Short Communication

Productive life cycle of adeno-associated virus serotype 2 in the complete absence of a conventional polyadenylation signal

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We showed that WT adeno-associated virus serotype 2 (AAV2) genome devoid of a conventional polyadenylation [poly(A)] signal underwent complete genome replication, encapsidation and progeny virion production in the presence of adenovirus. The infectivity of the progeny virion was also retained. Using recombinant AAV2 vectors devoid of a human growth hormone poly(A) signal, we also demonstrated that a subset of mRNA transcripts contained the inverted terminal repeat (ITR) sequence at the 3' end, which we designated ITR in RNA (ITRR). Furthermore, AAV replication (Rep) proteins were able to interact with the ITRR. Taken together, our studies suggest a new function of the AAV2 ITR as an RNA element to mediate transgene expression from poly(A)-deleted mRNA.

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Adeno-associated virus (AAV) was first discovered as a contaminant of adenoviral stocks in the 1960s (Atchison et al., 1965). Recombinant AAV (rAAV) vector-based gene therapy has attracted increasing interest worldwide (Ling et al., 2014). The most extensively studied serotype of AAV is type 2 (AAV2), which serves as a prototype for the AAV family. The AAV2 genome is a ssDNA of approximately 4.7 kb (Srivastava et al., 1983). It is flanked by a 145 nt inverted terminal repeat (ITR) that forms a T-shaped, base-paired hairpin structure, and that contains cis-elements required for viral genome replication, encapsidation (McLaughlin et al., 1988) and integration into, as well as rescue from, host chromosomal DNA (Nennenmacher & Weber, 2012). As illustrated in Fig. 1(a), the AAV2 genome contains two ORFs. The left of these, rep, encodes four non-structural proteins required for replication (Rep78, Rep68, Rep52 and Rep40), whereas the right one, cap, encodes three structural proteins that make up the viral capsid (VP1, VP2 and VP3). Three viral promoters were identified by their relative map position within the viral genome: p5, p19 and p40. Productive AAV2 infection requires helper functions that can be supplied either by co-infecting helper viruses or by DNA damaging agents. Helper viruses include adenovirus (Hoggan et al., 1966), herpes simplex virus (Buller et al., 1981), vaccinia virus (Schlehofer et al., 1986), cytomegalovirus (McPherson et al., 1985), varicella-zoster virus (Georg-Fries...
et al., 1984) and human papillomavirus (Cao et al., 2012). Early studies have documented that, in the presence of adenovirus, all major AAV2 viral transcripts are polyadenylated (Carter & Rose, 1974; Laughlin et al., 1979). Subsequently, a potential 250 nt polyadenylation [poly(A)] signal, containing the critical sequence 5'-AATAAA-3', was identified in the AAV2 genome (Srivastava et al., 1983). However, it is also well known that the conventional AAV2 poly(A) signal is weak. Thus, in all rAAV vectors, stronger poly(A) signal sequences such as the human growth hormone (hGH) poly(A) signal, rather than the relatively weak AAV2 poly(A) sequences, are used (Liu et al., 2013c) to mediate transgene expression. In the present study, we wished to examine experimentally whether the WT AAV2 could also maintain its productive life cycle when devoid of its conventional poly(A) signal.

![Gene expression, genome replication and progeny virus production from plasmids containing WT and poly(A)-deleted AAV2 genomes.](http://vir.sgmjournals.org/2781)

**Fig. 1.** Gene expression, genome replication and progeny virus production from plasmids containing WT and poly(A)-deleted AAV2 genomes. (a) Schematic outline of plasmids containing WT and poly(A)-deleted AAV2 genomes and the location of quantitative reverse transcription-PCR (qRT-PCR) primer sets. Arrows indicate WT AAV2 promoters; bold, italic, underlined letters indicate the stop codon of the cap gene; italic letters indicate the NotI restriction site; and underlined letters indicate the ITR sequence. (b) WT and poly(A)-deleted AAV2 genome rescue and replication. HEK293 cells were transfected with indicated plasmids. Low-molecular-mass DNA was isolated at 72 h post-transfection and subjected to DpnI digestion for 4 h, followed by Southern blot analysis using Rep-specific, ^32P-labeled probes. (c) WT and poly(A)-deleted AAV2 gene expression. HEK293 cells were transfected with indicated plasmids. Western blot assays were performed at 48 h post-transfection using anti-Rep (clone 259.5, upper panel) and anti-Cap (clone B1, middle panel) mAbs (American Research Products). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mAb was used to quantify the protein loading (lower panel). (d) HEK293 cells were transfected with plasmids pHelper and pSub201-AD. Total RNA was isolated at 24 h post-transfection. One microgram of RNA was subjected to qRT-PCR assays using different sets of primers indicated in (a). DNase- and RNase-free water and 1 ng plasmid were used as negative and positive controls, respectively. (e–g) Highly purified viral stocks were generated by transfection of HEK293 cells with pSub201 and pHelper (lane 1), pSub201, pACG2 and pHelper (lane 2), pSub201-AD and pHelper (lane 3) and pSub201-AD, pACG2 and pHelper (lane 4). Ten microlitres of each viral stock was used for analysis. (e) Quantitative DNA slot blots for determining viral titre. Twofold serial dilutions of the viral stocks were applied to membranes and hybridized with Rep-specific, ^32P-labelled probes. Plasmid pSub201 (1 and 10 ng) was used as an appropriate control. (f) Alkaline Southern blot analysis of the nature of the WT and poly(A)-deleted AAV2 genomes. (g) Western blot analysis of the denatured viral capsids from viral stocks.
A recombinant plasmid containing the poly(A)-deleted AAV2 genome, designated pSub201-AD, was generated based on a PCR strategy (Zhang et al., 2014b). Its parental plasmid, pSub201, has been previously reported (Samulski et al., 1987) and contains the entire WT AAV2 genome (Fig. 1a). The stop codon (TAA) for the cap gene in pSub201-AD is adjacent to the right ITR, with a NotI restriction enzyme site between them. There is no poly(A) signal (‘-AATAAA-3’-like sequence) within the ITR. Although the WT AAV2 genome contains an internal ‘-AATAAA-3’ sequence in the rep gene, a number of previously published studies have established that this pseudo-poly(A) site is not used in the context of the AAV2 genome (Fasina & Pintel, 2013; Qiu & Pintel, 2002, 2004; Qiu et al., 2004). Rescue and replication assays were performed following transfection of the AAV plasmid, with pHelper and/or pACG2 in HEK293 cells. Plasmid pHelper provides essential adenoviral genes (e2a, e4orf6 and vrn1) to support the AAV life cycle in HEK293 cells, whilst plasmid pACG2 provides WT AAV2 rep and cap genes. Together, they facilitate replication and viral encapsidation of any DNA materials flanked by two ITRs and thus served as a positive control in our experiments. At various time points post-transfection, low-molecular-mass DNA samples were isolated, digested extensively with DpnI to degrade input plasmids and subjected to Southern blot analysis as described previously (Ling et al., 2015). As seen in Fig. 1(b), no rescue or replication of the AAV2 genome from either plasmid occurred in the absence of helper plasmids (lanes 1 and 4). However, in the presence of only pHelper, efficient rescue and replication of the AAV2 genomes occurred from both pSub201 and pSub201-AD plasmids (Fig. 1b, lanes 2 and 5). As it is well-accepted that rescue and replication of the AAV2 genome absolutely requires the presence of functional AAV2 Rep proteins, these results suggested that adequate levels of the AAV2 Rep proteins were expressed from the poly(A)-deleted pSub201-AD plasmid. This was further confirmed by Western blot analysis (Lin et al., 2013a) at 48 h post-transfection, as shown in the upper panel in Fig. 1(c). Similarly, the viral Cap proteins were expressed from both pSub201 and pSub201-AD plasmids (Fig. 1c, middle panel), in the presence of pHelper. To rule out the possibility that transcription may utilize alternative poly(A) signals in the plasmid backbone, HEK293 cells were transfected with plasmids, pSub201-AD and pHelper. Total RNA was isolated at 48 h post-transfection using the Trizol method (Han et al., 2015) and an RNeasy Mini kit (Qiagen) (Liu et al., 2013a). One microgram of RNA was reverse transcribed using random primers and subjected to quantitative PCR (qPCR) assays using four sets of primers, as shown in Fig. 1(a). The first set was located at the start of the rep gene, the second at the start of the cap gene, the third upstream of the left ITR and the last downstream of the right ITR. In addition, 1 ng placid DNA and DNase- and RNase-free water were used as appropriate controls and subjected to quantitative reverse transcription-PCR (qRT-PCR) assays using the same sets of primers. The results clearly indicated that transcripts from the poly(A)-deleted AAV2 plasmid did not utilize alternative poly(A) signals in the plasmid backbone (Fig. 1d).

We next examined whether poly(A)-deleted AAV2 genomes could undergo successful encapsidation into viral capsids, which are expressed from the same genome. To this end, a standard double- or triple-plasmid transfection and purification protocol was used to generate AAV progeny virions (Liu et al., 2013b). Following digestion with Benzonase (Roche) to degrade any unencapsidated DNA, equivalent amounts of viral stocks were deproteinized to release the rAAV genomes. Twofold serial dilutions were then analysed on quantitative DNA slot blots as described previously (Wang et al., 2012). These results, shown in Fig. 1(e), demonstrated that the presence of pHelper plasmid was necessary and sufficient for efficient packaging of the poly(A)-deleted AAV2 genomes, whilst an additional supply of AAV proteins by the plasmid pACG2 may further increase the progeny virion production. Analysis of purified DNA from both viral stocks on alkaline agarose gels, followed by Southern blot assays revealed similar levels of the >4.5 kb viral genomes (Fig. 1f), as well as the viral capsid proteins on SDS-PAGE gels (Mondal et al., 2014), followed by Western blot analysis. The conventional 1 : 1 : 10 ratio of VP1 : VP2 : VP3 proteins (Fig. 1g) was revealed.

Rescue and replication of the AAV2 genomes from recombinant plasmids might not truly reflect a natural course of infection. Thus, we performed viral DNA replication and protein expression assays using viral stocks generated from plasmids pSub201 and pSub201-AD. HEK293 cells were infected at 5000 viral genomes per cell for 2 h with each viral stock, with and without co-transfection with plasmids pACG2 and/or pHelper using lipofectamine (Wang et al., 2014). Low-molecular-mass DNA samples isolated at 72 h post-infection were analysed on Southern blots. The results demonstrated that both viral stocks were biologically active but only in the presence of adenoviral genes (Fig. 2a, lanes 2 and 3, and lanes 5 and 6, respectively), further corroborating the suggestion that pHelper plasmid alone is necessary and sufficient for efficient replication of poly(A)-deleted AAV2. The kinetics and the extent of poly(A)-deleted AAV2 genome replication were also determined by time-dependent accumulation of the AAV DNA replicative intermediates and comparison with their parental WT AAV2 genome (Fig. 2b). Furthermore, Western blot analyses revealed that reduced but detectable levels of viral rep and cap genes were expressed from poly(A)-deleted viral stocks (Fig. 2c).

Adenovirus, and not pHelper plasmid, is the natural helper for WT AAV2. To further mimic a natural infection, we also performed viral DNA replication and gene expression assays using both AAV2 viral stocks in the absence or presence of co-infection with WT adenovirus serotype 2 (Ad2). Whereas no replication occurred in the absence of Ad2 (Fig. 2d, lanes 1 and 4), an m.o.i. of as low as 0.5 was sufficient for efficient replication of the poly(A)-deleted AAV2 genome (Fig. 2d, lanes 2 and 5). The
**Fig. 2.** Gene expression, genome replication and progeny virus production from virus containing WT (vSub201) and poly(A)-deleted AAV2 (vSub201-AD) genomes. (a, b) WT and poly(A)-deleted AAV2 genome replication. HEK293 cells were infected with vSub201 or vSub201-AD virus and then mock transfected or transfected with the indicated plasmids. Low-molecular-mass DNA was isolated at 72 h (a) or at various time points (b) post-transfection and subjected to DpnI digestion for 4 h, followed by Southern blot analysis using Rep-specific, $^{32}$P-labelled probes. The monomeric (m) and dimeric (d) forms of replicative DNA intermediates are indicated. (c) WT and poly(A)-deleted AAV2 gene expression. HEK293 cells were infected with vSub201 or vSub201-AD virus and then transfected with the indicated plasmids. Western blot assays were performed at 48 h post-transfection using anti-Rep (clone 259.5, upper panel) and anti-Cap (clone B1, middle panel) mAbs (American Research Products). Anti-GAPDH mAb was used to quantify the protein loading (lower panel). (d, e) WT and poly(A)-deleted AAV2 genome replication and gene expression in the presence of WT adenovirus serotype 2 (Ad2). HEK293 cells were infected with vSub201 or vSub201-AD virus and co-infected with Ad2 at various m.o.i. (d) Low-molecular-mass DNA was isolated at 72 h post-transfection and subjected to DpnI digestion for 4 h, followed by Southern blot analysis using Rep-specific, $^{32}$P-labelled probes. (e) Western blot assays were performed at 48 h post-transfection. (f–h) Highly purified viral stocks were generated by co-infection of HEK293 cells with vSub201 and Ad2 (lane 1) and vSub201-AD and Ad2 (lane 2). Ten microlitres of each secondary viral stock was used for the analysis (lanes 3 and 4). (f) Quantitative DNA slot blots for determining viral titre. Twofold serial dilutions of the viral stocks were applied to membranes and hybridized with Rep-specific, $^{32}$P-labelled probe. Plasmid pSub201 (1 and 10 ng) was used as an appropriate control. (g) Alkaline Southern blot analysis of the nature of the WT and poly(A)-deleted AAV2 genomes. (h) Western blot analysis of denatured viral capsids from viral stocks following secondary infection.
AAV2 Rep and Cap protein were also expressed (Fig. 2e, lanes 2 and 5), although a relatively higher m.o.i. of Ad2 co-infection was required for the poly(A)-deleted viral stocks (Fig. 2e, lane 6). These results nonetheless showed that, in the presence of Ad2, the poly(A)-deleted AAV2 was capable of expressing both viral gene products as well as undergoing successful DNA replication. Following secondary infections, the progeny virions were both generated from parental WT and poly(A)-deleted AAV2, as determined by quantitative DNA slot blots (Fig. 2f). The characteristics of the genomes and capsid proteins from the secondary progeny virions were also confirmed by Southern blot assays (Fig. 2g) and Western blot assays (Fig. 2h), respectively.

To study the function of the ITR at the 3’ end of the mRNA, we generated a single-stranded, poly(A)-deleted rAAV2 vector, ssAAV2-hrGFP-AD, derived from a commercially available recombinant vector, ssAAV2-hrGFP, carrying a humanized recombinant GFP (hrGFP) driven by a cytomegalovirus promoter and a strong hGH poly(A) signal (Fig. 3a). To examine whether the right ITR was transcribed as part of the mRNA, both HEK293 and HeLa cells were transduced with ssAAV2-hrGFP or ssAAV2-hrGFP-AD vectors at 5000 viral genomes per cell under conditions described previously (Cheng et al., 2012). Total RNAs were isolated at 24 h post-transduction and subjected to qRT-PCR using standard protocols (Li et al., 2015). The use of random primers (Fig. 3b, left) demonstrated that in either cell type, both vectors expressed similar levels of mRNAs, after adjustment to β-actin mRNA levels. In contrast, the use of oligo-d(T) primers (Fig. 3c, right) allowed the detection of mRNAs expressed from ssAAV2-hrGFP vectors but not those from ssAAV2-hrGFP-AD vectors, indicating that the transcripts generated by the latter contained no poly(A) tail. Most importantly, using primers specific for the terminal ITR sequence (5’-TTGGCCACCTCCTCTCTGCG-3’), we revealed that the mRNA transcripts derived from the ssAAV2-hrGFP-AD vectors contained the whole ITR sequence (Fig. 3c). Surprisingly, a small fraction of transcripts produced by the ssAAV2-hrGFP vectors also contained these sequences. Little signal could be detected in these assays in the absence of reverse transcriptase, representing the background levels of contaminating DNA. Taken together, we designated this novel RNA element as the ITR in RNA (ITRR).

As AAV2 Rep proteins are known to interact with the ITRs in the viral DNA genome (Ashktorab & Srivastava, 1989; Im & Muzyczka, 1989), we reasoned that such interaction might also occur with the ITRR sequences in the mRNA transcripts. To this end, a recombinant plasmid, pRep-TAP, was used (Nash et al., 2009), in which a TAP tag was placed at the C terminus of the rep ORF. A series of co-immunoprecipitation (co-IP) assays were carried out as described previously (Lin et al., 2013b) using the TAP tag to pull down protein–mRNA complexes. The efficiency of IP assays was first evaluated. HEK293 cells were either mock transfected (Fig. 3d, lanes 1 and 2) or transfected with pRep-TAP and pHelper (Fig. 3d, lanes 3 and 4). At 48 h post-transfection, whole-cell extracts were subjected to IP assays using anti-TAP antibody (Fig. 3d, lanes 2 and 4). Immunoprecipitates were separated by electrophoresis and detected on Western blots using Rep-specific antibody. A volume of 5 % of the whole-cell lysates was also separated by electrophoresis as a protein loading control (Fig. 3d, lanes 1 and 3). Thus, we concluded that ~20–25 % of total Rep-TAP proteins could be immunoprecipitated. HEK293 cells were then transfected with pRep-TAP and pHelper plasmids, followed by transduction with ssAAV2-hrGFP or ssAAV2-hrGFP-AD vector at 10000 viral genomes per cell for 2 h. Consistent with our previous data, both vectors resulted in similar levels of mRNA transcripts at 24 h post-transduction (Fig. 3e). Rep-TAP proteins from whole-cell lysates were subsequently immunoprecipitated using anti-TAP antibody, followed by total RNA extraction from the co-immunoprecipitates. IgG was used as an appropriate control. RNA samples were subjected to reverse transcription assays using either random primers or oligo-d(T) primers (Fig. 3f), followed by qPCR assays (Zhang et al., 2014a) using primers specific for hrGFP. As can be seen, when random primers were used, the levels of co-immunoprecipitated mRNA transcripts generated from the ssAAV2-hrGFP-AD vectors were approximately fourfold higher than those from the ssAAV2-hrGFP vectors, which is consistent with our previous data showing that ~15 % of mRNA transcripts produced by ssAAV2-hrGFP vectors contained the ITR sequences (Fig. 3c). In addition, the results using oligo-d(T) primers indicated that the co-immunoprecipitated mRNA transcripts from the ssAAV2-hrGFP-AD vectors did not contain a poly(A) tail. In contrast, the IgG control groups showed no differences using either random or oligo-d(T) primers, reflecting the background of the co-IP assays. Thus, we concluded that AAV2 ITRR sequences present at the 3’ ends of mRNA transcripts have the ability to recruit AAV2 Rep proteins. To obtain direct evidence that the ITRR is functional in permitting translation of poly(A)-deleted mRNA, in vivo transfection assays were performed. PCR products were generated using pAAV2-hrGFP-AD plasmid as template and primer F and primer R1/R2 as primers. Alternatively, PCR products were generated using pAAV2-hrGFP plasmid as a template and primer F and primer R1/R2 as primers. Following gel purification, PCR products were transfected into HEK293 cells. As shown in Fig. 3g, it was evident that the transgene cassette containing no poly(A) signal resulted in no hrGFP expression. However, the poly(A)-deleted transcripts with the ITRR sequences at the 3’ end resulted in visible hrGFP expression. Thus, we concluded that ITRR is functional in permitting translation of the mRNA and its efficiency is approximately 3 % of a conventional hGH poly(A) sequence.

The addition of a poly(A) tail in 3’ processing is an essential step in the maturation of all mRNAs. However, metazoan replication-dependent histone mRNAs are exceptions, as
Fig. 3. Characterization of the 3’ end of rAAV2 transcripts and their ability to bind to AAV Rep proteins. (a) Schematic structures of rAAV2 vectors and the location of PCR primers. CMVp, cytomegalovirus promoter. (b, c) HEK293 or HeLa cells were infected with ssAAV2-hrGFP or ssAAV2-hrGFP-AD virus at 5000 viral genomes per cell for 2 h. Total RNAs were extracted at 24 h post-infection and subjected to reverse transcription using random primers (left graph), oligo-d(T) primers (right graph) (b) or ITR primers (c), followed by qPCR assays specific for hrGFP. (d) Efficiency of co-IP assays. HEK293 cells were either mock transfected (lanes 1 and 2) or transfected with plasmids pRep-TAP and pHelper (lanes 3 and 4). IP was performed at 48 h post-transfection with an anti-TAP antibody (lanes 2 and 4) and Western blot analysis was performed with a Rep-specific antibody. A volume of 5% of the whole-cell lysates was used as a protein loading control (lanes 1 and 3). (e, f) HEK293 cells were transduced with ssAAV2-hrGFP or ssAAV2-hrGFP-AD vector at 10 000 viral genomes per cell for 2 h, followed by transfection with pRep-TAP and pHelper plasmids. (e) Total RNA was isolated at 24 h post-transfection, followed by qRT-PCR assays using hrGFP-specific primers. Data were normalized with β-actin mRNA. (f) Protein–RNA complex was immunoprecipitated by either IgG or anti-TAP antibody. Co-immunoprecipitated RNAs were reverse transcribed using random primers or oligo-d(T) primers. qRT-PCR assays were performed using hrGFP-specific primers. Data were normalized against the mean mRNA copy numbers in each group infected with ssAAV2-hrGFP vectors. (g) PCR products using plasmid pAAV-hrGFP or pAAV-hrGFP-AD as a template and Primer F and R1/R2/R3 as primers were gel purified. The same amount of DNA was transfected into HEK293 cells. hrGFP expression was determined by fluorescence microscopy and the ImageJ program (http://imagej.nih.gov/ij/) at 48 h post-transfection.
they are cleaved but not poly(A)denylated, displaying a stem–loop structure at their 3′ end. Without the help of poly(A) sites, stem–loop-binding protein binds the stem–loop and regulates canonical histone mRNA metabolism. Similarly, we demonstrated here that poly(A)-deleted AAV2 virus can undergo its whole life cycle successfully, and the stem–loop structure of the ITR can bind with Rep to mediate gene expression. Moreover, the data presented in this study on poly(A)-deleted AAV2 virus and poly(A)-deleted rAAV2 vectors complement and corroborate each other. Using rAAV2 vectors, we were able to demonstrate that, in the absence of a conventional poly(A) signal, the viral 3′-end ITR is transcribed as part of the mRNA, designated ITRR, which interacts with the AAV Rep proteins. Similarly, poly(A)-deleted AAV2 has the ability to maintain a complete life cycle in the presence of co-infection with adenovirus. The use of plasmid pHelper, instead of adenovirus associated virus (AAV) type 8 vectors for transduction of target cells.

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