COMPARISON OF VARIOUS METHODS OF DETECTION OF DIFFERENT FORMS OF DENGUE VIRUS TYPE 2 RNA IN CULTURED CELLS

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Summary. – In this report, the sensitivity of various methods of detection of dengue virus type 2 (DEN-2) sense, antisense, replicative intermediate (RI) and replicative form (RF) RNAs in infected mosquito *Aedes pseudoscutellaris* AP-61 and mammalian baby hamster kidney BHK-21 cells is compared. LiCl precipitation was used for separation of viral RF RNA from RI RNA. Our results show that reverse transcription-polymerase chain reaction (RT-PCR) followed by Southern blot analysis and slot blot hybridisation of LiCl-fractionated RNA were the most sensitive methods of detection of viral RNA and determination of its single-stranded form. Northern blot analysis was the least sensitive method of detection of any form of viral RNA. Using slot blot hybridisation of LiCl-precipitated RNA, viral RI RNA containing *de novo* synthesised negative strand viral RNA was first detected 30 mins after virus inoculation in both cell lines. This is the earliest time of detection of DEN viral RNA synthesis in host cells so far reported. However, RF RNA could not be detected until 24 hrs post infection (p.i.) in AP-61 and 2 days p.i. in BHK-21 cells, respectively. The sequential order of individual forms of viral RNA detected in the infected cells was RI, RF and genomic RNAs. Viral RNA was detected in AP-61 cells always earlier than in BHK-21 cells. Moreover, the level of viral RNA in AP-61 cells was higher than that in BHK-21 cells, suggesting that the virus replicated more actively in AP-61 cells. In conclusion, the LiCl separation of viral RNA followed by slot blot hybridisation was found to be the most sensitive and reliable method of detection of DEN virus RI, RF and genomic RNAs in the infected cells. Moreover, this method can be applied to determine the replication status of any single-stranded RNA virus in the host.

Key words: dengue virus; RNA synthesis; RT-PCR; AS-PCR; Southern blot analysis; Northern blot analysis; slot blot hybridisation

Introduction

Currently, the rate of viral RNA synthesis has been investigated by pulse-chase experiments with radioisotope-labelled viral RNA followed by sucrose gradient fractionation (Cleaves *et al.*, 1981; Stollar *et al.*, 1967; Wengler and Gross, 1978). Northern blot analysis was utilized to monitor viral sense (+) RNA synthesis using an antisense (–) RNA probe by asymmetric PCR (AS-PCR) (Cleaves *et al.*, 1981; Liu *et al.*, 1994). With PCR, also the study of antisense viral RNA synthesis using viral sense RNA probe became available (Shimizu *et al.*, 1992). Human epidermoid carcinoma KB cells infected with DEN-2 virus synthesised three types of RNA of sedimentation coefficients greater than 18 S. The first type is RF RNA which is soluble in 2 mol/l LiCl, sediments as a discrete species at about 20 S, and is RNase-resistant in more than 95%. RF RNA is a double-stranded molecule which acts as a recycling template for semiconservative and asymmetric replication (Baltimore and Girand, 1966). The second type is a discrete 40 – 42 S species which is insoluble in 2 mol/l LiCl and RNase-sensitive in more than 95%.

Abbreviations: AS-PCR = asymmetric PCR; DEN = dengue; DEN-2 = DEN virus type 2; DEPC = diethyl pyrocarbonate; DHFR = dihydrofolate reductase; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; RF = replicative form; RI = replicative intermediate; RNase = ribonuclease; RT = reverse transcription; SDS = sodium dodecyl sulfate

This RNA is the single-stranded genomic RNA. The third type is RI RNA insoluble in 2 mol/l LiCl, heterogeneous in size (20 – 28 S) and RNA-sensitive in 50% – 70% (Franklin, 1966). RI RNA consists at any time of the minus strand (template), a displaced full length plus strand, and an almost completed base-paired plus strand. As the release of the displaced plus strand progresses, the growing plus strand is completed but remains attached along its entire length to the minus strand template, and the whole RF molecule is regenerated. It has been estimated that 12 – 15 mins is needed for the synthesis of a complete plus strand (Cleaves *et al.*, 1981). Separation of flavivirus RI RNA from RF RNA by the LiCl precipitation represented an important advance in the study of viral replication because the two RNA species could not be satisfactorily resolved by gel electrophoresis or sedimentation (Chu and Westaway, 1985). However, the LiCl precipitation can not separate RI RNA from genomic RNA (both are precipitated) and the detection sensitivity of current electrophoresis is very low.

The temporal sequence in the appearance of various forms of DEN viral RNA, e.g. RI, RF and genomic RNAs was demonstrated by pulse-chase studies of [^{3}H]-uridine labelled viral RNAs, (Cleaves *et. al.*, 1981), and by RNA polymerase assay *in vitro* (Chu and Westaway, 1985).

DEN viruses transmitted between mosquitoes and mammals demonstrate complex interactions of these viruses with invertebrate and mammalian cells (Schlesinger *et al.*, 1986; Sinarachatanant and Olson, 1973). Although RNA synthesis of DEN viruses in different hosts is similar, the activity of DEN viruses, the response of host cells, and the antigenic response in different hosts vary widely (Westaway, 1987). It is known that flaviviruses can acutely infect mammalian cells but only persistently infect mosquito cells. A study on the relationship between viral multiplication and host cell response at molecular level in different hosts should pave the way to the understanding of the interaction of DEN viruses with mosquito and human.

In this report, we detected the accumulated DEN virus sense and antisense RNAs by Northern blot analysis and RT-PCR combined with Southern blot analysis. We also distinguished DEN viral RF RNA from RI RNA by LiCl precipitation, and detected the RNAs by slot blot hybridisation. In comparison, RT-PCR followed by Southern blot analysis and slot blot hybridisation of LiCl-precipitated RNA were found to be the most sensitive methods of detection of DEN viral RNA. Moreover, the latter method could be used to monitor replication of RNA viruses in general.

Materials and Methods

Virus. DEN-2 (New Guinea C) virus was grown in mosquito *Aedes pseudoscutellaris* AP-61 cells. To prevent selection of mutants, the virus was passaged not more than three times in these cells. After 6 to 7 days of incubation, the supernatant fluid harvested from infected cultures was clarified by centrifugation at 600 x g for 10 mins. The virus was pelleted at 63,000 x g for 2 hrs and resuspended in 1/100 of original volume of phosphate-buffered saline (PBS). The virus was further purified by sucrose density gradient centrifugation by resuspending the virus pellets in 1/100 of original volume of 0.01 mol/l Tris.HCl pH 7.8, 0.15 mol/l NaCl and 0.005 mol/l EDTA. The virus sample was layered on a density gradient of 5% – 25% (w/v) sucrose and centrifuged for 2.5 hrs at 65,000 x g in a SW41 rotor. The virus-containing band was saved and served as stock virus (10^6 PFU/ml).

Cell lines. Aedes pseudoscutellaris AP-61 cells (Varma *et al.*, 1974) were grown in L-15 Medium (Hazleton, USA) with 10% of foetal calf serum at 28°C. Baby hamster kidney BHK-21 cells were grown in Eagle's Minimum Essential Medium with 10% of foetal calf serum at 37°C in a CO_2 incubator.

Experimental cell infection. DEN-2 virus was used at a multiplicity of 1 PFU per cell for infection of AP-61 and BHK-21 cells. After 2 hrs of virus adsorption to cells, the virus inoculum was removed, a fresh medium was added, and this time was regarded as the 0 hr p.i.

Virus titration was conducted in BHK-21 cells by a standard plaque assay (Lanciotti *et al.*, 1992).

Virus harvesting. AP-61 cells grown in T-75 flasks (Costar, USA) were collected six days p.i. and centrifuged at 15,000 x g for 10 mins to remove cellular debris. The supernatant was then ultracentrifuged in a Ti50 rotor at 110,000 x g at 4°C for 3 hrs. The viral pellets were resuspended in PBS pH 7, filtered through a 0.2 µm filter (Poretics, USA), and stored at -70°C.

RNA extraction was carried out according to Chomczynski and Sacchi (1987) with slight modification. In brief, the supernatant from virus-infected cells was mixed with an equal volume of lysis buffer (8 mol/l guanidine isothiocyanate, 50 mmol/l sodium citrate, 100 mmol/l 2-mercaptoethanol, 1% Sarkosyl, and 1 mg/ml yeast tRNA). For RNA extraction from infected cells, the lysis buffer was used in 50% concentration. The sample was sequentially mixed with a 1/10 volume of 3 mol/l sodium acetate pH 5.2, an equal volume of water-equilibrated phenol, and a 1/5 volume of chloroform, and shaken. The mixture was centrifuged at 20,000 x g for 15 mins, and the aqueous phase was removed and mixed with 2.5 volumes of 95% ethanol. After centrifugation, the resulting RNA pellet was washed with 75% ethanol and dissolved in bidistilled water treated with diethyl pyrocarbonate (DEPC).

Northern blot analysis. Total RNA (30 µg) extracted from cells was first denatured in glyoxal solution (1 mol/l glyoxal and 10 mmol/l sodium phosphate pH 6.5) for 1 hr at 50°C, electrophoresed in 1% agarose gel at 80 V and 20 mA for 2 hrs, and blotted to Hybond-N membrane (Amersham, USA) in 25 mmol/l sodium phosphate pH 6.5. The blot was then hybridised with a [α-^{32}P]dATP probe (Sambrook *et al.*, 1989) prepared by AS-PCR (Bednarczuk *et al.*, 1991) and labelled to a specific activity of 4 x 10^8 dpm/µg. The blot was exposed to Kodak X-OMAT AR film.

Primers and probes. All the primers were synthesised by use of an Applied Biosystems DNA Synthesizer (Model 391) and purified through an oligonucleotide purification cartridge. DEN virus primers (Henchal *et al.*, 1991) were as follows:

AD3 (5'-CTGATTTCCATCCCGTA-3') and AD4 (5'-GATATGGGTTATTGGATAGA-3'). Dihydrofolate reductase (DHFR) gene primers (Shotkoski and Fallon, 1991) were as follows: DHFR1 (5'-ATCAAAGGCGATCTTCCATGG-3') and DHFR2 (5'-GCCACCAACGATCCATACGTT-3').

The viral sense and antisense probes (419 bp) were synthesised by AS-PCR using AD4 and AD3 primers. The PCR mixture contained 50 mmol/l dCTP, dGTP and dTTP, 1U of *Taq* polymerase (Amersham), 1 µl (10 µCi) of [α-^{32}P]dATP, 1 µmole AD3 or AD4 primer, and 50 ng of DNA template (419 bp PCR fragment) in the reaction buffer. The PCR proceeded in a thermal cycler (Perkin-Elmer Cetus, USA) in 30 cycles of denaturation at 94°C for 1.5 min, annealing at 50°C for 30 secs, and extension at 72°C for 5 mins (Liu *et al.*, 1995).

RT-PCR. Target viral RNA extracted from DEN-2 virus-infected AP-6 or BHK-21 cells was converted to cDNA by RT prior to enzymatic DNA amplification (Liu *et al.*, 1994). The primers AD3 and AD4 were used to initiate the antisense (–) and sense (+) cDNA synthesis, respectively. The reverse transcription and the subsequent amplification were performed in a single reaction tube. This method consistently yielded an equal or a greater amount of double-stranded DNA product than separate RT and PCR reactions. The target RNA reacted in a 50 µl volume containing 50 mmol/l KCl, 10 mmol/l Tris pH 8.5, 1.5 mmol/l $MgCl_2$, 0.01% gelatine, 500 µmoles each of dNTPs, 10 mmol/l dithiothreitol, 50 pmoles each of primers, 200 U of maloney murine leukaemia virus reverse transcriptase (Gibco, BRL, USA), and 1 U of *Taq* polymerase (Amersham). The reactions procedeed in the thermal cycler mentioned above for 1 hr at 37°C and then in 35 cycles of denaturation (94°C, 30 secs), primer annealing (45°C, 1 min), and primer extension (72°C, 2 mins) (Liu *et al.*, 1995).

To increase the yield and specificity of PCR products, a "hot start" PCR procedure was used, i.e. the reaction mixture without *Taq* polymerase was heated to 65°C for 5 mins, then *Taq* polymerase was added and only then the proper PCR cycling was started (Siebert and Larrick, 1992).

LiCl precipitation. RNA sample (30 µg) was mixed thoroughly with an equal volume of 4 mol/l LiCl, kept at 4°C for 18 hrs and centrifuged at 20,000 x g for 20 mins at 4°C. Both the supernatant and pellet were collected and 2 volumes of ethanol were added to each component. The RNA was precipitated at -70°C for 30 mins, centrifuged, and the pellets were resuspended in DEPC-treated water. To clarify the RNA samples, they (20 µl) were treated with RNase (1 µg/ml, 180 µl) at 37°C for 60 mins in high salt (2 x SSA, 0.3 mol/l NaCl, and 0.03 mol/l sodium acetate, pH 7.0) or low salt (0.01 x SSA) conditions. EDTA (5 mmol/l) in 0.5% sodium dodecyl sulfate (SDS) and 2 x SSA were then added to stop the reaction. The RNA samples were further treated with 20 mg/ml proteinase K (1 µl per sample) (Boehringer Mannheim, Germany) at 37°C for 30 mins. Then there followed phenol/chloroform extraction and ethanol precipitation. The pellets were finally dissolved in DEPC-treated water (Cleaves *et al.*, 1981; Halstead, 1988).

Slot blot hybridisation. Total RNA (30 µg) was depolarised in 1 mol/l glyoxal and 10 mmol/l sodium phosphate, and blotted onto a BioDot SF membrane (Biorad, USA) in a Bio-Dot SF Blotter (Biorad). The remaining procedures were the same as those mentioned before.

Results

Detection of DEN viral sense and antisense RNA in infected AP-61 and BHK-21 cells by Northern blot analysis

The viral sense RNA in infected cells was initially detected as a heterogeneous RNA (around 28 S, 18 S and below) at 0 hr p.i. by Northern blot analysis using the antisense viral probe. The level of viral sense RNA was very low at day 1 p.i. in AP-61 cells, and at day 3 p.i. in BHK-21 cells (Figs. 1A and 2). The amount of viral sense RNA started to increase from day 2 p.i. in AP-61 cells (Fig. 1B), and from day 3 p.i. in BHK-21 cells (Fig. 2).

To detect the viral antisense RNA, the blot used above for the detection of the viral sense RNA was deprobed and rehybridised with viral sense probe (Liu *et al.*, 1994). The viral antisense RNA was detected also as a heterogeneous RNA (around 28 S and 18 S) first at day 2 p.i. in AP-61 cells (Fig. 3) and at day 3 p.i. in BHK-21 cells (Fig. 4).

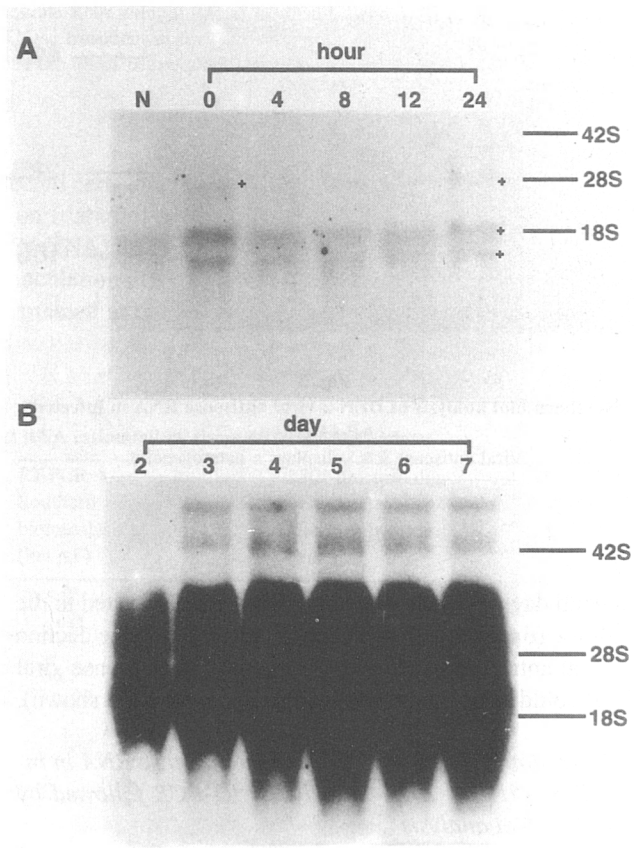

Fig. 1
Northern blot analysis of DEN-2 viral sense RNA in infected AP-61 cells
A: early viral sense RNA (marked by +). B: late viral sense RNA. 42 S: DEN-2 viral genomic RNA. The bands below 42 S are the heterogeneous RNA; 28 S and 18 S: ribosomal RNAs (markers). N: uninfected control.

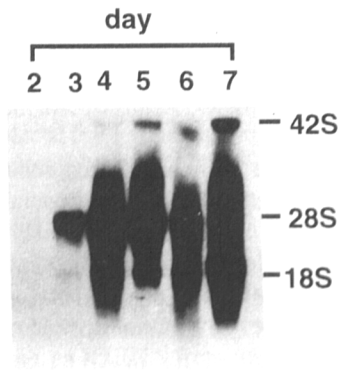

Fig. 2
Northern blot analysis of DEN-2 viral sense RNA in infected BHK-21 cells
42 S: DEN-2 viral genomic RNA.

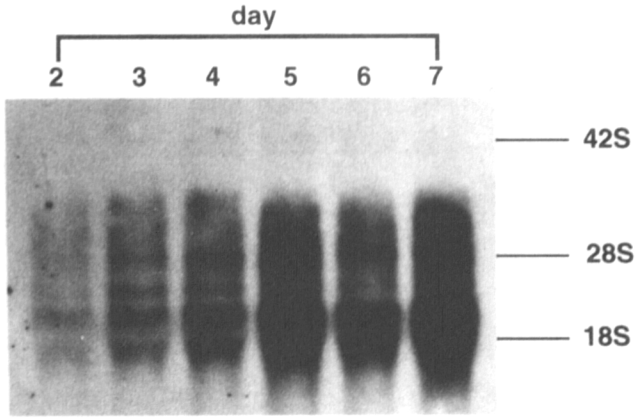

Fig. 3
Northern blot analysis of DEN-2 viral antisense RNA in infected AP-61 cells
Viral antisense RNA displays a heterogeneity.

Beyond day 6 p.i., the infected BHK-21 cells started to die and lyse (data not shown), which contributed to the decline of viral antisense RNA (Fig. 4, lane 6). No antisense viral RNA could be detected in uninfected cells (data not shown).

Detection of DEN viral sense and antisense RNA in infected AP-61 and BHK-21 cells by RT-PCR followed by Southern blot analysis

RT-PCR was chosen to detect small amounts of viral RNA synthesised during the early stage of virus infection. The detection of a 419 bp PCR fragment by the primer pair AD3/AD4 proves the presence of DEN viruses (Henchal *et al.*, 1991). Compared to Northern blot analysis, RT-PCR followed by Southern blot analysis dramatically shortened the detection time for viral antisense RNA to 30 mins post virus inoculation in AP-61 and BHK-21 cells (Fig. 5 and Table 1). The intensity of the bands correlated with the amount of the RF-PCR products.

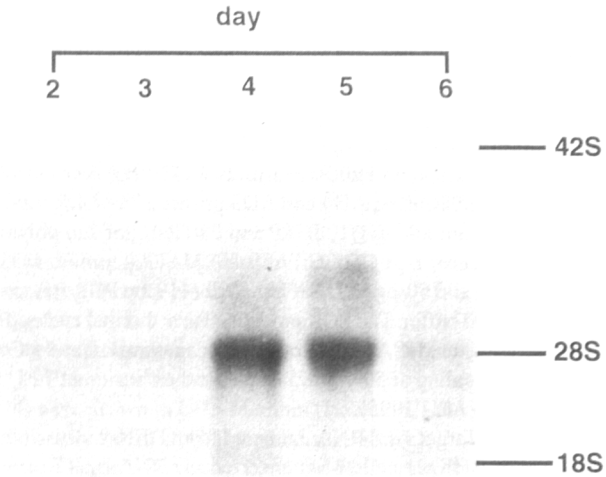

Fig. 4
Northern blot analysis of DEN-2 viral antisense RNA in infected BHK-21 cells
Viral antisense RNA was not detected day 3 p.i.

Detection of DEN viral RI and RF RNAs in infected AP-61 and BHK-21 cells by slot blot hybridisation of LiCl-precipitated viral RNA

RI and RF RNAs are the intermediate products of DEN viral replication (Cleaves *et al.*, 1981). In this study, RI and genomic RNAs precipitated by LiCl and separated from RF RNA that remained in the supernatant were treated with RNAse. The obtained results demonstrated that the nucleic acid precipitated was RNase-sensitive (Fig. 6, lane 2). The precipitated RNA contained the single-stranded genomic RNA of 42 S and heterogeneous RI RNA (below 42 S) (Fig. 6, lane 3). The unprecipitated RF RNA was RNase-resistant in high salt condition (Fig. 6, lane 4) but was RNase-sensitive in low salt condition (Fig. 6, lane 6).

The kinetics of appearance of each form of viral RNA was then monitored by slot blot hybridisation by use of specific probes.

The viral sense probe was applied specifically to detect and distinguish RI RNA from genomic RNA in the LiCl precipitate on a blot. In viral RNA added as the negative control (Figs. 7A and 7C), no antisense viral RNA was detected indicating the absence of RI RNA in the stock virus. Moreover, no antisense viral RNA was detected in unin-

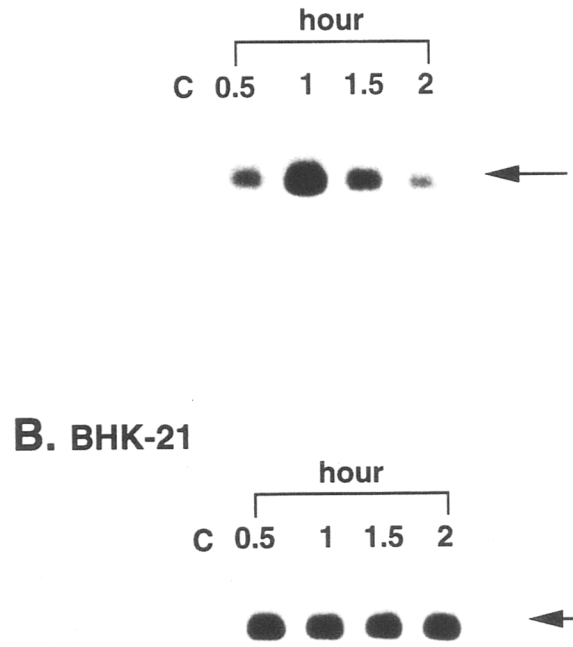

Fig. 5
Southern blot analysis of DEN-2 viral antisense RNA during virus adsorption to AP-61 and BHK-21 cells
To detect viral antisense RNA, the cells were harvested at 0.5, 1, 1.5 and 2 hrs post virus inoculation. The RT-PCR products were subjected to Southern blot analysis. C: purified DEN-2 viral genomic RNA (negative control).

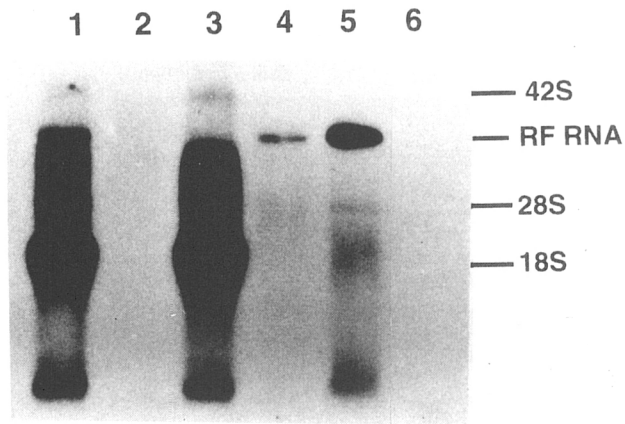

Fig. 6
Detection of DEN-2 viral RI, RF and genomic RNAs in infected AP-61 cells by Northern blot analysis
Various products of LiCl precipitation were subjected to Northern blot analysis. Lane 1: Heterogeneous viral RNA without any treatment. Lane 2: LiCl-precipitated RNA containing RI RNA and genomic RNA after RNase treatment under high salt condition. Lane 3: LiCl-precipitated RNA without RNase treatment. Lane 4: LiCl-soluble RNA containing RF RNA after RNase treatment in high salt condition. Lane 5: LiCl-soluble RNA without RNase treatment. Lane 6: LiCl-soluble RNA after RNase treatment in low salt condition. 42 S represents genomic RNA, and the bands below are heterogeneous viral RNA.

fected cells and in infected cells at 0 hr post virus inoculation (data not shown). Low level of antisense viral RNA (RI RNA) was first detected as early as 30 mins post virus inoculation in the two cell lines (Figs. 7A and 7C). RI RNA remained at the same level until 2 hrs post virus inocula-

Table 1. The earliest detection time of DEN-2 viral RNA estimated by three different methods

RNA form	Cells	Northern blot analysis (hrs p.i.)	RT-PCR + Southern blot analysis (hrs p.i.)	LiCl-precipitation + slot blot hybridisation (hrs p.i.)
sense RNA	AP-61	0	0[a]	
sense RNA	BHK-21	48	48[a]	
antisense RNA	AP-61	48	0.5[b]	
antisense RNA	BHK-21	72	0.5[b]	
RI	AP-61			0.5[b]
RI	BHK-21			0.5[b]
RF	AP-61			24
RF	BHK-21			48

The cells were inoculated with virus (time 0 post virus inoculation), the virus-cell adsorption was left to proceed for 2 hrs (time 2 hrs post virus inoculation), the virus was washed off and fresh medium was added (time 0 hr p.i.). The infection was left to proceed for 3 days.
[a]Data not documented in this study.
[b]The time post virus inoculation.
p.i. = post infection.

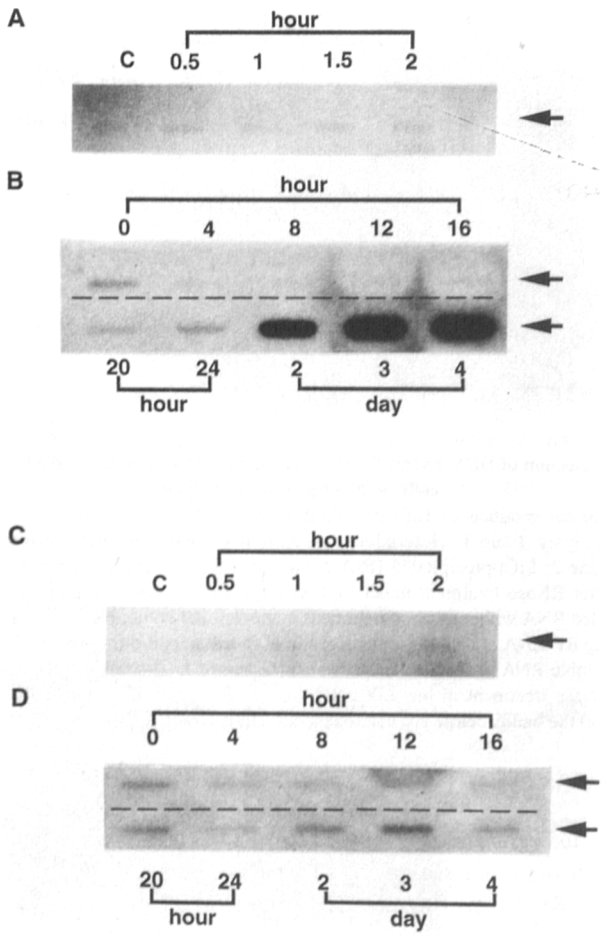

Fig. 7
Slot blot hybridisation of LiCl-precipitated DEN-2 viral RI RNA in infected AP-61 and BHK-21 cells
The LiCl-precipitated fraction contained viral genomic and RI RNAs. The viral sense probe was used to detect RI RNA but not genomic RNA. A and C: at various intervals post virus inoculation. B and D: at various intervals p.i. C: purified DEN-2 viral genomic RNA (10 μg). A and B: AP-61 cells. C and D: BHK-21 cells.

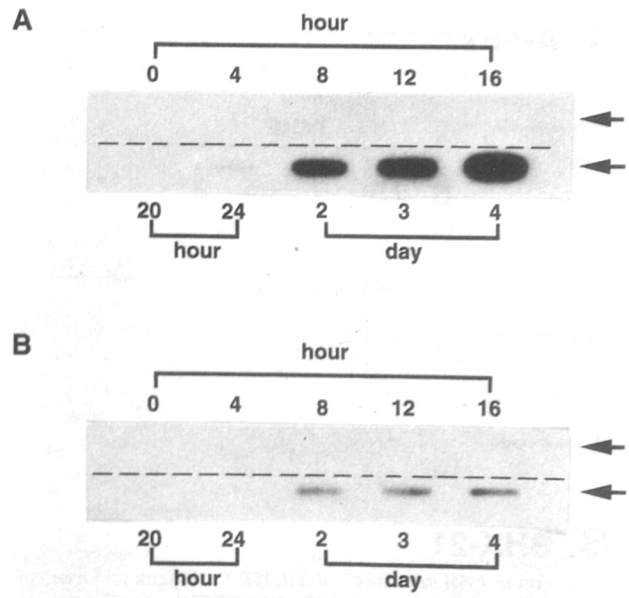

Fig. 8
Slot blot hybridisation of LiCl-soluble DEN-2 viral RF RNA in infected cells
A: AP-61 cells; B: BHK-21 cells.

tion (0 hr p.i.) and then dropped to undetectable levels before rising again at 20 hrs p.i. in both cell lines (Figs. 7B and 7D). The decline of viral RNA at 24 hrs p.i. in BHK-21 cells (Fig. 7B) was caused by unequal sample loading. The decline of viral RI RNA beyond day 4 p.i. was due to the death of infected BHK-21 cells (Fig. 7D).

The viral antisense probe was used to identify RF RNA. The earliest detection time for RF RNA was at 24 hrs p.i. in AP-61 cells and at day 2 p.i. in BHK-21 cells (Figs. 8A and 8B). A large amount of RF RNA was detected beyond day 2 p.i. in both cell lines. Levels of RF RNA were higher in AP-61 than in BHK-21 cells (Fig. 8). Unlike RI RNA, RF RNA did not decline but kept rising during the infection period in both cells.

Discussion

To increase the detection sensitivity, we utilised single-stranded probes which were synthesised by AS-PCR and labelled with [α-^{32}P]dATP. The labelling sensitivity of a single-stranded AS-PCR probe is at least 8-fold higher than that of a current double-stranded random priming probe (Bednarczuk *et al.*, 1991). Moreover, by choosing either AD4 or AD3 as the AS-PCR primer, a strand-specific probe was generated. In this way, we could detect both DEN viral sense and antisense RNAs on a simple blot by changing probes in Northern blot analysis (Liu *et al.*, 1994).

The reason for low sensitivity of the Northern blot analysis is that a lot of RNA is lost during the blotting. In our RT-PCR analysis, by choosing AD4 or AD3 as the primer, either the sense or antisense viral RNA could be detected following PCR amplification. We found that the detection sensitivity of RT-PCR followed by ethidium bromide staining was equivalent to that of the Northern blot analysis (data not shown). However, the RT-PCR followed by the Southern blot analysis dramatically enhanced the sensitivity for viral RNA detection. LiCl precipitation has been currently used to separate DEN viral RI RNA plus genomic RNA from RF RNA (Cleaves *et al.*, 1987). We modified this method by adding the slot blot hybridisation to enhance its detection sensitivity. By this procedure, the earliest detection time for RI RNA (containing a complete antisense RNA

and nascent sense RNA) was 30 mins post virus inoculation in both cell lines, indicating that viral RNA synthesis started 30 mins after viral invasion. This is consistent with our results with the RT-PCR followed by the Southern blot analysis. It has been reported that alphavirus replication occurs during the first hour p.i. (Strauss and Strauss, 1983), but it could not be detected until 3 hrs p.i. in the labelling studies of Schlesinger and Schlesinger (1986). In flaviviruses, Saint Louis encephalitis virus RNA synthesis was first detected 6 hrs p.i. (Trent *et al.*, 1969). The earliest time of DEN virus RNA synthesis reported so far was 3 – 6 hrs p.i. by [^{3}H]uridine incorporation (Stroller *et al.,* 1961; Takeda *et al.*, 1978) and 10 hrs p.i. by antigen analysis (Kimura *et al.*, 1985). Thus the detection time of DEN viral RNA of 30 mins after the first contact of cells with virus in our conditions is so far the earliest. Our data also demonstrate that RI RNA was detected earlier than RF RNA. The temporal sequence in the appearance of RI and RF RNAs in our study is consistent with the *in vivo* and *in vitro* studies of Chu *et al.* (1985) and Westaway (1987), in which the pulse-labelled RNA was detected first in RI RNA, then in RF RNA, and finally in genomic RNA.

DEN-2 virus showed different appearance of each form of viral RNA in AP-61 and BHK-21 cells. It seems that the virus replicated more actively in AP-61 than in BHK-21 cells, in contrast to the report that acute flavivirus infections in vertebrate cells are progressively and rapidly cytocidal, whereas similar infections in arthropod cells are often persistent (Westaway, 1987). Sinarachatanart and Olson (1973) have found that the mechanisms of DEN virus maturation in mosquito and vertebrate cells are different. Vector, host, and environmental factors may all play important roles in this complicated interaction (Ludwig and Iacona-Connor, 1993). This unique phenomenon may be reflected in complicated mechanisms of virus transmission. It is probable that while the virus is in the intermediate mosquito vector, it multiplies actively and massively produces infectious virus but keeps the host alive to continue the virus production. However, when the virus reaches its human host, it replicates slowly but steadily in permissive cells, spreading the virus and eventually destroying the host. Further research is required to identify those host-specific factors which determine the efficiency of virus replication.

In summary, (1) RT-PCR followed by Southern blot analysis and (2) slot blot hybridisation of LiCl-precipitated RNA were the most sensitive and reliable methods of detection of RNA viruses of those tested by us. Besides, slot blot hybridisation of LiCl-precipitated RNA with a specific probe could be specifically used to investigate the replication of single-stranded RNA viruses in the host in general.

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