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Expression of all six human Torque teno virus (TTV) proteins in bacteria and in insect cells, and analysis of their IgG responses ☆

Laura Kakkola *, Heidi Bondén, Lea Hedman, Niina Kivi, Susanna Moisala, Jaakko Julin, Jussi Ylä-Liedenpohja, Simo Miettinen, Kalle Kantola, Klaus Hedman, Maria Söderlund-Venermo

Department of Virology, Haartman Institute and Helsinki University Central Hospital Laboratory, Haartmaninkatu 3, P.O. Box 21, University of Helsinki, FIN-00014, Finland

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ABSTRACT

Torque teno virus (TTV) is a non-enveloped human virus with a circular (~3800 nt) ssDNA genome. TTV transcription results in three viral mRNAs and six proteins, the function or antigenicity of which are unknown.

The six open reading frames of TTV genotype 6 were expressed in bacteria and insect cells. Expression of the ORF1/1-encoded protein was inefficient, while expression of the others was successful, with ORF1 and ORF1/2 as arginine-rich region depleted.

All six recombinant TTV proteins were antigenic. Of healthy adults, 11/25 (44%) showed strong IgG reactivity with one or more proteins. Four subjects, two of whom were genotype-6-DNA positive, were followed. One of the latter showed concurrently a strong IgG response against the ORF1 protein. The other showed appearance of IgG against the ORF2 protein concomitantly with resolution of the genotype-6 viremia. The genotype-6 sequences remained unaltered for years, suggesting that some mechanisms other than amino acid substitutions play a role in TTV immune evasion.

Introduction

Torque teno virus (TTV) was found in 1997 from a Japanese patient with hepatitis of unknown etiology (Nishizawa et al., 1997). TTV is a small, non-enveloped virus containing an approximately 3.8 kb long, circular negative-sense single-stranded DNA genome (Miyata et al., 1999; Mushahwar et al., 1999). By genomic organization, TTV resembles the chicken anemia virus (CAV) of the *Circoviridae* family, and is currently classified as a member of a new, floating genus *Anellovirus* (Biagini et al., 2004).

TTV shows a very high sequence variation, both at nucleotide and amino acid levels. Several genotypes (differing by more than 30%) have been identified, and form five major phylogenetic clusters (Biagini et al., 2004). Also other TTV-like viruses have been characterized: Torque teno minivirus (TTMV) (Takahashi et al., 2000) and Torque teno midivirus (TTMDV) or small anellovirus (SAV) (Jones et al., 2005; Ninomiya et al., 2007a). All these viruses have genomes of similar structure, yet with different sizes: 3.7–3.8 kb for TTV, 3.2 kb for TTMDV/SAV, and 2.8–2.9 kb for TTMV (Biagini et al., 2007; Jones et al., 2005; Ninomiya et al., 2007a; Takahashi et al., 2000).

Nearly all TTV research has been hitherto based on the detection of viral DNA by PCR. It has become evident that this virus is spread worldwide, with a ~90% DNA prevalence in blood of asymptomatic individuals (Abe et al., 1999; Huang et al., 2001; Kakkola et al., 2002; Okamoto et al., 1999; Simmonds et al., 1999). Persistent infections and co-infections with several genotypes are common (Ball et al., 1999; Biagini et al., 1999; Irving et al., 1999; Lefrere et al., 2000; Sugiyama et al., 1999). In addition, co-infections have been shown to occur with all three TTV-like viruses in asymptomatic subjects (Biagini et al., 2006; Ninomiya et al., 2007b).

The TTV genome consists of a 2.6 kb coding and 1.2 kb non-coding region, the latter containing a GC-rich region, a promoter and transcriptional enhancer elements (Kamada et al., 2004; Miyata et al., 1999; Mushahwar et al., 1999; Suzuki et al., 2004). A TATA-box and a poly-A sequence define the coding area, in which overlapping open reading frames (ORF) in all three frames are located (Erker et al., 1999; Hijikata et al., 1999; Miyata et al., 1999). TTV has been shown to produce by alternative splicing three mRNA species (Kamahora et al., 2000), from which by alternative translation initiation six proteins are produced (Qiu et al., 2005). The splice sites are well conserved among various isolates (Peng et al., 2002). Even though variation between the TTV genotypes is higher at the amino acid than at the nucleotide level (Tanaka et al., 2000), the proteins encoded by different genotypes have similar motifs, thus suggesting similar functions.

☆ Nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession number AY666122.

* Corresponding author. Fax: +358 9 19126491.
E-mail addresses: laura.kakkola@helsinki.fi (L. Kakkola), heidi.bonden@nextbiomed.com (H. Bondén), lea.hedman@helsinki.fi (L. Hedman), niina.kivi@helsinki.fi (N. Kivi), mmoisala@cc.hut.fi (S. Moisala), jaako.julin@uta.fi (J. Julin), jylalied@mappi.helsinki.fi (J. Ylä-Liedenpohja), simo.miettinen@helsinki.fi (S. Miettinen), kalle.kantola@helsinki.fi (K. Kantola), klaus.hedman@helsinki.fi (K. Hedman), maria.soderlund-venermo@helsinki.fi (M. Söderlund-Venermo).

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The longest open reading frame, ORF1, has been suggested to encode a capsid protein (Takahashi et al., 1998). ORF1 has circovirus-like replication-associated motifs (Erker et al., 1999; Tanaka et al., 2001) and a CAV-like arginine-rich N-terminus (Mushahwar et al., 1999; Okamoto et al., 1998; Takahashi et al., 1998) that has been suggested to have DNA-binding activity and to function in packaging of the viral DNA (Erker et al., 1999). ORF1 contains hypervariable regions in which mutations leading to amino acid changes occur more frequently than in the remaining part of the protein (Jelcic et al., 2004; Nishizawa et al., 1999), suggesting immune evasion by hypermutations.

ORF2 encodes a putative 200-amino-acid protein that has a conserved amino acid motif (Hijikata et al., 1999; Peng et al., 2002). This motif is part of the dual specificity phosphatase sequence, the activity of which has been shown also in CAV and TTMV proteins (Peters et al., 2002). In several genotypes a stop codon divides ORF2 into two smaller frames, ORF2a and ORF2b. Even though the ORF2b protein is less conserved than the ORF2a at the amino acid level, it contains a CAV-like motif (Kakkola et al., 2002; Tanaka et al., 2000; Ukita et al., 2000). Very recently the ORF2 protein was shown to suppress the NF-κB pathway and thus to have putative regulatory effects on innate and adaptive immunity (Zheng et al., 2007).

The genotype-1a ORF3 protein [corresponding to ORF2/2 in (Qiu et al., 2005), and ORF2-4 in (Kamahora et al., 2000)] has similarities with a non-structural protein NS5A of hepatitis C virus (HCV). In Cos-1 cells, this protein is produced in two forms, of which the slower-migrating is phosphorylated at the C-terminal serine residues (Asabe et al., 2001). The remaining three TTV proteins are essentially unknown, as are the functions of all the six TTV proteins.

Only a few publications exist on TTV immunology and on the expression of TTV proteins. Tanaka et al. (2000) expressed by an *in vitro* transcription/translation method the putative ORF2 proteins, showing the functional stop codon dividing the ORF2 into smaller frames (Tanaka et al., 2000). ORF3 of genotype 1a was expressed for phosphorylation studies in Cos-1 cells (Asabe et al., 2001). For immunological studies, the putative capsid protein encoded by ORF1 has been produced in fragments. Lo et al. (1999) expressed in prokaryotes ~120 amino acids of ORF1 (the N22 region within the C-terminal half), and found by immunoblots no IgG antibodies in asymptomatic adults (Lo et al., 1999). Ott et al. (2000) expressed in prokaryotes the C-terminal (aa 504–752) and Handa et al. (2000) the N-terminal (aa 1–411) part of genotype-1 ORF1. These two groups found by immunoblots antibodies in 98.6% of hepatitis patients, blood donors, and asymptomatic children, and in 38% of blood donors, respectively (Handa et al., 2000; Ott et al., 2000). We have previously expressed in prokaryotes the two ORF2 proteins, ORF2a and ORF2b, and detected IgM and IgG in 9–10% of asymptomatic adults (Kakkola et al., 2002). In addition to immunoblotting, the existence of TTV antibodies in human sera has also been shown by other methods, including immunoprecipitation and immunocapture combined with PCR (Tsuda et al., 1999; Tsuda et al., 2001).

As the methods in current use for detection of TTV infections (restricted to acute and persisting, but not past) are PCR-based, little is known of the TTV proteins and their antigenic potential. Serological tests (measuring also past infections) would give valuable information on immunological reactions in humans against this highly prevalent, persisting, and potentially non-pathogenic virus.

In our previous studies we have cloned in full length the genome of TTV genotype 6 (Kakkola et al., 2007), and have identified the proteins encoded (Qiu et al., 2005). The aim of the current study was to express all the six TTV proteins in prokaryotic or baculoviral expression systems for use as antigens in immunological and diagnostic studies.

Results

Expression of the proteins in bacteria

Of the six protein coding regions (Fig. 1), altogether 11 constructs were cloned into bacterial expression plasmids: ORF1ΔArg, ORF1-N, ORF1-NΔArg, ORF1-C, ORF2, ORF2/2, ORF2/3, ORF1/1, ORF1/ΔArg, ORF1/2 and ORF1/2ΔArg. The proteins were expressed in fusion with GST in BL21 cells, and were analyzed by immunoblotting with GST antibody. The Rosetta strain was attempted with the arginine-rich proteins ORF1-N, ORF1/1 and ORF1/2, and with one arginine rich region-depleted protein ORF1/1ΔArg, however, with no improvement in expression levels. The successful expression conditions for each construct are given in Table 1.

Since the expression of the arginine-rich ORF1 protein was unsuccessful in our previous study (Kakkola et al., 2002), we decided to express the protein in bacteria as arginine-depleted constructs (ORF1ΔArg, ORF1-NΔArg) or in two parts (ORF1-N, ORF1-C). The expressions of the ORF1-NΔArg (~63 kDa) and ORF1-C (~69 kDa) proteins were successful, as was the expression of ORF1ΔArg (~100 kDa), however, shorter forms of the latter protein were also produced (Fig. 2). The expression of ORF1-N (~70 kDa) was not successful with any of the three media regardless of induction time. For this construct only the Rosetta strain was used.

The expression of ORF1/1 (~54 kDa) was of low level with any of the three media, with any of the induction times, and with both of the bacterial strains. Removal of the arginine-rich part (ORF1/1ΔArg; ~47 kDa) did not significantly improve the expression (Fig. 2). The expression of ORF1/2 (~44 kDa) was likewise low in any of the expression conditions; however, the removal of the arginine-rich part (ORF1/2ΔArg; ~37 kDa) resulted in major improvement of expression level (Fig. 2).

In our previous study we had expressed the fp2a (nt 104–253) and fp2b (nt 237–707) proteins from the ORF2 coding area (Kakkola et al., 2002). The ORF2 (nt 354–707; ~44 kDa) encoded protein expressed in this study corresponds to amino acids 40–118 of the previously expressed fp2b, and was successfully expressed in prokaryotes (Fig. 2). Also the expressions of ORF2/2 (~57 kDa) and of ORF2/3 (~56 kDa) were successful (Fig. 2).

For expression of some constructs, room temperature and IPTG concentration of 0.2 mM were also tried, but with no significant improvement. However, with the constructs that were prone to forming shorter fragments in standard conditions, shifting of the growth/induction time e.g. from 4/2 to 2/4 h brought a substantial gain in protein yield (less fragmentation; data not shown).

Expression of the proteins in insect cells

Of the six protein coding regions, altogether 7 constructs, covering all the reading frames in the coding area, were cloned into a

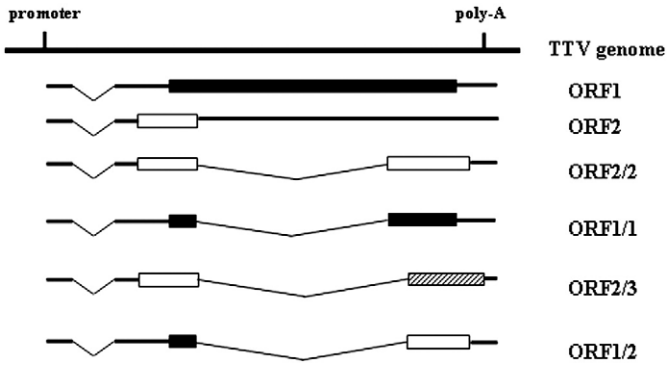

Fig. 1. Schematic presentation of the transcription map of TTV genotype 6 (adapted from Qiu et al., 2005). Thin lines represent introns, thick lines mRNA, and boxes translated areas, the shading of which indicate different reading frames.