GROWTH OF HEPATITIS A VIRUS IN MURINE CELLS

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Summary. – In order to investigate the growth of hepatitis A virus (HAV) in murine cells, L929 cells of the established mouse cell line were transfected with the virion RNA or infected with the virions and examined for the formation of negative-strand RNA and the rise of the viral infectivity titer. In both the transfected and infected cells, the formation of negative-strand HAV RNA was assayed by the reverse transcription-polymerase chain reaction (RT-PCR). In the transfected cells, infectious HAV of an average titer of $10^{1.8}$ $TCID_{50}$/dish was obtained. The experiment with the virion infection was further extended by using other mouse cell lines, namely Balb/3T3 clone A31, NIH/3T3, and Swiss/3T3. Here, only NIH/3T3 cells were found capable to support the formation of negative-strand HAV RNA. Thus some murine cell lines are considered to have a complete cellular machinery for supporting the growth of HAV, though the efficiency of virus growth therein was considerably lower as compared to that in the susceptible primate cells.

Key words: hepatitis A virus; murine cells; negative-strand viral RNA; reverse transcription-polymerase chain reaction

Introduction

HAV is an unenveloped small spherical virus belonging to the *Picornaviridae* family. The virus particle has a genome of single-stranded RNA of positive polarity, which replicates *via* negative-strand RNA of virus, that is *de novo* synthesized after infection by virus-coded RNA polymerase using the viral genome as template (Siegl and Frösner, 1978; Gauss-Müller *et al.*, 1984).

For experimental propagation of HAV *in vivo*, the initial host was non-human primates as chimpanzees (Dienstag *et al.*, 1975), marmosets, tamarins (Deinhardt *et al.*, 1975), lesser bush babies (Grabov and Prozesky, 1975), stump-tailed monkeys (Mao *et al.*, 1981), and owl monkeys (Lemon *et al.*, 1982). Later, the virus adaptation to the cultured primate cells enabled serial propagation of virus *in vitro* (Provost and Hilleman, 1979; Daemer *et al.*, 1981; Binn *et al.*, 1984) and provided with sufficient amounts of viral materials for experimental as well as vaccination purposes.

On the other hand, the host animals for experimental model of HAV infection are still confined to the non-human primates. Although the animals are well susceptible to the virus and manifest apparent signs of the acute hepatitis (Zachoval and Deinhardt, 1993), they are quite expensive. This aspect restrains investigators from frequent use of these animals for experimental purposes.

In an attempt to expand the model system for HAV infection to conventional experimental animals, we investigated the growth of HAV in an established mouse cell line, L929, by pursuing the formation of the negative-strand viral RNA and the rise of the virus infectivity titer. For detection of the negative-strand RNA, cells were transfected with the virion RNA or infected with the virions and subjected

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Abbreviations: DEAE-dextran = diethylaminoethyl-dextran; EDTA = ethylendiamine tetraacetate; EIA = enzyme immunoassay; HAV = hepatitis A virus; MoAb = monoclonal antibody; MuLV = murine leukemia virus; PBS = phosphate-buffered saline; p.i. = post infection; RT-PCR = reverse transcription-polymerase chain reaction

to RT-PCR. For assaying the virus infectivity titers, cells were transfected with the virion RNA. The obtained results indicated that the replication of viral RNA and the rise of virus infectivity titer were evident in the mouse cell line employed, though the efficiencies thereof were considerably low. In addition, another mouse cell line, NIH/3T3, was also found to support the replication of HAV RNA after infection with the virions. The technical as well as theoretical aspects of these observations are discussed for their implications.

Materials and Methods

Cells of an established mouse cell line, L929, were mainly employed; in addition, other murine cell lines, Balb/3T3 clone A31, NIH/3T3, and Swiss/3T3, were also tested. All these cells were obtained from ICN Biomedicals. As susceptible cells for propagation of HAV were used S.la/Ve-1 cells, a fused cell line of marmoset liver and Vero cells (Ashida *et al.*, 1989). They were grown in 150 cm^2-plastic tissue culture flasks (Corning) using Dulbecco's Minimum Essential Medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% foetal bovine serum (FBS; Gibco BRL), glutamine (100 mmol/l), penicillin G (100 U/ml), and streptomycin (100 µg/ml) (growth medium, below) at 37°C in 5% CO_2 atmosphere. After obtaining cell monolayers, the concentration of FBS in the medium was reduced to 1% (maintenance medium, below).

Virus. HAV, T.T. strain, a faecal isolate from an apparent case of hepatitis type A (Kojima *et al.*, 1981), adopted to S.la/Ve-1 cells was used. For virus growth, cell monolayers in 150 cm^2-plastic tissue culture flasks (Corning) were infected with virus (2 ml) at multiplicity of 1. Virus adsorption proceeded for 2 hrs at 37°C in 5% CO_2 atmosphere. The cultures were then washed three times with DMEM, fed with 50 ml of the maintenance medium, and kept in the same condition for 2 weeks with periodical medium changes. The cells were then scraped into the culture medium, pelleted by light centrifugation, resuspended into 2 ml of the medium, and sonicated for 1 min to release the virus from cells. The obtained cell homogenates were combined with the remaining culture medium and centrifuged at 2,000 rpm for 10 mins. The resulting supernatants were stored at -70°C as the virus stock. Its infectivity titers ranged at $10^{7.0-7.5}$ $TCID_{50}$/ml.

Virus infectivity titration. The end-point dilution method coupled with enzyme immunoassay (EIA) was adopted (Ashida *et al.*, 1989). The serial 10-fold dilutions of virus samples in DMEM were inoculated into S.la/Ve-1 cell cultures in microtiter plates (Corning) using 10 wells per dilution (100 µl of inoculum/well). Infected plates were kept at 37°C in 5% CO_2 atmosphere for 2 hrs, then 100 µl of the maintenance medium per well was added and the cultivation with periodical medium changes continued for 2 more weeks. Then the plates were fixed with 80% methanol and subjected to EIA: incubation with 1% skim-milk solution for 1 hr, reaction with horseradish peroxidase-tagged HAV monoclonal antibody (MoAb, 1:500) for 1 hr, and reaction with 0.04% ortho-phenylenediamine solution containing 0.02% H_2O_2 for color development. The wells were assayed for their A_{492} values. As positive were regarded those exceeding the two-fold of the mean value of the uninfected sample. The $TCID_{50}$ titers were determined according to the incidence of the antigen-positive wells by the method of Reed and Muench (1938). The anti-HAV MoAb was prepared in our laboratory and labelled with horseradish peroxidase according to Wilson and Nakane (1978).

Virus purification. HAV was purified from infected S.la/Ve-1 cell lysates to density homogeneity by the method of Lemon *et al.* (1991). Briefly, the cells 2 weeks post infection (p.i.) were sonicated in TN-buffered solution (TN buffer:50 mmol/l Tris-HCl pH 7.5 and 50 mmol/l NaCl) to release the virus. The obtained cell lysates were treated subsequently with DNase I (50 µg/ml; Takara Shuzo, Otsu, Japan), 0.5% trypsin (Difco), and 0.1% sodium lauroyl sarcosinate at 37°C for 30 mins each, and centrifuged at 150,000 x g for 4 hrs. The pelleted virus was applied to a linear CsCl gradient (21 – 45%) centrifugation at 150,000 x g for 18 hrs. The fractions at a buoyant density of 1.33 g/cm^3 were collected and pooled as purified HAV.

Extraction of HAV RNA from virus and cells. The Total RNA Separator Kit (Clontech Laboratories) was used. Density-purified HAV specimens containing 6 x 10^9 $TCID_{50}$ or 3 x 10^6 infected cells were extracted according to the manufacturer's instructions. Briefly, they were denaturated by guanidinium thiocyanate, extracted by phenol-chloroform mixture, and precipitated by isopropanol (Chomczynski and Sacchi, 1987). The RNA fractions obtained were subjected to immediate use, so as to avoid a loss of their biological activities.

Transfection of cells with virion RNA. A modification of the method of Van der Werf *et al.* (1986) was used. One ml of HEPES-buffered saline solution (21 mmol/l HEPES, 137 mmol/l NaCl, 5 mmol/l KCl, 0.7 mmol/l Na_2HPO_4, and 6 mmol/l dextrose) pH 7.05, supplemented with 0.05% diethylaminoethyl (DEAE)-dextran (Sigma) and 16 U of RNase inhibitor (Toyobo Co., Osaka, Japan) contained 2 µg of virion RNA. Cell monolayers in 6 cm-plastic Petri dishes (Becton Dickinson), each containing about 3 x 10^6 cells, were transfected with 2 ml of the virion RNA and kept at room temperature for 1.5 hr. The dishes were then washed once with phosphate-buffered saline (PBS) pH 7.2 and kept in the maintenance medium at 37°C in 5% CO_2 atmosphere

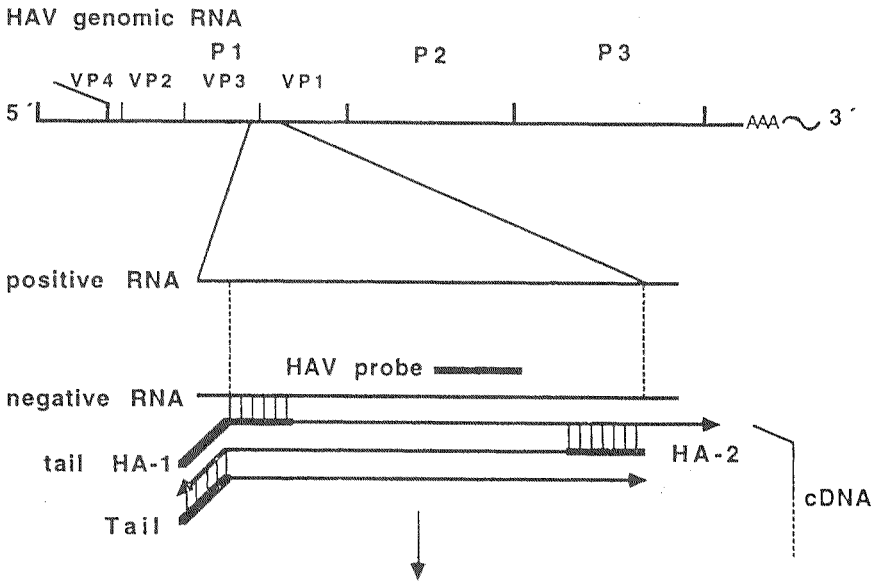

Fig 1

Schematic illustration of the genomic HAV RNA and the strategy to detect negative-strand HAV RNA by RT-PCR

P1-3: subgenomic regions; VP1-4: genes coding for structural proteins; AAA: poly(A)-tail; positive HAV RNA: indicator stretch for the RT-PCR; negative HAV RNA: direct target for the RT-PCR; tail HA-1 (bold line): sense primer; HA-2 (bold line): antisense primer; tail (bold line): another sense primer; HAV probe: probe region for Southern blot hybridization.

for 3 weeks. Thereafter, the cells were separately processed. For the RT-PCR, cells from one dish were taken into 500 µl of denaturing solution (4 mol/l guanidium thiocyanate, 25 mmol/l sodium citrate, 0.5% N-lauroyl sarcosinate, and 0.1 mol/l 2-mercaptoethanol) pH 7.0 subjected to RNA extraction described above. For the EIA of virus infectivity titer, cells from one dish were harvested into 2 ml of PBS by a treatment with ethylenediamine tetraacetate (EDTA)-trypsin mixture. The cell suspensions were then extracted by two cycles of freeze-thawing in acetone-dry ice mixture and sonication for 1 min, and centrifuged at 2000 rpm for 10 mins. The resulting supernatants were subjected to EIA.

RT-PCR. The strategy of RT-PCR for detection of negative-strand HAV RNA followed Chaves *et al.* (1994) (Fig. 1). The nucleotide sequence nt 2,172-2,414 (VP3 3'-tail to VP1 5'-head) of the viral genome was chosen for amplification (Margolis and Nainan, 1990). The primer ("tail HA-1") for the initiation of reverse transcription was 5'-TTGGGATTAGCGAGTATG-GCTCCTCTTTATCATGCTATG-3' (nt 2,172-2,192 for the non-underlined sequence) including the underlined tail structure lacking sequence complementarity to HAV genome (Chaves *et al.*, 1994); the sense primer ("tail" to read cDNA) was 5'-TTGGGATTAGCGAGTATG-3', which was equivalent to the tail structure of the "tail HA-1"; and the antisense primer ("HA-2") was 5'-GGAAATGTCTCAGGTACTTTCTTTG-3' (nt 2,390-2,414). All these primers were purchased from Kurabo Co., Osaka, Japan.

Thirty µg of RNA extracted from the infected cells was dissolved in 10 µl of distilled water and subjected to reverse transcription with 40 µl of RT-reaction buffer (50 mmol/l Tris-HCl pH 8.3, 5 mmol/l $MgCl_2$, and 70 mmol/l KCl), supplemented with 2.5 mmol/l dithiothreitol, 20 U of RNase inhibitor (Perkin-Elmer), 2.5 mmol/l each of dNTPs in mixture, 5 pmoles of the "tail-HA-1" primer, and 50 U of murine leukemia virus (MuLV) reverse transcriptase (Perkin-Elmer). The reverse transciption proceeded for 1 hr at 42°C and ceased by heating of the mixture at 99°C for 1.5 hr (the inactivation of the reverse transciptase). Then followed the treatments with 1 µg of RNase A (Worthington Biochemical) and 2 U of RNase H (Toyobo) at 37°C for 1 hr to digest the RNA template.

The cDNA sample obtained was then diluted to 50 µl with PCR buffer solution (10 mmol/l Tris-HCl pH 8.3, 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, and 0.1% gelatin) supplemented with 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer) and 50 pmoles each of the "tail" and "HA-2" primers for PCR. The latter consisted of 30 cycles of heatings at 94°C for 1.5 min (denaturation), at 55°C for 1.5 min (annealing), and at 72°C for 2 mins (elongation). The products obtained were analyzed by electrophoresis in 2% agarose gel with ethidium bromide staining.

Southern blot hybridization. PCR products were blotted onto nitrocellulose membranes and hybridized with the "HAV probe". The latter [5'-TCAACAACAGTTTCTACAGACAGAATGTT-3', nt 2,232-2,262 of HAV genome (Co-

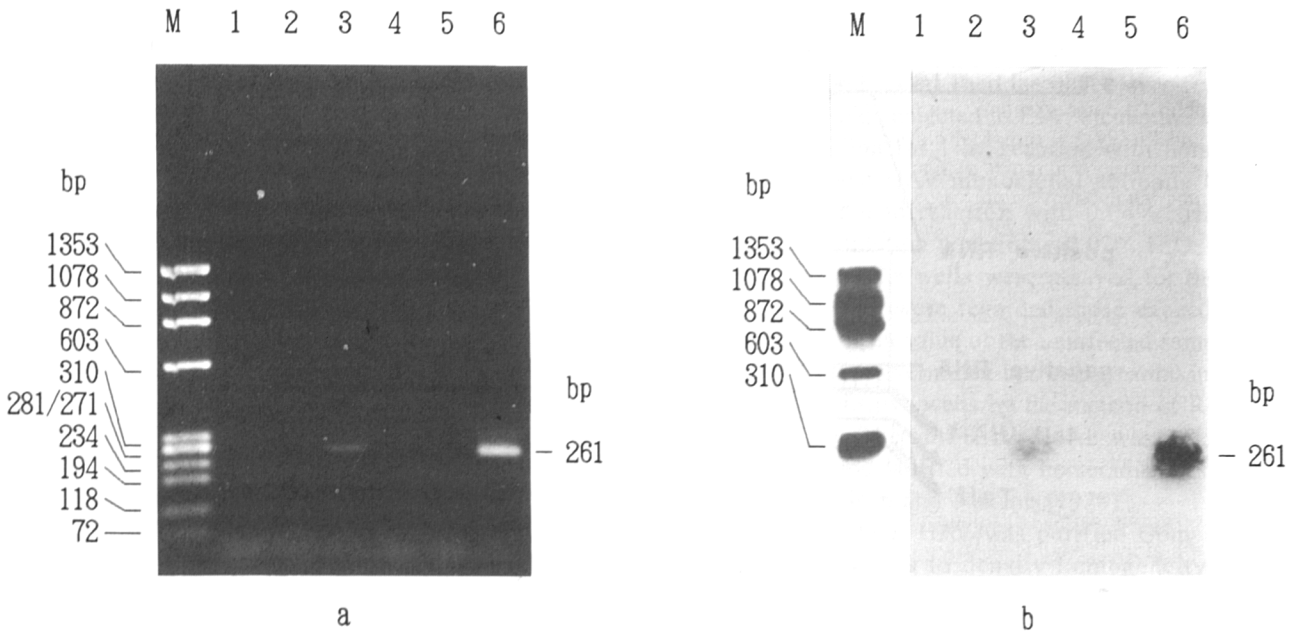

Fig. 2
Detection of negative-strand HAV RNA in L929 cells transfected with virion HAV RNA

RT-PCR products were electrophoresed in 2% agarose gel at 9 V/cm for 50 mins and visualized by ethidium bromide staining and UV-light illumination (a). The products were transferred onto nitrocellulose membrane for Southern blot hybridization using the "HAV-probe" (b). M: *Hae*III fragments of øX174DNA as molecular size markers; 1: L929 cell lysate; 2: mixture of virion HAV RNA and L929 cell lysate; 3: lysate of L929 cells transfected with virion HAV RNA; 4: S.la/Ve-1 cell lysate; 5: mixture of virion HAV RNA and S.la/Ve-1 cell lysate. 6: lysate of S.la/Ve-1 cells transfected with virion HAV RNA.

hen *et al.*, 1987) (Fig. 1)] was prepared by Kurabo Co. and labelled with fluorescein-modified dUTP by employing the ECL 3'-oligolabelling system (Amersham) and the manufacturer's protocol. The hybridized blots were subjected to fluorography by use of X-ray films.

Results

Detection of negative-strand HAV RNA in L929 cells transfected with the virion RNA

L929 cells in 6 cm-plastic Petri dishes were transfected with 2 μg of the virion RNA and assayed for the negative-strand HAV RNA. Results of the RT-PCR at 2 weeks post transfection are given in Fig. 2a. The product of RT-PCR derived from the transfected cells formed a faint but discrete band of approximately 260 bp (lane 3), which was identical in size with the indicator stretch plus the "tail" primer (261 bp). The band reacted specifically with the HAV probe in Southern blot hybridization (Fig. 2b, lane 3). Thus the presence of HAV sequence in this band was proved.

As a positive control, the same experiment was carried out with the susceptible host, S.la/Ve-1 cells. An identical band with stronger signal was seen in both the RT-PCR (Fig. 2a, lane 6) and Southern blot hybridization (Fig. 2b, lane 6).

Another control was included to monitor a possible false RT-PCR product. Host cell lysates and mixtures of the virion RNA and host cell lysates were subjected to the RT-PCR, but they gave no discernible signals of the products (Fig. 2a, lanes 1, 2, 4, and 5). The RT-PCR system employed thus proved well reliable in specific detection of negative-strand HAV RNA under the experimental conditions used.

Table 1. Growth of HAV in L929 and S.la/Ve-1 cells transfected with virion HAV RNA

Transfected cells	HAV titer ($TCID_{50}$/dish)			
	Dish No.			Average
	1	2	3	
L929	$10^{1.5}$	$10^{2.2}$	$10^{1.7}$	$10^{1.8}$
S.la/Ve-1	$10^{6.9}$	$10^{7.4}$	$10^{6.8}$	$10^{7.0}$

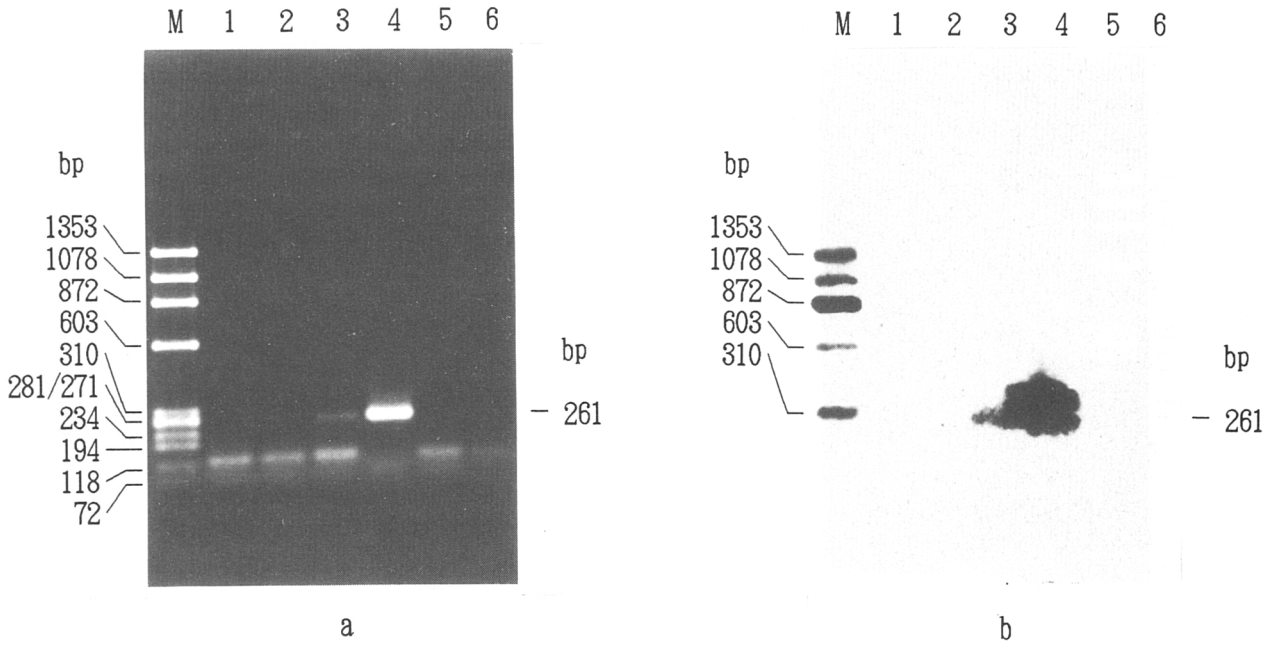

Fig. 3
Detection of negative-strand HAV RNA in L929 cells infected with HAV virions
RT-PCR were electrophoresed in 2% agarose gel (a) and tested by Southern blot hybridization (b). 1: L929 cell lysate; 2: mixture of HAV virions and L929 cell lysate; 3: lysate of L929 cells infected with HAV virions; 4: lysate of S.la/Ve-1 cells infected with HAV virions; 5: S.la/Ve-1 cell lysate; 6: mixture of HAV virions and S.la/Ve-1 cell lysate. For the rest of the legend see Fig. 2.

Recovery of infectious HAV from L929 cells transfected with the virion RNA

Detection of negative-strand HAV RNA in the transfected L929 cells prompted us to examine the production of infectious virus in cells. Cells were transfected with the virion RNA in the same manner as before. Cellular extracts prepared at 2 weeks post transfection were subjected to EIA of the virus infectivity titers. The 3 dishes of transfected cells yielded infectious HAV titers of $10^{1.5\text{-}2.2}$ $TCID_{50}$/dish (Table 1). For comparison, the susceptible S.la/Ve-1 cells gave titers of $10^{6.8\text{-}7.4}$ $TCID_{50}$/dish in the same experiment.

Detection of negative-strand HAV RNA in L929 cells infected with the virions

Upon transfection with virion RNA, L929 cells supported the replication of viral RNA, and eventually yielded infectious virus. The cells were further examined for the susceptibility to HAV virions. The cells in 6 cm-plastic Petri dishes were inoculated with $10^{6.0}$ $TCID_{50}$ of HAV in 0.5 ml (multiplicity of infection (MOI) of 10), the cells were subjected to the RT-PCR. In this experiment too, the formation of negative-strand RNA was evident as a discrete band of approximately 260 bp (Fig. 3a, lane 3). The authenticity of this result was also confirmed by the Southern blot hybridization test (Fig. 3b), which included several controls.

Detection of negative-strand HAV RNA in other murine cells infected with the virions

The findings of the formation of negative-strand HAV RNA and the rise of infectivity titer in L929 cells led us to examine other mouse cells for their susceptibility to the virus infection. Cell monolayers of Balb/3T3 clone A31, NIH/3T3, and Swiss/3T3 in 6 cm-plastic Petri dishes were inoculated with $10^{6.0}$ TCID of HAV in 0.5 ml (MOI of 10) and assayed for the negative-strand HAV RNA by the RT-PCR (Fig. 4a).

Among the cell lines examined, only NIH/3T3 cells were positive for the signal of the negative-strand HAV RNA (Fig. 4a, lane 3). The intensity of the signal was identical with that of L929 cells (lane 1), indicating that both the L929 and NIH/3T3 cell lines are equaly susceptible to HAV. All of the bands of the RT-PCR products in Fig. 4a were confirmed for their HAV specificity by the Southern blot hybridization test (Fig. 4b).

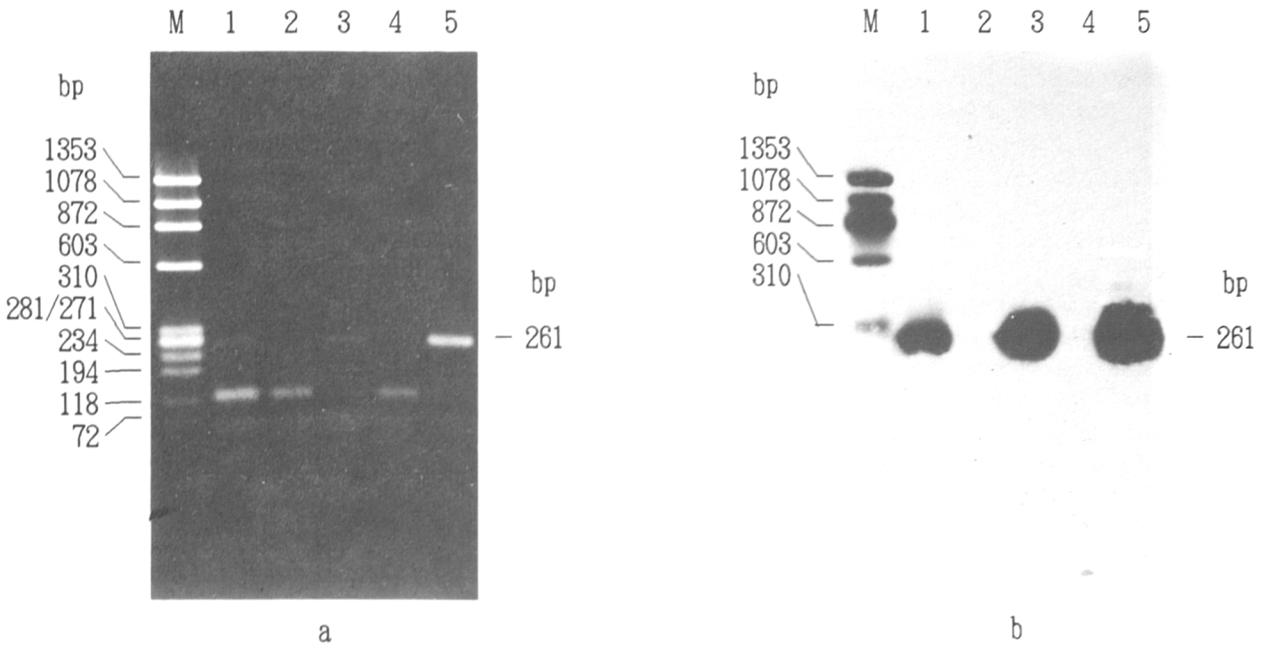

Fig. 4
Detection of negative-strand HAV RNA in other murine cells infected with HAV virions
Lysates of different cell lines infected with HAV virions were used. RT-PCR products were electrophoresed in 2% agarose gel (a) and tested by Southern blot hybridization (b). 1: L929; 2: Balb/3T3 clone A31; 3: NIH/3T3; 4: Swiss/3T3; 5: S.la/Ve-1. For the rest of the legend see Fig. 2.

Discussion

In this study, cells of the established mouse cell line L929 were transfected with HAV RNA or infected with HAV virions, and observed for the formation of the negative-strand HAV RNA as an indicator of virus growth. The method employed for this purpose was the RT-PCR, which followed the strategy of Chaves *et al.* (1994) to adopt two primers, "tail HA-1" and "tail", for specific detection of the negative-strand RNA (Fig. 1). The "tail HA-1" primer first primed the reverse transcription according to the HAV sequence specificity. The cDNA produced was then amplified on the basis of the HAV complementarity of the "HA-2" primer and the "tail" sequence complementarity between the "tail" primer and the "HA-2"-primed DNA. As the "tail" sequence lacked any homology with HAV genome (Chaves *et al.*, 1994), the primer prevented the system from non-specific amplification of unrelated sequences.

In this RT-PCR system, another prerequisite of the reliability of the reaction was a total inactivation of the reverse transcriptase at shifting from the reverse transcription to cDNA amplification. In our preliminary experiment, when the inactivation of the enzyme followed the original protocol, 99°C for 10 mins, also virion HAV RNA was amplified in the indicator stretch giving a clear signal of the product. This was due to a residual activity of the enzyme, which drove the reverse transcription of the positive-strand RNA by the "HA-2" primer. At the time of completion of the reverse transcription, the protocol was modified to inactive the enzyme to zero activity by heating the specimen at 99°C for 1.5 hr. After this heating, the signal of the positive-strand RNA disappeared totally (data not shown).

In the experiments, L929 cells, either transfected with HAV RNA or infected with HAV virions, showed the formation of the negative-strand HAV RNA. After transfection with HAV RNA, the cells yielded infectious virus. In the EIA of virus infectivity titers, each of the culture supernatants of viral antigen-positive wells was passaged in S.le/Ve-1 cell cultures, where all of the supernatants gave a full growth of the virus (data not shown). The formation of the negative-strand HAV RNA in L929 cells is thus considered a direct indication of the authentic replication of viral RNA leading to the production of HAV virions.

Regarding the susceptibility of non-primate animals and their cells to HAV, there is a lack of information on the relevant findings. There is a report documenting the HAV attachment of non-primate cells in culture (Zajac, 1991), however, its experimental evidence is obscure as no strict

criteria of the autonomous growth of virus in cells were observed. Dotzauer *et al.* (1994) reported recently that the cell lines derived from guinea pig, dolphin, and probably from pig, were capable of supporting the growth of HAV after the adaptation of the virus to cell cultures. In this report, mouse cells were also included, but with negative results. Thus, there are no reports on the growth of HAV in murine cells except for the present paper.

The present study was motivated by an idea to extend the experimental model of HAV infection to the murine species. The results obtained indicate that L929 cells are capable to support the growth of HAV, and that NIH/3T3 cells can replicate HAV RNA. Thus, it is likely that some murine cells have a complete cellular machinery needed for the growth of HAV. On the other hand, the efficiency of HAV growth in the murine cells was considerably low as compared with that in the primate cells. The HAV strain employed was adapted to primate but not murine cells. It is clear that HAV cannot grow efficiently in cell cultures without adaptation by repeated passages. This was exemplified in the case of HAV field strains growing in primate cell cultures (Provost, 1984) and of primate cell culture-adapted strains growing in non-primate cell cultures after overcoming the species barriers (Dotzauer *et al.*, 1994). In conclusion, it seems worthwhile to make further trials for adapting HAV to murine cell cultures by repeated passages.

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