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SARS_CoV2 RBD gene transcription cannot be driven by CMV promoter

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ABSTRACT

Cytomegalovirus (CMV) promoter drives various gene expression and yields sufficient protein for further functional investigation. Receptor binding domain (RBD) on spike protein of the SARS_CoV2 is the most critical portal for virus infection. Thus native conformational RBD protein may facilitate biochemical identification of RBD and provide valuable support of drug and vaccine design for curing COVID-19. We attempted to express RBD under CMV promoter *in vitro*, but failed. *RBD*-specific mRNA cannot be detected in cell transfected with recombinant plasmids, in which CMV promoter governs the RBD transcription. Additionally, the pCMV-Tag2B-SARS_CoV2_RBD *trans*-inactivates CMV promoter transcription activity. Alternatively, we identified that both Chicken β-actin promoter and Vaccinia virus-specific medium/late (M/L) promoter (pSYN) can highly precede *SARS_CoV2 RBD* expression. Our findings provided evidence that *SARS_CoV2 RBD* gene can be driven by Chicken β-actin promoter or Vaccinia virus-specific medium/late promoter instead of CMV promoter, thus providing valuable information for RBD feature exploration.

COVID-19 is spreading across the world now. The confirmed case number around the world is soaring over thirty-five million so far (https://coronavirus.jhu.edu/map.html). The causative agent SARS_CoV2 belongs to *Coronavirus* family β, which also contains other two seriously infectious and highly deadly pathogens, SARS_CoV and MERS_CoV. Respiratory tract is considered the major route for rapid SARS_CoV2 transmission between human to human. However currently there are no commercial vaccine and drug preventing the ongoing disease.

Like another *Coronavirus* family β member SARS_CoV, SARS_CoV2 infects host by utilizing human cell surface angiotensin converting enzyme-2 (ACE2) as receptor as well (Zhou et al., 2020; Yan et al., 2020). Spike protein (S) on SARS_CoV2 particle associates with ACE2, initiating the spike conformational change and protein cleavage, such as S1–S2 cleavage and S2 exposing on the viron. And then, exposed fusion peptide in S2 inserts to the host cell membrane, consequently completing the fusion between virus membrane and host cell membrane (Shang et al., 2020a, 2020b). During the binding and fusion of SARS_CoV2 to the host cell, the virus attachment attracts intensive attention, most likely this entrance process being the critical hotspot that could be interfered (Xia et al., 2020a, 2020b). More specifically, receptor binding domain (RBD) on SARS_CoV2 spike is the promising target of vaccine development and drug design (Tai et al., 2020; Zhao et al., 2018; Jiang et al., 2012; Zhu et al., 2013).

Fully deciphering the precise characteristics of a protein is strictly dependent on *in vitro* expression and purification of the protein. Numerous commercial ready-for-use plasmids can be used for interest gene expression with high efficiency. A large proportion of commercial plasmids are artificially equipped with a most commonly used Cytomegalovirus (CMV) promoter and enhancer in the front of multiple cloning site (MCS), which extremely strongly directs the expression of downstream gene.

We previously attempted to construct a recombinant plasmid, in which a gene *SARS_CoV2 RBD* was at downstream of the CMV promoter, aiming to obtain sufficient RBD protein and intensively study its character. Unexpectedly, the expression of RBD cannot be detected *in vitro*. In this brief study, we provided evidence for the rare expression of RBD under CMV promoter, explored the possible reasons for the incapability of expression for *RBD* gene, and gave two alternative workable expression strategies for gene *SARS_CoV2_RBD*.

1. CMV promoter cannot driven *SARS_CoV2_RBD* expression

To verify the expression of *SARS_CoV2_RBD*, we constructed pCMV-SARS_CoV2_RBD-EGFP and confirmed the recombinant plasmid by sequencing. 293T in six-well plate were transfected with 2 μg of

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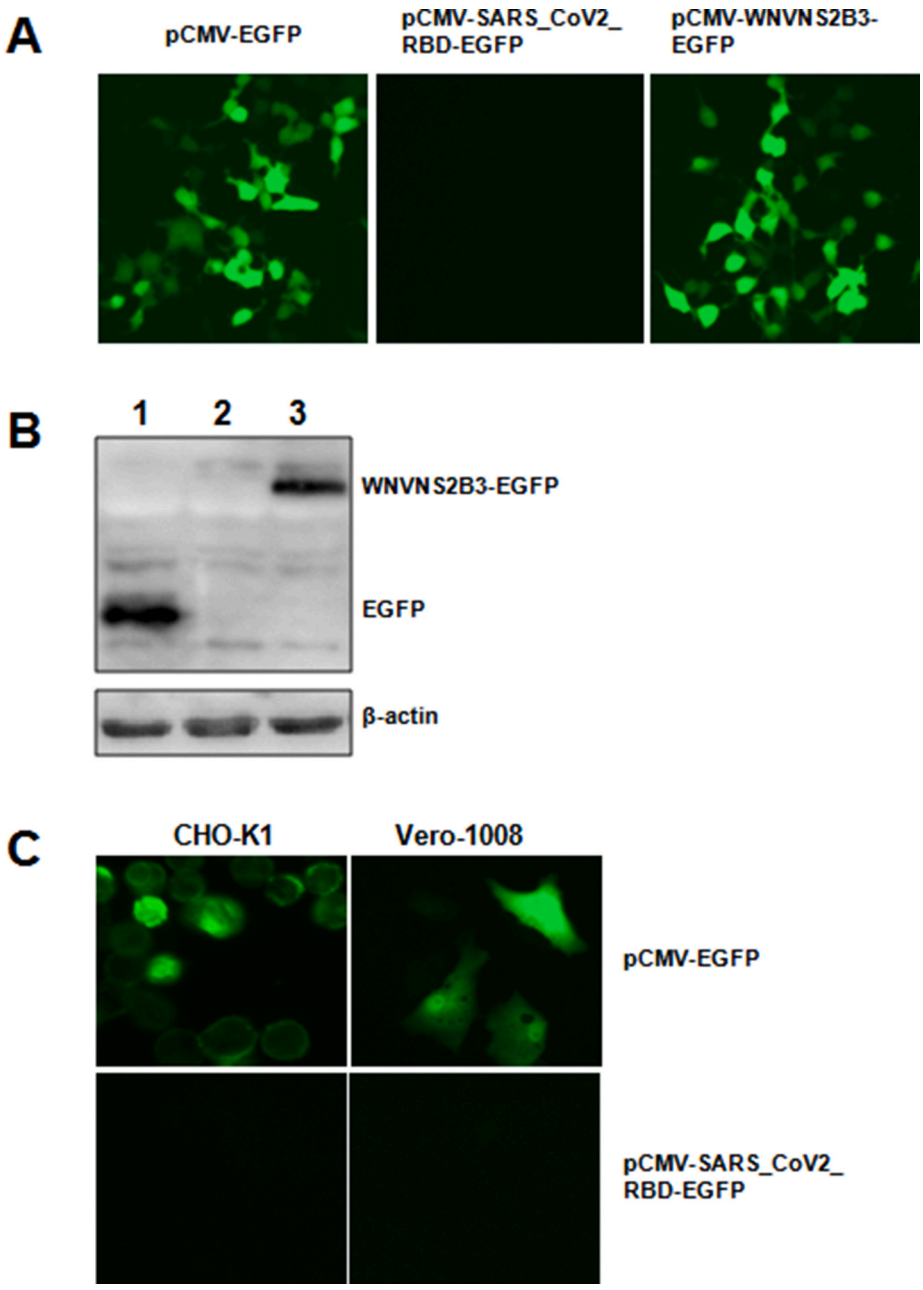

Fig. 1. CMV promoter cannot drive SARS_CoV2 RBD transcription and expression. (A) 293T cells were transfected with recombinant plasmids pCMV-EGFP, pCMV-SARS_CoV2_RBD-EGFP, and pCMV-WNVNS2B3-EGFP, respectively. At 48 h post-transfection, the cells were observed under fluorescent microscope. No green fluorescent signal was observed in cell transfected with pCMV-SARS_CoV2_RBD-EGFP. While cells transfected with pCMV-EGFP or pCMV-WNVNS2B3-EGFP yielded obvious green fluorescent signal. (B) Cell samples mentioned above were collected and subject to Western blotting. Mouse anti-EGFP served as detection antibody. Lane 1, 293T transfected with pCMV-EGFP; Lane 2, 293T transfected with pCMV-SARS_CoV2_RBD-EGFP; Lane 3, 293T transfected with pCMV-WNVNS2B3-EGFP. Resultantly, no specific band can be detected in cell sample transfected with pCMV-SARS_CoV2_RBD-EGFP. Whereas EGFP and WNVNS2B3-EGFP can be detected in cells transfected with plasmids pCMV-EGFP or pCMV-WNVNS2B3-EGFP, respectively. (C) CHO–K1 and Vero-1008 cell lines were transfected with pCMV-EGFP or pCMV-SARS_CoV2_RBD-EGFP, respectively. At 48 h posttransfection, the cells were observed under fluorescent microscope.

recombinant plasmid pCMV-SARS_CoV2_RBD-EGFP, pCMV-EGFP, or pCMV-WNVNS2B3-EGFP (containing the West nile virus *NS2B3* gene), respectively. At 48 h posttransfection, the cell fluorescent signal were observed under microscope. As shown in Fig. 1A, green fluorescent signal was not able to be detected when pCMV-SARS_CoV2_RBD-EGFP was transfected. Whereas transfection of pCMV-WNVNS2B3-EGFP and pCMV-EGFP yielded obvious fluorescent signal. Then the corresponding cell samples mentioned above were collected and subject to Western blotting assay. Mouse anti-EGFP served as detection antibody. As shown in Fig. 1B, the individual EGFP and fusion protein WNVNS2B3-EGFP can be detected. With the similar method, pVAX1-SARS_CoV2_RBD and pCMV-Tag2B_SARS_CoV2_RBD, both of which harbor the same CMV promoters for driving *RBD* gene expression, were constructed and assessed the expression upon CMV promoter by transfecting into CKO–K1 or Vero 1008. Resultantly, RBD protein cannot be detected indirect fluorescent assay (IFA) (Fig. 1C). Collectively, the expression of *SARS_CoV2_RBD* gene upon CMV promoter was extremely impeded.

2. *RBD* gene-specific mRNA cannot be detected in recombinant pcmv-SARS_CoV_RBD-EGFP-transfected cells

Given that *SARS_CoV2_RBD* cannot express upon CMV promoter, the inhibitory effect may occur at two consecutive steps, namely, transcription or translation process. Therefore we next carried out experiments to assess the existence of *RBD*-mRNA in plasmid transfected cell. Briefly, 293T in six-well plate were transfected with 2 μg of recombinant plasmids pCMV-SARS_CoV2_RBD-EGFP, pCMV-EGFP, or pCMV-WNVNS2B3-EGFP, respectively. At 24 h posttransfection, transfected

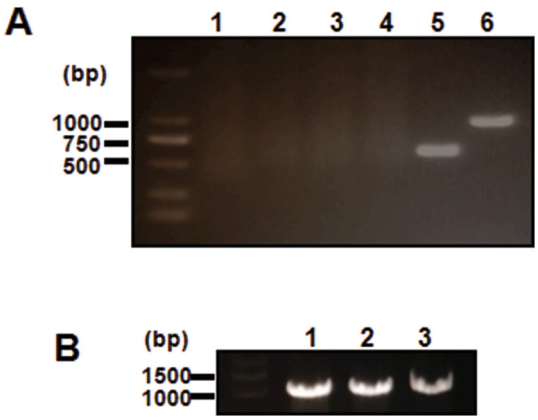

Fig. 2. No RBD-specific mRNA can be detected in pCMV-SARS_CoV2_RBD-EGFP-transfected cells. 293T cells were transfected with plasmids, pCMV-EGFP, pCMV-SARS_CoV2_RBD-EGFP, or pCMV-WNVNS2B3-EGFP, respectively. At 24 h posttransfection, the mRNAs were extracted and detected by PCR with different primer pairs for distinct genes. (A) Lane 1, 293T transfected with pCMV-EGFP for PCR detection of gene *SARS_CoV2_RBD*; Lane 2, 10-circle PCR product of gene *SARS_CoV2_RBD* using cDNA as template from 293T transfected with pCMV-SARS_CoV2_RBD-EGFP; Lane 3, 20-circle for gene *SARS_CoV2_RBD*; Lane 4, 30-circle for gene *SARS_CoV2_RBD*; Lane 5, plasmid pCMV-SARS_CoV2_RBD-EGFP served as template for gene SARS_CoV2_RBD multiplication; Lane 6, gene *WNVNS2B3* detection using cDNA as template from 293T transfected with pCMV-WNVNS2B3-EGFP. (B) 293T cells transfected with pCMV-EGFP (Lane 1), pCMV-SARS_CoV2_RBD-EGFP (Lane 2), or pCMV-WNVNS2B3-EGFP (Lane 3), respectively. *β-Actin* genes were detected by PCR as control in above three groups. Unless otherwise specified, PCR process ran thirty circles.

cells were collected and subject to mRNA extraction and subsequent reverse transcription. The *RBD* gene open reading frame (ORF) was amplified using cDNA as template by PCR. As shown in Fig. 2, the *RBD* gene cannot be detected even at up to 30 times' replication. Whereas as for the plasmid pCMV-WNVNS2B3-EGFP, the *WNVNS2B3* gene was able to be detected by PCR following transfection. Therefore *RBD* gene may not be transcripted by CMV promoter, indicating the very low content of mRNA of *RBD* should be the crucial reason for the absence of RBD protein in pCMV-SARS_CoV2_RBD-EGFP-transfected cells.

3. pCMV-Tag2B-SARS_CoV2_RBD *trans*-inactivates CMV promoter activity

Since the CMV promoter lost its preceding activity for exogenous gene transcription when followed with gene *SARS_CoV2 RBD*, it was warrant investigating whether the gene *SARS_CoV2 RBD* or the corresponding protein RBD would *trans*-inactivate CMV promoter activity. 293T in six-well plate were cotransfected with 2 μg of plasmids pCMV-EGFP, along with pCMV-Tag2B, pCMV-Tag2B-SARS_CoV2_RBD, or pCMV-Tag2B-WNV_NS2B3, respectively. At 48 h postcotransfection, cells were collected and subject to Fluorescence activated cell sorting (FACS) assay for analyzing the quantity of green fluorescent cell. As observed under fluorescent microscope in Fig. 3A, green fluorescent cells seemed less in cell transfected pCMV-Tag2B-SARS_CoV2_RBD compared with that transfected pCMV-Tag2B or pCMV-Tag2B-WNV_NS2B3. As shown in Fig. 3B and C, the fluorescent cell number of cell transfected with pCMV-Tag2B_SARS_CoV2_RBD decreased significantly compared with that transfected with plasmid pCMV-Tag2B-WNV_NS2B3 ($p < 0.05$) (Student's *t*-test), indicating that pCMV-Tag2B-SARS_CoV2_RBD *trans*-inactivates transcription activity of CMV promoter. Based on the above-mentioned data, we deduced that the very small quantity of RBD protein inhibited the process of other gene further transcription under CMV promoter in a feedback way.

Unexpectedly, the full length of spike gene (S) of SARS_CoV2 or the fusion formation of SARS_CoV2 RBD with human immunoglubin IgG Fc portion (RBD-hFc) can express at a very low level under the driving of CMV promoter (data not shown). In a most recently published finding, Shang revealed that RBD portion hides in the entire S protein with high frequency (Shang et al., 2020b). Therefore, we inferred that the core domain of RBD, which inhibits the process of transcription of CMV, is embedded in the full length of S protein, thus making the full S protein to express to some degree with less inhibition of RBD by the CMV promoter. Additionally, we also found that the RBD of spike protein of porcine transmissible gastroenteritis virus (TGEV) that also belongs to the *Coronavirus* family β cannot express under the CMV promoter (data not shown), thus raising a possible universal mechanism by which RBD gene expression of *Coronavirus* family β was prohibited due to the CMV promoter.

4. Chicken β-actin promoter and vaccinia virus-specific medium/late promoter introduce SARS_CoV2 RBD transcription and expression

The commercial eukaryotic expression DNA vector pCAGGS has a Chicken β-actin promoter, which precedes exogenous gene expression in MCS. *SARS_CoV2_RBD* gene was inserted into pCAGGS at MCS. 293T in six-well plate were transfected with 2 μg of recombinant plasmids pCAGGS, pCAGGS-SARS_CoV2_RBD or pCAGGS-WNVNS2B3, respectively. At 48 h posttransfection, cell samples were collected and subject to IFA and Western blotting. As seen in Fig. 4A, expression of *SARS_CoV2_RBD* were able to be easily detected. Similarly in Western blotting, as shown in Fig. 4B, the SARS_CoV2_RBD with approximately 25 kDa can be detected as expected.

Vaccinia virus-specific promoter can precede a lot of gene expression with high efficiency (Xie et al., 2019; Yu et al., 2018; Zhao et al., 2020). Similarly, the constructed plasmid pVV-M/E-SARS_CoV2_RBD contains a Vaccinia virus-specific promoter pSYN (a late promoter) and SARS_CoV2_RBD. 1×10^6 Vero-1008 in the six-well plate was infected with Vaccinia virus (Tiantan strain). At 2 h postinfection, Vero-1008 was transfected with pVV-M/E-SARS_CoV2_RBD. At 24 h posttransfection, transfected cells were collected and subject to IFA and Western blotting. As shown in Fig. 4C and D, the SARS_CoV2_RBD protein also can be detected.

We have demonstrated that the gene *SARS_CoV2_RBD* could express when constructed into plasmid pCAGGS instead of pCMV-Tag2B. Whether the promoters are the crucial element for the transcription of gene *SARS_CoV2_RBD* was not clear. The promoters between pCMV-Tag2B and pCAGGS in front of gene *SARS_CoV2_RBD* were reciprocally exchanged by overlapping PCR and ligation. Recombinant plasmid pCMV-Tag2B_SARS_CoV2_RBD with an already replaced Chicken β-actin promoter, and a pCAGGS-Tag2B_SARS_CoV2_RBD with a CMV promoter were constructed. When the above plasmids were transfected to 293T,

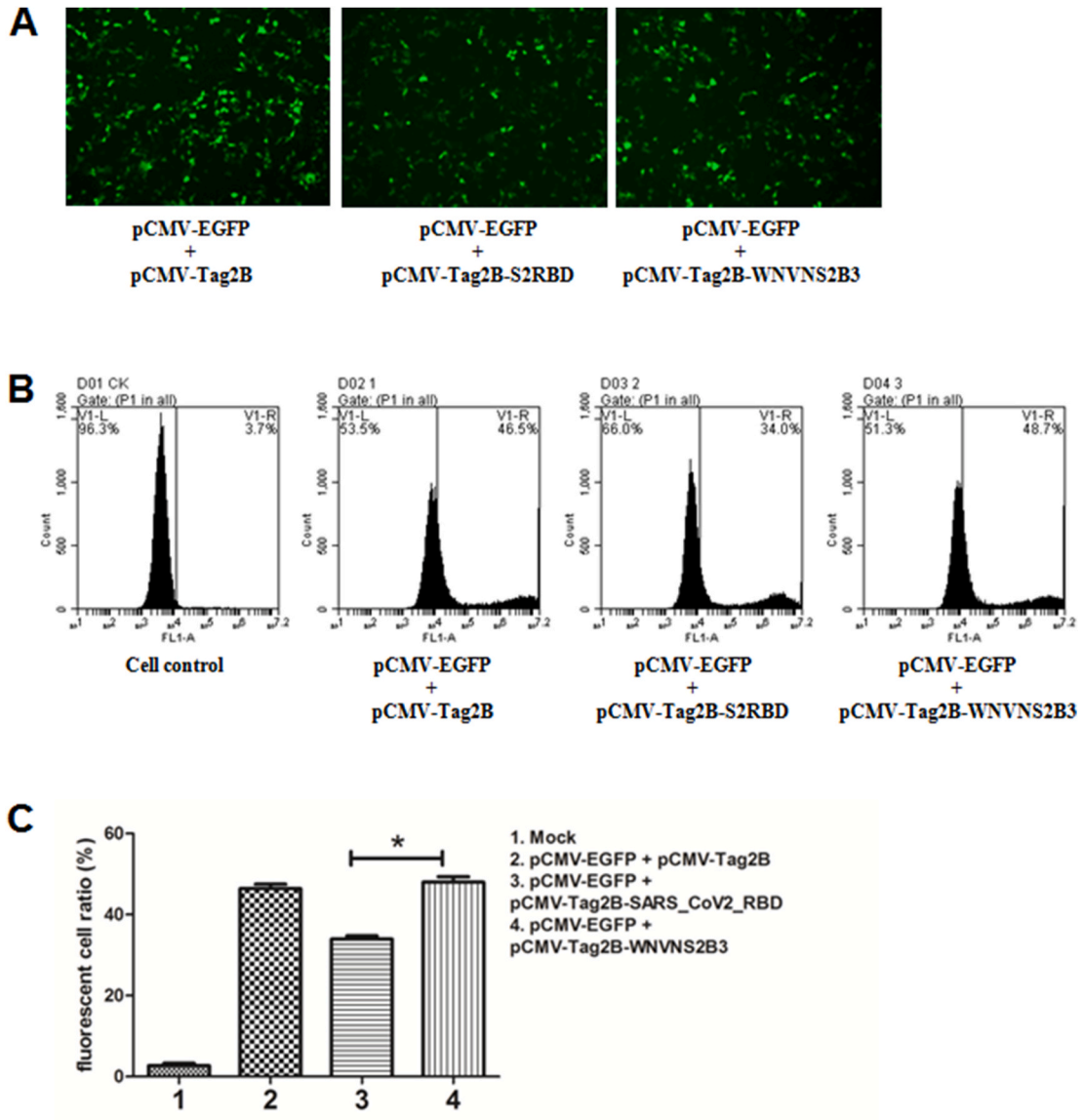

Fig. 3. SARS_CoV2 RBD *trans*-inactivates CMV promoter activation. 293T cells were cotransfected with recombinant plasmid pCMV-EGFP, along with pCMV-Tag2B, pCMV-Tag2B-SARS_CoV2_RBD, or pCMV-Tag2B-WNVNS2B3, respectively. (A) After 48 h transfection, the cells were observed under fluorescent microscope. (B) Cell samples mentioned above were collected and subject to Fluorescence activated cell sorting (FACS) assay. (C) The quantity of fluorescent cells were calculated and analyzed. Column 1, 293T mock transfected. Column 2, 293T cotransfected with pCMV-EGFP, along with pCMV-Tag2B; Column 3, 293T cotransfected with pCMV-EGFP, along with pCMV-Tag2B-SARS_CoV2_RBD; Column 4, 293T cotransfected with pCMV-EGFP, along with pCMV-Tag2B-WNVNS2B3. (*, $p < 0.05$).

fluorescent signal could be observed when the Chicken β-actin promoter was set in front of the gene *SARS_CoV2_RBD* following IFA (Fig. 4E), thus highlighting the critical role of Chicken β-actin promoter in driving the gene *SARS_CoV2_RBD*.

Collectively, *SARS_CoV2_RBD* gene can be preceded by Chicken β-actin promoter and a Vaccinia virus-specific promoter pSYN. Meanwhile, the results also demonstrated that *SARS_CoV2_RBD*-mRNA presenting in the cytoplasm can be translated into protein RBD, thus illustrating that CMV promoter-mediated inhibitory effect for RBD expression mentioned earlier is not at the translation step.

RBD on SARS_CoV2 spike protein engages with the ACE2 receptor on host cell, initiating the fusion between viron and cell. Therefore, RBD is deemed the most attractive target for drug and vaccine development against COVID-19. All the basic research regarding RBD and RBD-associated vaccine and drug developments should be rigidly based on the sufficient RBD protein expressed in eukaryotic cell, which maintains its natural structure and property. Our findings demonstrated that CMV promoter is not a suitable DNA element for driving expression of *RBD* of SARS_CoV2. Alternatively, it was suggested in this investigation that both Chicken β-actin promoter and Vaccinia virus-specific medium/late promoter are more feasible promoters to introduce efficient expression for *RBD* of SARS_CoV2 *in vitro*, thus providing new avenue for DNA vaccine or Vaccinia virus-based vaccine development against COVID-19.

5. Conflict of interest disclosure

No competing financial interests exist.

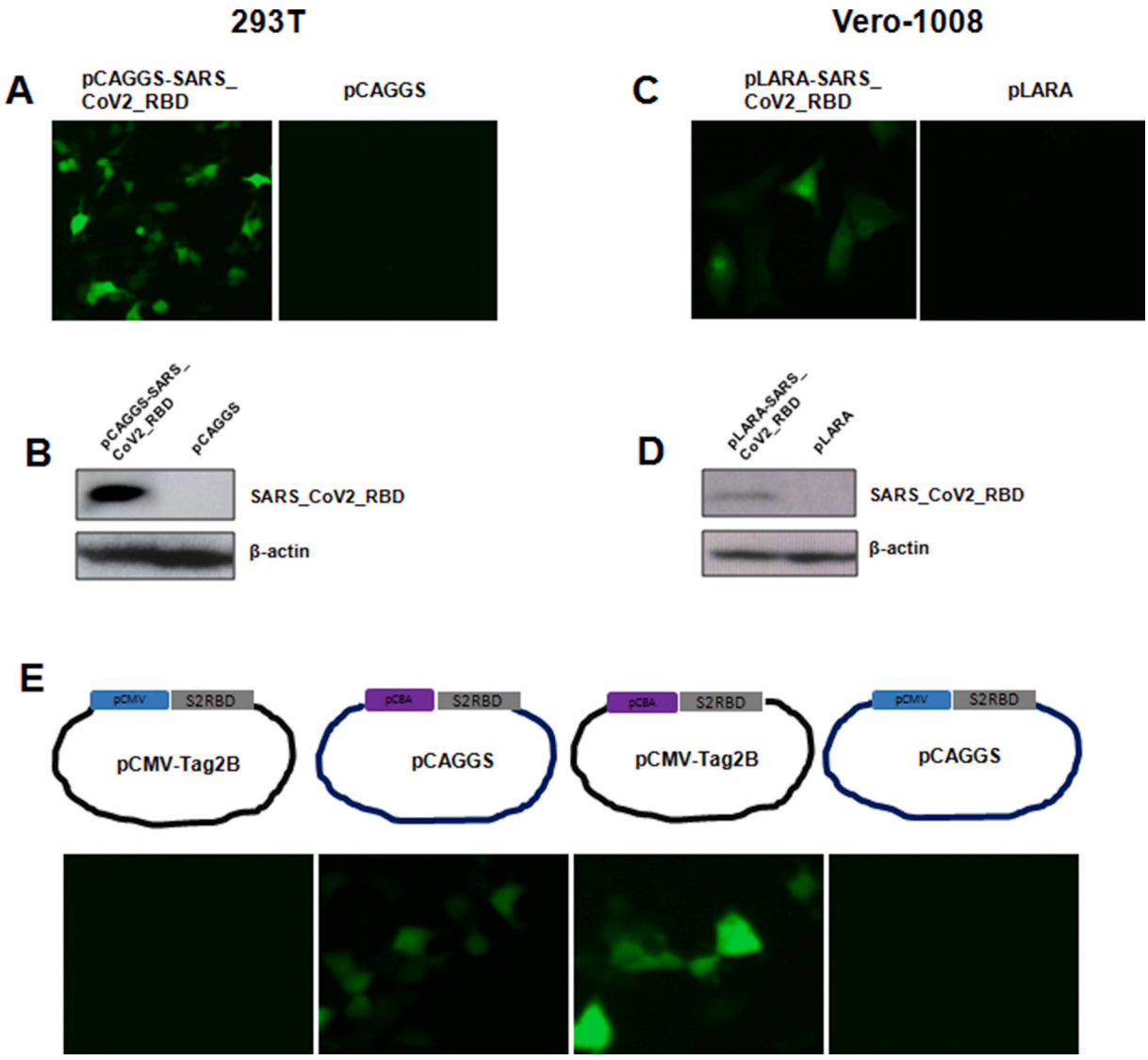

Fig. 4. Chicken β-actin promoter and Vaccinia virus-specific medium/late promoter can both precede *SARS_CoV2 RBD* transcription and expression. 293T cells were transfected with plasmids, which contains a Chicken β-actin promoter in the front of MCS, pCAGGS-SARS_CoV2_RBD or pCAGGS vector, respectively. At 48 h posttransfection, cell samples mentioned above were collected and subject to IFA (A) and Western blotting (B). Mouse anti-HA tag served as detection antibody. SARS_CoV2_RBD protein can be detected in both assays. Recombinant plasmid pVV-M/E-SARS_CoV2_RBD that contains a Vaccinia virus-specific promoter pSYN and target gene *SARS_CoV2_RBD* was transfected into Vaccinia virus-infected Vero-1008. At 24 h posttransfection, transfected cells were collected and subject to IFA (C) and Western blotting (D). Mouse anti-His tag served as detection antibody. The SARS_CoV2_RBD were able to be detected both in IFA and Western blotting. (E) The promoters between pCMV-Tag2B-SARS_CoV2_RBD and pCAGGS-SARS_CoV2_RBD were reciprocally exchanged. The resultant plasmids were transfected to 293T, and fluorescent signal was evaluated following IFA toward RBD. pCBA, Chiken β-actin promoter; pCMV, CMV promoter. Black line represents pCMV-Tag2B backbone, while dark blue line represents pCAGGS backbone.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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