but not in the skin of healthy individuals. It was suggested that TTV infection could, for example by immunosuppression, have some effect on the replication of human herpes virus 8 and thus have a role in the disease pathogenesis (Girard et al., 2007). TTV has been shown to occur in 100 times higher loads in the PBMCs of any cancer patient tested than in healthy controls. However, it is not clear if this is due to the

cancer or represents something in common with the seriously ill patients (Zhong et al., 2001b).

Autoimmune disorders: Several autoimmune disorders still lack a definitive causative agent, and TTV has been studied in this regard as well. Gergely and coworkers noticed peptides from ORF1 and ORF2 areas to resemble human endogenous retrovirus-encoded nuclear protein, a common autoantibody-generating epitope of systemic lupus erythematosus (SLE). They showed that patients, in addition to having high TTV DNA prevalence, had antibodies against the peptides. However, it is not clear whether TTV has a role in autoantibody generation, or whether, due to immunological-dysfunction, the patients could be more prone to TTV infections (Gergely et al., 2005b). Sospedra and coworkers showed that T-cells obtained from multiple sclerosis patients reacted against arginine-rich peptides similar to the TTV and TTMV ORF1 N-terminus. It was suggested that these T-cells could be expanded due to repeated TTV infections, and in conjunction with some other predisposing factors (genetic/microbial) could have some effect on the development of the disease (Sospedra et al., 2005). In addition, TTV has been found in patients with rheumatoid arthritis (Gergely et al., 2005a; Hirata et al., 1998)and with idiopathic inflammatory myopathy (IMM) (Gergely et al., 2005a). However, the TTV prevalence did not differ from that of blood donors (Gergely et al., 2005a). Maggi and coworkers have shown that rheumatoid arthritis patients, when compared to healthy subjects, did not have elevated TTV loads, whereas patients with arthritis related to other autoimmune diseases and SLE, did. The authors suggest that there could be a connection between TTV replication and arthritis, but as with the other disorders mentioned, causality is not known (Maggi et al., 2007).

Interestingly, the expression of genotype-1 ORF1 in transgenic mice, leading to production of a spliced protein, caused pathological changes in the kidneys. Expression of the TTV protein seemed to interfere with differentiation of the renal epithelial cells, the importance of which in TTV pathology remains to be revealed (Yokoyama et al., 2002).

Although TTV is potentially related to many diseases, due to its global presence in healthy subjects (i.e. lack of controls) and due to the lack of abnormalities in TTV-infected cells, conflicting opinions exist on TTV's disease-causing potential. It has been suggested that smaller sub-genomic fragments of TTV identified in human sera could have some role in diseases as is the case with plant geminiviruses (Leppik et al., 2007). In addition, it could be possible that certain genotypes/genogroups could be more pathogenic than others (Maggi et al., 2003b; Maggi et al., 2007), as seen for example with human papillomavirus. On the other hand, before anything definitive can be said about the pathogenicity of TTV, it must be noted that TTV replicates in PBMC that are frequently found in infected or

destructed tissues. Thus, the presence or variation of loads of TTV DNA in tissue samples that contain PBMCs may not be direct causal evidence for a disease. At the site of infection/tissue destruction, it is also possible that the TTVs in dying cells begin to replicate in order to rescue themselves. Thereby it is possible that the TTV loads in patients can be higher as a secondary phenomenon, giving thus a statistical relationship without a causal relationship with the disease (Biagini et al., 2006b). Vaccination with a new antigen seems to boost TTV replication, suggesting that TTV loads could be increased due to co-infection with other pathogens (Maggi et al., 2005b). TTV infections with multiple genotypes, as well as low and fluctuating levels of viremia, make the discovery of potential disease associations with the currently existing methods extremely difficult. PCR systems for detecting, differentiating, and quantifying all the genotypes and/or genogroups should be set up, as should methods to detect past infections and immunological responses to anellovirus infections.

AIMS OF THE STUDY

In 1998 when this study began, no information existed on the worldwide prevalence, cell biology, or the immunology of the newly detected virus, TTV. We wanted to answer these questions by setting these aims for our study:

- To reveal the prevalence of acute, past and persistent TTV infections in the Finnish population
- To clone (at least) one genotype in full length for cell biological studies
- To analyze the genome, transcription, proteins and replication of TTV
- To find the host cells for cell-biological research and for growth of the virus for diagnostic purposes
- To express all TTV proteins
- To reveal the immunology in TTV infections by studying B- and T-cell responses
- To set up diagnostic tests for TTV infections (DNA and antibody based tests)
- To seek possible disease associations

SUMMARY OF MATERIALS AND METHODS

This section briefly summarizes the materials and methods used in this thesis work. More detailed descriptions are found in the original publications (I-V). The nucleotide numbering in this thesis is according to the GenBank submission AY666122 (supplement 1).

Northern blot analyses, RNase protection assays, mRNA 3'- and 5'-end sequencing, 9-amino-acid hemagglutinin (HA)- and green fluorescent protein (GFP)-tagging of the proteins, Western blotting with α-HA antibody, confocal microscopy, and Southern analysis with radioactive probe were done by the collaborators, and are explained in detail in the original publications (III, IV).

1 SAMPLES

1.1 Patient samples (I, II, V)

Serum samples for study I were obtained from 89 non-symptomatic adults (80 of those were Finnish), of which 25 were used in study V. For study II, plasma, peripheral blood mononuclear cells (PBMC) and bile samples were obtained from 31 Finnish patients during abdominal operations in conjunction with cholecystectomy. Liver, plasma and PBMC samples were obtained from 3 Finnish patients with ulcerative colitis. Liver DNA preparations were obtained from 17 B19 DNA positive German patients. Skin, synovial and serum samples were obtained from 13 Finnish patients, synovial tissue from joint trauma patients. Adenoidal and tonsillar tissue samples were obtained from 6 patients with adenoidectomy and 10 with tonsillectomy for hyperplasia or chronic infection. More detailed descriptions of the patients are found in the original publications (I, II, V). Samples were collected from the patients with informed consent. The studies involving patient material were approved by The Ethical Review Committee of the Helsinki University Central Hospital.

1.2 Processing of the samples (I, II, V)

Sera were collected from coagulated blood. Bile samples were centrifuged for 2 minutes before DNA isolation from the supernatant. Plasma and PBMC were separated with Vacutainer Cell Preparation Tubes (Becton Dickinson), and the cells possibly remaining were removed from plasma by centrifugation, and PBMC were washed three times with PBS followed by re-suspension into PBS. All the samples were stored at -20°C until DNA isolation.

1.3 DNA isolation from clinical material and cultured cells (I, II, IV, V)

The total DNA for PCR studies (I, II, V) was isolated from the patient samples with proteinase-K treatment and phenol-chloroform extraction, was precipitated with Na-acetate and ethanol, and was re-suspended into water (except in study II the 17 German liver samples, see reference therein). The total DNA for Southern analyses (IV) was isolated from the transfected cells with phenol-chloroform method, or with QIAamp DNA Blood Mini Kit (Qiagen). The low molecular weight DNA was also isolated with the Hirt protocol (Hirt, 1967).

2 PCR

2.1 TTV PCRs and B19 PCRs (I, II, IV, V)

Universal-PCR (UTR-PCR) (I, II, IV, V): The highly conserved area of TTV, the untranslated region (UTR), was amplified (nt 88-230) by nested-PCR with the primers NG133, NG147, NG134 and NG132 (Table 1) (Okamoto et al., 1999b). PCR mixtures contained 200 μM each dNTP, 600 nm each primer, 1.5 mM $MgCl_2$, and 2.5 U AmpliTaq Gold polymerase. Amplification conditions for 35 cycles were as described by Okamoto and coworkers. Briefly, annealing was at 60°C, and elongation was for 40 seconds (Okamoto et al., 1999b).

N22-PCR (I): The N22 region within the ORF1 coding area was amplified (nt 1840-2169) by nested-PCR with the primers TT6, TT7, TT8 and TT9 (Table 1) (Höhne et al., 1998). PCR conditions were as in UTR-PCR, except that the annealing was at 42°C, and elongation was for 45 seconds.

Genotype-6 PCR (I, II, IV, V): The nested PCR, overlapping the N22 region, was designed (nt 1803-2200) to amplify HEL32-like sequences. The primer sequences are in the Table 1. Amplification conditions were as with UTR-PCR, except that the annealing was at 55°C for the outer PCR and at 52°C for the inner PCR.

B19 PCR (II): The nested-PCR for human parvovirus B19 (nt 2407-2797) was done with primers p6f, p3r, p8f and p5f (Söderlund et al., 1997).

The semi-quantification of the DNA amount was done by serial ten-fold dilutions of the DNA for the PCRs.

3 CLONING

3.1 Cloning of the PCR products (I, V)

The amplicons were isolated from PCR mixtures or from agarose gels, and cloned into the pSTBlue-1 AccepTor vector in *Escherichia coli* DH5α (I).

3.2 Construction of the full-length TTV plasmid clone (I, IV)

The 3381-bp region of the HEL32 isolate was amplified by PCR from the serum of a Finnish non-symptomatic female, and cloned in two overlapping pieces into the pSTBlue-1 AccepTor vector. A 3269-bp product (nt 111-3380) was amplified with nested-PCR using primers NG133, NG135, NG134 and NG136 (Table 1) (Okamoto et al., 1999b). The PCR reactions contained 200 μM dNTP, 320 nM primers, 1.5 mM $MgCl_2$, and 2.6 U of Expand High Fidelity enzyme mix (Boehringer Mannheim/Roche). The PCR programs consisted of annealing at 54°C and extension at 68°C for 2.5 min for the first 10 cycles, with 5 seconds/cycle added during each of the last 20 cycles. The PCR product was isolated from agarose gel, and cloned for sequencing into the pSTBlue-1 AccepTor vector in *E. coli* DH5α. The region of (-)1-220 nucleotides of HEL32 was amplified with semi-nested PCR using primers NG054, NG147 and NG132 (Table 1) (Okamoto et al., 1999b). The PCR program was that of UTR-PCR, except that annealing was at 50°C, extension was for 45 seconds and the cycles were repeated 30 times. The resulting 222-bp product was isolated and cloned for sequencing as the 3269-bp product (I).

To sequence the whole genome of the HEL32, and to clone the complete genome into a plasmid, the GC-rich region (nt 3206-216) was amplified from the original serum with semi-nested primers TTVGCF, TTVGCR1 and TTVGCR3 (Table 1). PCR reactions contained 200 μM dNTP, 400 nM primers, 1.5 mM $MgCl_2$, 10% dimethylsulfoxide, 1 M betaine and 17.5 U of Expand High Fidelity enzyme mix. PCR programs consisted of annealing at 55°C and extension at 72°C for 2 min for the first 10 cycles, with 5 seconds/cycle added during each of the last 20 cycles. The resulting 759 bp amplicon was isolated and cloned for sequencing as the 3269 bp product. For cloning of the entire HEL32 isolate a 396 bp product [nt (-)1-394] was amplified from the original serum with primers NG054 (Okamoto et al., 1999b) and TTVGCR6 (Table 1). With restriction enzyme digestions followed by ligations the three overlapping genomic parts were combined into pSTBlue-1 AccepTor vector that then contained the entire genome of the HEL32 isolate (named as pTTV), flanked by 175 bp areas.

3.3 Sequence analyses (I, IV, V)

Sequencing of the purified PCR amplicons and of the cloned constructs (cloned PCR amplicons, ORFs and TTV clone) was done at the sequencing core facility of Haartman Institute at University of Helsinki, Finland (I, IV). Sequencing of the cloned GC-rich region of HEL32 was done at the DNA sequencing facility of the Institute of Biotechnology at the University of Helsinki, Finland (IV).

For database searches the program Blast was used (NCBI) (I). For genomic organization analyses programs MapDraw (DNAStar) and DNAStrider were used (I). Multiple sequence alignments and evolutionary distance analyses were done with Pileup, GrowTree and Distance programs (I). Phylogenetic trees were constructed with the program TreePuzzle using the maximum likelihood approach, with 10 000 puzzling steps using the HKY model of substitution, and with the program Phylip using the neighbour-joining algorithm with 500 bootstrap replicates (references for these are found in the original publication I).

4 CELLS AND TRANSFECTIONS

4.1 Cell lines and cell culture (III, IV)

Altogether seven cell lines were used in these studies: Cos-1, Huh7, Chang liver, KU812Ep6, UT/Epo-S1, 293T and 293. Cos-1 cells (African green monkey kidney cells, transformed with SV40) were maintained in D-MEM with 10 mM HEPES Buffer, Huh7 cells (human hepatoma cell line) and Chang liver (human liver cell line) in MEM, and 293 cells (human kidney derived cell line) and 293T cells (expressing SV40 T antigen) in D-MEM. KU812Ep6 cells (human erythroid leukaemia cell line) (Miyagawa et al., 1999) were maintained in RPMI1640 with 6 U/ml of erythropoietin (Janssen-Cilag) and UT7/Epo-S1 cells (human erythroblastoid cell line) (Morita et al., 2001; Shimomura et al., 1992) were maintained in Iscove's modified Dulbecco's medium with 2 U/ml of erythropoietin. All cell lines were grown in the presence of 10% FBS and antibiotics, and at 37°C in 5% CO_2. Cells were monitored for the presence of TTV DNA by UTR-PCR and by genotype-6 PCR.

4.2 Aphidicolin treatment (IV)

For aphidicolin treatment of 293T cells, 2 μg/ml of aphidicolin (Sigma) was added to the growth medium. The cells were transfected in the presence (calcium phosphate), or in the absence (lipofection) of aphidicolin, and grown further in the presence of aphidicolin. The non-treated 293T cells were included as controls (IV).

4.3 Transfection (III, IV)

The adherent cells (Cos-1, Huh7, Chang liver, 293T and 293) were transfected with 5 µg of DNA per 60 mm^2 plate at approximately 95% confluence on day 1 post subculture. For lipofection, the amount of Lipofectamine2000-reagent was optimized for each cell line (the amounts used are found in publication IV). 293 and 293T cells were also transfected by the calcium phosphate technique.

Cells growing in suspension were transfected at the exponential growth phase (days 3-4 post subculture). For KU812Ep6 cells the conditions of electroporation were optimized; $2x10^6$ cells were transfected with 5 µg of DNA by electroporation at 300 or 350 V, and 960 µF. For transfection of UT7/Epo-S1 cells the Amaxa Nucleofector system was used with Kit R and program T-24 (Amaxa Biosystems).

For study III, the localization of the GFP-constructs was analyzed in transfected 293T and HuH7 cells. For study IV, the pTTV was constructed so that the complete genome with overlaps was inserted between *Eco*RI sites in the pSTBlue-1 AccepTor vector, and that *Bsp*EI could be used to excise in a single unit the complete TTV genome (without overlaps) in linear form. The cells were transfected with two forms of cloned TTV genome: an uncut plasmid clone, pTTV, containing overlaps, and a linear construct, linTTV (full-length genome without overlaps, excised with *Bsp*EI).

The transfection efficacy in each experiment was optimized and observed with the pEGFP-Luc vector encoding the green fluorescent protein (GFP), and the percentage of transfected cells was estimated with a fluorescence microscopy on day 1 post transfection (III, IV).

4.4 Microscopy (III, IV)

The GFP signal in the transfected cells was observed with fluorescence microscopy (III, IV).

5 HYBRIDIZATION-BASED ANALYSES

5.1 Dot blot assay (I)

The products of UTR-PCR were confirmed by a dot-blot assay. The probe for hybridization was prepared by PCR from the cloned HEL32 isolate. The probe was labelled with the inner UTR-PCR by incorporating digoxigenin-11 dUTP. The products of genotype-6 PCR were confirmed also by the dot-blot assay. The probe was prepared similarly to the previous probe, but with the inner genotype-6 PCR.

5.2 Southern hybridization (IV, V)

For replication assays the total DNA isolated from the transfected cells was digested with *Bam*HI and *Dpn*I, separated by gel electrophoresis and transferred to a nylon membrane (Hybond-N+, Amersham Biosciences). The membranes were hybridized with a DIG-labelled PCR-probe covering nucleotides 1803-2200 (prepared with the outer genotype-6 PCR) (IV). For replication assays with radioactive label, the ^{32}P-labelled probe was prepared to cover nucleotides 2788-3348 (560 bp) of the untranslated region (IV).

For circularization experiments total isolated DNA was digested with various enzymes, separated by gel electrophoresis and transferred to the nylon membrane. The membranes were hybridized with a DIG-labelled probe prepared by nick translation using DIG-Nick Translation Mix (Roche) to cover the entire TTV genome (IV).

To confirm the genotype-6 PCR and dilution series results, the obtained amplicons in the gels were transferred to the nylon membrane and were hybridized with the genotype-6 PCR generated probe (V).

For the hybridization procedures involving a DIG label, the membranes were hybridized at 42°C overnight. The DIG label was detected with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) forming a coloured precipitate (I, IV). For the hybridization procedures involving a ^{32}P-label, the signal from the hybridized probe was detected by film exposure (IV).

6 RNA WORK

6.1 RNA isolation and RT-PCR (IV)

The cells for RNA analyses were collected post transfection and total RNA was isolated with TRIzol Reagent (Invitrogen Life Technologies). When necessary, residual DNA was removed with DNases. For RT-PCR, primers RT1F and RT1R (Table 1) were designed to flank the common 101 bp spliced intron (Qiu et al., 2005). This separated the amplicons originating from DNA and RNA by size. RT-PCRs were done with RobusT II RT-PCR Kit (Finnzymes) in the presence of an RNase inhibitor. The PCR program consisted of reverse transcription at 42°C for 30 minutes, followed by 35 cycles of PCR with annealing at 65°C and elongation at 68°C for 45 seconds. RT-PCR for retinoblastoma mRNA was done with the primers published previously (Brunstein et al., 2000).

7 PROTEIN EXPRESSION

7.1 Cloning of ORFs for expression systems (I, V)

Cloning of ORF1, ORF2a and ORF2b (I): In-frame primers (Table 1) with restriction sites (underlined in Table 1) were designed for each ORF. The reverse primers were located downstream of the stop codons. ORFs were amplified from the 3269-bp HEL32 plasmid-clone, and the agarose-purified amplicons were cloned in-frame to GST (glutathione S-transferase) into a pGEX-4T-1 expression plasmid (Amersham Pharmacia) in *E. coli* DH5α.

Cloning of all six cDNAs (V): In-frame primers (Table 1) were designed for all six ORFs or cDNAs. Those were amplified either with PCR from the existing clones, or with RT-PCR from the RNA isolated from pTTV-transfected cells. For PCR amplification either Ampli Taq Gold or Expand High Fidelity PCR System, and for RT-PCR either OneTube Titan RT-PCR System (Roche) or RobusT II RT-PCR Kit (Finnzymes), was used. The purified amplicons were cloned in-frame to GST into a pGEX-4T-1 expression plasmid, and in-frame to GST-6xHis into pAcGHLT-A Baculovirus Transfer Vector (BD Biosciences) in *E. coli* DH5α.

7.2 Expression of proteins in *E. coli* (I, V)

Cloned ORF amplicons from pGEX-4T-1 expression plasmids were expressed as GST fusion proteins in *E. coli* strain BL21 (Amersham Pharmacia). Arginine-rich proteins were expressed also in strain Rosetta (Novagen). For the expression of each construct, growth medium (L-Broth, YT or Terrific Broth), IPTG (isopropyl β-D-1-thiogalactopyranoside) concentration, IPTG induction time, and induction temperature were optimized.

7.3 Expression of proteins in insect cells (V)

The pAcGHLT-A Baculovirus Transfer Vectors containing the cloned cDNA constructs were purified from bacteria with GenElute Endotoxin-free Maxiprep Plasmid Purification Kit (Sigma), and transfected together with Linearized Baculovirus DNA (BD Biosciences) into *Spodoptera frugiperda* (Sf9) insect cells. For large scale protein production, the recombinant baculoviruses produced by Sf9 cells were used to infect either Sf9 and/or *Tricoplusia ni* (HighFive) insect cells.

8 PROTEIN AND ANTIBODY ANALYSES

8.1 SDS-PAGE and immunoblotting (I, V)

To analyze the expressed proteins, the proteins of the whole cell lysates were separated by a 10% SDS-PAGE and the proteins were visualized by staining with Coomassie stain (I).
For immunological detection, the proteins were transferred from the SDS-PAGE to a Protran nitrocellulose membrane (Schleicher & Schuell). The membranes were blocked with 5% fat-free milk powder and 0.2% Triton X-100 in PBS. For immunodetection, primary antibodies [goat α-GST (I), mouse α-GST (V), human sera (I)] and HRP (horseradish peroxidase)-conjugated secondary antibodies [rabbit α-goat IgG (I), rabbit α-mouse IgG (V), rabbit α-human IgG (I, V), rabbit α-human IgM (I)] were diluted with blocking liquid into appropriate concentrations (dilutions are in the original publications I and V).

The bound antibodies were detected with hydrogen peroxide and DAB (3,3'-diaminobenzidine). For IgM detection, to avoid possible interference by rheumatoid factor, the IgG was removed from sera with Gullsorb (Meridian Diagnostics) (I).

8.2 B19 serology (II)

The second-generation epitope-type enzyme immunoassay (IgG-ETS EIA) was used for analyzing the B19 infection status in certain patients. Briefly, acute and past B19 infections were diagnosed by calculating the ratio of IgG binding to conformational and linear epitopes (Kaikkonen et al., 1999).

Table 1. Primers for TTV used in this study. Restriction sites are underlined, translation initiation sites and stop codons are marked with capital letters. In primers used for cloning of ORFs the numbering of nucleotides is according to those corresponding AY666122, and thus excludes restriction enzyme sites and 5' sequences from them.

Name of the primer	5' – 3' sequence	Nucleotide position according to AY666122	Reference
NG133	gtaagtgcacttccgaatggctgag	88-112	Okamoto et al., 1999
NG147	gccagtcccgagcccgaattgcc	230-208	Okamoto et al., 1999
NG134	agtttccacgcccgtccgcagc	111-133	Okamoto et al., 1999
NG132	agcccgaattgcccctgac	220-201	Okamoto et al., 1999
TT6	acagacaggaggaaggcaa	1840-1859	Höhne et al., 1998
TT7	taccayttagctctcatt	2169-2152	Höhne et al., 1998
TT8	aacatgytatggatagactgg	1859-1879	Höhne et al., 1998
TT9	ctggcatttaccatttcca	within 1840-2169	Höhne et al., 1998
Gen6 PCR outer forward	cccctggagcataccaag	1803-1820	I
Gen6 PCR outer reverse	catgacctctagctggtggaac	2200-2179	I
Gen6 PCR inner forward	cagtatactcagaaaagcaaag	1896-1917	I
Gen6 PCR inner reverse	catttccacctcattcttatagg	2166-2144	I
NG135	gcggctgtgacgtcactggaacgg	3389-3366	Okamoto et al., 1999
NG136	acgtcactggaacggccatttgg	3380-3357	Okamoto et al., 1999
NG054	ttgctacgtcactaaccac	(-)1-18	Okamoto et al., 1999
TTVGCF	cagactccgagatgccattg	3206-3225	IV
TTVGCR1	cgaattgccccttgactg	216-199	IV

Name of the primer	5' – 3' sequence	Nucleotide position according to AY666122	Reference
TTVGCR3	gggatcacccttcgaggt	157-140	IV
TTVGCR6	cgttcgagttgggttccatt	394-375	IV
RT1F	gcagcggcagcacctcgaa	129-147	IV
RT1R	gtctagcaggtcctcgctgcgag	683-660	IV
ORF2a forward	cgtggatccgctgagtttccacgcccgtc	107-127	I
ORF2b forward	tcggaattcATGggcaaggctcttag	237-253	I
ORF2a reverse/ORF2b reverse/2R	tctaataaaggcggccgcccactg	802-797	I, V
noARG	tttgaattcATGcgcagacacagaaaaaaac	767-785	V
RepCR	tttcccgggTTAtgcatgggaagatagt	2791-2773	V
3F/ORF1 forward	tcggaattcATGgcctggtactggt	581-596	I, V
O1aSTOP	tttcccgggTTAtccttgggacaggaatatg	1786-1768	V
O1endATG	tttgaattcATGaacacatggtacagaggcaatg	1613-1634	V
Nsf	ttttgaattcATGtggcagccacctacccag	354-374	V
Ns1rkor	tttcccgggttaaacataaagaccttgtt	2810-2791	V
ORF3srkorj	ctggagaaagtgtaagagcatctg	2504-2481	V
ORF3sf	gccgcagaatcgtccgac	696-703 -> 2314-2324	V
Ns2r	tttcccgggTTAatgaaagccaagtttg	2979-2964	V
3R/ORF1 reverse	gctttgggcagcggccgctatgtgg	2921-2915	I, V
RepCF	tttgaattcaacacatggtacagaggcaatg	1613-1634	V
noARG2	tttgaattcATGcgcgctcgccgcaga	689-703	V

RESULTS AND DISCUSSION

1 EPIDEMIOLOGY OF TORQUE TENO VIRUS

1.1 TTV DNA prevalence in sera (I, II)

In the beginning of this study, in 1998, it was not known how common TTV was in Finland. Thus, we initiated our TTV research by testing, with two PCR methods (i.e. N22 and UTR PCRs) (Höhne et al., 1998; Okamoto et al., 1999b), the TTV-DNA prevalence in healthy adults in the Finnish population. By the UTR-PCR (amplifying the majority of TTV genotypes) 74/87 (85%) of the subjects were TTV-DNA positive, and additionally, two subjects became TTV DNA positive during the follow-up. By the N22 PCR (amplifying genotypes 1-6), 14/80 (18%) of the subjects were TTV DNA positive. Our results were in accordance with the results presented concurrently by Simmonds and coworkers, showing a 73% DNA prevalence among Finnish blood donors (Simmonds et al., 1999), and in line with others showing high TTV DNA prevalences all over the world.

In our subsequent studies we focused especially on TTV of genotype 6. By genotype-specific PCR we observed genotype-6 DNA prevalence in sera/plasma of the subjects studied to be in the order of 4% (2/47) – 8.6% (3/35). In the literature the first reference to genotype 6 is from Tanaka and coworkers identifying genotype-6 DNA in serum from a Japanese patient (Tanaka et al., 1998b). Subsequently, genotype-6 isolates have also been detected in nasal and blood samples in an Italian study (Maggi et al., 2003b), in saliva and PBMC of Chinese patients (Chan et al., 2001a), in serum of a Japanese hemophiliac (Takayama et al., 1999b), in serum of a Hungarian hepatitis patient (Takacs et al., 2003), and in serum of a chimpanzee that was used in hepatitis research (Verschoor et al., 1999). In addition, genotype 6 has been shown to establish persistent infection in PBMC and persistent viremia in plasma (Maggi et al., 2006).

1.2 TTV DNA and genotype-6 DNA in human tissues (II)

To analyze the tissue prevalence of TTV DNA and genotype-6 DNA, we studied various tissue samples with TTV-specific PCRs. The prevalence of another ssDNA virus, human parvovirus B19, was included as a reference. Table 2 shows the results of viral DNA prevalences in various tissues.

Table 2. TTV DNA, genotype-6 DNA and human parvovirus B19 DNA prevalences in tissues of various patients (more detailed descriptions of the patients are in publications I, II, V).

	TTV	Genotype 6	B19	Relative titers of TTV DNA
Finnish surgical patients				
Bile	24/31 (77%)	1/31 (3.%)	0/31 (0%)	10^{0}-10^{-3}
PBMC	28/31 (90%)	0/31 (0%)	NT*	10^{0}-10^{-4}
Plasma	29/31 (94%)	1/31 (3%)	NT	10^{0}-10^{-3}
Liver samples from Finnish patients				
Liver	3/3 (100%)	NT	NT	10^{1}-10^{-4}
PBMC	3/3 (100%)	0/3 (0%)	NT	10^{0}-10^{-3}
Plasma	2/367%)	0/3 (0%)	NT	10^{0}-10^{-1}
Liver samples from German patients				
Liver	17/17 (100%)	1/17 6%)	15/17 (88%)	10^{0}-10^{-3}
Skin and synovial samples from Finnish patients				
Skin	5/13 (38%)	1/13 (8%)	8/13 (62%)	10^{0}-10^{-1}
Synovia	5/13 (38%)	0/13 (0%)	6/13 (46%)	10^{0}-10^{-1}
Serum	11/13 (85%)	1/13 (8%)	NT	10^{0}-10^{-3}
Adenoidal and tonsillar samples from Finnish patients				
Adenoid	6/6 (100%)	0/6 (0%)	1/6 (17%)	10^{0}-10^{-5}
Tonsil	10/10 (100%)	0/10 (0%)	3/10 (30%)	10^{0}-10^{-5}

*NT = not tested

Plasma, bile and PBMC of 31 Finnish surgical patients were studied by UTR-PCR, and TTV DNA was found simultaneously in all three sample types in 24/31 (77%) of the subjects. The remaining seven patients had no detectable TTV DNA in bile, yet three were positive in plasma and in PBMC, two in plasma, one in PBMC, and one was negative for TTV DNA in all three sample types. Genotype-6 DNA was detected in one patient, both in bile and in plasma, but not in PBMC. All the bile samples were negative for our reference virus B19 DNA, while 45% of the patients were seropositive for IgG against B19. Liver samples from 3 Finnish and 17 German patients were all found to be TTV DNA positive. One German liver sample contained genotype-6 DNA. B19 DNA was found in 15/17 (88%) of German liver samples, including the genotype-6 positive one. TTV DNA was detected in 38% of skin samples and of synovial samples. Two subjects had TTV DNA in both skin and synovia, and six in either sample type, all of those being TTV DNA positive also in sera. One subject had genotype-6 DNA in skin and in serum. Those that were B19 DNA positive in tissue, had past immunity to B19. TTV-DNA was found in all the adenoidal and tonsillar samples. None of the samples contained genotype-6 DNA, and 25% had B19 DNA.

To summarize our results, we detected TTV DNA and genotype-6 DNA in a number of different tissues. The relative viral DNA titers did not vary significantly, and there was no tissue or symptom specificity in association with genotype-6 infection. These results, and those of others, demonstrate that genotype 6 is spread worldwide, and can be found in various tissues and/or cell types. In agreement with our results, TTV DNA has been amplified from bile, PBMC, skin and liver (Jiang et al., 2000; Okamoto et al., 1999a; Okamura et al., 1999; Osiowy et al., 2000; Rodriguez-Inigo et al., 2000; Ukita et al., 1999; Yamamoto et al., 1998; Yu et al., 2002), while the presence of TTV DNA in synovia, adenoids and tonsils has not been studied by others. In fact, TTV DNA has been detected by PCR in nearly all tissues studied. However, the occurrence of TTV DNA does not necessarily reflect productive TTV infection in the particular tissue, but instead could either indicate the presence of TTV in some tissue-invading cells, or silent persistence of TTV DNA in those tissues. The latter scenario has been confirmed with human parvovirus B19, that has been shown to persist in tissues for decades (Norja et al., 2006). The high prevalence of TTV per se is not an uncommon phenomenon among viruses, for example, papillomaviruses and herpesviruses are frequently found in healthy individuals. However, TTV differs from all the other viruses known with its ability to sustain viremia, i.e. to actively replicate and produce viruses into blood continuosly for years or decades in healthy individuals.

2 CELL BIOLOGY OF TORQUE TENO VIRUS

In order to study the cell biology of a virus, identification of host cells sustaining viral growth is essential. In addition, an infectious plasmid clone capable of producing viruses and easy to manipulate would greatly assist the research. We approached the cell biology questions in the TTV field by cloning the TTV genotype 6 for use in cell biological assays.

The research on several viruses has been greatly assisted by infectious plasmid clones. For example the replication of CAV and PCV have been extensively studied with infectious clones and with permissive cell lines (Claessens et al., 1991; Meehan et al., 1997; Meehan et al., 1998; Noteborn et al., 1991). The plasmid clones are easy to grow, can be manipulated in bacteria (e.g. mutated at specific places, genome parts changed between isolates etc.), and can be introduced into cells of any type by transfection, thereby by-passing the need for virus receptors. The plasmid clones can be used to study a variety of virus functions; e.g. phosphatase functions of the CAV protein were successfully revealed by mutating the infectious clone (Peters et al., 2005; Peters et al., 2006). In addition, with infectious plasmid clones, progeny virions can be produced. However, even though the plasmid clone would not be infectious, several features of the virus can still be studied, as is shown in this thesis with a full-length clone of TTV.

2.1 Cloning and phylogenetic analysis of the HEL32 isolate (I, IV)

To investigate the coding capacity and the cell biological issues of TTV, we amplified, with previously published primers (Okamoto et al., 1999b), the complete coding region of a TT-virus from one TTV-DNA positive serum. The coding region was amplified in two overlapping PCR products (3269 bp and 222 bp) that were cloned and sequenced. The cloned TTV was found to be 3381 bp in length, and to diverge by more than 40% from the prototype TTV, isolate TA278 (GenBank #AB017610). When we compared the ORF1 sequence of the clone to 29 TTV isolates published (Okamoto et al., 2001a), our clone was found to be unique. By comparison of the N22-area to the 42 TTV sequences (representing 20 genotypes) (Muljono et al., 2001), our clone was closely related to the representative of genotype 6 (Fig. 9), of which only one sequence of 222 bp had been deposited into GenBank (#AB017777). We named our genotype-6 isolate as HEL32 (I).

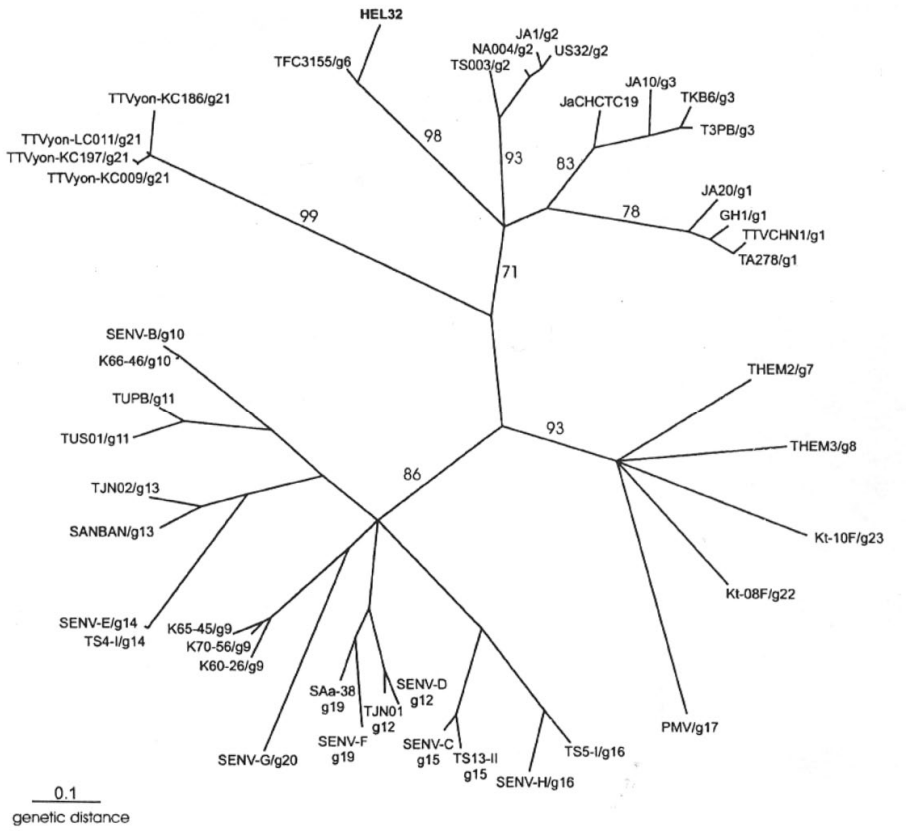

Figure 9. A phylogenetic tree based on the N22 sequence of TTV isolates. Numbers indicate the support values. Names and genotypes of the isolates are indicated. (Reproduced from Kakkola et al., 2002, with permission.)

Subsequently, after successful amplification of the GC-rich region, a full-length clone of the HEL32 was completed (IV). The complete genome of the HEL32 isolate was found to be 3748 nt in length, containing the GC-rich area of 107 nucleotides; this was the first full-

length sequence reported for genotype 6. The full-length clone of genotype 6 was constructed in such a way that it could be used for transfection experiments in two forms: an intact plasmid pTTV containing 175 bp overlapping areas at both ends, which would potentially aid in the recombination rescue of the viral genome from the backbone plasmid; and a linear *Bsp*EI-excised, genome-sized form containing the 3748 nt TTV genome (Fig. 10). A similar full-length clone, with overlapping ends, had been constructed from genotype 1, and used successfully in the investigation of TTV RNA transcription (Kamahora et al., 2000).

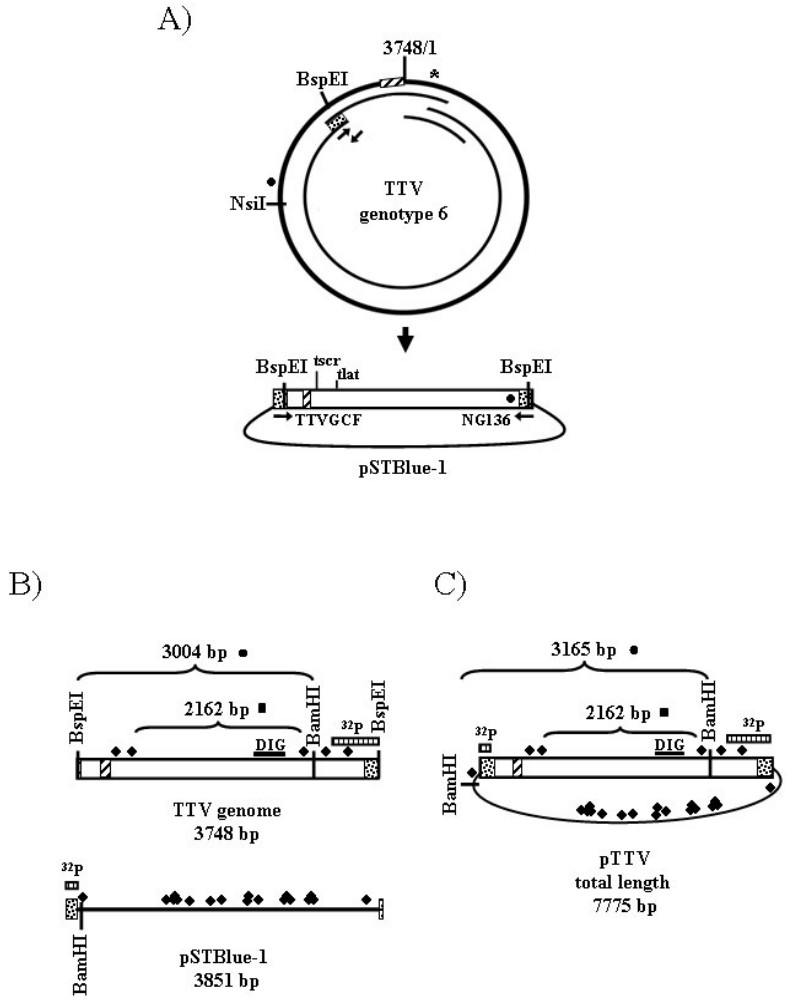

Figure 10. The full-length clone of HEL32 and the constructs used for transfection experiments. A) The clone was constructed from three overlapping PCR products (inner black lines). B) The excised linear construct and C) the intact plasmid construct were used for transfection experiments. The GC-rich region is shown as striped area, the TATA-box with *, poly-A with a black dot, primers with arrows, and *Dpn*I restriction enzyme sites with diamonds. The sites for DIG- and ^{32}P-probes are indicated with bars. The predicted sizes of products with *Bam*HI and *Dpn*I based replication analyzes are indicated (the same symbols are used in Figure 11 for Southern analysis). (Reproduced from Kakkola et al., 2007, with permission.)

2.2 Analysis of the HEL32 genome (I, IV)

The genomic organization of our genotype 6 isolate was found to be similar to that of other TTVs: a GC-rich region in the non-coding area, and putative ORFs surrounded by a TATA-box (ATATAA, nt 82-87) and by a poly-A sequence (ATTAAA, nt 2978-2983). Sequence analysis of the coding area of the HEL32 isolate revealed at least three possible open reading frames (ORFs). Sequence comparison with 41 TTV isolates showed that in 34 a similar genomic organization was seen as in our HEL32, i.e. ORF2 in two parts, ORF2a and ORF2b, while seven representatives of genotype 1 had ORF2 singly. ORF2a of HEL32 was predicted to encode for 49 amino acids, and ORF2b 156 amino acids. The uninterrupted ORF2 and the spliced ORF2b products contain the amino acid motif $WX_7HX_3CX_1CX_5H$ that is found in all TTVs and in the CAV VP2 protein sequence (Hijikata et al., 1999b). We found the ORF2a to be highly conserved in all isolates (divergence at the amino acid level to TA278 was 12%), whereas the ORF2b showed higher divergence (>50%). All TTV genotypes, including our genotype 6, as well as TTMVs and TTMDVs/SAVs, contain one large ORF1 encoding a protein with an arginine-rich N-terminus. The ORF1 of our HEL32 was predicted to encode a 736 amino acids protein, however, we found the ORF1 area among the 41 TTV isolates to vary in length, and identified also stop codons within the ORF1. The ORF2/3 protein of our isolate had a conserved amino acid motif, $E\text{-}X_8\text{-}R\text{-}X_2\text{-}R\text{-}X_6\text{-}P\text{-}X_{12\text{-}19}\text{-}F\text{-}X_1\text{-}L$ (unpublished), that is found in the C-terminus of the same protein among other TTV isolates (Okamoto et al., 2000b; Peng et al., 2002).

2.3 RNA transcription and DNA replication (IV)

In order to examine the cell biology of TTV infection, we used our full-length TTV clone for studies of RNA transcription and DNA replication. Prior to the studies, the cell lines (Cos-1, Huh7, Chang liver, KU812Ep6, UT/Epo-S1, 293T and 293) were confirmed by PCR to be free of endogenous TTV DNA and of genotype-6 DNA.

We detected TTV mRNA production in all seven cell lines after transfection with either pTTV or the linear construct. TTV DNA replication, indicated by *Dpn*I-resistant TTV DNA forms, was shown to occur in 293T, 293, Huh7, UT/Epo-S1 and Chang liver cells (Fig. 11). TTV DNA replication was not detected in Cos-1 or KU812Ep6 cell lines. Interestingly, the 293T cells showed more pronounced replication than 293 cells. The 293T cells express a large T antigen-protein of a polyomavirus, simian virus 40 (SV40). This multifunctional protein binds to the cell-cycle control proteins pRb and p53, and inactivates them. Subsequently, the cells enter the S-phase more readily and the SV40 genome replication can proceed (Skern, 2006; Sullivan et al., 2002). Likewise, the replication of our

TTV clone could have been promoted by the rapidly dividing 293T cells, which would explain the superiority of the 293T cells over the 293 cells in the replication of TTV DNA.

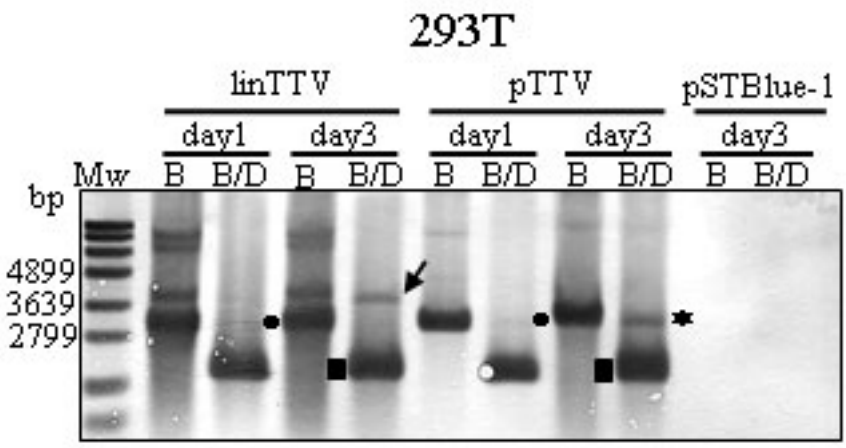

Figure 11. Southern analysis of TTV replication in 293T-cells transfected with linear (lin) and plasmid (pTTV) constructs. Replication was analyzed on days 1 and 3 post-transfection. The arrow and the asteriks indicate *Dpn*I-resistant and replicating TTV DNA. Other symbols indicate the input TTV-DNA. Compare to Figure 10, which schematically illustrates the DNA fragments obtained with the restriction enzymes used in the replication analysis. Cells transfected with the backbone plasmid (pSTBlue-1) were included as negative control. (Reproduced from Kakkola et al., 2007, with permission.)

Our results indicated that in the transfected cells the linear construct had circularized. Replicated forms of the linear construct were shown to accumulate with time, however, the *Dpn*I-resistant DNA declined after cell passage. After transfection with pTTV, replication of the entire plasmid in the transfected liver-, kidney- and erythroid-derived human cell lines was observed, however, without release of the TTV genome from the backbone plasmid. The replication experiments were confirmed with two probes (DIG and radiolabelled) and with two DNA isolation methods (isolation of total DNA; and isolation by Hirt method) in two laboratories (Helsinki, Finland; and Leiden, The Netherlands). Although we were able to demonstrate the replication of TTV DNA in the transfected cells, we were not able to show production of infectious progeny virions, which would be the ultimate proof of a clone being infectious. A similar, *Dpn*I restriction enzyme-based method was very recently used by Leppik and coworkers to show the replication of their full-length clone of a genogroup-5 TTV isolate in a Hodgkin's lymphoma cell line. In addition to DNA replication, they detected with electron microscopy virus-like particles in transfected cells. However, the infectivity of those particles remains to be shown (Leppik et al., 2007).

With PCR-based methods, others have shown replicating TTV DNA in several tissues, especially in PBMC, bone marrow and liver (Okamoto et al., 2000e; Okamoto et al., 2000f; Zhong et al., 2002). Furthermore, low-level TTV production, and infectivity of TTV, was shown in activated PBMC and in a few cell lines (Desai et al., 2005; Maggi et al., 2001b; Mariscal et al., 2002). Desai and coworkers documented in the replication-permissive Chang liver cells a cytopathic effect post-infection. In contrast to their results, we did not observe any cytopathic effect in our transfected cells supporting TTV DNA replication, not even in the Chang liver cells. Whether this is due to a potential lack of virus production is not known. In any case, the amount of TTV released from the infected Chang cells has been shown to be very low (Desai et al., 2005), suggesting that either the virus production is tightly controlled, or the Chang liver cells are not the optimal host cells for TTV infection.

2.4 Dependence of TTV DNA replication on cellular polymerases (IV)

Most small DNA viruses are dependent on the host cell replication machinery for virus replication. Thus, they either infect actively dividing cells (e.g. parvoviruses) or induce the cells to enter S-phase, and subsequently prevent apoptosis (e.g. papillomaviruses). We examined the host cell dependence of the replication of our full-length clone by blocking the cellular DNA polymerase with the drug aphidicolin. In the presence of aphidicolin, TTV DNA replication did not occur, indicating that TTV utilizes for genome replication the cellular replication machinery. Probably, as has been shown with other circoviruses, viral proteins are needed to interact with cellular proteins for the initiation of replication; however, with TTV these issues remain to be resolved.

Nothing else is known about the cell biology of TTV. Furthermore, little is known of the cell biology of animal circoviruses on the whole. PCV2 has been shown to enter the cells via clathrin-mediated endocytosis, the infection requiring the acidic environment provided by endosomes (Misinzo et al., 2005). This entry pathway is used for example by canine parvovirus (Basak et al., 1992; Vihinen-Ranta et al., 1998), and could also be used by TTV. However, these issues of TTV infection remain to be elucidated, hopefully in the near future.

3 TRANSCRIPTION MAP AND SUB-CELLULAR LOCALIZATION OF TORQUE TENO VIRUS PROTEINS

3.1 Transcription map of TTV genotype 6 (III)

By sequence analysis we identified three putative ORFs in our genotype-6 clone (I), however, the effect of splicing on the mRNAs was not yet known. With a genotype-1 full-length clone, Kamahora and coworkers showed that TTV produces three species of mRNA by alternative splicing (Kamahora et al., 2000). In order to study if similar splicing occurs with our genotype, and to obtain a more detailed transcription map, our full-length clone was transfected into 293 cells, and the produced mRNAs were analyzed.

Three species of mRNAs were identified (Fig. 12): an intron from nt 182 to nt 284 was spliced from all of them, and by alternative splicing a second splicing joined the common 5' splice site (2D, nt 703) to alternative 3' splice sites (2A1, nt 2315 or 2A2, nt 2505). Furthermore, with alternative translation initiation codon usage, altogether six putative proteins were predicted to be translated. From the 2.8 kb mRNA the full-length ORF1 (737 aa) could be translated. Splicing in the middle of ORF1-encoding mRNA created also an ORF cabable of encoding a shorter protein, ORF1/1 (199 aa), containing the N- and the C-terminal parts of the ORF1-encoded protein. For translation of protein ORF1/2, alternative splicing combined the 5'-sequence of ORF1 into a different reading frame, resulting in an ORF cabable of encoding a protein that has an N-terminus identical to that of the ORF1-encoded protein but with a C-terminus encoded in another reading frame.

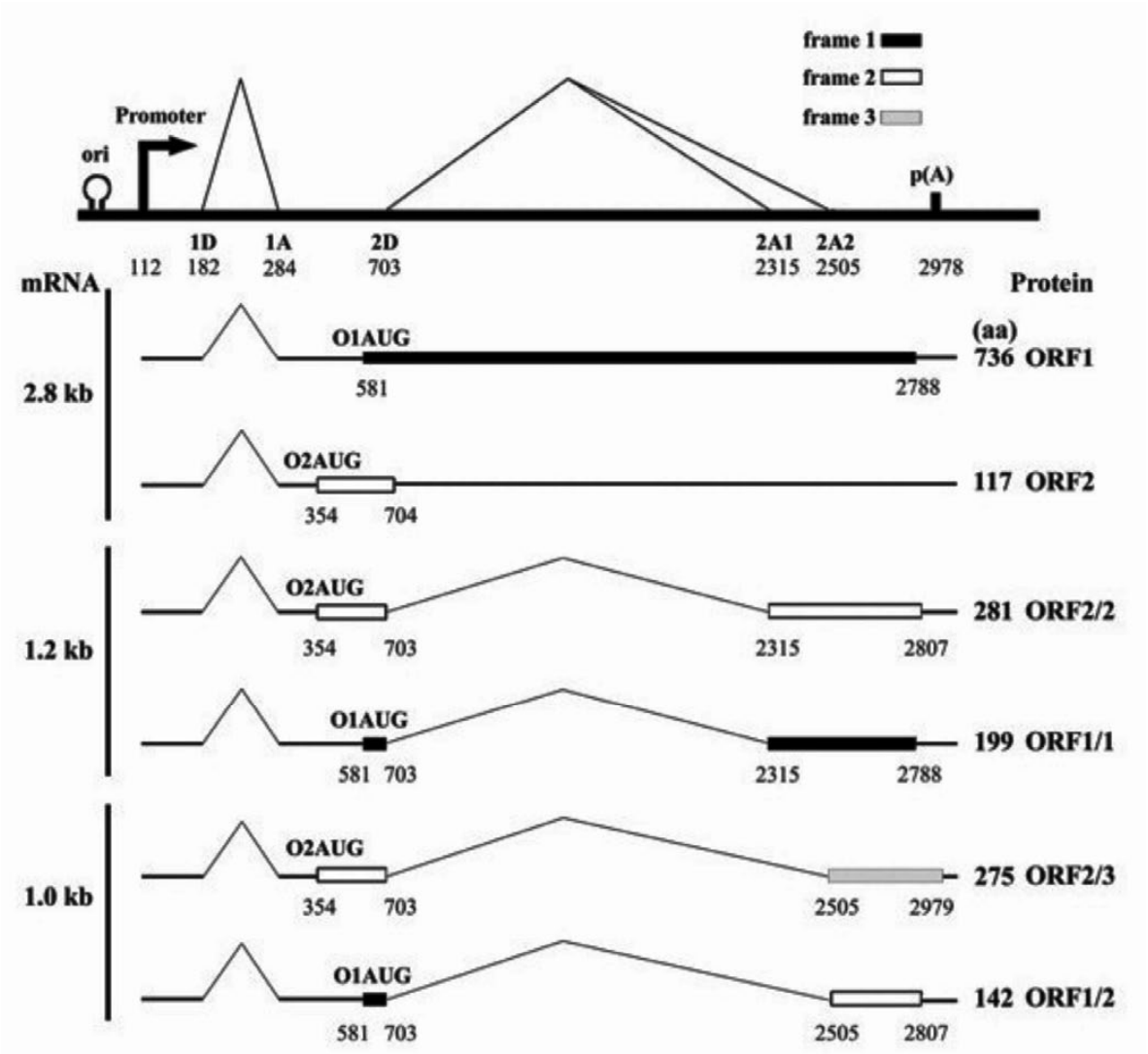

Figure 12. The transcription and translation map of TTV isolate HEL32. Translation initiation sites, splice junctions and poly-A sites are indicated with numbers. (Reproduced from Qiu et al., 2005, with permission.)

The ORF2-encoding region of several TTV genotypes has been shown to be interrupted by a stop codon dividing ORF2 into two parts: a smaller, more conserved ORF2a, and a larger, more variable ORF2b (Erker et al., 1999; Tanaka et al., 2000b). Our genotype-6 isolate also had a stop codon within ORF2, dividing it into ORF2a and ORF2b (I). Based on the transcription map of genotype 6 (III), ORF2a would not be expressed since the splicing removes ORF2a and also 39 amino acids from the N-terminus of ORF2b, resulting in an ORF2 protein that is 79 amino acids long. However, only 95% of the mRNAs were spliced at the first common intron deleting ORF2a (III), thereby leaving the remaining 5% of mRNAs with a potential to encode the ORF2a protein. Of note, the ORF2a sequence is

highly conserved among all TTV genotypes (I), and could serve some important function for the virus.

The ORF2 region is included also in the ORF2/2 and ORF2/3 encoded proteins: alternative splicing combines it into two reading frames, resulting in proteins with identical N-termini but different C-termini. The complete transcription map of genotype 6 is presented in Fig.12.

3.2 Translation and subcellular localization of the proteins (III)

Translation of the six TTV proteins within cells was verified by immunoblotting and by visualization of the HA or GFP-tagged proteins with fluorescence microscopy.

The ORF1-encoded protein was not detected by HA-antibodies in immunoblot, though its mRNA levels comprised over half of the total cellular RNA (for discussion, see 4.1). However, the ORF1 protein was detected as a GFP-tagged construct in the cytoplasm of the living cells, thus confirming its expression. ORF1 of TTV is predicted to encode a capsid- and replication-associated protein (Erker et al., 1999; Luo et al., 2002; Mushahwar et al., 1999; Takahashi et al., 1998; Tanaka et al., 2001). Like a similar protein in circoviruses, the TTV ORF1-encoded protein has an arginine-rich N-terminus (Hijikata et al., 1999b; Mushahwar et al., 1999; Okamoto et al., 1998b; Takahashi et al., 1998; Tanaka et al., 2001) that is supposed to be involved in DNA binding (Erker et al., 1999) and to contain putative nuclear localization signals (Bendinelli et al., 2001). Our GFP-tagged ORF1-encoded protein was localized in the cytoplasm (III), regardless of putative nuclear localization signals. However, when the protein was expressed from the cDNA, instead of the full-length clone, in 293T, HuH7 and 911 cells it localized into spot-like structures within the nucleus (our unpublished data). Of note, the cDNA construct was not mutated at the splice site, and thus in addition to the ORF1-encoded protein, it also was cabable to encode the ORF1/1 protein. It is known that viral and/or cellular proteins interact with and regulate the expression and localization of their associated proteins. For example PCV proteins show variable localization during infection: the Rep protein is consistently nuclear, while the Cap protein is first localized in the nucleoli and then dispersed throughout the nucleoplasm and the cytoplasm (Finsterbusch et al., 2005). It is probable that during TTV infection and protein production, interactions of viral proteins take place, affecting their subcellular localization, and possibly their function.

The ORF2-encoded protein translated from our full-length clone was localized in the cytoplasm of the transfected cells. The ORF2-encoded protein of TTMV has been shown to act as a phosphatase, proteins known to regulate mitogenesis, transcription, signal transduction, cell-cell interactions, cellular differentiation and cytokine responses of

lymphocytes (Peters et al., 2002). This activity is located within a motif in common with the ORF2 proteins of CAV, TTV, TTMV, and TTMDV/SAV. The same motif was also found in the ORF2-encoded protein of our HEL32 isolate (I), suggesting that it also may have phosphatase activity.

In addition to the ORF1- and ORF2-encoded proteins, four more proteins were confirmed to be produced in transfected 293 cells, from our genotype-6 clone: The ORF2/2 protein was localized in the nucleus, as was also the ORF2/3 protein, while the ORF1/1 and ORF1/2 proteins were distributed evenly in the cytoplasm and in the nucleus. The ORF3-encoded protein (corresponding to ORF2/2 of this study) of genotype 1a has at the amino acid level similarities to a non-structural protein of HCV (Asabe et al., 2001). However, nothing is known of the functions of the TTV proteins described.

It is possible that, in different cell types or under certain circumstances, mRNA splicing and protein production of TTV could be altered. As shown by Leppik and coworkers it is possible that even additional mRNAs and proteins could be produced by intragenomic rearrangements. Furthermore, they observed cell type-specific transcription of TTV mRNA, potentially leading to production of different mRNAs in different cell types (Leppik et al., 2007). It should be investigated whether the six proteins that are produced by our TTV clone, are produced also by other genotypes. For example the non-pathogenic PCV1 and the pathogenic PCV2 differ in the numbers of mRNAs produced (12 vs. 9, respectively) and in the splice junction selection, thus leading to transcription of both shared and unique mRNAs (Cheung, 2003a; Cheung, 2003b). Whether similar differences could exist between the TTV genotypes, and what their function would be, are interesting questions for further research.

Our results, demonstrating the translation of six proteins, have revolutionized the TTV proteomics. We have shown that instead of three to four proteins produced by overlapping reading frames and by splicing, the coding capacity of this small virus is even further expanded by alternative translation initiation. Apart from the localization, nothing is known of the functions of these proteins. Thus the transcription map, the full-length clone, and the cell lines analyzed in this thesis work open a new path for explorers of the functions of TTV proteins.

4 IMMUNOLOGY

The immunology of TTV infections is an intriguing issue. Since TTV establishes persistent viremia in healthy immunocompetent individuals, it must have effective mechanisms for evasion of the immune system. CAV of chickens is known to be immunosuppressive, and it has been speculated whether TTV also could have some effects on our immunity. In order to study the immunology of a virus, the viral proteins need to be produced for use as antigens in immunological assays. Our Study III revealed that the HEL32 isolate is capable of producing altogether six proteins, whereby we pursued to express them all.

4.1 Expression of TTV proteins in prokaryotes and in insect cells (I, V)

We cloned the ORFs in frame with GST, and expressed the GST-fusion proteins in *E. coli*. In addition, we cloned ORF1, ORF1ΔArg, ORF1-N, ORF1-C, ORF2/2, ORF2/3 and ORF1/1 (covering all the reading frames) for expression in insect cells.

The expression of a complete ORF1 protein was not successful (I, V), which could be due to its relatively large size, or to the arginine-rich N-terminus, or to cytotoxicity. Even the eukaryotic expression of ORF1 in 293 cells was noticed to occur at very low levels (III), suggesting that the ORF1 protein production is tightly regulated or that the protein is unstable, i.e. rapidly degraded. It is also possible that, to achieve proper folding, co-expression of other viral proteins is needed, as it is with the CAV capsid protein VP1, which for proper folding needs the function of the scaffold protein VP2 (Noteborn et al., 1998; Todd et al., 1990). Nevertheless, we were able to express ORF1 in two parts, as have others (Handa et al., 2000; Ott et al., 2000), and furthermore as an arginine-rich-region-deleted, near-complete (lacking only 62 amino acids from the N-terminus) construct (V).

The ORF2 proteins, both ORF2a and ORF2b, as well as the spliced ORF2, ORF2/2 and ORF2/3-encoded proteins were successfully produced in bacteria (the two latter also in insect cells) (I, V). The ORF2/2 and ORF1/2-encoded proteins are highly enriched in serine residues at their C-termini. Asabe and coworkers have shown that ORF3 (which equals to our ORF2/2) produced by TTV genotype 1a is expressed as two forms that differ in their phosphorylation state (Asabe et al., 2001). Similarly, we have noted two forms of the eukaryotically produced ORF2/2 protein in the immunoblots, which probably represent the differentially phosphorylated forms. Whether similar phosphorylation occurs on the ORF1/2-encoded protein is not known. In our expression studies, we noted that the arginine-rich N-terminus affects the expression of the proteins in bacteria: the expression level of the complete ORF1/2 was quite low, however, when the arginine-rich part was deleted, it increased dramatically. A similar phenomenon has been noted in the expression

of the BFDV capsid protein (Johne et al., 2004). On the other hand, the expression of ORF1/1 was not very successful, neither in the absence of the arginine-rich part nor in insect cells; the reason for this is not known. It is possible that some characteristic in the mRNA or in the protein itself makes it unstable or difficult to translate. It is also possible that with some other expression vector and/or some other fusion part, the expression could be more prominent, as has been shown with the PCV2 capsid protein (Liu et al., 2001).

Taken together, we were able to express all the proteins of TTV genotype 6. The expression of ORF1 as a complete protein was not successful, but the entire coding region became covered with partial constructs. This is the first time that all six proteins of TTV have been expressed in any system.

4.2 Detection of antibodies against the TTV proteins (I, V)

To study the prevalence of antibodies against the proteins expressed, we used them as antigens in immunoblotting, and screened sera of nonsymptomatic subjects for the presence of TTV specific antibodies.

Although ORF2a mRNA has not been detected (III), antibodies against the encoded protein were detected in human sera (I). We analyzed by immunoblotting, using the ORF2a and ORF2b products (fp2a and fp2b, respectively), serum samples of 89 subjects. We found strong IgM reactivities against fp2a in 2/87 (2%) subjects, and against fp2b in 6/87 (7%). Of these 8 subjects, 6 were TTV-DNA positive with UTR-PCR. Strong IgG reactivities against fp2a were not detected, whereas strong IgG reactivities against fp2b were detected in 9/87 (10%) of the subjects, who all had also TTV DNA in blood. Of the 63 TTV antibody-negative subjects, 55 (87%) had TTV DNA in serum. Of 35 selected subjects (17 seropositive and 18 seronegative), three had TTV DNA of genotype 6: two were IgG positive for fp2b and one was seronegative. For example of immunoblots done with human sera, see Figure 5 in I.

We also studied 25 human sera by immunoblotting for the presence of IgG antibodies against all the six TTV proteins (expressed in bacteria and/or in insect cells). Of the sera, 20/25 (80%) were TTV DNA positive, and 4/25 (16%) were genotype-6 DNA positive. Altogether 11/25 (44%) had a strong IgG responses and 5/25 (20%) had no responses for any of the proteins. However, no universal pattern in the protein-specific immune reactions could be seen: IgG antibodies against the ORF2 protein were detected in 6/21 (28.6%), against ORF2/2 in 1/19 (5.3%), against ORF2/3 in 5/21 (23.8%), against ORF1/1 in 1/18 (5.6%) and against ORF1/2 in 0/24 (0%) subjects. IgG antibodies against ORF1 were detected in 2/21 (14.3%) of the subjects, all of whom showed reactivity against the C-terminus. Ott and coworkers have also detected antibodies against the C-terminus of

genotype-1 ORF1, yet at a higher prevalence of 98.6% (Ott et al., 2000). Handa and coworkers have detected antibodies against the N-terminus of ORF1 of genotype 1b in 38% of blood donors in the USA (Handa et al., 2000). In contrast to this, we found no antibodies against the N-terminus of genotype 6 ORF1. The reason could be an immunological difference between the genotypes; for example genotype 1 could be more prevalent, could be more immunogenic, or could more readily cross-react with antibodies against other genotypes. However, no studies on genotype-specific antibody affinities nor on antibody cross-reactivities between TTV genotypes exist.

The relatively low IgG prevalence in general observed in our TTV studies could reflect that genotype 6 proteins elicit genotype-specific antibodies, and/or that some of them, e.g. intracellular non-structural viral proteins, are not regularly accessible to the immune system. In any case, the simultaneous presence of TTV DNA and antibodies indicates that the antibodies are not neutralizing and/or cross-protective. It remains to be investigated whether some of these proteins, or of their epitopes, could be used as general or genotype/genogroup-specific markers for TTV infections, i.e. as diagnostic tools.

4.3 Follow-up studies (I, V)

We retrospectively analyzed select subjects for the presence of TTV DNA and antibodies. In Study I, we had follow-up samples from four subjects, spanning 1.5 to 15 years of time (Table 3). Two subjects had stable TTV DNA and antibody status: strong IgM reactivity for fp2b and TTV DNA positivity. Two subjects had an altering phenotype: subject #1 showed strong IgM against fp2b for three years, and also strong IgG reactivity for fp2a throughout the 12-year follow-up, at the end of which she became positive for TTMV DNA; #97 was followed up for 1.5 years and he had fluctuating borderline reactivities for fp2b IgM and fp2b IgG, converting to positivity for TTMV DNA at the end of the follow-up period.

Table 3. Prevalence of TTV DNA and antibodies against two ORF2 proteins in four subjects followed for 1.5 to 15 years.

	TTV DNA	-				-	-	-			-	-		-		+			
	IgM/fp2a	-				-	-	-			-	-				-			
#1	IgM/fp2b	+				+	+	+/-			+/-	+/-		-		-			
	IgG/fp2a	+				+	+	+			+	+		+		+			
	IgG/fp2b	-				+/-	-	-			+/-	-		-		-			
	TTV DNA	+					+	+		+	+							+	
	IgM/fp2a	-					-	-		-	-							-	
#26	IgM/fp2b	+					+	+		+	+							+	
	IgG/fp2a	-					-											-	
	IgG/fp2b	-					-											-	
	TTV DNA	+	+					+	+								+		
	IgM/fp2a	-	-					-	-								-		
#55	IgM/fp2b	+	+					+	+								+		
	IgG/fp2a	-															-		
	IgG/fp2b	-															-		
	TTV DNA	-		-	+														
	IgM/fp2a	-		-															
#97	IgM/fp2b	+/-		+/-	-														
	IgG/fp2a	-		-															
	IgG/fp2b	+/-		-	-														
Time in years		0	0.5	1	1.5	2	3	4	5	6	7	8	9	10	11	12	13	14	15

The continued presence of TTV DNA in sera of healthy subjects is a well-documented phenomenon. However, others have shown, with different serological methods, the occurrence of short-lived IgM antibodies (Tsuda et al., 2001), as opposed to our long-lasting IgM. The reason for the longevity of the IgM detected in our study is not known, but one possibility could be impaired T-helper cell activity caused by T-cell cross-tolerance against multiple TTV genotypes.

In Study V, we had follow-up samples from four additional subjects. In two subjects no changes in virus DNA or in antibodies was seen: subject #2 was TTV DNA negative and seropositive for the ORF1, ORF1/1 and ORF1/2-encoded proteins; #80 was TTV-DNA negative and borderline seropositive for ORF1. Of the remaining two subjects, #86 had TTV DNA in serum through the follow-up of 13 years. She became genotype-6 DNA positive and sero-converted to IgG seropositivity for the ORF2-encoded protein in a sample taken two years later, and remained IgG seropositive thereafter. Interestingly, in the sample taken three years later, the genotype-6 DNA had disappeared. Subject #32 from whom the HEL32 clone originated, remained genotype-6 DNA positive throughout the follow-up, for at least 8 years. She was also continuously strongly IgG seropositive for the ORF1-encoded

protein. Both the sequences and the relative titers of genotype-6 DNA remained unaltered in both of the genotype-6 DNA positive subjects.

In conclusion, all the proteins were shown to be immunogenic. However, no correlation in antibody patterns between individuals was seen. Our follow-up results showed that 1) IgM against ORF2b can co-exist with TTV DNA for years, 2) superinfections with anelloviruses can occur regardless of pre-existing antibodies, 3) ORF1 antibodies and the DNA of the same genotype can co-exist for several years, and 4) the appearance of IgG against the ORF2-encoded protein occurred in conjunction with the disappearance of the DNA of the same genotype (for further discussion, see 4.4). In addition, the genotype-6 sequence remained unchanged throughout the follow-up, indicating that some mechanisms other than mutations could be used by TTV for immune evasion. However, it has to be kept in mind that immunoblotting tends to favour linear over conformational epitopes, and during the course of infection this proportion, i.e. the conformational dependence of antibodies, might change, as has been shown with human parvovirus B19 (Kaikkonen et al., 1999; Söderlund et al., 1995). Therefore the antibody responses detected in immunoblots could represent only a one time-window during the course of infection. Other techniques, such as ELISA, need to be developed to get the complete picture of antibody responses during TTV infections.

4.4 Immunological scenarios for TTV infections

The response to and the outcome of a virus infection depends on viral parameters such as cytopathogenicity, kinetics, cell and tissue tropism, host reservoirs, and susceptibility to other resistance mechanisms (such as interferons); as well as on the immune system including the specificity, kinetics and duration of humoral and cell-mediated immunity, in conjunction with the actions of the complement system, interleukins and phagocytes. For a virus it is optimal to achieve a clinically inapparent infection, or a balance between the host immune system and a tolerable disease state (Hilleman, 2004; Zinkernagel, 1996).

Another small DNA virus, human papillomavirus, is known to escape and control immune reactions in several ways: the innate immunity has difficulties in recognizing the virus because, for example, it is not cytolytic, it has no envelope, and it does not express proteins on the plasma membrane of the infected cells. Due to the difficulties in the initiation of an innate immune response, the adaptive immune response may remain defective also. However, a bacterial superinfection may activate the immune system to recognize also the papillomaviruses and the cytotoxic T cells to clear the viral infection (Skern, 2006). In general, the persistent viruses with low cytopathogenicity can evade or delay neutralizing B cell responses via 1) low numbers of activated B-cells, 2) alteration of the B-cell repertoire, 3) alteration of T-helper cell function, or 4) immunopathological changes in lymphoid

tissues (Hangartner et al., 2006). Perhaps TTV could use some or several of these mechanisms for evasion of the immune system.

Based on our and others' serological data it is evident that some form of immune responses against TTV occur in humans (Tsuda et al., 1999; Tsuda et al., 2001; I; V), and that TTV may occur in sera as immunecomplexes with IgG (Itoh et al., 2000), especially during persistent infection (Nishizawa et al., 1999). Interestingly, a superinfection with a new TTV strain has been shown to decrease the amount of immunecomplexes, followed by clearance of the new infection. The authors proposed that secondary, as opposed to persistent infections, might be more effectively cleared by preformed antibodies (Maggi et al., 2006). It has also been shown that over 90% of TTVs are cleared from the circulation and replenished by progeny viruses, thus indicating chronic active infection (Maggi et al., 2001c). In any case, frequent superinfections in healthy individuals with several genotypes suggest that (some) immune responses are neither neutralizing nor cross-protective.

The disappearance of genotype-6 DNA from the circulation after appearance of antibodies against the ORF2-encoded protein, which most probably is intracellular, was quite intriguing. It is tempting to speculate that the antibodies could clear the virus by helping in the destruction of the infected cells; whereas the humoral response against the ORF1-encoded putative capsid protein could be non-neutralizing and therefore unable to block, and eradicate, the viral infection. In addition, the antibodies could opsonise the virus for cellular intake. The low expression of the putative capsid protein in eukaryotic cells could furthermore represent one mechanism of immune response control; the less capsid protein is produced, the weaker is the antigen presentation. In addition, fragmented ORF1s may yield defective virus particles (Jelcic et al., 2004; Luo et al., 2002), which could exhaust the immune system. Although our results point to stability of the genotype-6 DNA sequence for years, it is possible that TTV evades the immune system also by mutating its critical epitopes on the capsid surface. Furthermore, transplacental transmission of TTV, potentially affecting self-nonself-discrimination, could also help the virus to avoid the immune response and to establish persistence. It is also possible that the brain and liver, expressing the HLA-I molecules in low levels, could avoid cell destruction by cytotoxic T cells and thereby could act as reservoirs for persistent TTV viremia (Griffiths, 1999).

As the closely related CAV is known to be immunosuppressive in chickens, TTV also has been investigated from this point of view. Maggi and coworkers have shown that the numbers of T cells decreased and B cells increased with increasing TTV loads, suggesting that TTV replication could cause immune imbalance (Maggi et al., 2003c). Zheng and coworkers have shown that TTV ORF2 protein blocks the NF-κB pathway, thus potentially suppressing immune responses and aiding in the establishement of persistent infection

(Zheng et al., 2007). In addition, CAV VP2 has been shown to cause down regulation of MHC-I class molecules on the plasma membrane of the infected cells, thereby reducing the presentation of viral peptides to the immune system. This function has been mapped to the dual specificity protein phophatase site of CAV VP2 protein (Peters et al., 2006). The same phosphatase activity has also been demonstrated in the TTMV ORF2 protein, a sequence present also in the ORF2 of TTV (Peters et al., 2002). Furthermore, whether infection of PBMCs by TTV exerts any effects on the virus-specific immunity remains to be studied.

TTV is a very small virus and does not possess the genetic capacity, as do for example herpesviruses, to encode a number of proteins for immune evasion. Nevertheless, TTV seems to have adapted very well to its host(s). This kind of co-existence and co-evolution is a very effective way for a virus to achieve its main goal: to replicate and spread. In order for us to achieve a full picture of TTV and its interplay with our immunity, we must produce and analyze proteins of also other genotypes for cross-reactivity, for conformational and linear epitopes, and for antibody isotypes and affinity/avidity. In addition, the role of T-cell function in TTV infections needs to be elucidated.

5 TORQUE TENO VIRUS: A PATHOGEN, A CONSTITUENT OF THE NORMAL FLORA, OR BOTH?

It is obvious that TTV infections are extremely prevalent even in healthy individuals, and no definitive causal association with TTV infection has been found for any of the diseases investigated. However, it should be noted that due to the lack of tools for immunological studies TTV research has focused mainly on detection of TTV DNA; and due to sub-optimal PCR methods, the detection and identification of many of the anelloviruses has not been possible.

Viruses can cause diseases by a number of different mechanisms: directly by cell destruction, or by inducing genetic or cellular alterations, and indirectly by interfering with the immune system. It has been said that viruses are guilty until proven innocent, and TTV, let alone the other human anelloviruses, certainly have not yet convinced the jury. It is possible that some genotypes of TTV are pathogenic, as is the case with PCV and HPV. In addition, many long-term chronic microbial infections are known to predispose to chronically evolving diseases, a phenomenon which shall be extremely difficult to elucidate with such a ubiquitous virus as TTV.

However, it is also possible that TTV is a genuinely harmless bystander with no adverse effects whatsoever on human health (Griffiths, 1999; Simmonds et al., 1999). Pathogens of the normal flora (typical for bacteriology) do not induce disease in every individual

infected. On the other hand, as with opportunistic pathogens the disease appears only under exceptional circumstances. In some virus infections the viral load is a critical determinant for development of the disease. It has been suggested that TTV could be a commensal in normal conditions not capable of exceeding the threshold of a disease-causing load (Griffiths, 1999). Commensal bacteria live on our surfaces, whereas viruses invade our body and our cells. Therefore the conclusion that anelloviruses are endosymbionts is justified (Mushahwar et al., 2001). However, for genuine symbiosis the virus should benefit the host – an intriguing aspect hitherto unexplored with TTV.

It is very probable that anelloviruses have co-evolved with us and other animals for millions of years. Evidence to support this comes from the study showing that TTV and TTMV sequences identified in non-human primates form their own sequence clusters, with increasing divergence from human isolates, suggesting co-evolution and co-specification within their respective species (Thom et al., 2003). Similar phylogenetic clustering and co-evolution has been shown with the species-specific evolution of e.g. the hantaviruses (Plyusnin et al., 1996). This long-term virus-host co-evolution could help understand the existence of the numerous TTV genotypes and the possible lack of strong immune responses.

In all, it is obvious that (nearly) all humans (in addition to many other species) carry anelloviruses. These viruses are actively replicating within us, and as it seems have achieved the euphoria of virus life: apparently unlimited replication and spread all over the world.

6 FUTURE ASPECTS IN TORQUE TENO VIRUS RESEARCH

To understand the TTV life cycle, the following key issues should be addressed: 1) methods should be developed to detect, to quantify, and to separate all the existing anelloviruses; 2) new genotypes and/or TTV-like viruses, as yet unknown, should be searched for; 3) proteins from various genogroups need to be expressed; 4) antibodies against the proteins should be produced; and 5) efficient cell culture systems for virus production should be set up. With these tools, some of which have been developed in this thesis work, the cell biology, immunology, pathology, and epidemiology of this new virus family can be uncovered.

Supplement 1. The GenBank submission #AY666122 containing all the information on the isolate HEL32 of genotype 6. (Reproduced from http://www.ncbi.nlm.nih.gov)

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TITLE       Cloning and sequencing of TT virus genotype 6 and expression
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JOURNAL     J. Gen. Virol. 83 (Pt 5), 979-990 (2002)
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REFERENCE   2  (bases 1 to 3748)
AUTHORS     Qiu,J., Kakkola,L., Cheng,F., Ye,C., Soderlund-Venermo,M.,
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TITLE       Human circovirus TT virus genotype 6 expresses six proteins
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JOURNAL     J. Virol. 79 (10), 6505-6510 (2005)
PUBMED      15858033
REFERENCE   3  (bases 1 to 3748)
AUTHORS     Kakkola,L., Tommiska,J., Boele,L.C., Miettinen,S., Blom,T.,
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TITLE       Construction and biological activity of a full-length
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JOURNAL     FEBS J. 274 (18), 4719-4730 (2007)
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AUTHORS     Kakkola,L., Hedman,K., Vanrobaeys,H., Hedman,L. and
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TITLE       Direct Submission
JOURNAL     Submitted (08-MAY-2001) Department of Virology, Haartman
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            P.O.B. 21, 00014, Finland
REFERENCE   5  (bases 1 to 3748)
AUTHORS     Kakkola,L., Tommiska,J., Hedman,K. and Soderlund-Venermo,M.
TITLE       Direct Submission
JOURNAL     Submitted (23-JUN-2004) Department of Virology, Haartman
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REFERENCE   6  (bases 1 to 3748)
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TITLE       Direct Submission
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2281 ctggaaatgg ggaggaaatc ctatctccca acagatcgtc cgacacccct gcaacggaca
2341 gacctcctca ggcaatagag tgcctcgctc agtacaagca gttgacccga aatacgtctc
2401 actccaactc gtgtggcact cgtgggactt cagaagaggc ctctttggcc aggcaggtat
2461 taagagaatg caacaagaat cagatgctct tacactttct ccagtccaca gacccaaacg
2521 ccccaagaga gacacccagg tcaaagaaaa aacgcccgaa aaagactcag attcagcagt
2581 ccaactcaga agactccagc cctggatcca ctccagtcaa gaaacaaaag acgaagagga
2641 ggagataccg gagggcccgg tacaagaaca acttctccag cagctccagc agcagcgact
2701 cctcagagtc cagctcgagt ccattgccca agaagtcctc aaaatcagga gggggcacag
2761 cctccacccc ctactatctt cccatgcata aacaaggtct ttatgtttaa ccctccaggt
2821 ccaaaacgca tcactgggta cgaggcctgg agggatgaat atgaaacatg caaggcctgg
2881 aacaggcccc ctagatcctt ttacacagat attcccacat acacctggat gcccaaagcc
2941 caagaccaat ttcgtgtaag cttcaaactt ggctttcatt aaaatttcaa ggccgtgcaa
3001 cattcacttg tcggtgtcta cctctaaagg tcactaagca ctccgagcgc aagcgaggag
3061 tgcgaccctc ccccctgggg gcaactccct cgaagtccgg cgctacgcgc ttcgcgctgc
3121 gccggacatc tcggaccccc cctccacccg aaacgcttgc gcgtttcgga ccttcggcgt
3181 cgggggggtc gggggcttta ctaaacagac tccgagatgc cattggacac tgaggggggca
3241 tacagcaacg aaagtgagtg gggccagact tcgccataag gcctttatct tcttgccatt
3301 tgtcagtata gggggtcgcc ataggcttcg gcctccattt taggccttcc ggactacaaa
3361 aatggccgtt ccagtaacgt cacggccgcc attttaagta ggtgccgtcc agcactgctg
3421 ttccgggtta aagggcatcc tcggcggaac ctatacaaaa tggccgccaa tttcttccgg
3481 gtcaaaggtc acgcctacgt catcgatgac gtggggaggc gtactatgaa acgcggaagt
3541 aggccccgct acgtcatcat cacgtgggtg ctgcgtcacg gccgccattt tgttttacaa
3601 aatggccgac ttccttcctg ttttttaaaa attaaccgtt agcggcggcg cgcgcgcttc
3661 gcgcgcgcgc cgggggctc cgccccccc ccgcgcatgc gcggggcccc ccccccgcgg
3721 ggggctccgc cccccggccc cccccccg
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CONCLUSIONS

Torque teno virus (TTV) is highly prevalent among the general population throughout the world. Virtually nothing is known of the cell biology or the proteins of TTV. In this work we have determined the prevalence of TTV in Finland, studied the cell biology, and expressed all the six proteins of TTV for immunological studies.

Torque teno viruses were found to be highly prevalent also in Finland; 85% of healthy adults had ongoing viremia. Genotype-6 DNA was detected in 4% of sera, and also in a number of different tissues, with no tissue-type or symptom specificity.

A full-length clone constructed of TTV genotype 6 was shown to transcribe mRNAs in all the cell lines tested. DNA replication was determined to occur in several human cell lines, with the highest expression levels observed in human kidney-derived 293T cells. Viral DNA replication was shown to use the cellular replication machinery, representing the first replication-related cell biological fact of the life cycle of this newly found human virus.

The transcription map of TTV genotype 6 was determined: this small virus was shown to produce altogether six proteins. To achieve this, the virus uses alternative splicing and alternative translation initiation. In addition, the sub-cellular localizations of the proteins were determined in cultured cells, leading to further need for investigations of potential protein interactions.

The six proteins were expressed in bacteria and in insect cells, and their antigenicity was studied. IgG antibodies against the proteins were detected in 44% of nonsymptomatic adults. Two genotype-6 DNA positive subjects were followed: one had persisting genotype-6 viremia with a concurrent IgG response against the putative capsid protein, and the other had a transient genotype-6 viremia with appearance of IgG against the ORF2 protein, correlating with the disappearance of genotype 6 from the circulation. The genomic DNA of genotype 6 remained constant throughout a follow-up of eight years, simultaneously with a strong IgG response against the putative capsid protein, indicating that some mechanism other than mutation is most probably used for TTV immune evasion.

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From the comic *Tiger*, Published with kind permission from Bulls Press, © KFS/distr. Bulls.

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