Virology 450-451 (2014) 258–265

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Translational readthrough in Tobacco necrosis virus-D

Laura R. Newburn, Beth L. Nicholson, Michael Yosefi, Peter A. Cimino, K. Andrew White *

Department of Biology, York University, Toronto, ON, Canada M3J 1P3

ARTICLE INFO

Article history:
Received 30 October 2013
Returned to author for revisions
23 November 2013
Accepted 8 December 2013
Available online 9 January 2014

Keywords:
Plant virus
RNA virus
Recoding
Readthrough
Frameshifting
Cap-independent translation
RNA structure
Tombusviridae
Necrovirus
Carmovirus

ABSTRACT

The plus-strand RNA genome of Tobacco necrosis virus-D (TNV-D) expresses its polymerase via translational readthrough. The RNA signals involved in this readthrough process were characterized *in vitro* using a wheat germ extract translation system and *in vivo* via protoplast infections. The results indicate that (i) TNV-D requires a long-range RNA-RNA interaction between an extended stem-loop (SL) structure proximal to the readthrough site and a sequence in the 3′-untranslated region of its genome; (ii) stability of the extended SL structure is important for its function; (iii) TNV-D readthrough elements are compatible with UAG and UGA, but not UAA; (iv) a readthrough defect can be rescued by a heterologous readthrough element *in vitro*, but not *in vivo*; and (v) readthrough elements can also mediate translational frameshifting. These results provide new information on determinants of readthrough in TNV-D and further support the concept of a common general mechanism for readthrough in Tombusviridae.

Introduction

Plus-strand RNA viruses use a variety of translational strategies for the expression of their encoded proteins. Some employ recoding mechanisms such as translational readthrough or frameshifting to further expand their coding capacity (Firth and Brierley, 2012). Translational readthrough occurs when a stop codon is read as a sense codon by a suppressor tRNA, resulting in a C-terminal extension of the initial protein. Translational frameshifting also results in a C-terminally extended protein, but this occurs when a translating ribosome shifts its reading frame. Frameshifting into the −1 reading frame is most common, and this event, as well as readthrough, are used to express the RNA-dependent RNA polymerases (RdRps) in many different viruses (Firth and Brierley, 2012; Firth et al., 2011; Cimino et al., 2011). For both of these recoding events, RNA sequences and/or structures that facilitate the processes are generally found 3′-proximal to the recoding sites (Firth and Brierley, 2012).

Interestingly, some viruses also require distal RNA elements in addition to those found proximal to recoding sites. Barley yellow dwarf virus (BYDV; genus *Luteovirus*, family Luteoviridae) uses −1 frameshifting to express its RdRp and this process requires an extended stem-loop (SL) structure located just downstream of the frameshift site as well as a sequence in the 3′UTR of the genome (Paul et al., 2001; Barry and Miller, 2002). The key sequence in the 3′UTR is located in the terminal loop of an RNA hairpin structure and it must base pair with a bulge in the extended stem-loop (SL) structure proximal to the frameshift site for efficient −1 frameshifting to occur (Barry and Miller, 2002). Frameshifting in Red clover necrotic mosaic virus (RCNMV; genus *Dianthovirus*, family Tombusviridae) has been shown to involve RNA structures and a long-range base pairing interaction similar to those present in BYDV (Tajima et al., 2011). Interestingly, comparable structures and interactions are also needed for readthrough in Carnation Italian ringspot virus (CIRV; genus *Tombusvirus*, Family Tombusviridae) and Turnip crinkle virus (TCV; genus *Carmovirus*, Family Tombusviridae) (Cimino et al., 2011). Accordingly, comparable sets of long-range RNA–RNA interactions are involved in different recoding events in these viruses and it has been proposed that other genera in Tombusviridae may have similar requirements (Cimino et al., 2011).

Tobacco necrosis virus-D (TNV-D) is a plus-strand RNA virus and the type member of the genus *Betanecrovirus* in the family Tombusviridae (Sit and Lommel, 2010). Its ~3.8 kb genome encodes five viral proteins (Coutts et al., 1991) (Fig. 1A). The RdRp, p82, is expressed from the genomic RNA via readthrough of the 5′-proximal open reading frame (ORF) coding for p22 (Molnár et al., 1997; Fang and Coutts, 2013). The movement (p7a and p7b) and capsid (p29) proteins are translated from two subgenomic (sg) mRNAs that are transcribed during infections (Offei et al.,1995;

* Correspondence to: Department of Biology, York University, 4700 Keele St. Toronto, Ontario, Canada M3J 1P3. Tel.: +1 416 736 2100x40890, +416 736 2100x70352; fax: +1 416 736 5698.
E-mail address: kawhite@yorku.ca (K.A. White).

0042-6822/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2013.12.006

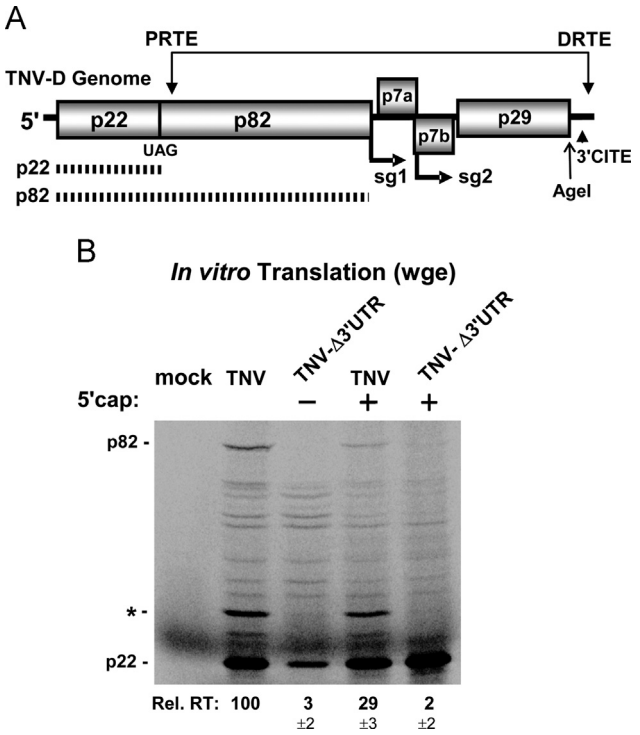

Fig. 1. Assessing the role of the 3′UTR in readthrough. (A) Schematic linear representation of the TNV-D RNA genome with boxes representing encoded proteins. p22 and its readthrough product p82 (shown by thick hatched lines) are translated directly from the genome. Initiation sites for subgenomic mRNA1 (sg1) and sg2 are indicated below the genome. The double-headed arrow above the genome connects the locations of two RNA sequences, the PRTE and DRTE, which base pair with each other to mediate readthrough. The relative positions of the 3′CITE and an AgeI restriction enzyme site are shown. (B) SDS-12%PAGE analysis of proteins translated from the TNV-D genome. Protein products in this, and all other subsequent experiments, were generated by translating 0.5 pmol of viral genomic RNA in wge for 1 h at 25 °C. The mock lane consists of a translation reaction using wge without any viral RNA added. The wild-type TNV-D genome (TNV) and a mutant TNV-D genome lacking a 3′UTR (Δ3′UTR) were tested with (+) and without (−) a 5′-cap. The positions of the viral proteins produced, p22 and p82 (RdRp), are indicated to the left and the asterisk denotes the position of p29 capsid protein. In this and subsequent experiments the corresponding means (±standard error) were determined from at least three independent experiments. Relative readthrough (Rel. RT) was calculated as the ratio of p82/p22, with that for wt TNV set as 100%.

Offei and Coutts, 1996; Molnár et al., 1997; Jiwan et al., 2011). The viral genome is neither 5′-capped nor 3′-polyadenylated and translation of viral proteins requires the activity of a 3′ cap-independent translational enhancer (3′CITE) that is located in the 3′UTR of the genome (Fig. 1A) (Shen and Miller, 2004). In addition to the 3′CITE, the 3′UTR also harbours RNA elements that are important for TNV-D genome replication (Shen and Miller, 2007).

In the present study we have investigated the readthrough process in TNV-D that mediates production of its RdRp. Our findings indicate that TNV-D requires a long-range RNA-RNA interaction for efficient translation of p82. Characterization of the readthrough signals along with comparative structural-function analyses provide novel insights into this translational recoding event.

Results

Efficient readthrough of p82 requires a 3′-proximal sequence

Translation of viral proteins from the TNV-D RNA genome was assessed using a wheat germ extract (wge) *in vitro* translation system. Translation of *in vitro* synthesized transcripts of the wt TNV-D genome yielded abundant p22 and lesser amounts of its readthrough product p82 (Fig. 1B, lane 2). The readthrough efficiency was calculated to be 2.5% ± 0.1. An additional prominent product of ~29 kDa was also observed (Fig. 1B, asterisk), which through additional analysis was determined to be the p29 coat protein that is likely expressed via an internal ribosome entry site (data not shown), as shown for some carmoviruses (Koh et al., 2003; Fernández-Miragall and Hernández, 2011). When the 3′UTR was deleted in TNV-Δ3′UTR, expression of all major products was dramatically reduced and the relative readthrough level of p82 (*i.e.* the ratio of p82/p22) was ~3% that of wt (Fig. 1B, lane 3). Adding a 5′-cap structure to TNV-Δ3′UTR led to revived p22 levels, but relative readthrough of p82 remained low at ~2% (Fig. 1B, lane 5). The presence of a 5′-cap on the wt TNV-D genome did reduce relative readthrough to ~29% of wt (Fig. 1B, lane 4), possibly due to interference with normal 3′CITE-mediated translation that may be important for efficient readthrough. However, ~29% was still ~15-fold higher than the ~2% level observed for capped TNV-Δ3′UTR. Collectively, these data suggest that the 3′UTR not only contains elements that are necessary for efficient cap-independent translation (*i.e.* a 3′CITE), as previously reported (Shen and Miller, 2004), it also contains a determinant for efficient readthrough of p82.

A long-range RNA–RNA interaction is required for readthrough

The requirement for an element in the 3′UTR of TNV-D (Fig. 1B), along with the previous observation of complementary sequences in the RNA structure 3′-adjacent to the readthrough site and a sequence in the 3′UTR (Cimino et al., 2011), suggested that readthrough production of p82 may require a long-range intra-genomic interaction. This interaction was predicted to occur between a sequence, the proximal readthrough element (PRTE), located in a bulge within an extended stem-loop structure, termed SL-PRTE, positioned 3′-proximal to the readthrough site and a sequence, the distal readthrough element (DRTE), located near the 3′-terminus of the viral genome (Figs. 1 and 2A). To test the possible involvement of the proposed interaction in readthrough, substitutions were introduced into the TNV-D genome that were predicted to disrupt (mutants TNV-2A and TNV-2B) and then restore (mutant TNV-2C) complementarity between the PRTE and DRTE (Fig. 2A). *In vitro* translation assays in wge revealed that the reduced pairing potential in mutants TNV-2A and TNV-2B lowered relative readthrough by ~10-fold, while re-establishing base pairing potential in mutant TNV-2C led to full recovery of readthrough (Fig. 2B). The effect of the mutations on virus viability was also assessed in protoplast transfections. Mutants TNV-2A and TNV-2B with reduced pairing accumulated to ~29 and ~66% the level of wt TNV-D, respectively, whereas mutant TNV-2C with restored pairing accumulated to wt levels (Fig. 2C). These results support an important role for the long-range interaction in both synthesis of p82 and viability of the viral genome.

The SL-PRTE structure is important for readthrough

The structural context of the PRTE places it within a bulge in an extended stem-loop structure, the SL-PRTE (Fig. 3A), and similar structural contexts have also been predicted for known and proposed PRTEs in all tombusvirids that utilize readthrough (Cimino et al., 2011). Comparably structured elements are also located 3′-proximal to −1 frameshift sites in BYDV and RCNMV (Barry and Miller, 2002; Tajima et al., 2011), thus similar secondary structures are required for both readthrough and frameshifting in these viruses. Despite their prevalence, the importance of the secondary structure within these RNA elements has not yet been investigated experimentally for any of these viruses. Fortunately, in the case of TNV-D, four base pairs in the lower part of SL-PRTE corresponded to opposing wobble positions (Fig. 3A), which allowed for substitutions of individual base pairs (Fig. 3B) or