JOURNAL OF VIROLOGY, Nov. 1988, p. 4201–4206
0022-538X/88/114201-06$02.00/0

Vol. 62, No. 11

Restricted Replication of Hepatitis A Virus in Cell Culture: Encapsidation of Viral RNA Depletes the Pool of RNA Available for Replication

DAVID A. ANDERSON,* BRUCE C. ROSS, AND STEPHEN A. LOCARNINI

Macfarlane Burnet Centre for Medical Research, Fairfield Hospital, Fairfield, Victoria 3078, Australia

Received 23 May 1988/Accepted 31 July 1988

The replication of hepatitis A virus (HAV) in BS-C-1 cells was examined under single-cycle growth conditions by using strand-specific probes for detection of viral RNA species. No measurable lag phase was demonstrated between accumulation of positive-strand HAV RNA and production of infectious virions, indicating that replication of virion RNA is rate limiting for the production of infectious virus. Intracellular viral RNA was further analyzed by using 2 M LiCl to fractionate the insoluble nonvirion 35S RNA and replicative intermediates (RI) from the soluble virions and double-stranded replicative forms, in conjunction with sucrose density gradient ultracentrifugation to separate the different forms of viral RNA. Throughout the productive phase of HAV infection, 95 to 97% of positive-strand HAV RNA was soluble in 2 M LiCl and was shown to be contained in mature virions. Of the LiCl-insoluble HAV RNA, more than 99% was positive-stranded 35S RNA, whereas 0.4% was negative stranded and had the sedimentation and partial RNase resistance characteristics of RI. The pattern of RNA accumulation in HAV-infected cells is thus very different from that seen in poliovirus-infected cells, where large pools of RI and mRNA are produced before RNA is sequestered into mature virions. The results of this study suggest that encapsidation of positive-strand HAV RNA inhibits transcription at all times during the growth cycle, thereby reducing the pool of replicating RNA and the final yield of infectious HAV.

The growth of hepatitis A virus (HAV) in cell culture has been studied extensively since it was first propagated by Provost and Hilleman in 1978 (19). In contrast to most picornaviruses, HAV generally establishes persistent infections of cell cultures, with the continuous production of low levels of infectious virus (10, 11, 19, 23, 27, 30) and very little effect on morphology (14) or host cell macromolecular synthesis (10, 11, 15). While the recently described cytopathic strains of HAV (1, 9, 22, 28) also produce low yields of virus, their growth cycles are short enough to allow comparative studies of the replicative cycle with other picornaviruses such as poliovirus type 1 (PV).

Poliovirus replication is highly efficient, with almost half the total cellular production of RNA and protein being found in progeny virions (7). The mechanisms which control the fate of viral RNA and proteins during the growth cycle are not well understood, but the sequential accumulation of progeny viral RNA in replicative intermediates (RI), polyribosomes, and finally mature virions is essential for the efficient replication of the virus (3, 7, 17).

We have previously shown that, with HAV strain HM175, accumulation of infectious virus and HAV RNA have similar kinetics, and we have suggested that the production of HAV RNA is rate limiting (2). However, the factors restricting RNA replication were not identified in that study. A similar pattern of RNA and virion accumulation for the noncytopathic strain of HAV, GBM, in MRC-5 cells has also been reported (10).

To determine the factors limiting RNA replication, we have examined the fate of different forms of viral RNA produced throughout the growth cycle of HAV strain HM175. The results indicate that while the very small amount of negative-strand HAV RNA is used efficiently as a template for RNA synthesis, HAV positive-strand RNA is preferentially encapsidated and only poorly utilized as a template for RNA synthesis. The control of HAV RNA replication and morphogenesis is thus dissimilar to that of other picornaviruses.

MATERIALS AND METHODS

Cells and viruses. The cytopathic HAV strain HM175A.2 and PV (Mahoney) were propagated in BS-C-1 cells as described previously (1), and after plaque purification, virus stocks were prepared from passages 8 and 3, respectively. BS-C-1 cells were used between passages 44 and 60. All cultures were maintained at 34°C in Eagle minimum essential medium (MEM).

The plaque assay for HAV (1) was modified by the use of an overlay medium consisting of MEM containing 0.5% SeaPlaque agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) and 25 μM 5,6-dichlorobenzimidazole riboside (Sigma Chemical Co., St. Louis, Mo.), which has been reported to enhance HAV growth in cell culture (32). Addition of this drug results in a clear plaque morphology but, in our hands, had no significant effect on the yield of virus (D. A. Anderson, unpublished observations). After 9 to 14 days of incubation, cultures were fixed with 4% formaldehyde in 0.85% NaCl for 1 h and stained with 0.2% crystal violet in 20% ethanol.

Synthesis of strand-specific probes. The molecular cloning of HAV cDNA has been described previously (20). A cDNA fragment representing map positions 2.0 (*Hin*dIII) to 5.0 (*Eco*RI) kilobases was subcloned into plasmid pGEM-3 (Promega Biotec, Madison, Wis.) between the *Eco*RI and *Hin*dIII sites of the polylinker (pGEMHAV). Plus strands were synthesized from *Eco*RI-digested pGEMHAV by using the SP6 promoter, and minus strands were synthesized from *Hin*dIII-digested pGEMHAV by using the T7 promoter.

* Corresponding author.

Labeled RNA was synthesized by using 200 ng of digested pGEMHAV for 1 h at 37°C in 30 μl containing 40 mM Tris (pH 7.5), 10 mM NaCl, 6 mM MgCl, 2 mM spermidine, 250 μM each of cold ribonucleotides (ATP, GTP, and CTP), 1 μM [α-^{32}P]UTP (1,000 Ci/mmol), and 10 U of SP6 polymerase (BRESA, Australia) or T7 polymerase (Amersham, England). After incubation, 10 U of RNase-free DNase (BRESA) was added and the mixture was incubated for 15 min at 37°C. The unincorporated ribonucleotides were removed by gel filtration chromatography through Sephadex G-75 (Pharmacia, Uppsala, Sweden). The column void volume was pooled, and the labeled RNA was stored at −20°C.

Single-cycle growth experiments. Cells were infected with 2 PFU per cell of HAV or PV for 1 h at 34°C prior to the addition of MEM. In some experiments, cultures were treated with MEM containing 4 μg of actinomycin D per ml (Merck Sharp & Dohme, West Point, Pa.) for 3 h before infection to suppress host cell RNA synthesis and were fed a similar medium containing 100 μCi of [5,6-^{3}H]uridine per ml (41 Ci/mmol; Amersham). At various times after infection, cells were harvested in 0.5 ml of NPT (0.5% Nonidet P-40, 0.3 M NaCl, 10 mM Tris [pH 7.4]) per 2 × 10^6 cells, and nuclei were removed by centrifugation at 13,500 × *g* for 1 min in an Eppendorf microcentrifuge. Clarified lysates were stored at −70°C. Parallel cultures on glass cover slips were fixed in acetone at 4°C for 2 min for indirect immunofluorescence staining of double-stranded RNA (dsRNA), as described previously (2), by using rabbit anti-dsRNA (a gift from R. Francki, University of Adelaide, Adelaide, South Australia).

Fractionation of RNA. Intracellular RNA was fractionated with 2 M LiCl by a modification of the method of Spector and Baltimore (25). Cell lysates in NPT were mixed with 10% sodium dodecyl sulfate (SDS), 0.3 M EDTA, and 10 M LiCl to give final concentrations of 1% SDS, 10 mM EDTA, and 2 M LiCl [pH 7.4] and were incubated at −20°C overnight. The precipitate containing single-stranded cytoplasmic RNA and partially single-stranded (RI) RNA was collected by centrifugation at 13,500 × *g* for 15 min at 4°C, washed with 2 M LiCl, and redissolved in 1% SDS–10 mM EDTA. Samples for nuclease digestion were then extracted by the phenol-chloroform method as described previously (8) and were redissolved in 0.3 M NaCl–10 mM Tris [pH 7.4].

Sucrose density gradient ultracentrifugation. Linear 10 to 30% sucrose gradients in 0.1% SDS–100 mM NaCl–10 mM EDTA–10 mM Tris [pH 7.4] were prepared in Beckman SW60 tubes over a 0.3-ml cushion of 90% glycerol–100 mM Tris [pH 7.4]. Gradients were allowed to equilibrate at room temperature for 4 h before use. Cell lysates in NPT or purified RNA samples were adjusted to 1% SDS, and 0.4 ml was loaded on gradients and centrifuged at 130,000 × *g* for 4 h at 18°C. Two fractions of 0.5 ml and fourteen fractions of 0.25 ml were collected from the bottom of each tube and were analyzed for viral RNA and infectious virus.

RNase digestion. The sensitivity of RNA samples and gradient fractions to RNase was determined by the addition of NaCl to 0.3 M and RNase to 0 (control) or 8 μg per ml (6,550 U/mg; Millipore Corp., Freehold, N.J.). After incubation for 30 min at 37°C, the reactions were stopped by the addition of 10% SDS to a final concentration of 1% for sucrose density gradient ultracentrifugation or 3 volumes of FSSC (6.1 M formaldehyde, 3 M NaCl, 0.3 M sodium citrate) for dot blot hybridization.

Hybridization of viral RNA. HAV RNA samples mixed with FSSC were heated at 65°C for 15 min and then applied to 0.45-μM-pore-size nitrocellulose membranes (Schleicher & Schuell, Dassel, Federal Republic of Germany) equilibrated in 20× SSC (pH 7.0) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) by using a Bio-Dot apparatus (Bio-Rad Laboratories, Richmond, Calif.). Filters were dried at 20°C, baked at 80°C for 2 h, and prehybridized overnight at 42°C with 200 μg of herring sperm DNA per ml in 50% formamide–5× SSC, 1× Denhardt solution (0.02% each Ficoll, polyvinylpyrrolidone, and bovine serum albumin)–5 mM $NaHPO_4$ [pH 6.5]. Plus- or minus-strand probes were added to the prehybridized filters and hybridized overnight at 42°C. Filters were then washed with two changes each of 2× SSC–0.1% SDS at room temperature and 0.1× SSC–0.1% SDS at 50°C. After drying in air the filters were exposed to preflashed X-Omat RP film (Eastman Kodak Co., Rochester, N.Y.) between Cronex intensifying screens (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) at −70°C. In some experiments, the filters were separated and the amount of labeled probe bound was measured in a liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

PV RNA samples were processed in the same way with the omission of the hybridization steps, and after washing, the samples were separated and mixed with Insta-Gel (Packard Instrument Co.) for the counting of incorporated [5,6-^{3}H]uridine.

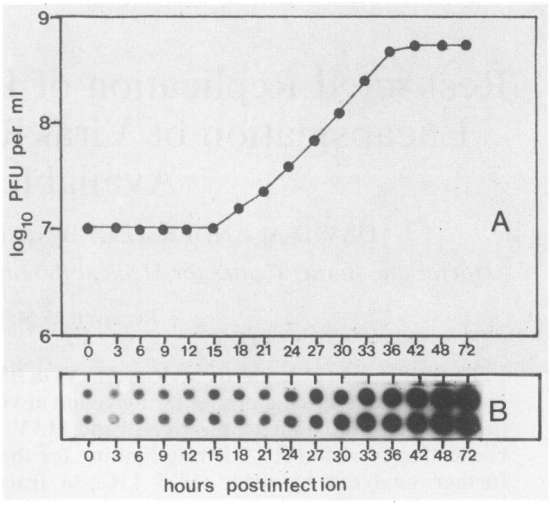

FIG. 1. Accumulation of positive-strand RNA during a single growth cycle of HAV strain HM175A.2. BS-C-1 cells were infected with 2 PFU of HAV per cell and assayed for intracellular infectious virus (A) and positive-strand RNA (B) as described in Materials and Methods.

RESULTS

Kinetics of RNA synthesis in HAV-infected cells. The replication of viral RNA during a single growth cycle of HAV in BS-C-1 cells was examined by dot blot hybridization with HAV-specific minus-strand probes (Fig. 1). As noted previously (2, 30), there was no clearly defined eclipse of the input HAV, as infectious virus can be isolated from the cells at all times after infection. An increase in the amount of positive-strand HAV RNA and infectious virus was first evident at 18 h postinfection (Fig. 1). Positive-strand RNA and infectious virus continued to accumulate in parallel to 36 h, after which there was no further increase of infectious virus despite continued RNA synthesis. This was in contrast to the differential accumulation of PV RNA and virions during

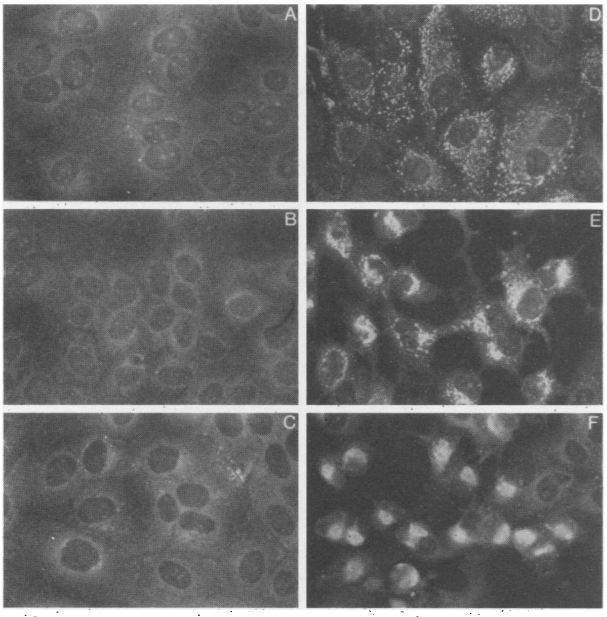

FIG. 2. Indirect immunofluorescence staining of double-stranded RNA in BS-C-1 cells. (A) Uninfected cells. (B) HAV-infected cells at 36 h postinfection. (C through F) PV-infected cells at 4, 6, 8, and 10 h postinfection, respectively.

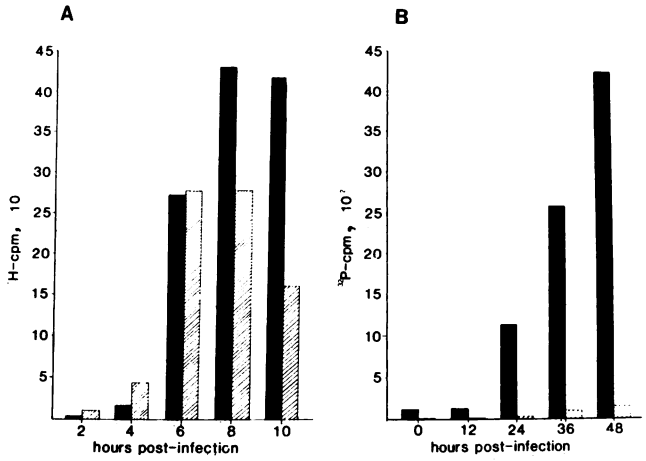

FIG. 3. Fractionation of intracellular viral RNA with 2 M LiCl. Single-stranded nonvirion RNA and partially single-stranded RI RNA were precipitated with 2 M LiCl as described in Materials and Methods. ▨, LiCl-insoluble RNA; ■, LiCl-soluble RNA. (A) PV-infected cells labeled with [5,6-^{3}H]uridine in the presence of actinomycin D from the beginning of infection. (B) HAV-infected cells probed for positive-strand RNA by using ^{32}P-labeled minus-strand probes as described in Materials and Methods.

infection (2, 7), where a large pool of replicating RNA is produced before virions start to accumulate.

Negative-strand RNA in RI and replicate forms (RF) represents approximately 10 to 20% of viral RNA in poliovirus-infected cells (7), but negative-strand HAV RNA was barely detectable in samples such as those in Fig. 1, where it was present in 2×10^6 HAV-infected cells (results not shown). HAV clearly utilizes a very small number of negative-strand templates for RNA synthesis.

Indirect immunofluorescence staining of dsRNA in cells infected with PV or HAV (Fig. 2) also demonstrated that HAV produces few RI. Specific staining of dsRNA in PV-infected cells is seen at 4 h and is very intense at 6 to 8 h (Fig. 2C, D, and E), when the accumulation of RNA and virions is most rapid. In contrast, little specific staining is seen in HAV-infected cells at 36 h (Fig. 2B), when RNA synthesis is at a peak. This antiserum presumably detects both RI and RF, since the addition of 2 mM guanidine hydrochloride to PV-infected cells for 30 min before acetone fixation, which would convert most RI to RF (17, 21), has no apparent effect on the distribution or intensity of staining (results not shown).

Fractionation and sedimentation of viral RNA. To determine why the replication of HAV RNA is at such a low level, the RNA in cells infected with HAV or PV was fractionated with 2 M LiCl (25). In PV-infected cells (Fig. 3A), most of the viral RNA is found in LiCl-insoluble forms (RI and nonvirion 35S RNA) from 2 to 6 h postinfection, while from 6 to 10 h it accumulates predominately in LiCl-soluble forms (virions and RF). However, in HAV-infected cells negative-strand RNA was undetectable in this experiment (results not shown); most of the positive-strand viral RNA is LiCl soluble at all times during infection (Fig. 3B), and only 3 to 5% is insoluble in 2 M LiCl. This indicates both the inefficiency of HAV uncoating and the high proportion of progeny positive-strand RNA found in virions. HAV RNA was shown to be insoluble in 2 M LiCl by heating the HAV lysates at 68°C for 10 min before precipitation. This treatment is sufficient to denature virions (24) and resulted in all of the HAV RNA becoming LiCl insoluble (results not shown).

Since these results suggested that encapsidation of positive-strand HAV RNA was more efficient than replication and translation, infected cell lysates were further examined by using sucrose density gradient ultracentrifugation (Fig. 4). Poliovirus RNA labeled with [5,6-^{3}H]uridine from 0 to 6 h postinfection resolved into three peaks under these conditions (Fig. 4A), representing infectious virions, RNase-sensitive nonvirion 35S RNA, and partially RNase-resistant material with lower sedimentation coefficients. Precipitation with LiCl removed most of the RNase-resistant material (results not shown), which is presumably RI, leaving a small amount of RF which is LiCl soluble. PV-infected cells labeled from 0 to 8 or 10 h postinfection yielded similar amounts of 35S RNA and RI but increased amounts of virions and RF (results not shown). Positive-strand HAV RNA in cells harvested at 48 h postinfection was detected in two peaks (Fig. 4B), representing infectious virions (major peak) and RNase-sensitive 35S RNA (minor peak). Qualitatively similar results were seen with cells infected for 24 or 36 h (results not shown). In addition, an extremely faint RNase-resistant peak was sometimes seen in fractions corresponding to the RI region of the PV gradients (results not shown). These results further demonstrate that most of the positive-strand HAV RNA is encapsidated.

Analysis of LiCl-insoluble HAV RNA. The RNA which is actively engaged in the production of virus-specific macromolecules in picornavirus-infected cells is expected to be insoluble in 2 M LiCl, being at least partially single stranded (RI and nonvirion 35S RNA). It was therefore of interest to examine this small fraction of HAV intracellular RNA in more detail. The LiCl-insoluble RNA from 2×10^8 uninfected cells and HAV-infected cells was extracted at 36 h postinfection with phenol-chloroform as described in Materials and Methods. Samples of RNA were treated with RNase and applied to nitrocellulose filters in doubling dilutions along with untreated RNA samples representing an

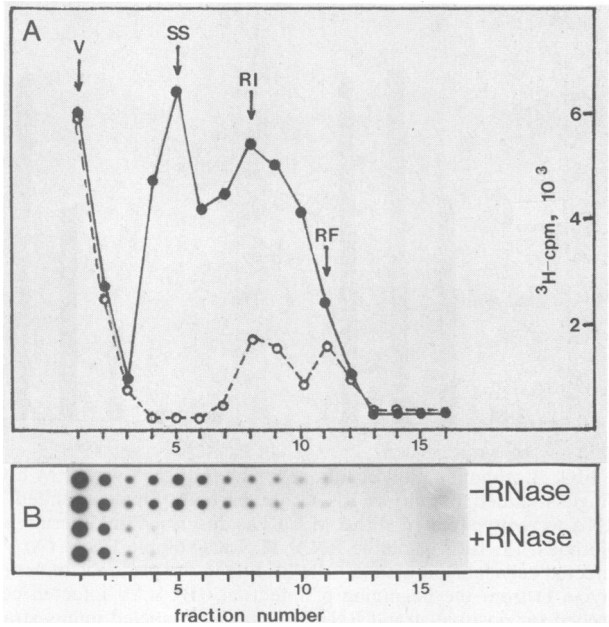

FIG. 4. Sucrose density gradient ultracentrifugation of intracellular viral RNA. Cell lysates were centrifuged at 130,000 × *g* for 4 h at 18°C on linear 10 to 30% sucrose gradients over a 90% glycerol cushion in Beckman SW61 tubes. Two fractions of 0.5 ml and fourteen fractions of 0.25 ml were collected from the bottom of each tube, and 0.1-ml aliquots of each fraction were blotted before (−RNase, ●) or after (+RNase, ○) digestion with RNase as described in Materials and Methods. (A) PV-infected cells labeled with [5,6-^{3}H]uridine from 0 to 6 h postinfection. (B) HAV-infected cells harvested at 48 h postinfection and probed for positive-strand RNA as described in Materials and Methods. V, Virions; SS, nonvirion 35S RNA.

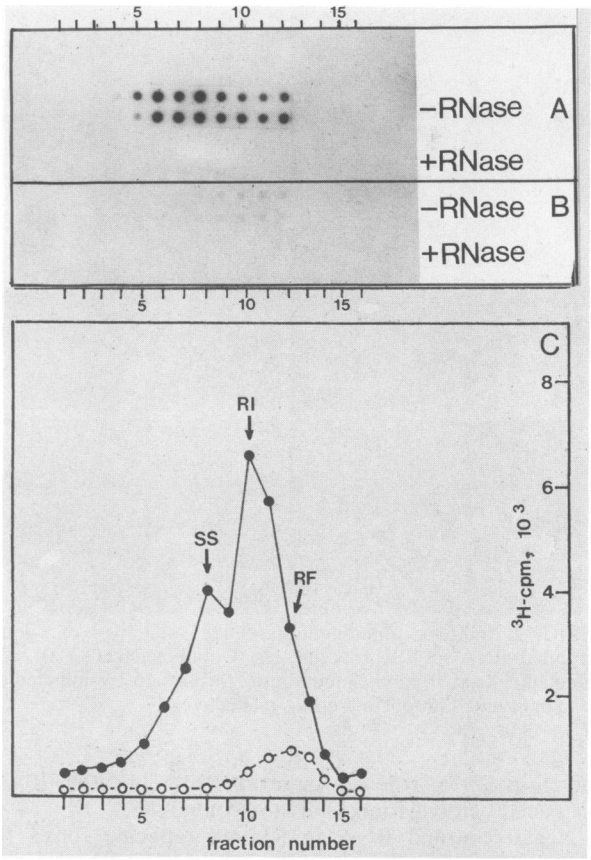

FIG. 6. Sucrose density gradient ultracentrifugation of LiCl-insoluble PV and HAV RNA. Samples were prepared as described in the legend to Fig. 5, mixed with RNase or water, and then centrifuged as described in Materials and Methods. Positive-strand HAV RNA (A) and negative-strand HAV RNA (B) are shown at 48 h postinfection. (C) PV RNA labeled with [5,6-^{3}H]uridine from 0 to 6 h postinfection. −RNase, ●, no RNase digestion; +RNase, ○, RNase digested; SS, nonvirion 35S RNA. Sedimentation was from right to left.

equal number of cells. These filters were probed for the presence of positive- and negative-strand RNA (Fig. 5). Approximately 0.4% of the total LiCl-insoluble HAV RNA was of negative polarity, and more than 25% of this RNA was resistant to RNase digestion (Fig. 5B). However, only 0.1% of the LiCl-insoluble positive-strand HAV RNA was RNase resistant (Fig. 5A); as expected, equal amounts of positive- and negative-strand RNA were RNase resistant. Electrophoresis of LiCl-insoluble HAV RNA on denaturing formaldehyde-formamide gels, followed by hybridization with strand-specific probes, showed that the RNase-sensitive positive-strand RNA is predominately of genome length, but the RNase-resistant positive- and negative-strand RNA were not detected in this experiment (results not shown). Sucrose density gradient ultracentrifugation of similar samples (Fig. 6) showed that the positive-strand RNA (Fig. 6A) sedimented in parallel with external marker 35S PV RNA, while the negative-strand RNA (Fig. 6B) was found in fractions corresponding to PV RI (Fig. 6C). Similar results were seen for positive-strand HAV RNA at 24 and 36 h postinfection, but negative-strand RNA was undetectable (results not shown). Furthermore, the putative RNase-resistant backbone of HAV RI was not detected in this experiment. Nevertheless, these results suggest that the very small amount of negative-strand HAV RNA in the cells is efficiently utilized as a template for positive-strand RNA synthesis, while the positive-strand HAV RNA which is not encapsidated is presumably used for translation and only rarely utilized for the synthesis of negative-strand RNA.

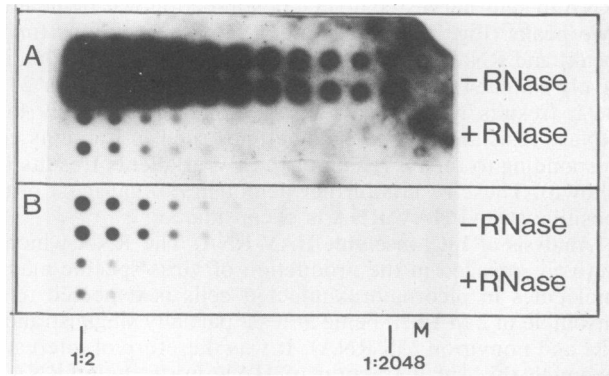

FIG. 5. Titration of LiCl-insoluble HAV RNA. Intracellular LiCl-insoluble RNA was prepared from 100 times the number of infected cells shown in Fig. 1 and 3 and mixed with water (−RNase) or RNase (+RNase) as described in Materials and Methods. Serial twofold dilutions of the reaction mixtures were probed for residual positive- (A) and negative- (B) strand RNA. M, Uninfected cell lysate.

DISCUSSION

This report provides the first detailed analysis of the replicative cycle of HAV and the factors which restrict the growth of this unique picornavirus in cell culture. We suggest that the slow rate of growth and low yield of HAV are direct results of highly efficient encapsidation of positive-strand RNA at the expense of the RNA pool available for transcription.

Poliovirus replication is controlled in such a way that the progeny RNA produced early in infection are predominantly used for further transcription and translation, thereby generating pools of 35S RNA and capsid precursors, while mature virus particles are not produced until a large pool of replicating RNA has accumulated (4, 7). In contrast, infectious HAV virions are produced as soon as RNA is available (2) (Fig. 1) and most of the positive-strand RNA is found within virions throughout infection (Fig. 3 and 4). Negative-strand HAV RNA is present in only minute amounts but is largely resistant to RNase (Fig. 5) with the sedimentation characteristics of RI (Fig. 6) and is presumably a functional and efficient template for RNA synthesis. Together, these results suggest that it is the regulation of HAV RNA replication and morphogenesis which limits the growth of the virus.

The pattern of RNA accumulation into virions throughout the HAV growth cycle (Fig. 1, 3, and 4) is similar to that seen during the linear phase of PV replication, when much of the newly synthesized RNA is encapsidated (7). The regulation of HAV growth may therefore differ from other picornaviruses only in kinetics rather than in the mechanisms of regulation. The control mechanisms in PV RNA replication are not fully understood (7, 21, 33), but the change from exponential accumulation of RNA to linear accumulation with the concomitant formation of mature virions suggests that morphogenesis and RNA replication are intimately associated (21).

The genome-linked protein VPg is probably involved in the control of picornavirus morphogenesis (21), since all encapsidated RNA molecules have VPg at the 5′ end whereas polyribosomal RNA does not contain VPg (13). VPg may also be involved in the initiation of RNA synthesis (16, 26). Interestingly, the VPg of HAV is unique among the picornaviruses, having an estimated isoelectric point of 7.15 compared to 10 to 11 for other picornaviruses (29). This could be expected to have some effect on the kinetics of HAV replication, although it should be noted that the initiation of positive-strand synthesis on negative-strand HAV RNA templates appears to be quite efficient, judging from the small number of RI synthesizing the progeny HAV RNA (Fig. 2, 5, and 6).

The viral capsid or a precursor of it is also likely to be a regulator of morphogenesis (7, 18). In this respect, two features of HAV which have been described now appear relevant. The HAV virion is extremely stable at elevated temperatures (1, 24) and is also very stable upon penetration of the cell, taking approximately 12 h to be uncoated (2, 30; Fig. 1). Interestingly, VP4 is involved in both uncoating and thermal inactivation, being lost as an early step in uncoating or upon heating of picornaviruses (21). The available nucleic acid sequence data suggest that HAV has a very small VP4 of only 23 amino acids (5), but this protein has not been convincingly demonstrated in virions, and there is conflicting evidence for the existence of VP0 or VP2 in mature HAV virions (12, 31; B. C. Ross, unpublished observations). The atypical VP4 of HAV is likely to contribute to the unusual stability of the virion. Even though the reasons for the stability of HAV are not clear, it is reasonable to speculate that there is a very high affinity between the viral subunits and RNA which could be expected to affect the kinetics of HAV morphogenesis and may account for the rapid encapsidation of newly synthesized positive-strand HAV RNA and the slow rate of uncoating.

There is clearly an advantage to HAV in the extreme stability of the virion, allowing it to survive in the environment for long periods. We propose a model for the restriction of HAV replication which suggests that this stability is achieved at the expense of replicative efficiency. The attachment and penetration of HAV are rapid and efficient (2, 30), but after penetration the virion is uncoated very slowly because of a high affinity of the capsid and RNA. VPg is probably removed from the input RNA, which is then translated to generate the viral RNA polymerase, proteases, VPg precursors, and structural proteins. Positive-strand RNA is only rarely transcribed into negative strands, but these negative strands are very efficient templates for positive-strand RNA synthesis. Most of the newly synthesized positive-strand RNA is immediately encapsidated as a consequence of the same mechanisms (involving VPg or VP4) which contribute to the stability of the mature virion. The virus therefore never produces a large pool of replicating RNA, but the small amount of virus produced is extremely stable.

Several predictions can be made on the basis of this hypothesis. It is likely that an excess of viral capsid precursors would be present in the cell to enable the encapsidation reactions to occur at such a high rate. We have preliminary data which suggest that such a pool exists (B. C. Ross and D. A. Anderson, unpublished observations), and further study of the HAV virion precursors may provide important information about the assembly of picornaviruses. Additionally, a reduction in the amount of viral protein in the cell by a partial translational block (such as pactamycin) might result in RNA replication and encapsidation becoming more equally balanced. Similarly, mutants of HAV which are unable to produce the correct capsid precursors would be expected to replicate their RNA more efficiently, as competition for the newly synthesized positive strands would be reduced. It should become possible to test this by using infectious RNA transcripts of HAV cDNA (6) with deletions in the capsid region. Substitution of PV or aphthovirus VPg for the unusual HAV VPg might also affect HAV replication. Indeed, we believe that manipulation of the unique features of the HAV replicative cycle outlined in this report may provide useful tools for the study of picornavirus replication and morphogenesis.

ACKNOWLEDGMENTS

We are grateful to D. S. Bowden and I. D. Gust for critical reading of the manuscript.

B.C.R. was supported by the Maroline M. Weatherley Scholarship. This work was supported in part by grants from the National Health and Medical Research Council and the World Health Organization.

LITERATURE CITED

1. **Anderson, D. A.** 1987. Cytopathology, plaque assay, and heat inactivation of hepatitis A virus strain HM175. J. Med. Virol. **22**:35–44.
2. **Anderson, D. A., S. A. Locarnini, B. C. Ross, A. G. Coulepis, B. N. Anderson, and I. D. Gust.** 1987. Single-cycle growth kinetics of hepatitis A virus in BSC-1 cells, p. 497–507. *In* M. A. Brinton and R. R. Rueckert (ed.), Positive strand RNA viruses.

Alan R. Liss, New York.
3. **Baltimore, D.** 1968. Structure of the poliovirus replicative intermediate RNA. J. Mol. Biol. **32**:359–368.
4. **Baltimore, D.** 1969. The replication of picornaviruses, p. 101–176. *In* H. B. Levy (ed.), The biochemistry of viruses. Marcel Dekker, Inc., New York.
5. **Baroudy, B. M., J. R. Ticehurst, T. A. Miele, J. V. Maizel, Jr., R. H. Purcell, and S. M. Feinstone.** 1985. Sequence analysis of hepatitis A virus cDNA coding for capsid proteins and RNA polymerase. Proc. Natl. Acad. Sci. USA **82**:2143–2147.
6. **Cohen, J. I., J. R. Ticehurst, S. M. Feinstone, B. Rosenblum, and R. H. Purcell.** 1987. Hepatitis A virus cDNA and its RNA transcripts are infectious in cell culture. J. Virol. **61**:3035–3039.
7. **Cooper, P. D., A. Steiner-Pryor, and P. J. Wright.** 1973. A proposed regulator for poliovirus: the equestron. Intervirology **1**:1–10.
8. **Coulepis, A. G., G. A. Tannock, S. A. Locarnini, and I. D. Gust.** 1981. Evidence that the genome of hepatitis A virus consists of single-stranded RNA. J. Virol. **37**:473–477.
9. **Cromeans, T., M. D. Sobsey, and H. A. Fields.** 1987. Development of a plaque assay for a cytopathic, rapidly replicating isolate of hepatitis A virus. J. Med. Virol. **22**:45–56.
10. **de Chastonay, J., and G. Siegl.** 1987. Replicative events in hepatitis A virus-infected MRC-5 cells. Virology **157**:268–275.
11. **Gauss-Müller, V., and F. Deinhardt.** 1984. Effect of hepatitis A virus infection on cell metabolism in vitro. Proc. Soc. Exp. Biol. Med. **175**:10–15.
12. **Gauss-Müller, V., F. Lottspeich, and F. Deinhardt.** 1986. Characterization of hepatitis A virus structural proteins. Virology **155**:732–736.
13. **Hewlett, M. J., J. K. Rose, and D. Baltimore.** 1976. 5′ terminal structure of poliovirus polyribosomal RNA is pUp. Proc. Natl. Acad. Sci. USA **73**:327–330.
14. **Kiernan, R. E., J. A. Marshall, A. G. Coulepis, D. A. Anderson, and I. D. Gust.** 1987. Cellular changes associated with persistent hepatitis A infection *in vitro*. Arch. Virol. **94**:81–95.
15. **Locarnini, S. A., A. G. Coulepis, E. G. Westaway, and I. D. Gust.** 1981. Restricted replication of human hepatitis A virus in cell culture: intracellular biochemical studies. J. Virol. **37**:216–225.
16. **Morrow, C. D., and A. Dasgupta.** 1983. Antibody to a synthetic nonapeptide corresponding to the NH_2 terminus of poliovirus genome-linked protein VPg reacts with native VPg and inhibits in vitro replication of poliovirus RNA. J. Virol. **48**:429–439.
17. **Perez-Bercoff, R.** 1979. The mechanism of replication of picornavirus RNA, p. 293–318. *In* R. Perez-Bercoff (ed.), The molecular biology of picornaviruses. Plenum Publishing Corp., New York.
18. **Phillips, B. A., R. E. Lindquist, and J. V. Maizel.** 1980. Absence of subviral particles and assembly activity in HeLa cells infected with defective-interfering (DI) particles of poliovirus. Virology **100**:116–124.
19. **Provost, P. J., and M. R. Hilleman.** 1979. Propagation of human hepatitis A virus in cell culture in vitro. Proc. Soc. Exp. Biol. Med. **160**:213–221.
20. **Ross, B. C., B. N. Anderson, A. G. Coulepis, M. P. Chenoweth, and I. D. Gust.** 1986. Molecular cloning of cDNA from hepatitis A virus strain HM-175 after multiple passages in vivo and in vitro. J. Gen. Virol. **67**:1741–1744.
21. **Rueckert, R. R.** 1985. Picornaviruses and their replication, p. 705–738. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, J. L. Melnick, B. Roizman, and R. E. Shope (ed.), Virology. Raven Press, New York.
22. **Shen, W., J. Lian-cal, S. Hui-xun, Z. Xi-tan, M. Olng-hal, and L. Xue-guang.** 1986. Strain of hepatitis A virus causing cytopathic effects isolated in A549 cell line. Chin. Med. J. **99**:387–392.
23. **Siegl, G., J. de Chastonay, and G. Kronauer.** 1984. Propagation and assay of hepatitis A virus in vitro. J. Virol. Methods **9**:53–67.
24. **Siegl, G., M. Weitz, and G. Kronauer.** 1984. Stability of hepatitis A virus. Intervirology **22**:218–226.
25. **Spector, D. H., and D. Baltimore.** 1975. Polyadenylic acid on poliovirus RNA. II. Poly(A) on intracellular RNAs. J. Virol. **15**: 1418–1431.
26. **Toyoda, H., C.-F. Yang, N. Takeda, A. Nomoto, and E. Wimmer.** 1987. Analysis of RNA synthesis of type 1 poliovirus by using an in vitro molecular genetic approach. J. Virol. **61**: 2816–2822.
27. **Vallbracht, A., L. Hofmann, K. G. Wurster, and B. Flehmig.** 1984. Persistent infection of human fibroblasts by hepatitis A virus. J. Gen. Virol. **65**:609–615.
28. **Venuti, A., C. DiRusso, N. del Grosso, A.-M. Patti, F. Ruggeri, P. R. De Stasio, M. G. Martiniello, P. Pagnotti, A. M. Degener, M. Midulla, A. Pana, and R. Perez-Bercoff.** 1985. Isolation and molecular cloning of a fast-growing strain of human hepatitis A virus from its double-stranded replicative form. J. Virol. **56**:579–588.
29. **Weitz, M., B. M. Baroudy, W. L. Maloy, J. R. Ticehurst, and R. H. Purcell.** 1986. Detection of a genome-linked protein (VPg) of hepatitis A virus and its comparison with other picornaviral VPgs. J. Virol. **60**:124–130.
30. **Wheeler, C. M., H. A. Fields, C. A. Schable, W. J. Meinke, and J. E. Maynard.** 1986. Adsorption, purification, and growth characteristics of hepatitis A virus strain HAS-15 propagated in fetal rhesus monkey kidney cells. J. Clin. Microbiol. **23**:434–440.
31. **Wheeler, C. M., B. H. Robertson, G. Van Nest, D. Dina, D. W. Bradley, and H. A. Fields.** 1986. Structure of the hepatitis A virion: peptide mapping of the capsid region. J. Virol. **58**:307–313.
32. **Widell, A., B. G. Hansson, E. Nordenfelt, and B. Öberg.** 1988. Enhancement of hepatitis A propagation in tissue culture with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. J. Med. Virol. **24**:369–376.
33. **Yin, F. H.** 1977. Involvement of viral procapsid in the RNA synthesis and maturation of poliovirus. Virology **82**:299–307.