Replication and in vivo repair of the hepatitis A virus genome lacking the poly(A) tail

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The precise role of the poly(A) tail at the 3' end of the picornavirus RNA genome and the cellular factors that control its homeostasis are unknown. To assess the importance of the poly(A) tail for virus replication, the genome of the slowly replicating hepatitis A virus (HAV) with and without a poly(A) tail was studied after transfection into cells maintained under various conditions. A tailless HAV genome had a shorter half-life than a poly(A)-containing genome and was unable to replicate in quiescent cells. In dividing cells, the tailless RNA gave rise to infectious virus with a restored poly(A) tail of up to 60 residues. Cells arrested at the G₀ and the G₂/M phase produced lower amounts of infectious HAV than cells in the G₁ phase. These data suggest that the 3' poly(A) tail of HAV can be restored with the help of a cellular and/or viral function that is regulated during the cell cycle.

The picornavirus genome is a positive-sense RNA molecule with a central open reading frame encoding a polyprotein that is flanked by highly structured, non-translated regions (5' NTR and 3' NTR) and a 3' poly(A) tail. The terminal regions of the viral genome – the 5' and 3' origins of replication (Paul, 2002) – were identified as important cis-acting replication elements that are required for initiation of plus- and minus-strand synthesis. As minus-strand synthesis is initiated internally within the 3' poly(A) tail (Herold & Andino, 2001), it is still an open question by what mechanism the length of the poly(A) tail is maintained. The poly(A) tail is hypothesized to be indispensable for the formation of a protein-bridged, ‘closed-loop’ structure by bringing the 5' and 3' RNA ends into close proximity. RNA circularization mediated by the poly(A)-binding protein (PABP) and other proteins was demonstrated for both cellular cap-dependent and polioviral cap-independent translation (Sachs et al., 1997; Bergamini et al., 2000; Kean, 2003). Removal or shortening of the poly(A) tail resulted in loss of infectivity or retarded replication of polioviral RNA (Sarnow, 1989; Herold & Andino, 2001). For mammalian gene expression, poly(A)-tail lengthening can increase mRNA stability and translatability, whereas translational repression and RNA degradation often follow poly(A) removal.

There is increasing evidence that the biological properties of the hepatitis A virus (HAV) are unique among those of the picornaviruses. HAV replication in cell culture is slow, persistent and highly asynchronous, and little is known about the rate-limiting steps in the virus life cycle. To gain further insight into the regulatory steps of HAV replication, we analysed the role of the cell cycle and the length of the viral 3' poly(A) tail.

To assess the role of the 3' poly(A) tail of the HAV genome, we examined the replication efficiency of HAV RNA with and without a poly(A) tail in quiescent cells. In vitro transcripts were prepared from linearized plasmids (Kusov & Gauss-Müller, 1999; Kusov et al., 2001) that encoded the genome of HAV strain 18f with a tail of 14 (18f-A14) or no (18f-A0) adenosine residues, followed by the genomic ribozyme of hepatitis δ virus (HDV). The 3' product of the HDV ribozyme was shown to serve as substrate for polyadenylation (Düvel et al., 2003). After RNA transfection into human hepatoma cells (Huh-7) that were rendered quiescent by serum starvation (Feuer et al., 2002), virus replication was assessed by RT-PCR amplification of a 0.3 kb fragment within the 5' NTR (Kusov et al., 2001) and by viral-particle identification using a particle-specific ELISA (Kusov & Gauss-Müller, 1999). RNA 18f-A0 was non-infectious, as neither viral particles (Fig. 1a, open circles) nor infectious virus (data not shown) was recovered [see also Kusov et al. (2001)]. In contrast, HAV RNA with a poly(A) tail of 14 residues was infectious, with viral antigen detectable approximately 14 days post-transfection (p.t.) (Fig. 1a, filled circles). Input RNA 18f-A0 was demonstrable by RT-PCR (day 0) and became undetectable at 6 days p.t. (Fig. 1b, upper panel). In contrast, RNA 18f-A14 was detectable for 3 days, proving its enhanced stability in vivo (Fig. 1b, lower panel). No or very small amounts of RT-PCR products, detectable on days 6 and 9 p.t., indicated loss of input RNA below the detection limit and a delay of de novo RNA synthesis. Newly synthesized viral
genomes of RNA 18f-A14 were found 15 days p.t. The poly(A)-tail length of the rescued virus was determined by a method described previously and consisted of about 60 residues, a tail length similar to that of HAV isolated from infected patients or cell culture (Siegl et al., 1981; Kusov et al., 2001, 2002). The results implicate that, possibly owing to its reduced stability, the tailless HAV RNA was unable to initiate genome replication in quiescent cells.

Infectious virus was rescued not only after transfection of in vitro RNA transcripts, but also after cDNA transfection into Huh-T7 cells, which express T7 RNA polymerase constitutively (Schultz et al., 1996). To compare the infectivity of HAV cDNAs in a reverse-genetics approach, plasmid DNAs encoding the HAV genome with and without a poly(A) tail (pT7-18f-A14 and pT7-18f-A0) were transfected into Huh-T7 cells (Gauss-Müller & Kusov, 2002; Kusov et al., 2001, 2002) and the rescue of infectious virus was assessed by the accumulation of viral particles. In vivo transcripts with a tail of 14 adenosine residues produced viral antigen and infectious virus 10 days after pT7-18f-A14 cDNA transfection (Fig. 1c, filled circles). Intriguingly, under the same transfection conditions, the poly(A)-lacking plasmid pT7-18f-A0 exhibited delayed replication kinetics, giving rise to infectious virus 30 days p.t. (open circles). When assayed by the G-tailing method for tail length (Kusov et al., 2001), the rescued viruses acquired a poly(A) tail with a length indistinguishable from that of the wild-type virus. No infectious virus was rescued from the lethally mutated pT7-18f-A0-mut cDNA (Fig. 1c, open triangles). Note that, possibly due to higher specific infectivity of the in vivo-compared with the in vitro-produced transcripts, infectious virus was detected at earlier time points when cDNA was used for transfection (Fig. 1c) than when synthetic RNA was transfected (Fig. 1a). As in vivo transcription of transfected DNA continues for several days (Y. Kusov & V. Gauss-Müller, unpublished observations; Graeber et al., 1998), the data imply that the continuously in vivo-generated transcripts could function as templates for translation and replication. The sustained in vivo transcription allowed the transcripts to become substrates of adenylating enzymes (see below).

For cytolytic picornaviruses (e.g. poliovirus and coxsackievirus), it was shown that the cell-cycle status affects virus replication, persistence and reactivation (Eremenko et al., 1972; Feuer et al., 2002). Moreover, stationary and dividing somatic cells differ in their content of factors that regulate the poly(A)-tail length (Kazazoglou et al., 1987; Colgan

Fig. 1. HAV replication of the HAV genome in quiescent cells depends on the presence of the poly(A) tail. (a) HAV-particle formation after transfection of the tailless RNA 18f-A0 (○) and RNA 18f-A14 (●) into non-dividing Huh-7 cells. No replication was detected for the replication-incompetent RNAs 18f-A0-mut and 18f-A14-mut, which have a mutation in the polymerase gene (data not shown). Freshly prepared in vitro transcripts (1 μg in 10 cm²) were transfected in duplicate or triplicate into Huh-7 cells with DMRIE-C reagent [3 μl (μg RNA)] as described by the supplier (Invitrogen). (b) RT-PCR detection of viral RNA found after transfection of RNA 18f-A0 and 18f-A14 into quiescent Huh-7 cells at various time points. Even though the amount of recovered RNA 18f-A0 was higher on day 0 than that of RNA 18f-A14, the tailless RNA disappeared by day 9 p.t. Viral RNA preparation and the RT-PCR protocol with primers amplifying a portion of the 5′ NTR were described previously (Kusov et al., 2001). (c) HAV-particle formation after transfection of equal amounts of cDNA pT7-18f-A0 (○), pT7-18f-A0-mut (△) or pT7-18f-A14 (●) into Huh-T7 cells. All plasmids encode the HDV ribozyme downstream of the poly(A) tail. pT7-18f-A0-mut carries a mutation in the HAV polymerase gene. HAV replication was determined by viral-antigen accumulation using an HAV particle-specific ELISA.
et al., 1996; Mendez & Richter, 2001). These reports and our observations (Fig. 1c, open circles) that the tailless vector-encoded viral genome was infectious prompted us to study the role of cell division in the infectivity of HAV transcripts lacking a poly(A) tail. In contrast to the experiment shown in Fig. 1(a), RNAs 18f-A0 and 18f-A14 were transfected into Huh-7 cells that were actively dividing by passaging them at 1 day p.t. and then every 3 days. The data show clearly that RNA 18f-A0 (Fig. 2a, open circles) was able to produce increasing amounts of viral particles, albeit with delayed kinetics compared to those of RNA 18f-A14 (filled circles).

Compared to quiescent cells (Fig. 1a), replication of RNA18f-A14 was faster in dividing cells. The poly(A)-tail length of viral isolates originating from RNA 18f-A0 transfection and rescued 30 days p.t. was found to be 36–60 residues, as determined by the G-tailing method described previously (Kusov et al., 2001). The replication-defective mutant RNA 18f-A0-mut was unable to initiate an infectious replication cycle (Fig. 2a, open triangles).

To substantiate the infectivity of the tailless transcripts, the subcellular locations of HAV RNA and of the non-structural protein 2B were visualized by fluorescence microscopy (Fig. 2c).

**Fig. 2.** Time course of virus rescue after transfection of HAV RNAs 18f-A0 (○ and grey bars), 18f-A14 (● and black bars) and 18f-A0-mut (▼) into actively dividing Huh-7 cells. (a) HAV replication was determined by viral-antigen accumulation using a particle-specific ELISA (A₄₅₀). (b) Number of Huh-7 cells that tested positive for plus-strand RNA [FISH(+), left-hand axis and lines] and positive for minus-strand RNA [FISH(−), right-hand axis and columns] by FISH (also see Fig. 3a). An FITC-labelled, strand-specific riboprobe covering nt 744–6998 of the HAV genome was used to detect minus-strand RNA. (c) Plus-strand (+) and minus-strand (−) HAV RNA was detected by FISH (left panels) and non-structural protein 2B was visualized by immunofluorescence (right panels) after transfection of RNAs 18f-A14 (upper row), 18f-A0 (middle row) or 18f-A0-mut (lower row) into actively dividing Huh-7 cells. RNA and protein 2B appear in a dot-like pattern and localize mainly to the periplasmic region.
protein 2B were determined in the same experiment. Immunofluorescence and fluorescent in situ hybridization (FISH) were applied (Gosert et al., 1996, 2000). At 9 days p.t., using a fluorescein isothiocyanate (FITC)-labelled, strand-specific riboprobe, plus-strand HAV RNA was detectable as small dots in the cytoplasm of approximately 5% of cells transfected with RNA18f-A14 (Fig. 2c, left panel, upper row; Fig. 2b, filled circles). The number of FISH-positive cells increased over time and, at 24 days p.t., plus-strand RNA had accumulated in virtually all cells (Fig. 2c, middle panel, upper row; Fig. 2b, filled circles). Late after transfection, similar levels of plus-strand RNA accumulated in cells transfected with RNA18f-A0. However, RNA accumulation was delayed by 5 days (Fig. 2c, middle panel, middle row; Fig. 2b, open circles). Immunofluorescence demonstrated that the number of protein 2B-positive cells at a given time point was similar to the number of plus-strand RNA-positive cells. At 9 days p.t., protein 2B was detectable in a dot-like pattern in the perinuclear space in 8% of cells transfected with RNA 18f-A14 (Fig. 2c, left panel, upper row), whereas <1% of the cells transfected with RNA 18f-A0 were immunofluorescence-positive (Fig. 2c, left panel, middle row).

Final evidence for HAV genome replication after RNA transfection was achieved by FISH detection of minus-strand RNA with a strand-specific riboprobe. Minus-strand RNA yielded a less strong signal than plus-strand RNA and was detectable only late after transfection in 2–6% of cells (Fig. 2c, right panels; Fig. 2b, grey and black columns, dimensions on the right-hand axis). Cells transfected with RNA 18f-A0-mut or mock-transfected cells were negative in all tests (Fig. 2b,c; data not shown). Thus, HAV RNA 18f-A0 is replication-competent when transfected into actively dividing cells, suggesting that the cell cycle might affect HAV replication.

Based on the observation that HAV replication is affected by host-cell replication, we next studied virus replication in cells arrested at various steps of the cell cycle. We employed widely used cell-cycle inhibitors that were applied, for example, in a study on coxsackievirus B3 replication (Feuer et al., 2002). To determine HAV genome replication independently of its uncoating and packaging, RNAs of the HAV replicon (pT7-18f-Luc) and its replication-deficient mutant 18f-Luc-mut, both containing a poly(A) tail of 20 residues, were transfected into arrested Huh-7 cells (Gauss-Müller & Kusov, 2002). Expression of the reporter gene 18 h p.t. was suppressed in cells arrested at the G0 phase and G2/M transition of the cell cycle, but was hardly affected in cells in the G1 and G1/S phases (Fig. 3a). Similar effects were observed when the reporter activity of a replication-defective, yet translation-competent replicon carrying a mutation in the polymerase gene was analysed (data not shown). When the transfected and arrested cells were permitted to divide after the removal of inhibitors, reporter-gene expression was restored to normal levels, indicating that the limited protein expression is not due to

**Fig. 3.** HAV and HAV genome replication depends on the cell-cycle status of Huh-7 cells. (a) Sixty to seventy per cent confluent Huh-7 cells in a six-well plate were arrested at various stages of the cell cycle by using the following inhibitors: L-mimosine (300 μM; G1), hydroxyurea (1 mM; G1/S transition) or paclitaxel (200 nM; G2/M transition). Serum starvation (0% FCS) was used to arrest cells in the G0 phase. One microgram of replicon RNA 18f-Luc produced by in vitro transcription was transfected into arrested Huh-7 cells grown on 10 cm2. Eighteen hours later, luciferase activity (relative luciferase units, r.l.u.) in the soluble cell extract was determined by using a luciferase assay kit (Promega). The inhibitory effects were independent of whether the replicon RNAs carried a poly(A) tail of 20 or 60 residues. (b) Arrested cells were infected with HAV strain 18f (at an m.o.i. greater than 1) and incubated for 6 days with the inhibitors. HAV antigen was measured by using a particle-specific ELISA (A450). Error bars represent SD of three experimental values.
irreversible damage of the cells by the inhibitors (data not shown). Similar results were obtained when HAV infection was analysed (Kusov et al., 1996). HAV replication, as detected by the accumulation of viral antigen, was reduced when the cells were arrested at the G0 phase and G2/M boundary, but not at the G1 or G0/S phases (Fig. 3b). In conclusion, our data show clearly that cell division favours efficient HAV RNA translation and replication.

Based on these data, it is clear that the tailless HAV genome could not initiate a productive infection in quiescent cells (see Fig. 1a), but was able to replicate in dividing cells, with the concomitant restoration of the poly(A) tail (Figs 2 and 3). In quiescent cells, the half-life of in vitro-produced tailless, and hence PABP-unprotected, HAV genome was too short to serve as a substrate for adenylylating enzymes that are more active in dividing cells (Kazazoglou et al., 1987; Zhao & Manley, 1998). The function of poly(A) polymerase may be involved or even required for maintenance of an optimal poly(A)-tail length that is lost during minus-strand synthesis and, thus, for efficient replication of HAV and possibly other poly(A)-containing RNA viruses (C. Polack, personal communication; Jupin et al., 1990; Tacahashi & Uyeda, 1999). A cell cycle-dependent isoform of poly(A) polymerase can add adenosine residues to the HAV 3′ NTR and render the tailless genome replication-competent (Thuresson et al., 1994). Thus, the in vitro transcripts have regained not only the cis-active replication element for initiation of minus-strand RNA synthesis, but also the ability to bind to PABP, which provides the transcripts with an increased half-life. Aside from a general increase in RNA stability after the addition of terminal adenosine residues and subsequent PABP binding, PABP will help to circularize viral RNA through its interaction with proteins (PCBP, eIF4G) that bind to the 5′ NTR of the RNA (Herold & Andino, 2001). The delayed replication of the tailless compared with the poly(A) tail-containing genome (Fig. 1c) might reflect the time required for adenylation. Our data unambiguously demonstrate replication of the tailless HAV genome and concomitant poly(A)-tail regeneration. Further investigations with potential host factors are required to dissect the mechanism by which HAV responds to the host cell cycle.

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