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Epitope mapping and functional analysis of sigma A and sigma NS proteins of avian reovirus

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Abstract

We have previously shown that avian reovirus (ARV) σA and σNS proteins possess dsRNA and ssRNA binding activity and suggested that there are two epitopes on σA (I and II) and three epitopes (A, B, and C) on σNS. To further define the location of epitopes on σA and σNS proteins and to further elucidate the biological functions of these epitopes by using monoclonal antibodies (MAbs) 62, 1F9, H1E1, and 4A123 against the ARV S1133 strain, the full-length and deletion fragments of S2 and S4 genes of ARV generated by polymerase chain reaction (PCR) were cloned into pET32 expression vectors and the fusion proteins were overexpressed in *Escherichia coli* BL21 strain. Epitope mapping using MAbs and *E. coli*-expressed deletion fragments of σA and σNS of the ARV S1133 strain, synthetic peptides, and the cross reactivity of MAbs to heterologous ARV strains demonstrated that epitope II on σA was located at amino acid residues ^{340}QWVMAGLVSAA^{350} and epitope B on σNS at amino acid residues ^{180}MLDMVDGRP^{188}. The MAbs (62, 1F9, and H1E1) directed against epitopes II and B did not require the native conformation of σA and σNS, suggesting that their binding activities were conformation-independent. On the other hand, MAb 4A123 only reacted with complete σNS but not with truncated σNS fusion proteins in Western blot, suggesting that the binding activity of MAb to epitope A on σNS was conformation-dependent. Amino acid sequence analysis and the binding assays of MAb 62 to heterologous ARV strains suggested that epitope II on σA was highly conserved among ARV strains and that this epitope is suitable as a serological marker for the detection of ARV antibodies following natural infection in chickens. On the contrary, an amino acid substitution at position 183 (M to V) in epitope B of ARV could hinder the reactivity of the σNS with MAb 1F9. The σNS of ARV with ssRNA-binding activity could be blocked by monoclonal antibody 1F9. The epitope B on σNS is required for ssRNA binding because its deletion fully abolished the ssRNA binding activity of σNS.

Keywords: Avian reovirus; σA and σNS proteins; Epitope; Gel-shift assay; Monoclonal antibody; Synthetic peptide; Conformation-dependent; ssRNA binding activity

Introduction

Avian reovirus (ARV) and mammalian reovirus (MRV) belong to the genus *Orthoreovirus*. Both share physical–chemical and morphological characteristics, including segmented genomes consisting of 10 genome segments of double-stranded (ds) RNA. The RNA is packaged into a non-enveloped icosahedral double capsid (Spandidos and Graham, 1976). The genomic segments can be separated by polyacrylamide gel electrophoresis (PAGE) into three size classes, large (L), medium (M), and small (S). However, ARV differs from its mammalian counterpart in its lack of hemagglutination activity (Glass et al., 1973), ability to induce cell fusion (Bodelon et al., 2001, 2002; Shmulevitz and Duncan, 2000), and association with naturally occurring pathological conditions (Robertson and Wilcox, 1986). ARV is an important cause of diseases in poultry. In particular, reovirus-induced arthritis, chronic respiratory diseases, and

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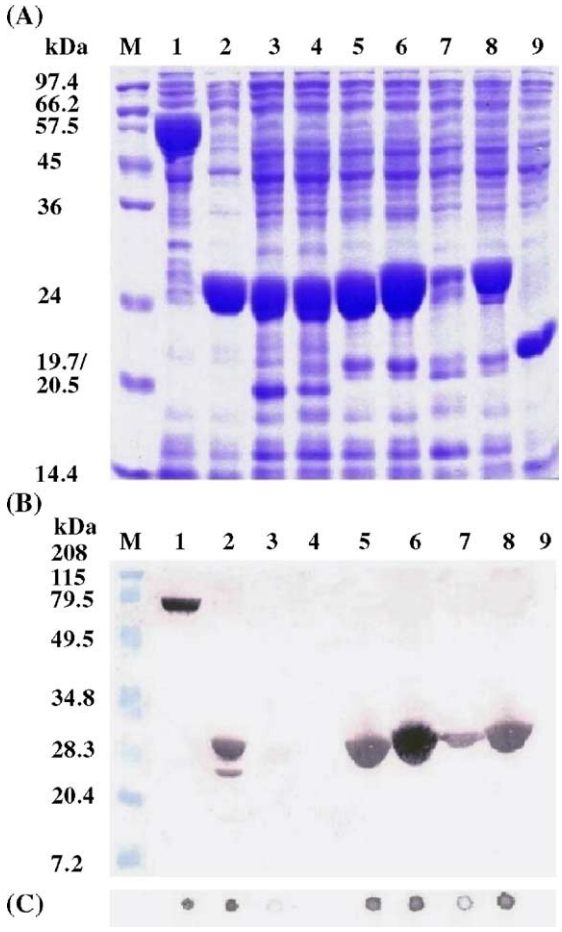

Fig. 1. Mapping of epitope II on σA recognized by monoclonal antibody 62. σA and its deletion fragments were expressed in *E. coli* BL21(DE3). The fusion proteins were stained with Coomassie blue after SDS–PAGE (A) and identified by Western blotting (B) and dot blot analysis (C) using MAb 62. Molecular weight markers (Bio-Rad) are shown in lane M. Lane 1, S2-all; lane 2, S2D13; lane 3, S2D14; lane 4, S2D15; lane 5, S2D16; lane 6, S2D17; lane 7, S2D18; lane 8, S2D19; lane 9, pET32a only. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

malabsorption syndrome (Fahey and Crawley, 1954) provoke considerable economic losses.

ARV-encoded proteins, including at least 10 structural proteins (λA, λB, λC, μA, μB, μBC, μBN, σC, σA, and σB), and 4 nonstructural proteins (μNS, P10, P17, and σNS) have been demonstrated (Bodelon et al., 2001; Varela and Benavente, 1994). Protein σC, encoded by the S1 gene (Shapouri et al., 1995), shows noticeably higher divergence than other σ-class proteins (Liu et al., 2003) and is a cell attachment protein (Martinez-Costas et al., 1997) and apoptosis inducer (Shih et al., 2004). Protein σC is also the target for type-specific neutralizing antibodies while antibodies against σB are group-specific (Wickramasinghe et al., 1993). Recently, ARV σA encoded by the S2 gene (Yin et al., 2000) has been identified as a double-stranded RNA (dsRNA) binding protein (Martinez-Costas et al., 2000; Yin et al., 2000) and possible involvement in resistance to interferon (Martinez-Costas et al., 2000). Another protein of ARV, σNS, encoded by the S4 gene (Chiu and Lee, 1997), has been reported for its single-stranded RNA (ssRNA) binding activity (Yin and Lee, 1998). We have previously shown that two epitopes on σA (I and II) and three epitopes (A, B, and C) on σNS (Hou et al., 2001; Pai et al., 2003). As part of our work to characterize epitopes on σA and σNS proteins of ARV, we further extended our past studies (Hou et al., 2001; Pai et al., 2003). In this communication, we first defined the locations of two epitopes on σA and σNS proteins and analyzed their biological function using monoclonal antibodies, *Escherichia coli*-expressed deletion fragments of σA and σNS, synthetic peptides, the binding assay of MAbs to heterologous ARV strains, and gel shift assay.

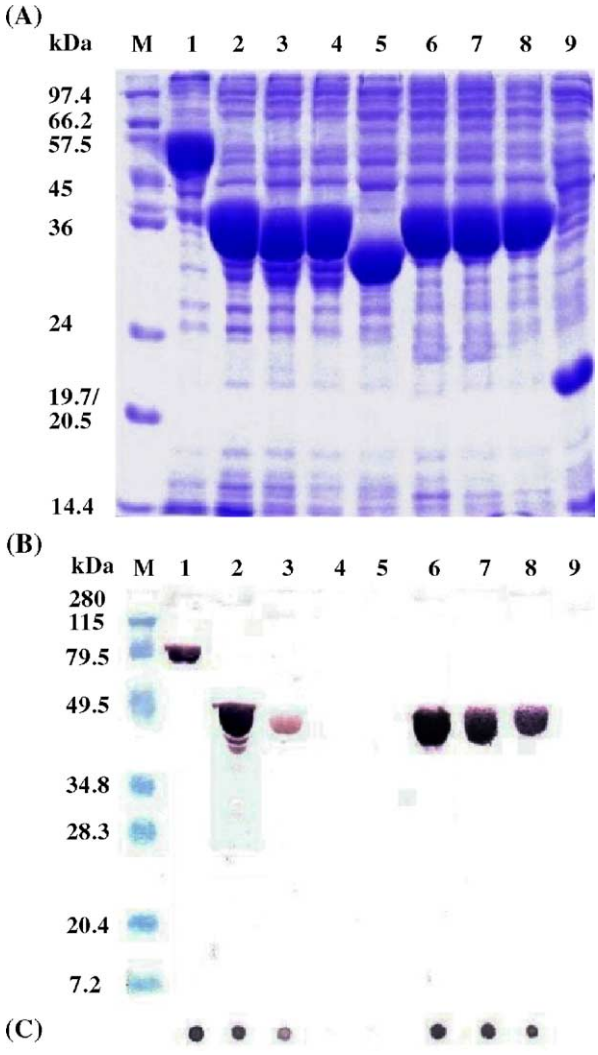

Fig. 2. Mapping of epitope B on σNS recognized by monoclonal antibody 1F9. σNS and its deletion fragments were expressed in *E. coli* BL21(DE3). The fusion proteins were stained with Coomassie blue after SDS–PAGE (A) and detected by Western blotting (B) and dot blot analysis (C) using MAb 1F9. Molecular weight markers (Bio-Rad) are shown in lane M. Lane 1, S4-all; lane 2, S4D1; lane 3, S4D13; lane 4, S4D14; lane 5, S4D20; lane 6, S4D16; lane 7, S4D17; lane 8, S2D18; lane 9, pET32a only. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Results and discussion

Expression and identification of σA and σNS proteins

The full-length σA- and σNS-encoding cDNAs of the expression plasmid constructs were successfully overexpressed in *E. coli*. Each plasmid DNA was introduced into *E. coli* strain BL21 (DE3), and the protein extracts of the induced recombinant cells were analyzed by 10% SDS–PAGE. Protein bands were subsequently visualized by Coomassie brilliant blue staining. Analysis of transformed and expression-induced bacteria by SDS–PAGE revealed the expressed σA and σNS fusion proteins with molecular weight of 64.75 kDa (Fig. 1A) and 60.24 kDa (Fig. 2A), respectively, which were consistent with the expected size of the fusion proteins. The purified fusion protein and protein extracts of the induced recombinant cells were then analyzed by Western blotting and dot blot assays with MAbs.

Monoclonal antibodies are useful for analyzing antigenic properties of viruses. The recognition of expressed σA and σNS proteins by MAbs 62, 1F9, H1E1, and 4A123, respectively, was analyzed by Western blotting and dot blot assays. Three MAbs 62, 1F9, and H1E1 were reactive in Western blotting and dot blot assays (Figs. 1B–C and 2B–C) when MAb 4A123 showed reactivity to σNS only in dot blot assays (Table 2B). These results indicated that epitopes, which were recognized by MAbs (62, 1F9, and H1E1) following denaturation by, for instance, SDS–PAGE and

Table 1
Primers used for the construction of the full-length and deletion fragments of σA- and σNS-encoding genes

A. Primers used for σ A-encoding gene of ARV

Construct	Sequence (5'-3')[ab]		Primer location	Expected size (bp)
S2-all	S2A	5'-TTCCACGATGGCGCGTGCCATATAC-3'	9-33	
	S2B	5'-AGCGTACGACCCTA CGCCTA GGC-3'	1283-1261	1275
S2D1	S2D1-A	5'-GTGAATTCTTTCATACATTGG-3'	666-686	
	S2D1-B	5'-AATAAG CTTACTTACGACCCT ACGCCT AG-3'	1292-1263	627
S2D2	S2D2-A	5'-CGCGGATCCACGATGGC GCGTGC CATATAC G-3'	4-34	
	S2D2-B	5'-CCAAAG CTTTCTATGAAA GAAGC CACAAAC-3'	691-662	688
S2D3	S2D3-A	5'-AAGAATT CGACAGTTGGACTTTATTCTA-3'	746-773	
	S2D1-B	5'-AATAAG CTTACTTACGACCCT ACGCCT AG-3'	1292-1263	547
S2D4	S2D4-A	5'-TGGAA TTCCGTCAACCG GCCG GTGTTCGC-3'	896-923	
	S2D1-B	5'-AATAAG CTTACTTACGACCCT ACGCCT AG-3'	1292-1263	397
S2D5	S2D5-A	5'-CCGAATTCGAATGTATTCGGCAGTGGG-3'	1091-1033	
	S2D1-B	5'-AATAAG CTTACTTACGACCCT ACGCCT AG-3'	1292-1263	202
S2D6	S2D6-A	5'-CAGAATT CATCCAGGCGGATC TAGGAC-3'	1103-1129	
	S2D1-B	5'-AATAAG CTTACTTACGACCCT ACGCCT AG-3'	1292-1263	190
S2D7	S2D2-A	5'-CGCGGATCCACGATGGC GCGTGC CATATAC G-3'	4-34	
	S2D7-B	5'-AAGAAGCTTTTAGTATTTAGATTGCAGCTCAC-3'	453-422	450
S2D8	S2D2-A	5'-CGCGGATCCACGATGGCGCGTGC CATATAC G-3'	4-34	
	S2D8-B	5'-GAGAAGCTTCTACCACTGAGC AGAGGC TGGC TG-3'	393-361	390
S2D9	S2D2-A	5'-CGCGGATCCACGATGGC GCGTGC CATATAC G-3'	4-34	
	S2D9-B	5'-CGAAA GCTTCTAGGGTGC TTCAGGC GTTGAAAC -3'	201-169	198
S2D10	S2D10-A	5'-CAGAATTCAAGGGG CAAGC CTTAAC GC-3'	1058-1084	
	S2D1-B	5'-AATAAG CTTACTTACGACCCT ACGCCT AG-3'	1292-1263	235
S2D11	S2D4-A	5'-TGGAA TTCCGTCAACCGGCCGGTGTTCGC-3'	896-923	
	S2D11-B	5'-GGCAAGC TTTTACGCTGCTGACACCAAACCGG-3'	1077-1046	182
S2D12	S2D4-A	5'-TGGAA TTCCGTCAACCGGCCGGTGTTCGC-3'	896-923	
	S2D12-B	5'-CGCAAGCTTTTACAAACCGGCCA TCACCCA CTG-3'	1056-1033	161
S2D13	S2D13-A	5'-GGGAATTCGTGATGGCCGGTTTGG-3'	1031-1054	
	S2D1-B	5'-AATAAG CTTACTTACGACCCT ACGCCT AG-3'	1292-1263	262
S2D14	2D14-A	5'-TGGAATTC TTGGTGTC AGC AGCGAAGG-3'	1043-1069	
	S2D1-B	5'-AATAAG CTTACTTACGACCCT ACGCCT AG-3'	1292-1263	250
S2D15	S2D15-A	5'-TGGAATTCGCAGCGAAGGGGCAAGC-3'	1052-1076	
	S2D1-B	5'-AATAAG CTTACTTACGACCCT ACGCCT AG-3'	1292-1263	241
S2D16	S2D4-A	5'-TGGAA TTCCGTCAACCGGCCGGTGTTCGC-3'	896-923	
	S2D16-B	5'-GTTAAGC TTTTAATTAGCCTCCTGCGTTA-3'	1107-1079	212
S2D17	S2D4-A	5'-TGGAA TTCCGTCAACCGGCCGGTGTTCGC-3'	896-923	
	S2D17-B	5'-ATCAAGCTTTTAGAGGTTTGAGAAGTCAT-3'	1122-1096	227
S2D18	S2D4-A	5'-TGGAA TTCCGTCAACCGGCCGGTGTTCGC-3'	896-923	
	S2D18-B	5'-TTGAAGC TTTTACGCCTGGATGAGGTTTG-3'	1131-1103	236
S2D19	S2D4-A	5'-TGGAA TTCCGTCAACCG GCCG GTGTTCGC-3'	896-923	
	S2D19-B	5'-CGCAAGCTTTTATCCTAGATC CGCCTGGA-3'	1140-1112	245

Table 1 (*continued*)

B. Primers used for σ NS-encoding gene of ARV

Construct	Sequences (5'-3')[ab]		Primer location	Expected size (bp)
S4-all	S4-1	5'-TTGAATTCTTGTGCAGCCATGGAC-3'	-1-22	
	S4-2	5'-GCGAATTCTCACCCGCACCATGGG-3'	1150-1127	1152
S4D1	S4D1-A	5'-TTGAATTCCCTTACATGCTTGAC-3'	540-562	
	S4D1-B	5'-ACCAAGCTTCCCGTCTACGCC-3'	1134-1114	625
S4D2	S4-1	5'-TTGAATTCTTGTGCAGCCATGGAC-3'	-1-22	
	S4D2-B	5'-GTCAAGCTTTTAAGGGAGCACAAACTTATTC-3'	562-532	564
S4D3	S4D3-A	5'-CTGAATTCGATTGATGCTTCATTTGG-3'	634-660	
	S4D1-B	5'-ACCAAGCTTCCCGTCTACGCC-3'	1134-1114	501
S4D4	S4D4-A	5'-CCGAATTCAAGGTTGAGTTGGACGCGC-3'	792-818	
	S4D1-B	5'-ACCAAGCTTCCCGTCTACGCC-3'	1134-1114	343
S4D5	S4D5-A	5'-GTGAATTCTGATCGTGAACTGATGGAAC-3'	902-929	
	S4D1-B	5'-ACCAAGCTTCCCGTCTACGCC-3'	1134-1114	233
S4D6	S4D6-A	5'-CGGAATTCCGTCGTTCCGGTCCTACTCG-3'	993-1020	
	S4D1-B	5'-ACCAAGCTTCCCGTCTACGCC-3'	314-1114	142
S4D7	S4-1	5'-TTGAATTCTTGTGCAGCCATGGAC-3'	1-22	
	S4D7-B	5'-TGCAAGCTTTTATAGATCCGAGGAAAATGGCAG-3'	292-260	294
S4D8	S4-1	5'-TTGAATTCTTGTGCAGCCATGGAC-3'	-1-22	
	S4D8-B	5'-AAGAAGCTTTTAGCATCGCACAGCACGTGAAAG-3'	196-164	298
S4D9	S4-1	5'-TTGAATTCTTGTGCAGCCATGGAC-3'	-1-22	
	S4D9-B	5'-TAGAAGCTTTTAGTTTCTAAAGAGTGTCTGAC-3'	100-69	102
S4D10	S4D10-A	5'-CTGAATTCGTCCTGCCGTCTCATACCGTAG-3'	578-608	
	S4D1-B	5'-ACCAAGCTTCCCGTCTACGCC-3'	1134-1114	557
S4D11	S4D11- A	5'-AAGAATTCACCAACACCAGCTTGC-3'	612-635	
	S4D1-B	5'-ACCAAGCTTCCCGTCTACGCC-3'	1134-1114	523
S4D12	S4D12-A	5'-CGGAATTCATCAACTACGCACACCATG-3'	273-299	
	S4D12-B	5'-CAAAAGCTTTTACAATTGGGCCGCCATC-3'	811-784	539
S4D13	S4D13-A	5'-ACGAATTCGACATGGTAGATGGTC-3'	552-575	
	S4D1-B	5'-ACCAAGCTTCCCGTCTACGCC-3'	1134-1114	583
S4D14	S4D14- A	5'-TGGAATTCGGTCGTCCTCAGATTG-3'	564-587	
	S4D1-B	5'-ACCAAGCTTCCCGTCTACGCC-3'	1134-1114	571
S4D15	S4D15-A	5'-GTGAATTCCAGATTGTCCTGCCGTC-3'	573-597	
	S4D1-B	5'-ACCAAGCTTCCCGTCTACGCC-3'	1134-1114	562
S4D16	S4-1	5'-TTGAATTCTTGTGCAGCCATGGAC-3'	-1-22	
	S4D16-B	5'-GGTAAGCTTTTATTCTACGGTATGAGACG-3'	622-594	624
S4D17	S4-1	5'-TTGAATTCTTGTGCAGCCATGGAC-3'	-1-22	
	S4D17- B	5'-GCTAAGCTTTTACAACATTTCTTCTACGG-3'	631-603	633
S4D18	S4-1	5'-TTGAATTCTTGTGCAGCCATGGAC-3'	-1-22	
	S4D18-B	5'-CGAAAGCTTTTAGCTGGTGTTGGTCAACA-3'	643-615	645
S4D19	S4-1	5'-TTGAATTCTTGTGCAGCCATGGAC-3'	-1-22	
	S4D19-B	5'-ACCAAGCTTTTAATCAATCGAGTTCAGC-3'	661-634	663
S4D20	S4D20- A	5'-GATCGAATTCATGGACAACACCG-3'	7-29	
	S4D20-B	5'-CGTCAAGCTTATCAGACGGCAGGACAAT-3'	661-584	605

[a] Underline indicates restriction sites (*Eco*RI and *Hin*dIII) introduced in each primer.
[b] Box indicates stop codons.

Western blotting, were conformation-independent. The reactivity of MAb 4A123 recognizing the epitope A on σNS was fully abolished by denaturation, suggesting that MAb 4A123 recognized a conformation-dependent epitope of which at least one disulfide bond is essential to maintain the three-dimensional integrity of the epitope. To map this epitope, specific procedures to increase binding of MAbs to conformation-dependent epitopes following Western blotting are required (Dunn, 1986). In addition, we had tested other MAbs from each of our previously-established competitive groups, with the exception of MAb H1E1 which was the only monoclonal antibody against epitope C on the σNS of ARV (Hou et al., 2001). The results were in agreement with our previous investigations.

Epitope mapping of anti-σA and -σNS monoclonal antibodies by reaction with deletion mutants of σA and σNS

To localize the epitopes II and B on σA and σNS proteins, respectively, a series of amino- and carboxy-terminal-deleted fragments of the σA- and σNS-encoding genes of S1133 strain were synthesized by PCR and subcloned into pET expression vectors to construct a series of expression constructs (Tables 1A–1B). Each plasmid was introduced into *E. coli* strain BL21 (DE3), and the protein extracts of the induced recombinant cells were analyzed by 10% SDS–PAGE. The series of truncated σA- and σNS-encoding genes were efficiently expressed in *E. coli* strain BL21 (DE3) and could be visualized by

Table 2A
The antibody reactivity of the complete and truncated σA proteins with MAb 62 in Western blotting and dot blot assays

Constructs	Monoclonal antibody	62	
	Amino acid residue positions	DB*	WB*
S2-all	1–417	+	+
S2D9	1–63	−	−
S2D8	1–127	−	−
S2D7	1–147	−	−
S2D2	1–226	−	−
S2D1	221–417	+	+
S2D3	247–417	+	+
S2D4	297–417	+	+
S2D5	336–417	+	+
S2D13	343–417	+/−	+/−
S2D14	346–417	−	−
S2D15	349–417	−	−
S2D10	352–417	−	−
S2D6	366–417	−	−
S2D12	297–345	−	−
S2D11	297–349	−	−
S2D16	297–360	+	+
S2D17	297–365	+	+
S2D18	297–368	+	+
S2D19	297–371	+	+

+, strong reactions; −, no reactions.
* WB, Western blotting; DB, dot blotting.

Coomassie brilliant blue staining. When induced bacteria expressing S2D1, S2D2, S2D3, S4D2, S4D16, S4D17, S4D18, and S4D19 were disrupted and the resulting extract was centrifuged, the expressed proteins could not be detected in the supernatant fraction, but it appears in the pellet fraction, suggesting that these expressed proteins were assembled into inclusion bodies. Proteins were solubilized from pelleted inclusion bodies by incubation with a buffer containing 8 M urea, and the expressed proteins were subsequently purified by affinity chromatography using Ni-NTA spin columns. With the exception of the deletion fragments of S2D6, S2D18, S4D7, and S4D8, which showed a low level of expression in *E. coli*, protein purification resulted in approximately 80–90% pure deletion fragments of σA and σNS, demonstrated by SDS–PAGE and Coomassie blue staining. To determine the nature of the epitopes, antibody reactivity to antigen subjected to SDS–PAGE, Western blotting, and dot blot assays was used for discrimination of linear from non-linear epitopes. In the present study, the recognition of recombinant deletion fragments of σA and σNS by MAbs 62, 1F9, H1E1, and 4A123 was analyzed, and the results are summarized in Tables 2A and 2B.

For further location of epitope II on ARV σA, Western blotting and dot blot assays of the amino- and carboxyl-terminal-deleted mutants using MAb 62 showed that the S4-all, S2D5, S2D16, S2D17, S2D18, and S2D19 retained the full binding activity to the antibody, while S2D13 and S2D14 almost lost its binding activity (Figs. 1B–C and Table 2A). In Western blotting, other visible bands with higher or lower molecular weight were also recognized. These could be protein molecules in samples that were not well dissociated and were probably generated as a result of the degradation of the fusion proteins. The results from deletion analysis and the cross-reactivity of MAb 62 to heterologous ARV variants indicated that the region of σA for interaction with MAb 62 was located at amino acid residues 336–360. Because the region of epitope mapped by subtractive analyses was too large to be a linear epitope, the synthetic peptides were used to confirm and to reduce the boundary of epitope. In synthetic peptide assay, MAb 62 can recognize three synthetic peptides (σA-1, σA-2, and σA-3) (Table 3). Based upon a combination of deletion mapping and reactivity of synthetic peptides with MAb 62, we further defined epitope II on σA to be ^{340}QWVMAGLVSAA^{350} (Fig. 3A). Our previous study suggested that the reactivity of MAbs against epitope I on σA was fully abolished after denaturation on σA protein and that this epitope was conformation-dependent (Pai et al., 2003). As mentioned above, the mapping of conformation-dependent epitopes needed specific procedures to increase binding of MAbs to conformation-dependent epitopes. Therefore, we did not attempt to map this conformation-dependent epitope on σA because the methods used in this study were not capable of mapping this epitope.

To define the epitope B on σNS, a series of truncated recombinant proteins of the σNS were expressed and

Table 2B
The antibody reactivity of the complete and truncated σNS proteins with MAbs 1F9 and H1E1, and 4A123 in Western blotting and dot blot assays

Constructs	Monoclonal antibodies	1F9/H1E1		4A123	
	Amino acid residue positions	DB*	WB*	DB*	WB*
S4-all	1–367	+	+	+	−
S4D9	1–29	−	−	−	−
S4D8	1–61	−	−	−	−
S4D7	1–93	−	−	−	−
S4D2	1–183	−	−	−	−
S4D20	1–194	+	+	−	−
S4D16	1–198	+	+	−	−
S4D17	1–201	+	+	−	−
S4D18	1–205	+	+	−	−
S4D19	1–211	+	+	−	−
S4D12	91–270	+	+	−	−
S4D1	178–367	+	+	−	−
S4D13	182–367	+/−	+/−	−	−
S4D14	186–367	−	−	−	−
S4D15	189–367	−	−	−	−
S4D10	191–367	−	−	−	−
S4D11	201–367	−	−	−	−
S4D3	210–367	−	−	−	−
S4D4	263–367	−	−	−	−
S4D5	296–367	−	−	−	−
S4D6	330–367	−	−	−	−
S4D6	330–367	−	−	−	−

+, strong reactions; +/−, weak reactions; −, no reactions.
* WB, Western blotting; DB, dot blotting.

Table 3
The synthetic peptides for epitope mapping

Label	Sequence	Epitope/protein	Monoclonal antibody[a]	
			62	1F9/H1E1
σA-1	^{338}IRQWVMAGLVSAAKG^{352}	II/σA	+[b]	*[c]
σA-2	^{340}QWVMAGLVSAAK^{351}	II/σA	+	*
σA-3	*^{340}QWVMAGLVSAA^{350d}*	II/σA	+	*
σNS-1	^{178}PYMLDMVDGRPQIVL^{192}	B/σNS	*	+
σNS-2	^{180}MLDMVDGRPQI^{190}	B/σNS	*	+
σNS-3	^{180}MLDMVDGRPQ^{189}	B/σNS	*	+
σNS-4	*^{180}MLDMVDGRP^{188e}*	B/σNS	*	+
σNS-5	^{179}YMLDMVDGRPQ^{189}	B/σNS	*	+
σNS-6	^{179}YMLDMVDGRP^{188}	B/σNS	*	+

[a] Reactivity of monoclonal antibodies with each synthetic peptide in dot blot assay.
[b] Strong reaction.
[c] Not determined.
[d] The minimum region for epitope II.
[e] The minimum region for epitope B.

purified. Western blotting and dot blot assay of truncation fragments of σNS using MAbs1F9 and H1E1showed that the S4-all, S4D1, S4D16, S4D17, and S4D20 retained full binding activity to the antibody, while S4D13 S4D14, and S4D11 showed weak or no binding activity to the antibody (Figs. 2B–C and Table 2B). The results from deletion analysis and the reactivity of heterologous virus strains with MAbs 1F9 and H1E1 suggested that the region of σNS for interaction with MAbs 1F9 and H1E1 was located at amino acid residues 178–194. The epitopes mapped above by deletion mapping using reactivity with the expressed protein fragments seem relatively large for linear epitopes. To further confirm epitope locations and to reduce the boundary of the epitope, the synthetic peptides related to these epitopes were used in dot blot assay. Monoclonal antibodies 1F9 and H1E1 can recognize six synthetic peptides (σNS-1, σNS-2, and σNS-3, σNS-4, σNS-5, and σA-6) tested in this study (Table 3). Based upon a combination of deletion mapping and reactivity of synthetic peptide with monoclonal antibody, we further defined epitope B on the σNS to be ^{180}MLDMVDGRP^{188} (Fig. 3B). The present results indicated that both MAbs 1F9 and H1E1 recognized the same epitope. It seems to be contradictive to our previous report that MAbs 1F9 and 1E1 recognized different epitopes B and C on σNS, respectively, based on competitive binding assay (Hou et al., 2001). In fact, when the competition was rated as significant if it was 25% (or even 30%) to 60% rather than 35% to 60% (Hou et al., 2001) to define epitope groups, the epitopes B and C could be grouped into the same epitope recognized by the MAbs 1F9 and H1E1.

The conformation-dependent epitope A on NS was not possible to map since no binding to σNS deletion fragments could be detected (Table 2B). However, a few studies have recently detected non-linear epitopes by the use of Pepscan techniques based on peptides coupled to cellulose membranes (Korth et al., 1997; Reineke et al., 1995, 1998) or to Immobilon (Gao and Esnouf, 1996).

Amino acid sequence analysis

The results of the Jameson–Wolf antigenicity index and sequence analysis indicated that 15 regions with a theoretical Jameson–Wolf antigenicity index of 1.5 or more were identified within the σA amino acid sequence of the S1133 virus strain and 9 regions were identified within the σNS amino acid sequence. Results indicated that epitope II has high antigenicity and hydrophilicity, while epitope B possess high antigenicity and low hydrophilicity. Sequence analysis of ARV σA revealed that σA appeared to contain a carboxyl terminal region (one-fourth of the protein) that contains α-helices and β-turns and a large amino-terminal region (three-fourth of the protein) that is mainly β-turns and β-strands. These results are similar to those for MRV σ2 described previously (Dermody et al., 1991), suggesting that both σA and σ2 proteins may have similar functions. Sequence analysis of ARV σNS revealed that σNS appeared to contain a carboxyl-terminal region that contains α-helices and a large amino-terminal region that is mainly β-turns and β-sheets.

Variations resulting in evolutionary changes are both a consequence of modifications in the genome and the result of environmental pressures placed on the virus. Factors determining genetic variability of ARV include point mutations and genetic reassortment (Liu et al., 2003; Ni and Kemp, 1995). However, RNA viruses which lack proofreading and postreplicative error correction mechanisms are expected to have a high mutation rate and therefore to evolve rapidly (Steinhauer and Holland, 1987). Therefore, it was interesting to know whether sequence variations of epitopes among heterologous ARV strains occurred. In the present study, the regions of amino acid 340–350 of σA and aa 180–188 of σNS among 13 ARV strains, related to the regions of epitopes II and B, were compared (Figs. 3A and 3B). Sequence analysis showed that only two amino acid substitutions at position 343 (from methionine to valine) and position 345 (from glycine to valine) were found in σA (Fig. 3A), while four amino acid substitutions at position 180

(A) Amino acid sequences of epitope II on σA among ARV strains

	340										350	MAb 62
S1133	Q	W	V	M	A	G	L	V	S	A	A	+
2408	.	.	.	.	.	.	.	.	.	.	.	+
1733	.	.	.	.	.	.	.	.	.	.	.	+
919	.	.	.	.	.	.	.	.	.	.	.	+
T6	.	.	.	.	.	V	.	.	.	.	.	+
750505	.	.	.	.	.	.	.	.	.	.	.	+
OS161	.	.	.	.	.	.	.	.	.	.	.	+
601SI	.	.	.	V	.	.	.	.	.	.	.	+
R2/TW	.	.	.	V	.	.	.	.	.	.	.	+
918	.	.	.	.	.	.	.	.	.	.	.	+
1017-1	.	.	.	V	.	.	.	.	.	.	.	+
916	.	.	.	V	.	.	.	.	.	.	.	+
601G	.	.	.	V	.	.	.	.	.	.	.	+

(B) Amino acid sequences of epitope B on σNS among ARVstrains

	180								188	MAb 1F9/ H1E1
S113 3	M	L	D	M	V	D	G	R	P	+
2408	.	.	.	.	.	.	.	.	.	+
1733	.	.	.	.	.	.	.	.	.	+
919	.	.	.	.	.	.	.	.	.	+
T6	.	.	.	.	.	.	.	.	.	+
750505	T	.	.	.	.	.	.	.	.	+
OS161	.	.	.	.	.	.	.	.	.	+
601SI	.	.	.	.	.	.	.	.	.	+
R2/TW	.	.	.	.	I	.	.	.	.	+
918	.	.	.	.	I	N	.	.	.	+
1017-1	.	.	.	.	I	.	.	.	.	+
916	.	.	.	.	I	.	.	.	.	+
601G	.	.	.	V	.	.	.	.	.	+/-

Fig. 3. Sequence comparison of epitope II on σA (A) and epitope B on σNS (B) of ARV strains. ARV S1133 sequences were shown on top and the differences were indicated. Reactivity of each virus strain with monoclonal antibodies in ELISA assays was shown at the right hand side. Strong and weak reactions were indicated by (+) and (+/−), respectively.

(from methionine to threonine), 183 (from methionine to valine), 184 (from valine to isoleucine), and 185 (from aspartic acid to asparagine) occurred in σNS among ARV strains (Fig. 3B). The results indicated that the type of amino acid changes in epitopes showed no correlation with either serotypes or pathotypes of ARV.

Antibody binding assay

Since the region related to epitope II on σA protein of Taiwanese strains showed sequence variation only at positions 343 (from M to V) and 345 (from G to V), we examined the importance of the residue in antibody binding. In the binding of MAb 62 to heterologous virus strains, it was found that MAb 62 recognized all tested strains which belonged to different serotypes (Fig. 3A), suggesting that the two-valine substitutions did not affect the reactivity of epitope II and that the residues were not critical in antibody binding. MAb 62 reacted with all heterologous ARV strains, suggesting that this epitope is commonly shared by the ARV strains. Recently, we developed an antigen capture enzyme-linked immunosorbent assay (ELISA) using MAbs prepared against the epitope II on σA synthesized in *E. coli*, demonstrating that the MAb-based ELISA is a useful method for the detection of ARV from chickens suspected to have ARV infections (Pai et al., 2003). Taken together, all the data indicated that epitope II is a good marker of ARV infection that could be used for the detection of ARV antibody response.

It was of particular interest to determine whether amino acid changes on epitope B of σNS could hinder antibody binding. Since the epitope B of σNS showed sequence variations at positions 180, 183, 184, and185, the importance of residue substitutions in antibody binding was examined. To test the binding abilities of MAb1F9 to heterologous ARV strains, the antibody was used to test for their cross-reactivity with other ARV strains in ELISA. With the exception of the 601G strain of ARV containing a substitution at amino acid residue 183 (M to V), which showed weaker reactivity (Fig. 3B), MAb 1F9 reacted with all heterologous ARV strains. These observations suggested that amino acid changes at 180 (M to T), 183 (M to V), 184 (V to I), and 185 (D to N), did not affect the binding activity of epitope B with MAb 1F9, but substitution at an amino acid residue 183 (M to V) could hinder the binding activity of epitope B (Fig. 3B).

Blocking of ssRNA binding activity of σNS and truncation mutants with MAb 1F9 as well as gel shift assay of the σNS and truncation mutants binding to ssRNA

To date, the region of ARV σNS which is responsible for ssRNA binding has not been defined. To further determine if the epitope B of σNS is involved in ssRNA binding, preparation of deleted mutant proteins of σNS along with gel shift assay was performed. It has been described for several viruses that the ssRNA-protein binding activity is important in genome assortment and initial stage of replication (Antczak and Joklik, 1992; Patton, 1995). Previous investigations suggested that σNS of MRV plays an important role in mRNA assortment and packaging because of its ssRNA-binding activity (Huisman and Joklik, 1976). In blocking assay, MAb 1F9 recognizing epitope B was used to block ssRNA binding activity of σNS and deletion mutants (S4 D1 and S4D2) that had been preincubated with MAb 1F9, followed by poly (A)-sepharose binding and bound protein analysis. The S4D2 mutant which did not possess epitope B showed no signals in the blocking assay (Fig. 4B). The results showed that MAb 1F9 at a dilution of 1:5 blocked binding of ssRNA to σNS and S4 D1 (Figs. 4A–B), indicating that the inhibition of 1F9 on the RNA binding of σNS could be a direct or indirect effect on this activity. To further address this issue, the gel shift assay of σNS and deletion fragments binding to

ssRNA was performed. The σNS and deletion fragments of S4D13, S4D18, and S4D19 with epitope B had ssRNA binding capacity while truncation fragments of S4D2 and S4D10 without epitope B almost lost their ssRNA binding capacity (Fig. 5). The results suggested that this epitope is required for ssRNA binding of σNS because its deletion fully abolished the ssRNA binding activity of σNS. In addition, the full-length σNS that displayed the strongest binding ability (Fig. 5, last lane) suggested that nature conformation of σNS could be important for ssRNA binding. The σNS mutants may lose some structure needed for ssRNA binding and therefore showed weaker binding ability than that of the full-length σNS of ARV. It is also observed that S4D13 containing the C-terminal of σNS showed much stronger ssRNA binding ability than that of S4D18 and S4D19 that contain the N-terminal of σNS, suggesting that the C-terminal of σNS could be important for ssRNA binding.

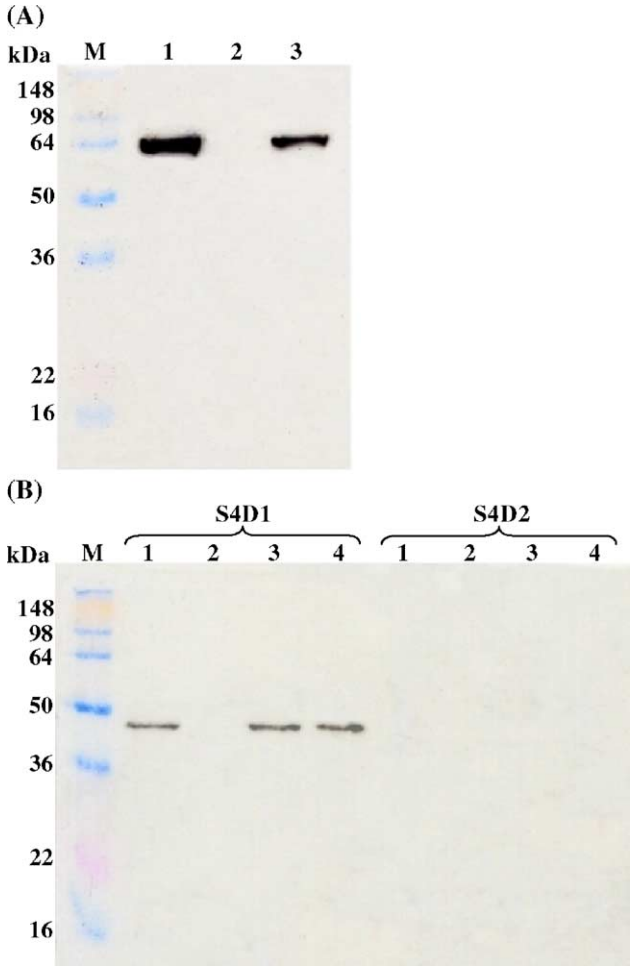

Fig. 4. Blocking of ssRNA binding activity of σNS with MAb 1F9. (A) Protein σNS was preincubated with monoclonal antibody at a dilution of 1:5 (lane 2) and 1:5000 (lane3). (B) Deletion fragments of S4D1 and S4D2 were preincubated with monoclonal antibody at a dilution of 1:5 (lane 2), 1:500 (lane 3), and 1:5000 (lane 4). Lane 1 represents the sample that contains poly (A)-sepharose and σNS without antibody. The deletion fragment of S4D2 which did not contain epitope B was used as a negative control. The reaction mixtures were mixed with poly (A)-sepharose. Proteins bound to poly (A) were separated by SDS–PAGE, electro-transferred onto nitrocellulose membranes, and probed with MAb 1F9.

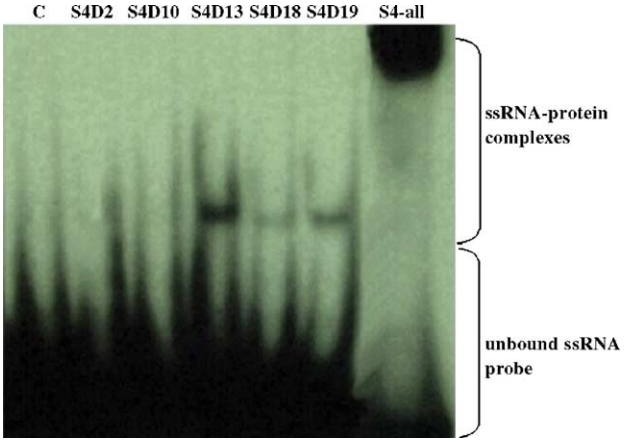

Fig. 5. Gel shift assay of σNS and truncation mutants (S4D2, S4D10, S4D13, S4D18, and S4D19) binding to ssRNA. Autoradiographs of binding reactions after electrophoresis through a native polyacrylamide gel are shown. Lane C represents the negative control.

In the present study, the conservative characteristics of epitope II on σA and epitope B on σNS seem to have functional constraints against amino acid changes. To further elucidate if these epitopes on σA and σNS of ARV have other biological function would be worthwhile to obtain more useful information concerning the roles of σA and σNS proteins in genome assortment and virus replication.

Materials and methods

Virus strains and RNA preparation

Thirteen ARV strains used in this study were described previously (Lee et al., 1992). These ARV strains were propagated in Vero cells. Upon development of 70–80% cytopathic effect (CPE), the cell cultures were frozen and stored at −70 °C. Viral particles were purified following methods described previously (Liu et al., 1999, 2002, 2004). Purified viral particles were resuspended in TNE buffer containing 5% sodium dodecyl sulfate (SDS). Proteinase K was added to a final concentration of 1 mg/ml. After incubation for 1 h at 37 °C, the viral RNA was isolated by two consecutive phenol/chloroform/isoamyl alcohol (25:24:1) extractions and recovered by precipitation with ethanol containing 0.4 M LiCl. The dsRNA was further purified by LiCl fractionation precipitation (Diaz-Ruiz and Kaper, 1978). Viral RNA was washed twice with 70% ethanol to remove LiCl and suspended in diethylpyrocarbonate (DEPC)-treated water.

Monoclonal antibodies

The σA-specific MAb (62) and σNS-specific MAbs (1F9, H1E1, and 4A123) against the S1133 strain of ARV

were described previously (Hou et al., 2001; Pai et al., 2003). These particular MAbs used in this study were based on the higher concentration of immunoglobulin secreted by the hybridoma cell lines (62, 1F9, H1E1, and 4A123).

Reverse transcription (RT)-polymerase chain reaction (PCR)

To determine the S2 and S4 gene nucleotide sequences of thirteen ARV strains, purified genomic dsRNA was used to generate the full-length cDNA clones by reverse transcription and polymerase chain reaction amplification (RT-PCR). The sequences and locations of primers used to generate the full-length and deletion fragments of S2 and S4 genes of S1133 strain of ARV are indicated in Tables 1A–1B. The primers for amplification of the full-length σA- and σNS-encoding genes were located at the 5′ and 3′ noncoding regions encompassing the σA and σNS open reading frame (ORF). The primers for construction of the deleted fragments of σA- and σNS-encoding genes were located at regions with a low Jameson–Wolf antigenicity index and hydrophilicity. In RT-PCR test, 1 μg of purified dsRNA was denatured in boiling water for 10 min, chilled on ice for 5 min, and then used as a template. RT-PCR reactions were carried out according to procedures provided by Perkin-Elmer Co. (Branchburg, NJ, USA). Reverse transcription (RT) was carried at 50 °C for 30 min. PCR reactions were subjected to 35 cycles consisting of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and extension for 90 s at 72 °C. An additional 7 min at 72 °C was included at the end of the program to ensure complete extension. After completion of PCR, 5 μl of reaction mixture was loaded onto a 1.5% agarose gel, containing 5 μg/ml ethidium bromide, for electrophoresis and subsequent visualization by UV transillumination.

Gene expression and protein purification

The full-length σA- and σNS-encoding genes of S1133 strain were cloned into pET32 expression vectors (Novagen, Madison, WI, USA). PCR products (1275 bp) from the σA-encoding gene were treated with Klenow polymerase and inserted into the *Sma*I site of pET32a expression vector. PCR products (1152 bp) derived from the σNS-encoding gene were digested with *Eco*RI and then ligated into the corresponding site of a pET32b bacterial expression vector. Recombinant plasmids were used to transform *E. coli*-competent cells (DE3). DNA minipreps using an alkaline lysis method were performed on white colonies suspected of containing an insert. Colonies with correct sizes were further identified by restriction enzyme analysis. Recombinant plasmid DNA was purified using a purification kit (Qiagen, Valencia, CA, USA) prior to sequencing. In order to assure that the sequences did not contain PCR-based errors, three clones of each gene were sequenced with an Automated Laser Fluorescence DNA Sequencer (ABI).

The procedures for σA and σNS expression in *E. coli* have been described previously (Liu et al., 2002; Yin and Lee, 1998; Yin et al., 2000). Briefly, after induction for 3 h with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM in culture medium, *E. coli* cells containing pET32a-σA and pET32b-σNS were induced, and the cell pellets were harvested by centrifugation (5000 rpm for 5 min), resuspended in TE buffer (50 mM Tris–HCl, pH 8.0; 2 mM EDTA) containing 0.1% Triton X-100 and 100 μg/ml chicken egg-white lysozyme (Sigma), and then incubated on ice for 15 min. To obtain soluble or insoluble fusion proteins, whole cell pellets from bacterial cultures were disrupted by sonication at 4 °C. The expressed proteins were subsequently purified by affinity chromatography using Ni-NTA spin columns (Qiagen, Valencia, CA, USA). The protein concentration was determined according to the Lowry method (Lowry et al., 1951). The supernatant or pelleted insoluble materials were separated on 10% acrylamide gels.

To generate the deletion fragments of σA and σNS of S1133 strain, a series of amino- or carboxy-terminal deletion mutants of the σA- and σNS-encoding genes synthesized by PCR were subcloned into pET expression vectors to construct a series of expression constructs (Tables 1A–B). The expression and purification of the truncated σA and σNS fragments were performed by the procedures described above.

SDS–PAGE, Western blotting, and dot blot assay

To localize the epitopes on σA and σNS of S1133 strain, SDS–PAGE, Western blotting, and dot blot analysis were performed. Purified σA, σNS, and a series of truncated σA and σNS fusion proteins and protein extracts of the induced recombinant cells were mixed with an equal volume of reducing Laemmli sample buffer, boiled for 3 min, and separated by SDS–PAGE. The gels were stained by standard methods using Coomassie brilliant blue or electroblotted onto nitrocellulose (NC) membranes (Towbin et al., 1979). Following post-coating of the NC membranes with 5% dry milk in phosphate-buffered saline, pH 7.4, with 0.05% Tween 20 (PBST) for 30 min at room temperature, the NC membranes were probed with a 1:200 dilution of monoclonal antibodies followed by reaction with HRP-conjugated goat anti-mouse immunoglobulin. The binding reaction was detected by the addition of the diaminobenzidine color development reagent (0.05% diaminobenzidine, 0.005% hydrogen peroxide in distilled water).

To determine the need for a native structure for antibody binding, an immuno-dot binding assay was performed. In dot blot assays, purified σA, σNS, and a series of truncated σA and σNS fusion proteins were dotted onto NC membranes and air dried for 30 min. Following post-coating of the NC membranes with 5% dry milk in PBST for 30 min

at room temperature, the NC membranes were probed with MAbs. HRP conjugate incubation and color development were performed as described for Western blotting.

Sequence analysis

To analyze the sequence variations of epitopes on σA and σNS, the Kyte–Doolittle hydrophilic plot and Jameson–Wolf antigenicity index analysis were performed using DNASTAR package software (DNASTAR, Madison, WI, USA). Amino acid sequences of these epitopes among ARV strains were compared using the same program described above. The sequences of σA and σNS of ARV were reported by our laboratory (Liu and Huang, 2001; Liu et al., 2003). Accession numbers for the σA- and σNS-encoding genes were as follows: 1733-σA, AF293773; 2408-σA, AF247724; OS161-σA, AF294770; 601SI-σA, AF294769; T6-σA, AF294768; 750505-σA, AF294767; 919-σA, AF294763; 601G-σA, AF311322; R2/TW-σA, AF294765; 918-σA, AF294766; 916-σA, AF294764; 1017-1-σA, AF294762; 1733-σNS, AF294722; 2408-σNS, AF213468; OS161-σNS, AF294777; 601SI-σNS, AF294773; T6-σNS, AF213469; 750505-σNS, AF213470; 919-σNS, AF294776; 601G-σNS, AF311322; R2/TW-σNS, AF294778; 918-σNS, AF294775; 916-σNS, AF294774; and 1017-1-σNS, AF294771. Accession numbers for other sequences were as follows: S1133-σA, AF104311 and S1133-σNS, U95952.

Synthetic peptide

Synthetic peptides have been a useful means for epitope mapping as described previously (Dietzschold et al., 1987; Goto et al., 2000). To further confirm the epitopes identified by deletion mapping method were real and not artifacts, the consecutive overlapping peptides were designed to mimic and cover the entire region of epitopes mapped by the subtractive analyses of deletion mapping. Synthetic peptides (Table 3) were synthesized and used in dot blot assay.

Cross-reactivity of MAbs to heterologous ARV strains

The procedures for cross-reactivity of the MAbs to heterologous ARV strains have been described previously (Hou et al., 2001; Pai et al., 2003). Briefly, to study the monoclonal antibodies (62 and 1F9) for their cross-reactivity with various ARV strains in an antigen-captured enzyme-linked immunosorbent assay (ELISA), a volume of 100 μl (1:200) of mouse anti-σA and -σNS antiserum was coated onto ELISA plates. After washing and blocking with 2% bovine serum albumin, 100 μl of cell extracts (30 moi) from Vero cells infected with each ARV strain or from mock-infected cells was added and incubated at 37 °C for 1 h. For the binding assay of MAbs with various ARV strains, 50 μl of HRP-conjugated MAbs 62 and 1F9 was added as primary antibodies. To determine if σA and σNS proteins present in each cell extract from Vero cells infected with each ARV strain were captured by anti-σA and -σNS antiserum, respectively, a 1:200 dilution of chicken anti-ARV S1133 hyperimmune serum and HRP-coupled goat anti-chicken antiserum (1:2000) was used as primary and secondary antibodies, respectively. Absorbance was measured at 405 nm. Binding to the heterologous virus is expressed as percentage by taking the absorbance obtained with ARV S1133 in the reaction as 100. The relative binding activity to heterologous ARV isolates was rated as described previously (Hou et al., 2001; Pai et al., 2003).

Binding of protein σNS and truncation mutants to poly (A)-sepharose in the presence of MAb 1F9

To determine if the epitopes of σNS were involved in ssRNA binding, in the initial experiments various concentrations of MAb 1F9 were used to test the blocking activities of binding of σNS to ssRNA probes in gel shift analysis (Richmond et al., 1998) or Northwestern blot (Dermody et al., 1991). In blocking assay, purified σNS (5 μg) and two deletion mutants (S4D1 and S4D2) were incubated with various dilutions of MAb 1F9 in a total volume of 10 μl at 37 °C for 1 h. The reaction mixtures and poly (A)-sepharose (Pharmacia) containing 55 μg poly(A) oligomer were combined with a binding buffer (150 mM NaCl, 1 mM DTT, 0.5% Tween 20, 20 mM Hepes, 5 mM MgOAc, 10% glycerol, pH 7.4) for a total volume of 150 μl and incubated at 25 °C for 15 min. After four washes with binding buffer, proteins bound to the poly (A)-sepharose were eluted with binding buffer containing 1 M NaCl. Eluted proteins were separated by SDS–PAGE and electrotransferred onto nitrocellulose membrane. The nature of σNS protein and deletion mutants (S4D1 and S4D2) immobilized on the membrane was detected by using a conformation-independent MAb 1F9 as probe.

Gel shift assay of protein σNS and truncation mutants binding to ssRNA

In gel shift assay, purified σNS protein (S4-all) and five deletion mutants and ^{32}P-radiolabeled ssRNA probes (10^4 to 10^5 cpm) were combined with a binding reaction buffer (150 mM NaCl, 1 mM DTT, 0.5% Tween 20, 20 mM Hepes, 5 mM MgOAc, 10% glycerol, pH 7.4) for a total volume of 15 μl and incubated at 30 °C for 15 min (Yin et al., 1997). The ^{32}p-radiolabeled virus-specific ssRNA probe by run-off transcription was prepared as described previously (Yin and Lee, 1998). The reaction mixtures were then separated by electrophoresis on 6% polyacrylamide nondenaturing gels (Konarska and Sharp, 1987) and subjected to autoradiography for 16–18 h. To ensure that the 16 kDa peptide which was fused to σNS did not affect the RNA-protein binding, the purified σNS and deleted mutants were treated with enterokinase.

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