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Analysis of a bovine herpesvirus 1 protein encoded by an alternatively spliced latency related (LR) RNA that is abundantly expressed in latently infected neurons

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ABSTRACT

The bovine herpes virus 1 (BoHV-1) encoded latency-related RNA (LR-RNA) is abundantly expressed in latently infected sensory neurons. A LR mutant virus with three stop codons at the amino-terminus of ORF2 does not reactivate from latency or replicate efficiently in certain tissues. ORF2 inhibits apoptosis, interacts with Notch1 or Notch3, and interferes with Notch mediated signaling. Alternative splicing of LR-RNA in trigeminal ganglia yields transcripts that have the potential to encode a protein containing most of ORF2 sequences and parts of other coding sequences located within the LR gene. In this study, we determined that an ORF2 protein fused with reading frame B (15d ORF) was more stable in transfected cells. ORF2 and the 15d ORF stimulated neurite formation in mouse neuroblastoma cells, interfered with Notch3 mediated trans-activation, and had similar DNA binding properties. Increased stability of the 15d ORF is predicted to enhance the establishment of latency.

Introduction

Bovine herpesvirus 1 (BoHV-1), an alpha-herpesvirinae subfamily member, causes significant economic losses to the cattle industry (Turin et al., 1999; Jones, 2009; Jones and Chowdhury, 2007). When acute infection occurs on mucosal linings within the ocular, nasal, or oral cavity, sensory neurons within trigeminal ganglia (TG) become the primary site for BoHV-1 latency. Abundant viral gene expression (Schang and Jones, 1997) and infectious virus (Inman et al., 2002) are detected during acute infection, but once latency is established and infectious virus is not readily detected (Jones et al., 2006; Jones and Chowdhury, 2007). Stress increases corticosteroid levels, and initiates reactivation from latency, which is crucial for virus transmission (Jones and Chowdhury, 2010). Administration of the synthetic corticosteroid dexamethasone to calves latently infected with BoHV-1 reproducibly induces reactivation from latency (Inman et al., 2002; Jones, 1998, 2003; Jones et al., 2006, 2000; Rock et al., 1992). Within 90 min after dexamethasone treatment, certain cellular transcription factors that stimulate productive infection and viral promoters are readily detected in trigeminal ganglionic sensory neurons (Workman et al., 2012). Dexamethasone also induces expression of two viral proteins, bICP0 and VP16, in sensory neurons of latently infected calves within 90 min after treatment (Frizzo da Silva et al., 2013). We suggest that increased corticosteroids, as a result of stress, stimulate lytic cycle viral gene expression by several distinct mechanisms: consequently reactivation from latency occurs.

The BoHV-1 encoded latency related (LR) RNA is abundantly expressed in latently infected neurons (Jones, 1998, 2003; Jones et al., 2006; Kutish et al., 1990; Rock et al., 1992; Rock et al., 1987). LR-RNA is anti-sense relative to the bICP0 gene and has a unique start site in TG (Bratanich et al., 1992; Hossain et al., 1995). Two open reading frames (ORF1 and ORF2) and two reading frames that lack an initiating ATG (RF-B and RF-C) are present in the LR gene (Kutish et al., 1990) (Fig. 1A). An LR mutant virus strain with 3 stop codons at the N-terminus of ORF2 exhibits diminished clinical symptoms, and reduced virus shedding from the eye, TG, or tonsils of infected calves (Inman et al., 2001b). Furthermore, the LR mutant virus does not reactivate from latency following dexamethasone treatment, in part because establishment of latency is reduced (Inman et al., 2002). ORF2 containing proteins promotes cell survival by inhibiting apoptosis (Ciacci-Zanella et al., 1999; Lovato et al., 2003; Shen and Jones, 2008; Sinani and Jones, 2011), which we predict is important for the latency-reactivation cycle. ORF2 also interacts with cellular transcription factors, Notch1, Notch3, or C/EBP-α (Meyer et al., 2007a,b; Meyer and Jones, 2008; Workman et al., 2011) and reduce Notch mediated trans-activation of the bICP0 early promoter and glycoprotein C

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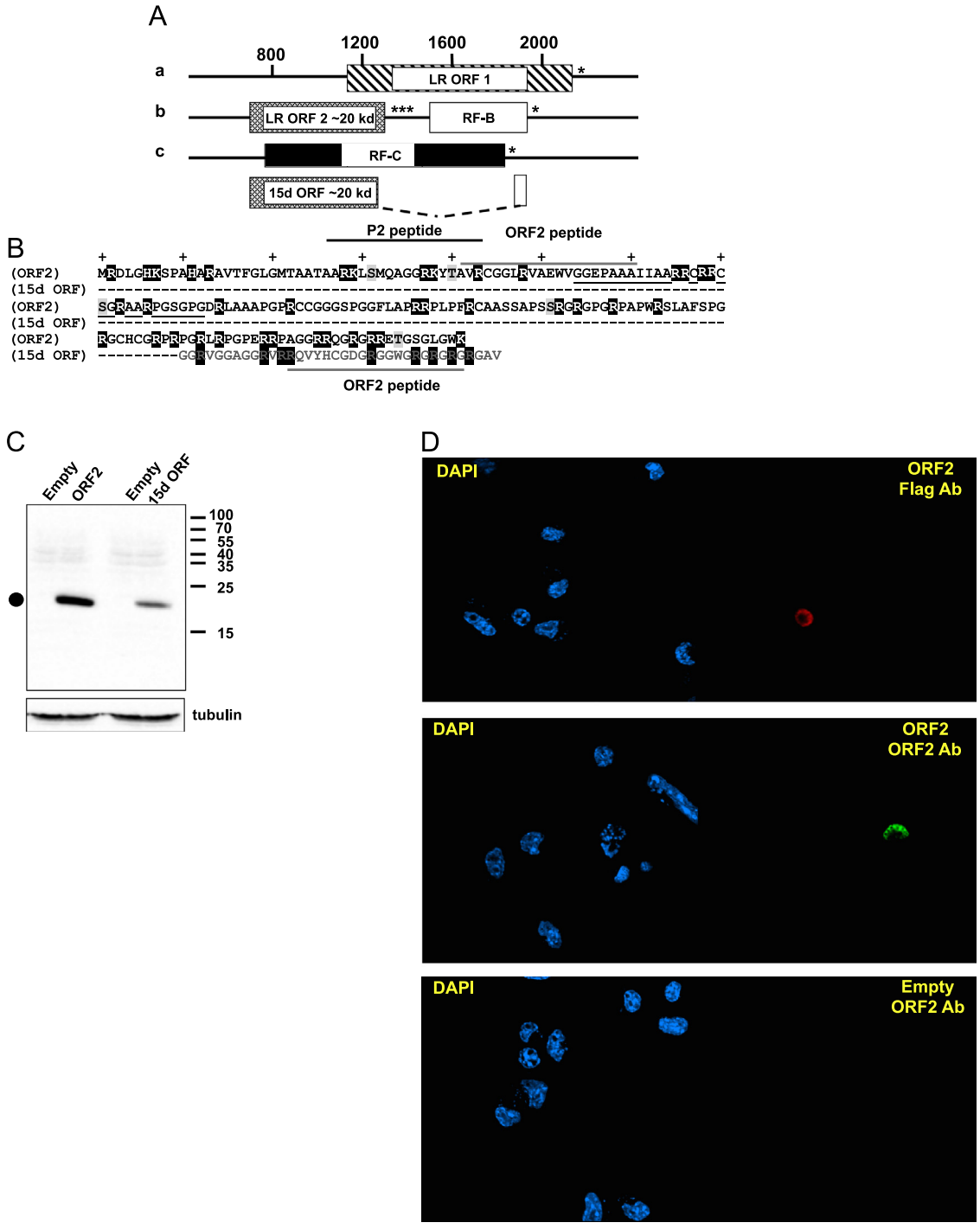

Fig. 1. Schematic of LR gene and comparison of ORF2 versus 15d ORF. Panel A: Location of ORFs within the LR gene. The numbering system of the LR gene and position of ORF2 was derived from a previous study (Kutish et al., 1990). Reading frames that lack an initiating methionine are designated RF-B and RF-C. LR ORF1, ORF2, and RF-C are located in reading frames a–c respectively. RF-B, like ORF2, is in reading frame b. The 15d ORF is comprised of ORF2 sequences and contains 36 amino acids derived from RF-B due to alternative splicing (Devireddy and Jones, 1998). Panel B: Comparison of ORF2 amino acid sequences versus the 15 d ORF. Amino acid sequence of ORF2 and 15 cDNA ORF: dashed lines in 15d ORF reflect identity with ORF2. The black shaded amino acids in ORF2 and the 15d ORF are basic amino acids, the underlined amino acids are the nuclear localization sequence, and the gray shaded amino acids are protein kinase A or protein kinase C phosphorylation sites. The 36 gray amino acids in the 15d ORF are from C-terminal sequences of RF-B. Consequently, the 15d ORF has no serine or threonine residues at its C-terminus: but it contains an additional tyrosine residue. The C-terminal ORF2 sequences have one threonine (PKA), one serine, and no tyrosine residues. The 15d ORF sequences contain 9 basic amino acids whereas ORF2 has 10 basic amino acids (shaded in gray) in the same domain, which results in a slight charge difference between the two isoforms. The+sign denotes every 10th amino acid. The location of peptides used to generate the P2 and ORF2 polyclonal antibody are denoted. Panel C: Neuro-2A cells were transfected with 2 μg of the designated plasmids that express a Flag-tagged ORF2 or 15d ORF. 48h after transfection total lysate was prepared and Western blot analysis performed using the ORF2 polyclonal antibody (1:2000 dilution): 100 μg total cell lysate was used for each lane. Molecular weight markers are denoted to the right and the closed circle denotes the position of ORF2 and the 15d ORF. Tubulin protein expression was used as a loading control for this study. The images are representative of 4 experiments. Panel D: Neuro-2A cells were transfected with 2 μg of the designated plasmids that express a Flag-tagged ORF2, 15d ORF, or the empty vector. Cultures were prepared for confocal microscopy at 48 h after transfection as described in the materials and methods. Cells expressing ORF2 were stained with the anti-Flag antibody (red), ORF2 polyclonal antibody (green), or DAPI (blue) to visualize ORF2 in the nucleus. Images are representative of 3 experiments.

promoter. An abundant spliced poly-A+LR-RNA was identified in TG of infected calves at 15 days post infection (dpi) and this cDNA was sequenced (Devireddy and Jones, 1998). As a result of splicing, the C-terminal sequences of ORF2 contain 36 amino acids derived from RF-B that replace the C-terminal 32 amino acids of ORF2 (Fig. 1B and C). We propose that LR gene products promote the establishment and maintenance of latency: but does not directly influence reactivation from latency because all LR gene products are repressed during the early stages of dexamethasone induced reactivation from latency (Jaber et al., 2010; Rock et al., 1992; Sinani et al., 2013).

In this study, we examined the properties of the 15d ORF to known functions of ORF2. We found that the 15d ORF was more stable than ORF2 in transfected Neuro-2A cells. As with ORF2, the 15d ORF localized to the nuclear periphery, interfered with Notch3-mediated trans-activation, re-localized Notch3 to the nuclear periphery, and stably interacted with DNA. We suggest that the enhanced stability of the 15d ORF is important during the establishment of latency.

Results

The 15 dpi cDNA has the potential to encode a protein fusing ORF2 with the C-terminus of RF-B

ORF2 is the first open reading frame downstream of the start site of LR-RNA (Fig. 1A) and ORF2 coding sequences completely overlap the bICP0 transcript (Rock et al., 1987). ORF2 is expressed in a subset of latently infected neurons (Jiang et al., 1998; Jones et al., 2011; Sinani et al., 2013) and its expression appears to be important for dexamethasone induced reactivation from latency and viral replication in the ocular cavity, tonsil, and TG (Inman et al., 2001a, 2002). We originally designed a peptide antibody (P2) that recognized a prominent 40 kd protein and several other proteins, including a 20–25 kd protein (Hossain et al., 1995), which is the approximate size of ORF2 (Rock et al., 1987) (see Fig. 1B for location of P2 peptide). We assumed that the 40 kd protein was a large protein comprised of ORF2, and as a result of splicing, one of the adjacent reading frames or ORF1 comprised the C-terminal part of this large protein. In trigeminal ganglia at 7 days after infection (dpi), an alternatively spliced LR transcript was discovered that has the potential to encode a protein comprised of ORF2 and ORF1 sequences (Devireddy et al., 2003; Devireddy and Jones, 1998). However, the major spliced LR-RNA identified in TG of cattle at 1 and 60 dpi has the potential to encode the intact ORF2 (Devireddy and Jones, 1998). Since ORF2 can inhibit apoptosis and interacts with Notch1 and Notch3 in the absence of other viral genes (Shen and Jones, 2008; Sinani and Jones, 2011; Workman et al., 2011), we have focused our studies on ORF2. Two immunodominant peptides were used to generate an additional ORF2 polyclonal antibody (for locations of ORF2 peptides, see Fig. 1B). The ORF2 polyclonal antibody recognized a protein migrating at approximately 21 kd following transfection of Neuro-2A cells with the ORF2 expression vector, but this band was absent in cells transfected with the empty vector (Fig. 1C). This result is consistent with that obtained with the Flag antibody (for example, see Fig. 2B). Furthermore, the ORF2 polyclonal antibody and Flag antibody recognized a protein that localized to the nuclear periphery in Neuro-2A cells when transfected with the ORF2 expression vector; conversely neither antibody recognized a protein in cells transfected with the empty vector (Fig. 1D).

A spliced polyA+LR-RNA in TG of calves, which is readily detected at 15 days after infection, has the potential to be translated into a protein that contains the last 36 amino acids of RF-B in place of the last 32 amino acids of ORF2 (Devireddy and Jones, 1998) (Fig. 1A and B; designated as the 15d ORF). The nuclear localization signal (NLS) within ORF2 and the 15d ORF (Fig. 1B) are identical to the NLS in the transcription factor Sp1 (Devireddy et al., 2003) and deletion of amino acids comprising the ORF2 NLS prevents nuclear localization (Sinani and Jones, 2011). ORF2 contains approximately 18% basic amino acids (Fig. 1B, black shaded amino acids). The 15d ORF contains one less basic amino acid, lacks a consensus protein kinase C (PKC)/PKA phosphorylation site at amino acid 174, and contains a novel tyrosine residue near the C-terminus. The Flag (Fig. 2D) and ORF2 polyclonal antibody (Fig. 1C) recognize a protein migrating near 20 kd. Relative to ORF2, the ORF2 polyclonal did not recognize the 15d ORF as well as ORF2 because the C-terminal immuno-dominant peptide used to generate the ORF2 polyclonal antibody is missing in the 15d ORF (Fig. 1B).

A panel of transposon insertion mutants spanning the 15d ORF was constructed to identify functional domains (see Fig. 2A for location of transposon insertions). The 15d ORF contains four consensus protein kinase A (PKA) and PKC phosphorylation sites (Fig. 1B; gray shaded amino acids). Activation of PKA stimulates HSV-1 reactivation from latency (Leib et al., 1991; Smith et al., 1992) suggesting PKA regulates certain functions of ORF2 or the 15d ORF. To test whether the consensus phosphorylation sites within the 15d ORF mutant are important, a mutant was prepared in which the serine or threonine residues in PKA/PKC consensus sites were substituted with alanine (15d-P). This mutant also contains alanine substitutions at Tyr39 and Ser120 because they are adjacent to the PKA/PKC sites.

Relative to the 15d ORF (Fig. 2B), reduced levels of the 20 kd protein was consistently detected in Neuro-2A cells transfected with the following transposon mutants, 15d-119, -159, -169, -524, and -525. As previously reported (Pittayakhajonwut et al., 2013; Sinani and Jones, 2011), the ORF2 mutant containing alanine substitutions in the PKA/PKC consensus motifs was expressed at higher levels compared to wt ORF2 (Fig. 2C). The 15d-P mutant was only expressed at a slightly higher steady state level relative to the 15d ORF (arrow) (Fig. 2D). The predominant band expressed by ORF2-P and the 15d-P mutant migrated as a slightly larger molecular weight compared to wt ORF2 or 15d ORF. The 15d ORF and all of the mutants constructed were generally localized to the nuclear periphery in transfected Neuro-2A cells (data not shown).

Neuro-2A cells transfected with the respective constructs were treated with cycloheximide (CHX) to examine the stability of the respective proteins in the absence of de novo protein synthesis. We have consistently observed that ORF2 was not as stable as the 15d ORF (Fig. 2E and F). The ORF2-P mutant appeared to be as stable as the 15d ORF, whereas the 15d-P mutant was not as stable. These studies revealed three important differences: 1) the C-terminal 36 amino acids within the 15d ORF increased the ½ life of the protein in transfected cells, 2) ORF2 C-terminal sequences decrease the ½ life of the protein, and 3) the missing PKA/PKC phosphorylation site in the 15d ORF appeared to stabilize protein expression in transiently transfected Neuro-2A cells.

The 15d ORF promotes neurite formation in Neuro-2A cells but interferes with Notch3 mediated trans-activation

To understand whether the 15d ORF and ORF2 have similar biological properties, we compared the ability of ORF2 to induce neurite formation in Neuro-2A cells expressing Notch3 to the 15d ORF. ORF2 promotes neurite formation in Neuro-2A cells overexpressing activated Notch1 or Notch3 intracellular domain (ICD) (Sinani et al., 2013), which correlates with the ability of ORF2 to bind Notch1 or Notch3 and interfere with Notch-mediated trans-activation of certain viral promoters (Workman et al., 2011). Notch expression maintains neuronal progenitors in an undifferentiated

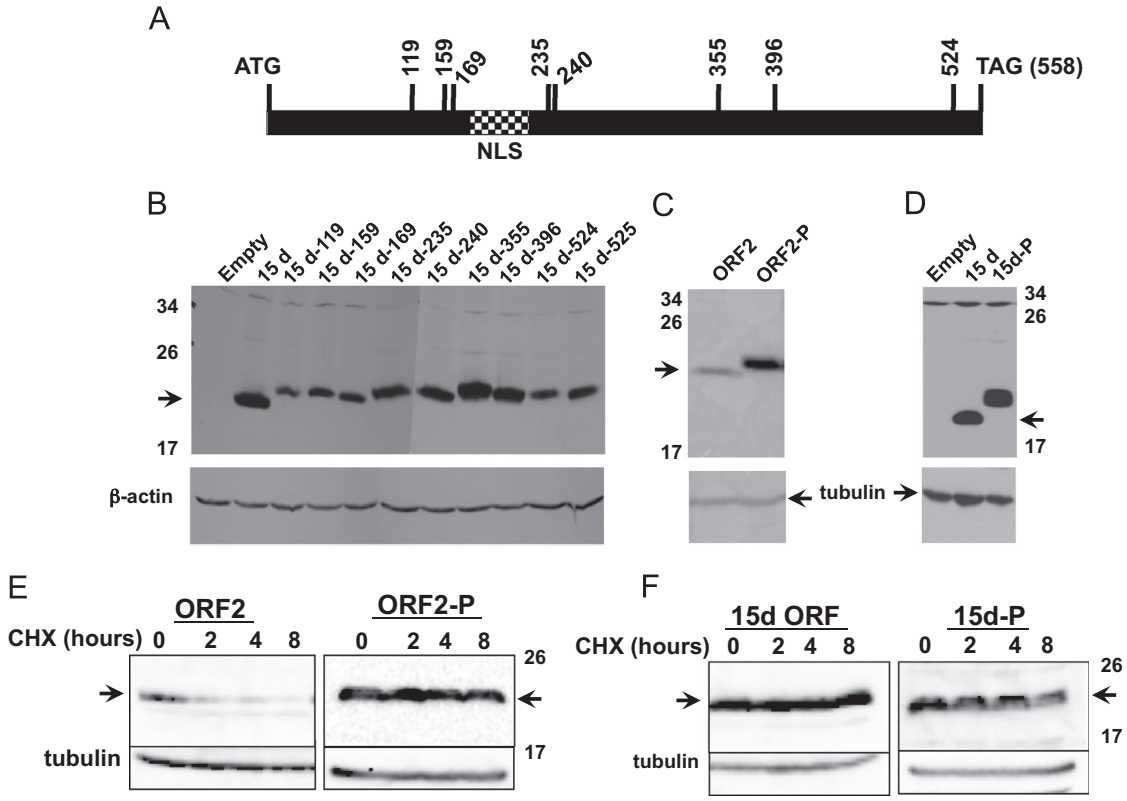

Fig. 2. Expression of the 15d ORF and the mutants in transfected Neuro-2A cells. Panel A: Location of transposon mutants in the 15d ORF. Vertical lines with the respective numbers indicate the nucleotide position of the respective transposon insertion. The position of the NLS, which spans nucleotides 192–210 (amino acids 64–70), is denoted by the checkered rectangle. Panel B: Neuro-2A cells were transfected with plasmids expressing ORF2, the 15d ORF, or the respective mutant constructs. At 48 h after transfection, cells were collected and processed for Western blot analysis as described in the materials and methods. 100 μg of protein was loaded in each lane. β-actin protein expression was used as a loading control. An arrow denotes the position of wt ORF2, the 15d ORF, or the designated mutants. Panel C: Relative levels of wt ORF2 versus ORF2-P mutant in transfected Neuro-2A cells. 100 μg protein of total cell lysate in each lane. Tubulin protein expression was used as a loading control for this study. Panel D: Relative levels of the 15d ORF versus the 15d-P mutant in transfected Neuro-2A cells. 100 μg protein of total cell lysate in each lane. Tubulin protein expression was used as a loading control for this study. Panel E: Neuro-2A cells were transfected with either the ORF2 expression plasmid or the ORF2-P mutant. At 24 h after transfection, cycloheximide (CHX; 100 μg/ml final concentration) was added to cultures for the indicated time. Cell lysate (40 μg for ORF2-P and 120 μg for ORF2 protein) was examined for ORF2 protein expression. Tubulin protein expression was used as a loading control for this study. Panel F: Neuro-2A cells were transfected with either the 15d ORF expression plasmid or the 15d-P mutant. At 40 h after transfection, cycloheximide (CHX; 100 μg/ml final concentration) was added to cultures for the indicated time. Cell lysate (40 μg for 15d-P or 120 μg for 15d ORF protein) was then examined for ORF2 protein expression. Tubulin protein expression was used as a loading control for this study.

state (Hitoshi et al., 2002). Activation of Notch signaling in differentiated neurons inhibits neurite sprouting (Berezovska et al., 1999; Franklin et al., 1999; Levy et al., 2002a,b; Sestan et al., 1999) and axon repair (El Bejjani and Hammerlund, 2012), which can result in neuronal cell death (Coleman and Freeman, 2010; Raff et al., 2002). The 15d ORF restored neurite formation in Neuro-2A cells when cotransfected with Notch3 ICD and then growth factors removed (Fig. 3A). As previously demonstrated (Sinani et al., 2013), ORF2 also restored neurite formation, but Neuro-2A cells transfected with Notch3 ICD plus the empty vector did not routinely sprout long neurites. Quantification of the data revealed that ORF2 and the 15d ORF stimulated neurite formation with similar efficiency (Fig. 3B).

Additional studies tested whether the 15d ORF inhibited trans-activation of the Hairy Enhancer of Split 5 (HES5) promoter with similar efficiency as ORF2. Hes5 protein expression is activated by Notch signaling and the Hes5 protein inhibits neuronal differentiation (Ohtsuka et al., 1999). The 15d ORF significantly inhibited the ability of the Notch3 ICD to trans-activate the HES5 promoter in Neuro-2A cells (Fig. 4A). ORF2 and the 15d ORF inhibited Notch3 ICD mediated trans-activation of the HES5 promoter with similar efficiency. Five of the nine-transposon mutants (15d-119, -159, -235, -396, and -525) were unable to significantly reduce Notch3 ICD mediated trans-activation of the HES5 promoter (Fig. 4B). The 15d-P mutant inhibited Notch3 ICD mediated trans-activation of the HES5 promoter to the same extent as the 15d ORF.

When the Notch3 ICD is expressed in Neuro-2A cells, it localizes throughout the nucleus {Fig. 5A and Workman et al., (2011)}. Re-localization of the Notch3 ICD to the nuclear periphery occurs in Neuro-2A cells that express ORF2 Workman et al., (2011) and Fig. 5B}. We have consistently observed that the 15d ORF also re-localized Notch3 ICD to the nuclear periphery in Neuro-2A cells (Fig. 5C). In summary, the 15d ORF stimulated neurite formation in cells expressing Notch3, inhibited Notch3 ICD mediated trans-activation of the HES5 promoter and re-localized the Notch3 ICD to the nuclear periphery in a similar fashion as ORF2.

The 15d ORF interacts with DNA

Studies were performed to compare the ability of the 15d ORF versus ORF2 to interact with single stranded (ss)-DNA or double stranded (ds)-DNA coupled to cellulose. The rationale for testing ss- and ds-DNA is certain DNA binding proteins preferentially bind ss- versus ds-DNA, reviewed in (Marceau, 2012) and ORF2 preferentially interacts with ss-DNA (Pittayakhajonwut et al., 2013). Since the ORF2-P mutant was expressed at higher levels in transfected cells compared to the 15d-P mutant or the 15d ORF, we adjusted the levels of cell lysate to ensure that similar levels of

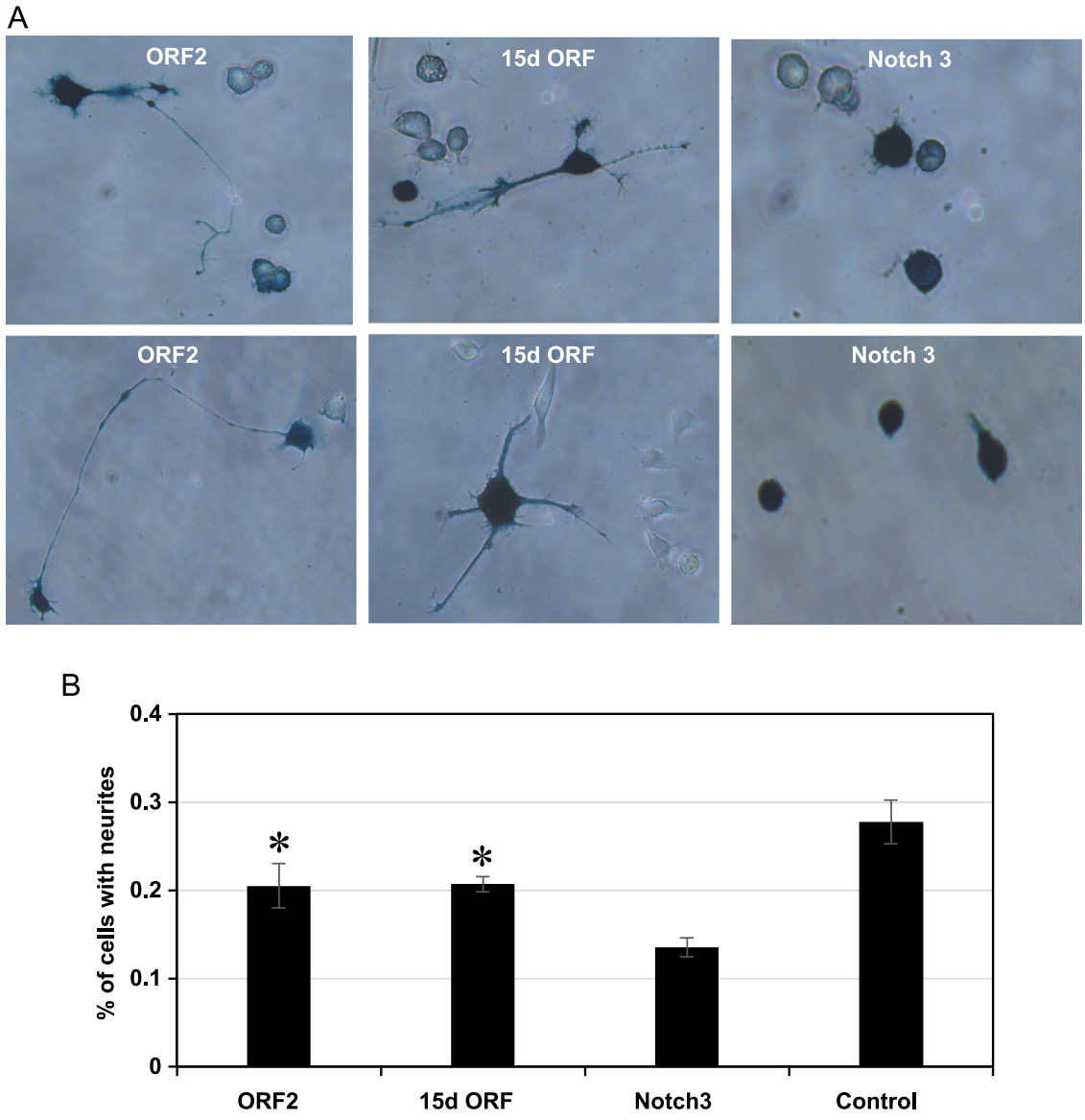

Fig. 3. The 15d ORF stimulates neurite formation in Neuro-2A cells transfected with Notch3. Panel A: Neuro-2A cells were co-transfected with 1 μg plasmid expressing Notch3 ICD, 1 μg plasmid expressing ORF2 or the 15d ORF, and 1 μg plasmid expressing the Lac Z gene (transfection control). To induce neurite sprouting, 24 h after transfection cells were seeded into new plates at a low density (2000 cells/cm^2) and then starved in media with 0.5% serum for 3 days. Cells were then fixed and β-gal+cells identified as described in the materials and methods. Panel B: The percent of β-gal+cells containing neurites was calculated by dividing the number of β-gal+cells with a neurite length at least twice the diameter of the cell by the total number of β-gal+cells. The average of 3 independent experiments is shown with the respective standard deviation. An asterisk denotes significant differences ($P < 0.05$) in β-gal+Neuro-2A cells containing neurites following cotransfection with the ORF2 reporter and a Notch1 or Notch3 expression plasmid relative to β-gal+Neuro-2A cells with neurites following transfection with a plasmid expressing Notch1 or Notch3 plus empty vector, as determined by the One-way ANOVA and Fisher's LSD multiple means comparison tests.

the respective ORF2 proteins were present in the DNA binding assays. Cell lysate was incubated with DNA cellulose beads, and binding of the 15d ORF or ORF2 to DNA cellulose beads was detected by Western blot analysis using a Flag specific monoclonal antibody. In five independent studies, the 15d ORF prepared from transfected Neuro-2A cells preferentially interacted with ss-DNA, which was similar to previous results for ORF2 (Fig. 6A). The 15d-P mutant also interacted with ss-DNA and ds-DNA beads (Fig. 6B). As a control, we examined the interaction between the ORF2-P mutant because it is expressed at high levels in transfected Neuro-2A cells and it binds preferentially to ds-DNA coupled to cellulose (Pittayakhajonwut et al., 2013). The ORF2-P mutant showed a slight preference for ds-DNA compared to ss-DNA (Fig. 6C), which was similar to previous studies (Pittayakhajonwut et al., 2013). In summary, these studies indicated that the 15d ORF interacted with DNA coupled to cellulose, and suggested that the 15d-P mutant did not interact with ds-DNA as efficiently as the ORF2-P mutant.

Discussion

In this study, we compared a novel protein, the 15d ORF to ORF2, which is generated by alternative splicing of poly-A+ LR-RNA in TG (Devireddy and Jones, 1998). We suggest that ORF2 N-terminal sequences contain important functions for certain aspects of the latency-reactivation cycle and that the respective ORF2 isoforms have slightly different functional properties. Support for this prediction comes from the finding that the ORF2-ORF1 fusion protein encoded by an alternatively spliced cDNA detected in TG at 7 days after infection interacted

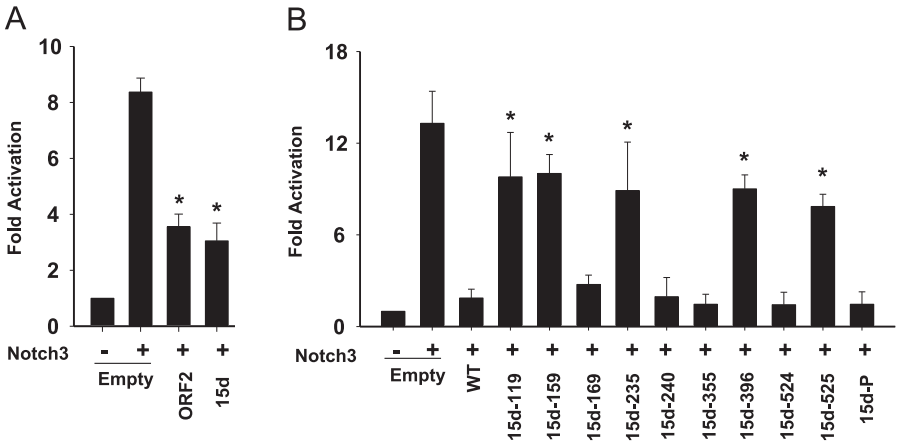

Fig. 4. The 15d ORF interferes with Notch3 mediated trans-activation of the HES5 promoter. Panel A: Neuro-2A cells were co-transfected with a plasmid containing the *Firefly* luciferase gene downstream of the HES5 promoter, a plasmid expressing Notch3 ICD and a plasmid expressing ORF2 or the 15d ORF. As a control, the HES5 promoter was transfected with an empty vector (pCMV-Tag2B) plus Notch3 ICD to demonstrate the effect that Notch3 ICD has on promoter activity. Panel B: Neuro-2A cells were cotransfected with a plasmid containing the *Firefly* luciferase gene downstream of the HES5 promoter, a plasmid expressing Notch3 ICD, and a plasmid expressing the 15d ORF or the respective mutants derived from the 15d ORF. As a control, the Notch3 ICD was cotransfected with an empty vector. 48 h after transfection promoter activity was measured using a dual luciferase assay. A plasmid expressing *Renilla* luciferase under control of a minimal herpesvirus TK promoter was used as an internal control. The results are the average of four independent experiments and the error bars denote standard deviation. An asterisk for Panel A denotes significant differences ($P<0.05$) in HES5 promoter activation by Notch3 in the presence of empty vector or WT ORF2 and 15d ORF, as determined by the Student *t* test. In Panel B, the asterisk denotes significant differences ($P<0.05$) in HES5 promoter activation by Notch3 in the presence of WT 15d ORF or the indicated 15d ORF transposon mutants.

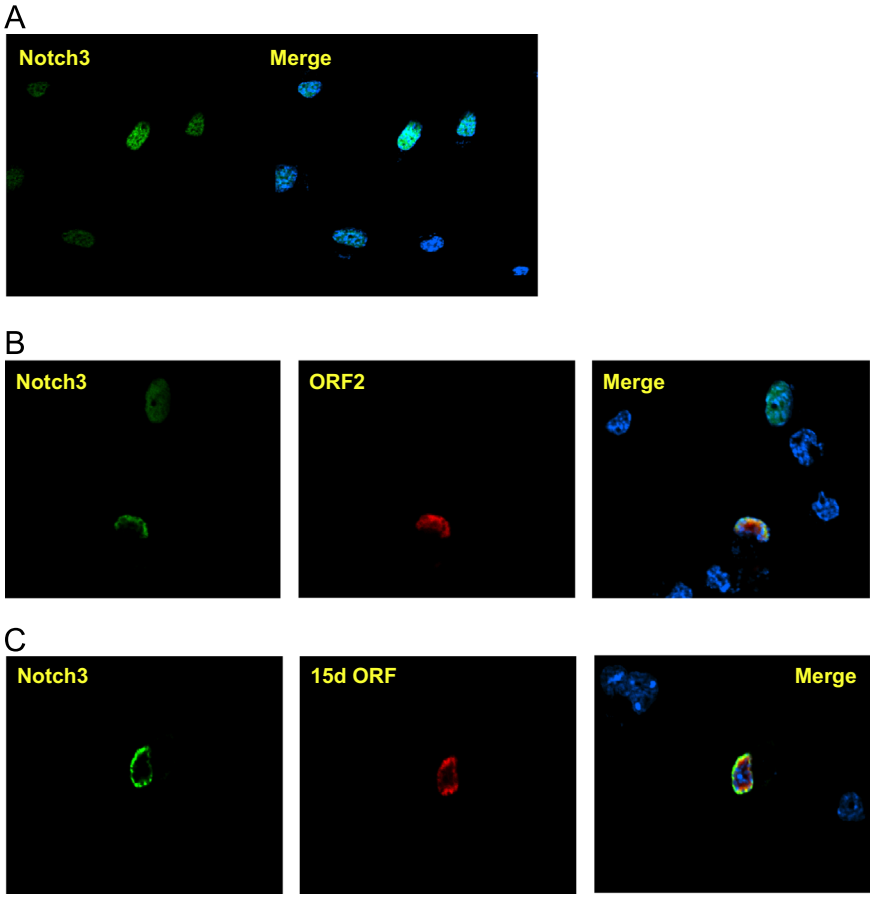

Fig. 5. The 15d ORF re-localizes Notch3 in transfected Neuro-2A cells. Neuro-2A cells were transfected with Notch3 ICD (Panel A), Flag-tagged ORF2 and the Notch3 ICD (Panel B), or Notch3 ICD and the 15d ORF plus empty vector (Panel C). Cultures were prepared for confocal microscopy at 48 h after transfection as described in the materials and methods. Cells were stained with anti-Flag antibody (red), Notch3 antibody (green) or DAPI (blue) to visualize ORF2, Notch3 and the nucleus respectively. DIC (differential interference contrast) was used to show the unstained cells. The images are representative of 6 experiments.

with C/EBP-alpha (Meyer et al., 2007b), but ORF2 does not. Although a previous study demonstrated that the 15d ORF inhibits cold-shock induced apoptosis in Neuro-2A cells with similar efficiency as ORF2 (Shen and Jones, 2008), it was of interest to compare the other known biological properties of these two proteins. None of the LR-RNA spliced isoforms that were identified can express an intact ORF1. Since ORF1 is detected in latently infected neurons (Meyer et al., 2007a), we

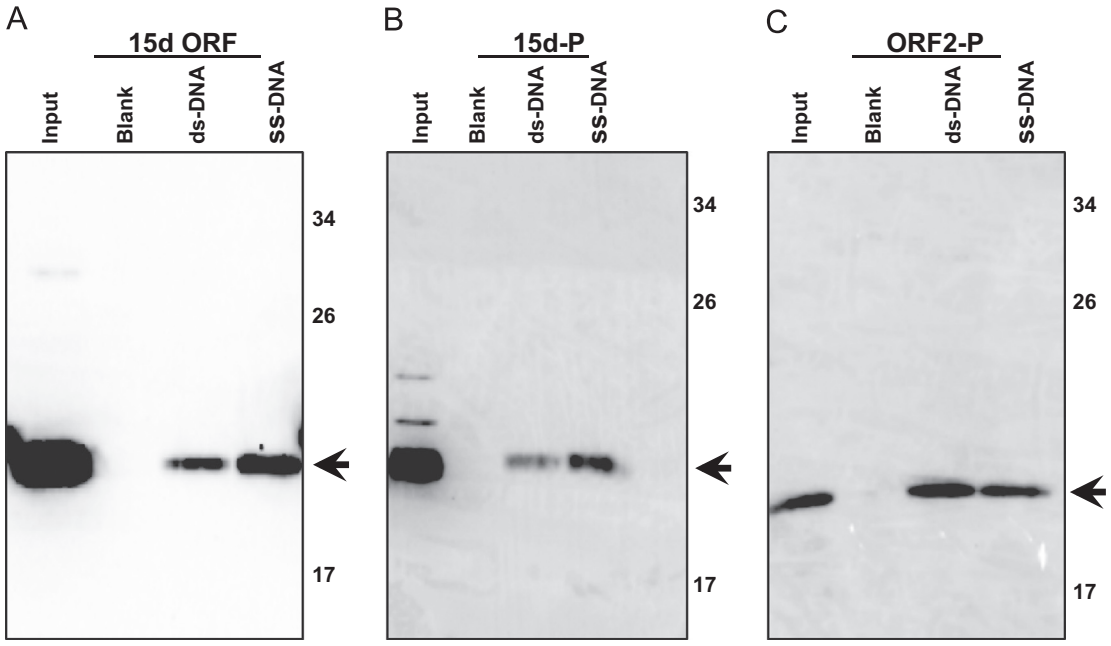

Fig. 6. DNA binding properties of the 15d ORF. Neuro-2A cells (5×10^6) were transfected with plasmids expressing the 15d ORF (Panel A), the 15d-P mutant (Panel B), or the ORF2P mutant (Panel C). DNA chromatography was performed as described in the materials and methods with ss-DNA, ds-DNA, or blank cellulose beads. 1 mg for 15d ORF, 300 μg for 15d-P and 100 μg for ORF2-P mutant of cell extract were used from cells to incubate with DNA cellulose. 10% of the total cell extract used to verify that ORF2 was expressed (lanes denoted Input). Expression of the 15d ORF, 15d-P, or ORF2-P in Neuro-2A cells was analyzed by Western blots using an anti-Flag antibody. An arrow denotes ORF2 specific bands and position of molecular weight standards is denoted.

suggest that low levels of un-spliced LR-RNA are expressed. The function of ORF1 is unknown; however, it does not inhibit apoptosis or interfere with Notch-mediated signaling (Sinani, unpublished results).

ORF2 and the 15d ORF have three functions that are predicted to play a role in the establishment and/or maintenance of latency. First, both proteins inhibit apoptosis (Ciacci-Zanella et al., 1999; Shen and Jones, 2008; Sinani and Jones, 2011). Secondly, both proteins interfere with Notch1 and Notch3 mediated transcription, in part, by interacting with these proteins. Thirdly, the ability of ORF2 to promote neurite growth in the presence of Notch (Sinani et al., 2013) may restore normal neuronal functions following infection. Most ORF2 transposon mutants tested inhibited trans-activation of the Hes5 promoter whereas five out of the nine-transposon mutants of the 15d ORF were inactive. For example, two ORF2 transposon mutants at position 95 and 134 inhibited Notch1 or Notch3 mediated trans-activation of the HES5 promoter with similar efficiency as wt ORF2 (Sinani et al., 2013). Conversely, two 15d ORF transposon mutants near the amino-terminus, 15d-119 and 15d-159, were unable to inhibit Notch3 mediated trans-activation of the HES5 promoter. Furthermore, the 15d-P mutant, but not ORF2-P mutant (Sinani et al., 2013), inhibited Notch3 mediated trans-activation of the HES5 promoter. Although these differences appear to be inconsistent with each other, the replacement of 32 C-terminal ORF2 amino acids with 36 amino acids derived from RF-B may regulate interactions with Notch3 or other Notch family members. Notch mediated trans-activation is complicated because it is controlled by interactions between RBP-Jκ (a sequence specific DNA binding protein), Mastermind, and other RNA polII transcription factors (P300/CBP, P/CAF, and GCN5 for example), reviewed in Borggrefe and Oswald (2009), Bray (2006) and Ehebauer et al. (2006). Consequently, it is conceivable that ORF2 and the 15d ORF have different affinities for Notch family members or their interactions with Notch influenced the association of Notch with other transcription factors. The enhanced stability of the 15d ORF is predicted to have biological significance because at 15 days after infection, the establishment of latency and repair of infected neurons, as a result of infection, would be important. Expressing a protein that is more stable, but has similar biological properties as ORF2, would appear to be advantageous during the transition from acute infection to establishment of latency.

Materials and methods

Cells

Murine neuroblastoma cells (Neuro-2A) were grown in Earle's modified Eagle's medium (EMEM) supplemented with 5% FCS, penicillin (10 U/ml), and streptomycin (100 μg/ml). The designated ORF2 or 15d ORF expression construct was transfected into Neuro-2A cells using TransIT-Neural (Mirus, Madison, WI), according to manufacturer's instructions. At 24 h after transfection, certain cultures were treated with 100 μg/ml cycloheximide in EMEM at 37 °C.

ORF2 expression constructs and mutants used in this study

The mammalian 15 dpi cDNA ORF expression construct was generated in the vector pCMV-Tag-2B (Stratagene). A Flag epitope is at the N-terminus of the ORF and the human IE CMV promoter drives its expression. The 15d-P mutant contains alanine substitutions in consensus cAMP-dependent protein kinase A (PKA) and/or the serine/threonine-specific protein kinase C (PKC) (Ser31, Thr40, Ser71, and Ser121). The 15d-P mutant also contains alanine substitutions in Tyr39 and Ser120, which are adjacent to the consensus PKA/PKC sites. This mutant was synthesized by IDT (Coralville, IA) and ligated into the pCMV-Tag-2B at the unique BamHI-HindIII restriction enzyme sites.

Transposon mutants of the 15d ORF were prepared as previously described (Sinani and Jones, 2011) (see Fig. 1C for location of the transposon insertions). In brief, a 57 base pair linker was randomly inserted into the 15d ORF DNA sequences according to the manufacturer's instructions (Epicentre Cat # EZ104KN). The

site of a transposon insertion was determined by DNA sequencing and the respective inserts re-cloned into the pCMV2B Flag-tagged vector at the BamHI-Sal site. The Flag-tagged mutant constructs were confirmed by sequencing. Each transposon mutation expresses a full-length 15d ORF with a 19 amino acid transposon insertion in frame with the ORF.

Western blot analysis

Neuro-2A cells in 60 mm dishes were transfected with the designated plasmids. 48 h after transfection cells were collected, washed once with PBS, lysed in RIPA buffer (50 mM Tris–HCl, pH 8150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitors (Thermo-Scientific). The respective samples were boiled in Laemmli sample buffer for 5 min and all samples were separated on a 12% SDS–polyacrylamide gel. Immunodetection of ORF2, the 15d ORF, and the designated 15d ORF mutants were performed using a mouse anti-Flag antibody (Sigma F1804) or the ORF2 polyclonal antibody, which were diluted 1:1000

Open Biosystems, which is now part of Thermo Fisher Scientific, generated the ORF2 polyclonal antibody. Open Biosystems predicted that two regions in ORF2 were immune-dominant. These immune-dominant peptides were synthesized, both peptides were co-injected into two rabbits, the crude sera was collected, and then ORF2-specific antibodies were affinity purified using both peptides. The location of the two ORF2 peptides is shown in Fig. 1B, denoted by the gray lines.

DNA binding assays by affinity chromatography

The designated ORF2 or 15d ORF expression construct was transfected into Neuro-2A. 48 h after transfection, monolayers were washed twice with cold phosphate-buffered saline, scraped from plates, and then lysed in cell lysis buffer {20 mM Tris–HCl (pH7.4), 10 mM $MgCl_2$, 500 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol and 100 μg/ml BSA}. The lysate was sonicated for 15 s prior to rotating at 4 °C for 30 min. Cell debris was centrifuged at 46,500*g* for 15 min. The supernatant was subsequently desalted against dialysis buffer {20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM mercaptoethanol, 10% glycerol and 100 μg/ml BSA} and then used for DNA chromatography. ORF2 was detected by Western blot analysis using an anti-Flag antibody (Sigma, St. Louis, MO).

DNA-cellulose chromatography was performed by incubating 100 μg of ds- or ss-DNA cellulose (Sigma, USA) with 1 mg of mammalian cell lysate containing 15d ORF, 300 μg for 15d-P or 100 μg for ORF2-P mutant for 16 h at 4 °C in binding buffer {20 mM Tris–HCl (pH 7.4), 50 mM NaCl, 1 mM β-mecaptoethanol, 1 mM EDTA, 100 μg/ml BSA, 10% glycerol, 1 mM Phenylmethanesulfonyl fluoride (PMSF) and 1 × protease inhibitor (Roche, USA)}. After the protein was incubated with the designated DNA cellulose beads, the beads were washed 5 times in binding buffer containing 125 mM NaCl and bound proteins were eluted with Laemmli SDS–PAGE buffer. Samples were subjected to electrophoresis by SDS–PAGE and analyzed by Western blot.

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