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# Identification of Basal Promoter and Enhancer Elements in an Untranslated Region of the TT Virus Genome

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**The regulation of TT virus (TTV) gene expression was characterized. Transient-transfection assays using reporter constructs revealed that a 113-nucleotide (nt) sequence within the untranslated region, proximal to the transcription initiation site and containing a TATA box motif, has a basal promoter activity. This sequence is well conserved among different TTV genotypes. Upstream stimulating factor bound to a consensus binding motif within this region and positively regulates TTV transcription. Furthermore, a 488-nt region upstream of the basal promoter exhibited enhancer activity, presumably in a cell type-specific manner. This study illustrates some of the mechanisms involved in the transcriptional regulation of TTV.**

TT virus (TTV), which was discovered in a patient with acute hepatitis, is an unenveloped, single-stranded, circular DNA virus, with a genome of approximately 3.8 kb (6). TTV is thought to be a new member of the *Circoviridae* family of viruses, and it was recently proposed that the virus be named Torque Teno virus (6). The TTV genome includes an untranslated region (UTR) of approximately 1.2 kb and a coding region of approximately 2.6 kb, including two major open reading frames which are sandwiched by the TATA box and polyadenylation signal motifs (11, 13, 15). Analyses of TTV transcripts have revealed three spliced mRNA species of 3.0, 1.2, and 1.0 kb with common 5′ and 3′ termini (9, 14). However, the molecular mechanisms controlling TTV transcription are still unknown. In this study, the basal promoter and enhancer of a TTV isolate, SANBAN of genogroup 3 (5, 18), were identified and functionally characterized.

First, we determined the transcription initiation sites of the TTV genome by 5′ rapid amplification of cDNA end (5′-RACE) analysis (Marathon cDNA amplification kit; Clontech) using poly(A)-rich RNA from a human hepatocellular carcinoma cell line, HepG2, transfected with a cloned TTV genome. The 5′-RACE PCR products were cloned and sequenced. We observed two potential transcription initiation sites, which map at nucleotides (nt) 121 and 110 (numbered according to the sequence deposited in DDBJ/GenBank/EMBL databases under accession number AB025946). Although transcription may be initiated at both sites, the upper site was designated position +1 in this study.

The UTR of the TTV genome contains a TATA box element between positions −40 and −35, as well as a number of putative transcription factor-binding motifs (Fig. 1A). Despite considerable genetic diversity throughout the whole genome, the UTR sequence was relatively conserved among the different TTV genotypes, presumably reflecting its functional constraints (15, 16). Thus, we analyzed transcriptional regulation of the UTR sequence.

To characterize TTV promoter activity, a firefly luciferase reporter plasmid, p(−890/+115), was constructed by subcloning the TTV sequence from positions −890 to +115, which was amplified by PCR using appropriate primers with restriction sites at the 5′ ends, into the promoterless pGL3-Basic (Promega). Eleven different cell lines were transfected with p(−890/+115), along with a *Renilla* luciferase expression vector, pRL-TK, as an internal standard for determining transfection efficiency. Luciferase activities in cell lysates prepared after 16 h of transfection were determined (2). It is of interest that the 1.0-kb fragment demonstrated a pronounced promoter activity in all the hepatocellular carcinoma cell lines tested. Human (Huh7, HepG2, and FLC4 [1, 2]) and mouse (Hepa1-clc7) hepatocellular carcinoma cells were tested (Fig. 1B). This fragment demonstrated the greatest activity in Huh7 cells (~10-fold greater than in other cells). We observed substantial promoter activity in GL37 (African green monkey kidney) and CHO (Chinese hamster ovary) cells, whereas limited activity was observed in Caco2 (human colon carcinoma), MOLT4 (human T-cell leukemia), CV1 (African green monkey kidney fibroblast), 3T3 Swiss (mouse fibroblast), and CMT93 (mouse rectal carcinoma) cells. These results indicate that the UTR of the TTV genome functions as a promoter in a cell type-specific manner.

To assess basal, proximal promoter activity in the UTR, a series of 5′ deletions fused to the luciferase gene were constructed and transfected into Huh7 and HepG2 cells (Fig. 2). A deletion extending to nt −601 [p(−601/+115)] enhanced promoter activity in both cell lines (by ~1.5-fold), while deletion of another 274 nt [p(−327/+115)] decreased promoter activity by more than 80%, suggesting that there is a negative regula-

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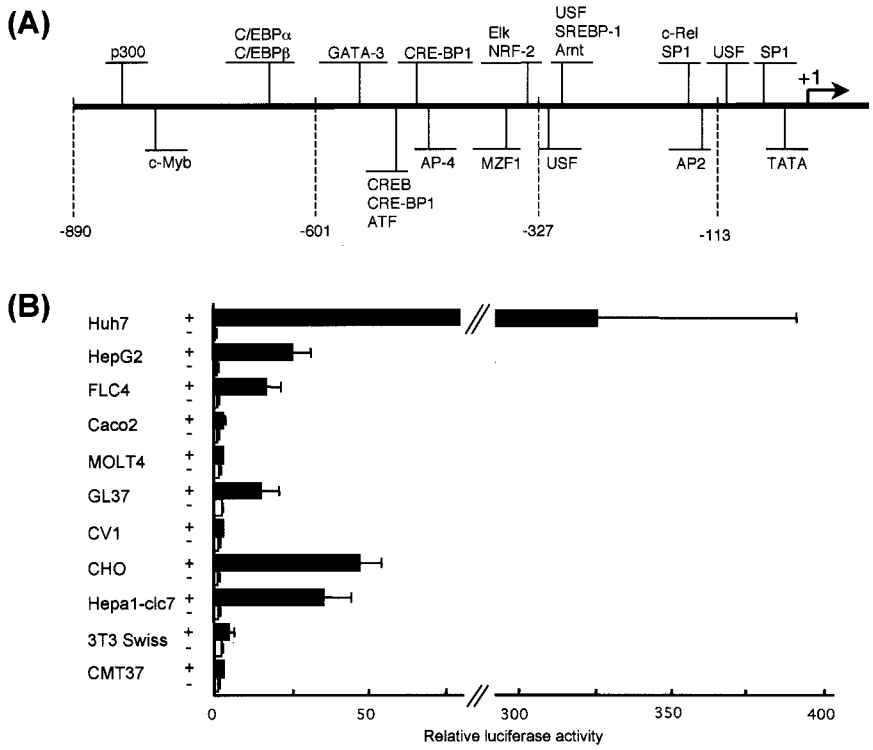


FIG. 1. Functional analysis of the TTV promoter in the viral UTR. (A) Schematic representation of the 1.0-kb UTR sequence. The TATA box element and putative transcription factor-binding sites are shown. Transcription factor-binding sites were identified using the TRASFAC database and a search program (http://motif.genome.ad.jp/). The transcription initiation site (+1) is indicated and corresponds to nt 121 (AB025946). The numbers at the bottom of vertical dotted lines indicate the start points of the full-length promoter construct and deletion mutants of the promoter constructs used in Fig. 1B and 2. (B) Cell type specificity of the TTV promoter activities. Cells were transfected with p(−890/+115) (+) or promoter-less pGL3-Basic (−) together with pRL-TK (*Renilla* luciferase). Cell extracts were prepared 16 h after transfection, and luciferase activities in the extracts were determined using a dual-luciferase reporter assay system (Promega) with the Lumat LB9501 luminometer (Berthold). All values were normalized to *Renilla* luciferase activities and are shown as means ± standard deviations (error bars) of three independent samples.

tory element between nt −890 and −601. A deletion extending to nt −113 [p(−113/+115)] resulted in a slight to moderate reduction in activity, but promoter activity still remained greater than that observed with p(+15/+113), in which the TATA box and the transcription start sites were deleted. These findings suggest that the 113 nt immediately upstream of the transcription initiation site contains a basal promoter region critical for TTV gene expression.

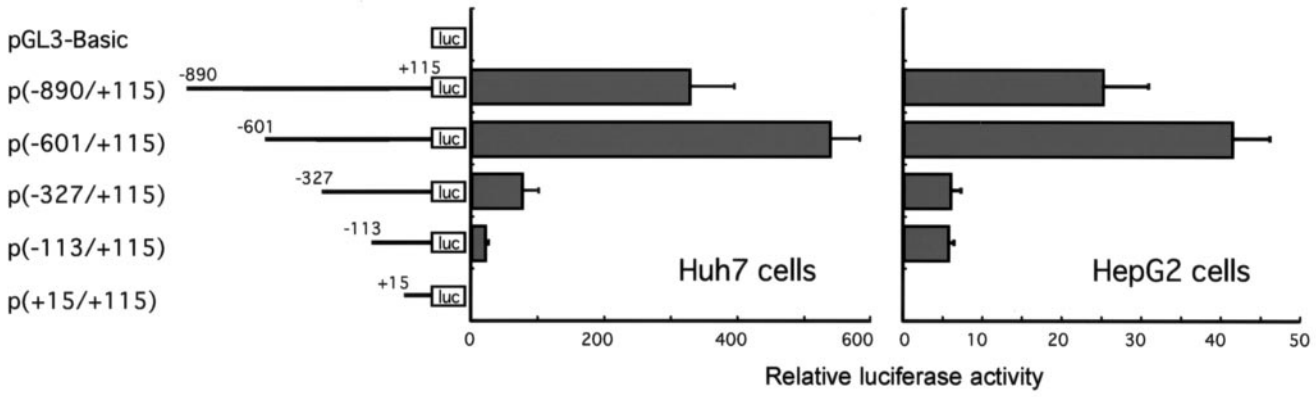


FIG. 2. Deletion analysis of the TTV promoter in Huh7 and HepG2 cells. The structures of the luciferase reporter constructs containing various lengths of the TTV UTR sequence are shown to the left. A series of DNA fragments with 5′ deletions of the TTV promoter were amplified by PCR using the full-length TTV DNA of SANBAN isolate as a template with the same reverse primer and various forward primers. The fragments were cloned into pGL3-Basic at XhoI and HindIII sites. The indicated constructs were transfected into Huh7 cells or HepG2 cells. Relative luciferase activity in each transfectant was determined as described in the legend to Fig. 1B. Results are shown as means ± standard deviations (error bars) of three independent samples.

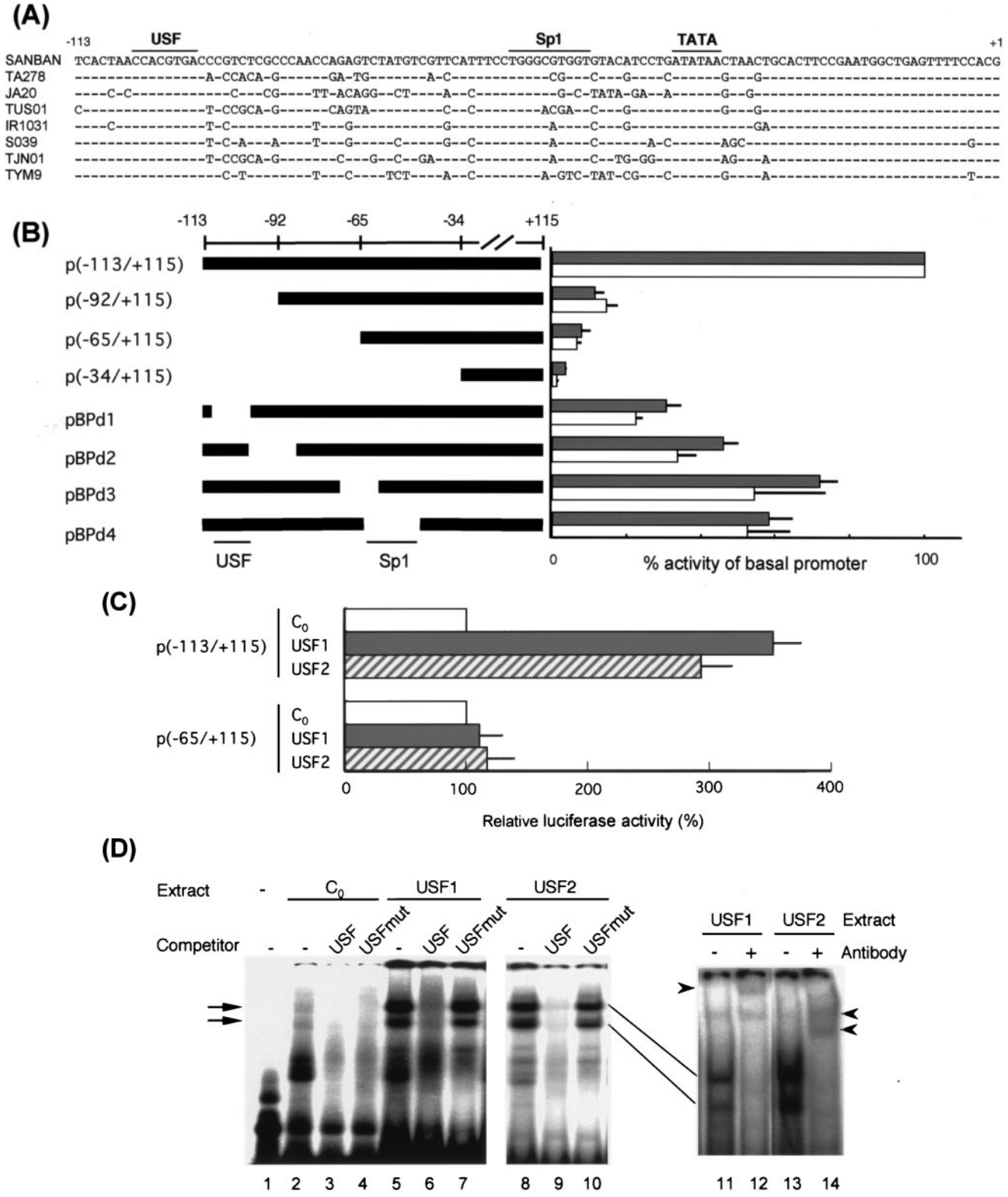


FIG. 3. Basal promoter activity of TTV regulated by USF. (A) Alignment of the putative basal promoter regions from TTV isolates SANBAN (DDBJ/GenBank/EMBL accession number AB025946), TA278 (AB017911), JA20 (AF122914), TUS01 (AB017613), IR1031 (AB038619), S039 (AB038620), TJN01 (AB028668), and TYM9 (AB050448). The transcription initiation site is numbered +1. The TATA box and positions of putative binding sites for USF and SP1 are indicated. Nucleotides that are identical to those in the SANBAN isolate (−) are indicated. (B) Effect of deleting DNA from the basal promoter region on the TTV basal promoter activity. A series of DNA fragments with 5′ or internal deletions of the basal promoter region were amplified by PCR and cloned into pGL3-Basic at XhoI and HindIII sites. The indicated constructs were transfected into Huh7 cells (gray bars) or HepG2 cells (white bars). Relative luciferase activity in each transfectant was determined as described in the legend

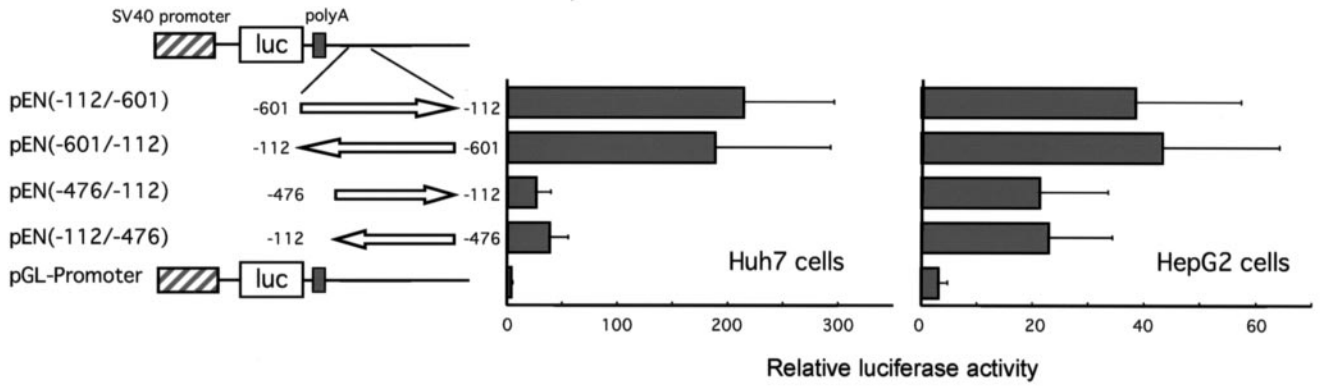


FIG. 4. Enhancer activity of the 488-nt fragment (−601/−114) of the TTV UTR. The 488-nt (−614/−114) and 363-nt (−476/−114) fragments were PCR amplified using primers with 5′ overhangs containing BglII (sense) and BamHI (antisense) sites. The fragments were then cloned into pGL3-Promoter at the BamHI site. The indicated constructs (left panel) were transfected into Huh7 cells or HepG2 cells. Relative luciferase activity in each transfectant was determined as described in the legend to Fig. 1B. Results are shown as means ± standard deviations (error bars) of three independent samples. SV40, simian virus 40.

Computer-assisted analysis of this basal promoter region identified potential binding sites recognized by upstream stimulating factor (USF) and Sp1, which are conserved among TTV genotypes (Fig. 3A). To determine whether these sequences contribute to TTV promoter activity, 5′ or internal deletion mutations were introduced into p(−113/+115), which was then examined for dual luciferase activity. Deletions, those found in p(−92/+115) and pBPd1, reduced promoter activity by 70 to 85%, suggesting that the USF-binding sequence is crucial for TTV promoter activity (Fig. 3B). Deletion of a Sp1-binding sequence (pBPd4) also conferred a decrease in promoter activity, although their effects were relatively moderate, suggesting that the Sp1-binding motif and/or its encompassing sequence may play a role in regulating TTV promoter activity by maintaining the structural integrity of the transcriptional machinery.

In genes where USF regulates transcription, cotransfection of USF expression vectors with reporter genes stimulates reporter activities. To further investigate the effect of USF on TTV promoter activity, we cotransfected USF1 or USF2 expression vectors (pCMV-USF1 and pCMV-USF2) (7) with p(−113/+115) into HepG2 cells. The cotransfection significantly increased promoter activity (by threefold), suggesting that USF proteins regulate TTV transcription (Fig. 3C).

USF is a family of basic-helix-loop-helix-leucine zipper transcription factors, initially identified by their ability to bind to the 5′-CACGTG-3′ sequence within the adenovirus major late promoter (3, 4, 10). USF1 and USF2 have been subsequently shown to bind to the promoters of various cellular and viral genes. To determine whether the TTV basal promoter was capable of USF binding, gel mobility shift assays were performed on an end-labeled oligonucleotide (nt −113 to −84) containing the putative USF-binding motif (Fig. 3D). DNA-protein-binding complexes were observed in nuclear extracts from cells transfected with pCMV-USF1 (Fig. 3D, lane 5), pCMV-USF2 (lane 8), and the empty vector (lane 2). An excess of unlabeled homologous probe competed with the protein binding (lanes 3, 6, and 10), whereas a mutated USF sequence failed to compete (lanes 4, 7, and 10). The addition of anti-USF antibodies to the binding reaction mixture supershifted the DNA-protein complexes (lanes 12 and 14). The combined data demonstrate that USF binds to its binding motif within the TTV basal promoter to up-regulate viral transcription.

On the basis of the results of the luciferase assays using 5′ deletions of the TTV UTR (Fig. 2), the positive regulatory element appears to be located immediately upstream of the basal promoter. To ascertain whether the 488-bp fragment between nt −601 and −113 functions as the enhancer region, this fragment or a 5′ deletion of this fragment was placed downstream of the polyadenylation signal in pGL3-Promoter (Promega), driven by the simian virus 40 promoter, in either

to Fig. 1B. Results are shown as a percentage of the activity in cells transfected with p(−113/+115); values are shown as means ± standard deviations (error bars) ($n = 3$ per group). (C) Effects of USF overexpression on basal promoter activity. HepG2 cells were cotransfected with each reporter construct with pCMV-USF1 (USF1), pCMV-USF2 (USF2), or empty $pC_0$ vector ($C_0$). Luciferase activity was determined 48 h after transfection. For each reporter construct, relative luciferase activity is presented as a percentage of the activity in $pC_0$-transfected cells. (D) Binding of USF proteins to the region from nt −113 to −84 in the TTV basal promoter. The electrophoretic mobility shift assays were performed as described previously (19). A double-stranded oligonucleotide corresponding to the TTV sequence from nt −113 to −84 was used as a probe. Nuclear extracts from the cells transiently transfected with pCMV-USF1 (USF1; lanes 5, 6, 7, 11, and 12), pCMV-USF2 (USF2; lanes 8, 9, 10, 13, and 14), or $pC_0$ ($C_0$; lanes 2 to 4) or no extract (lane 1) were mixed with $^{32}$P-labeled probe for the binding reaction mixtures. Competitors, unlabeled probe (USF), and a mutant with the USF-binding motif (USFmut) were added at a 25-fold molar excess. The sense sequence (5′-TCACTAAC CAATTGACCCGTCTCGCCCAAC [the mutated nucleotides are underlined]) and complementary sequence of the mutant with the USF-binding motif were added. A supershift experiment was also performed by incubating antibody against USF1 (lane 12) or USF2 (lane 14) (+) with the nuclear extracts before the probe was added. The positions of specific binding complexes (arrows) and supershifted complexes (arrowheads) are indicated.

the sense or antisense orientation. Luciferase activity of constructs containing the 488-bp fragment [pEN(−601/−114) and pEN(−114/−601)] led to 50- and 10-fold stimulation in Huh7 and HepG2 cells, respectively. The 5′ deletion extending to nt −476 [pEN(−476/−114) and pEN(−114/−476)] reduced enhancer activity (Fig. 4). No enhancement was observed by transfection of MOLT4 cells with pEN(−601/−114) and pEN(−114/−601) (data not shown). These results demonstrate that the 488-bp region upstream of the basal promoter contains an enhancer element, suggesting cell-specific transcription of the TTV genome. It is noteworthy that the enhancer element is conserved among TTV genotypes. For example, 72% homology has been observed between clones SANBAN and TA278, and the database search has revealed at least 20 potential transcription factor-binding sites within this element, including CREB and CRB, which are activated upon cyclic AMP signaling-dependent phosphorylation (12, 17).

While the manuscript was being prepared, Kamada et al. reported the promoter and enhancer activities in the UTR of TTV, clone VT416 whose genome is 98% similar to that of TA278, and its cell tropism (8). However, they did not identify transcription factors that bind to the region and regulate TTV transcription. In summary, the findings reported by Kamada et al. and the findings of our present study emphasize the important role of the UTR as a basal promoter and enhancer within the UTR. Other areas of interest for further study include the identification of additional factors involved in tissue-specific TTV transcription and determining the significance of polymorphism of the regulatory elements.

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