The *cis*-responsive element of Foot-and-mouth disease virus interacts with host cellular factor PCBP2 dependent on host specificity

Jeong A Jang[1], Bok Kyung Ku[2], and JaeHun Cheong[1]*,

[1] *Department of Molecular Biology, Pusan National University, Busan 46241, Republic of Korea.*

[2] *Foot-and-Mouth Disease Research Division, Animal and Plant Quarantine Agency, Gimcheon, Gyeongsangbuk-do 39660, Republic of Korea.*

*Corresponding authors: JaeHun Cheong, Ph.D.
Tel: 82-51-510-2277, Fax: 82-51-513-9258
E-mail: molecule85@pusan.ac.kr

Abstract

I. Background: Foot-and-mouth disease virus (FMDV) is a highly contagious viral pathogen in cloven-hoofed animal including cattle and pig, yet progress in the molecular mechanisms of FMDV genome replication is notably lagging behind that for many RNA viruses. A positive single stranded RNA of FMDV encodes a single long open reading frame flanked by a long 5'-untranslated region (5'UTR) and a short 3'-UTR. The *cis*-responsive element (CRE) of 5'UTR is critical for FMDV genome replication.

II. Methods and Results: Here, we described that poly(C)-binding protein 2 (PCBP2) is revealed as a CRE-binding cellular factor. The RNA immunoprecipitation experiment confirmed that the FMDV CRE interacts with PCBP2 protein. CRE derived from FMDV infection in pig bound stronger to PCBP2 protein of pig than cattle PCBP2, showing host specific RNA-protein interaction. In addition, PCBP2 interacts with FMDV 3B protein together with CRE. The interaction of PCBP and 3B protein with CRE also showed host-specific manners.

III. Conclusions: These data suggest that cellular PCBP2 may serve as a host cellular factor of FMDV to facilitate viral replication through interaction with the viral genome and contribute to determine host susceptibility of FMDV variants. The inter-molecular interaction between cellular PCBP2 and FMDV 3B and CRE provides perspectives for antiviral strategy.

Key words: FMDV, CRE, PCBP2, RNA-protein interaction, viral replication

Declarations

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Conflicts of interest/Competing interests

The authors declare that they have no conflicts of interest with the contents of this article.

Availability of data and material

Not applicable

Code availability

Not applicable

Author's contributions

Jeong A Jang: Methodology, Validation, Investigation, Resources

Bok Kyung Ku: Validation, Investigation, Resources, Funding acquisition

JaeHun Cheong: Conceptualization, Resources, Methodology, Writing-Original Draft

Ethics approval

All authors agree to ethics approval.

Consent to participate

All authors agree to participate.

Consent for publication

All authors agree to publication.

Introduction

The genomes of FMDV encode the viral proteins required for viral genome replication and contain *cis*-acting RNA sequences and structures at the end of 5' and 3' of the genome structure [1] Host intracellular factors that interact with the RNA elements are critical for successful maintenance of the virus life cycle and this functional interaction between host factors and viral RNA elements can be applied to manipulate the introduction of mutations into the viral genome for approach to rational attenuation [2].

The 5'-UTR plays a critical role in initiation of replication and translation of the FMDV genome [3]. FMDV RNA, which consists of a long 5'-UTR containing over 1,300 nucleotides (nt), shows extensive secondary structure with individual five regions. Cis-acting replication element (CRE), which is one of 5'-UTR, is a highly conserved stem-loop of 55 nt length. CRE is essential for FMDV genome RNA replication. Different picornavirus RNAs also have this element with a conserved "AAACA" motif in the loop region [4]. This element structure acts as the template for uridylylation of 3B (VPg) protein to produce 3BpU and /or 3BpUpU by the RNA polymerase 3D, providing a platform as a primer that initiates FMDV RNA synthesis [5]. The 3B uridylylation reaction requires that 3B binds to 3D and 3D RNA polymerase transfers UMP to 3B [6]. The interactions between 3B and 3D only reflect a partial aspect of the formation of the 3B uridylylation complex. The combination of protein–protein of 3B and 3D and protein–RNA interactions of 3B and CRE facilitate the formation of a functional 3B-3D replication complex. However, the direct interaction between 3B and 3D is not fully sufficient for the formation of the complete replication complex. The efficient formation of 3B-3D replication complex with CRE RNA substrate may require additional host cellular factors. To date, our understanding of host cell factors that are involved in the FMDV life cycle is limited.

PCBP2 (hnRNP E2), a host cellular protein known to bind to the 5'UTR of the poliovirus genome, is involved in switching between viral translation and replication [7]. PCPB2 is a member of the cellular heterogeneous nuclear ribonucleoprotein (hnRNP) family, which is expressed in both the nucleus and cytoplasm [8]. hnRNPs are well known for their abilities to bind to cellular proteins and RNAs to facilitate many biological processes, such as mRNA stabilization, transcriptional regulation, translational control, and apoptotic program activation.

In this study, we used RNA-protein interaction assay to identify PCBP2 interacts with the

CRE RNA differentially dependent on host species of pig and cattle. Usage of a distinct host cell factor may provide additional targets for host susceptibility discrimination of FMDV. The CRE of FMDV functions as a platform to recruit viral and cellular proteins, which regulates viral RNA replication. Some of the host factors regulate FMDV RNA replication either by participating in the formation of RNA replication complex or binding to viral RNA. Here, we demonstrate that PCBP2 serves as a host cellular factor to facilitate the formation of FMDV RNA replication complex through interaction with CRE RNA and 3B protein.

Materials and Methods

Cell culture

IBRS-2 (pig kidney epithelial cell) and MDBK (cattle kidney epithelial cell) were obtained from Korea Animal and Plant Quarantine Agency) and maintained in Dulbecco's modified Eagle's medium (DMEM) with 5% heat-in-activated fetal bovine serum (FBS; GIBCO BRL, grand island, NY, USA) and 1% (v/v) penicillin-streptomycin (PS; GIBCO BRL) at 37℃ in a humidified atmosphere containing 5% CO_2.

FMDV stock production

Viruses were isolated from a porcine cell line (LFBK, porcine kidney) or a goat cell line (ZZR-127, fetal tongue epithelium), which were obtained from the ATCC (LGC Standard). To produce an amplified stock of inoculum for viral serotypes A and O, two steers per serotype were inoculated with plaque forming units (PFU) of field-collected vesicular fluid by an intraepithelial lingual route. Vesicular fluid was collected in tubes and an equal volume of MEM with 25 mM HEPES was added to each tube before freezing at -70°C. After harvest, vesicular fluids were thawed, pooled, diluted 1:50 in MEM with 25 mM HEPES, clarified, filtered using a 0.45 μm filter, aliquoted, and stored at -70°C. One aliquot of each viral stock was subsequently thawed for titration in cells and animal inoculation.

RT-PCR and FMDV RNA sequencing

FMDV RNA was extracted using an automatic RNA extraction machine (MagNA Pure 96, Roche) according to the manufacture's instruction. The RNA was stored at -70℃ until use. The cDNA was synthesized using the PrimeScript™ II 1st strand cDNA Synthesis Kit (TAKARA). In brief, a 10 µL reaction mixture was prepared containing 10 mM dNTP, 1 µL of oligo dT primer (50 µM), 3 µL RNase Free dH_2O and 5 µL of viral RNA. The mixture was incubated for 5 min at 65°C then cooled immediately on ice. Next, the reaction was mixed with 10 µL of a second reaction mixture containing 5X PrimeScript II buffer, 0.5 µL of 40U/µL RNase inhibitor, 1 µL of enzyme, and RNase-free dH_2O. The mixture was then incubated at 42°C for 45 min and then 70°C for 15 min. The entire genome was amplified using AccuPower® ProFi Taq PCR PreMix (BIONEER, Korea) according to the manufacturer's instructions with 9 overlapping pairs of FMDV-specific primers. RT-PCR products were analyzed by QIAxcel (Qiagen). Purified PCR products were either sequenced directly or after cloning into the pGEM-T easy vector (Promega, USA). DNA sequencing was performed using automatic DNA sequencer (ABI3730) using the BigDye Terminator v3.1 cycle sequencing kit (ABI, USA). Analysis of sequence identity and divergence was carried out using BioEdit software (version 7.2.5.). PCR product sequences were assembled with SeqMan Pro software (DNASTAR, Inc., Madison, WI, USA) using default parameters.

Preparation of CRE RNA of FMDV

The regions of interest, namely nucleotides 622-687 of the FMDV genome, were amplified using PCR with the FMDV cDNA clone. T7 promoter sequence was conjugated the 5'- end of viral RNA specific sequence for the forward primer. A control RNA was applied to PCR amplification using the primers VP3 coding region (2630-CGTGCAGCGACGGTTACGGCGGTCTGGTGACCA) and (2730-CCCGGGCAACATGTTGCGAGGGGGGTTAA). The PCR DNA were purified in gels and subjected to *in vitro* transcription using T7 RNA polymerase. The *in vitro* transcription reactions were carried out in the buffer containing 40 mM Tris (pH 8.0), 40 mM DTT, 6 mM $MgCl_2$, 0.5 mM NTPs, 500 ng of PCR product, 2.5 µg T7 polymerase, and 100 units of RNasin in 100 µl volume) at 37°C for 4 hours. The purified RNA from gel was dissolved in RNA storage buffer for store until use. For checking the RNA integrity prior to use, we checked it

by PAGE.

Preparation of RNA affinity beads

150 μg RNA was covalently coupled to cyanogen bromide (CNBR)-activated Sepharose beads. Preswollen CNBR-activated Sepharose beads (Sigma) 150 μl was mixted with 100 μg of RNA at 4°C for 12 hrs. After reaction, the beads were washed three times with buffer (50 mM Tris (pH 8.0), 50 mM KCl, 5 mM MgO-acetate, 125 mM NaCl, 2 mM DTT, 10% glycerol) and stored for RNA-protein interaction assay.

RNA immunoprecipitation

Cells in a 60-mm culture dish were washed thrice with 1 ml of ice-cold PBS and lysed in 100 μl of lysis buffer (25 mM Tris-HCl [pH 7.4] containing 150 mM NaCl, 1% NP-40, 1 mM EDTA, and 5% glycerol) supplemented with a protease inhibitors cocktail at 4°C. Cell lysates were incubated at 4°C for 30 min, and centrifuged at 12,000 x *g* for 15 min. The lysates of 1.8 x 10^6 IBRS-2 cells, a pig cell line previously shown to be highly permissive for FMDV replication, were reacted with 100 ng of CRE RNA. The cell lysate containing 300 μg total protein was incubated with protein G beads precoated with the indicated antibodies at 4°C overnight. The cells lysates were diluted to a concentration of 2 μg/μl, and the protein-RNA complexes binding to beads were eluted in lysis buffer at 70°C for 45 min. RNA was extracted from the precipitate complex using TRIzol reagent and reverse-transcribed by PrimeScript RT reagent kit gDNAEraser (Perfect Real Time) (TaKaRa). SYBR RT-PCR kit (Roche) was used for real-time qRT-PCR analysis. The protein from the precipitate complex was boiled in 2x Laemmli sample buffer and then subjected to SDS-PAGE.

Results

PCBP2 interacts with the CRE RNA of FMDV

The RNA structures present in CRE play a role in the FMDV life cycle and viral replication. Mutational analysis of the RNA structures at the CRE has indicated that the stem-loop structures are essential for initiation of FMDV RNA replication. FMDV 3B protein binds to CRE at the priming stage of FMDV RNA replication. In addition to 3B protein, host cellular protein might involve the formation of CRE-3B complex and lead to efficient initiation of RNA replication. CRE RNA (O/JC/SKR/2014/P, which was derived from FMDV-infected pig in Korea at 2014) was prepared and coupled to Sepharose beads with CNBR activation (Fig. 1A). We used VP3 gene of FMDV capsid protein as a control RNA or Sepharose beads only. The RNA-coupled beads were incubated with cytoplasmic lysates of IBRS-2 cells. Since PCBP2 was known to bind to the 5'UTR of the poliovirus, which is one of picornaviruses, genome, the interacting cellular proteins with CRE RNA were loaded in gels of SDS-PAGE and confirmed by immunoblotting with specific antibody to PCBP2 protein. As shown in Figure 1B, PCBP2 protein was identified in the CRE-bound fraction, not at the non-specific RNA (VP3) bound fraction and control IgG immunoblotting. This result indicates that PCBP2 is a host cellular protein interacting CRE RNA of FMDV.

Different PCBP2 derived from cattle and pig show differential binding to CRE dependent on host specificity

In other study, we identified different RNA secondary structures of CRE along with different host species of cattle and pig. We compared the amino acid variation of PCBP2 protein between cattle and pig. As shown in Fig. 2A, the amino acid composition of pig PCBP2 was different at one position with cattle one. Pig PCBP2 protein has an additional four amino acids (TLSQ) at position from 169 to 172 compared with cattle one (Fig. 2A).

To analyze the differential interaction of pig-derived PCBP2 and cattle one to the CRE RNA, we detected the direct interaction between two different PCBP2 proteins and FMDV CRE RNA. We performed RNA immunoprecipitation (RIP) of CRE RNA and the lysate of PCBP2-transfeced IBRS-2 cells. The precipitant complex was analyzed by RT-PCR and immunoblotting. After RT-PCR detection using primers specific to FMDV CRE, PCBP2 protein enriched in RNA-protein complexes was confirmed by immunoblotting. As shown in

Figure 2B, pig-derived PCBP2 bound stronger to CRE RNA (O/JC/SKR/2014/P) than cattle one. These results indicated that the intermolecular interaction affinity of CRE RNA and PCBP2 protein showed host specificity.

To further verify whether PCBP2 differentially binds to CRE RNA dependent on host species, we performed the reverse way of experiment done by Figure 2B using two CRE RNAs (from cattle and pig). In the experiment of Figure 3, we applied two different CRE RNAs from O/JC/SKR/2014/P (pig) and A/PC/SKR/2010/C (cattle). There were eight different nucleotides between two CRE RNAs (Fig. 3A). Using the two CRE RNAs, we applied RIP experiment in the presence of pig PCBP2 protein expression in IBRS-2 cells (Fig. 3B). CRE RNA derived from pig infection (O/JC/SKR/2014/P) showed a strong intermolecular interaction with pig-PCBP2 compared to CRE RNA from FMDV infection of cattle (A/PC/SKR/2010/C). Next, we tried an alternative experiment of Figure 3B using cattle PCBP2 in MDBK cells (Fig. 3C). Contrasting with Fig. 3B, the same CRE RNA from pig infection, which was used Fig. 3B, bound weakly to cattle PCBP2. However, the CRE RNA of A/PC/SKR/2010/C (cattle infection) strongly interacted with cattle PCBP2 (Fig. 3C). These results indicate that the specificity of intermolecular interaction between CRE RNA and host cellular protein PCBP2 might contribute to host susceptibility of FMDV replication.

PCBP2 interacts with FMDV 3B protein together with CRE

The 5'UTR of FMDV functions as a platform to recruit viral and cellular proteins and not only directs IRES-dependent protein synthesis but also plays roles in CRE-dependent RNA replication. For the efficient formation of FMDV replication complex including CRE RNA and FMDV 3B protein, PCBP2 might associate with not only CRE RNA but also 3B protein. To verify the protein-protein interaction of PCBP2 and FMDV 3B proteins, we performed co-immunoprecipitation after 3B gene transfection in IBRS-2 cells. The His-3B gene transfected cell were harvested and immunoprecipitated with anti-His antibody. PCBP2 protein from the precipitate complex was detected by immunoblotting with the PCBP2 antibody. As shown in Fig. 4A, PCBP2 protein was detected in the FMDV 3B-immunoprecitated cell extract. In order to further confirm whether PCBP2 binds to CRE RNA together with 3B protein, the 3B gene-transfected cells were harvested and immunoprecipitated with control IgG or anti-PCBP2 antibody in the presence of CRE RNA. CRE RNA was obtained from the precipitated complex

and subjected to RT-PCR analysis. Immunoprecipitated 3B protein was determined by immunoblotting using anti-His antibody. In the presence of pig PCBP2, pig infection-derived CRE recruited 3B protein stronger than cattle infection-derived CRE (Fig. 4B). These results indicate that formation of the functional replication complex of CRE RNA, 3B protein and PCBP2 is associated with host species susceptibility.

Discussion

FMDV genome uses its RNA for the translation of viral proteins and forms conserved cis-acting RNA structures required for various stages of the viral multiplication. The distinct secondary structures of non-coding RNA are associated with many important roles, including regulating encapsidation, replication, viral translation, and viral pathogenesis. Positive-strand RNA viruses, including FMDV, amplify their genomes to hundreds of copy numbers in a short time. The viral replication efficiency is attributed to the characteristics of the viral replication components. Initial stage of viral replication starts translation of viral proteins directly by the viral genomic RNA. Subsequently, complementary negative strand RNA of FMDV is generated from the 3'-end and next a positive strand RNA can be transcribed [9]. For viral multiplication, both the viral replication machinery and the translation apparatus should are necessary to recognize the viral genomic RNA. The regulation between these processes is very important and may be affected by various host factors interacting with the viral RNA or viral proteins. Recent report shows that host cellular proteins as well as viral factors play a key role in the replication of positive-strand RNA viruses [10].

We identified a potential factor PCBP2 that bind to the replication region, CRE, of the FMDV 5' UTR. PCBP2 previously was known to interact with the 5' UTR of the HCV genome [11]. Signals required for the replication and translation of positive-strand RNA viruses usually are located in the 5'- and 3'-terminal regions of the viral RNA. The sequences required for RNA replication and translation often overlap, and the regulatory mechanisms can be separated or shared. Some research indicates that the role of PCBP2 in HCV is to mediate IRES-

dependent translation [12]. Also, a study showed that the HCV RNA level was reduced by PCBP2 short interfering RNA (siRNA) knockdown [13].

PCBP2 also functioned in RNA replication and viral protein translation of poliovirus [14-16]. Furthermore, PCBP2 regulated the transition of poliovirus translation into viral genome replication [17]. PCBP2 binding to the IRES of poliovirus increased the initiation of translation of poliovirus, resulting in connection of RNA to the ribosomes. The process of poliovirus RNA polymerase in the initiation of negative strand RNA elongation was prohibited by ribosomes on viral RNA. After PCBP2 was cleaved by the accumulated viral proteinase 3CD, the ribosome-IRES binding was inhibited. The translation process was stopped, but the cleaved form of PCBP2 still could increase the interaction of the 3' poly(A) tract with the 5' RNP complex in the presence of poly(A)-binding protein (PABP). Therefore, the translating ribosomes on the RNA template are cleared, and the functional interaction of 5' RNP complex and 3' poly(A) might induce the initiation of negative strand RNA replication [18].

It is possible that PCBP2 recruits other factors to facilitate the assembly of the replication complex. It is particularly interesting that it interacts with 3B, an essential protein in FMDV RNA replication. Genome circularization may be a common feature of positive-strand RNA viruses, but the molecular regulation of circularization is different from virus species to species. Specific RNA-RNA interaction is necessary for flavivirus genome circularization [19] and picornaviruses require RNA-protein interaction [20], and cRNA-RNA sequences for dengue virus [21]. Circularization of FMDV Genome might coordinate viral translation and RNA synthesis and make a platform for the viral polymerase localization at the start site of RNA synthesis (allowing the viral and cellular proteins to interact with the terminal ends of the viral genome and, in turn, initiating translation or replication efficiently). In addition, the circularization may maintain the integrity of FMDV genome. In this study, PCBP2 was found to bind to both the 5' ends of the FMDV genome and 3B protein and also formed an RNA-protein complex.

This study indicates that PCBP2 association in the replication complex of FMDV is responsible for understanding of the finer replication initiation of FMDV. Further study on the identification of interaction details of PCBP2 and CRE or/and 3B might provide the molecular targets for development of vaccine and therapeutic interventions.

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Figure Legends

Figure 1. PCBP2 interacts with CRE RNA of FMDV. (A) The RNA sequences of FMDV CRE are described and applied to RNA-protein interaction assay. The CRE sequences were derived from FMDV of Korea endemic in 2014, which was infected in pig with O genotype. (B) The interacting cellular proteins with CRE RNA were loaded in gels of SDS-PAGE and confirmed by immunoblotting with specific antibody to PCBP2 protein or control IgG. NS indicated a non-specific RNA (VP3).

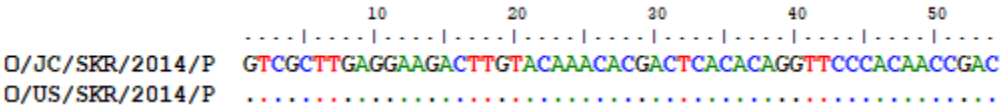
Figure 2. Different PCBP2 derived from cattle and pig binds to CRE dependent on host specificity. (A) Sequence comparison of PCBP2 was analyzed with pig (Sus scrofa), cattle (Bos Taurus), and rat (Mesocricetus auratus). The red box indicates pig-specific variation and the blue box shows rat-specific difference of amino acid composition. (B) Protein from the precipitate complex was detected by immunoblotting with the PCBP2 antibody. Purified PCBP2 protein and CRE RNA were incubated in binding buffer followed by RIP analysis. The designation of pigP2 is PCBP2 from pig and cattleP2 is cattle PCBP2 protein.

Figure 3. Different CRE RNA interacts with PCBP2 protein dependent on host species. (A) Sequence comparison of FMDV CRE is described and applied to RNA-protein interaction assay. Two CRE sequences (O/JC/SKR/2014/P and O/US/SKR/2014/P) are derived from FMDV of pig infection. One CRE sequence of A/PC/SKR/2010/C is one of cattle infection of FMDV. (B and C) IBRS-2 (B) or MDBK (C) cells were harvested and immunoprecipitated with control IgG or anti-PCBP2 antibody. Different CRE RNAs of pig or cattle-infected FMDV were applied to RIP assay.

Figure 4. PCBP2 binds to FMDV 3B protein together with CRE. (A) co-immunoprecipitation of PCBP2 and FMDV 3B proteins. The His-3B gene transfected cell were harvested and immunoprecipitated with anti-His antibody. PCBP2 protein from the precipitate complex was detected by immunoblotting with the PCBP2 antibody. (B) The cells were harvested and immunoprecipitated with control IgG or anti-PCBP2 antibody. Different CRE RNAs of pig or cattle-infected FMDV were applied to RIP assay. Finally, 3B protein was determined in the immunoprecipitated fraction by immunoblotting using anti-His antibody.

Figure 1

A


10
20
30
40
50
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O/US/SKR/2014/P

B

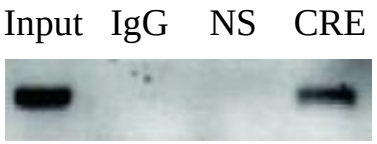
Input
IgG
NS
CRE

Figure 2

A


PCBP2 Bos taurus
PCBP2 Sus scrofa
PCBP2 Mesocricetus auratus
MDTGVIEGGLNVTLTIRLLMHGKEVGSIIGKKGESVKKMREESGARINISEGNCPERIITLAGPTNAIFKAFAMIIDKLEEDISSSMTNSTAASRPPVTL
RLVVPASQCGSLIGKGGCKIKEIRESTGAQVQVAGDMLPNSTERAITIAGIPQSIIECVKQICVVMLE----SPPKGVTIPYRPKPSSSPVIFAGGQDRY
TLSQ
STGSDSASFPHTTPSMCLNPDLEGPPLEAYTIQGQYAIPQPDLTKLHQLAMQQSHFPMTHGNTGFS----------AGLDASAQTTSHELTIPNDLI
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B

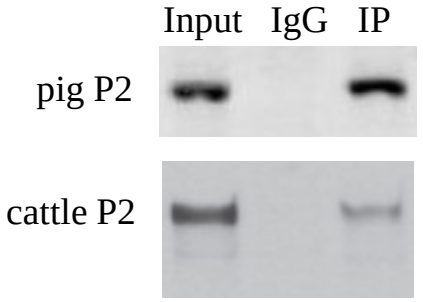
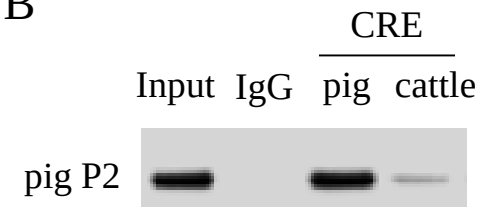

Input
IgG
IP
pig P2
cattle P2

Figure 3

A


10
20
30
40
50
O/JC/SKR/2014/P GTCGCTTGAGGAAGACTTGTACAAACACGACTCACACAGGTTCCCACAACCGAC
O/US/SKR/2014/P .....
A/PC/SKR/2010/C .....G.....TCT..G.....CT.....T...

B


CRE
Input
IgG
pig
cattle
pig P2

C

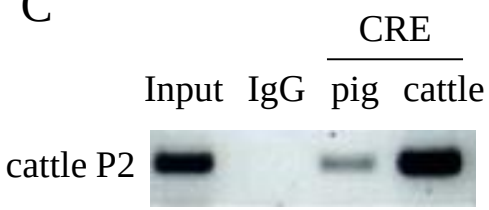
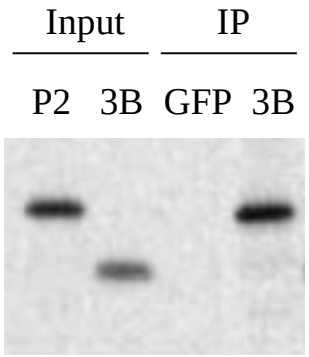
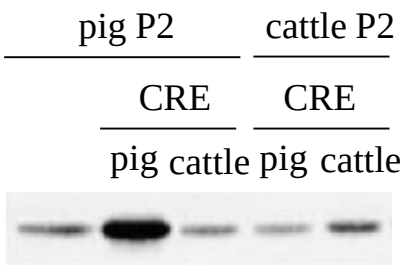

CRE
Input
IgG
pig
cattle
cattle P2

Figure 4

A


Input
IP
P2
3B
GFP
3B

B


pig P2
cattle P2
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