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Apoptosis induced by a cytopathic hepatitis A virus is dependent on caspase activation following ribosomal RNA degradation but occurs in the absence of 2′–5′ oligoadenylate synthetase

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Abstract

We have presented previously evidence that the cytopathogenic 18f strain of hepatitis A virus (HAV) induced degradation of ribosomal RNA (rRNA) in infected cells [Arch. Virol. 148 (2003) 1275–1300]. In contrast, the non-cytopathogenic parent virus HM175 clone 1 had no effect on rRNA integrity. We present here data showing that rRNA degradation is followed by apoptosis accompanied by characteristic DNA laddering in the cytoplasm of 18f infected cells. The DNA laddering coincided with the detection of caspase 3 and PARP-1 cleavage and was dependent upon activation of the caspase pathway, since treatment with Z-VAD-FMK, a pan-caspase inhibitor, inhibited both events. RNase L mRNA was present in both virus-infected and uninfected cells. Messenger RNA for the interferon inducible enzyme 2′–5′ oligoadenylate synthetase (2′–5′ OAS), which polymerizes ATP into 2′–5′ oligo adenylate (2–5A, the activator of RNase L) in the presence of double-stranded RNA, was not detected following virus infection. 2′–5′ OAS mRNA was induced by treatment of the cells with interferon- β (IFN- β). IFN- β mRNA was marginally induced following infection. However, phosphorylated STAT 1, a key regulator of interferon-stimulated gene transcription was not detected in virus infected cells. STAT 1 phosphorylation in response to IFN treatment was lower in virus-infected cells, compared to uninfected cells treated with interferon, suggesting that 18f virus infection interferes with interferon signaling. The results suggest that 18f infection causes the induction of a 2–5A independent RNase L like activity.

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Keywords: Hepatitis A virus; Apoptosis; RNA degradation; Caspase

1. Introduction

Hepatitis A virus is a *picornavirus* and is the only known member of the genus *hepatovirus*. It is the major causative agent for infectious hepatitis worldwide and is easily disseminated by person to person contact as well as via contaminated foods and water, due to its resistance to environmental factors such as heat, low pH, etc. (Siegl et al., 1984; Gust, 1988). Most of the known HAV strains establish a slowly replicating and non-cytopathogenic persistent infection in permissive cells in vitro (Provost and Hilleman, 1979; Flehmig, 1980; Binn et al., 1984; Vallbracht et al., 1984). This is in contrast to in vivo infection, where persistence is restricted to a few weeks (Lemon, 1985; Vallbracht et al., 1989), although sporadic cases of chronic infection

have been reported (Fagan et al., 1990; Inoue et al., 1996). Several laboratories have isolated rapidly replicating cytopathogenic (cp) strains that produce visible morphological changes primarily in continuous cell lines of monkey kidney origin (Divizia et al., 1986; Venuti et al., 1986; Anderson, 1987; Cromeans et al., 1987; Nasser and Metcalf, 1987; Zhang et al., 1995; Brack et al., 1998). The mechanism of the cytopathogenic effect (cpe) produced by the rapidly replicating HAV strains has been intensely investigated during the past few years to understand why wild type (wt) and cell culture adapted (cc) strains replicate slowly and are unable to induce cpe in these cell lines. These studies have revealed that the genome of the cpe-inducing (cp) strains contain point mutations, compared to the wt strains from which they were derived, scattered throughout the genome, as well as a 14-bp repeat in the 5′ non-translated region (NTR), which harbors the internal ribosomal entry site or IRES (Lemon et al., 1991; Brack et al., 1998). The biological significance of these mutations for the rapid replication

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phenotype of the cp strains is not completely understood. Experiments with chimeric viral genomes containing mutated regions of cp strains on a genetic background of cc strains suggested an effect of these mutations on the expression of the viral genome, possibly due to altered interaction of the mutated genomes with cellular proteins that either positively or negatively regulate viral gene expression (Brown et al., 1994; Whetter et al., 1994; Zhang et al., 1995; Funkhouser et al., 1999; Yi et al., 2000). However, the view that HAV IRES is inefficient compared to other picornaviruses is not universally accepted (Jia et al., 1996; Graff and Ehrenfeld, 1998; Gauss-Müller and Kusov, 2002).

We have recently reported that in the permissive monkey kidney cell line FrhK4 infection with the cp strain HM175 18f (hereafter referred to as 18f) resulted in degradation of ribosomal RNAs (rRNAs), whereas viral genomic RNA was not degraded. In contrast, the parental cc strain did not cause such degradation either after an acute infection or extended persistent infection (Kulka et al., 2003). Based on the pattern of rRNA degradation in intact ribosomes, we suggested that the 18f virus activates the interferon (IFN) controlled 2′–5′ oligoadenylic acid-dependent RNase L pathway (for recent reviews, see Stark et al., 1998; Barber, 2001; Sen, 2001). Since this pathway is essentially an antiviral mechanism mounted by cells in defense against viral infection, the usurpation of this pathway by the 18f virus could be a mechanism employed by this virus to reduce competition from cellular mRNAs for the host protein synthetic machinery. This virus lacks an active 2A protease (Schultheiss et al., 1994; Harmon et al., 1995) capable of degrading either eIF-4G or other cellular translation factors required for translation of capped cellular mRNAs, therefore, the importance of such an RNA degradative mechanism cannot be overstated.

The activation of the RNase L pathway is controlled by IFN (Stark et al., 1998; Barber, 2001; Sen, 2001) through the induction of 2′–5′ oligoadenylate synthetase (2′–5′ OAS), which synthesizes 2′–5′ oligoadenylic acid or 2–5A, the proximal activator of the RNase L from ATP, in the presence of double stranded RNA (dsRNA). Overexpression of RNase L has been shown to inhibit the growth of diverse RNA viruses (Zhou et al., 1998). Whether HAV infection can induce synthesis of type I IFN (IFN α/β) in cultured cells or intact organisms and consequently activate the 2′–5′ OAS/RNase L pathway remains unclear. Cell cultures persistently infected with culture-adapted HAV do not induce type I IFN, and the replication of the virus in persistently infected cells remains sensitive to exogenously added IFN (Vallbracht et al., 1985; Brack et al., 2002). Acute infection with cc or cp strains of HAV did not cause induction of IFN α/β mRNA transcription and cc strains also blocked IFN mRNA transcription and secretion of IFN in response to dsRNA in FrhK4 and MRC-5 cells (Brack et al., 2002). Exogenous IFN inhibited virus replication when added either before or after exposure to virus in a dose dependent manner in PLC/PRF/5 cells (Crance et al., 1995). Inhibi-

tion of virus replication in the above study also showed a strong dependence on the multiplicity of infection (moi). A significant increase in the activity of the IFN-induced 2′–5′ OAS was observed following IFN treatment, suggesting that the RNase L pathway may be involved in the inhibition of virus replication (Crance et al., 1995). Constitutive expression of 2′–5′ OAS results in the inhibition of picornavirus replication (Chebath et al., 1987), and ectopic expression and activation of RNase L induces apoptosis in animal cells (Diaz-Guerra et al., 1997; Castelli et al., 1998).

The goal of this investigation was to study the role of the IFN-regulated 2–5A activated RNase L on RNA degradation and apoptosis in 18f infected cells.

2. Materials and methods

2.1. Cells and viruses

FrhK4 monkey kidney cell line was a kind gift of Dr. G. Kaplan (FDA, Center for Biologics Evaluation and Research, Bethesda, MD). The cells were grown in Eagles Minimal Essential Medium (EMEM) containing 5% heat inactivated fetal bovine serum (FBS), MEM non-essential amino acids and sodium pyruvate (all from Invitrogen, Carlsbad, CA), with routine weekly sub-culturing. Under these conditions the cells increase about 20-fold in 6–7 days. The cell line is contact inhibited and can be kept in growth medium or maintenance medium (1% FBS) for several weeks without degeneration of the monolayer. HAV strains HM175/18f (cytopathogenic in FrhK4 cells) and HM175/clone1 (non-cytopathogenic in FrhK4 cells) were obtained from ATCC. Viruses were grown in FrhK4 cells. Virus stocks were prepared as described before (Kulka et al., 2003). 18f was titered by plaque assay, while clone 1 virus was titered by EIA in 96-well plates as described previously (Goswami et al., 2002) using the HAVAB EIA Diagnostic Kit (Abbott Laboratories, Abbott Park, IL). Human IFN- β and the pancaspase inhibitor Z-VAD-FMK were obtained from Sigma (St. Louis, MO).

2.2. Virus infection for RNA isolation

Confluent cultures of FrhK4 cells in 75 cm² flasks (5×10^6 cells) were infected with a multiplicity of infection (moi) of 5 pfu/cell of 18f or 15 TCID₅₀/cell of clone 1 in 1.5 ml of MEM containing 1% heat inactivated FBS or were mock infected. After 2 h of adsorption, 13.5 ml of the same medium was added to each flask and incubation continued until the desired time of harvest. Cells were harvested by scraping, centrifuged to remove medium, and the pellets washed once with PBS (Ca²⁺ and Mg²⁺ free) and stored frozen at –70 °C or used immediately. RNA isolation was carried out as previously described (Kulka et al., 2003).

2.3. Persistent infection with HM175/clone 1

A persistent infection of FrhK4 cells with clone 1 virus was established as described (Kulka et al., 2003). The cells were maintained by routine subculturing in the same manner as for normal FrhK4 cells. To monitor virus replication, cells were periodically seeded into 12-well plates along with uninfected cells, and viral antigen in methanol fixed cells measured by the HAVAB EIA procedure (Goswami et al., 2002; Kulka et al., 2003). All experiments with this cell line (hereafter referred to as clone 1) were carried out between 6 months and 1 year of routine subculture.

2.4. Isolation of cytoplasmic RNA and DNA and gel analysis

Cell pellets of uninfected or virus infected cells were re-suspended in 10 mM Tris-HCl, 20 mM EDTA, pH 8.0 and lysed by the addition of Triton X-100 to 0.5% (Brack et al., 1998). Cell lysates were kept on ice for 20 min with intermittent vortex mixing, followed by centrifugation at 12,000 × g for 10 min. The supernatant was carefully removed and digested with proteinase K (0.1 mg/ml) in the presence of 0.5% SDS. The digest was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (PCIA), and the aqueous phase removed to a fresh tube. Nucleic acids were precipitated by the addition of sodium acetate to 0.3 M and 2.5 volume of ethanol. The precipitates were collected by centrifugation, washed once with 70% ethanol, dried and dissolved in RNase/DNase free water. Nucleic acids were quantitated by A₂₆₀ measurement. Equal amounts of sample were incubated at 37 °C for 15 min in 15 µl TE with or without 50 U of cloned RNase 1 (Ambion, Austin, TX), and analyzed by 1% agarose gel electrophoresis in TBE.

2.5. Denaturing agarose gel electrophoresis

Denaturing agarose gel electrophoresis was performed as described before (Kulka et al., 2003; Chomczynski and Mackey, 1992). The gel was photographed under UV light.

2.6. TUNEL assay

The induction of apoptosis was investigated by the incorporation of biotin labeled deoxyribonucleotides and detection of biotin incorporation according to the manufacturer's instructions (DeadEnd colorimetric TUNEL system, Promega). Cells were seeded in four well chamber slides, and infected at confluency with 18f at 5 pfu/cell. Cells were fixed for TUNEL assay at 48 h pi. Persistently infected cells were seeded in four well chamber slides, and processed when the monolayers reached confluence. Viral antigen-positive cells were displayed by immunohistochemistry, using an antibody to VP1 (Kulka et al., 2003). Briefly, cells were fixed in buffered formalin, permeabilized with 0.1% Tween 20 in PBS, and reacted with 1:200 dilution of

rabbit anti-VP1 antibody in PBS containing 1% nonfat dry milk (Nadala and Loh, 1990). After several washes with PBS containing 0.1% Tween 20, the cells were incubated with HRP labeled anti-rabbit IgG diluted in 1% non-fat dry milk. Following several washes to remove unbound antibody, color was developed using CN-DAB substrate (Pierce Chemical Co.).

2.7. Reverse transcription-PCR

Total cytoplasmic RNAs were digested with RNase free DNase (Promega) in the presence of RNasin RNase inhibitor followed by PCIA extraction and ethanol precipitation, as above, to remove any contaminating DNA. RNA samples (2 µg) each were reverse transcribed using AMV reverse transcriptase in a total volume of 20 µl using 1 µg oligo(dT)₁₅ as primer as described previously (Goswami et al., 1994). IFN-β mRNA was amplified using the primer pairs 5'ACCAACAAGTGTCTCCTCCA3' (sense) and 5'GAGGTAACCTGTAAGTCTGT3' (antisense), with an annealing temperature of 60 °C. This primer pair amplifies a 552-bp fragment (Brack et al., 2002). Amplification of the p40/46 and the p69/71 forms of 2'–5' OAS were carried out with the primers p40/46 sense (5'GAAGCCTGTCAAAGAGAGAG3') and p40/46 antisense (5'TGTGTTTTTCATGCTCCCTCG3') which amplifies a 424-bp fragment (nucleotides 382–806), p69/71 sense (5'CAATCAGCGAGGCCAGTAATC3'), and p69/71 antisense (5'CTTGACGATTTTGTGCCGCTC3'), which amplifies a 447-bp fragment (nucleotides 872–1318), with annealing at 56 and 59 °C, respectively (Takahashi et al., 2002). To amplify RNase L mRNA, the following primers were synthesized based on the sequence of human RNase L (GenBank accession number NM 021133): RNase L sense (5'AGATGAGGAACTAAGGACCTC3'), and RNase L antisense (5'GAGGTTTGTGGACTGTGGG3'), which amplifies a 512-bp fragment (nucleotides 1768–2279). The annealing temperature was 60 °C. γ-Actin mRNA levels were used to normalize the RT-PCR signal generated by the other mRNAs. The primers for γ-actin were 5'AAGTACCCCATGAGCATGGC3' (sense) and 5'CACAGCTTCTCCTTGATGTCGC3' (antisense). The annealing temperature was 60 °C. Viral RNA in total or cytoplasmic RNA samples were amplified using primers 5'CCGTTTGCCTAGGCTATAGGCTA3' (sense) and 5'CAGCTCCATGCTAATCATGGAGT3' (antisense). This primer pair is directed to the 5' end of the HAV genome and can differentiate between the genomes of 18f and clone 1 strains of HAV based on the presence of an additional 14 bases in the 5' NTR of the 18f genome (Goswami et al., 2002; Kulka et al., 2003). All PCR amplifications were carried out in a total volume of 50 µl, and contained 2–5 µl of the cDNA pool, 2.5 mM MgCl₂, 200 µM of each deoxynucleoside triphosphate, 50 pmol of each primer, and 2 U of Taq DNA polymerase (Promega) for 30–35 cycles.

2.8. Virus infection and Z-VAD-FMK treatment for protein extraction

FrhK4 cells were mock infected or infected with 5 pfu/cell of 18f, 15 TCID₅₀/cell of clone 1 or 5 pfu/cell of coxsackievirus B1 (CBV1) in MEM containing 1% heat inactivated FBS. After 2 h adsorption period, medium was added to each flask and incubation continued for the indicated time period. For infected cultures treated with Z-VAD-FMK, the virus adsorption media was removed prior to the addition of MEM/1% FBS containing 0–50 µM inhibitor and subsequently incubated for 48 h. At the indicated times post-treatment, cell culture monolayers were scraped into their media and pelleted by centrifugation (1500 × g, 4 °C). Cell extracts were prepared and the protein content estimated as described previously (Kulka et al., 2003).

2.9. Western blot analysis

Equal concentrations of each protein extract were adjusted to equivalent volumes in denaturing sample buffer and heated at 95 °C for 5–10 min and subjected to SDS-PAGE under reducing conditions, followed by electrophoretic transfer onto nitrocellulose membranes in transfer buffer containing methanol. The membranes were blocked with TBS-T (Tris buffered saline/0.1% Tween 20) containing 5% nonfat dried milk (NFD) for 90 min at room temperature (rt) and washed four times with TBS-T. Membranes were incubated either overnight at 4 °C or 1 h at rt with primary antibody diluted in TBS-T containing either 5% BSA or 5% NFD. Primary antibodies were used that recognize (i) STAT1 (phosphorylation state independent) or STAT1 phosphorylated on tyrosine at position 701 (Cell Signaling Technologies), used at a 1:1200 dilution (5% BSA) for incubation overnight at 4 °C; (ii) full length/large (cleaved) fragment caspase-3 (Cell Signaling Technologies), used at a 1:1200 dilution (5% NFD) for overnight incubation at 4 °C; (iii) full length/large (cleaved) fragment PARP (Sigma), used at a 1:2000 dilution (5% BSA) for 1 h incubation at rt, and (iv) β-actin (Sigma), used at a 1:1000 dilution (5% NFD) for 1 h incubation at rt. Secondary antibody-HRP conjugates (Pierce-Endogen) were used at a 1:30,000 dilution. All other steps including detection were performed as previously described (Kulka et al., 2003).

2.10. Viral replication assay

Confluent cultures of FrhK4 cells in 12-well plates were infected with 18f at 0.2, 1, or 5 pfu/cell, as described previously. Replication of virus was assessed by the level of viral antigen in each well as described before using the HAVAB EIA kit (Goswami et al., 2002; Kulka et al., 2003).

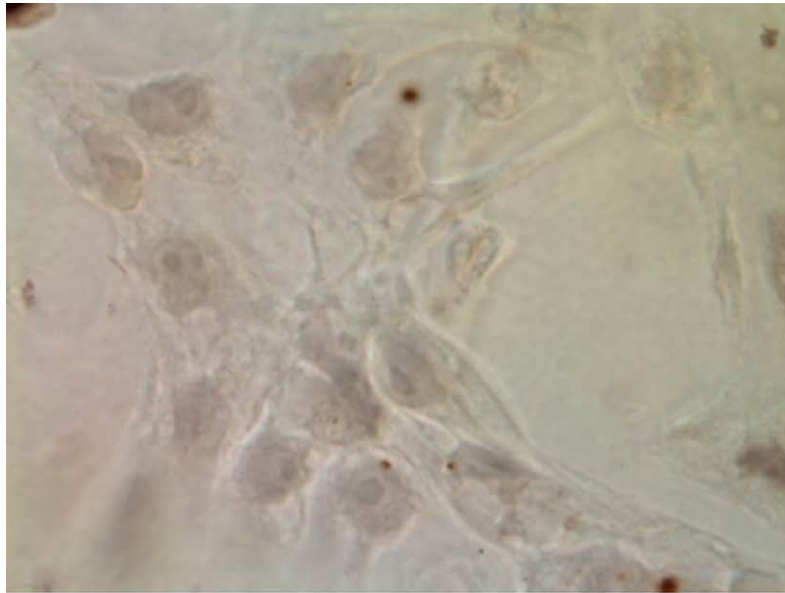
3. Results

3.1. Virus replication and apoptosis in infected cells

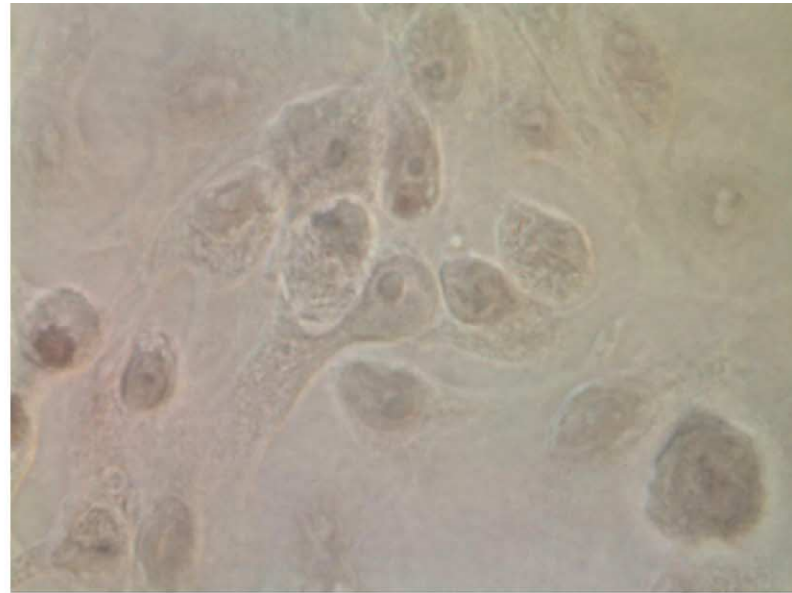
Previous investigations have demonstrated that a cp strain of HAV, HAV_{cyt}/HB1.1, derived from the cc strain HM175, induced apoptosis in 2–5% of the cells by 120 h post-infection (pi), increasing to 37.5% by 14 days pi (Brack et al., 1998). Similar slowly developing cpe and apoptosis were reported in cells infected with another cp strain, HM175/24a (Gosert et al., 2000). In contrast, cells infected with the 18f strain develop extensive cpe in approximately 15% of the cells by 48 h, and an almost total cpe is observed by 96 h (Kulka et al., 2003). Using a colorimetric TUNEL assay at 48 h pi we investigated whether cpe induced by the 18f strain is a result of apoptosis (Fig. 1). TUNEL positive cells were present in the 18f infected monolayers, whereas positively stained cells were only occasionally observed in the absence of virus infection. Persistently infected clone 1 cells at 35 days or 330 days pi also showed an occasional positively stained cell. The culture remained viable, however, and 18f like cpe was not observed until more than a year of weekly passages. Immunohistochemical staining was performed on the persistently infected clone 1 cells to determine if the persistently infected status of these cells is due to the lack of actively replicating virus in a majority of the cell population (Fig. 2). Almost all of the cells showed positive staining for viral antigen. In addition, replication of the virus as measured by EIA confirmed the presence of actively replicating virus (data not shown). Taken together, these results show that the 18f strain causes apoptosis in infected cells much earlier than cells infected with other cp strains, and that the lack of apoptosis in persistently infected cells is not due to lack of active virus replication.

3.2. RNA degradation occurs prior to apoptotic DNA fragmentation

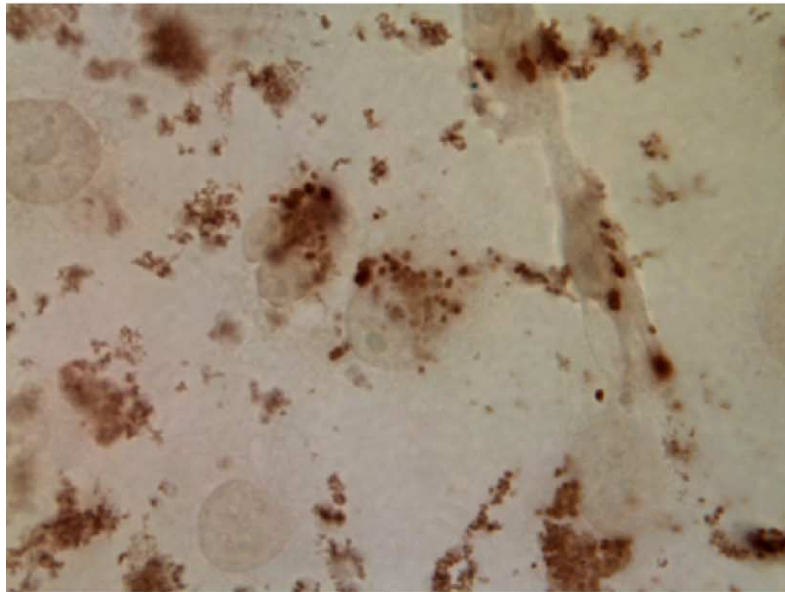
We have previously shown that rRNA degradation is dependent upon replication of 18f, since treatments that inhibited replication also resulted in inhibition of rRNA degradation. However, certain cell lines are sensitive to perturbation of cellular macromolecule synthesis and can be driven to apoptotic cell death by treatment with metabolic inhibitors. In such instances, apoptosis causes 28S rRNA fragmentation (Houge et al., 1995). Therefore, to determine whether rRNA degradation is a cause, or the effect of apoptosis in 18f infection, it was necessary to establish the temporal sequence of these two events. FrhK4 cells were infected with 18f and total nucleic acids from the cytoplasm were prepared at different times as described in Section 2. As control, cytoplasmic nucleic acids were also isolated from mock infected cells and persistently infected (clone 1) cells. Nucleic acids were analyzed by agarose gel electrophoresis before and after digestion with RNase 1 (Fig. 3). Degradation of the 28S and 18S molecules into discrete products was



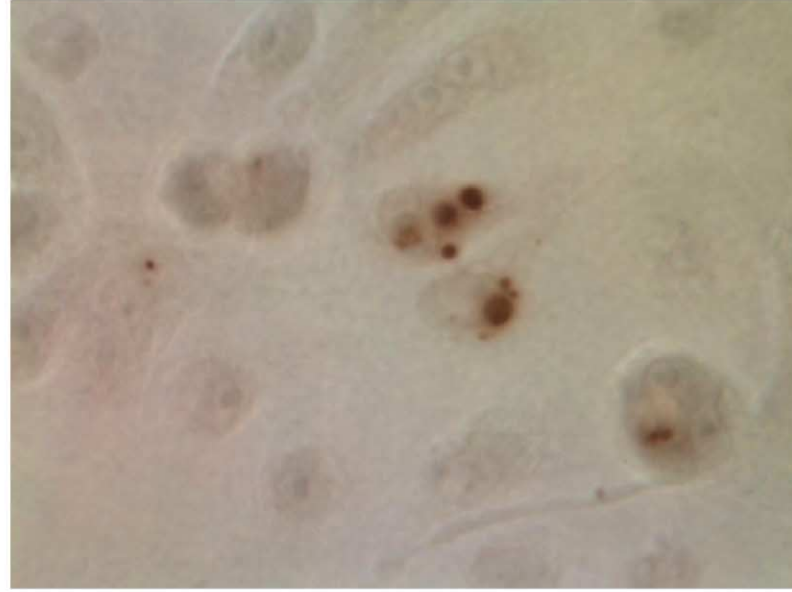
FrhK4 cells - 48h mock infected



FrhK4 cells - 35d pi, HAV clone1

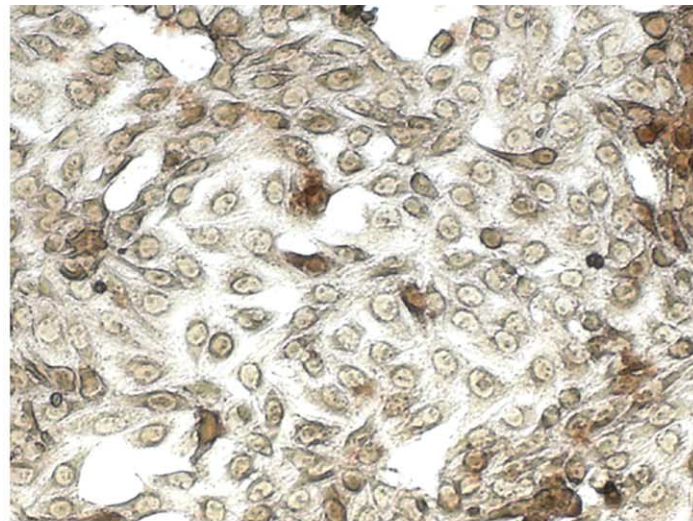


FrhK4 cells - 48h pi, HAV 18f

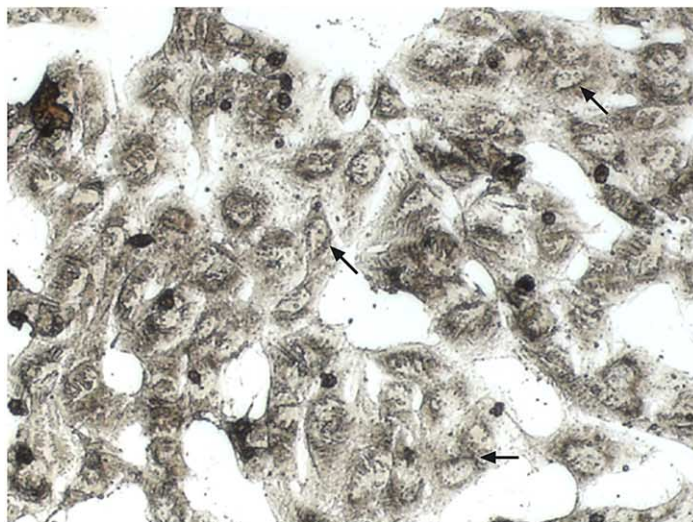


FrhK4 cells - 330d pi, HAV clone1

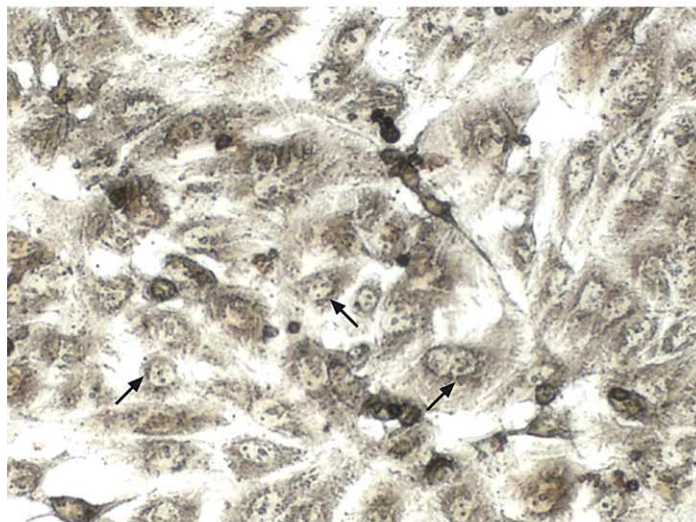
Fig. 1. Rapid induction of apoptosis in 18f infected FrhK4 cells. Confluent cultures of FrhK4 cells were mock infected or infected with the cytopathic 18f strain of HAV. Cells were processed for the detection of TUNEL positive cells at 48 h pi as described in Section 2. Persistently infected cultures following infection by the non-cytopathic HM175 (clone 1) strain of HAV were established as described in Section 2. Cells were processed for TUNEL positive cells at 35 days or 330 days post infection.



FrhK4 cells - 48h mock infected



FrhK4 cells - 48h pi, HAV 18f



FrhK4 cells - 330d pi, HAV clone 1

Fig. 2. Immunoperoxidase staining of uninfected and virus infected cells for the detection of HAV antigen, using an antiserum against the capsid protein VP1. Cultures were fixed and stained 48h after mock infection, or 18f infection. Persistently infected cultures (clone 1) were processed at 330 days pi. Examples of positive staining (perinuclear punctuate) for viral antigen in 18f-infected and clone 1 cells are identified by arrows.

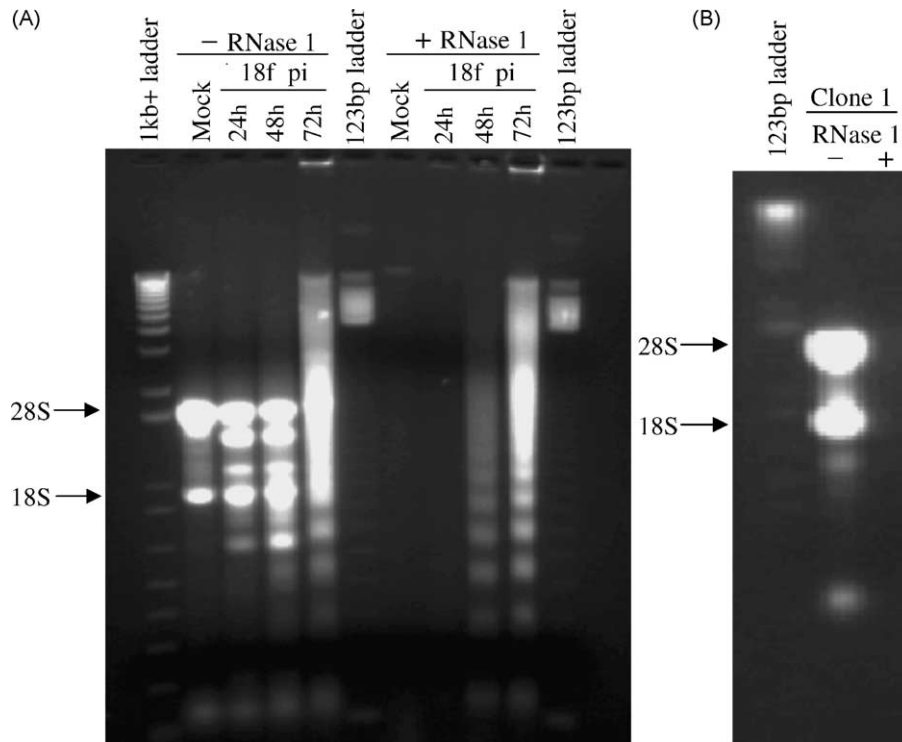


Fig. 3. Degradation of rRNA and appearance of apoptotic DNA laddering in the cytoplasm of infected cells. Total nucleic acids from the cytoplasm of mock infected or virus infected cells were prepared as described in Section 2. Equal amounts of total nucleic acids from mock or 18f infected cells (A), and persistently infected clone 1 cells (B), were separated in a 1% agarose gel containing ethidium bromide with (+) or without (–) treatment with RNase 1.

evident at 24 h pi, and by 72 h pi very little intact rRNAs could be detected. The appearance of apoptotic DNA laddering in the cytoplasm could be detected following elimination of rRNAs from total nucleic acid preparations by RNase 1 and clearly lagged behind rRNA degradation as a result of 18f infection (Fig. 3, Panel A). Neither mock infected cells nor persistently infected (clone 1) cells showed any evidence of RNA or DNA degradation (Fig. 3, Panel B). No evidence of RNA or DNA degradation could be seen in nucleic acid preparations from cells infected with 18f virus, at 6 or 12 h pi (data not shown). The data indicate that rRNA degradation in 18f infected cells occurs prior to apoptosis, and is not a consequence of apoptosis or DNA fragmentation.

3.3. Caspase is activated in 18f infected cells

Previous studies have shown that rRNA degradation in cells over-expressing the IFN regulated 2–5A activated RNase L results in apoptosis due to activation of caspase 3 (Rusch et al., 2000). Caspase 3 is the terminal enzyme in the caspase cascade, and activation of caspase 3 by upstream caspases occurs due to proteolytic cleavage (for a review, see Roulston et al., 1999). To investigate if a similar activation of caspase 3 takes place due to infection with the 18f virus, we investigated caspase 3 activation by western blotting experiments (Fig. 4). Proteolytic cleavage of caspase 3 could be detected in 18f infected cells at 48 h pi,

which coincided with the appearance of a DNA ladder in the cytoplasm (compare Fig. 3, Panel A with Fig. 4, Panel A). Proteolysis of caspase 3 was not detectable at earlier times, nor in persistently infected (clone 1) cells (Fig. 4, Panel A), confirming that caspase activation follows rRNA degradation (Fig. 3). Activation of caspase 3 was accompanied by the degradation of PARP-1 (poly ADP-ribose polymerase 1), a target for caspase 3 during the apoptotic response (Kaufmann et al., 1993). Cleavage of PARP-1 was evident at 48 h pi and coincided with the appearance of activated caspase 3; PARP was no longer detectable in 18f infected cells at 72 h pi (Fig. 4, Panel B). Commensurate with the lack of caspase activation or RNA degradation, there was no cleavage of PARP in persistently infected cells.

3.4. Effects of caspase inhibitor Z-VAD-FMK

Having shown the proteolytic activation of caspase 3 in 18f infected cells, it was of interest to determine if apoptotic DNA degradation was a consequence of caspase activity. FrhK4 cells were infected with 18f and then treated with different concentrations of the caspase inhibitor Z-VAD-FMK for 48 h. Cultures were processed for the analysis of caspase activation by western blotting as described in Section 2. Caspase activation was prevented in a dose dependent manner by treatment with Z-VAD-FMK (Fig. 5). Significant inhibition of caspase activation was achieved with 25 μ M

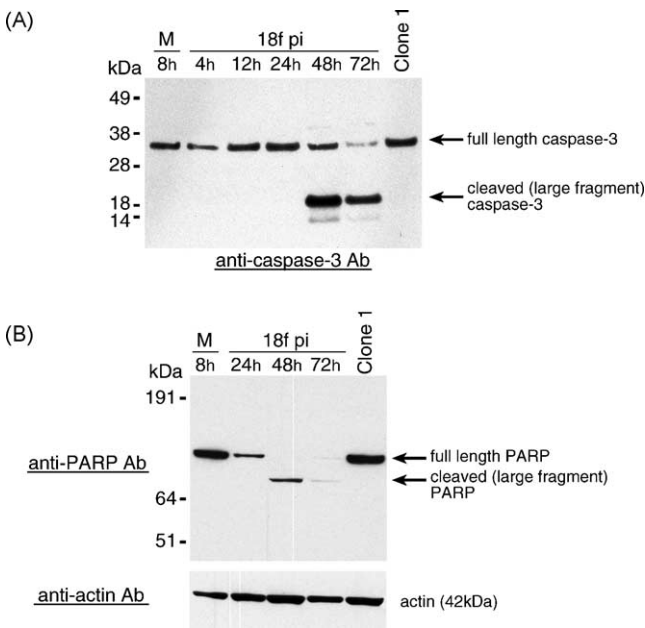


Fig. 4. Activation of caspase 3 and degradation of PARP in response to virus infection. Cells were infected with 18f virus and harvested at different times during an interval from 4 to 72 h pi. Clone 1 cells were processed between 300 and 330 days pi. Total proteins were extracted as described in Section 2. Equal amounts of proteins were run on 4–12% Bis-Tris gels (Invitrogen) under reducing conditions and transferred to nitrocellulose. The membranes were probed with antibody against caspase 3 (A), PARP (B), or actin (B).

Z-VAD-FMK, and complete inhibition was observed at 50 μ M of the inhibitor. RNA and DNA analyses by agarose gel electrophoresis in the cytoplasm of 18f infected cells were carried out to establish a connection between caspase activation and apoptotic DNA laddering (Fig. 6). Treatment of 18f infected cells with 50 μ M Z-VAD-FMK did not prevent rRNA degradation (Panel A). The appearance of apoptotic DNA laddering in the cytoplasm was completely prevented (Panel B). These results show that rRNA degradation is independent of caspase induced DNA degradation,

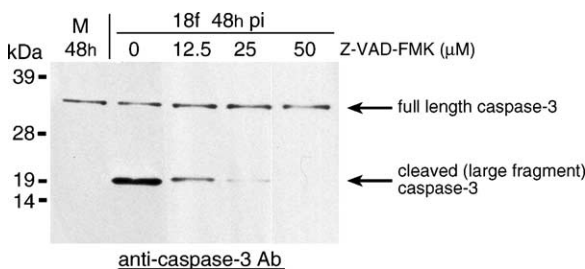


Fig. 5. Effect of the caspase inhibitor Z-VAD-FMK on proteolytic activation of caspase 3 in response to 18f infection. Cells were infected with 18f (5 pfu/cell), and incubated in growth medium containing 0–50 μ M of the inhibitor for 48 h, prior to protein extraction. Electrophoresis, transfer, and immunodetection with antibody against caspase 3 were performed as described in Section 2. The lane marked M contains protein extracts from mock infected cells harvested and processed 48h following mock infection in the absence of inhibitor.

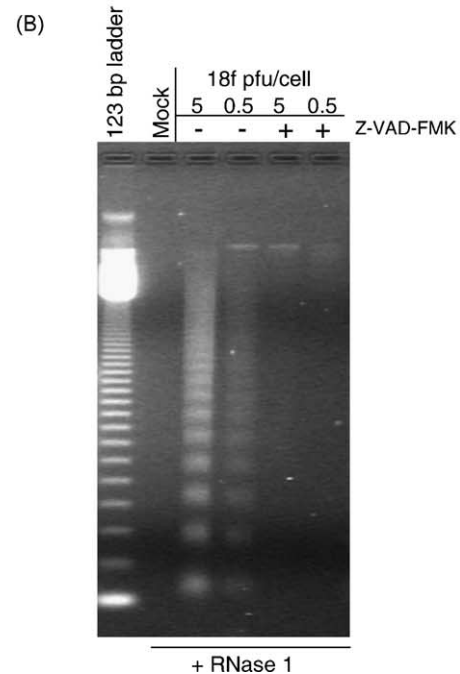
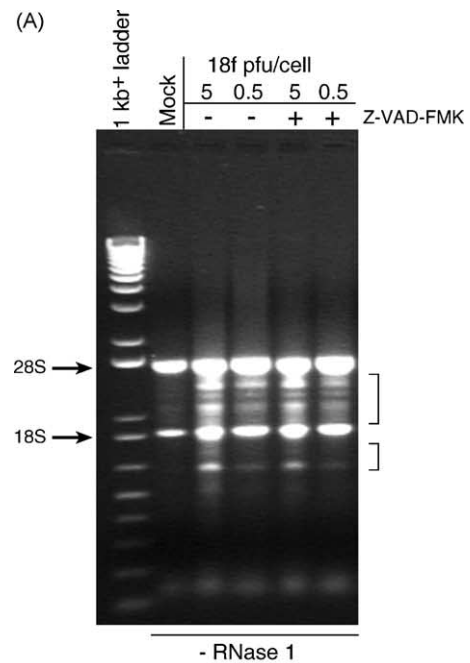


Fig. 6. Effect of Z-VAD-FMK on rRNA degradation and apoptotic DNA laddering in the cytoplasm of infected cells. Cells were infected with 18f (5 or 0.5 pfu/cell) or mock infected, and further incubated in growth medium containing 50 μ M Z-VAD-FMK for 48h, prior to isolation of total cytoplasmic nucleic acids. Samples were separated in a 1% agarose gel before (A) or after (B) digestion with RNase 1.

and is not a consequence of apoptosis due to perturbation of cellular macromolecule synthesis as a result of virus infection. We have previously shown that despite rRNA degradation, cellular protein synthesis remains unaffected in 18f infected cells between 48 and 72 h pi (Kulka et al., 2003). In agreement with the lack of effect of Z-VAD-FMK

Table 1
Effects of Z-VAD-FMK and IFN-β on the replication of cytopathic 18f strain of HAV

moi	HAV antigen titer (A_{495}) (means ± S.D.)		
	No treatment	Z-VAD-FMK (50 μM)	IFN-β (500 U/ml)
5.0	0.66 ± 0.03	0.58 ± 0.025	0.68 ± 0.024
1.0	0.58 ± 0.02	0.57 ± 0.015	0.54 ± 0.037
0.2	0.58 ± 0.023	0.57 ± 0.024	0.40 ± 0.019

FrhK4 cells were pretreated with IFN-β for 24 h prior to infection with HAV 18f at an moi of 0.2, 1.0 or 5.0. After virus adsorption for 2 h, growth medium containing IFN was added. For Z-VAD-FMK treatment, cells were infected with 18f virus followed by the addition of growth medium containing Z-VAD-FMK. As a control (no treatment), cells were infected in the absence of any treatment with either IFN-β or Z-VAD-FMK. Cells were analyzed at 48 h pi for determination of viral antigen by EIA as described in Section 2. Uninfected cells had a background A_{495} value of 0.08. Statistical significance between the means of untreated and treated, infected cultures was determined using the Student's *t*-test (one-tailed). Significance at $P < 0.025$ exists for IFN-β treatment at moi = 0.2.

on rRNA degradation which requires replication of the virus (Kulka et al., 2003), virus replication was not inhibited by treatment with this compound (Table 1).

3.5. Effect of interferon pretreatment on RNA degradation

Ribosomal RNA degradation is one of the three antiviral mechanisms controlled by IFN. It is brought about by the activation of a latent ribonuclease RNase L by 2–5A, and requires IFN treatment of cells prior to virus infection

(Stark et al., 1998; Barber, 2001; Sen, 2001). In contrast, RNA degradation or apoptosis induced by 18f did not require IFN pretreatment of the cells (Figs. 1 and 3). To investigate whether IFN is involved in 18f induced rRNA degradation and apoptosis, cells were pretreated with 500 U/ml IFN-β (human) for 24 h, prior to exposure to 18f virus at a moi of 1 or 0.2 pfu/cell. DNA and RNA from the cytoplasm of infected cells were analyzed at 48 h pi as described in Section 2 (Fig. 7). Pretreatment with IFN had a modest inhibitory effect on both rRNA degradation and apoptotic DNA degradation, particularly at low moi. When IFN was added to the cells following the 2 h virus absorption period, neither RNA degradation nor DNA laddering was affected by IFN treatment (Fig. 7). IFN treatment had a significant growth inhibitory effect on 18f only at low moi (0.2 pfu/cell) infection (Table 1), and had no effect on virus replication in clone 1 cells over a 72 h treatment period (data not shown). Thus, the involvement of the canonical IFN regulated 2–5A system seemed unlikely. The relatively slow replication of even the 18f strain might allow the infected cells to synthesize and excrete IFN, however, which may stimulate the 2–5A pathway in neighboring uninfected cells causing RNA degradation. We addressed this question by two different approaches. First, the level of IFN, 2′–5′ OAS and RNase L mRNAs were investigated by RT-PCR to determine if any of these RNAs were induced following virus infection (Fig. 8). In agreement with previous reports that HAV infection does not induce IFN, we did not observe significant induction of IFN message up to 24 h pi with 18f virus (Fig. 8, Panel A).

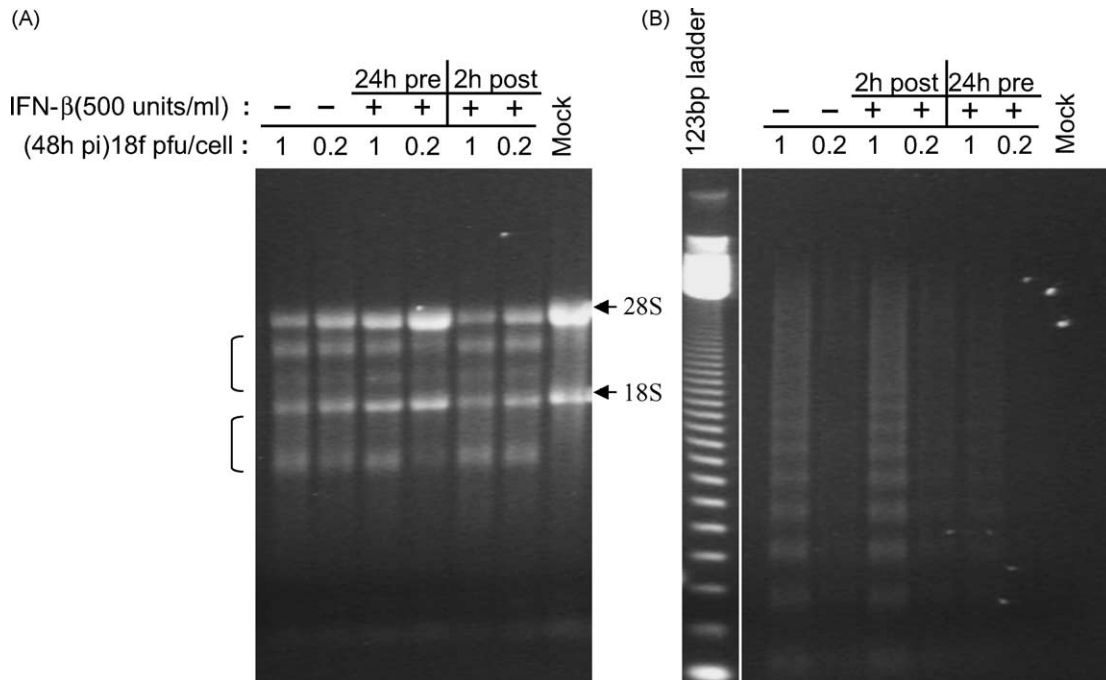


Fig. 7. Effect of IFN on rRNA degradation and DNA ladder formation in response to 18f infection. Cells were either pretreated with 500 U/ml IFN-β for 24 h (24 h pre) before infection or treated with IFN following virus infection (2 h post). At 48 h pi, cytoplasmic nucleic acids were isolated as described in Section 2 and analyzed by agarose gel electrophoresis before (A) or after (B) treatment with RNase 1 to assess RNA or DNA degradation. The bracketed areas indicate the position of the degradation products of 28S and 18S rRNA.

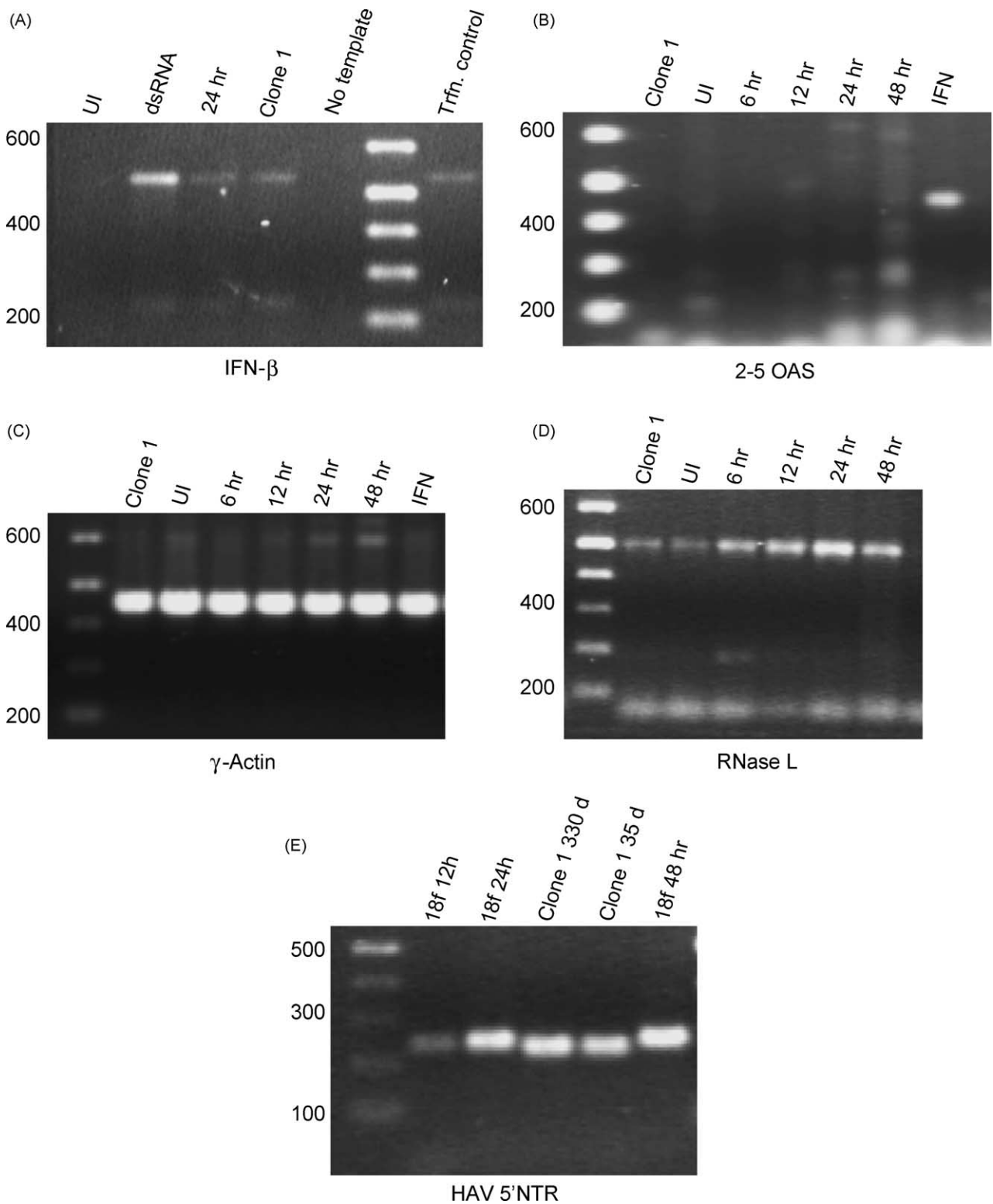


Fig. 8. RT-PCR amplification of selected cellular and viral mRNAs. Two microgram of RNA isolated from virus infected, IFN- β , or dsRNA treated cells were reverse transcribed in a volume of 20 μ l using oligo(dT)₁₅ as primer as described in Section 2. Five microliter of each RT reaction was amplified for 35 cycles with sense and antisense primers for IFN- β (A), 2'-5' OAS (B), γ -actin (C), RNase L (D), or HAV genomic RNA (E) as described in Section 2, and 15 μ l PCR reactions were analyzed by 1% agarose gel electrophoresis. RNA from uninfected cells (UI) and cells transfected in the absence of dsRNA (Trfn. Control) were included in the RT-PCR analyses.

Takahashi et al. (2002) successfully demonstrated the application of RT-PCR for the detection of mRNAs, which encode the 2'–5' OAS isoforms that synthesize the biologically active forms of 2–5A. Using these primers, we determined that the mRNAs for either the p69/71 form (Fig. 8, Panel B) or the p40/46 form (data not shown) of 2'–5' OAS was not induced following virus infection. Treatment of the cells with 500 U/ml IFN- β induced the message for the 69/71 form (Panel B), but not the 40/46 form, although the primer pair is capable of amplifying this mRNA from both mouse and human cells (data not shown). Thus, the FrhK4 cells are competent to mount an IFN response by inducing the 69/71 form of 2'–5' OAS. RT-PCR experiments with primers for RNase L, on the other hand, revealed that this mRNA was present in cells constitutively, with some increase in the level of this mRNA following virus infection (Panel D). To ascertain that both the 18f and clone 1 cells were actively replicat-

ing virus, viral genomic RNA was amplified using primers directed to the 5'NTR (Panel E). The results clearly show the presence of undiminished amount of viral genome in clone 1 cells after several months of cultivation, with no indication that the virus has acquired the 14 base repeat present in the genome of 18f and other cp strains (Brack et al., 1998; Zhang et al., 1995).

Second, we determined whether STAT 1 phosphorylation occurs following virus infection. Phosphorylation of STAT1 on Y701 is indicative of the activation of the JAK-STAT pathway, and is a prerequisite for the transcriptional induction of ISGs by IFN (for a review, see Stark et al., 1998). To investigate if this signaling pathway is activated following viral infection due to synthesis of low levels of IFN, phosphorylation of STAT1 was investigated by immunoblot analysis (Fig. 9). In IFN treated FrhK4 cells, STAT1 phosphorylation was observed as early as 10 min post-treatment (data

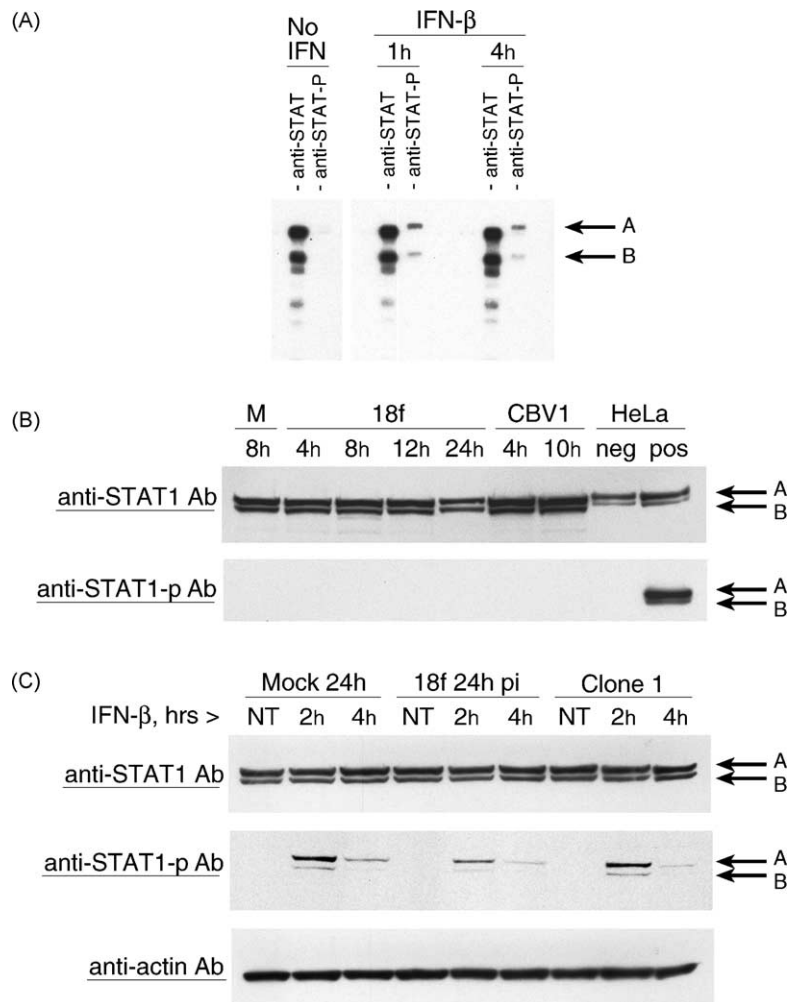


Fig. 9. STAT 1 phosphorylation in response to virus infection and/or IFN- β treatment. (A) FrhK4 cells were treated with IFN- β for 1 or 4 h prior to total protein extraction. (B) FrhK4 cells were mock infected (M), infected with 18f for various times, or infected with coxsackie B1 (CBV1) prior to protein extraction. (C) FrhK4 cells were infected with 2 pfu/cell 18f for 2 h followed by the addition of growth medium. After 24 h, cells were treated with 500 U/ml IFN- β in growth medium. NT means not treated with IFN- β . Proteins were extracted after 2 or 4 h of IFN treatment. Clone 1 cells were used at 330d pi. Western blotting with an antibody to unphosphorylated STAT 1 (anti-STAT), phosphorylated STAT 1 (anti-STAT-P), or actin was performed as described in Section 2.

not shown), and the peak level of phosphorylation was detected at 1 h post-treatment (Panel A). These results confirm that FrhK4 cells are capable of responding to IFN (Panel A). However, no phosphorylation was detectable following infection with 18f, up to 24 h pi (Panel B), when rRNA degradation is occurring. Previously, we followed STAT 1 phosphorylation for up to 72 h pi and found no evidence of activation of this pathway (Kulka et al., 2003). Surprisingly, cells infected with the 18f virus for 24 h showed diminished phosphorylation of STAT 1 when treated with IFN, compared to uninfected or clone 1 cells treated with IFN (Panel C). Thus, the cp strains of HAV not only interfere with the induction of IFN by dsRNA (Brack et al., 2002), but can also interfere with IFN signaling. Similarly, coxsackievirus B1, a fast-replicating picornavirus that induces similar RNA degradation in FrhK4 cells, did not induce phosphorylation of STAT1 (Panel B). The data clearly suggest the existence of a virus inducible, 2–5A independent RNA degradative pathway that is activated by 18f infection in the apparent absence of IFN induction of 2′–5′ OAS.

4. Discussion

In this study, we report a novel pathway for rRNA degradation in cytopathogenic hepatitis A virus infected cells. The observed cleavage of both species of rRNA suggests that this phenomenon is different from the cleavage of 28S rRNA in mouse hepatitis A virus (a coronavirus) infected cells (Banerjee et al., 2000). We have also shown that the virus induced rRNA cleavage is not a consequence of apoptosis, as has been reported to occur as a result of perturbation of cellular macromolecular synthesis (Houge and Døskeland, 1996). In a previous study, we have shown that the cleavage of rRNAs is also accompanied by a reduction in the levels of some cellular mRNAs, while viral RNA was not significantly affected (Kulka et al., 2003). The appearance of specific sizes of rRNA degradation products is reminiscent of IFN regulated 2–5A activated RNase L. The activation of this pathway, which is part of the cellular defense against viral infection, requires prior exposure of cells to IFN (Stark et al., 1998; Barber, 2001; Sen, 2001). RNA degradation during 18f infection, however, occurs without prior treatment of the cells with IFN. It is conceivable that a slowly replicating virus such as HAV could induce production of low levels of IFN to activate the 2–5A pathway in surrounding uninfected cells. We showed that this is not the case, since the JAK/STAT pathway was not activated following virus infection, even though IFN- β mRNA was marginally induced in 18f and clone 1 infected cells. These results make it unlikely that the canonical 2–5A dependent RNA degradation pathway is activated during 18f infection. We also showed that RNA is not degraded in persistently infected cells despite the presence of comparable levels of viral RNA and antigens (proteins). Our results complement the results of Brack et al. (2002) and Vallbracht

et al. (1984), that IFN- β does not play a major role in HAV infection.

The RNase L enzyme is one member of a multi-component system for RNA degradation, consisting of IFN, 2′–5′ OAS (the enzyme that synthesizes 2–5A from ATP in the presence of dsRNA), and the latent endoribonuclease RNase L. RNase L is generally present in most cells and may function in situations other than virus infections, particularly during differentiation, apoptosis, hormonal regulation, chronic fatigue syndrome, and certain types of prostate cancer (for a review, see Silverman, 2003). RNase L levels are usually unaffected by virus infection or IFN treatment, but it is activated only in the presence of the proximal activator 2–5A, a series of unique 2′–5′ phosphodiester linked oligoadenylic acid molecules. The synthesis of 2–5A is dependent upon the induction of the enzyme 2′–5′ OAS which is activated by dsRNA or other activator molecules such as the hepatitis C virus core protein (Naganuma et al., 2000). We showed that 2′–5′ OAS was not induced in 18f infected cells, despite the occurrence of massive RNA degradation. We also showed that the failure to detect 2′–5′ OAS mRNA was not due to a failure of the RT-PCR protocol or the genetic background of the FrhK4 cells, since treatment of this cell line with IFN- β resulted in an induction of the 69/71 form of 2′–5′ OAS mRNA. We also showed that the mRNA for RNase L was present in both virus infected and uninfected cells, with some increase in the level of its mRNA following virus infection. We conclude that either RNase L may be activated by factors other than 2–5A, or that the enzyme responsible for RNA degradation is a novel RNase specifically induced or activated in 18f and coxsackievirus infected cells. In this regard, IFN treatment has been shown to stimulate the expression of a second ribonuclease, identified as ISG20 (Nguyen et al., 2001). However, this enzyme is an exonuclease, and the negative effect of IFN treatment on 18f-induced RNA degradation (Fig. 7) argues against the role of this enzyme.

Unlike most picornaviruses, persistent infection is the norm rather than the exception for HAV, at least in vitro in tissue culture cells. Persistent infection by most cc strains of HAV has been suggested to be the result of inefficient translation initiation from its IRES (Whetter et al., 1994; Funkhouser et al., 1999; Yi et al., 2000). Recent results suggest that HAV IRES is as efficient as the IRES of other picornaviruses in initiating translation, and the switch from translation to negative strand synthesis is the rate-limiting step in HAV replication (Gauss-Müller and Kusov, 2002). It has also been suggested that the slow replication (even the replication of cp strains are slow compared to other picornaviruses) of HAV is a result of down regulation of its replication by the virus itself by unknown mechanisms (De Chastonay and Siegl, 1987). In the discussion of slow replication of HAV, the lack of a functional 2A protease has often been overlooked. Picornaviral 2A protease is responsible for promoting cap-independent translation by inactivating eIF4G, and possibly other translation factors (Ziegler et al.,

1995; Kerekatte et al., 1999). 2A protease is also a strong inducer of apoptosis when expressed ectopically (Goldstaub et al., 2000). In the absence of a functional 2A protease, HAV viral RNA has to compete with cellular mRNAs for the host cell translation machinery. We suggest that induction of an RNase activity may partially compensate for the absence of an active 2A protease, by reducing competition from cellular mRNAs and contributing to the rapid replication phenotype of 18f and possibly other cp strains of HAV.

Apoptosis is generally thought to be a host defense response to virus infection (for a review, see Barber, 2001). Premature induction of apoptosis results in abortive virus replication. However, a late induction of apoptosis might favor the virus by allowing it to evade the host immune system. Many viruses have thus evolved mechanisms to delay the onset of an apoptotic response (Roulston et al., 1999). Similarly, induction of IFN mediated antiviral pathways is a defensive response of the host to virus infection, and many viruses have evolved mechanisms to block the induction of IFN mediated antiviral pathways (for reviews, see Barber, 2001; Sen, 2001). It is therefore not surprising that the two major IFN mediated antiviral pathways, namely the 2–5A/RNase L pathway and the dsRNA activated PKR pathway, are also mediators of apoptosis.

Among the picornaviruses, HAV is unique in its slow replication in permissive cells, and also in its ability to initiate and maintain a persistent infection. Persistent infection is known for other picornaviruses, but usually requires restriction of virus replication and gene expression, through the use of inhibitors in cultured cells and presumably the host IFN regulated system or the immune system in the intact organism (Agol et al., 2000). Moreover, cp strains of HAV kill cells by apoptosis in cell culture, while in the whole organism, a role for apoptosis in the destruction of infected cells, or evading the host immune response is uncertain. The late depletion of PARP-1 clearly indicates that HAV is capable of avoiding the induction of necrosis in infected cells and evade a host immune response.

In conclusion, we present evidence that unlike some other picornaviruses, notably encephalomyocarditis (EMC) virus, HAV is relatively insensitive to the antiviral effect of IFN. Furthermore, the degradation of rRNA in HAV 18f infected cells occurs in the absence of 2′–5′ OAS, the key enzyme that regulates the 2–5A/RNase L antiviral pathway. The 18f virus is able to suppress the expression of ISGs including 2′–5′ OAS by interfering with JAK/STAT signaling by an unknown mechanism, and it utilizes the degradation of RNA as a means to augment its replication and subsequently induce apoptosis in the host cell.

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