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Molecular characterization of virus-specific RNA produced in the brains of flavivirus-susceptible and -resistant mice after challenge with Murray Valley encephalitis virus

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Natural resistance to flaviviruses in mice is controlled by a single genetic locus, *Flv*, on chromosome 5. Although the mechanism of this resistance is not fully understood, it is believed to operate at the level of virus replication rather than the immune response. It has been hypothesized that enhanced production of viral defective interfering (DI) particles is responsible for a substantial reduction in the titres of infectious virus in resistant mice. However, this has never been established at the molecular level since such particles have not been isolated and characterized. We have studied the products of virus replication in the brains of flavivirus-susceptible C3H/HeJ (*Flv*^{s}) and -resistant congenic C3H/RV (*Flv*^{r}) mice after an intracerebral challenge (i.c.) with Murray Valley encephalitis (MVE) virus and have found no evidence for the accumulation of truncated viral RNA in the brains of resistant mice. All three major viral RNA species, the replicative intermediate (RI), replicative form (RF) and virion RNA (vRNA) together with a subgenomic RNA species of 0·6 kb, which has not been previously described, were present in the brains of both mouse strains. However, the viral RF and RI RNA forms preferentially accumulated in the brains of resistant mice. Thus, we confirm that the resistance allele *Flv*^{r} interferes with discrete steps in flavivirus replication, although the precise mechanism remains to be determined.

Introduction

Innate resistance to flaviviruses, which is under the control of a single genetic locus, a flavivirus resistance locus (*Flv*) (Green, 1989), is responsible for the reduction in virus titres in the brains and several other organs of resistant mouse strains (Sabin, 1952). Although the mechanism of this resistance is not known in detail, it has been postulated to operate at the level of virus replication (Darnell & Koprowski, 1974). The three allelic forms of the *Flv* locus that have been described so far confer either susceptibility (*Flv*^{s}), resistance (*Flv*^{r}) or minor resistance (*Flv*^{mr}) to flavivirus infection (Goodman & Koprowski, 1962; Groschel & Koprowski, 1965; Sangster *et al.*, 1993). We have recently mapped this locus to mouse chromosome 5 and have produced a low resolution genetic map of the chromosomal region around *Flv* (Sangster *et al.*, 1994; Urosevic *et al.*, 1995).

The flaviviruses are positive-strand RNA viruses carrying a single-stranded (ss) RNA of 11 kb which both encodes and directs the synthesis of the viral polyprotein (Wengler *et al.*, 1994). Polyprotein synthesis and cleavage, and the synthesis of minus-strand RNA, are required for the synthesis of virion RNA (Wengler *et al.*, 1994). Flavivirus replication has been studied only in cell culture, and viral RNA products have been characterized by either pulse-chase (Cleaves *et al.*, 1981; Chu & Westaway, 1985) or cell-free RNA-dependent RNA polymerase (RDRP) assays (Chu & Westaway, 1987; Bartholomeusz & Wright, 1993). Three major viral RNA species have been detected in flavivirus-infected cells: an ss 40–44S virion RNA (vRNA), a double-stranded replicative form (ds RF), which consists of plus and minus viral RNAs, and a replicative intermediate (RI) containing ds RF with ss vRNA attached to it (Cleaves *et al.*, 1981; Chu & Westaway, 1985). A model has been proposed in which ds RF serves as the template

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for the synthesis of new positive-strand RNA which replaces the existing one in the RF (Chu & Westaway, 1985). It has yet to be clarified whether the same replicative complex catalyses the synthesis of both positive- and negative-strand RNA and why the positive strand is synthesized at the higher rate (Chu & Westaway, 1985).

Although genetically determined resistance to flaviviruses has been extensively studied in a mouse model of flavivirus-susceptible C3H/HeJ inbred mice and the flavivirus-resistant, congenic C3H/RV mouse strain (Goodman & Koprowski, 1962; Darnell & Koprowski, 1974; Jacoby & Bhatt, 1976; Sangster *et al*., 1993), detailed molecular characterization of total viral RNA produced in these mice has never been performed. Only limited biochemical analyses of intracellular viral RNA and extracellular virus particles produced by cell cultures from resistant and susceptible mice have been previously reported (Brinton, 1983). Based on these and virus interference studies, it has been proposed that an increased production of viral defective interfering (DI) particles is responsible for the reduced synthesis of infectious virus in resistant mice (Darnell & Koprowski, 1974; Brinton, 1983). Similar interference was obtained when brain suspension from Banzi virus-infected resistant C3H/RV mice was used in the virus interference assay, although no biochemical evidence for the presence of viral DI particles was provided (Smith *et al*., 1980). The aim of this study was to clarify whether truncated viral RNAs, which are components of viral DI particles, accumulate in resistantmice. In addition, we wanted to find out whether any other difference in the production of viral RNA existed as an effect of the *Flv*^{r} gene expression *in vivo*. This is the first time that the total viral RNA produced in the brains of flavivirus-susceptible C3H/HeJ and -resistant C3H/RV mice after challenge with a flavivirus [Murray Valley encephalitis (MVE)] virus has been characterized at the molecular level. Size and conformation of virus-specific RNAs were analysed after electrophoretic separation under denaturing and non-denaturing conditions by Northern blot hybridization using either MVE virus-specific cDNA or strand-specific oligonucleotide probes.

Methods

■ **Mice.** Specific pathogen-free inbred congenic C3H/HeJ and C3H/RV mice were obtained from the Animal Resources Centre (Murdoch, Western Australia) and housed under minimal disease conditions. Male and female mice of 8–10 weeks of age were used.

■ **Virus and cell cultures.** The Australian flavivirus MVE virus strain OR2 was used at a dose which was expressed as number of intracerebral (i.c.) 50% lethal doses (LD_{50}) for 3-month-old female flavivirus-susceptible C3H/HeJ mice. The virus was propagated in Vero cells using MEM as growth medium, enriched with 20 mM HEPES and 10% FCS (Sangster *et al*., 1993). Vero cells, used either for virus quantification ($TCID_{50}$ assay) or for isolation of viral RNAs, were grown in M199 medium supplemented with L-glutamine and 2% FCS.

■ **Infection of mice and collection of brains.** Mice were inoculated i.c. with 10^3 LD_{50} of MVE virus strain OR2, as described previously (Sangster *et al*., 1993). This corresponds to $10^{7\cdot4}$ $TCID_{50}$ units. Control, non-infected mice were inoculated with 5 µl of MOBS (buffered saline adjusted to the osmolarity of mouse serum) containing 2% FCS. On various days post-infection brains were removed aseptically, immediately placed in cold M199 containing 2% FCS, homogenized and stored at −70 °C before use.

■ **Virus titration.** Virus titres in 10% (w/v) brain homogenates were determined by the $TCID_{50}$ assay in Vero cells as described previously (Sangster *et al*., 1993) except that M199 medium and 2% FCS were used.

■ **Total brain RNA isolation.** Total tissue RNA was isolated from freshly collected brains of either infected or non-infected mice, using Total RNA Isolation Reagent (Integrated Sciences, Sydney, Australia) according to the manufacturer's instructions, dissolved in sterile DEPC-treated double-distilled water, aliquoted and stored at −70 °C. The concentration of RNA was spectrophotometrically determined by reading optical density (OD) at 260 nm. All RNA preparations were pure as confirmed by an OD_{260}/OD_{280} ratio in the range 1·8–2·0.

■ **Viral RNA preparation.** Single-stranded virion RNA (vRNA) and double-stranded replicative form (RF) RNA, which were used as standards during agarose gel electrophoresis and Northern blot analysis, were isolated from Vero cells infected with MVE virus OR2 strain. Confluent monolayers of cells were infected at either an m.o.i. of 1·0 for vRNA isolation, or at a m.o.i. of 5–10 for RF RNA isolation. Viral particles present in the supernatant, which was collected from cultures of infected Vero cells showing strong cytopathic effect at 64 h post-infection (p.i.), were pelleted by centrifugation at 10000 ***g*** following precipitation by PEG 6000 and NaCl. vRNA was isolated from pelleted viral particles using the same procedure as described above for total brain RNA isolation. Intracellular viral RNAs were extracted directly from the infected Vero cells at 40 h p.i. using the same procedure as above. Double-stranded viral RF RNA was recovered in the supernatant after LiCl fractionation as described by Cleaves *et al*. (1981), and ethanol precipitated. Both vRNA and RF RNA were dissolved in sterile DEPC-treated double-distilled water, aliquoted and stored at −70 °C.

■ **Agarose gel electrophoresis of RNA.** Viral RNAs were separated in 0·8% agarose gels (size 15 × 15 × 0·5 cm) for 2–3 h at 80 V. Two different types of agarose gel electrophoresis were used in order to allow separation of viral RNAs according to either conformation (non-denaturing) or size (denaturing). All the solutions used in both types of agarose gel electrophoresis were prepared with DEPC-treated double-distilled water.

(*a*) Non-denaturing electrophoresis. Separation of viral RNAs by electrophoresis under non-denaturing conditions was performed using 1 × TBE (89 mM Tris base, 89 mM boric acid and 2 mM EDTA, pH 8·0) as an electrophoresis buffer. The gel-loading buffer consisted of 0·04% bromophenol blue, 0·04% xylene cyanol and 5% glycerol mixed with 0·1–20 µg of total brain RNA in a final volume of 20 µl (Maniatis *et al*., 1982). vRNA and RF RNA isolated from infected Vero cells were used as standards for electrophoretic separation of ss and ds viral RNA forms.

(*b*) Denaturing electrophoresis. An agarose gel of the same percentage and size as used for non-denaturing electrophoresis was prepared in an alternative electrophoresis buffer consisting of 1 × MOPS (20 mM MOPS, 8 mM sodium acetate and 1 mM EDTA pH 7·0), with the addition of 0·37 M formaldehyde. Twenty µg of pelleted RNA was dissolved in 15–20 µl of the sample buffer (1 × MOPS buffer, 2·2 M formaldehyde and 50% formamide) and heated at 65 °C for 15 min. The RNA samples were quickly chilled on ice and before loading into gel were mixed with 5 µl of tracking dye mixture (0·5% SDS, 0·025%

bromophenol blue, 0·025% xylene cyanol, 25% glycerol and 25 mM EDTA) (Ausubel *et al.*, 1988). During electrophoresis the running buffer (1 × MOPS) was recirculated. RNA size standards purchased from Promega were sometimes used in addition to flavivirus vRNA and RF RNAs.

■ **Northern blot hybridization.** Electrophoretically separated RNA was vacuum transferred to a nylon membrane (Hybond N+, Amersham) in 50 mM NaOH for 90 min and immobilized by baking at 65 °C for 60 min. Hybridization was performed in a hybridization oven (Hybaid) at 65 °C overnight following 4–5 h of pre-hybridization. Two MVE virus cDNA clones, 1/1/12 (Dalgarno *et al.*, 1986) and 2/2/38 (Lee *et al.*, 1990) were random-prime labelled using the GIGAprime Random Labelling Kit (Bresatec) and [α-^{32}P]dCTP (Amersham) and used in hybridization either separately or together. Both pre-hybridization and hybridization buffers consisted of 7% SDS, 0·5 M sodium phosphate buffer pH 7·0, 1 mM EDTA and 1% BSA. In addition, the hybridization buffer contained radioactively labelled probe. The membranes were washed in 2 × SSC − 0·1% SDS twice for 5 min at room temperature, twice for 15 min at 37 °C and twice for 30 min at 65 °C, followed by a stringent wash in 0·1 × SSC − 0·1% SDS twice for 30 min at 65 °C, and then exposed to X-ray film (Fuji X-ray or Kodak X-Omat AR) in the presence of an intensifying screen for 1–5 days at −70 °C.

■ **Densitometry.** Densitometric analysis was performed using a Molecular Dynamics Computing Densitometer in conjunction with the ImageQuant software program.

■ **Statistical analysis.** Student's *t*-test was used to determine the statistical significance of the difference in the titre of the infectious virus and the amount of viral RNA between susceptible C3H/HeJ and resistant C3H/RV mice.

Results

Replication of MVE virus in the brains of susceptible and resistant mice

Adult susceptible C3H/HeJ and resistant C3H/RV mice were inoculated i.c. with 10^3 LD_{50} of the OR2 strain of MVE virus, and the virus titres in the brains were determined from day 2 to day 10 p.i. As shown in Fig. 1, at day 2 p.i. viral titres were similar in the susceptible and resistant mice, but from day 3 p.i. the production of infectious virus in these two strains differed. This difference in titre was statistically significant from day 4 p.i. (Student's *t*-test; $P < 0·001$). While C3H/HeJ mice succumbed to the infection from day 6, and showed signs of disease from day 4, mice of the C3H/RV strain showed no outward signs of disease and had cleared virus from the brain by day 10 (Fig. 1).

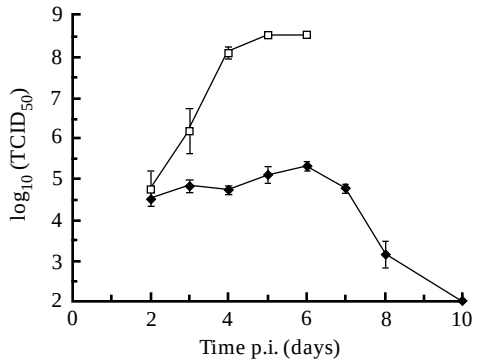

Fig. 1. Replication of MVE virus in the brains of flavivirus-susceptible (C3H/HeJ) and -resistant (C3H/RV) mice. At various days p.i. the titre of the virus in the brains of C3H/HeJ (□) and C3H/RV (◆) mice was determined by $TCID_{50}$ assay. Two to three animals of each strain were used for each time-point. The virus titre was expressed as $\log_{10}TCID_{50}$/0·01 g brain tissue. The limit of sensitivity of the assay was 2 $\log_{10}TCID_{50}$/0·01 g.

Qualitative analysis of viral RNA species produced *in vivo*

In order to find out whether such a profound difference in the virus titres between the resistant and susceptible mice is a result of impaired virus replication, the virus-specific RNA species produced in the brains of susceptible and resistant mice were studied in detail by a Northern blot hybridization analysis. Virus-specific cDNA probe 1/1/12, which covers the 5′ portion of the viral genome (Dalgarno *et al.*, 1986), and probe 2/2/38, covering the 3′ portion of the genome (Lee *et al.*, 1990), were labelled with ^{32}P and used in the analysis (Fig. 2). Total brain tissue RNA was isolated from both non-infected and infected resistant and susceptible mice at different time-points p.i. as indicated in the legend to Fig. 2, and separated using two different types of agarose gel electrophoresis which allowed separation of viral RNAs according to either conformation (Fig. 2*a*) or size (Fig. 2*b*, *c*). When samples were separated under non-denaturing conditions, three flavivirus-specific RNA species were detected in the brains of both resistant and susceptible mice (Fig. 2*a*). These were replicative intermediate (RI), replicative form (RF) and virion RNA (vRNA), as confirmed in co-migration studies using standard viral RF and vRNA forms isolated from Vero cells (Fig. 2*a*, lanes M_1 and M_2). Fractionation of viral RNA species by LiCl and RNase A treatment confirmed the presence of ss and ds RNA (data not shown). It is evident that the amounts of both the ds RF and ss vRNA species increased with time in both groups of mice with a very profound accumulation of RF form at later times p.i. in the resistant mice (Fig. 2*a*, C3H/RV, day 7 p.i.). Single-stranded virion RNA present in the samples isolated from resistant mice exhibited retarded mobility compared to the mobilities of similar forms isolated from Vero cells (Fig. 2*a*, lane M_2) and susceptible mice (Fig. 2*a*, C3H/HeJ, days 4, 5, 6 p.i.). It has been demonstrated that the increased amount of total tissue RNA present in the sample of resistant mice affected the electrophoretic mobility of ss vRNA separated under both denaturing and non-denaturing conditions (data not shown). No hybridization signal was detected with total RNA from non-infected susceptible and resistant mice (data not shown).

The presence of positive or negative viral RNA strands in different viral RNA forms produced in the brains of both resistant and susceptible mice was also analysed by Northern blot hybridization using four MVE virus-specific 20-mer oligonucleotides. The probes were of either sense orientation

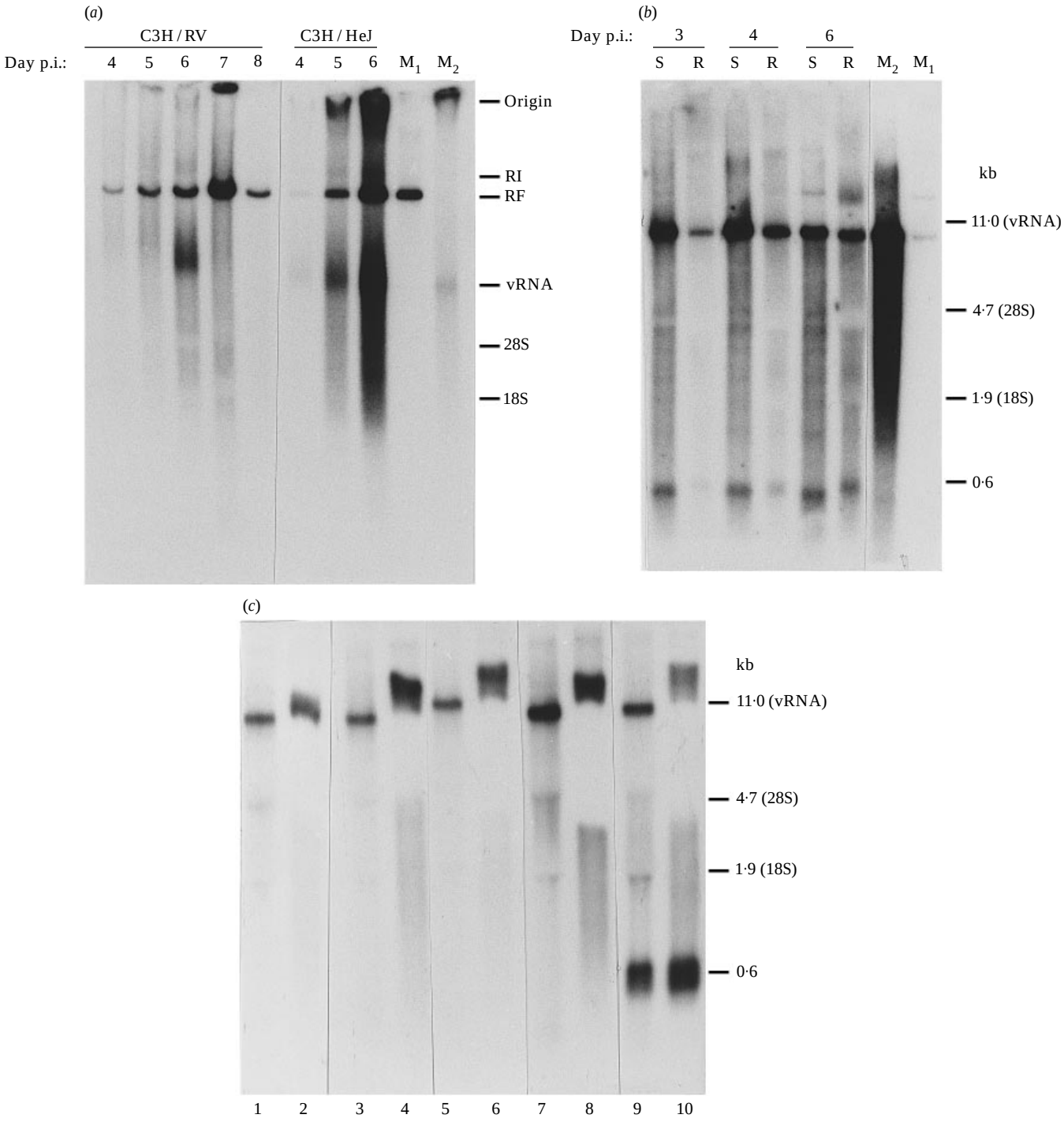

Fig. 2. Northern blot analysis of different viral RNA species. Total brain RNA isolated from non-infected mice (data not shown), and from infected resistant C3H/RV mice (20 μg) and susceptible C3H/HeJ mice (0·1 μg) was resolved on 0·8% agarose gels under non-denaturing (*a*) and denaturing conditions (*b*, *c*), transferred to Hybond N+ and probed with either the MVE virus cDNA clone 1/1/12 (*a*) or 2/2/38 (*b*, *c*). The samples were separated on the same gel together with the standard viral RNAs isolated from infected Vero cells, ds RF (lane M_1) and ss vRNA (lane M_2), respectively (*a* and *b*). (*a*) Total viral RNA isolated at different days p.i., as indicated by the numbers at the top of the figure, was hybridized to the viral clone 1/1/12. (*b*) Total brain RNA isolated from susceptible C3H/HeJ (S lanes) and resistant C3H/RV (R lanes) mice at various days p.i. as shown on the top of the figure, was separated under denaturing conditions and probed with the viral cDNA clone 2/2/38. (*c*) Total brain RNA from infected C3H/RV (lanes 1, 3, 5, 7 and 9) and C3H/HeJ mice (lanes 2, 4, 6, 8 and 10), at day 6 p.i., was separated on a denaturing 0·8% agarose gel and probed with *Eco*RI fragments of cDNA clone 2/2/38 as follows: 0·15 kb (lanes 1 and 2), 0·40 kb (lanes 3 and 4), 1·1 kb (lanes 4 and 6), 1·4 kb (lanes 7 and 8) and 1·7 kb (lanes 9 and 10).

Table 1. Relationship between the infectious virus titre and the amount of total ss viral RNA produced in the brains of susceptible and resistant mice

Values are means (±SE) obtained with nine mice of each strain.

	C3H/HeJ	C3H/RV
Virus titre ($\log_{10}$ $TCID_{50}$/0·01 g brain tissue)	8·81 ± 0·06	5·44 ± 0·13
Total ss viral RNA (ng/0·01 g brain tissue)	163·93 ± 19·64	0·32 ± 0·07
Virus titre/ssRNA ($TCID_{50} \times 10^6$/ng total ss viral RNA)	4·5 ± 0·6	1·3 ± 0·3

corresponding to the regions of the viral genome between nucleotides 430–449 and 5969–5988, or anti-sense orientation corresponding to the regions between nucleotides 1386–1405 and 7037–7056. This analysis has revealed similar distribution of positive and negative strands among different viral RNA forms produced in the brains of resistant and susceptible mice, and in Vero cells (data not shown).

The size of viral RNA molecules synthesized *in vivo* in susceptible and resistant mice was estimated by Northern blot hybridization analysis after total brain RNA had been separated under denaturing conditions together with RNA markers (Fig. 2*b*). Twenty μg of total brain RNA from resistant and 0·1 μg from susceptible mice were separated on denaturing gels and probed with two viral cDNA probes, clone 1/1/12 (Dalgarno *et al.*,1986) (data not shown), and 2/2/38 (Lee *et al.*, 1990) (Fig. 2*b*). Both probes revealed a single RNA species of 11 kb corresponding to the viral genomic RNA in all samples deriving from either susceptible C3H/HeJ (Fig. 2*b*, S lanes) or resistant C3H/RV mice (Fig. 2*b*, R lanes), and Vero cells (Fig. 2*b*, lanes M_1 and M_2). They also revealed bands which corresponded to ds non-denatured viral RF RNA (Fig. 2*b*, lanes 6S, 6R, M_1). The viral 3′-end clone, 2/2/38, revealed a novel viral RNA species of 0·6 kb in the samples deriving from both infected susceptible and resistant mice in addition to the 11 kb vRNA (Fig. 2*b*, lanes S and R), but not in the samples deriving from non-infected mice (data not shown). This RNA, isolated at day 6 p.i., was further characterized and was shown to hybridize to the 1·7 kb *Eco*RI fragment of the MVE clone 2/2/38, which corresponds to the 3′-terminal portion of the viral genome (Fig. 2*c*). Since there was no difference in this RNA form between resistant and susceptible mice we have not studied it further.

Quantitative analysis of total viral RNA in the brains of susceptible and resistant mice

As shown above and in the work of others (Sabin, 1952; Goodman & Koprowski, 1962; Sangster *et al.*, 1993), the titre of the virus in the brains of resistant mice, determined by either plaque or $TCID_{50}$ assays, was several logs lower than in the brains of susceptible mice, regardless of which flavivirus was used. Since those analyses were limited to the detection of only the packaged infectious virus, the amount of total viral RNA produced in the brains of resistant and susceptible mice was not determined. We have developed a slot-blot hybridization assay to determine the amount of viral RNA produced in the brains of susceptible C3H/HeJ and resistant C3H/RV mice. Total brain RNA samples isolated from infected C3H/HeJ and C3H/RV mice at day 6 p.i. were applied onto a nylon membrane together with the 6·4 kb viral cDNA fragment of clone 1/1/12, and hybridized to the same viral cDNA. Total brain RNA from non-infected mice was included on every blot as a negative control and used as a correction factor if any non-specific hybridization occurred (data not shown). A standard curve, in which intensities of signals of various dilutions of viral cDNA were plotted against their known concentrations, was constructed for each blot and used to quantify viral RNA in different samples (data not shown). When this procedure was applied to LiCl-fractionated samples, no signal was detected in the LiCl-soluble fraction (which contains viral ds RF RNA), suggesting that the conditions used to apply RNA onto the slot blots were not sufficiently stringent to allow denaturation of dsRNA (data not shown). Therefore, the method we have developed was limited to the quantification of total ss viral RNA only.

To determine whether the difference in the amounts of viral ss RNA in resistant and susceptible mice corresponds to the difference in the virus titres from the same animals, two groups of nine susceptible C3H/HeJ or nine resistant C3H/RV mice were used. The brains were removed aseptically at day 6 p.i. and the left hemisphere from each mouse was used for the titration of infectious virus by $TCID_{50}$, whilst the right hemisphere was used for the quantification of total ss viral RNA by slot-blot hybridization. The spread of the virus from the site of inoculation (the right hemisphere) throughout the entire brain was monitored by the titration of the virus in both hemispheres independently and shown to be uniform by day 6 p.i. (data not shown). As shown in Table 1, the virus titre and the amount of ss viral RNA differ significantly between susceptible and resistant mice (*t*-values 25·3 and 8·4, respectively, df = 8, $P < 0{\cdot}001$). When the $TCID_{50}$ value was

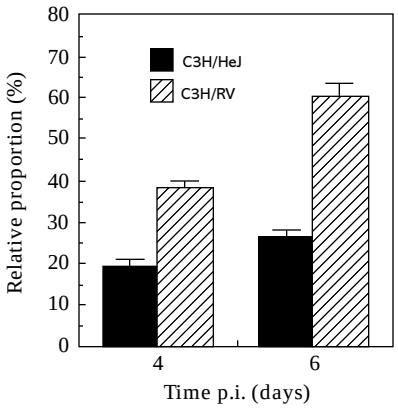

Fig. 3. Densitometric analysis of the relative proportion of RF RNA. The amount of RF RNA in total viral RNA detected in susceptible and resistant mice was estimated densitometrically and expressed as a percentage of total viral RNA. Two to three mice of either strain were used in this analysis for each different time-point.

expressed per ng of ss viral RNA for every individual animal of either susceptible or resistant genetic backgrounds, the ratio obtained for susceptible mice appeared to be significantly greater than that for resistant mice ($t = 4{\cdot}6$, df $= 8$; $0{\cdot}002 > P > 0{\cdot}001$) (Table 1).

Ratio between ds and ss viral RNA

A number of Northern blots of non-denaturing gels on which the viral ds RF was clearly separated from ss vRNA were used for densitometric analysis in order to determine the ratio between the ds and ss viral RNA. As presented in Fig. 3, the relative proportion of the ds RF form was consistently greater in the brains of resistant than in the brains of susceptible mice on days 4 and 6 p.i. Furthermore, the viral ds RF RNA also appeared to be the predominant viral RNA species in the brains of resistant mice at day 6 p.i. It has been demonstrated that the increased amount of total brain tissue RNA affected the mobility of ss vRNA, although no such effect on the intensity of hybridization signals was observed (data not shown).

Discussion

Molecular analyses of viral RNA species produced in the brains of flavivirus-susceptible C3H/HeJ and -resistant C3H/RV mice during the course of infection with MVE virus revealed the presence of three major species, RI, RF and vRNA (Fig. 2*a*), which had previously only been detected *in vitro* in cells infected with Uganda S, West Nile (Wengler *et al.*, 1978; Grun & Brinton, 1986), dengue (Cleaves *et al.*, 1981; Bartholomeusz & Wright, 1993) or Kunjin (Chu & Westaway, 1985) viruses. We have also detected for the first time a subgenomic RNA of 600 nucleotides which hybridized to the 1·7 kb *Eco*RI fragment of MVE cDNA clone 2/2/38 (Fig. 2*b*, *c*). The origin and role of this RNA in virus replication is not known at present. Two subgenomic viral RNA species of 190 and 120 nucleotides have been previously detected in vertebrate and insect cells infected with either Uganda S or West Nile viruses, respectively (Wengler *et al.*, 1978). It is not known whether either of these RNAs, including the one detected in our study, are related to viral DI particles. On the basis of the data presented in this report (Fig. 2*b*, *c*) we conclude that there is no preferential accumulation of this or any other truncated viral RNA in the brains of resistant mice. Therefore, we find no evidence for the involvement of viral DI particles in the expression of flavivirus resistance *in vivo*.

As presented in Table 1, the ratio $TCID_{50}$/ng total viral ss RNA is significantly greater in susceptible than in resistant mice. This indicates the reduced infectivity of ss viral RNA produced in the brains of resistant mice, which could result from either the accumulation of ss viral RNA other than vRNA, or the impaired packaging of vRNA in the same mice. However, the data obtained in this study, although the analysis was only qualitative, suggest that there was more viral RI RNA relative to the amount of ss vRNA in the brains of resistant than in the brains of susceptible mice (data not shown). In addition, densitometric analysis performed on several Northern blots revealed significantly greater accumulation of RF RNA in the brains of resistant than in the brains of susceptible mice during the course of infection (Fig. 3). These observations suggest the interference of the Flv^r gene product with discrete steps in flaviviral replication (packaging), although the precise mechanism will be difficult to resolve unless the gene is isolated and cloned. This interference may slow down the rate of flavivirus replication and create the conditions for the immune mechanisms to step up and clear the virus in resistant mice. We are currently using two major approaches to study flavivirus resistance further; one is 'reverse genetics' to clone the Flv^r gene and the other is *in situ* hybridization and immunohistological study of the virus spread in the brains of resistant and susceptible mice during acute infection with flaviviruses.

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