Transgenic Mice with Antisense RNA against the Nucleocapsid Protein mRNA of Mouse Hepatitis Virus

Nobuya SASAKI, Masanobu HAYASHI*, Shiro AOYAMA, Tadashi YAMASHITA, Ichiro MIYOSHI[1)], Noriyuki KASAI[1)], and Shigeo NAMIOKA

Department of Laboratory Animal Science, Faculty of Veterinary Medicine and [1)]Institute for Animal Experimentation, Hokkaido University, Sapporo 060, Japan

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ABSTRACT. In this study transgenic mice which expressed antisense RNA against the nucleocapsid protein gene of mouse hepatitis virus (MHV) under the control of RSV LTR were produced. These transgenic mice were able to transmit the foreign gene to their progeny in a Mendelian fashion. Antisense RNA was detected in various tissues from the transgenic mice including liver and brain, the target organs of MHV infection. One strain of transgenic mice derived from founder mouse No. 19 was more resistant to the lethal challenge of MHV than non-transgenic mice. The results of the present study show the ability of antisense RNA against the viral gene to protect agaisnt viral infection *in vivo*.—KEY WORDS: antisense RNA, MHV, N protein, transgenic mouse.

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It has been shown that antisense RNAs and oligonucleotides against viral genes can be used as tools for inhibiting viral gene expression and viral replication *in vitro* [10, 15, 22, 29, 31, 38]. The molecular mechanisms underlying the phenomenon are however still unclear. These results gave rise to the concept of unnatural intracellular antiviral immunity driven by specific antisense RNA. Indeed, it has been reported that the transgenic mice which express antisense RNA against proviral packaging sequences of Moloney murine leukemia virus (MMLV) do not develop any symptoms of leukemia after infection with MMLV [11]. Also, cell lines established from transgenic mice which express the antisense RNA against adenovirus h5 (Ad5) are more resistant to Ad5 than a normal kidney cell line [7]. We recently reported that multiplication of mouse hepatitis virus (MHV) was inhibited by an oligonucleotide complementary to the leader RNA sequence of MHV [23]. Consequently, transgenic mice which expressed antisense RNA against MHV gene were produced and the effect of antisense RNA on the MHV infection was examined to provide further information concerning the ability of antisense RNA to inhibit multiplication of the virus *in vivo*.

MHV is a member of the Coronaviridae, which causes a variety of diseases including hepatitis and encephalomyelitis in laboratory mice [13, 39]. MHV is an enveloped virus containing a helical nucleocapsid structure composed of a single-stranded, positive-polarity RNA of approximately 31 kilobase (kb) in length [27]. During infection, virion RNA is initially transcribed into full-length negative-stranded RNA [4, 16]. In turn, the negative-stranded RNA is transcribed into a genomic mRNA and six species of subgenomic mRNAs [16]. The mRNAs form 3'-coterminal nested-set extending for different lengths in the 5' direction [18]. The 5'-ends of each mRNA and the genomic mRNA contain an identical leader sequence of approximately 70 nucleotides, which are encoded only at the 5'-end of genomic RNA [17, 34]. The free leader RNA is synthesized initially, dissociates from the negative-stranded template, and rebinds to the full-length negative-stranded RNA at the initiation sites of the six subgenomic mRNAs. The leader RNA thus takes part in a leader-primed transcription [3]. However it is suggested that subgenomic negative-stranded RNA is also synthesized as a template for the transcription of mRNAs [30]. In MHV-infected cells, three major proteins are detected. The glycoproteins, M and S, of ca. 23,000 and 90,000 to 180,000 Da, are translated from mRNA 6 and 3, respectively [37]. A nucleocapsid (N) protein of ca. 60,000 Da is the most abundant and translated from mRNA 7 [35]. It has been shown that a specific interaction occurs between the N protein and the sequence in the leader RNA. The N protein also plays an important role in viral transcription and replication [2, 36]. The treatment of mice with neutralizing monoclonal antibodies (MAbs) to the N protein protects the mice against a lethal challenge of MHV, but not with MAbs to the M protein [25]. Therefore, the N protein may also be an important factor affecting the pathogenesis of MHV. Since the genomic sequence of the N protein of MHV was conserved among several strains of MHV [1, 32], transgenic mice which express the antisense RNA complementary to the N protein sequence of one strain of MHV may be resistant to the another strain of MHV. We therefore selected the N protein mRNA sequence as a target sequence of antisense RNA in the present study.

* CORRESPONDENCE TO: HAYASHI, M., Department of Laboratory Animal Science, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan.

MATERIALS AND METHODS

Reagents: Restriction endonuclease and agarose S were purchased from Nippon Gene. Nitrocellulose membrane (Hybond-C) and ^{32}P-dCTP (3000 Ci/mmole) were from Amersham. Reverse transcriptase was from Seikagaku Kogyou, Japan. Oligo $(dT)_{15}$, and primer oligonucleotides were synthesized using the phosphoamidite method on a Beckman DNA synthesizer (model System plus 1), and purified by HPLC.

Plasmid: The recombinant plasmid used to produce the transgenic mice consisted of Rouse sarcoma virus (RSV) LTR as a promoter/enhancer [9, 26], a 1.8 kilobase pair (kbp) full length of N protein cDNA fragment of MHV-JHM strain [32], the chloramphenicol acetyltransferase (CAT) gene, the SV40 splice site and polyA signal (Fig. 1). The cDNA fragment of N protein was inserted into the plasmid with antisense orientation relative to the RSV LTR. This recombinant gene was referred to as Rs-Nanti.

Transgenic mouse productions: C57BL/6//JCl mice were purchased from CLEA, Japan, at 8 weeks of age, and used at 10 weeks. The recombinant plasmid was digested with *Bam*HI and *Nde*I, and the 4.4 kbp fragment was separated from the vector sequence by agarose gel electrophoresis. The resulting fragment was microinjected into male pronuclei of fertilized mouse eggs according to the procedure of Gordon *et al.* [9]. Research was conducted according to the principles defined in the "Guide for the Care and Use of Laboratory Animals"

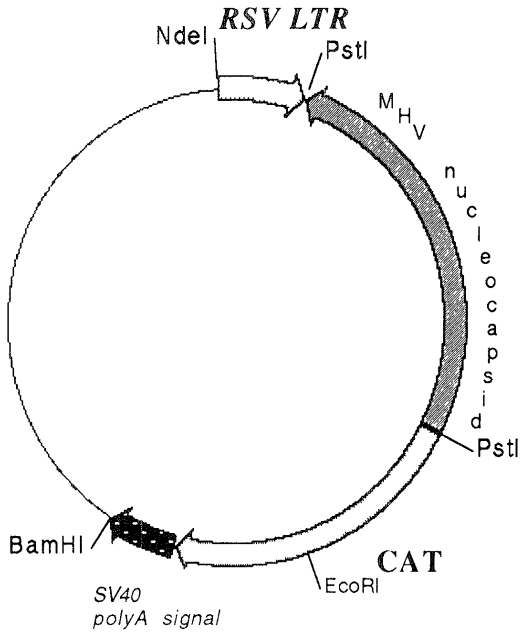

Fig. 1. Recombinant plasmid with antisense RNA against the N protein gene of MHV. The plasmid contains RSV LTR, cDNA of N protein of MHV, CAT gene, and SV40 T antigen polyadenylation region. A cDNA fragment of N protein was inserted with antisense orientation relative to the RSV LTR.

prepared by Hokkaido University.

Southern blotting hybridization analysis: DNA samples were prepared from tail segments of transgenic mice essentially by the method of Maniatis *et al.* [20]. The DNA samples were digested with restriction endonuclease and the DNA fragments were electrophoretically separated on a 0.6% agarose gel. The separated DNA fragments were transferred onto nitrocellulose paper by the method of Southern [35]. A cDNA fragment of N protein of MHV was ^{32}P-labelled by nick translation [28]. Hybridization was carried out under the conditions described previously [15], i.e. 6X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate), 5X Denhardt's solution (1X Denhardt's solution is 0.05% bovine serum albumin, 0.05% Ficoll and 0.05% polyvinylpyrrolidone) [6], 100 μg/m*l* of yeast tRNA, and 50% formamide at 42°C. Autoradiography using Fuji RX X-ray film (Fuji Photo Film) was carried out with an intensifying screen at −80°C for 24 hr.

Reverse transcriptase-polymerase chain reaction (RT-PCR): Total RNA samples were prepared from various tissues of transgenic mice according to the method of Chairgwin *et al.* [5]. RT-PCR was carried out under the conditions described previously [15], *i.e.* cDNA was synthesized in a 50 μ*l* reaction mixture which contained 5 μg of tissue RNA, 4 ng of synthetic oligo $(dT)_{15}$, 100 mM Tris-HCl (pH 8.3), 4 mM dithiothreitol, 10 mM $MgCl_2$, 140 mM KCl, 20 μg actinomycin D/m*l*, 1 mM each of dTTP, dCTP, dATP and dGTP, and 80 U of avian myeloblastosis virus reverse transcriptase. The RNA was used for the reaction after treatment with RNase-free DNaseI (Promega Biotec). The reaction mixture was heated briefly at 90°C and then reacted at 42°C for 2 hr.

Two primer oligonucleotides for the PCR, P1; TGCCGACATAGGATTCATTCTCT and P2; ATATTGGTACAGACACAACCGAC were used for the amplification of the 1.1 kbp region of the N protein cDNA of MHV. The PCR was performed according to the protocol provided with the GeneAmp kit (Perkin-Elmer Cetus) using synthesized cDNA as a template.

Infection: The JHM strain of MHV [19] used in this study was propagated in DBT cells [14]. After 10 μ*l* of inoculum was placed on the external nares of each 5–14 transgenic and non-transgenic mice pups at 1–2 days of age, animals were kept in a separate vinyl isolator with sterile food, water and corncob bedding, and inspected for survival at least twice a day.

RESULTS

The male pronuclei of 1,465 fertilized eggs were microinjected with several hundred copies of the recombinant gene, Rs-Nanti, and 1,060 eggs were then transplanted to the oviducts of pseudopregnant foster recipient mice for further development. The presence of the Rs-Nanti gene in mice developing from the eggs injected with Rs-Nanti DNA was determined by Southern blotting hybridization of DNA extracted from the tail segments (Fig. 2a). Five transgenic mice, designated as No. 15, 16,

17, 19 and 20, were identified from a total of 42 offspring and they carried different copy numbers of the Rs-Nanti genes (Table 1). Four out of five transgenic mice were able to transmit the foreign gene to their progeny and both male and female were fertile (Table 1). We developed two independent strains of transgenic mice derived from founder mice No. 19 and No. 20 which carried 20 and 30 copies of the Rs-Nanti gene per haploid genome, respectively (Table 1, Fig. 2). The inheritance pattern of the Rs-Nanti gene in the two pedigrees showed that both male and female mice were able to transmit the transgene to their progeny in a Mendelian fashion (data not shown).

To analyze the expression of the antisense RNA against the N protein mRNA of MHV, cDNAs were synthesized from the RNA samples prepared from several tissues of F3 mice of strain No. 19 which were homozygotes for the Rs-Nanti gene and then PCR was carried out using the

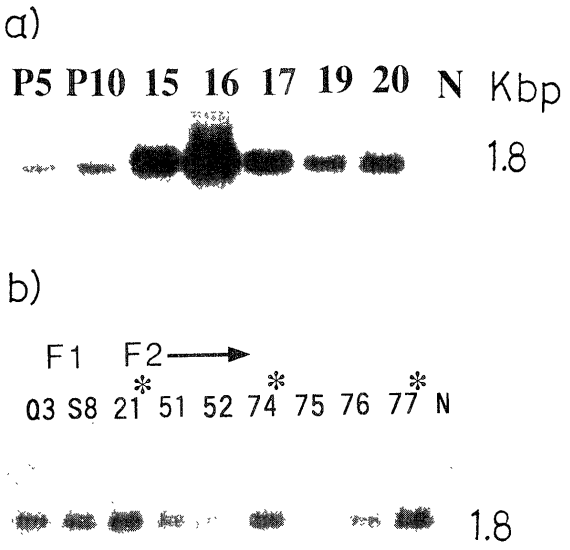

Fig. 2. Southern blot analysis of transgenic mice. (a) Ten micrograms of DNA samples prepared from founder mice (No. 15, 16, 17, 19 and 20) and non-transgenic mouse (N) were digested with *Pst*I, and analyzed by Southern blotting hybridization using cDNA of N protein as a probe. P5 and P10 represent positive controls (5 and 10 copies of *Pst*I-digested Rs-Nanti gene per haploid genome). (b) Ten micrograms of DNA samples prepared from F1 (O3 and S8) and F2 (21, 51, 52, 74, 75, 76 and 77) mice of strain No. 19, and non-transgenic mouse (N) were digested with *Pst*I and analyzed as described in (a). * represents homozygote for the Rs-Nanti gene.

Table 1. Transgenic mice with Rs-Nanti gene

Founder mouse	Copy number of transgene (per haploid genome)	F1[a]	F2[b] homozygote	F3[c] homozygote
No. 15(f)	50	m 2/ 3 f 4/ 8	3/24	NT
No. 16(f)	>50	m 0/ 5 f 0/ 7		
No. 17(f)	40	m 1/ 6 f 6/14	14/53	NT
No. 19(f)	20	m 8/ 9 f 6/ 6	28/78	+
No. 20(f)	30	m 4/ 5 f 2/ 4	24/63	+

a) F1 mice were obtained from the mating of founder mouse and parental C57BL/6 mouse and represented as transgenic mice/total male or female offsprings. f and m represent female and male, respectively.
b) F2 mice were obtained from the mating of F1 mice which carried the Rs-Nanti gene, and represented as homozygotes/total offsprings.
c) F3 mice were obtained from the mating of F2 mice which were homozygote for the Rs-Nanti gene. + represents the appearance of transgenic mice with homozygote of transgene. NT represents not tested.

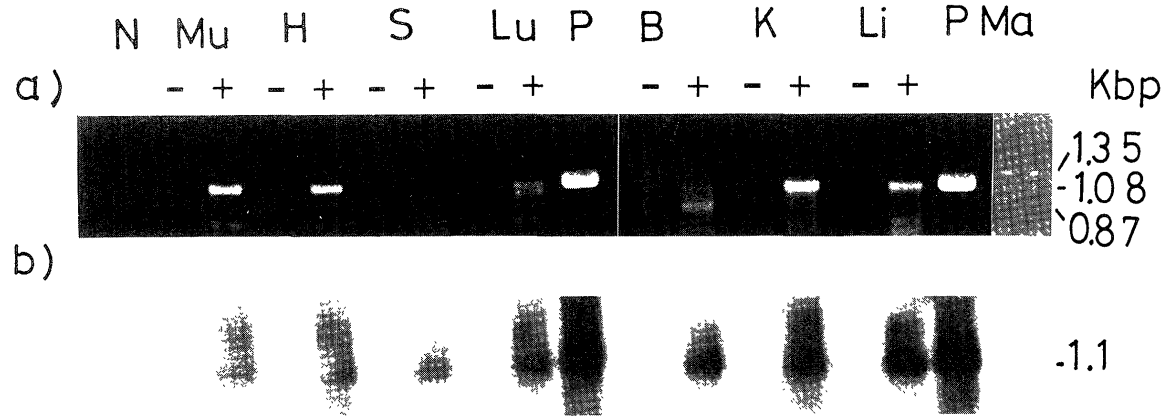

Fig. 3. The expression of antisense RNA in transgenic mice. (a) Five micrograms of the total RNA samples were prepared from various tissues of F3 mice of strain No. 19 and non-transgenic mice (N). cDNA was synthesized from each of the RNA samples and amplified by PCR. The amplified products were electrophoresed on agarose gel and stained with ethidium bromide at a concentration of 0.5 μg/m*l*. + represents that the RNA samples were reacted with reverse transcriptase before PCR analysis. − represents that the RNA samples were not reacted with reverse transcriptase. *Hae*III-digested phiX174 DNA was used as a size marker (Ma). (b) The amplified products as shown in (a) were transferred onto the nitrocellulose membrane and hybridized with cDNA of N protein as a probe. Tissues are shown: Mu, muscle; H, heart; S, spleen; Lu, lung; B, brain; K, kidney; Li, liver. P represents positive control (amplified product from the recombinant plasmid as a template).

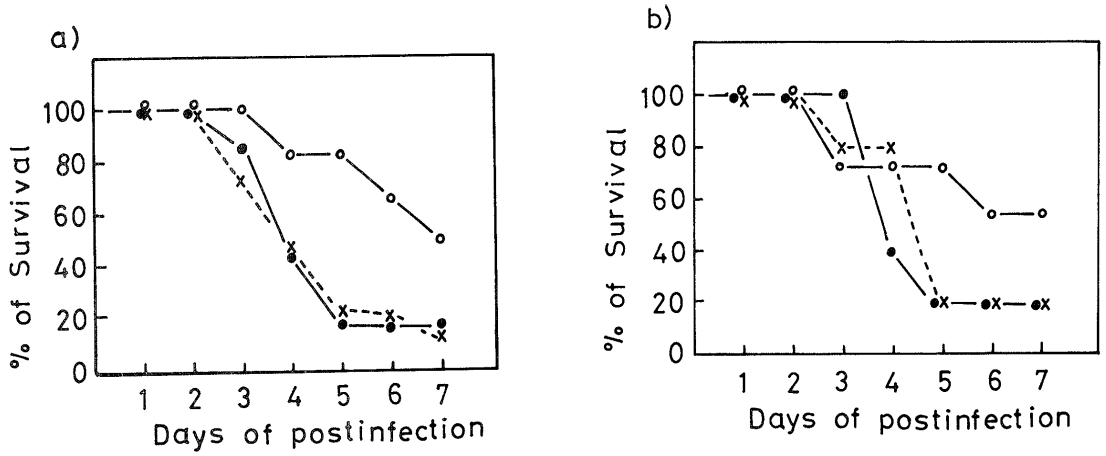

Fig. 4. The survival rate of the transgenic mice infected with MHV. (a) Twelve mice of No. 19 strain (○- ○), twenty mice of No. 20 strain (× -- ×) and eleven non-transgenic mice (● - ●) were infected with MHV at 1.0×10^3 PFU. (b) Eleven mice of No. 19 (○ - ○), five mice of No. 20 (× -- ×) and five non-transgenic mice (● - ●) were infected at 6.8×10^2 PFU.

synthesized cDNA as a template (Fig. 3). The expected 1.1 kb amplified fragment was observed in PCR products from all 7 tissues examined in the present study, and the 1.1 kbp amplified fragment was hybridized with the cDNA of N protein. The 1.1 kb amplified fragment was observed in PCR products of the RNA samples from F3 mice of strain No. 20 by RT-PCR analysis (data not shown).

To examine the effect of antisense RNA on the infection of MHV *in vivo*, the transgenic mice were infected with MHV at 5 to 10×10^2 PFU. The results are shown in Fig. 4. In the case of No. 19 strain of transgenic mice, higher survival rates were observed after 4 days of post infection compared with those of non-transgenic mice. The survival rate of non-transgenic mice was approximately 20% at 7 days of post infection. In contrast, 50% of transgenic mice (No. 19) survived. After 8 days of post infection, death of the infected mice was not observed. No significant difference in survival rate or day of death-incidence was observed between non-transgenic mice and transgenic mice of strain No. 20.

DISCUSSION

It has been reported that antisense RNA against viral genes inhibits the expression of the gene and viral multiplication *in vitro* [10, 22, 29, 31, 38]. However, at present only two research groups have reported successful production of transgenic animals with antisense RNA against viral genes [7, 11]. We produced transgenic mice which expressed antisense RNA against the N protein gene of MHV under the control of RSV LTR and examined the ability of antisense RNA to inhibit the viral infection *in vivo*. It is well known that RSV LTR contains the regulatory sequence of the gene expression and directs an active expression in various mammalian cells and tissues [9, 26]. In the present study, the expected 1.1 kbp amplified fragment was observed in PCR products of the RNA samples from various tissues including liver and brain of transgenic mice by RT-PCR analysis (Fig. 3). The results showed that RSV LTR directed the expression of antisense RNA in these tissues, the target organs of MHV infection [13, 39].

Since four out of five transgenic mice were able to transmit the Rs-Nanti gene to their progeny (Table 1), the Rs-Nanti gene was integrated into the host chromosome in each of the four transgenic mice. Transgenic mouse No. 16 did not transmit the foreign gene to its progeny. Therefore, this founder mouse should be mosaic concerning the transgene.

The transgenic mice derived from founder No. 19 were more resistant to the lethal infection of MHV than non-transgenic mice and the transgenic mice of strain No. 20 (Fig. 4). The present result and the reports of other workers [7, 11] show the ability of antisense RNA against viral gene to protect against viral infection *in vivo*, although the present study does not confirm a mechanism for the inhibition of antisense RNA complementary to the N protein gene against lethal infection of MHV in transgenic mice. The N protein plays an important role in transcription and replication [2, 36], and the pathogenesis [25] of MHV. In the transfected DBT cell lines which expressed antisense RNA against the N protein gene of MHV, viral transcription and multiplication were inhibited (24 and data not shown). It has been proposed that antisense RNA can work by interfering with translation due to the formation of hybrids between mRNA and antisense RNA [12, 21]. Therefore, antisense RNA against the N protein gene may inhibit the synthesis of the N protein and the viral multiplication, and this results in the protection of transgenic mice against the lethal infection of MHV. However, the reason why there was no significant difference in the sensitivity to MHV infection between non-transgenic mice and the transgenic mice of strain No. 20 remains still unclear. The expression level of antisense RNA may be an important factor for the inhibitory effect. Further studies are now in progress to

confirm the mode of inhibition by the MHV antisense sequence.

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