Recurrent Mutations Associated With Isolation and Passage of SARS Coronavirus in Cells From Non-Human Primates

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Four clinical isolates of SARS coronavirus were serially passaged in two primate cell lines (FRhK4 and Vero E6). Viral genetic sequences encoding for structural proteins and open reading frames 6–8 were determined in the original clinical specimen, the initial virus isolate (passage 0) and at passages 5, 10, and 15. After 15 passages, a total of 15 different mutations were identified and 12 of them were non-synonymous mutations. Seven of these mutations were recurrent mutation and all located at the spike, membrane, and Orf 8a protein encoding sequences. Mutations in the membrane protein and a deletion in ORF 6–8 were already observed in passage 0, suggesting these amino acid substitutions are important in the adaptation of the virus isolate in primate cell culture. A mutation in the spike gene (residue 24079) appeared to be unique to adaptation in FRhK4 cells. It is important to be aware of cell culture associated mutations when interpreting data on molecular evolution of SARS coronavirus. J. Med. Virol. 76:435–440, 2005.

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INTRODUCTION

Severe acute respiratory syndrome coronavirus (SARS-CoV) is a novel coronavirus which is an outlier of the group 2 coronaviruses in the order of Nidovirales [Snijder et al., 2003]. The genome is a 29.7 kb, single-stranded RNA and contains five major open reading frames (Orfs) that encode the replicase polyprotein (Orf 1a and Orf 1ab), spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins, in that order. The genomic RNA itself is mRNA and is responsible for Orf 1a and Orf 1ab polyprotein expression. Proteins encoded by the rest of the genome are generated by subgenomic mRNA. In addition to the structural proteins and the replicase, the RNA also encodes other proteins with unknown functions [Marra et al., 2003; Rota et al., 2003].

The S, E, M, and N proteins are the structural proteins of the virus. The N protein is an internal protein and it binds to viral RNA to form the ribonucleoprotein complex [Huang et al., 2004]. By contrast, S, E, and M are surface proteins. E and M are integral membrane proteins and are the minimal set of proteins required for virus assembly [Bos et al., 1996]. S protein is responsible for receptor binding and a metallopeptidase named angiotensin-converting enzyme 2 (ACE2) has been identified as a key functional receptor of the protein [Li et al., 2003]. The receptor-binding domain of the S protein is located between amino acid residues 303 and 537 [Xiao et al., 2003]. Recently, CD209L (L-SIGN) is also shown to facilitate SARS-CoV entry [Jeffers et al., 2004].

The natural reservoir of this pathogen is not known. But, the identification of a related virus in Himalayan palm civets and other small mammals suggested that SARS-CoV may have emerged from a non-primate mammalian species. Being a virus newly introduced to humans, it had several sequence changes that might be associated with adaptation to the human host [Guan et al., 2003; Chinese SARS Molecular Epidemiology Consortium, 2004]. In particular, sequence analyses of SARS-CoV isolated from different phases of the outbreak indicated the S gene had the highest mutation rate and that it was adapting to the new host [Chinese SARS Molecular Epidemiology Consortium, 2004; Yeh et al., 2004]. Initial isolation of the SARS-CoV was made
in cells derived from non-human primates [Ksiazek et al., 2003; Peiris et al., 2003]. We serially passaged four clinical isolates in two primate cell lines and looked for recurrent mutations in their progeny viruses in comparison with the viral genetic sequence obtained from the original clinical specimen.

MATERIALS AND METHODS

Viruses

Four isolates (strains A–D) used in this study originated from different clinical specimens and patients (Table I). Viruses first isolated in FRhK4 cells and passaged once in Vero E6 cells were the starting viral stocks and are designated as “passage 0” in this study.

Cells

FRhK4 or Vero E6 cells were maintained by Eagle’s minimal essential medium (MEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (PS). Cultures were incubated at 37°C with 5% CO2.

Viral Culture

One hundred microliters of viral culture with approximately 10^6 TCID_{50} was used to infect a monolayer of cells seeded in a 25 cm² culture flask. Infected cells were maintained in Eagle’s MEM supplemented with 1% PS and incubated at 37°C with 5% CO2. Progeny viral particles in culture supernatants at each passage level were harvested at day 3 of post-infection and were stored at −80°C for future analysis.

RNA Extraction

RNA from 140 μl of the culture supernatant was extracted by QIAamp virus RNA mini kit (Qiagen, Germany) as instructed by the manufacturer. Extracted RNA was eluted in 50 μl of RNase-free water and stored at −20°C. Complementary DNA was generated by using random primers as described [Poon et al., 2003]. Briefly, 10 μl of eluted RNA samples was reverse transcribed by 200 U of Superscript II reverse transcriptase (Invitrogen, USA) in a 20 μl reaction containing 0.15 μg of random hexamers, 10 mM DTT, 0.5 mM dNTP. Reactions were incubated at 42°C for 50 min, followed by a heat inactivation step (72°C for 15 min).

PCR and Sequencing

PCR primers specific for S, E, M, Orfs 6–8, and N gene sequences (Table II) were used in this study. In a typical PCR reaction, 1 μl of cDNA was amplified in a 50 μl reaction containing 0.1 mM dNTPs, 3 mM MgCl₂, 1 μM forward primer, 1 μM reverse primer, 0.5 U AmpliTaq Gold (Applied Biosystems, USA). Reactions were thermal-cycled with the following condition: 95°C, 10 min followed by 40 cycles of 95°C, 30 sec; 50°C, 30 sec; 72°C, 90 sec. Amplified products were purified by QIAquick PCR purification kit (Qiagen, Germany) as instructed by the manufacturer. Purified products were sequenced by using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA). Sequencing products were analyzed by ABI PRISM 3700 DNA Analyzer (Applied Biosystems, USA). Both sense and anti-sense sequences of these PCR products were analyzed at least once.

Sequence Analysis

Deduced viral sequenced were analyzed by BioEdit, version 5.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

RESULTS

SARS-Cov isolated from lung biopsy, nasopharyngeal aspirate, throat swab, and stool from four different patients were serially passaged 15 times in FRhK4 or Vero E6 cells, respectively. Viral RNA from the original clinical sample, the initial virus isolate (passage 0), passage 5, passage 10, and passage 15 were sequenced. Orfs for all structural proteins (i.e., S, E, M, and N) were examined. Because previous studies had suggested that deletions in between the M and N genes might be associated with adaptation of the virus to primates [Guan et al., 2003; Thiel et al., 2003], the Orfs 6–8 (i.e., 27074–28118 nt of TOR2 strain) was also sequenced.

Fifteen different mutations were identified and these are summarized in Table III. All mutations were confirmed by independent sequencing reactions. Of these mutations, there were three synonymous mutations (residues 27995, 28161, and 28557), five recurrent non-synonymous mutations (residues 23412, 23473, 23518, 24079, and 24677), and one recurrent deletion (residues 27808–27809). Three of these 15 mutations (residues 24978, 26477, and 26600) were also reported by others (http://www.ncbi.nlm.nih.gov/genomes/SARS/sarsnuc.html). Apart from the mutation at residue 26477, all other point mutations were transition mutations.

Two non-synonymous mutations (residues 26477 and 26600) in the M gene and a deletion in the Orf 8a were already observed from viruses in passage 0. In subsequent passages, viruses grown in both Vero E6 and FRhK4 cells developed identical mutations at residues 23412, 23473, and 23518 of the spike protein gene. By contrast, a mutation at residue 24079 occurred in all four viruses passaged in FRrhk4 cells but in none of Vero E6 cell passaged viruses.

DISCUSSION

In the early phase of the SARS outbreak, the S protein of the virus was under strong selection pressure and...
evolving rapidly [Chinese SARS Molecular Epidemiology Consortium, 2004]. The virus isolates used in our study were from the middle phase of the outbreak by which time SARS-CoV was better adapted to the human host. In this study, we investigated the selection pressures imposed by the process of virus isolation in two primate kidney epithelial cell lines, FRhK4 and Vero E6 commonly used to grow the virus [Ksiazek et al., 2003; Peiris et al., 2003]. We used four independent viral isolates and serially passaged these viruses in these two cell lines. The sequence of the virus in the original clinical specimen was compared with that of the initial

### TABLE II. PCR Primer Used in the Study

| Targeta | PCR reaction | Primer sequence (5′–3′)b | Nucleotide positionc |
|---|---|---|---|
| S (Orf 2) | 1 | FP: TCTCTTCTGGAAAAAGGTAGGC | 21405 |
| | | RP: CAGGACATTGTGTAGTGCAGCA | 22359 |
| | 2 | FP: CAAATTTCATAGGCCATTCTTACG | 22177 |
| | | RP: AAATATGACCTGACTGACAGTGAT | 23157 |
| | 3 | FP: CCAGGACAAATCTGTTAT | 22686 |
| | | RP: CAGCATCAGCGAGTGTCAC | 23931 |
| | 4 | FP: GAGCTGCGATTGTGCTAGT | 23449 |
| | | RP: AGAAGGCCGTATTGCAGCAG | 24500 |
| | 5 | FP: GAGGGAGGGTACAAATTTGGA | 24399 |
| | | RP: AACGCCAATACACAGGCATC | 25412 |
| E (Orf 4) | 6 | FP: GGATCCATGTACTCATTCGTTTCG | 26117 |
| | | RP: AAGCTTGGACCAGAAGATCAGGAA | 26344 |
| M (Orf 5) | 7 | FP: GGATCCATGGCAGACAACGGTACTA | 26398 |
| | | RP: GAATTCTTACTGTACTAGCAAAGCAA | 27063 |
| Orf 6–8 | 8 | FP: TGTGTTCTATCCACCCCAAA | 26720 |
| | | RP: GCTGAGTGAGAGCTGTGAACC | 28297 |
| N (Orf 9a) | 9 | FP: TCTAGTCAGGATCCATGAAGGTCACCAAACT | 28049 |
| | | RP: TCAGACTAAAGCTTGCTGATGCAG | 29385 |

*Labeling system is referenced to Snijder et al. [2003].
Forward primer (FP) and reverse primer (RP) used in the corresponding PCR reactions. Sequences in bold are linker sequences.
Refer to the viral nucleotide position recognized by the 5′ end of the primer (excluding the linker sequences).
Reference sequence: TOR2 (Genebank accession number: AY274119).

### TABLE III. Mutations in S, E, M, Orf7-11, and N Sequences of SARS-CoV Passaged in Vero E6 and FRhK4 Cells

| Genea | Nucleotide positionb | Mutation | Amino acid position (mutation)c | Found in isolates (passage)d |
|---|---|---|---|---|
| Vero E6 | | | | |
| S | 23412 | C → T | 641 (His → Tyr) | B (10), C (10) |
| | 23473 | A → G | 661 (His → Arg) | A (10) |
| | 23518 | C → T | 671 (Ala → Val) | D (15) |
| | 23632 | C → T | 714 (Ala → Val) | C (10) |
| | 24864 | C → T | 1125 (Pro → Ser) | B (10) |
| | 24978e | A → G | 1163 (Lys → Glu) | B (15) |
| E | No mutation | | | |
| M | 26477 | G → T | 27 (Cys → Phe) | D (0) |
| | 26600f | C → T | 68 (Ala → Val) | A (0), D (0) |
| Orf 6–8 | 27806–27809 | TT deletion | Frame shift in Orf 8a | A (0), D (0) |
| | 27995 | T → C | 44 (Val, Silent mutation) in Orf 8b | B (15) |
| N | 29294 | C → T | 392 (Thr → Ile) | B (15) |
| | 29366 | C → T | 416 (Ser → Phe) | B (15) |
| FRhK4 | | | | |
| S | 23412 | C → T | 641 (His → Tyr) | B (10), C (10) |
| | 23473 | A → G | 661 (His → Arg) | A (10) |
| | 23518 | C → T | 671 (Ala → Val) | D (15) |
| | 24079 | C → T | 863 (Thr → Ile) | A (10), B (10), C (10), D (10) |
| E | No mutation | | | |
| M | 26477 | G → T | 27 (Cys → Phe) | D (0) |
| | 26600f | C → T | 68 (Ala → Val) | A (0), D (0) |
| Orf 6–8 | 27806–27809 | TT deletion | Frame shift in Orf 8a | A (0), D (0) |
| N | 28161 | C → T | 9 (Pro, Silent mutation) | A (15) |
| | 28557 | C → T | 146 (His, Silent mutation) | B (10) |

*Labeling system is referenced to Snijder et al. [2003].
Nucleotide position is referenced to SARS-CoV, TOR2 (Genebank accession number: AY274119).
Amino acid position is referenced to the initiation codon (ATG) of the corresponding gene.
The earliest passage number when the mutation was first identified.
Polymorphism that were also observed in original specimens or viral cultures (http://www.ncbi.nlm.nih.gov/genomes/SARS/sarsnuc.html).
isolate and subsequent passages. To avoid possible bias generated from clone selection, we did not plaque purified the progeny viruses during the passage. Instead, we used supernatants from virus cultures for subsequent infection and for sequencing. Thus, our results represented the dominant viral species in these passages. Previous studies indicated that the overall mutation rate for SARS-CoV in cell culture was found to be low [Vega et al., 2004]. It was therefore expected that, the recurrent mutations identified in this study would not be a random event and might be highly relevant to host adaptation or to tissue tropism.

We observed a total of 15 different mutations in this study. Seven point mutations (residues 23412, 23473, 23518, 23632, 24079, 24864, and 24978) were observed in the S gene. The M gene was found to contain 2 point mutations (residues 26477 and 26600). There was 1 point mutation (residue 27995) and 1 deletion (residues 27808–27809) in the Orf 6–8 region. Four mutations were identified in the N gene (residues 28161, 28557, 29294, and 29366). By contrast, no mutation was observed in the E gene.

The majority of these mutations were non-synonymous mutations (12 out of 15), suggesting that replication in non-human primate cells imposes a high selection pressure on these viruses. This selection pressure was further highlighted by the fact that 7 out of these 12 non-synonymous mutations were recurrent mutations. All of these recurrent mutations were located at the S, M, and Orf 8a sequences. While a number of these recurrent mutations occurred in both Vero E6 and FRhK4 cell passaged viruses, one mutation (residue 24079) appeared unique to FRhK4 cell culture.

Three of these recurrent mutations (point mutations in residue 26477, and a dinucleotide-deletion in residues 27808–27809) were already apparent in the early passages, might be critical for adaptation of the virus in culture.

In the S gene, seven non-synonymous point mutations were found. All of these mutations were located at the ectodomain of the protein (Fig. 1). Interestingly, none of these mutations were within the receptor binding domain [Li et al., 2003; Xiao et al., 2003], suggesting there is no major selection pressure on this domain. As the strains we studied were all isolated from the middle phase of the SARS outbreak [Chinese SARS Molecular Epidemiology Consortium, 2004], it is likely that the receptor-binding site of these isolates was already adapted to primate receptors. The mutation at position 24978 has been previously found by direct sequencing of the virus in the original clinical specimens [Chinese SARS Molecular Epidemiology Consortium, 2004]. This result suggests that this particular position is not just a cell culture artifact but is highly relevant in adaptation to primates.

The recurrent mutations in the S gene (i.e., positions 23412, 23473, 23518, and 24079) were all located in between the receptor binding and heptad repeat 1 domains. Mutations at positions 23412, 23473, and 23518 were observed in viruses propagated in both Vero E6 and FRhK4 cells. By contrast, all viruses passaged in FRhK4 viruses had the same mutation at position 24079. None of the Vero E6 cell passaged viruses had acquired this mutation. As this mutation is located at the putative fusion peptide of the S protein [Bosch et al., 2004], our results suggested this cell type-specific mutation might facilitate fusion and entry of these viruses in FRhK4 cells. Nonetheless, further study is required to elucidate the biological significance of these mutations.

Two mutations (26477 G/T; 26600 C/T) observed in the M protein encoding sequence in early passages were also found in previously reported genetic sequences from clinical samples or from human isolates. The polymorphism at position 26477 could be observed from direct sequencing of viruses from original clinical samples [Chinese SARS Molecular Epidemiology Consortium, 2004]. The polymorphism at position 26600 was also found in some published viral sequences (http://www.ncbi.nlm.nih.gov/genomes/SARS/sarsnuc.html). However, there was insufficient information on cell culture passage history of these viruses to know whether this mutation is a natural sequence variation or a mutation selected by cell culture in vitro. Interestingly, based on the polymorphisms in these two positions, all of our clinical isolates and published viral sequences (http://www.ncbi.nlm.nih.gov/genomes/SARS/sarsnuc.html, N = 111) could be classified into GC, TT and TC genotypes. By contrast, no GT genotype was observed in all available sequences, suggesting the virus does not tolerant this genotype. In our study, both strains A and D in our study have a C to T mutation at position 26600. This mutation converted strain A from a TC to a TT genotype. For the strain D, an additional G to T

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**Fig. 1.** Schematic diagram of SARS-CoV S protein. The S protein can be cleaved and generate S1 and S2 domains as shown. Recurrent mutations were highlighted by open circles. Mutations found in an unique viral strain were highlighted by open circles. The locations of the signal peptide (SP, amino acids 1–13), receptor binding site (RBS, amino acids 303–537), fusion peptide (F, amino acids 858–866), heptad repeat 1 (HR1, amino acids 892–1014), heptad repeat 2 (HR2, amino acids 1153–1198), transmembrane domain (TM, amino acids 1186–1215), and cytoplasm domain (C, amino acids 1215–1255) are shown as indicated. The diagram is not drawn to scale.
mutation at position 26477 was observed, these double mutations thereby converting strain D from a GC to a TT phenotype. It should be noted that these mutations resulted in changing the amino acid residues in the first and second transmembrane helix domains [Marra et al., 2003]. It is therefore tempting to speculate that the amino acids at these positions are important for the topology of the protein and that the virus would introduce a complementary mutation in these positions to prevent the generation of a GT genotype. Further structure studies on this protein are required to prove this hypothesis.

None of the viruses we studied had mutation in the Orf of the E gene suggesting that culture in non-human primate cells does not impose a major selection pressure on the E gene. Alternatively, it is possible that mutations in such a small protein would not be well tolerated by the virus. This latter possibility is supported by a previous finding that the E proteins are highly conserved among different strains of mouse hepatitis viruses [Fischer et al., 1998].

Of the four mutations identified in the N protein encoding sequence, two were non-silent mutations. These two mutations occurred simultaneously in the same virus (strain B at passage 15). Whether these mutations are complementary to each other requires further investigation. Nonetheless, no recurrent mutation was found in this gene, indicating the gene is not subjected to a high selection pressure.

When compared to SARS-CoV found in animals, a 29-nucleotide (nt) deletion in the ORF 8b was found in most of human SARS-CoV sequences [Guan et al., 2003]. Subsequent investigations further revealed similar nucleotide deletions in most of human isolates [Chinese SARS Molecular Epidemiology Consortium, 2004; Vega et al., 2004]. Besides, Thiel et al. [2003] had reported the emergence of a 45-nucleotide deletion in the Orf 7b following passage in Vero E6 cells. These findings suggested that a deletion in these regions might be associated with host adaptations. We therefore sequenced the Orf 6–8 of all our viruses and identified a silent mutation in the Orf 8b (Table I). All of our original clinical isolates maintained the 29-nt deletion throughout the passages and we did not observe deletion in Orfs 6 and 7. However, a dinucleotide deletion at nt 27808–27809 in strains A and D was identified. This deletion leads to a frame shift mutation in Orf 8a. The same deletion was found in two published sequences (strains TWC and WHU). These published sequences were derived from viruses passed in Vero E6 cells [Yan et al., 2004; Yeh et al., 2004]. It should be noted that, apart from the above deletions, several other deletions in the Orf 8 sequence were detected from other isolates (http://www.ncbi.nlm.nih.gov/genomes/SARS/sarsnuc.html). Although the function of Orf 8a is not clear, these observations clearly suggested that Orf 8a and its protein were dispensable in cell cultures.

The identification of SARS-like coronavirus in Himalayan palm civet and other small mammals suggested human SARS-CoV might grow in cells from these mammals [Guan et al., 2003]. However, attempts in culturing human isolates in primary lung and kidney cells derived from Himalayan palm civets were not successful (data not shown), indicating human SARS-CoV are not adapted to these primary cells.

In summary, we report several mutations that might be relevant to the adaptation of SARS-CoV to cell cultures of non-human primates. Of all the sequenced regions, recurrent mutations were only identified in the S, M, and putative Orf 8a protein encoding sequences. Further studies on these regions might advance our understanding of pathogenesis, host adaptation, and tissue tropism. Besides, our results demonstrated that mutations can be cell type-specific and can occur rapidly during viral passage. Mutations at these sites in phylogenetic and epidemiological analyses may reflect adaptations in cell culture rather than true changes in the human host. Therefore mutations identified in virus isolates cultured in vitro should ideally be confirmed by the direct sequencing of original clinical specimens.

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