Virology 454-455 (2014) 1–10

Contents lists available at ScienceDirect

Virology

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

Presence of poly(A) and poly(A)-rich tails in a positive-strand RNA virus known to lack 3' poly(A) tails

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ARTICLE INFO

Article history:
Received 13 November 2013
Returned to author for revisions
8 December 2013
Accepted 1 February 2014
Available online 20 February 2014

Keywords:
Tobacco mosaic virus
Poly(A) tails
RNA polyadenylation

ABSTRACT

Here we show that *Tobacco mosaic virus* (TMV), a positive-strand RNA virus known to end with 3' tRNA-like structures, does possess a small fraction of gRNA bearing polyadenylate tails. Particularly, many tails are at sites corresponding to the 3' end of near full length gRNA, and are composed of poly(A)-rich sequences containing the other nucleotides in addition to adenosine, resembling the degradation-stimulating poly(A) tails observed in all biological kingdoms. Further investigations demonstrate that these polyadenylated RNA species are not enriched in chloroplasts. Silencing of cpPNPase, a chloroplast-localized polynucleotide polymerase known to not only polymerize the poly(A)-rich tails but act as a 3' to 5' exoribonuclease, does not change the profile of polyadenylate tails associated with TMV RNA. Nevertheless, because similar tails were also detected in other phylogenetically distinct positive-strand RNA viruses lacking poly(A) tails, such kind of polyadenylation may reflect a common but as-yet-unknown interface between hosts and viruses.

Introduction

RNA polyadenylation involves addition of the homopolymeric poly(A) tails composed exclusively of adenosines, or sometimes heteropolymeric poly(A)-rich stretches containing the other three nucleotides as well, to 3' end of RNA substrates (Edmonds, 2002; Slomovic et al., 2006, 2008a, 2010). This post-transcriptional modification is a common event present in almost all organisms, but shows somewhat paradoxical roles. Stable poly(A) tails found on mature 3' end of most nucleus-encoded mRNAs in eukaryotes play roles in nucleocytoplasmic export and translation initiation, in addition to their more known role in stabilizing transcripts (Edmonds, 2002; Moore and Proudfoot, 2009). In bacteria, archaea and organelles, however, polyadenylation occurs mainly on the degradation intermediates of both coding and non-coding RNAs, thereby generating transient poly(A) or poly(A)-rich stretches as 'landing pads' to recruit 3'→5' exoribonucleases for further degradation (Slomovic et al., 2006a, 2008b; Condon, 2007; Regnier and Hajnsdorf, 2009; Schuster and Stern, 2009). Hence, the transient polyadenylate tails have been considered a tell-tale sign of the presence of polyadenylation-stimulated RNA degradation pathway (Slomovic et al., 2006a, 2008b).

The polyadenylation-stimulated RNA degradation mechanism, however, is not limited to the bacterial and bacterial-like genetic systems, non-abundant truncated nuclear transcripts having destabilizing poly(A) or poly(A)-rich tails were also detected in nuclei or cytoplasm of eukaryotes such as budding yeast (Kuai et al., 2004, 2005; Wyers et al., 2005; LaCava et al., 2005; Vanácová et al., 2005), fission yeast (Win et al., 2006), fruit fly (Nakamura et al., 2008), trypanosome (Cristodero and Clayton, 2007), *Arabidopsis thaliana* (Lange et al., 2008; Zakrzewska-Placzek et al., 2010) and human cells (Slomovic et al., 2006b, 2010). The fact that the poly(A)-stimulated RNA degradation occurs throughout the prokaryotes and eukaryotes reflects that stimulating RNA degradation is an ancestral role of polyadenylation (Slomovic et al., 2008a, 2008b; Houseley and Tollervey, 2009). Thus far, this evolutionarily conserved mechanism has been shown to play critical roles in rapidly removing 'unwanted RNAs', such as maturation byproducts, cryptic transcripts as well as the over-expressed, mutated, incorrectly folded or misprocessed RNAs, thereby maintaining the RNA quality of genome expression (Slomovic et al., 2010; Houseley and Tollervey, 2009; Lange et al., 2009; Lange and Gagliardi, 2011).

Viruses typically are not considered to be organisms, but are small infectious entities that can replicate only within the living cells of organisms. It is so far clear that RNA of many eukaryotic viruses, ranging from DNA to RNA viruses, has 3' poly(A) tails (King et al., 2012), which, however, are synthesized *via* not merely

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http://dx.doi.org/10.1016/j.virol.2014.02.002
0042-6822

a post-transcriptional manner, also the direct or reiteritive transcription of a poly(U) stretch in the template strand (Sanfaçon et al., 1991; Weichs an der Glon et al., 1993; Silver and Pagano, 1997; Poon et al., 1999; Chen et al., 2005; Steil et al., 2010; Bier et al., 2011; Ogram and Flanegan, 2011). Regardless of the synthesis mechanism used, the viral poly(A) tails have long been considered as a player in RNA stability and translation, mimicking roles of the stable poly(A) tails in eukaryotic mRNA (Dreher, 1999; Barr and Fearns, 2010). No evidence had come to light that the poly(A) tails function in destabilizing the viral RNA until recently. In HeLa cells infected with vaccinia virus (VV), a double-stranded DNA virus of the poxvirus family, types of non-abundant truncated mRNA molecules of VV containing poly(A) or poly(A)-rich tails were detected, resembling the polyadenylated degradation intermediates of the poly(A)-stimulated RNA decay pathway (Slomovic et al., 2010). This indicates that the VV mRNA is subjected to the poly(A)-stimulated degradation pathway. However, due to the sole piece of evidence as of today, it remains an open question whether the transient polyadenylation is widespread in viruses.

Positive-strand RNA viruses constitute the largest group of viruses, whose genome serves directly as mRNA for protein synthesis (Ahlquist et al., 2003; King et al., 2012). It is known, however, that many viruses within this group, do not have poly(A) tails but evolve 3'-termini as tRNA-like structure (TLS) or non-TLS heteropolymeric sequence (Het) instead (Dreher, 1999). In contrast to the hitherto widely accepted view, here we show that *Tobacco mosaic virus* (TMV), a positive-strand RNA virus believed to terminate with TLSs (Creager et al., 1999; Scholthof et al., 2011), does possess a certain number of gRNA molecules that are polyadenylated at sites corresponding to both full length and near full-length viral gRNA. Particularly, in addition to some poly(A) tails, most of the polyadenylate tails are of poly(A)-rich stretch resembling the degradation-stimulating poly (A)-rich tails previously observed in all biological kingdoms (Lisitsky et al., 1996; Mohanty and Kushner, 2000, 2010; Bralley and Jones, 2002; Campos-Guillén et al., 2005; Slomovic et al., 2006a, 2008b, 2010; Zimmer et al., 2009; Germain et al., 2011). In addition, similar types of polyadenylate tails were also found in *Odontoglossum ringspot virus* (ORSV), *Cucumber green mottle mosaic virus* (CGMMV), *Cucumber mosaic virus* (CMV), *Tobacco rattle virus* (TRV), *Turnip crinkle virus* (TCV) and *Tobacco necrosis virus* (TNV), six more positive-strand RNA viruses known to terminate with either TLSs or Hets. This, to our knowledge, is the first discovery of poly(A) and poly(A)-rich tails in positive-strand RNA viruses known to lack poly(A) tails, and suggest a so-far-unknown virus-host interacting but probably with respect to the polyadenylation-assisted RNA degradation.

Results

RNA-Seq of TMV-inoculated Chenopodium amaranticolor suggested TMV RNA bearing poly(A) tails

Using RNA sequencing (RNA-Seq), we recently constructed a foliar transcriptome database containing 112,453 unigenes based on the poly(A)$^+$ RNA extracted from the *Chenopodium amaranticolor* leaves that include healthy leaves, as well as TMV- and CMV-inoculated leaves (Zhang et al., 2012). Whilst the unigenes were searched against the NCBI NR database, we surprisingly noticed that one assembled sequence (Unigene 24636), which has a length of 6232 bp, matches nearly the complete genome of TMV U1 (#NC_001367). In contrast, none of the unigenes matched any of the tripartite genomes of CMV. The immediate application of this transcriptome data to analyze the digital gene expression (DGE) profiles of the TMV-inoculated *C. amaranticolor* leaves identified numbers of tags mapped to Unigene24636. In concert with the course of virus multiplication in host plants, the number of these tags increased sharply from 86 at 6 hours post inoculation (hpi) to 2776 at 28 hpi.

The presence of TMV RNA in the transcriptome and DGE profiles implied that the TMV RNA might bear poly(A) tails. If so, this will greatly challenge the traditional view that the TMV RNA terminate with TLSs rather than poly(A) tails (Creager et al., 1999; Scholthof et al., 2011). However, an alternative possibility we could not rule out was that the poly(A)$^+$ RNA isolated from the TMV-inoculated leaves might have some contamination with the TMV RNA probably due to its nonspecific interaction with oligo(dT) magnetic beads. Further experiments were therefore carried out here below to clarify if TMV RNA has 3' poly(A) tails indeed.

Characterization of the polyadenylate tails associated with TMV RNA

The approach of oligo(dT) primed RT-PCR was pursued to this end (Fig. 1A). Total RNA extracted from the TMV-inoculated leaves of *C. amaranticolor*, *Arabidopsis thaliana* Col-0 and *Nicotiana benthamiana* were individually reverse transcribed with an anchored oligo(dT) primer PT_{18}, and then PCR amplified with two nested primer pairs P1/TMV-5372-94 and P2/TMV-6023-44 (Fig. 1A and Table S1). The resulting PCR products were subcloned and sequenced. Taking this approach, we indeed isolated the TMV RNA molecules bearing ploy(A) or poly(A)-rich tails in each of the aforementioned plant species infected with TMV (Fig. 1B and Fig. S1). By contrast, no such TMV RNA molecules were identified in a control experiment, in which 1 μg RNA mixture containing 0.1 μg *in vitro* TMV RNA transcripts lacking poly(A) tails and 0.9 μg total RNA from uninfected *N. benthamiana* leaves was analyzed with the same approach (data not shown), ensuring that the polyadenylate tails of TMV RNA was not of amplification artifacts. Taken together, the data conclusively demonstrated that TMV RNA had 3' poly (A) tails. Moreover, the presence of the polyadenylated TMV RNA in various hosts belonging to evolutionarily distant plant families indicated that the viral RNA polyadenylation is not host-dependent but occurs widely in hosts of TMV.

In an effort to decipher nature of the poly(A) and poly(A)-rich tails associated with TMV RNA, we totally analyzed 65 polyadenylated viral RNA molecules that were cloned from the TMV-inoculated *N. benthamiana* as described above. In addition to 15 RNA molecules bearing poly(A) tails comprised exclusively of adenosines, a large number of RNA had poly(A)-rich tails, a mixture of predominantly adenosines (88.68%), guanosines (5.66%), uridines (5.53%) and cytosines (1.57%). Notably, these poly(A)-rich tails with heterogeneity confined to their 5' ends, but terminated in homogenous stretches of adenosines (Fig. 1B), possibly due to the 3' bias of oligo(dT)-dependent detection.

The homopolymeric poly(A) tails ranged in size from 12 nt to 37 nt, while the length of the poly(A)-rich tails varied significantly from 14 nt to 78 nt. However, considering that the oligo(dT)-dependent RT-PCR does not allow determination of the maximum length of the poly(A) and poly(A)-rich tails as previously discussed (Lisitsky et al., 1996; Bralley and Jones, 2002; Slomovic et al., 2010), the real tails associated with TMV RNA should be longer than those we detected here. In addition, the adenosines within the poly(A)-rich tails, in contrast to the pure adenosines of the poly(A) tails, were most often clustered with other residues interspersed. Length of the contiguous adenosines in poly(A)-rich tails varied, with 16∼20-nt stretches occurring most frequently, while almost all stretches of the clustered non-adenosine residues was not longer than four residues (Fig. 1B). These features are similar with those of the previously observed poly (A)-rich tails in *Escherichia coli* (Mohanty and Kushner, 2000), spinach, Arabidopsis and Chlamydomonas chloroplasts (Lisitsky et al., 1996; Zimmer et al., 2009), *Streptomyces coelicolor* (Bralley and Jones, 2002), *Bacillus subtillis* (Campos-Guillén et al., 2005) and human cells