รายงานโครงการวิจัยฉบับสมบูรณ์

(1 กรกฎาคม 2545 – 30 ธันวาคม 2546)

โครงการ การศึกษาแผนผัง epitopes ของ capsid protein ของ porcine circovirus type 2 (PCV2) และคุณสมบัติของไวรัสลูกผสม (PCV1/PCV2) ที่ประกอบด้วย epitopes ของ PCV2 (TRG 4580023)

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บทคัดย่อ

ทำการศึกษาแสดงหา epitopes ของ capsid protein (ORF2) ของ porcine circovirus type 2 (PCV2) โดยการใช้ โมโนโคลนอล แอนติบอดี้ ที่เฉพาะ จำนวนเจ็ดโคลน ในการตรวจหาตำแหน่งที่เป็นแอนติเจน บน capsid protein ของไวรัสลูกผสม ระหว่าง PCV1และ PCV2 ไวรัสลูกผสมสร้างจากการตัดส่วนของ PCV2-ORF2 เป็นลำดับ และเชื่อมส่วนที่ถูกตัดด้วย ORF2-PCV1 จากนั้นทำการ transfect ไวรัสลูกผสม PCV1/PCV2 เข้า PK-15 cells และทดสอบการมีชีวิตของไวรัสลูกผสมโดย immunofluorescent assay (IFA) กับ convalescent PCV2 swine antiserum ไวรัสลูกผสมที่กรดอมิโน จำนวน 47 หน่วยขาดหายไปจาก ปลายด้าน NH_2 สามารถจับกับ โมโนโคลนอล แอนติบอดี้ ทั้งเจ็ดโคลนได้ดี เมื่อทำให้กรดอมิโนที่ปลาย NH_2 ของ PCV2-ORF2 ขาดหายไปจาก 47 เป็น 57 (r175) เป็นผลให้ไวรัสลูกผสม ไม่สามารถจับกับโมโนโคลนอล แอนติบอดี้ 3B7, 3C11, 6H2 และ 8F6 ได้ ไวรัสลูกผสมที่มีกรดอมิโนที่ปลาย NH_2 ของ PCV2-ORF2 ขาดหายไป 62 หน่วย ไม่ สามารถจับกับ โมโนโคลนอล แอนติบอดี้ ทั้งเจ็ดโคลนได้ ถ้ากรดอมิโนลำดับที่ 165 ถึง 233 ที่ปลายด้าน COOH ของ PCV2-ORF2 ถูกแทนที่ด้วยลำดับกรดอมิโนของ PCV1-ORF2 ไวรัสลูกผสมดังกล่าวไม่สามารถจับกับ โมโนโคลนอล แอนติบอดี้ ทั้งเจ็ดโคลน ได้ เมื่อยืดลำดับกรดอมิโนของ PCV2-ORF2 จากหน่วยที่ 165 (r464) เป็น 185 (r526), 200 (r588) หรือ 224 (r652) ทำให้ไวรัสลูกผสมสามารถจับกับ โมโนโคลนอล แอนติบอดี้ 3C11, 6H2, 9H7 และ 12G3 ได้ เมื่อกรดอมิโนจำนวนสี่ หน่วยที่ปลายด้าน COOH ของ r588-ORF2 ถูกแทนที่ด้วย กรดอมิโนของ PCV2-ORF2 ทำให้ได้ไวรัสลูกผสม r588F ซึ่ง สามารถจับกับ โมโนโคลนอล แอนติบอดี้ ทุกโคลนได้ ผลที่ได้ชี้ให้เห็นว่า โมโนโคลนอล แอนติบอดี้ ทั้งเจ็ดโคลน จดจำ epitopes จำนวน อย่างน้อยห้าตำแหน่ง บน capsid protein ของ PCV2 ซึ่ง epitopes เหล่านี้น่าจะซ้อนทับอยู่ภายในช่วงกรดอมิโนลำดับที่ 47 ถึง 63, 165 ถึง 200 และ กรดอมิโน สี่หน่วยสุดท้ายทางปลาย COOH ของ capsid protein ของ PCV2 epitopes ทั้งห้านี้ น่าจะเป็น conformational epitopes ซึ่งอยู่บนผิวภายนอกของอนุภาคไวรัส

Abstract

Seven monoclonal antibodies (MAb) against the capsid protein of type 2 porcine circovirus (PCV2) were used to map the antigenic sites using PCV2 infectious DNA clones containing PCV1/PCV2- open reading frame (ORF) 2 chimeras. The chimeric PCV1/PCV2-ORF2 cassettes were constructed, by serial deletions of the PCV2-ORF2 and substitution of the deletions with the corresponding sequences of the PCV1-ORF2. The viability of the chimeras in transfected PK-15 cells was confirmed with a convalescent PCV2 swine antiserum. The chimeric PCV1/PCV2 clones were transfected into PK-15 cells, and their reactivities with the seven MAbs were detected by immunofluorescent assay (IFA). The chimera with a deletion of 47 amino acids at the N-terminus of PCV2-ORF2 (chimera r140) reacted strongly to all seven MAbs. Expanding the deletion of PCV2-ORF2 from amino acid residues 47 to 57 (r175) abolished the recognition of MAbs 3B7, 3C11, 4A10, 6H2 and 8F6 to the chimera. Further deletion of PCV2-ORF2 to 62 residues disrupted the binding of this chimera to all seven MAbs. Reactivities with all MAbs were absent, when amino acid residues 165-233 at the C-terminus of ORF2 was replaced with the corresponding PCV1-ORF2 sequence. Extending the sequence of PCV2-ORF2 from residues 165 (r464) to 185 (r526), 200 (r588) or 224 (r652) restored the ability of the three chimeras to bind MAbs 3C11, 6H2, 9H7 and 12G3 but not MAbs 8F6, 3B7, or 4A10. When the four amino acids at the C termini of r588 were replaced with that of PCV2-ORF2, the resulting chimera (r588F) reacted with all seven MAbs. The results from this study suggest that these seven MAbs recognized at least five different but overlapping epitopes within residues 47 to 63, 165 to 200 and the last four amino acids at the C-terminus of the PCV2 capsid protein. These epitopes are likely conformational epitopes on the exterior surface of the capsid protein.

1. ปัญหาที่ทำการวิจัย ความสำคัญและของปัญหา

Porcine circovirus (PCV), classified in the family *Circoviridae*, is a small non-enveloped DNA virus. There are two genotypes of PCV, the non-pathogenic strain-PCV1 and the pathogenic strain-PCV2. PCV2 was isolated from pigs with postweaning multisystemic wasting syndrome (PMWS), a recently emerged disease characterized by progressive weight loss, emaciation, difficult breathing and jaundice. PMWS has been recognized as one of the important diseases of swine, and PCV2 infection has been reported worldwide, including Canada, the United States, Europe and Asia. In addition, PCV2 was also isolated from PMWS cases in Thailand. The disease frequently occurs in nursery or grower pigs. Morbidity is usually low, but case fatality can be higher than 50% in epidemic herds. As the impact of PMWS to swine industry has increased dramatically, the World Health Organization for Animal Health (Office International des Epizootics-OIE) is considering PMWS to the list of pig diseases that must be declared internationally by infected countries.

While PMWS and PCV2-related diseases increase threat in swine population throughout the world, an effective mean of prevention and control has not been established. **Our long-term goal** is to develop effective strategies for prevention and control of PMWS and PCV2-related diseases. Therefore, information regarding this virus is required to the understanding of its interaction with the host. Securing background information related to viral coat/capsid protein(s) is essential, because coat proteins play an important role in biological activities such as binding to cellular receptors/ligands for infection and eliciting immune responses. The latter function suggests the possible use of the coat/capsid protein(s) for vaccine development and diagnostic purpose.

2. วัตถุประสงค์

The goals of this study are to map antigenic determinants of the major capsid protein of PCV2 using PCV1/PCV2 chimeras and characterize the chimeras biologically. We hypothesize that the major capsid protein of PCV2 contains neutralizing epitope(s), and the PCV1/PCV2 chimeric capsid protein containing neutralizing epitope is able to protect PCV2 infection. To accomplish these objectives, we will pursue three specific aims:

1. Map antigenic epitopes on the major capsid protein of PCV2 using PCV1/PCV2 chimeras.
2. Examine infectivity of PCV1/PCV2 chimeras in *vitro*.

Test ability of the PCV1/PCV2 chimera containing the neutralizing epitope to induce neutralizing antibodies.

3. วิธีดำเนินการวิจัย

1. Map antigenic epitopes on the major capsid protein of PCV2 using PCV1/PCV2 chimeras.

Chimeric ORF2 cassettes containing ORF2 segments of PCV1 and PCV2 will be constructed by PCR using an elongase enzyme (Gibco BRL) for high fidelity amplification. The templates for amplification are the prototype PK-15 cell contaminant, PCV1, and PCV2 ISU31. PCR will be performed using pairs of internal primers, each containing an overlapping sequence of approximately 10 to 20 nucleotides at the recombination junction. The joining sites will be determined according to the homology of ORF2-PCV1 compared with ORF2-PCV2. Primers at the 3' and 5' ends of the ORF2 gene will be similar to those used for amplification of ORF2-PCV1 or ORF2-PCV2 and will be engineered to contain *Xho*I and *Hind*III sites for cloning.

The remaining PCV2 genomic sequence, called the ORF1-PCV2 fragment (containing ORF1 and non-coding regions), will be amplified with primers flanked with *Bam*HI and *Xho*I sites at the 5' end, and a *Hind*III site at the 3' end. Both ORF2 cassettes and the ORF1-PCV2 fragments will be cloned into plasmid pKSII+ (Stratagene) at *Xho*I-*Hind*III and *Hind*III-*Bam*HI sites, respectively. As a result, the insert consists of a full length PCV genome flanked by *Xho*I sites. The integrity of the base composition of the constructs will be verified by sequence analysis.

To test reactivities of each chimera with MAb, the chimeras will be excised with a restriction enzyme, *Xho*I, to create *Xho*I fragments containing the entire sequence of the PCV2 or PCV chimeras (Fig. 1). One microgram of the purified *Xho*I fragments will be self-ligated at at room temperature for one hour using T4 DNA ligase (Gibco BRL). The ligation conditions will be optimized to facilitate the formation of a circularized DNA containing only a single genomic copy resembling the natural PCV replicative form rather than a multi-genomic copy. The self-ligated *Xho*I fragments will be transfected into PK-15 cells grown overnight using Lipofectamine2000 (Gibco BRL) according to the manufacturer's instructions. At 24 hours

post-transfection, cells will be fixed with absolute methanol and used for immunofluorescent assay with the polyclonal antibodies or MAbs.

2. Examine infectivity of PCV1/PCV2 chimeras in vitro.

The circularized chimeric PCV1/PCV2 clones will be transfected into PK-15 cells as described previously. At 72 hours post transfection, the transfected cells will be washed before frozen and thawed three times. The transfected cell suspension will be clarified and the supernatant will be use for inoculating PCV-free PK-15 cells. The process will be repeated at a total of three times. The third passage of the chimeric PCV will be tested for the present of the infectious virus by immunofluorescent assay. The genomic DNA of each infectious chimera will be isolated, amplified and sequenced to verify the integrity of base composition. The clone that showed positive results will be called infectious clone.

3. Test ability of the PCV1/PCV2 chimera containing the neutralizing epitope to induce neutralizing antibodies.

PCV1/PCV2 chimera reacting with neutralizing MAb(s) will be propagated in PK-15 cells and purified by isopycnic centrifugation. Each purified chimeric PCV will be inoculated into 2 rabbits at a total of three times with 3 weeks interval to produce polyclonal antibodies to the chimeras. The antibodies will be examined for viral neutralization activity using a foci reduction assay. Viral neutralizing activity is the ability of antibodies to reduce the number of positive foci by more than 80% when compared with the control.

4. Time-table with six months interval

Tentative accomplishment	Period within the first year		Period within second year	
	1-6	7-12	1-6	7-12
1. Construct series of chimeric ORF2 using PCR techniques	←	→		
2. Clone ORF2 cassettes into plasmid pKSII+		←	→	
3. Subclone ORF1 and non-coding region into plasmid pORF2		←	→	
4. Sequence insert of the each selected clone			←	→
5. Produce self-ligated PCV chimeric clone			←	→
6. Test reactivities between each PCV chimera and MAbs				← →

Tentative accomplishment	Period within the first year		Period within second year	
	1-6	7-12	1-6	7-12
7. Test infectivity of each PCV chimera	←	→		
8. Propagate selected PCV chimera containing neutralizing epitope(s) in PK-15 cells and purified the PCV chimera to prepare antigen for inoculation			←	→
9. Produce rabbit hyperimmune sera using purified PCV chimera as antigens			←	→
10. Test the rabbit sera for antibodies to PCV2			←	→
11. Test the rabbit hyperimmune sera for neutralizing antibodies to PCV2 by foci reduction assay			←	→
12. Prepare manuscripts for publication in international journal(s)				← →
13. Prepare report for TRF				← →

5. Topic to be published in international journal

1. Epitope mapping of the major capsid protein of porcine circovirus type 2 using PCV1/PCV2 chimeras. To be published in journal of virology
2. Chimeric porcine circovirus (PCV) types 1 and 2 induce neutralizing antibodies against PCV2. To be published in journal of general virology

6. Budget

1. Salary: Monthly salary for researcher 10,000 X 24 = 240,000 bahts
2. Equipments and materials:

-Disposable plastic ware (cell culture flasks, plastic tubes, microfuge tubes, pipette tips, transfer pipettes, polyallomer tubes, syringes, needles, ect.) 42,000

-PCR and gel electrophoresis related materials (Primers, Taq polymerase, Elongase, 1 kb plus, Agarose IV, ect.) 22,000

-Sequencing expense 18,000

-Cloning materials (Competent cells for subcloning, T4 DNA ligase, Restriction enzymes, Bacto Agar, Yeast extract, IPTG, β-Gal, etc.)	20,000
-Kits (DNA purification kit, Plasmid miniprep kit, ect.)	15,000
-Transfection reagents (Lipofectamine2000)	10,000
-Immunological reagents (anti-mouse IgG FITC conjugate, anti-rabbit IgG FITC conjugate,)	16,000
-Media (MEM, FBS, antibiotic-antimycotic, Trypsin, HBSS, etc.)	35,000
-Chemical reagents and ultra-pure water	28,000
-Rabbits, husbandry and care	15,000
3. Publication cost and office materials	26,000
Total	247,000

Summary of Research

1. Objectives

The goals of this study are to map antigenic determinants of the major capsid protein of PCV2 characterize the PCV2 chimeras biologically. The proposed objectives of the research in the past year were followed.

1. Map antigenic epitopes on the major capsid protein of PCV2 using PCV1/PCV2 chimeras.
2. Examine infectivity of PCV1/PCV2 chimeras in *vitro*.

2. Research Procedures

Map antigenic epitopes on the major capsid protein of PCV2 using PCV1/PCV2 chimeras.

Chimeric ORF2 cassettes containing ORF2 segments of PCV1 and PCV2 were constructed by polymerase chain reaction (PCR). The templates for amplification are the prototype PK-15 cell contaminant, PCV1, and PCV2 ISU31. PCR were performed using pairs of internal primers, each containing an overlapping sequence of approximately 10 to 20 nucleotides at the recombination junction. The joining sites were determined according to the homology of PCV1-ORF2 compared with PCV2-ORF2. Primers at the 3' and 5' ends of the ORF2 gene were similar to those used for amplification of PCV1-ORF2 or PCV2-ORF2 and were engineered to contain *Xho*I and *Hind*III sites for cloning.

The remaining PCV2 genomic sequence, called the PCV2-ORF1fragment (containing ORF1 and non-coding regions), were amplified with primers flanked with *Bam*HI and *Xho*I sites at the 5' end, and a *Hind*III site at the 3' end. Both ORF2 cassettes and the PCV2-ORF1 fragments were cloned into plasmid pKSII+ (Stratagene) at *Xho*I-*Hind*III and *Hind*III-*Bam*HI sites, respectively. As a result, the insert consisted of a full length PCV genome flanked by *Xho*I sites. The integrity of the base composition of the constructs was verified by sequence analysis. Twenty-three PCV1/PCV1 chimeras were constructed using PCR cloning. The first set of the recombinant contained deleted ORF2 of PCV2 from its 3' end. The deletions were replaced with the corresponding portions of PCV1-ORF2. They were named according to the nucleotide positions at joining junctions as r39, r140, r175, r192, r256, r327, r382 and r427. The second set of the chimeras included r464, r526 and r652. Their ORF2 were deleted from 5' end, and replaced with ORF2 of PCV1 at nucleotide positions 464, 526, 588 and 652, respectively. The

ORF2 of r526F and r588F were engineered similarly to those of r526 and r588 but the last four amino acids at their 5'end were replaced with the sequence of PCV2-ORF2.

The third set of the recombinants contained ORF2 of PCV2 in the middle flaked by ORF2 of PCV1. These chimeras are r526/588F, r175/526, r140/588 and r140/588F, which the number represents the joining junctions between PCV1-ORF2 and PCV2-ORF2 at 3'/5' portions. Each chimeric ORF2 was transfected into porcine kidney cells (PK-15 cells).

To test reactivities of each chimera with MAb, the chimeras were excised with a restriction enzyme, *Xho*I, to create *Xho*I fragments containing the entire sequence of the PCV2 or PCV chimeras (Fig. 1). One microgram of the purified *Xho*I fragments were self-ligated at room temperature for one hour using T4 DNA ligase (Gibco BRL). The ligation conditions were optimized to facilitate the formation of a circularized DNA containing only a single genomic copy resembling the natural PCV replicative form rather than a multi-genomic copy. The self-ligated *Xho*I fragments were transfected into PK-15 cells grown overnight using Lipofectamine2000 (Gibco BRL) according to the manufacturer's instructions. At 24 hours post-transfection, cells were fixed with absolute methanol and used for immunofluorescent assay with the polyclonal antibodies or MAbs.

3. Results

The results of epitope mapping of the major capsid protein of PCV2 are demonstrated in Figure 1. All chimeras showed positive signals when reacted with the swine serum. The recombinant with a deletion of 140 nucleotides at 3' end of ORF2 still reacted strongly to all seven MAbs. Expanding the deletion of PCV2-ORF2 from 140 to 175 or 192 nucleotides abolished the binding of MAbs 3B7, 3C11, 4A10, 6H2 and 8F6 to the chimeras. When 256 nucleotides at 3' portion of ORF2 were replaced with PCV1-ORF2 sequence, they lost reactivities with all MAbs. The chimeras, r526, r588 and r652, which at least 47 nucleotides at 5' end of ORF2 were deleted, could not reacted with MAbs 8F6, 3B7 and 4A10. Further deletion of ORF2 at 5' end from 173 to 235 nucleotides completely abolished reactivity of the recombinant and all seven MAbs. Substitution of four amino acids of PCV1-ORF2 by those of PCV2 at 5' end restored reactivity of the chimeras with MAbs 8F6, 3B7 and 4A10. r526/588F did not react with all MAbs while r175/526, r140/588 and 1r40/588F reacted with some MAbs.

A number of PCV chimeras containing ORF1 of PCV2 and non-coding regions and PCV2-ORF2, PCV1-ORF2 or a series of PCV1/PCV2-ORF2 recombinant cassettes, were constructed to determine the antigenic sites on the capsid protein of PCV2. The composition of the ORF2 genes of chimeric PCV constructs is shown in Figure 1. Each DNA fragment representing a full-length genome of PCV recombinant was isolated after digestion with *Xho*I, self-ligated and transfected into PK-15 cells. Each type of transfected cell was probed separately with anti-PCV2 swine serum, anti-PCV2 rabbit hyperimmune serum and the panel of seven MAbs. Reactivity of each recombinant with polyclonal antibodies and MAbs was detected by IIF.

Reactivity of anti-PCV2 swine serum with the engineered PCV clones 31/31 and 31/15 detected by IIF is shown in Figure 1. At a dilution of 1:50, the pig serum recognized the antigens produced by PCV clones 31/31 and 31/15 and served as a transfection control. In contrast, the anti-PCV2 rabbit hyperimmune serum and the seven MAbs produced a strong signal in cells transfected with PCV clone 31/31. However, neither the rabbit serum nor MAbs reacted with PCV clone 31/15. These results confirmed that both the rabbit serum and all seven MAbs were specific to the capsid protein of PCV2.

A set of PCV chimeras was constructed in which the NH_2 portion of PCV2-ORF2was replaced with the corresponding portion of PCV1-ORF2 (Fig. 3). The PCV chimeras: clones r39, r140, r175, r192, r256, r382 and r427 contained 14, 47, 58, 63, 85, 133 and 151 amino acid residues of PCV1-ORF2 recombined with the remaining amino acid sequence of PCV2-ORF2, respectively. The swine serum reacted strongly with each chimera. The rabbit serum reacted strongly with r39, r140, r175 and r192, but no reactivity was observed with r256. All seven MAbs produced strong signals with r39 and r140 transfected cells similar to their reactivities with cells transfected with clone 31/31. When the next 11 amino acids of PCV2-ORF2 of r140 (residues 47-57) were replaced with those of PCV1-ORF2 to create r175, MAbs 3B7, 3C11, 4A10, 6H2 and 8F6 lost reactivity with this chimeric capsid protein. However, it reacted strongly with MAb 9H7 but weakly with MAb 12G3. Further replacement of residues 58 to 62 of PCV2-ORF2 with those of PCV1-ORF2 (r192) completely abolished the binding of MAbs 9H7 and 12G3 to the chimeric capsid protein.

A second set of PCV chimeras contained the amino terminus of PCV2-ORF2, joined with the remaining C terminus of PCV1-ORF2. In chimeras r464, r526, r588 and r652, the C

terminus of PCV2-ORF2was replaced with the corresponding sequences of PCV1-ORF2 at amino acid positions: 165, 185, 200 and 224 (68, 48, 33 and 9 residues from C termini), respectively. The results of the antibody reactivity to these chimeric capsid proteins are summarized in Figure 1. The anti-PCV2 swine serum produced strong signals with all of the PCV chimeras. The rabbit serum reacted strongly with chimeric capsid proteins expressed by r526, r588 and r652, but it did not react with r464 antigens. None of the MAbs bound to the chimera r464, in which 68 amino acids at the C terminus of PCV2-ORF2was replaced with the PCV1-ORF2 sequence. Extending the C terminus of PCV2-ORF2 from amino acid 165 (r464) to 185 (r526) fully restored the reactivity of MAb 9H7 and 12G3 with the chimeric capsid protein. MAb 6H2 reacted weakly with r526; however, when the length of PCV2-ORF2 was increased from 185 (r526) to 200 (r588) amino acids, full reactivity of this MAb was observed. MAb 3C11 reacted weakly with r526 and r588. When the PCV2-ORF2 amino acid sequence was expanded from 200 (r588) to 224 (r652), the reactivity of MAb 3C11 with r652 was only slightly increased.

To further confirm that the aforementioned amino acid residues at the C- and N-termini of the PCV2 capsid protein play roles in binding with the seven MAbs, a third set of chimeric ORF2 cassettes was produced. These mosaic chimeras contained the amino acid sequences of PCV2-ORF2, encompassing residues 58 to 185 in chimera r175/526; 47 to 200 in chimera r140/588; 47 to 200 plus the four amino acids at the C terminus for chimera r140/588F, 47 to 224 for chimera r140/652, and 47 to 224 plus the four amino acids at the C terminus for chimera r140/652F, flanked by the remaining amino acid sequence of PCV1-ORF2. The convalescent swine antiserum produced strong signals for all of the mosaic chimeras, indicating all chimeras are viable. The polyclonal rabbit serum specific for the PCV2 capsid protein reacted strongly with chimeras r175/526, r140/588, r140/588F, r140/652, and r140/652F.

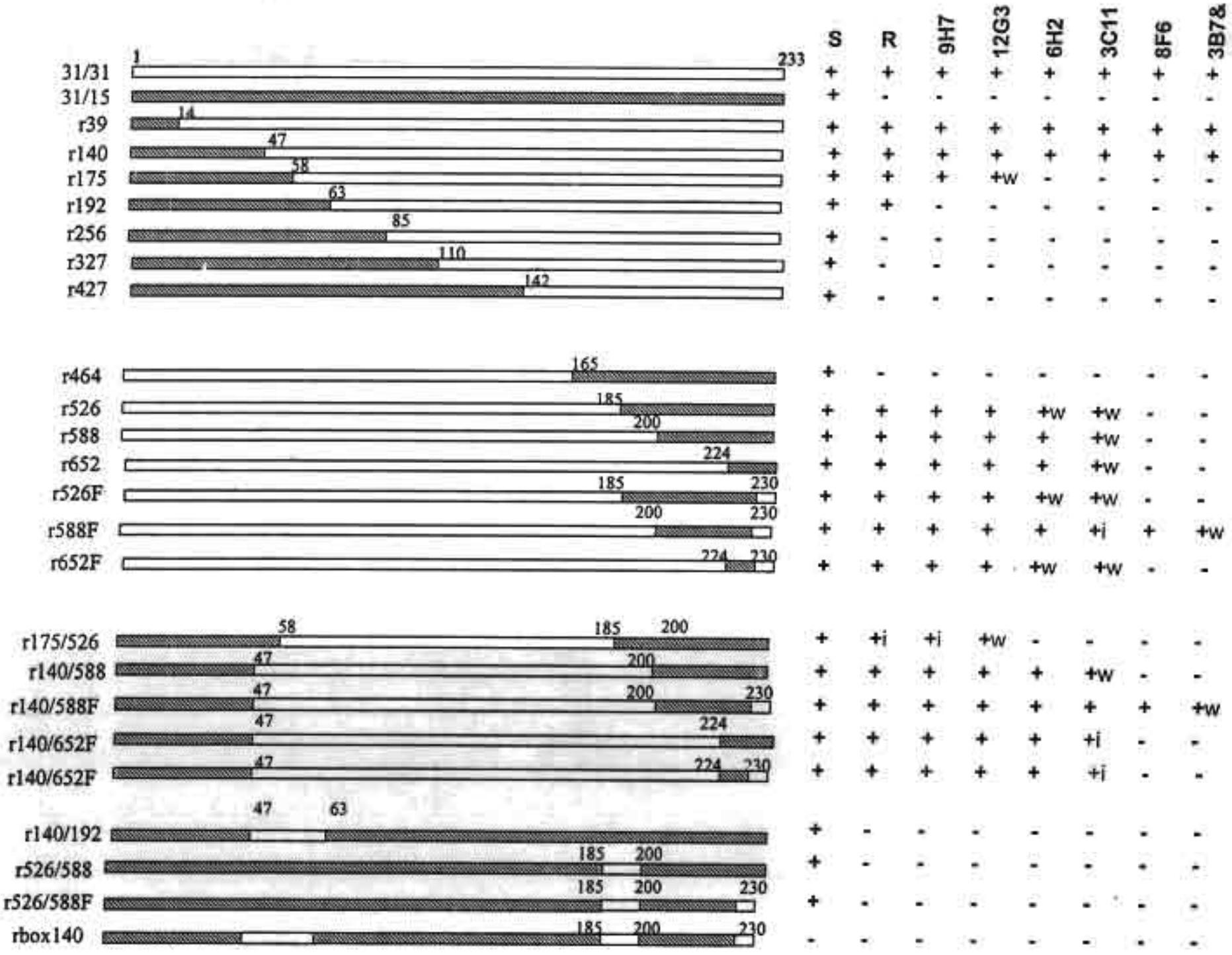
The results of reactivities between MAbs and these mosaic chimeras are shown in Figure 1. Strong reactivities of MAb 9H7 with chimeras r175/526, r140/588, r140/588F, r140/652, and r140/652F confirmed that the epitope recognized by this MAb resides between residues 58 and 185. MAb 12G3 reacted weakly with chimera r175/526, but strongly with chimeras r140/588, r140/588F, r140/652 and r140/652F, indicating that it recognized amino acids residues 47 to 185, and possibly extending to residues 200. MAb 6H2 showed full reactivity with chimeras r140/588, r140/588F, r140/652 and 140/652F but had no reactivity with chimera r175/526,

suggesting that the region spanning amino acid residues 47 to 200 is responsible for binding to this MAb. MAb 3C11 produced a moderate positive signal with chimera r140/588F, a weak positive signal with chimera r140/588 and no signal with chimera r175/526. Therefore, this epitope may contain amino acids residing between residues 47 and 200, as well as the last four residues at the C terminus. The absence of the binding of MAb 3C11 to chimeras r140/652 and 140/652F implied that improper folding of these chimeric capsid proteins may have disrupted the epitope structure. The lack of reactivity observed among this set of mosaic chimeras with MAbs 8F6, 3B7 or 4A10 may be due to the incorrect formation of three-dimensional structures of the chimeric capsid proteins, thus disrupting the epitope structure.

In an attempt to further pinpoint the precise locations of the reacting epitopes, a set of chimeric ORF2 cassettes was constructed, which consisted of block(s) of PCV2-ORF2 sequences in the context of PCV1-ORF2 polypeptide. These recombinants contained PCV2-ORF2 residues 47 to 62 in chimera r140/192, 185 to 200 in chimera r526/588, 185 to 200 plus C-terminal four amino acids in chimera r526/588F, and 47 to 62 plus 185 to 200 for chimera rbox140. No IFA reactivity was detected using the polyclonal rabbit serum, or the seven MAbs for chimera r526/588F, indicating that the amino acid residues at the C terminus (185 to 200 plus PLPK) of the PCV2 capsid protein alone are not sufficient for recognition of these MAbs. The chimeras r140/192 and r526/588 showed reactivities with the convalescent swine antiserum but did not react with the polyclonal rabbit serum or any of the MAbs. The chimera rbox140 failed to replicate in the PK-15 cells as the transfected cells did not react with the convalescent swine antiserum.

Figure 1. Schematic diagram of PCV1/PCV2–ORF2 chimeras and their reactivities with antibodies. PCV clone p31/31 (PCV2) and p31/15 contained the intact 233 amino acid sequences of PCV2-ORF2 (empty block) and PCV1-ORF2 (hatched block), respectively. The PCV1/PCV2-ORF2 chimeric cassettes contain serial deletions of the PCV2-ORF2 sequence joined with the remaining sequence of PCV1-ORF2 at a similar position. (A) The first set of chimeras consists of the N-terminal sequence of PCV1-ORF2 fused with the C-terminal sequence of PCV2-ORF2 at amino acid positions 14 (r39), 47 (r140), 58 (r175), 63 (r192), 85 (r256), 110 (r327), and 142 (r427), respectively. (B) For the second set of constructs, the N-terminal sequence of PCV2-ORF2 is joined with the C-terminal sequence of ORF2 of PCV1 at amino acid positions 165 (r464), 185 (r526 & r526F), 200 (r588 & r588F), and 224 (r652 & r652F). The F chimeras (r526F, r588F and r652F) differ from their corresponding ORF2 chimeras in that they contained the last four amino acid residues of PCV2-ORF2 (PLKP) rather than those of PCV1-ORF2 (-LNK) at the C-termini. (C) The thrid set of the mosaic chimeras contains the sequence of PCV2-ORF2 encompassing residues 58-185 (r175/526), 47-200 (r140/588), 47-200 plus the last four amino acids at C-terminus (140/588F), 47-224 (r652), and 47-224 plus the last four amino acids at C terminus (140/652F). (D) The last set of the chimeras contains block(s) of PCV2-ORF2 sequences at residues 47 to 62 (r140/192), 185-200 (r526/588), 185-200 plus the last four amino acids at C terminus (r526/588F), or 47 to 62 plus 185 to 200 (rbox140) flanked by PCV1-ORF2 sequences.

The results of IFA reactivities between each antibody and PK-15 cells transfected with each PCV construct were indicated next to each construct. IFA reactivities of the constructs were demonstrated by PCV2 convalescent swine antiserum (S), rabbit hyperimmune serum (R) or MAbs (9H7, 12G3, 6H2, 3C11, 8F6, 3B7 and 4A10). +, a strong reactivity; +w, a weak reactivity; +I, a moderate reactivity; and –, no reactivity.


S
R
9H7
12G3
6H2
3C11
8F6
3B7&
4A10
31/31 1 233 + + + + + + + +
31/15 + - - - - - - -
r39 14 + + + + + + + +
r140 47 + + + + + + + +
r175 58 + + + +w - - - -
r192 63 + + - - - - - -
r256 85 + - - - - - - -
r327 110 + - - - - - - -
r427 142 + - - - - - - -
r464 165 + - - - - - - -
r526 185 + + + + +w +w - -
r588 200 + + + + + +w - -
r652 224 + + + + + +w - -
r526F 185 230 + + + + +w +w - -
r588F 200 230 + + + + + +i + +w
r652F 224 230 + + + + +w +w - -
r175/526 58 185 200 + +i +i +w - - - -
r140/588 47 200 + + + + + +w - -
r140/588F 47 200 230 + + + + + + + +w
r140/652F 47 224 + + + + + +i - -
r140/652F 47 224 230 + + + + + +i - -
r140/192 47 63 + - - - - - - -
r526/588 185 200 + - - - - - - -
r526/588F 185 200 230 + - - - - - - -
rbox140 185 200 230 - - - - - - - -

การดำเนินงาน

1. การดำเนินงาน ☐ ได้ดำเนินงานตามแผนที่วางไว้

☐ ได้ดำเนินงานล่าช้ากว่าแผนที่วางไว้

X ได้เปลี่ยนแผนงานที่วางไว้ดังนี้

ได้ทำการศึกษาแผนผัง epitopes ของ capsid protein ของ porcine circovirus type 2 (PCV2) เป็นผลสำเร็จ ดัง manuscript ที่แนบมากับรายงานฉบับนี้ ขณะนี้กำลังเลือก clones ที่น่าสนใจมาทดสอบว่า infectious หรือไม่ แต่เนื่องจากงบประมาณที่ได้รับไม่เพียงพอที่จะดำเนินการวิจัยต่อ จึงขอยุติการวิจัยเพียงแค่ epitope mapping ส่วนการ propagate chimeric clones เพื่อเตรียม antigens สำหรับ inoculate กระต่าย และผลิต hyperimmune sera รวม ถึงการตรวจสอบว่า clones ดังกล่าวสามารถกระตุ้น neutralizing antibodies ได้หรือไม่ ไม่มีงบประมาณที่จะสานต่อ

1. รายละเอียดผลการดำเนินงานของโครงการ

2.1 กิจกรรมที่วางแผนไว้

- Construct series of chimeric ORF2 using PCR techniques
- Clone ORF2 cassettes into plasmid pKSII+
- Subclone ORF1 and non-coding region into plasmid pORF2
- Sequence insert of the each selected clone
- Produce self-ligated PCV chimeric clone
- Test reactivities between each PCV chimera and MAbs
- Test infectivity of each PCV chimera
- Propagate selected PCV chimera containing neutralizing epitope(s) in PK-15 cells and purified the PCV chimera to prepare antigen for inoculation
- Produce rabbit hyperimmune sera using purified PCV chimera as antigens
- Test the rabbit sera for antibodies to PCV2
- Test the rabbit hyperimmune sera for neutralizing antibodies to PCV2 by foci reduction assay

2.2 กิจกรรมที่ทำได้จริง

- Construct series of chimeric ORF2 using PCR techniques
- Clone ORF2 cassettes into plasmid pKSII+
- Subclone ORF1 and non-coding region into plasmid pORF2
- Sequence insert of the each selected clone
- Produce self-ligated PCV chimeric clone
- Test reactivities between each PCV chimera and MAbs
- Test infectivity of each PCV chimera

2.3 แผนกิจกรรมเดิม เปรียบเทียบกับแผนกิจกรรมใหม่ และเหตุผลในการเปลี่ยนแผนงาน

Tentative accomplishment	Original plan				Adjusted plan			
	1-6	7-12	13-18	19-24	1-6	7-12	13-18	19-24
1. Construct series of chimeric ORF2 using PCR techniques	←→				←→			
2. Clone ORF2 cassettes into plasmid pKSII+	←→				←→			
3. Subclone ORF1 and non-coding region into plasmid pORF2	←→				←→			
4. Sequence insert of the each selected clone	←→				←→			

Tentative accomplishment	Original plan				Adjusted plan			
	1-6	7-12	13-18	19-24	1-6	7-12	13-18	19-24
5. Produce self-ligated PCV chimeric clone	←→					←→		
6. Test reactivities between each PCV chimera and MAbs		←→					←→	
7. Test infectivity of each PCV chimera			←→					
8. Propagate selected PCV chimera containing neutralizing epitope(s) in PK-15 cells and purified the PCV chimera to prepare antigen for inoculation			←→				←→ •	
9. Produce rabbit hyperimmune sera using purified PCV chimera as antigens				←→			•	
10. Test the rabbit sera for antibodies to PCV2				←→			•	
11. Test the rabbit hyperimmune sera for neutralizing antibodies to PCV2 by foci reduction assay				←→			•	
12. Prepare manuscripts for publication in international journal(s)				←→				←→
13. Prepare report for TRF				←→				←→

*หมายเหตุ ไม่ได้ดำเนินการวิจัยต่อเพราะขาดงบประมาณ

ภาคผนวก

Epitope Mapping of the Major Capsid Protein of Type 2 Porcine Circovirus (PCV) Using Chimeric PCV1 and PCV2

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Running title: epitope mapping of PCV2 capsid protein

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Abstract

Seven monoclonal antibodies (MAb) against the capsid protein of type 2 porcine circovirus (PCV2) were used to map the antigenic sites using PCV2 infectious DNA clones containing PCV1/PCV2- open reading frame (ORF) 2 chimeras. The chimeric PCV1/PCV2-ORF2 cassettes were constructed, by serial deletions of the PCV2-ORF2 and substitution of the deletions with the corresponding sequences of the PCV1-ORF2. The viability of the chimeras in transfected PK-15 cells was confirmed with a convalescent PCV2 swine antiserum. The chimeric PCV1/PCV2 clones were transfected into PK-15 cells, and their reactivities with the seven MAbs were detected by immunofluorescent assay (IFA). The chimera with a deletion of 47 amino acids at the N-terminus of PCV2-ORF2 (chimera r140) reacted strongly to all seven MAbs. Expanding the deletion of PCV2-ORF2 from amino acid residues 47 to 57 (r175) abolished the recognition of MAbs 3B7, 3C11, 4A10, 6H2 and 8F6 to the chimera. Further deletion of PCV2-ORF2 to 62 residues disrupted the binding of this chimera to all seven MAbs. Reactivities with all MAbs were absent, when amino acid residues 165-233 at the C-terminus of ORF2 was replaced with the corresponding PCV1-ORF2 sequence. Extending the sequence of PCV2-ORF2 from residues 165 (r464) to 185 (r526), 200 (r588) or 224 (r652) restored the ability of the three chimeras to bind MAbs 3C11, 6H2, 9H7 and 12G3 but not MAbs 8F6, 3B7, or 4A10. When the four amino acids at the C termini of r588 were replaced with that of PCV2-ORF2, the resulting chimera (r588F) reacted with all seven MAbs. The results from this study suggest that these seven MAbs recognized at least five different but overlapping epitopes within residues 47 to 63, 165 to 200 and the last four amino acids at the C-terminus of the PCV2 capsid protein. These epitopes are likely conformational epitopes on the exterior surface of the capsid protein.

Introduction

Porcine circovirus (PCV), classified in the family *Circoviridae* (17), is a small non-enveloped DNA virus (32). PCV was first isolated as a contaminant of porcine kidney cell line PK-15 (32) and shown to be nonpathogenic in swine (33, 2). Recently, a new disease designated as postweaning multisystemic wasting syndrome (PMWS) has emerged in pigs (7, 12). Clinical signs of the disease include progressive weight loss, emaciation, difficult breathing and jaundice (7, 12). A variant strain of PCV, designated PCV2, was isolated from pigs with PMWS, and PCV2 antigen and nucleic acid were detected in the tissues of the affected pigs (3, 8, 22). Further genetic and pathogenesis studies revealed that there exist 2 genotypes of PCV: nonpathogenic PCV1 and PMWS-associated PCV2 (11, 20, 21, 22).

PMWS associated with PCV2 infection has been recognized as one of the most important swine diseases, and potentially has a serious economic impact on the global swine industry. Incidence of PMWS/PCV2 has been reported worldwide (3, 6, 8, 16, 22, 25, 30, 34, 36). The disease frequently occurs in nursery or grower pigs (12). Morbidity is usually low, but case fatality can be more than 50% in epidemic herds (12). A recent study indicated that pathogenesis of PCV2-induced PMWS may be mediated by host immune response (15). PMWS/PCV2 cases in Midwestern swine farms increased sharply from 16 affected herds in 1997 to more than 400 affected herds in 1999 (31). More recently, PCV2 was also found to be associated with porcine dermatitis and nephropathy syndrome (4, 28).

PCV contains a single-stranded, close-circular DNA genome of 1,759 bp for PCV1 and 1,768 bp for PCV2 (11, 20, 21, 22). The genomic DNA of both PCV1 and PCV2 consists of two major open reading frames (ORFs), ORF1 and ORF2, oriented in opposite directions. The ORF1 of PCV1 and PCV2 is 936 bp and 942 bp in length, respectively, and the nucleotide sequence

identity between these two strains is about 86%. Amino acid sequence (11, 20, 21) and transcriptional analyses of PCV2 (5) as well as the demonstrated ability of the ORF1 protein to drive the replication of plasmids with the PCV origin of replication (19) suggested that ORF1 encodes a replication-associated protein. The ORF2 of both PCVs is 699 nucleotides in length (11, 20, 21, 22) and encodes for a major capsid protein of approximately 30 kDa (23). The sequence identity between the ORF2 of PCV1 and PCV2 is about 67% and 65% at the nucleotide and amino acid levels, respectively (22).

Reactivities between anti-PCV2 swine sera and synthetic peptides revealed at least three immunoreactive regions on the capsid protein (18). These B cell epitopes localized within amino acid residues 65-87, 113-147, and157-183. However, conformational epitopes on the capsid protein also exist since all monoclonal antibodies (MAbs) we produced recognize conformational epitopes. We have previously reported the generation and characterization of infectious DNA clone of PCV2 (9) and chimeric PCV1/PCV2 infectious DNA clones (10). In this study, we mapped the conformational epitopes of the PCV2 capsid protein by analyses of chimeric PCV1/PCV2 ORF2 genes in the context of the PCV2 genome using seven MAbs recognizing conformational epitopes.

Materials and Methods

Cells and viruses

Porcine kidney cell line (PK-15) free of PCV contamination was kindly provided by Dr. K. M. Lager of the National Animal Disease Center, Ames, Iowa. Both PCV-free PK-15 cells and PK-15 cells permanently infected by PCV1 (ATCC-CCL 33) were maintained at 37°C with 5% CO_2 in minimum essential medium (MEM) (Gibco BRL) and 5% heat-inactivated fetal

bovine serum (FBS) (Gibco BRL). PCV2 was propagated in PK-15 cells as previously described (23).

Generation of monoclonal antibodies (MAbs)

To generate MAbs against PCV2, PCV2 virions were purified through CsCL gradient, and used as the antigen for the immunization of mice. Briefly, CsCL gradient-purified PCV2 virions were diluted with PBS buffer to a final concentration of 1 mg/ml. Thirty μg of the antigen mixed with complete Freund's adjuvant were used to immunize each female BALB/c mouse intraperitoneally. A total of four mice were immunized, each for three times at 2-week intervals. The appearance of antibodies to PCV2 in immunized mice was tested on PCV2-infected PK-15 cells using IFA. Three days prior to fusion, the mice with anti-PCV2 antibody response were primed by intravenous injection of 30 μg of the PCV2 antigen. The mice were euthanized and their splenocytes were collected and fused with SP2/O myeloma cells as described elasewhere (38). Hybridoma supernatants were screened by IFA for the presence of specific antibodies to PCV2. Positive hybridomas were recloned by the cell-sorter method, and their supernatants were retested for confirmation. Seven clones of lymphocytes secreting MAbs to the capsid protein of PCV2 were selected. These MAbs were designated 3B7, 3C11, 4A10, 6H2, 8F6, 9H7, and 12G3.

Molecular cloning of PCV2 and construction of PCV1/PCV2 chimeras

Three sets of constructs were produced for epitope mapping. Plasmid p31/31 contains the entire genomic sequence of PCV2 (strain ISU 31). To produce p31/31, the ORF2 of PCV2 strain ISU 31 was amplified with forward primer (5'gagcaagcttttagggtttaagtgggggggtc3') and reverse primer (5'gagtctcgagatgacgtatccaaggaggcg3') containing *Xho* I and *Hind* III sites, respectively. The remaining PCV2 genomic sequence, designated PCV2-ORF1 fragment

(containing ORF1 and non-coding regions), was engineered to contain *Bam*H I and *Xho* I sites at the 5′ end, and a *Hind* III site at the 3′ end with primers 5′gacaggatccctcgagagctgaaaacgaaagaagtgcg3′ and 5′gacaaagcttatgaataaaaacaattacg3′, respectively. The PCV2-ORF2 and the PCV2-ORF1 fragments were ligated into plasmid pKSII+ (Stratagene) at *Xho* I/*Hind* III and *Hind* III/*Bam*H I sites, respectively, to produce PCV2 DNA clone p31/31 (Fig. 1).

Plasmid p31/15 contains a chimeric PCV1 and PCV2 genome, which consisted of the PCV2-ORF1 fragment and the ORF2 of PCV1 sequence. The PCV1-ORF2 sequence was amplified with primers 5′gagcaagcttttatttatttagagggtcttttag3′ and 5′gagtctcgagatgacgtggccaaggaggcg3′. The construction of clone p31/15 was performed essentially the same as described for clone p31/31, except that the PCV1-ORF2 was inserted in the plasmid instead of the ORF2 of PCV2.

The next constructs are series of chimeric ORF2 cassettes containing sequential deletions of PCV2-ORF2 fused with the remaining ORF2 sequence of PCV1 (Fig. 2). Chimeric ORF2 cassettes containing various ORF2 segments of PCV1 and PCV2 (Fig. 2) were constructed by PCR using an elongase enzyme (Gibco BRL) for high fidelity amplification. PCR was performed with pairs of internal primers, each containing an overlapping sequence of approximately 10 to 20 nucleotides at the recombining junction. Primers at the 3′ and 5′ ends of the ORF2 gene were complementary to the template sequences. Plasmid pORF2 chimeras were constructed essentially the same as described for plasmid p31/31, except that the recombinant ORF2 cassettes were used for cloning instead of the PCV2-ORF2. The integrity of the base composition of the constructs was verified by sequencing and sequence analyses.

Transfection

Plasmid p31/31, p31/15 and pORF2 chimeras were excised with *Xho* I digestion to produce *Xho* I fragments containing the entire sequence of the PCV2 or PCV2 chimeras (Fig. 1). One µg of the purified *Xho* I fragments was self-ligated using T4 DNA ligase (Gibco BRL), and subsequently transfected into PK-15 cells using Lipofectamine 2000 (Gibco BRL) according to the manufacturer's instructions. Briefly, 100 ng of DNA was incubated for 20 minutes with 0.8 µl of Lipofectamine 2000 in 50 µl MEM. Fifty microliters of the mixture were applied onto PK-15 cells in each well of a 96-well plate. After incubation for 5 hours at 37°C, 100 µl of MEM containing 10% FBS and 2% antibiotics were added into each well and incubated at 37°C with 5% CO_2. At 24 hours post transfection, the cells were fixed with absolute methanol and processed for IFA.

Indirect immunofluorescent assay (IFA)

PK-15 cells transfected with PCV2 clone or chimeras were incubated with a 1:50 dilution of convalescent PCV2 swine serum (13), 1:1,000 dilution of anti-PCV2 rabbit hyperimmune serum (23) or a 1:50 dilution of each MAb culture supernatant. PK-15 cells transfected with clones p31/31 (PCV2), p31/15 (PCV2 with PCV1-ORF2) as well as non-transfected cells were included as positive and negative controls for each transfection. After incubation at 37°C for 1 hour, cells were stained with FITC-labeled secondary antibodies of corresponding species (Sigma). At low dilutions, the convalescent swine antiserum recognized both PCV1 and PCV2 antigens in clones p31/31 and p31/15, and served as a confirmation for replication of PCV2 and chimeras. The anti-PCV2 rabbit hyperimmune serum reacted with clone p31/31 but not with clone p31/15. The reactivities of each MAb to each ORF2 recombinant were tested and compared with the controls.

Results

Reactivity of PCV clones p31/31 and p31/15

IFA reactivities of convalescent PCV2 swine antiserum with the engineered PCV clones p31/31 and p31/15 are shown in Figure 4. At a dilution of 1:50, the convalescent pig antiserum recognized the antigens produced by PCV clones p31/31 and p31/15, and thus served as a positive transfection control. The convalescent PCV2 swine antiserum likely recognized ORF1 proteins of both PCV1 and PCV2. The swine antiserum produced strong and homogeneous intra-nuclear and cytoplasmic staining in cells transfected with PCV clone p31/31 (Fig. 4A). Within cells transfected with clone p31/15, the positive signals appeared predominantly as small dense, granular intra-nuclear inclusion bodies resembling the pre-replication sites reported in herpes simplex viruses (27) (Fig. 4D). All PCV1/PCV2 chimeras, with the exception of the chimera rbox140, reacted with PCV2 convalescent pig antiserum (Fig. 2).

IFA with anti-PCV2 rabbit hyperimmune serum and the seven MAbs produced strong signals in the nucleus and cytoplasm of cells transfected with PCV clone p31/31, similar to that observed in IFA with the convalescent swine antiserum (Fig. 4B & C). However, neither the rabbit serum nor MAbs reacted with PCV clone p31/15 (Fig. 4E & F). These results confirmed that both the rabbit hyperimmune serum and all seven MAbs were specific to the PCV2 capsid protein and the convalescent swine antiserum recognized both PCV1 and PCV2 antigens.

Epitope mapping with chimeras containing the N-terminus of PCV1-ORF2 fused with the C-terminus of PCV2-ORF2

To explore the antigenic sites on the capsid protein of PCV2, a set of PCV1/PCV2 chimeras was constructed in which the N-terminus of the PCV2-ORF2 was replaced with the corresponding portion of the ORF2 of PCV1 (PCV1-ORF2) (Fig. 2A & 3). The PCV chimeras,

clones r39, r140, r175, r192, r256, r327 and r427, contained 14, 47, 58, 63, 85, 110, and 142 amino acid residues of PCV1-ORF2 recombined with the remaining amino acid sequence of PCV2-ORF2, respectively (Fig. 2A). The IFA reactivity of the chimeras, when transfected into PK-15 cells, with polyclonal antibodies and MAbs, are shown in Figure 2. The convalescent swine antiserum, which recognized ORF1 antigens of both PCV1 and PCV2, reacted strongly with all of the chimeras. The rabbit hyperimmune serum reacted strongly with chimeras r39, r140, r175 and r192, but not with r256, r327 or r427.

All seven MAbs yielded strong IFA staining signals with chimeras r39 and r140 transfected cells, similar to their reactivities with cells transfected with PCV clone p31/31. The IFA reactivities of MAbs with r140 and other chimeras are shown in Figure 5. When the next 11 amino acids (residues 47-57) of PCV2-ORF2 in clone r140 were replaced with those of PCV1-ORF2 to produce the chimera r175, MAbs 3B7, 3C11, 4A10, 6H2 and 8F6 lost their reactivities with the r175chimeric capsid protein. This indicates that amino acid residues 47-57 are crucial for the recognition of MAbs 3B7, 3C11, 4A10, 6H2 and 8F6. However, MAb 9H7 still reacted strongly with clone r175, indicating that the 57 amino acids at the amino terminus of the capsid protein were not involved in binding of this MAb. A weak IFA reactivity between MAb 12G3 and chimera r175 was observed, suggesting that MAb 12G3 may recognize an epitope overlapping the joining region. Further replacement of residues 58 to 62 of PCV2-ORF2 with those of PCV1-ORF2 in clone r192 completely abolished the binding of MAbs 9H7 and 12G3 to the chimeric capsid protein. Hence, residues 58 to 62 are important for the recognition of MAb 9H7, and the epitope for MAb 12G3 encompassed residues 47 to 62.

Although clone r192 did not bind with the MAbs, it reacted strongly with the anti-PCV2 rabbit serum (Fig. 2A), indicating that residues 63 to 84 of the capsid protein contain at least one

epitope. Indeed, the rabbit hyperimmune serum is specific to the capsid protein of PCV2. Clones r256, r327 and r427, which 84, 109 and 141 amino acid residues, respectively, at the N-terminus of PCV2-ORF2 were substituted with PCV1-ORF2, did not react with the anti-PCV2 rabbit serum or any of the MAbs. Therefore, the chimeric capsid proteins of clones r256, r327 and r427 may not contain epitopes for PCV2-ORF2. This observation also confirms that most of the immunodominant epitopes at the N-terminus of the capsid protein resides within residues 47 to 84.

Epitope mapping with chimeras containing the N-terminus of PCV2-ORF2 joined with the C-terminus of PCV1-ORF2

A second set of PCV chimeras containing the amino terminus of PCV2-ORF2 joined with the remaining C terminus of PCV1-ORF2 was constructed for further epitope mapping (Fig. 2B). In chimeras r464, r526, r588 and r652, the C terminus of PCV2-ORF2 was replaced with the corresponding regions of PCV1-ORF2 at amino acid positions 165, 185, 200 and 224 (68, 48, 33 and 9 residues from the C termini), respectively (Fig. 2B). The results of IFA reactivities of various antibodies to these chimeric capsid proteins are shown in Figure 2B. The PCV2 convalescent swine serum produced strong signals with all of the chimeras. The rabbit serum reacted strongly with chimeric capsid proteins expressed by r526, r588 and r652, but not r464.

None of the MAbs reacted to the chimera r464, in which the 68 amino acids at the C terminus of PCV2-ORF2 were replaced with the corresponding PCV1-ORF2 sequence (Fig. 2B). Therefore, the replacement of amino acids 165 to 233 of PCV2-ORF2 with those of PCV1-ORF2 completely disrupted the formation of immunodominant epitopes on the PCV2 capsid protein. Extending the C terminus of PCV2-ORF2 from amino acid 165 in chimera r464 to 185 in chimera r526 fully restored the reactivity of MAb 9H7 and 12G3 with the chimeric capsid

proteins, suggesting that these 21 residues (position 165 to 185) were also involved in epitope formation for both MAbs. MAb 6H2 reacted weakly with chimera r526, however, when the length of PCV2-ORF2 was extended from amino acid position 185 in chimera r526 to 200 in chimera r588, full reactivity of this MAb was restored. Thus, the epitope for MAb 6H2 at the C terminus included amino acid residues 165 to 200. MAb 3C11 reacted weakly with chimera r526 and r588. When the PCV2-ORF2 amino acid sequence was extended from position 200 in chimera r588 to 224 in chimera r652, the reactivity of MAb 3C11 with r652 was only slightly increased. The IFA reactivity of each MAb with the chimeras is shown in Figure 5.

Since some MAbs did not recognize chimeric capsid antigens produced by the second set of chimeras, three amino acids (LNK) at the C terminus of chimeras r526, r588 or r652 were replaced with the corresponding amino acid PLPK of PCV2-ORF2 (Fig. 3) to create new chimeras r526F, r588Fand r652F, respectively (Fig. 2B). Both convalescent swine and polyclonal rabbit sera yielded strong signals in cells transfected with these three chimeras. MAb 3C11 reacted with chimera r588F but not with chimeras r526F or r652F (Fig. 2B). MAb 3C11 reacted moderately with chimera r588F and weakly with chimeras r526, r588 and r652; the signals were less intense compared to the reactivity of this MAb with the PCV clone p31/31(Fig. 5). Hence, the epitope for MAb 3C11 possibly included amino acid residues 165 to 200 plus the four amino acids at the C terminus of the PCV2 capsid protein. The correct conformation of the capsid protein may be essential for the full reactivity of MAb 3C11 to this epitope.

In contrast to MAb 3C11, MAb 8F6 did not recognize chimeric capsid antigens in cells transfected with chimeras r464, r526, r588 or r652. However, MAb 8F6 reacted strongly with the chimera r588F, similar to that of PCV clone p31/31 (Fig. 2B and 5). Reactivity of the MAb 8F6 with chimera r526F was not observed. These findings indicated that residues 185 to 200 as

well as the four amino acids at the C terminus of the PCV2 capsid protein are important for the recognition of MAb 8F6. The absent of reactivity of MAb 8F6 with chimera r652 and r652F may be due to improper folding of these two chimeric capsid proteins.

The reactivities of both MAbs 3B7 and 4A10 with this panel of PCV chimeras were similar to those of MAb 8F6 (Fig. 2B & 5). These two MAbs did not react with chimeras r464, r526, r588, r652, r526F or r652F, although they produced a weaker positive signal with cells transfected with chimera r588F than with clone p31/31. The four residues at the C- terminus of the capsid protein may be partially responsible for MAb 3B7 and 4A10 recognitions, and the substitution of these four amino acids may partially restore the conformation of the epitopes. The weaker positive signal between the two MAbs with the chimera r588F could be due to improper formation of the tertiary structure of the epitope.

Epitope mapping with mosaic PCV1/PCV2 capsid chimeras

To further confirm that the aforementioned amino acid residues at the C- and N-termini of the PCV2 capsid protein play roles in binding with the seven MAbs, a third set of chimeric ORF2 cassettes was produced (Fig. 2C). These mosaic chimeras contained the amino acid sequences of PCV2-ORF2, encompassing residues 58 to 185 in chimera r175/526; 47 to 200 in chimera r140/588; 47 to 200 plus the four amino acids at the C terminus for chimera r140/588F, 47 to 224 for chimera r140/652, and 47 to 224 plus the four amino acids at the C terminus for chimera r140/652F, flanked by the remaining amino acid sequence of PCV1-ORF2 (Fig. 2C and 3). The convalescent swine antiserum produced strong signals for all of the mosaic chimeras, indicating all chimeras are viable (Fig. 2C). The polyclonal rabbit serum specific for the PCV2 capsid protein reacted strongly with chimeras r175/526, r140/588, r140/588F, r140/652, and r140/652F (Fig. 2C).

The results of reactivities between MAbs and these mosaic chimeras are shown in Figure 2C and 5. Strong reactivities of MAb 9H7 with chimeras r175/526, r140/588, r140/588F, r140/652, and r140/652F confirmed that the epitope recognized by this MAb resides between residues 58 and 185. MAb 12G3 reacted weakly with chimera r175/526, but strongly with chimeras r140/588, r140/588F, r140/652 and r140/652F, indicating that it recognized amino acids residues 47 to 185, and possibly extending to residues 200. MAb 6H2 showed full reactivity with chimeras r140/588, r140/588F, r140/652 and 140/652F but had no reactivity with chimera r175/526, suggesting that the region spanning amino acid residues 47 to 200 is responsible for binding to this MAb. MAb 3C11 produced a moderate positive signal with chimera r140/588F, a weak positive signal with chimera r140/588 and no signal with chimera r175/526. Therefore, this epitope may contain amino acids residing between residues 47 and 200, as well as the last four residues at the C terminus. The absence of the binding of MAb 3C11 to chimeras r140/652 and 140/652F implied that improper folding of these chimeric capsid proteins may have disrupted the epitope structure. The lack of reactivity observed among this set of mosaic chimeras with MAbs 8F6, 3B7 or 4A10 (Fig. 2C) may be due to the incorrect formation of three-dimensional structures of the chimeric capsid proteins, thus disrupting the epitope structure.

Attempts to narrow down the regions of the reacting epitopes

In an attempt to further pinpoint the precise locations of the reacting epitopes, a set of chimeric ORF2 cassettes was constructed, which consisted of block(s) of PCV2-ORF2 sequences in the context of PCV1-ORF2 polypeptide (Fig. 2D). These recombinants contained PCV2-ORF2 residues 47 to 62 in chimera r140/192, 185 to 200 in chimera r526/588, 185 to 200 plus C-terminal four amino acids in chimera r526/588F, and 47 to 62 plus 185 to 200 for chimera

rbox140. No IFA reactivity was detected using the polyclonal rabbit serum, or the seven MAbs for chimera r526/588F (Fig. 2D), indicating that the amino acid residues at the C terminus (185 to 200 plus PLPK) of the PCV2 capsid protein alone are not sufficient for recognition of these MAbs (Fig. 2D). The chimeras r140/192 and r526/588 showed reactivities with the convalescent swine antiserum but did not react with the polyclonal rabbit serum or any of the MAbs. The chimera rbox140 failed to replicate in the PK-15 cells (Fig. 2D) as the transfected cells did not react with the convalescent swine antiserum.

Figure 6 summarized regions on the capsid protein of PCV2 that are essential for the recognitions of MAbs and polyclonal rabbit serum as demonstrated in this study. The polyclonal rabbit serum against PCV2 capsid recognized most epitopes residing on the PCV2 capsid protein. Since deletions of the 84 amino acids at the N-terminus in chimera r526 or the 68 amino acids at C-terminus in chimera r464 of the capsid protein completely abolished the reactivity with the rabbit serum, these residues may involve in epitope formation of the capsid protein. In addition, the regions spanning amino acid residues 47 to 62, 165 to 200, and 230 to 233 are essential for epitope recognition of all seven MAbs generated in this study.

Discussion

By using PCV1/PCV2 chimeras, we demonstrated that the seven MAbs generated in this study recognize conformational epitopes on the PCV2 capsid protein. Our results showed that the immunodominant epitopes of PCV2 capsid protein likely located within amino acid residues 47 to 84, 165 to 200, and the last four amino acids of the capsid protein. The epitope for MAb 9H7 likely comprises residues 58 to 62 at the N-terminus and residues 165 to 185 at the C-terminus of the capsid protein. MAb 12G3 recognizes an epitope that resides within amino acids

47 to 62, and 165 to 185 of the capsid protein. Furthermore, amino acid sequences spanning residues 47 to 57 and residues 165 to 200 of the capsid protein may be involved in constituting the epitope recognized by MAb 6H2. An epitope recognized by MAb 3C11 resides from the residues 47 to 57, 165 to 200 and 230 to 233. MAb 8F6 likely recognizes an epitope comprising amino acids 47 to 57, 185 to 200 and the C-terminal last four amino acids of the capsid protein.

The region essential for binding of MAbs 3B7 and 4A10 appears to be similar to that for MAb 8F6. MAbs 3B7 and 4A10 may recognize the same or similar epitope, since the patterns of their IFA reactivities with all chimeras are indistinguishable. However, it is unlikely that MAb 8F6 and MAbs 3B7 (or 4A10) would bind to a similar epitope, since MAb 8F6 produced a very strong signal with chimera r588F whereas MAbs 3B7 and 4A10 reacted only weakly with this chimera. Analyses of the reactivities of the PCV1/PCV2 chimeras suggests that the amino acid sequences from residues 47 to 62 and residues 165 to 200 as well as the C-terminal last four amino acids of the capsid protein were likely in close proximity to form a cluster of epitopes on the surface of a PCV2 virion.

MAbs 8F6, 3B7 and 4A10 reacted with chimeras r140 or r588F and thus they were expected to react with chimera r140/588F. On the contrary, all three MAbs failed to react with cells transfected with the chimera r140/588F. These negative results could be due to the incorrect formation of the three-dimensional structures of chimeric capsid proteins, and thus disrupting the epitope structure.

Fourty-six residues at the N-terminus of the capsid protein are likely not involved in the formation of conformational epitope(s) since full reactivity with anti-PCV2 rabbit hyperimmune serum and the seven MAbs was observed in chimera r140 (the first 46 amino acids replaced with the ORF2-PCV1 sequence). This region also contains basic amino acid rich residues (11, 21,

22), thus may involve in the formation of the interior surface of the virion and interact with the negative charges of genomic DNA during virus assembly as reported in many icosahedral symmetric viruses (29). Indeed, sequence analyses revealed that the basic amino acid stretch at the N-terminus of the capsid proteins of PCVs, other members of the *Circoviridae* (psittacine beak and feather disease virus and chicken anemia virus), and plant viruses with a circular DNA genome (nanoviruses and geminiviruses) all resembles that of a sobemovirus, southern bean mosaic virus (SMBV), which has the DNA-binding activity in this lysine-arginine, basic amino acid, rich region (24). Three-dimensional structure of SMBV demonstrated that the basic amino acids at the N-terminus of the capsid protein were in close contact with the packaged genomic DNA in the native virion (14). However, this does not imply that the 46 amino acid residues at the N-terminus of the PCV2 capsid protein are not immunogenic, since a linear epitope was found between residue 25 and 43 using synthetic peptides (18).

The 187 amino acids within the ORF2 protein, from residue 47 to the C-terminus, may be important for capsid formation of the PCV2 virions. In most spherical DNA and RNA viruses, about 200 amino acid residues at the C-termini of the capsid proteins fold into an eight-stranded β barrel to form the core of the capsid, and each strand connects to one another with an α helix or loop constituting the exterior surface of a protomer (1, 26, 29, 35, 37). The minimum number of residues required for the formation of the β barrel domain is approximately 150 (29). The 187 residues involved in reacting with antibodies of the PCV2 capsid protein are also within this range. Portion of the amino acid sequences of PCV2-ORF2 within this region possibly constitutes the exterior surface of the PCV2 virion since it contains sequences essential for recognition by polyclonal as well as monoclonal antibodies. This finding is in agreement with the results of a published study using PEPSCAN and anti-PCV2 swine sera to map epitopes on

the capsid protein of PCV2 (18). Reactivity between the polyclonal antibodies and the synthetic peptides revealed that at least three immunodominant epitopes resided from residues 65 to 87, 113 to 147, and 157 to 183, respectively. Furthermore, few amino acid residues at the C-terminus of the PCV2 capsid protein may be exposed on the exterior surface of the virion, as found in some RNA viruses (29), since they were required for the reactivity of MAbs 3B7, 4A10 and 8F6. Structural analyses of the PCV2 capsid protein using cryo-electron microscopy and x-ray crystallography are required to confirm these assumptions.

We showed in this study that, in general, the chimeric PCV1/PCV2 ORF2 cassettes help, to some extend, maintain the structure of the chimeric capsid protein and its conformation epitopes. The IFA signals of the PCV2 chimeras in transfected cells with MAbs were produced solely from PCV2-ORF2 product, since all seven MAbs reacted with PCV2 but not with PCV1. The PCV1/PCV2 chimera system reported in this study is also useful in other studies to elucidate the pathogenesis of PCV2 (9, 10). The MAbs generated in the present study should be very useful for further in-depth studies of the molecular biology of PCV2 capsid protein and for virus-cell interaction. The antigenic sites on the capsid protein of PCV2 identified in this study provide valuable information for further in-depth mapping or structural analyses of PCV2 capsid protein.

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Figure Legends

Figure 1. Construction of PCV clone p31/31 (PCV2 strainISU31). The ORF2 gene, ORF1 fragment containing ORF1, and the remaining genomic DNA of PCV2 strain ISU31 were amplified by PCR and cloned into plasmid pKSII+ (hatched line) as shown. B, X and H are the *Bam*H I, *Xho* I and *Hind* III restriction sites used for cloning. The full-length PCV2 genome is excised with *Xho* I, and re-circularized with T4 DNA ligase to form a double-stranded circular DNA for transfection.

Figure 2. Schematic diagram of PCV1/PCV2–ORF2 chimeras and their reactivities with antibodies. PCV clone p31/31 (PCV2) and p31/15 contained the intact 233 amino acid sequences of PCV2-ORF2 (empty block) and PCV1-ORF2 (hatched block), respectively. The PCV1/PCV2-ORF2 chimeric cassettes contain serial deletions of the PCV2-ORF2 sequence joined with the remaining sequence of PCV1-ORF2 at a similar position. (A) The first set of chimeras consists of the N-terminal sequence of PCV1-ORF2 fused with the C-terminal sequence of PCV2-ORF2 at amino acid positions 14 (r39), 47 (r140), 58 (r175), 63 (r192), 85 (r256), 110 (r327), and 142 (r427), respectively. (B) For the second set of constructs, the N-terminal sequence of PCV2-ORF2 is joined with the C-terminal sequence of ORF2 of PCV1 at amino acid positions 165 (r464), 185 (r526 & r526F), 200 (r588 & r588F), and 224 (r652 & r652F). The F chimeras (r526F, r588F and r652F) differ from their corresponding ORF2 chimeras in that they contained the last four amino acid residues of PCV2-ORF2 (PLKP) rather than those of PCV1-ORF2 (-LNK) at the C-termini. (C) The thrid set of the mosaic chimeras contains the sequence of PCV2-ORF2 encompassing residues 58-185 (r175/526), 47-200 (r140/588), 47-200 plus the last four amino acids at C-terminus (140/588F), 47-224 (r652), and

47-224 plus the last four amino acids at C terminus (140/652F). (D) The last set of the chimeras contains block(s) of PCV2-ORF2 sequences at residues 47 to 62 (r140/192), 185-200 (r526/588), 185-200 plus the last four amino acids at C terminus (r526/588F), or 47 to 62 plus 185 to 200 (rbox140) flanked by PCV1-ORF2 sequences.

The results of IFA reactivities between each antibody and PK-15 cells transfected with each PCV construct were indicated next to each construct. IFA reactivities of the constructs were demonstrated by PCV2 convalescent swine antiserum (S), rabbit hyperimmune serum (R) or MAbs (9H7, 12G3, 6H2, 3C11, 8F6, 3B7 and 4A10). +, a strong reactivity; +w, a weak reactivity; +I, a moderate reactivity; and –, no reactivity.

Figure 3. Comparison between deduced amino acid sequences of the ORF2 of PCV1 (derived from PK-15 cells) and PCV2 strain ISU31. Both PCV1-ORF2 and PCV2-ORF2 are comprised of 233 amino acid residues. Dots (.) represent an identical amino acid in both strains. Dashes (-) represent deletions. Arrowheads demonstrate the recombination position of each chimera, preceded or followed by the name of the chimera. The chimeras are named according to the nucleotide position at the joining junction.

Figure 4. IFA reactivity between PK-15 cells transfected with PCV clone p31/31 or p31/15 and polyclonal antibodies or a representative MAb, 3B7. The upper panel indicates reactivities between cells transfected with clone p31/31 and PCV2 convalescent swine serum (A), rabbit hyperimmune serum (B) or MAb 3B7 (C). The lower panel showed reactivities between clone p31/15 and the swine antiserum (D), and no reactivitiy between clone p31/15 and the rabbit hyperimmune serum (E) or MAb 3B7 (F).

Figure 5. Positive reactivity between each MAb and each PCV chimera. MAb **9H7** gives a strong positive signal with chimeras r140 (A), r175 (B), r526 (C), and r175/526 (D). MAb **12G3**, reacts strongly with chimeras r140 (E), and r526 (F), but weakly with r175 (G), and r175/526 (H). MAb **6H2** shows strong IFA reactivity with chimeras r140 (I), r588 (J), and r140/588 (K). MAb **8F6** also reacts strongly with chimeras r140 (L), and r588F (M). MAb **3C11** reacted strongly with chimera r140 (N) and moderately with chimeras r588F (O) and r140/588F (P), and weakly with chimeras r588 (Q), r652 (R) and r140/588 (S). MAbs **4A10** and **3B7** have a similar IFA staining pattern: a strong reactivity of MAb **3B7** with chimera r140 (T), but weak reactivity with chimera r588F (U); a strong reactivity between MAb **4A10** and chimera r140 (V), but a weak reactivity with chimera r588F (W).

Figure 6. Schematic diagram of immunoreactive epitopes on the capsid protein of PCV2 responsible for binding of polyclonal and monoclonal antibodies. Each bar represents a full-length of the PCV2 capsid protein. Filled boxes within bars are the regions important for epitope recognitions of MAbs. All seven MAbs recognized different but overlapping conformational epitopes comprised of amino acids resided in both N- and C-portions of the PCV2 capsid protein. The regions recognized by seven MAbs are as follows: amino acids 58 to 62, and 165 to 185 for MAbs **9H7**; amino acids 47 to 62, and 165 to 185 for MAb **12G3**; amino acids 47 to 57, and 165 to 200 for MAb **6H2**; amino acids 47 to 57, 165 to 200, and 230 to 233 for MAb **3C11**; 47 to 57, 185 to 200, and 230 to 233 for MAbs **8F6**, **3B7** and **4A10**. Reactivities between the anti-PCV2 rabbit hyperimmune serum with PCV chimeras reveal an additional immunoreactive region within amino acids 63 to 84 on the capsid protein. The collective reactivities of seven MAbs and the rabbit hyperimmune serum with the PCV chimeras indicates that the immunodominant

epitopes encompassing amino acids 47 to 84, 165 to 200 and the last four amino acids at the C-terminus of the PCV2 capsid protein.

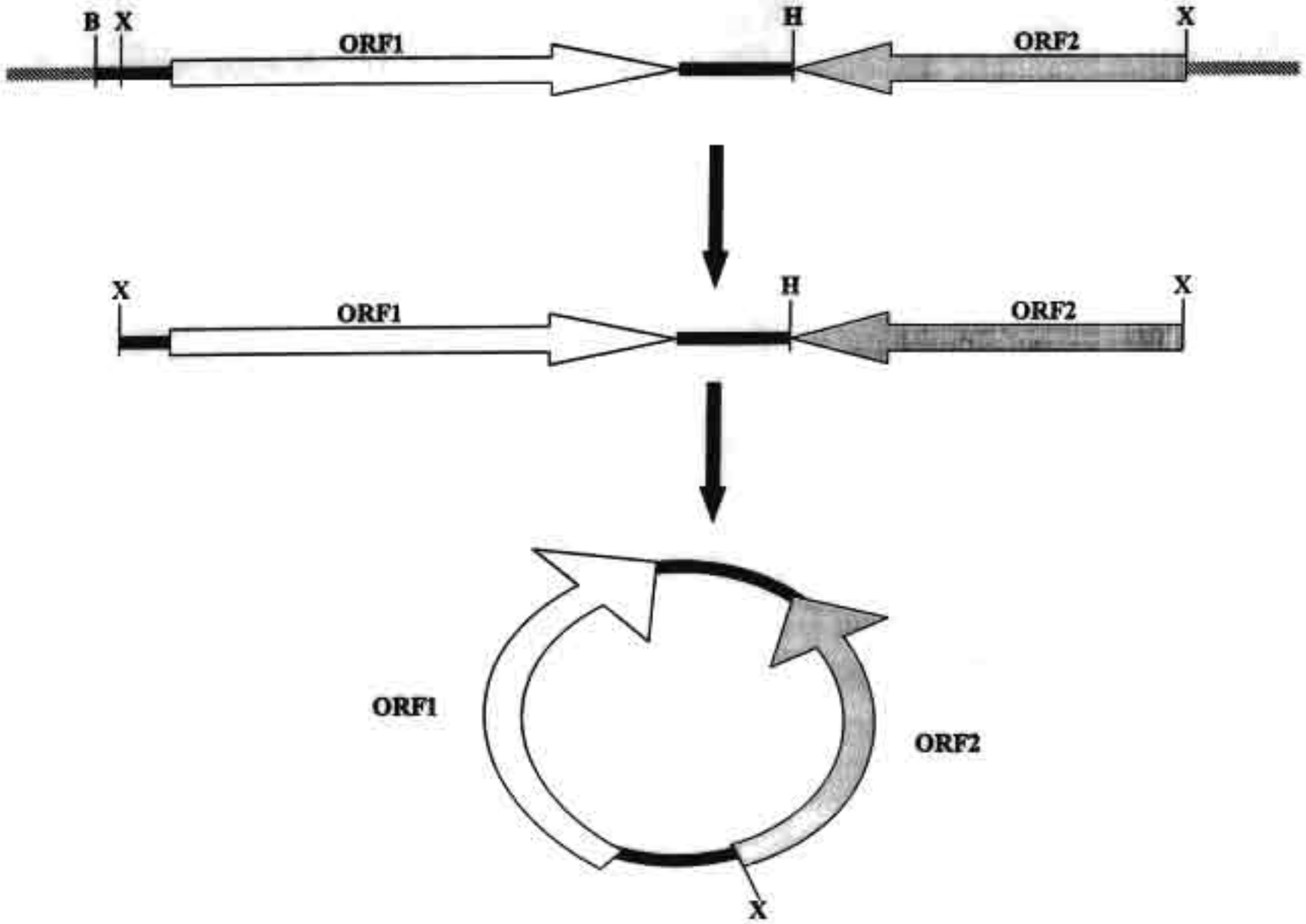
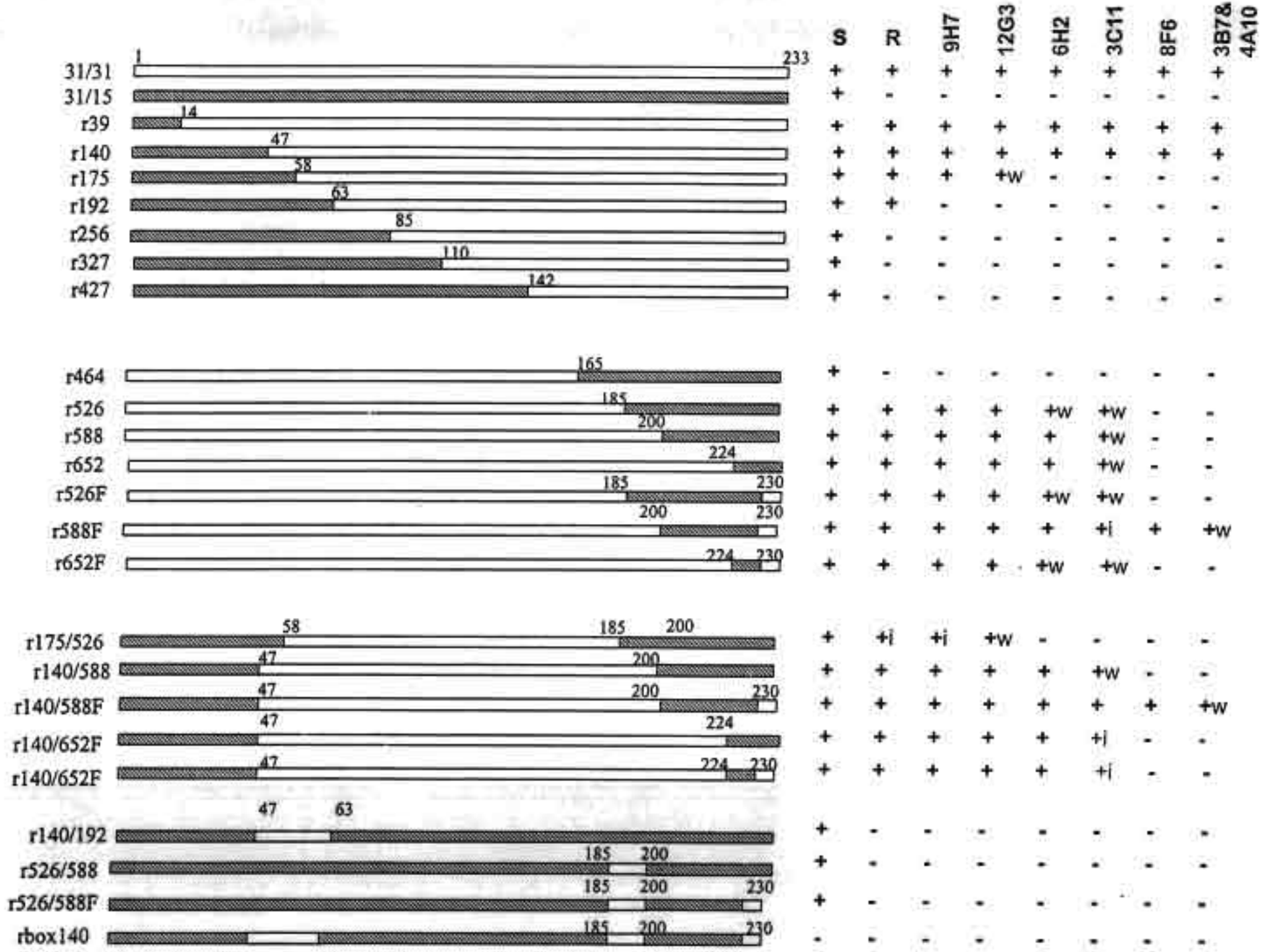


Figure 1


S
R
9H7
12G3
6H2
3C11
8F6
3B7&
4A10
31/31 1 233 + + + + + + + +
31/15 + - - - - - - -
r39 14 + + + + + + + +
r140 47 + + + + + + + +
r175 58 + + + +w - - - -
r192 63 + + - - - - - -
r256 85 + - - - - - - -
r327 110 + - - - - - - -
r427 142 + - - - - - - -
r464 165 + - - - - - - -
r526 185 + + + + +w +w - -
r588 200 + + + + + +w - -
r652 224 + + + + + +w - -
r526F 185 230 + + + + +w +w - -
r588F 200 230 + + + + + +i + +w
r652F 224 230 + + + + +w +w - -
r175/526 58 185 200 + +i +i +w - - - -
r140/588 47 200 + + + + + +w - -
r140/588F 47 200 230 + + + + + + + +w
r140/652F 47 224 + + + + + +i - -
r140/652F 47 224 230 + + + + + +i - -
r140/192 47 63 + - - - - - - -
r526/588 185 200 + - - - - - - -
r526/588F 185 200 230 + - - - - - - -
rbox140 185 200 230 - - - - - - - -

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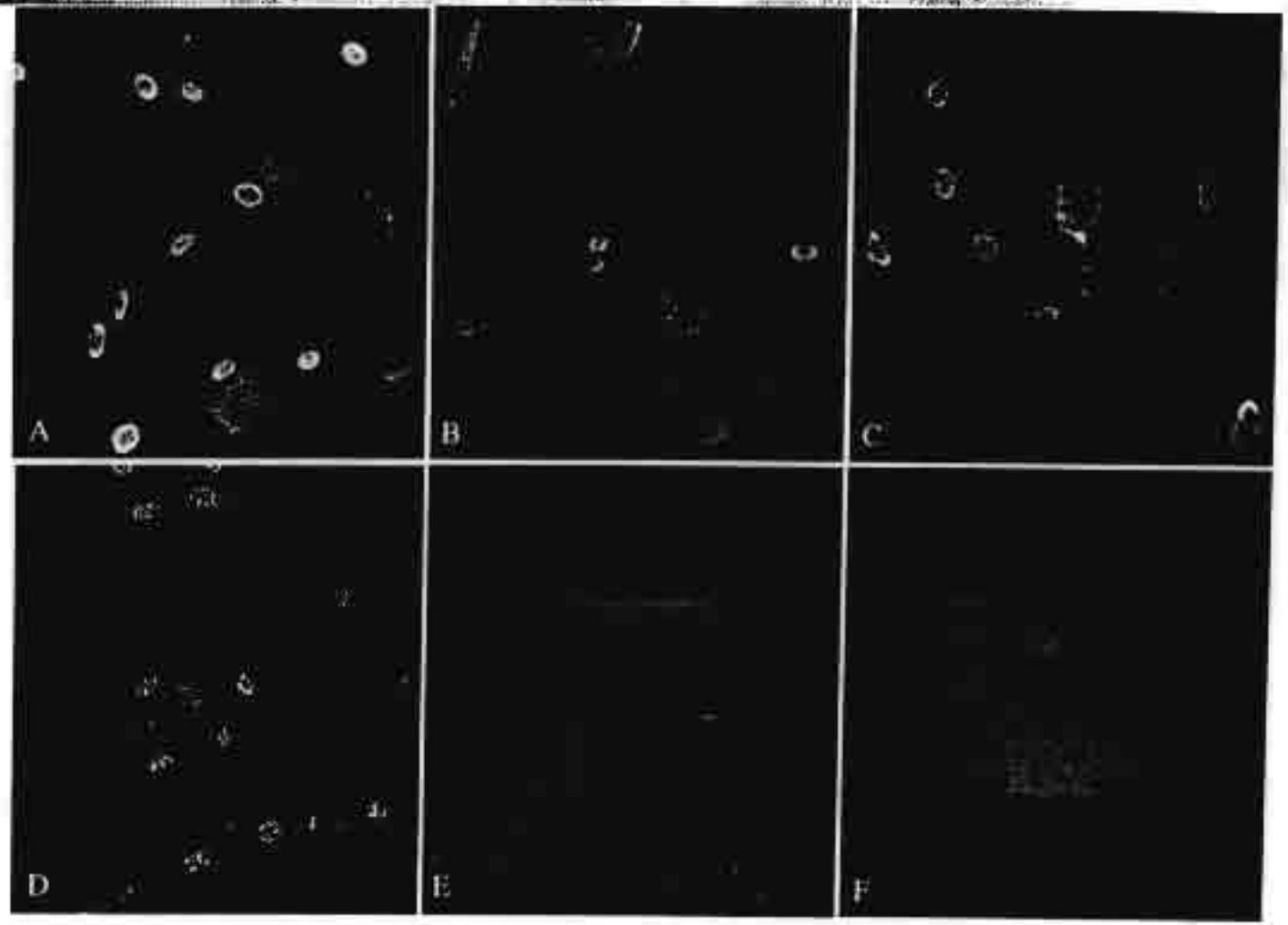
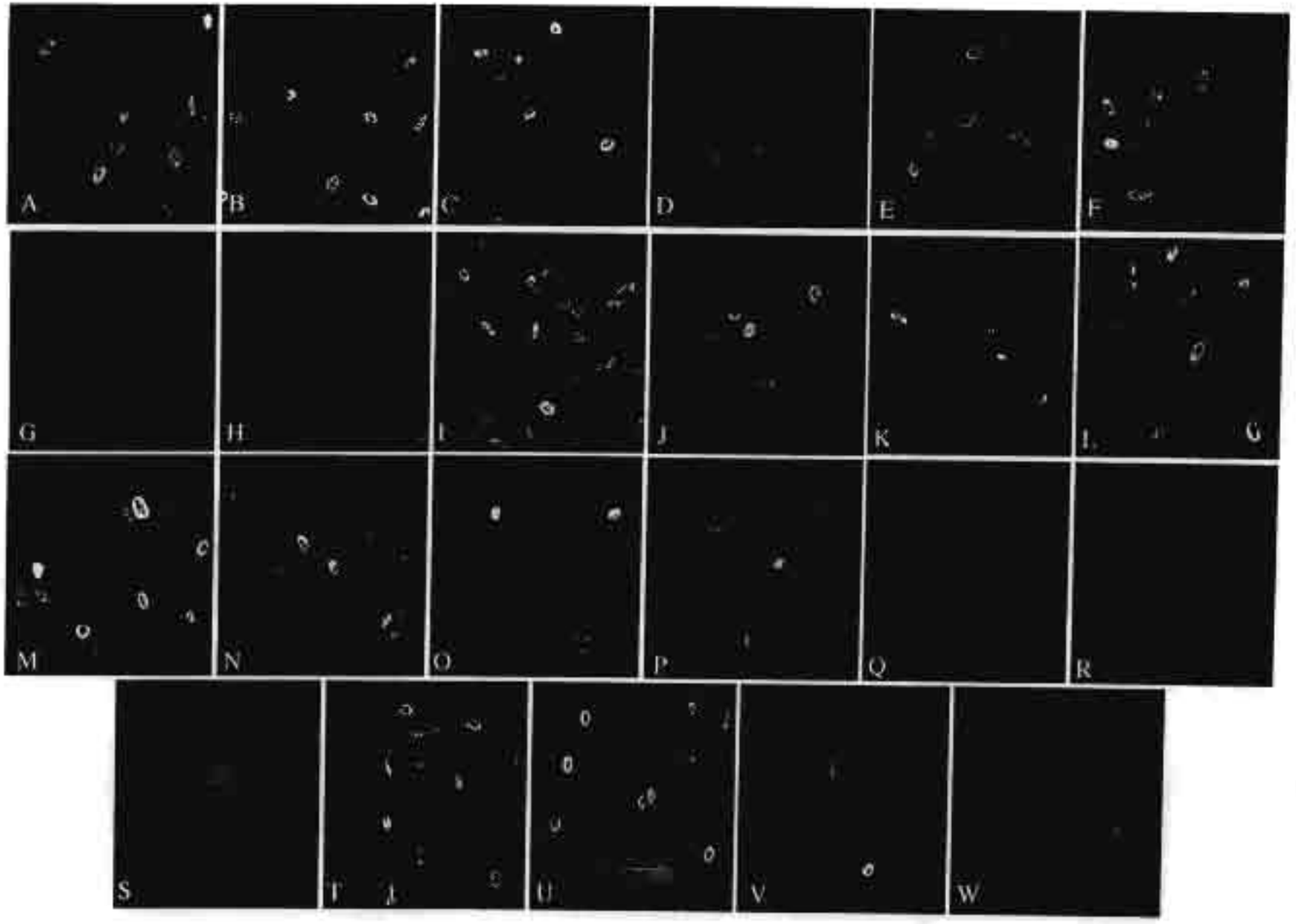
                       ▾r39                                        ▾r140
ISU-31ORF2   ..Y....... ..H....... Q......W.V ..RH.Y.W.R KN--....T.     48
PK-15 ORF2   ..W....... ..T....... N......Y.A ..AF.N.Y.W RRKT....S.     50
Consensus    MT.PRRRYRR RR.RPRSHLG .ILRRRP.L. HP..R.R.R. ....GIFN.R     50
                      ▾r175▾r192                   ▾r256
ISU-31ORF2   ..RT.GY.V. ATTVRT...A .DMMR...DD .V..G....K ISI..E....     98
PK-15 ORF2   ..TE.VL.I. G-GYSQ...N .NYLK...GQ .L..S....P LPL..Q....     99
Consensus    LS..F..T.K ......PSW. V....FNI.. F.PP.GGTN. ...PF.YYRI    100

ISU-31ORF2   ..V.V..W.C S...QGD... ...A....D. ...KA.A.T. ...V......    148
PK-15 ORF2   ..A.Y..Y.R D...SNQ... ...V....A. ...PS.N.A. ...I......    149
Consensus    RK.K.EF.P. .PIT...RGV GST.VILD.N FVT..T.L.Y DPY.NYSSRH    150
                        r464▾                 r526▾        r588▾
ISU-31ORF2   ..P...S... .......V.. S...Y.Q... .......R.Q .SR..D.V..    198
PK-15 ORF2   ..R...T... .......E.. Q...W.H... .......H.N .HT..E.T..    199
Consensus    TI.QPF.YHS RYFTPKP.LD .TID.F.PNN KRNQLWL.L. T..NV.H.GL    200
                                    r652▾      ▾F
ISU-31ORF2   .T.FE.SKYD .D.NI.V.M. ......N... .P.KP             233
PK-15 ORF2   .Y.LQ.AATA .N.VV.L.I. ......I... .-.NK             233
Consensus    G.A..N.... Q.Y..R.T.Y VQFREF.LKD P.L..             235
```

Figure 3


A
B
C
D
E
F


A
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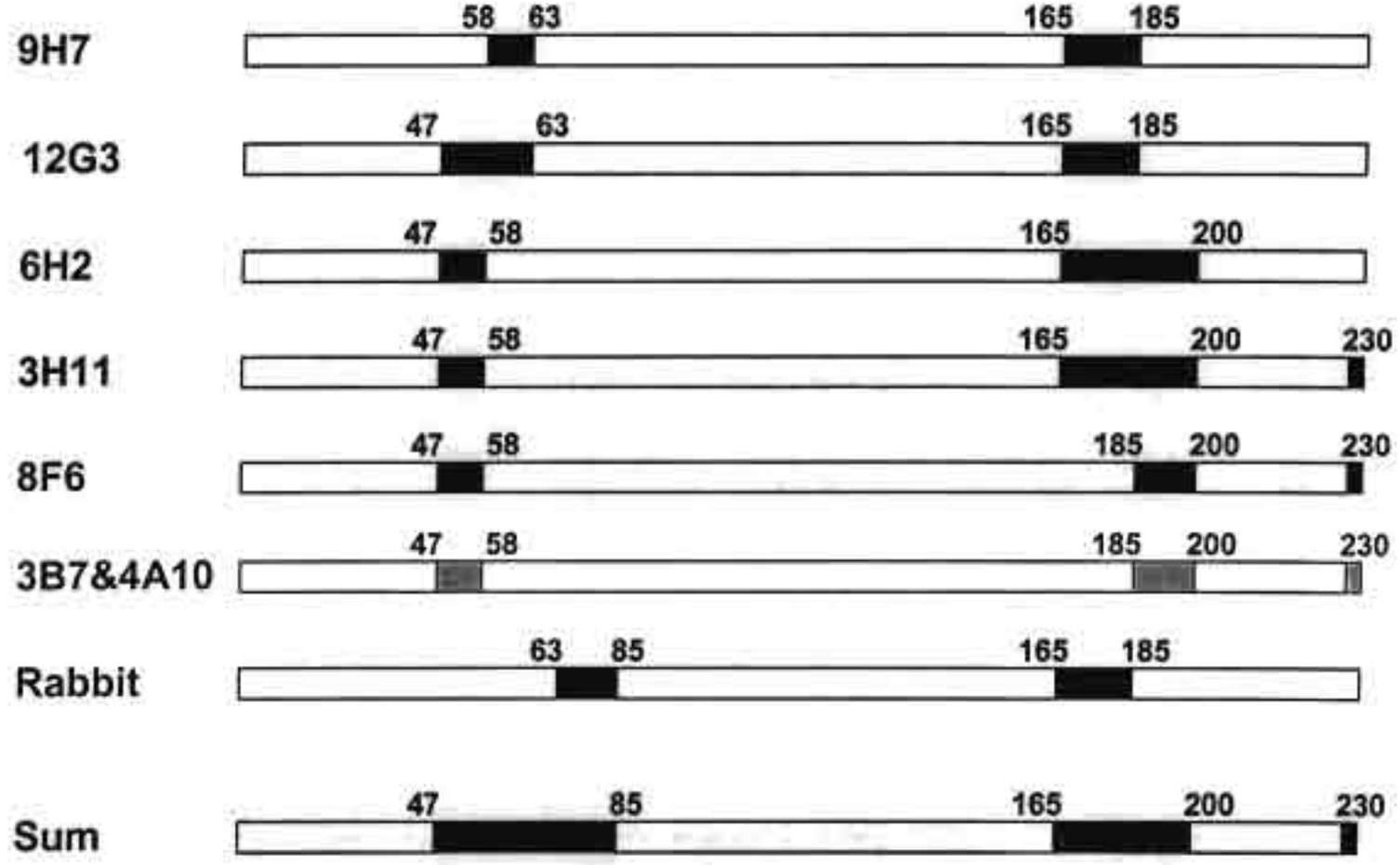
9H7
58
63
165
185
12G3
47
63
165
185
6H2
47
58
165
200
3H11
47
58
165
200
230
8F6
47
58
185
200
230
3B7&4A10
47
58
185
200
230
Rabbit
63
85
165
185
Sum
47
85
165
200
230

Figure 6