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Sequence Analysis of the Nucleocapsid Protein Gene of Rat Coronavirus SDAV-681¹SATOSHI KUNITA,² MIKI MORI, AND EIJI TERADA*Department of Laboratory Animal Science, School of Hygienic Sciences, Kitasato University, Kitasato, Sagami-hara 228, Japan**Received September 14, 1992; accepted October 28, 1992*

The nucleotide sequence of the 3'-end of the genomic RNA of sialodacryoadenitis virus strain 681 (SDAV-681) was determined. A large open reading frame encoding a 454-amino-acid protein was identified as the nucleocapsid protein (N) gene, since the predicted protein is similar in size, chemical properties, and amino acid sequence to the N proteins of other coronaviruses. The amino acid variance of the N proteins between SDAV and mouse hepatitis virus (MHV) is not markedly different from that among MHV strains. A high degree of genetic relatedness between SDAV and MHV was revealed in the intergenic and 3'-noncoding sequences as well as in the N gene. © 1993 Academic Press, Inc.

Sialodacryoadenitis virus (SDAV) is a member of the family Coronaviridae and is known to be an important pathogen which infects laboratory rats at high prevalence, causes clinically significant disease associated with rhinitis and sialodacryoadenitis, and interferes with research that uses rats. It shares antigenic relationships with other rodent coronaviruses, such as Parker's rat coronavirus (PRCV) and mouse hepatitis virus (MHV) (1). The genomic organization and the unique strategy of replication of MHV have been well characterized (2). The virion contains a single-stranded, positive-sense RNA genome of approximately 32 kilobases (kb). The genome is polyadenylated and organized into seven regions, each containing one or more open reading frames (ORFs). In infected cells, a genomic-sized mRNA and six subgenomic mRNA species are synthesized which form a nested-set with a common 3'-terminus but extending to different lengths in the 5'-direction. Only the 5'-proximal gene of each mRNA is utilized for translation. The genes encoding the structural viral proteins have been identified; the spike glycoprotein (S) gene is third from the 5'-end (gene 3), the membrane glycoprotein (M) gene is sixth from the 5'-end (gene 6), and the nucleocapsid protein (N) gene is the 3'-terminal gene (gene 7). Additionally, the hemagglutinin-esterase glycoprotein (HE) gene (gene 2-1) is translated by only some of the MHV strains (3). In contrast to MHV, the molecular biology of SDAV is not understood. To elucidate the structure and the molecular biological properties of SDAV, we have initiated sequence analysis of the SDAV ge-

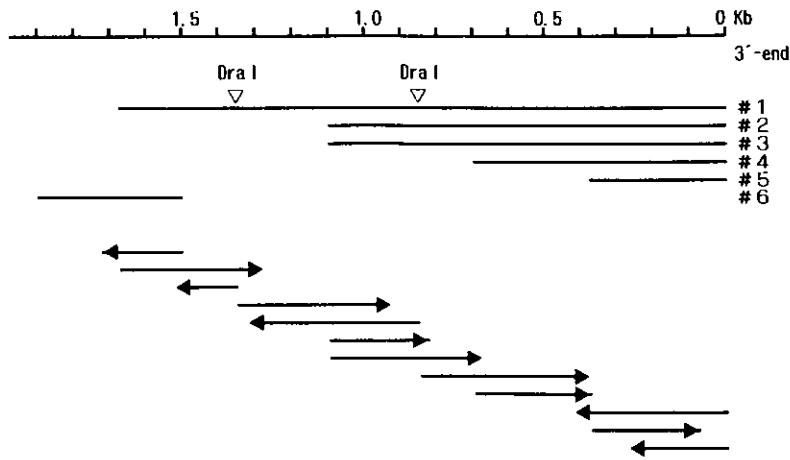
nome. We report here the complete nucleotide sequence of the N gene of SDAV-681 and its relatedness to other coronaviruses.

SDAV-681 (1) was propagated in a rat mammary tumor cell line (LBC) as described by Hirano *et al.* (4). Virus was purified by centrifugation on a discontinuous sucrose gradient consisting of 60 and 30% (w/w) sucrose in TNE buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) at 110,000 g for 2 hr at 4°. The virus recovered from the interface between 60 and 30% sucrose was incubated in TNE buffer containing 0.5 mg/ml proteinase K and 0.5% SDS for 10 min at 56°. The genomic RNA was extracted with phenol-chloroform-isoamyl alcohol and precipitated with ethanol. Double-stranded cDNA was synthesized from SDAV genomic RNA primed with oligo(dT), using cDNA synthesis system plus (Amersham). After addition of *Bam*HI adaptor (Pharmacia) to blunt-ended cDNA, the cDNA was purified on a cDNA spun column (Pharmacia) and ligated into the *Bam*HI-site of plasmid vector pUC19 (Takara). The ligated molecules were used to transform *Escherichia coli* JM109 and cells containing recombinant plasmid were selected on LB agar plates that contained 50 µg ampicillin/ml, 1 mM IPTG, and 0.01% X-gal. Recombinant colonies were transferred to nitrocellulose filters and probed with ³²P-labeled MHV-N gene cDNA (1362 nucleotides) prepared from MHV-A59 genomic RNA by polymerase chain reaction (PCR) described previously (5). Colonies yielding strong signals were analyzed for insert size. Five clones containing 0.4- to 1.7-kb inserts (#1-#5 in Fig. 1A) were sequenced in both directions by the dideoxy method using modified T7 DNA polymerase (Sequenase, U.S. Biochemical). An additional cDNA clone (#6 in Fig. 1A) was obtained using a synthetic oligonu-

¹ Sequence data from this article have been deposited with the DDBJ, EMBL, and GenBank Libraries under Accession No. D10760.

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A



B

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5' -TTGTTGAGAATCTAACTAAACTTTAAGGATGTCCTTTTGTCCCGGACAA 50
GAAAACGCCGGTAGCAGAAGCTCCTCTGGAAACCGCGCTGGTAATGGAATCCTCAAGAAG 110
ACCACTTGGGCTGACCAAACCGAGCGCGGACAAAATAATGGAAATAGAGGCAGAAGGAAT 170
CAGCCCAAGCAGACTGCAACTACTCAGCCCAATACCGGAGTGTGGTCCCCATTACTCT 230
TGGTTTTCGGGCATTACCCAATTCAGAAGGGAAAAGAGTCCAGTTTGAGGTGGACAA 290
GGAGTGCCATTATGCCAATGGAATCCACCTTCTGAGCAAAAGGGATATTGGTATAGACAC 350
AACCGTCGTTCTTTTAAACACCTGATGGGCAGCAGAAGCAACTACTCCCAGATGGTAT 410
TTTTACTATCTTGGGACGGGCCCCATGCTGGAGCCAGTTTCGGAGACAGCATTGAGGGA 470
GTCCTCTGGGTTGCAAAATAGTCAGGCGGATACCAACACCTCTGCTGACATTGTTGAAAG 530
GACCAAGTAGCCATGAGGCTATTCTACTAGGTTTGCGCCCGGTACGGTATTGCCTCAG 590
GGTTTCTATGTTGAAGGCTCGGGAAGGTCTGCACCTGCTAGTCGATCTGGTTCCGGTCA 650
CAATCCCGTGGGCCAAATAATCGCGCTAGAAGCAGTTCCAACCAGCGCCAGCCTGCCTCT 710
ACTGTAAAACCTGATATGGCCGAAGAAATTGCTGCTCTTCTTTGGCTAAGCTAGGCAA 770
GATGCCGGACAGCCTAAGCAAGTAACTAAGCAAAGTGCCAAAGAAGTCAGGCAGAAAATT 830
TTAAATAAGCCTCGCCAAAAGAGGACTCCAAACAAGCAGTGCCAGTCAGCAGTGTFTT 890
GGAAGAGAGAGCCCCAATCAGAATTTTGGAGGCCCTGAAATGTTAAACTTGGAACTAGT 950
GATCCACAGTCCCCATTCTTGCAGAGTTGGCCCCAACACCTGGTGCCTTCTTCTTTGGA 1010
TCTAAATTAGAATTGGTCAAAAAGAAATTCGGTGGCGTTGATGAACCCACCAAAGATGTG 1070
TATGAGCTGCAATATTCAGGTGCAGTCAGATTTGATAGTACTCTACCTGGTTTTGAGACT 1130
ATCATGAAAGTGTGAATGAGAATTTGAACGCCTACCAGAATCAAGCTGGTGGTGCAGAT 1190
GTAGTGAGCCCAAAGCCCCAAAGAAAGAGAGGGACGAAACAACGGCTCAGAAAGAAGAA 1250
TTAGATAGTATAAGCGTTGCAAAGCCCCAAAAGTGCCGTGCAGCGAAATGTAAGCAGAGAA 1310
TTAACCCAGAGGATAGAAGCCTGTTGGCGCAGATCCTAGATGATGGCGTTGTGCCTGAT 1370
GGGTTAGATGACTCTAATGTGTAAAGAGAATGAATCCTATGTGCGCGCTCGGTGGTAACC 1430
CCTCGCGAGAAAGTCGGGATAGGACACTCTCTATCAGAATGGATGTCTTGCTGTCATAAC 1490
AGATAGAGAAGTGTGGCAGACCCTGTATCAATTAGTTGAAAGAGATTGCAAAATAGAG 1550
AATGTGTGAGAGAGTGAAGGTCTACGTCTAACCATAAGAACGGCGATAGGCGCCCCCT 1610
GGGAAGAGCTCACATCAGGGTACTATTCTGCAATGCCCTAGTAAATGAATGAAGTTGAT 1670
CATGGCCAATTGGAAGAATCAC - POLY (A) - 3' 1692

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Fig. 1. Sequencing of the 3'-end of the SDAV-681 genome. (A) cDNA clones and sequencing strategy. Clone #1 was sequenced completely from subcloned fragments generated by restriction endonuclease *Dra*I. Clones #2-#6 were sequenced partly their 5'- or 3'-termini. (B) The primary nucleotide sequence of the 3'-end (1692 nucleotides) of the SDAV-681 genome. The initiation and termination codons for the N open reading frame are underlined and those for the internal open reading frame are shown by the wavy lines. The intergenic consensus sequence (TCTAAAC) and the 3'-conserved sequence (GGAAGAGC) are shown by the double lines. The region indicated by the dotted line was used for preparing a complementary oligonucleotide primer to obtain clone #6.

SDAV-681	MSFVPGQENA	GSRSSSGNRA	GNGILKKTW	ADQTERGQNN	GNRGRRNQPK	QTATTQPNTG	SVVPHYSWFS	70	
MHV-A59		G V		P Q		S		70	
MHV-JHM				L Q	K	S		70	
MHV-2				---	H	A		86	
MHV-S		S S		AG		S		90	
MHV-Nu67				PT Q		S		90	
SDAV-681	GITQFQKGKE	FQFAGGQGVF	IANGIPPSEQ	KGYWYRHNRR	SFKTPDGGQR	QLLPRWYFYF	LGTPHAGA	S 140	
MHV-A59		E	A					140	
MHV-JHM		Q	A Q			Y		140	
MHV-2		Q	A	S				136	
MHV-S		VQ	A		H			140	
MHV-Nu67		D	A					140	
SDAV-681	FGDSIEGVFW	VANSQADTNT	SA	DIVERDPS	SHEAIPTRFA	PGTVLPQGFY	VEGSGRSAPA	SRGSGRSQSR	210
MHV-A59	Y		RS						210
MHV-JHM	Y D V	SQ E R					P		210
MHV-2	Y D V	SQ K T	V						206
MHV-S	Y D V	SQ K T							210
MHV-Nu67	Y		RS						210
SDAV-681	GPNNRARSSS	NQRQPASTVK	PDMAEEIAL	VLAKLGKDG	QPKQVTKQSA	KEVRQKILNK	PRQKRTPNKQ	280	
MHV-A59								280	
MHV-JHM								280	
MHV-2		A				T		276	
MHV-S								280	
MHV-Nu67								280	
SDAV-681	CPVQQCFGKR	GPNQNFQGGPE	MLKLGTSDPQ	FPILAEALPT	PGAFFFGSKL	ELVKKNSGGV	DEPTKDVYEL	350	
MHV-A59		S			V	A		350	
MHV-JHM					A	A G		350	
MHV-2		S			S	A		346	
MHV-S		S			A	A	H	350	
MHV-Nu67		S						350	
SDAV-681	QYSGAVRFDS	TLPGFETIMK	VLNENLNAYQ	NQAGGADVVS	PKPQRKRGTK	QTAQKE	ELDS	ISVAKPKSAV	420
MHV-A59				K-D	GRRQ	AQEK D	V N V	S	419
MHV-JHM				D		K D	V N V	S	420
MHV-2	I			D SV L	P RRRQ	AQEK D	V N V	L	416
MHV-S				K-D	GRRQ	AQEK D	V N V	S	419
MHV-Nu67	I			T V	GRRQ	AQEK D	V N V	S	420
SDAV-681	QRNVSRELTP	EDRSLLAQIL	DDGVVPDGL-	DDSNV				454	
MHV-A59			E					454	
MHV-JHM			E					455	
MHV-2			E					451	
MHV-S			E					454	
MHV-Nu67	D		L	E				455	

Fig. 2. Amino acid sequence comparison of the N proteins of SDAV-681 and five MHV strains. The deduced sequence of the SDAV-681 N protein is from this work. The sequences for MHV-2 and -Nu67 were obtained from recent work (5). The MHV-A59 and -S sequences are taken from Parker and Masters (6) and the MHV-JHM sequence is taken from Skinner and Siddell (7). Spaces indicate amino acids identical to the SDAV-681 sequence. Hyphens indicate gaps introduced to maximize alignment. The spacer regions A and B (amino acid positions 140-162 and 381-406 in the SDAV-681 sequence, respectively) proposed by Parker and Masters (6) are bracketed.

cleotide primer complementary to nucleotides 183-202 (Fig. 1B) for cDNA synthesis and then sequenced in the same manner.

The nucleotide sequence of the 3'-end of SDAV-681 genome covering the complete ORF of 1362 nucleotides is presented in Fig. 1B. This ORF extends from nucleotides 30 to 1391 and potentially encodes for a protein of 454 amino acids. The predicted protein is highly homologous to the N proteins of MHV strains (MHV-A59, -JHM, -2, -S, and -Nu67) aligned in Fig. 2. Nucleotide and amino acid sequences of SDAV-681 and MHV strains are 89.0 to 92.7% and 90.1 to 93.8% identical, respectively. Incidentally, the amino acid sequence identity among the N proteins of MHV strains is 91.8 to 97.1%. Coronaviruses can be divided into several antigenic groups. SDAV belongs to a group including MHV, bovine coronavirus (BCV), human coronavirus strain OC43 (HCV-OC43), and hemagglutinating encephalomyelitis virus of swine (HEV). The N protein of MHV shows an amino acid sequence homology of approximately 70% with BCV (8) and HCV-OC43 (9). The high degree of sequence identity between SDAV

and MHV shown in this study confirmed the close relationship of these two viruses as revealed by serological analysis (1, 10). The predicted N protein of SDAV possesses some features which are characteristic of the N proteins of coronaviruses. That is to say, the protein is highly basic, suggesting RNA-binding property of this protein (11), and is rich in serine residues, which are presumed to be sites of phosphorylation (12). Sixty of the amino acid residues (13%) are basic and forty-two residues (9%) are acidic, giving the protein a net charge of +18 at neutral pH. There are thirty-nine serine residues (9%) located at the almost same positions as MHV.

SDAV-681 has an internal ORF in the +1 reading frame within the N gene (Fig. 1B). This ORF potentially encodes a protein of 207 amino acids, which is basic (a net +7 charge at neutral pH) and rich in hydrophobic amino acids (50%) especially leucine residues (18%). An internal ORF of identical size has been found within the N genes of MHV and BCV (6, 8). Recently, it was demonstrated for BCV that a 23-kDa protein encoded by the internal ORF lying within the N gene was ex-

pressed in virus-infected cells (13). Further studies are required to reveal the functional significance of this protein and whether similar proteins can be detected for other coronaviruses.

Noncoding regions flanking the N gene on SDAV genome were also compared with those of MHV. The 5'-noncoding sequence of 17 nucleotides (at nucleotide positions 13–29 in Fig. 1B) immediately before the initiation codon, including a stretch of consensus sequence (TCTAAAC), is identical to the intergenic sequence between the M and N genes of MHV (14, 15). This stretch of consensus sequence is conserved among most coronaviruses, and among the 5'-end of genome, the intergenic regions, and the mRNA leader sequences of the same coronavirus. Thus it is the proposed leader RNA binding site for the initiation of mRNA transcription (2, 14, 15). The SDAV N gene is flanked on its 3'-side by a noncoding region of 301 nucleotides (at nucleotide positions 1392–1692 in Fig. 1B) and a poly(A) tail. The 3'-noncoding sequence of SDAV is highly homologous (more than 97% sequence identity) to MHV (6, 7) and contains the conserved sequence, GGAAGAGC, 81 nucleotides from the 3'-end, which has been proposed as a recognition signal for negative-stranded RNA synthesis (2).

It has been reported that high frequency RNA recombination occurs among MHV strains during coinfection both *in vitro* (16) and *in vivo* (17). This recombination event is thought to be caused by reattachment of pausing RNA intermediates during RNA synthesis at sites of high homology (2). The conservation of nucleotide sequences between SDAV and MHV in the intergenic and 3'-noncoding regions as well as in the N gene suggests that these two viruses are at least structurally able to undergo RNA recombination with each other. Under experimental conditions, mice are susceptible to intranasal and contact infection with SDAV (18, 19) and rats are susceptible to MHV by intranasal inoculation (20), but the cell tropisms of two viruses are considerably different. Therefore, RNA recombination between SDAV and MHV is unlikely to occur frequently, it may be possible, in natural infections. Parker and Masters (6) suggested that the N protein of MHV consists of three domains separated by two spacer regions A and B, which have less constraint on their amino acid sequences, and these spacers tend to vary among a limited set of two or three alternatives. Interestingly, SDAV-681 has an A59-like spacer A and a JHM-like spacer B (Fig. 2), implying that the N gene of SDAV-681 may have been derived from two prototype

N genes of MHVs. It is possible that SDAV may be a rat-adapted virus arisen by recombination between two ancestral MHVs possessing different N genes or that recombination among an ancestral rat coronavirus and two ancestral MHVs may have given rise to SDAV-681.

The results of the present study suggest a high degree of genetic relatedness between SDAV and MHV which is about the same as that among MHV strains. Comparison of the remaining genes with SDAV and MHV and detailed analysis of SDAV structural proteins will provide a clue to understand their evolution and the molecular basis of host cell tropism.

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