Characterization of Temperature-sensitive (ts) Mutants of Coronavirus Infectious Bronchitis Virus (IBV)

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1. INTRODUCTION

Coronavirus infectious bronchitis virus is a positive-stranded RNA virus, which synthesises a 3'-coterminal nested set of six subgenomic RNAs. Subgenomic RNA transcription and genome replication are directed by the viral replicase, which is expressed in the form of polyproteins 1a and 1a/b and subsequently processed into smaller nonstructural proteins by the virus-encoded proteinases. Major structural proteins S (spike protein), E (envelope protein), M (membrane protein) and N (nucleoprotein) are translated from subgenomic RNAs 2, 3, 4 and 6 and involved in nucleocapsid and virion assembling (Lai & Cavanagh 1998). Previous studies demonstrated that mutations and deletions on the structural proteins conferred the coronavirus mouse hepatitis virus ts phenotypes (Masters *et al* 1994 & Luytjes *et al* 1997). However, little is known about the mechanisms involved. In this study, ts mutants were generated by growing wild type virus at progressively lower temperatures from 35⁰C to 28⁰C on Vero cells. Two ts mutants were isolated from passages grown at 29⁰C (ts291602) and 28⁰C (ts282902). Sequence analysis reveals that mutations emerged in the S protein and an insertion occurred in the N protein. Biological and biochemical properties of ts mutants were characterised.

2. MATERIALS AND METHODS

2.1 Virus and cells

An IBV strain, Beaudette, was first adapted to grow on Vero cells at 37°C in our laboratory. In this study, the virus was plaque-purified and then passaged on Vero cells at progressively lower temperatures from 35°C to 28°C. Cells were maintained in complete DMEM medium (GIBCO BRL) supplemented with 10% newborn calf serum and infected with viruses at low multiplicity of infection (0.1). Plaque assays were performed as described previously (Shen *et al* 1994).

2.2 RT-PCR and sequencing

Viral RNA was extracted from partially purified viruses using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription and polymerase chain reaction (RT-PCR) were performed using the Expand Reverse Transcription and High Fidelity PCR Kits (Boehringer Mannheim). Annealing and extension times of PCR were optimised for amplification of PCR products with different sizes using different primers. More than 100 specific primers were used for amplification, sequencing and cloning. Automated sequencing was carried out using PCR products and specific primers as previously described (Shen & Liu 2000). Sequence analyses were carried out using the GCG suite of programs.

2.3 Northern blot

Plasmid pGEMN1, covering nucleotides 26539 to 27608 under control of the SP6 promoter at the 3'-end of the IBV genome, was linearised with restriction enzyme PvuII (at position 27034). Transcription was performed using Dig Labelling Kit (Boehringer Mannheim) and the linearised template. The transcripts were treated with 20U/ml of RNase-free DNase (Boehringer) for 15 min at 37°C, extracted with phenol/chloroform and precipitated with equal volume of 4M ammonium acetate and 6 volumes of ethanol. The pellet was washed once with cold 70% ethanol, resuspended in 50µl of H₂O and stored at -20°C. Northern blot was performed as described previously (Shen *et al* 1999).

2.4 Analysis of viral structural proteins

Viral structural proteins were labelled, immunoprecipitated and separated on polyacrylamide gels as described previously (Liu *et al* 1998).

3. RESULTS

3.1 Isolation of ts mutants of IBV

An IBV strain Beaudette, grown on Vero cells at 37°C, was plaque-purified and adapted to progressively lower temperatures by growing at 35°C, 34°C, 33°C, 32°C, 30°C, 29°C and 28°C for 4, 5, 7, 44, 12, 21 and 29 times, respectively. Plaque assays of virus stocks were performed at both 32°C and 40°C. As shown in Table 1, viruses from early passages could form plaques at both temperatures at almost the same levels, but those from late passages only produced plaques at 32°C. The results suggested that ts mutants emerged in the cold-adaptation process and dominated quickly when temperature was shifted to 28°C. Two ts mutant clones, designated ts291602 and ts282902, were plaque-purified from passage 16 (29°C), and passage 29 (28°C), respectively. Plaque assays showed that ts291602 produced at least 1,000 time less plaques at nonpermissive temperature (40°C) than at permissive temperature (32°C), while ts282902 formed plaques only at permissive temperature. We believed that partially lethal and lethal ts mutants were generated through cold adaptation of the wild type on Vero cells.

3.2 Analysis of subgenomic RNA synthesis

Vero cells were infected with viruses at different temperatures. Viral subgenomic RNAs were extracted and detected with a probe complementary to the 3'-end of the positive-sense viral RNA from nucleotides 27034 to 27608 (nucleotide and amino acid numbering refer to those described by Bournsnel *et al*, 1987). As shown in Figure 1, synthesis of viral subgenomic RNAs of ts mutant 28P29 (passage 29 at 28°C) was hardly detectable under these conditions at 40°C. On the other hand, normal amounts of the subgenomic RNAs were detected when the ts mutant 28P29 was grown at 32°C and the wild type was grown at both 37°C and 40°C.

Table 1. Plaque assays of passages grown at different temperatures. Selected passages were assayed at both 32°C and 40°C.

Temperature	Passages	Passages assayed	Titre [#]	
			32°C	40°C
37°C	Wild type	wt63	5.0x10 ¹	2.0x10 ⁶
32°C	P1 to P44	P44	2.7x10 ⁵	2.0x10 ⁶
30°C	P1 to P12	P12	1.1x10 ⁵	1.4x10 ⁵
29°C	P1 to P21	P10	1.5x10 ⁴	5.0x10 ³
		P13	2.5x10 ⁵	2.5x10 ²
		P21	5.0x10 ⁵	1.3x10 ³
28°C	P1 to P29	P1	1.0x10 ⁵	5.0x10 ¹
		P5	3.3x10 ⁶	NP*
		P29	2.9x10 ⁶	NP

indicated as PFU/ml; * no plaques formed.

Figure 1. Subgenomic RNA synthesis. Northern blot analysis was carried out to detect viral subgenomic RNA synthesis of *ts* mutant 28P29 (passage 29 at 28°C) at 28°C, 32°C and 40°C (as indicated at the top). Cultures infected wild type wt63, passages 32P44 (passage 44 at 32°C) and 29P16 (passage 16 at 29°C) were used as controls and mock-infected culture as negative control. Subgenomic RNAs 2 to 6 are indicated.

3.3 Analysis of viral structural proteins

Cells were infected with wild type virus and *ts* mutants and viral proteins were labelled with ³⁵S-methionine. Viral structural proteins were immunoprecipitated with anti-IBV and antisera against the M and N proteins (Fig 2). The results revealed that the N proteins of the two mutants migrated slightly more slowly than that of the wild type, indicating mutation or insertion in the N protein of the *ts* mutants.

Figure 2. Structural protein profiles of plaque-purified wild type wt63, ts mutants ts291602 and ts282902. Labeled viral proteins were immuno-precipitated with antisera as indicated and separated on a 12.5% SDS polyacrylamide gel. Spike (S), nucleocapsid (N) and membrane (M) proteins are indicated. Numbers indicate molecular masses in kilodaltons.

3.4 Sequence analysis of the wild type virus and ts mutants

Nucleotide sequences of the wild type virus and ts mutants were determined using RT-PCR products of the three viruses. An insertion of 9 nucleotides, located near the 5'-end of the N gene of both mutants, was identified. This resulted in an in-frame Arg-Thr-Leu insertion in the N protein between the tenth and the eleventh residues of the wild type (Table 2). The insertion corresponds to the slow migration of the N protein of the ts mutants. In addition, three point mutations (Table 2), which caused two amino acid substitutions (a Gln²⁹⁴ to Leu²⁹⁴ and an Ile⁷⁶⁹ to Met⁷⁶⁹) in the S protein of both mutants, were identified. To date, sequences of the 5'- and 3'-NCR, half of the 1a region, whole 1b and structural regions of the wild type and ts mutant have been determined and no other mutations, insertions and deletions of the deduced gene products were identified except those mentioned above.

Table 2. Sequence analysis of the plaque-purified wild type and ts mutants.

Nucleotide position	Wt63		ts291602		ts282902	
	Nt [#]	Aa*	Nt	Aa	Nt	Aa
21247/8	CA	Q	UU	L	UU	L
22674	A	I	G	M	G	M
25902/3	-	-	AGGACCUUA	RTL	AGGACCUUA	RTL

[#] nucleotide; * amino acid; - locus corresponding to the insertion in the mutants.

4. CONCLSION

In this study, ts mutants emerged in the process of passaging the wild type IBV at progressively low temperatures. Under this selective pressure, ts mutants dominated soon in the viral population when the temperature shifted from 29⁰C to 28⁰C. To date, 80% of the genomes of the mutants have been sequenced and two mutations, Glu²⁹⁴ to Leu²⁹⁴ and Ile⁷⁶⁹ to Met⁷⁶⁹, in the S protein and an Arg-Thr-Leu insertion in the N protein of the ts mutants have been identified. The insertion of the mutants was confirmed by PAGE analysis. It is noted that synthesis of the viral subgenomic RNAs of the ts mutants was hampered at the nonpermissive temperature, although no mutations were identified in the 1b polyprotein including the polymerase domains. Further studies are underway to map the defective gene(s) in structural and/or nonstructural regions for ts phenotypes and to understand the mechanisms involved.

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