is the null allele only for the *T* gene [1, 9]. Both alleles interact with *tct* to cause the tailless phenotype. The TBAC transgene used in this study for the first time rescued the embryonic lethality associated with the T^{137}/T^{137} or T/T^{137} mutations. Differences between our result and that of Stott *et al.* [18] can be reconciled as follows, 1) the 23-kb transgene confers only partial or reduced activity of the *T* gene product, which is sufficient for complementation of the *T*/+ tail defect, but not for the tailless defect or embryonic lethality, whereas the TBAC transgene has full activity of the *T* product; or 2) there may be other gene(s) involved in the tailless defect or embryonic lethality located near the *T* gene. Since the original *T* mutation deletes the *T* and other neighboring genes, the phenotype of the original *Brachyury* mutation may be a sum of those of the *T* and other mutations, and these combined phenotypes can be rescued by the 170-kb BAC, but cannot be corrected by the 23-kb DNA containing the *T* gene only. Rennebeck *et al.* [15, 16] reported the presence of a gene designated as *T2* located ~40 kb upstream of the *Brachyury* gene, and that a transgene-insertional mutation of the *T2* gene resulted in embryonic lethality at 11.5 dpc. Rennebeck *et al.* [16] considered the *T2* gene as a strong candidate for *tct*. Whether *tct* is an allele of the *T* locus or represents a distinct locus adjacent to the *T* gene, such as *T2* gene, remains to be elucidated [12, 14–16]. Detailed characterizations of the genomic region covered by the TBAC19 and further analysis of genes in and around this genomic region will be important for understanding the functional relationships between the *T*, *T2* and possibly *tct* genes. The BAC transgenesis method presented in this paper should facilitate functional analyses of the genomic region.

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—Note—

Detection of MHV-RNAs in Mouse Intestines and in Filter Dust in Mouse Room Ventilation Duct by a Modified RT-Nested PCR

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Abstract: *We applied RT-nested PCR for the detection of MHV genomic RNA in a modified manner to obtain RNA from the intestines of mice and from filter dust in the ventilation ducts of the room in which a contaminated mouse colony was kept. Since the sequences of MHV-RNA that were extracted from the intestine of a serologically MHV-positive mouse in room No. 2 (MS2) and from the filter dust in a ventilation duct in the same room (FD2) were identical, amplified product from filter dust was demonstrated to come from the MHV contaminated room. Furthermore, sequences of FD2 and of filter dust from another contaminated mouse room, No. 7 (FD7) showed 38 nucleotide exchanges among 368-bp (10.3%), suggesting that two different MHV strains were contaminating our facilities. SSCP analysis of Dra I-digested PCR product of 393 bp also showed different patterns in FD2 and FD7 samples.*

Key words: *mouse hepatitis virus, MHV-RNA genome in filter dust, RT-nested PCR*

Mouse hepatitis virus (MHV) contamination in transgenic and knockout mouse colonies has remained prevalent despite continuing efforts to protect against it in animal facilities. Transfer of infected mice to other facilities must be inhibited to avoid the spread of MHV infection among laboratories. It is also absolutely necessary to detect MHV in a mouse colony as early as possible. Reverse transcription and nested polymerase chain reaction (RT-nested PCR) has been shown to be reliable in detecting MHV excreted in the feces during the early stages of a natural outbreak [1–3].

Many types of transgenic or knockout mice have been maintained in our animal facilities in a SPF condition. Recently, however, we noticed MHV contamination in mouse colonies with serological detection of MHV-specific IgG antibody by ELISA (ICLAS, Kanagawa Japan). We used the RT-nested PCR method to detect viral RNA in mice intestines and fresh feces. We also tried to detect MHV-RNA in filter dust in the mouse room ventilation duct by means of RT-nested PCR. The present experiments were undertaken under the Guiding Principles for Animal Experimentation of Niigata

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In the present experiments, some modifications were performed to obtain clear viral genomic RNA. Briefly, total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method described by Chomczynski, P. *et al.* [4], from intestine homogenates after passing DNA through an 18-gauge needle. RNA in fresh feces and feces left at room temperature for one hour after excretion was extracted by the AGPC method. RNA in filter dust (ca. 17–129 mg) in mouse room ventilation ducts was also extracted in the same manner. All RNA samples were subjected to additional phenol and chloroform extraction and ethanol precipitation, which gave a good A260/A280 ratio of 2.0. Extracted RNA was finally dissolved in 50 μl of diethyl pyrocarbonate (DEPC)-treated water and stored at –80°C.

The extracted RNA was checked by electrophoresis before RT-nested PCR. The electrophoresis tank was filled with 2% hydrogen peroxide overnight and rinsed with RNase-free water before use. RNA (5–10 μg) was loaded onto 1% agarose gel which had been autoclaved at 121°C for 5 min and stocked at room temperature (24°C). Electrophoresis was performed in 1xTris-borate/EDTA (TBE) buffer and stained with ethidium bromide. As shown in Fig. 1A, total RNA from mice intestines was 28s/18s eukaryotic ribosomal RNA and those from fresh feces and feces kept at room temperature for one hour were 23s/16s prokaryotic ribosomal RNA indicating that RNA preparation was successful. RNA from filter dust did not show such ribosomal RNA bands.

RNA (2–10 μg) and reverse transcription (RT) primer (KOR1: 0.4 μM) were denatured at 70°C for 5 min and chilled on ice before reaction. RT reaction was performed with a cDNA synthesis kit (1st strand with AMV reverse transcriptase, Life Science, Florida USA). RT reactant was extracted with phenol/chloroform, and then precipitated in 2 volumes of chilled ethanol. RT reactant was finally dissolved in 20 μl of DEPC-treated water (cDNA sample) and stored at –20°C.

In the first PCR, 1 μl of the cDNA sample was mixed with 0.4 μM each of KOF1 [5] and KOR2 primer [5], 0.2 mM of dNTPs and 0.25 U Taq DNA polymerase (TaKaRa Ex Taq, Takara Shuzo Co, Ltd, Japan) in a total volume of 10 μl reaction buffer. These primers were expected to amplify the N gene of MHV-1, 2, 3, S, A59, JHM, RI, Y, DVIM, TY, Nu67, TM, TH, IR, OP, JD, K1000, KX1, KQ6E, KV306, TK and KQT2 strains [6]. With a thermal cycler (ASTEC, Fukuoka, Japan), the PCR reaction was started by initial denaturation at 94°C for 3 min, and then amplified for 30 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and polymerization at 72°C for 2 min, followed by final polymerization at 72°C for 5 min. The final reactant was kept at 4°C. In the second PCR, the first PCR products (1 μl) and primer sets of 7P148 and 7N540, 7P148 and 7N722 were used (Table 1). The elongation time in a cycle time was changed to 30 s from the 1st PCR condition.

MHV-RNA was detected in the intestine of a C57BL/6J mouse from the serologically MHV-positive mouse room No. 2 (MS2) and in the filter dust in a ventilation duct in the same room (FD2), but was not detected in the feces. The amplified band was obtained only in the final RT-nested PCR product using 1 μl of cDNA

Table 1. Primers for RT-nested PCR

	Primer	Sequence (5' to 3')	Expected PCR product	Ref.
RT reaction	KOR1	5'GTGATTCTTCCAATTGGCCA 3'	(bp)	5
1st PCR	KOF1 (F)	5' ATGTCTTTTGTTCCTGGGCA 3'		5
	KOR2 (R)	5' CTACTTACATTTCGCTGCAC 3'	1277	5
2nd PCR	7P148 (F)	5' AAGCAGACTGCAACTACTCA 3'		1
	7N540 (R)	5' CGCAAACCTAGTAGGAATAG 3'	393	6
	and			
	7P148 (F)	5' AAGCAGACTGCAACTACTCA 3'		1
	7N722 (R)	5' ACAAGAGCAGCAATTTCTTC3'	575	1

KOR1, KOF1 and KOR2 primers were reported by Watanabe *et al.* (5), and 7P148, 7N540 and 7N722 were reported by Yamada *et al.* (1, 6).

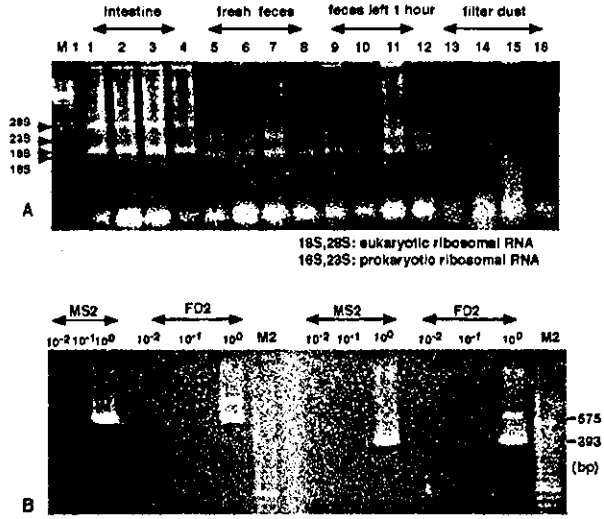

Fig. 1. Agarose gel electrophoresis of total RNA and RT-nested PCR product. (A) Agarose gel electrophoresis of extracted RNAs. RNA was extracted by the AGPC method (Chomczynski, P. *et al.*), with additional phenol, chloroform extraction, and ethanol precipitation. The gel was stained with ethidium bromide. Lane 1–4: RNAs from intestine. Lane 5–12: RNAs from both fresh feces and feces after 1-hour. Lane 13–16: RNA from filter dust in a ventilation duct of the mouse room. Lane M1: λ/ Hind III, marker. (B) MHV titers in RNA preparations from intestine and filter dust. RT-nested PCR was carried out. On the left halves of lanes are products with 7P148 and 7N722 second PCR primers sets. The right halves of lanes are those with 7P148 and 7N540 primer sets. PCR products were loaded onto 3.5% NuSieve agarose gel for electrophoresis and stained with ethidium bromide. FD2: RNA from filter dust from mouse room No. 2. MS2: RNA from intestine of a C57BL/6J mouse in mouse room No. 2. Lane 10^0: cDNA 1 μl. Lane 10^{-1}: cDNA 0.1 μl. Lane 10^{-2}: cDNA 0.01 μl. Lane M2: 20 bp ladder.

sample as the template in both the primer sets. When 0.1 μl of cDNA was used, the amplified band was not obtained. The results of filter dust in another contaminated mouse room No. 7 (FD7) were same as those of FD2 (data not shown).

PCR products obtained from FD2 and FD7 were further purified for sequencing and SSCP analysis. The products of a second PCR with a primer set of 7P148 and 7N540 were logged from the gel, floated in 3 volumes of TE buffer, and melted at 75°C in a water bath. After extraction of DNA by mixing well with an equal volume of phenol, the DNA (PCR-393) was precipitated with ethanol and dissolved in 20 μl water. The purified PCR-393 from FD2 and FD7, and PCR-393 from MS2 were directly sequenced with primer 7P148 (Sawady Technology Co., Ltd, Tokyo, Japan). The results of sequence analysis were compared among them (Fig. 2). Those of MS2 and FD2 were completely identical. Those of FD2 and FD7 had 38 bp exchanges among 368 bp (10.3%).

Since Yamada *et al.* reported that sequence analysis and nucleotide comparison of the N gene would be a useful tool to identify the origin of MHV prevalence (6), FD2 and FD7 seem to originate from different sources.

For further SSCP analysis of FD2 and FD7, the purified PCR-393 was digested with 10 unit of Dra I in a final volume of 50 μl, followed by mixing well with phenol. The DNA (PCR-393/Dra I) was precipitated with ethanol and the precipitant was finally dissolved in 10 μl of TE buffer. A half volume of PCR-393 after digestion with Dra I was loaded on non-denatured gel and two bands, 202 bp and 191 bp, were obtained (data not shown). Another volume of PCR-393/Dra I was heated at 95°C for 5 min to denature, immediately set on ice, then loaded on 6% polyacrylamide gel in 0.5 × TBE buffer. Electrophoresis was performed at 150 V constantly for 4 h (ATTO, Tokyo Japan) and then the gel was stained with ethidium bromide for 20 min. The results are shown in Fig. 3. Lane FD2 showed five bands (arrows 1, 3, 4, 5 and 7) and lane FD7 showed three bands (arrows 2, 4 and 6). Arrow 4 indicates 393 bp, arrows 1, 3, 5 and 7 are two fragments, 202 and 191 bp in lane FD2. On the other hand, only 2 bands (arrows 2 and 6) other than 393 bp were observed in lane FD7. From these results, SSCP analysis seems to be convenient for indirectly discriminating the sequence differences between various MHV strains as an alternative to complete sequencing.

In this experiment, RT-nested PCR was improved remarkably by evaluating the quality of RNA extracted from tissue and a fecal pellet by means of a simple RNA agarose gel electrophoresis. As shown in Fig. 1A, RNA extraction was demonstrated to be good when the ribosomal RNA of intestinal-bacteria origin from the fecal pellet was detected as a marker for the effect of RNase in the feces, even after the feces had been left for one hour in a cage.

Watanabe *et al.* [5] reported that MHV-RNA was detected by RT-nested PCR from dust in a cage in which mice had been kept after oral inoculation with

```
                                                                                      72
MS2: ATTCCGGGAGTGTGGTTCCCTACTACTCCTGGTTTTCCGGCATTACCCAGTACCAAAAGGGAAAGGAGTTTC
FD2: **T*****************T*C*****C********C***********G*AC**A*A***A**G*****T*
FD7: **C*****************C*T*****T********G***********A*TT**G*G***T**A*****C*
                                                                                     144
MS2: AGTTTGCAGATGGACAAGGAGTGCCTATTGCCAATGGAATCCCCGCTTCAGAGCAAAAGGGATATTGGTATA
FD2: *******************************************C*****A**********************
FD7: *******************************************A*****T**********************
                                                                                     216
MS2: GACACAATCGACGTTCCTTTAAAACACCTGATGGGCAATACAAGCAATTACTCCCCAGATGGTATTTTTACT
FD2: *******T********C********************AT*C**G****************************
FD7: *******C********T********************GC*G**A****************************
                                                                                     288
MS2: ATCTTGGCACGGGGCCCCATGCTGGAGACAGTTACGGAGACAGCATTGAAGGAGTCTTCTGGGTTGCAAACA
FD2: ************G******C****T*G**A**G**AC******A****************************
FD7: ************A******T****A*A**C**T**TT******C****************************
                                                                                     360
MS2: GCCAAGCGGACACCAACGCCCCCTCTGATATTCTTGAAAGGACCCAAGTAGCCATGAGGCTATTCCTACTA
FD2: GC***************G*****T****T***CT************A*************************
FD7: AT***************A*****G****C***TC************T*************************
            368(nt)
MS2: GGTTTGCG
FD2: ********
FD7: ********
```

Fig. 2. Comparison of partial sequence of N gene. MS2: MHV genomic RNA from mouse MS2. FD2: MHV genomic RNA from filter dust in mouse room No.2. FD7: MHV genomic RNA from filter dust in mouse room No.7. Asterisks represent the same nucleotide.

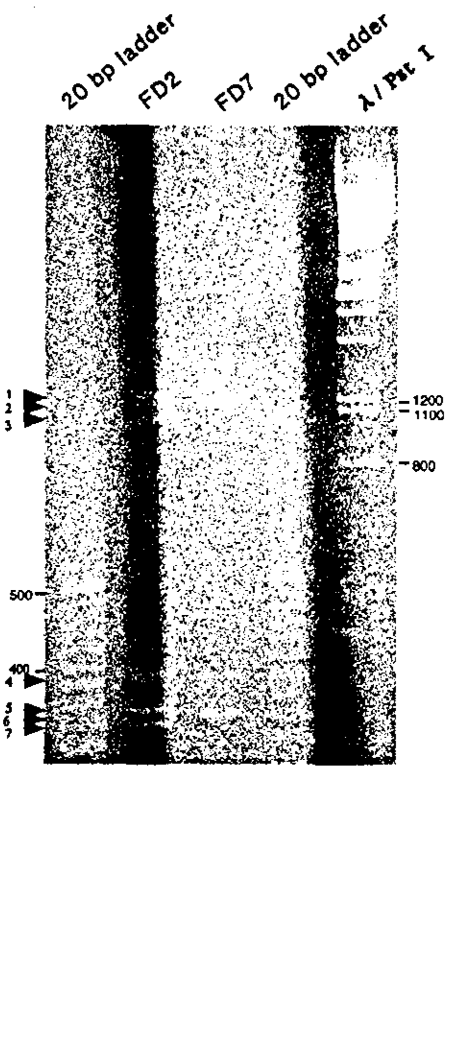

Fig. 3. SSCP analysis of PCR-393/Dra I. Fragments of PCR-393/Dra I from FD2 and FD7 were analyzed by 6% polyacrylamide gel at room temperature (approximately 24°C). Ethidum bromide staining. In lane FD2: PCR-393/Dra I of FD2. In lane FD7: PCR-393/ Dra I of FD7. Arrow No.4 indicates 393 bp.

the MHV-Ku strain. We kept cages without any filter in a laminar flow rack with positive pressure in a ventilated room. The number of ventilations was about 13 times/H. Since all fresh air is supplied to each mouse room through a filter and the air from each mouse room is exhausted through a pre-filter to the exterior, the risk of cross contamination being caused by airborne dust from room to room is considered to be very low. Particulates, such as bedding waste (wood), hair waste, and feed waste, are collected by a filter in the ventilation duct. Most contain bedding waste in our experience. MHV detected from filter dusts shown in Fig. 1B is considered to come from feces carried out of a cage in the air as dust caused by movements of the mice, and collected in the pre-filter.

The ELISA test was done in twenty randomly selected C57BL/6J mice including young, old, male and female among about 450 mice kept in Room No. 2.

All of the tested mice were serologically positive for MHV, but no MHV-RNA was detected by RT-nested PCR in a fecal pellet. Finally, viral RNA was detected from the intestine of one mouse (MS2), even though the virus titer was very low (Fig. 1B). It seems that almost all mice in Room No. 2 had been infected and obtained anti-MHV antibody, and that the virus was excluded from mice at the time of sampling.

Since a serological test is not suitable for immunocompromised mice, sentinel animal methods should be used for such mice. Matthaei *et al.* [3] reported that immunocompetent C57BL/6J mice were kept as sentinels for 20 days in a cage with dirty bedding from cages of nude mice clinically affected with MHV, and then sera from the sentinel mice were tested by ELISA for the antibody. Compared with such time-consuming sentinel animal serologic testing, RT-nested PCR of duct dust can be completed within 2 days.

The results of the sequence analysis and nucleotide comparison of some (368 bp) of the N genes of MS2 and FD2 were completely identical as shown in Fig. 2. It becomes clear that detection of the virus in the mouse population of a room unit will be possible by using dust. Detection of the MHV will be attained at an early stage by carrying out the monitoring of MHV in dust. Since Yamada *et al.* [6] reported that sequence analysis and nucleotide comparison of the N gene would be a useful tool to identify the origin of MHV prevalence, FD2 and FD7 seem to come from different strains existing simultaneously in two different rooms. Furthermore, we guess from the 10.3% rate of variation, that the two strains were introduced by different routes into our facilities rather than that one strain varying with the passage of time. The MHV invasion route, however, is unknown.

SSCP analysis is a simple and powerful technique for the detection of single base changes in amplified DNA [7], and is applied in various fields as the method for screening. We performed SSCP analysis to detect nucleotide exchanges along with sequence analysis. Figures 2 and 3 show the nucleotide difference between the FD2 and FD7 strains was clearly demonstrated by both methods. SSCP analysis is particularly useful for processing a lot of samples and is a simple technique to distinguish two different strains.

It is quite necessary to use the two methods, ELISA and RT-nested PCR, co-operatively for routine monitoring of MHV contamination in animal facilities. The findings of our research should improve the accuracy of routine monitoring by RT-nested PCR. We recommend routine inspection of filter dust for MHV-RNA in room ventilation ducts.

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