INTRODUCTION

Gallid herpesvirus type 2 (GaHV-2), previously known as Marek’s disease virus (MDV-1), is an oncogenic alphaherpesvirus of chickens that is considered to be a relevant animal model of virus-induced lymphoma (Burgess et al., 2004; Osterrieder et al., 2006). GaHV-2 induces aggressive T-cell lymphoma, leading to the death of the chicken in a few weeks. The genome of this virus is organized as in most alphaherpesviruses, including the prototype of the alphaherpesvirus subfamily, HHV-1 (human herpesvirus 1 or herpes simplex virus 1), and consists of a unique long (UL) region and a unique short (US) region, containing the core genes, flanked by the long (UL) and short (US) terminal and internal repeats, respectively (Fig. 1). These repeated regions contain, in particular, the genes involved in latency, lymphomagenesis, and transformation, such as the gene encoding the viral telomerase subunit (vTR) (Fragnet et al., 2003), meq, the major oncogene of GaHV-2 (Jones et al., 1992) and the latency-associated transcript (LAT lncRNA), a long non-coding RNA (lncRNA) specific to the latency phase (Cantello et al., 1994; Strassheim et al., 2012). Moreover, the RL and RS repeat regions harbour 13 pre-miRNAs grouped into three clusters: the mdv1-miR-M9-M4 and mdv1-miR-111-M1 clusters in the TR/IR regions upstream and downstream from the meq gene, respectively, and the mdv1-miR-18-M10 cluster in the first intron of the LAT gene in the TR/IR regions (Morgan et al., 2008).

The overproduction of IFN-α, IFN-β and IFN-γ has been reported during early cytolytic GaHV-2 infection in chickens (first week post-infection) (Heidari et al., 2010; Quére...
associated with an upregulation of the IFN-stimulated genes (ISGs) MDA-5 (melanoma differentiation-associated protein 5), iNOS (inducible nitric-oxide synthase), IFIT5 (IFN-induced protein with tetratricopeptide repeats 5) and Mx1 (myxovirus resistance protein 1) (Feng et al., 2013; Heidari et al., 2008, 2010). This ISG family also includes PKR (protein kinase RNA-activated), RNaseL, OAS (2′-5′-oligoadenylate synthase) and ADAR1 (adenosine deaminase acting on RNA 1), which play a key role in innate immunity to viral infections. ADAR1 belongs to the ADAR family of proteins, together with ADAR2 and ADAR3, which are encoded by genes not stimulated by IFNs (non-ISGs). ADAR proteins edit dsRNA substrates by catalysing the C-6 deamination of adenosine (A) to inosine (I), which is then recognized as guanine (G). Editing may be site-specific, with modifications limited to just one or a few adenosines, or non-specific and extensive as for the lymphocytic choriomeningitis virus glycoprotein sequence, which may display up to 67% mutated sites in hyperedited RNAs from infected mice (Zahn et al., 2007).

The mammalian ADAR1 gene encodes two major forms of the protein generated through alternative promoter usage (George & Samuel, 1999): a short form, ADAR1-S and a long form, ADAR1-L. ADAR1-S is constitutively produced and located in the nucleus, whereas ADAR1-L transcription is driven by an IFN-inducible promoter. This form shuttles between the nucleus and the cytoplasm, and seems to play a greater role in viral RNA editing than ADAR1-S (Samuel, 2012). By contrast, the ADAR1 genes of fish and amphibians each produce only one form, similar to the human ADAR1-L, which is induced in response to viral infections (Yang et al., 2012). In the chicken, neither the gene nor the protein has been well characterized but the chicken ADAR1 is phylogenetically closely related to the amphibian protein (Jin et al., 2009), and there is some evidence to suggest that it may be involved in viral RNA editing, as shown during the infection of chicken embryo fibroblasts (CEFis) with influenza virus (Suspène et al., 2011). ADAR1-L-mediated RNA editing contributes to the innate antiviral immune response by decreasing viral replication or leading to the production of defective virions (Zahn et al., 2007). However, several viruses have also been shown to hijack editing, and this process may act as a proviral factor, even becoming essential for completion of the replication cycle, as in the hepatitis delta virus, in which the editing of one adenosine residue leads to a shift from replication to encapsidation of the genome (Wong & Lazinski, 2002). In HHV-8 (human herpesviruses 8 or Kaposi’s sarcoma-associated herpesvirus) and HHV-4 (human herpesviruses 4 or Epstein–Barr virus), site-specific editing events have been reported to eliminate the transformation activity of the HHV-8 K12 transcript (Gandy et al., 2007) and to decrease processing of the HHV-4 pri-miR-BART6 (Iizasa et al., 2010). Finally, a HHV-4 IncRNA, which supports viral transcription during lytic reactivation, has recently been shown to be hyperedited by ADAR1 (Cao et al., 2015).

IFN-sensitive ADAR1 expression is induced via the JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway (Fenner et al., 2006). This activation leads to the phosphorylation of STAT-1/-2, which binds, in association with IFN regulatory factor 9 (IRF-9), to the IFN-stimulated response element (ISRE) in ISG promoters. This pathway is controlled by the suppressor of cytokine signalling 1 (SOCS1), which blocks STAT-1/-2 phosphorylation (Fenner et al., 2006). In mammals, SOCS1 is repressed by the microRNA miR-155 (Wang et al., 2010), promoting the IFN-signalling pathway and leading to the upregulation of ISGs, including ADAR1. MiR-155, which is classically deregulated in lymphoma and leukaemia, targets at least 140 genes, including regulatory proteins for leukaemogenesis, inflammation and tumour suppressors (Neilsen et al., 2013). Infection of human B cells by the lymphotropic gammaherpesvirus HHV-4 has been shown to be associated with the induction of hsa-miR-155 (Linnstaedt et al., 2010), whereas the infection of lymphoid cells by two other lymphotropic herpesviruses, HHV-8 and GaHV-2, is associated with the strong downregulation of hsa-mir-155 and gga-mir-155, respectively (Skalsky et al., 2007; Yao et al., 2009). This decrease in miR-155 levels is associated with strong expression of the viral orthologues KSHV-miR-K12-11-3p and mdv1-miR-M4-5p, for HHV-8 and GaHV-2, respectively. We have shown that, in addition to targeting the viral genes UL28 and UL32, which seems to be involved in the cleavage/packaging of virion DNA, mdv1-miR-M4-5p downregulates the expression of all tested mRNAs targeted by gga-mir-155-5p (Muylken et al., 2010).

In this study, we identified a new alternatively spliced GaHV-2 IncRNA, the edited repeat-long IncRNA (ERL IncRNA). This IncRNA is a natural anti-sense transcript (NAT) of 14KD genes, the meq gene and two of the three clusters of miRNAs: mdv1-mir-M9-M4 and mdv1-mir-M11-M1. It was hyperedited by ADAR1 during GaHV-2 infections in vitro and in vivo, and the editing rate was much higher in lytic infections than in latent infections. We found that ADAR1 expression, which was stronger in lytic than in latent infections, was controlled by an IFN-inducible promoter. Finally, we demonstrated that mdv1-mir-M4-5p, the most strongly expressed miRNA of the mdv1-mir-M9-M4 cluster encoded by the anti-sense strand of the ERL IncRNA, promoted the overexpression of ADAR1 by downregulating SOCS1.

**RESULTS**

**ERL IncRNA is transcribed as a spliced and hyperedited RNA**

Building on our previous work characterizing long RNAs from the TR4/IRL region (Coupeau et al., 2012), including transcripts of the miRNA clusters and the products of the meq and vIL8 genes, we searched anti-sense transcripts of this region in order to complete the TR4/IRL transcriptome. We identified, by various reverse transcription (RT-PCR) with
pairs of primers, a new transcript with alternatively spliced variants using canonical splice sites: as-a1 and as-a2 (primers 636–M774), as-b1 to as-b4 (primers M420–A230) and as-c1 to as-c3 (primers 636–A230) (Fig. 1). Moreover, we identified a hyperedited form of the as-b5 variant displaying the characteristic A-to-G substitutions for 48% of its adenosine residues (as-b5-edit). As no conserved domain has been identified in the various small ORFs carried by the complete transcript (Pfam database; Finn et al., 2016), and according to the definition (Dinger et al., 2008), we defined this transcript as a lncRNA, which we named ERL lncRNA, transcribed from a gene at least 4.9 kbp long (from position 4868 to 9788).

We characterized the 5' and 3' ends of the ERL lncRNA by 5' and 3' RACE-PCR on total RNA extracted from MSB-1 cells, from the first and last identified exons of the as-c sequences (A969a–A982) (Fig. 2a). A single transcriptional start site (TSS) at position 3577, within a potential initiator element (INR: CTACAATA), was identified in the 24 representative 5' RACE-PCR clones sequenced. For 3'RACE-PCR, the sequencing of 67 representative clones led to the identification of a major polyadenylation site at position 11 068 (91% of the sequences), and two minor sites at positions 11 064 and 11 077 (3 and 6% of the sequences, respectively). As expected, a consensus poly(A) signal (AAUAAA) was identified 19 nt upstream from the major polyadenylation site. These findings led to the characterization of the 7.5 kb-long ERL lncRNA gene, which codes for the ERL lncRNA split into nine exons and a set of alternatively spliced transcripts, mapping to the transcripts of the mdv1-mir-M9-M4 and mdv1-miR-M11-M1 clusters and of the meq and 14kD genes. Three alternative splicing events have been observed: the splicing of exons 4 (as-b3/-b5/-b5-edit/
Fig. 2. Identification of the extremities and hyper-editing of the ERL lncRNA. (a) Identification of the 5′ (◦) and 3′ (AAA) ends of the ERL lncRNA by 5′ and 3′ RACE-PCR with the A969b and A982 primers (black arrows) on total RNA from MSB-1 cells. Black lines and black dotted lines represent exons (E1–E9) and introns (I1–I8), respectively. The INR is underlined with the TSS (+1) shown in bold. For the 3′ end, intron 8 is represented by dotted lines. The consensus poly-A signal (AATAAA) is shown in bold italic typescript. The three polyadenylation sites are shown in bold, with the positions shown below the sequence and the corresponding percentages shown above the sequence. For both RACE-PCRs, a ‘G’ above the sequence indicates A-to-G editing. (b) Editing of the ERL lncRNA and effect on splicing. RT-PCR was performed with primers binding to introns (white triangles) or exons (black triangles) (Table S1). The edited regions of transcripts, as identified by sequencing, are represented by grey lines and the unedited regions are represented by black lines. The percentage of adenosines edited (A-to-G) for each transcript is indicated on the right. Edited and suppressed splice sites are indicated by (*) and their sequences are
shown underneath, with the edited base in bold. New identified splice sites are indicated by (a). (c) ERL IncRNA expression during the different phases of infection. Electrophoretic analyses of RT-PCR products generated from total RNA from MSB-1 cells, MSB-1 cells treated with 3 mM sodium n-butylate for virus reactivation (MSB-1-R), lytically infected CEFs (CEF-GaHV-2) and non-infected CEFs (CEF-Ni). Migration of the 265 bp amplicon (primers B017–F818) of the ERL IncRNA after specific reverse transcription (primer A13), the 283 bp amplicon for VP5 (432–433 primers) and the 220 bp amplicon for GAPDH (primers M450–M451) obtained by reverse transcription with hexa(dT). M, molecular weight marker.

We observed hyperediting events for 4 % of the 5′ RACE-PCR clones and 3 % of the 3′ RACE-PCR clones, with 44 and 17 % A-to-G modifications, respectively (Fig. 2a), suggesting that the ERL IncRNA transcript might be edited over its entire length. Finally, four libraries have been constructed by RT-PCRs with four couples of primers (M420/M774, M479/A230, M775/M774 and M775/A323). Sequencing of 96 random clones of each library led to the identification of four hyperedited clones (1 %): as-edit-2/-3/-4/-5 sequences, with a rate of A-to-G modification of 59, 37, 6 and 64 %, respectively (Fig. 2b). These sequences showed classic alterations of the splicing induced by the A-to-G modifications: introns 4 and 5 were retained following the suppression of A5b-SS (CAG/GA → CGGGA) in as-edit-3 and -4, and the suppression of A4-SS (UAG/AU → UGGAU) in as-edit-4 and -5 and the creation of a new SS (UAAA/U → UAG/AU), used in as-edit-2 but also observed in as-edit-4.

Expression of the ERL IncRNA is independent of the infection phase

We investigated the phase dependence of ERL IncRNA expression by studying total RNA from latently infected MSB-1 cells, MSB-1 cells treated with 3 mM sodium n-butylate for virus reactivation) and lytically infected CEFs. Two different RT reactions were carried out with hexa(dT) nucleotides, for the subsequent PCR amplification of GAPDH (M450/M451 primers) and VP5 (433/432 primers), or with the ERL IncRNA-specific A13 primer for its amplification (B017/F818 primers), respectively. We compared the level of the ERL IncRNA transcript to that of the transcript for the major capsid protein VP5, representative of lytic replication. Unlike VP5, which was expressed during lytic infection and reactivation, the ERL IncRNA was equally expressed in all phases of infection (Fig. 2c).

ERL IncRNA is hyperedited during infection in vivo and in vitro

We assessed the similarity of the hyperediting observed in MSB-1 cells to that of in vivo infections, using DNA and total RNA extracted from the peripheral blood leucocytes (PBLs) and feather follicles (FFs) of chickens infected with the highly virulent GaHV-2-RB-1B strain from a previous study (Stik et al., 2013). We standardized quantification of the editing rate, by focusing detection on the representative short and highly edited region corresponding to the as-edit-5 amplicon. We first checked that no editing of the DNA had occurred, by performing PCR with the degenerate A342/A13 primers (Table S1), corresponding to the positions of the previously used M775 and A323 primers, and then sequencing 48 clones. The same pair of primers was used for RT-PCR on total RNA. The sequencing of 96 random clones revealed that 2 % of the sequences in the PBL library and 1 % of those in the FF library were hyperedited, with 9 and 24 % A-to-G modifications, respectively (Table 1). Thus, hyperediting of the ERL IncRNA seems to be a natural part of the GaHV-2 replication cycle in infected chickens.

We then used a similar approach to detect editing events for the ERL IncRNA during the lytic in vitro infection of CEFs. By contrast to the 1 % hyperedited transcripts found in latently infected MSB-1 cells and in sodium n-butylate-treated MSB-1 cells, similar to the percentages observed in vivo, 26 % of the clones from GaHV-2-infected CEFs were edited, with a mean of 36 % A-to-G modifications (range: 7–72 %; Table 1, Figs 3a, S1). Moreover, an analysis of the substitution frequency of each adenosine in 174 hyperedited sequences of the as-edit-5 region of the ERL IncRNA revealed that editing was not uniform along the sequence, with substitution rates ranging from 2 to 83 % (Fig. 3b). We identified 21 editing hotspots among the 82 editing sites studied (50–75 %) and five highly edited sites (>75 %); 16 adenosines were rarely edited (5–10 %) and four were very rarely edited (<5 %). As almost all the substitutions observed (98.49 %) were A-to-G

<table>
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<th>Hyperediting</th>
<th>Edited clones</th>
<th>A-to-G substitutions</th>
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<tr>
<td>In vivo†</td>
<td>PBL 2 % (2/96)</td>
<td>8 % (4–12)</td>
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<td></td>
<td>FF 1 % (1/96)</td>
<td>24 %</td>
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<tr>
<td>In vitro‡</td>
<td>MSB-1 1 % (2/192)</td>
<td>35 % (5–65)</td>
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<td></td>
<td>MSB-1-R 1 % (2/192)</td>
<td>27 % (7–47)</td>
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<tr>
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<td>CEF 26 % (50/192)</td>
<td>36 % (7–72)</td>
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* n(A)=82.
† n(seq)=96.
‡ n(seq)=192.
substitutions (Fig. 3c), we investigated whether the surrounding nucleotide context was characteristic of the 5' and 3' preferential environment described in previous publications for the adenosine-editing events mediated by an ADAR protein: 'U>A>C>G' and 'U=A=C=G', respectively (Eggington et al., 2011). The analysis of 4832 editing events showed a significant preference in 5' for U or A (42 and 38 % of A-to-G modifications, respectively) rather than C and G (8 and 11 %, respectively), with no marked preference for the 3' nucleotide (Fig. 3d), entirely consistent with the involvement of an ADAR protein in the detected editing.

**ADAR1 is upregulated and edits the ERL IncRNA during GaHV-2 infection**

We initially carried out quantitative RT-PCR (RT-qPCR) with appropriate primers (Table S1) to compare the differences in ADAR1 and ADAR2 mRNA levels between non-infected or GaHV-2-infected CEFs and CEFs treated directly with chicken IFN-α, which is known to induce the overexpression of ISGs, or poly(I:C)/LyoVec, a strong inducer of IFN-α expression. As expected, no significant differences in mRNA levels were observed for the classically constitutively expressed ADAR2 gene, but ADAR1
transcript levels were significantly (1.8, 2.2 or 4.2 times) higher in infected CEFs and in CEFs treated with IFN-α or poly(I:C), respectively, than in untreated CEFs (Fig. 4a).

We assessed the effect of increases in ADAR1 expression on the level of ERL IncRNA editing, by treating GaHV-2-infected CEFs and MSB-1 cells with poly(I:C). RT-qPCR on total RNA showed that there was 3.1 and 4.5 times more ADAR1 mRNA in treated GaHV-2-infected CEFs and MSB-1 cells, respectively, than in untreated cells (Fig. 4b). RT-PCR with the A342/A13 primers was then performed to amplify the as-edit-5 hyperedited region of the ERL IncRNA from total RNA. The sequencing of 96 random clones from the corresponding libraries showed that poly(I:C) treatment increased the proportion of hyperedited sequences: from 24 to 44 % for GaHV-2-infected CEFs and from 0 to 13 % for MSB-1 cells, respectively (Fig. 4c). Thus, the rate of ERL IncRNA hyperediting is clearly correlated with ADAR1 expression, which increases during the infection of CEFs with GaHV-2, but not with expression of the constitutive ADAR2 gene.

ADAR1 overexpression is controlled by an inducible promoter

We expanded the partial description of the 14-exon mRNA for the chicken ADAR1 (GenBank accession number AM179858.1) by identifying the TSS, through 5′ RACE-PCR on total RNA from CEFs, with the A691 and A356 primers, which bind to two different exons. We characterized a new intron and a new exon starting from the two neighbouring TSSs, with 79 % of sequences starting 989 nt upstream from the ATG, and 21 % starting 13 nt further upstream (Fig. 4d). The full-length chicken ADAR1 sequence thus actually consists of 15 exons and 14 introns. Moreover, we identified only two closely related TSSs, suggesting that the chicken ADARI gene is transcribed only from an IFN-inducible promoter, as in Xenopus laevis, whereas the human and murine ADARI genes are transcribed under the control of two promoters. Consistent with the previous observation of ADAR1 overexpression in IFN-stimulated CEFs (Fig. 4a), an analysis of the promoter sequence upstream from the major TSS identified two overlapping consensus ISRE (core RE: AANNNGAAA) (Fig. 4d).

We investigated the functionality of these consensus ISREs, by inserting the 375 bp upstream from the major TSS (B034-B025 primers) from the CEFs genomic DNA into a promoter-less luciferase reporter plasmid (pGL3basic) to generate the pGL3-prADAR1-wt construct. DF-1 fibroblast cells transfected by the pGL3-prADAR1-wt and pGL3-prADAR1-mut constructs, in which the ISREs were mutated by PCR (B034-B211 primers), were then treated or not by poly(I:C). In untreated cells, the promoter containing the mutated ISRE yielded 30 % lower levels of relative luciferase activity than the wt promoter, indicating significant involvement of these response elements in the activity of the promoter (Fig. 4e). Furthermore, the wt promoter yielded 2.57 times higher relative luciferase activities in poly(I:C)-stimulated cells than in unstimulated cells, whereas no significant difference between stimulated and unstimulated cells was observed for promoters containing mutated ISREs, suggesting a role for these elements in poly(I:C) stimulation and, therefore, in the IFN response.

Mdv1-miR-M4 indirectly deregulates ADAR1 by targeting SOCS1 mRNA

IFN-inducible promoters containing ISREs, like prADAR1, are generally under the control of the JAK/STAT/SOCS pathway. Human SOCS1 expression is downregulated by hsa-miR-155. We therefore investigated the potential role of the chicken homologue gga-miR-155 and its viral analogue mdv1-miR-M4-5P in the regulation of SOCS1 mRNA levels. The sequences of the human and chicken SOCS1 ORFs were 67 and 65 % identical at the nucleotide and amino acid levels, respectively, but an alignment of the human and chicken SOCS1 mRNA 3′-UTR sequences showed the overall level of identity to be only 37 %. However, despite this low identity, the custom-built program TargetScan (release 6.2) predicted, as for the human SOCS1, a potential gga-miR-155/mdv1-miR-M4-5p target in the chSOCS1 mRNA (Fig. 5a). The sequence match between the miRNA seed region and the target was more extensive for mdv1-miR-M4-5p, as this seed displayed more complete complementarity (nucleotides 1–12 of the miRNA), than for gga-miR-155-5p (nucleotides 1–8 of the miRNA).

We assessed the functionality of this gga-miR-155/mdv1-miR-M4-5p target, by amplifying the surrounding 103 bp region from CEFs genomic DNA by PCR and inserting it into the pRL-TK vector downstream from the luciferase reporter gene to generate the pRLmiRE-155-M4 construct. We then constructed pRLmiRE-155-M4mut, with a mutated microRNA response element (miRE). We co-transfected DF-1 fibroblast cells and DT40 B-lymphocyte cells with these two reporter constructs, and either an empty pcDNA vector or one of the previously described pcDNA-mdv1-miR-M4, pcDNA-gga-miR-155 or pcDNA-gga-miR-21 constructs as a negative control (Muylkens et al., 2010). We found that gga-miR-155 and mdv1-miR-M4 significantly decreased luciferase activity, by 52 and 36 % in DF-1 cells and by 31 and 40 % in DT40 cells, respectively, relative to the corresponding miRE-mutated constructs, following transfection with wt constructs (Fig. 5b). No significant differences were found between wt 3′-UTRs and the corresponding miRE-mutated constructs if co-transfections were carried out with an empty pcDNA vector or with the negative pcDNA control vectors expressing gga-miR-21. The predicted gga-miR-155/mdv1-miR-M4-5p target in the SOCS1 mRNA is, therefore, equally functional for both miRNAs.

We checked the efficiency of SOCS1 silencing for three different siRNAs. DF-1 cells were transfected with the si-SOCS1 (si-a/-b/-c) or a negative control siRNA (si-neg). RT-qPCR and Western blotting were then performed to assess SOCS1 gene expression. Relative to si-neg, transfections with si-a, si-b and si-c resulted in 34 % (non-
Fig. 4. Increase in hyperediting is correlated with IFN-induced ADAR1 overexpression. (a) Relative levels of ADAR1 and ADAR2 mRNAs in CEFs. Untreated CEFs (NT) and GaHV-2-infected CEFs (GaHV-2) were cultured for 72 h and harvested. In parallel, uninfected cells were cultured for 64 h and were then stimulated for 8 h with 100 U ml\(^{-1}\) chicken IFN-\(\alpha\) or 1 \(\mu\)g ml\(^{-1}\) poly(I:C)/LyoVec. Total RNA was isolated for RT-qPCR. The relative levels of the ADAR1 and ADAR2 mRNAs were normalized with respect to GAPDH mRNA. Asterisks (*) indicate significant differences between stimulated cells and untreated cells (\(P<0.01\)). Error bars indicate the SD for three independent experiments. (b) ADAR1 expression in infected cells after stimulation of the IFN response pathway with poly(I:C)/LyoVec. Infected CEFs and MSB-1 cells were cultured for 64 h and were then incubated for 8 h in medium with (P1) or without (P0) 1 \(\mu\)g ml\(^{-1}\) poly(I:C)/LyoVec. Total RNA was isolated for RT-qPCR. Relative levels of ADAR1 mRNA were normalized with respect to GAPDH mRNA and are expressed relative to the level of ADAR1 in unstimulated, infected CEFs. Asterisks (*) indicate significant differences between stimulated cells and untreated cells (\(P<0.01\)). Error bars indicate the SD for three independent experiments. (c) Percentages of hyperedited transcripts of the ERL lncRNA in infected cells after stimulation of the IFN response pathway with poly(I:C)/LyoVec. Infected CEFs and MSB-1 cells were cultured for 64 h and then for 8 h in medium with (P1) or without (P0) 1 \(\mu\)g ml\(^{-1}\) poly(I:C)/LyoVec. Following specific reverse transcription with the A13 primer (Table S1) and amplification of the as-edit-5 hyperedited region, 96 random clones were sequenced for each set of conditions. Histograms show editing rates of a representative experiment. *\(\chi^2\) analysis, \(P\)-value <0.05. (d) Identification of the 5' ends (\(\ddagger\)) of the chicken ADAR1 mRNA by 5' RACE-PCR from the total RNA of CEFs with the A356 and A691 primers (black arrows). Exons (1–15) are indicated by grey and black rectangles for previously (AM179858.1) and newly described exons, respectively. Introns are represented by dotted lines. The major TSS (TSS-M) and a minority TSS (TSS-m), the 3' end of exon-1, and the 5' ends of exon-2 and the START codon are indicated in bold on the sequence, together with their positions relative to the TSS-M and their relative usage percentages. (e) Relative luciferase activity for the wt ADAR1 promoter (prADAR1-wt) and the promoter with mutated ISREs (prADAR1-mut) in the presence (P1) or absence (P0) of 1 \(\mu\)g ml\(^{-1}\) poly(I:C)/LyoVec. Error bars indicate the SD for three independent experiments carried out in triplicate. *Student's t-test, \(P\)-value <0.05 versus prADAR1-wt without poly(I:C)/LyoVec.
ADAR1 hyperediting of the GaHV-2 ERL IncRNA

Fig. 5. Effectiveness of mdv1-miR-M4/gga-miR-155 targeting of SOCS1 3'-UTR. (a) Schematic diagram of pRL-TK constructs (left) and alignment of wt (SOCS1-wt) and mutated (SOCS1-mut) target sequences with the sequences of gga-miR-155 and mdv1-miR-M4 (right). A wt or mutated target (miRE) from SOCS1 3'-UTR (GenBank reference: NM_003745.1 and NM_001137648.1), from position 595 to 698, was inserted into the 3'-UTR of the Renilla luciferase reporter gene. For targets, the initial seed sequence match is underlined and complementarities between the target and miRs are highlighted. (b) Impact of the ectopic expression of mdv1-miR-M4 and gga-miR-155 on luciferase expression. The fibroblastic DF-1 and lymphoid DT40 cell lines were co-transfected with luciferase reporter constructs and miRNA expression vectors: pcDNA-mdv1-miR-M4, pcDNA-gga-miR-155, pcDNA-gga-miR-21 or pcDNA3.1 empty vector. Reporter activities were standardized by measuring Renilla-to-firefly luminescence ratios. Each relative luciferase activity was normalized with respect to the empty pcDNA3.1 vector, and for each miRNA expression assay the wt values were then reported to the corresponding mut values (standardized to 1). Error bars indicate the sd for triplicate experiments. *Student’s t-test, P-value < 0.05 versus mutated target for each set of conditions.

DISCUSSION

The GaHV-2 IncRNA, which gave its name to the LAT family, was the first viral IncRNA to be described more than 20 years ago (Cantello et al., 1994). It is expressed specifically during latency and maps to the anti-sense transcript of
the ICP4 gene, in TR3/IR3. In this study, we identified the ERL lncRNA, a second GaHV-2 lncRNA encoded by the RL region, the second repeated region of the GaHV-2 genome (Fig. 1). Like the LAT lncRNA (Strassheim et al., 2012), the ERL lncRNA is a 5’-capped polyadenylated RNA, as shown by RACE-PCR on RNA from MSB-1 cells (Fig. 2a) and GaHV-2-infected CEFs (data not shown). Furthermore, like the LAT lncRNA, the ERL lncRNA exists in different lengths, due to alternative splicing of the 7.5 kb native pre-RNA transcript. In addition, like the LAT lncRNA, which is anti-sense to the ICP4 gene (Cantello et al., 1994; Strassheim et al., 2012), the ERL lncRNA forms a NAT (Lavorgna et al., 2004) overlapping with the transcripts of R-LORF5a, meq and 14kD genes and the two clusters of miRNAs of the RL region: mdv1-mir-M9-M4 and mdv1-mir-M11-M1 (Fig. 2b).

Other herpesvirus lncRNAs are detected principally in one phase of infection: in latency for the GaHV-2 and HHV-1 LAT transcripts and HHV-4 EBER1/EBER2 RNAs (da Silva & Jones, 2013; Wu et al., 2007), and during lytic reactivation of the virus from latently infected cells for the HHV-8 PAN and ALT lncRNAs, HHV-4 BHLF1 RNA, MuHV-1 MAT RNA and HHV-4 oriP transcript (Cao et al., 2015; Chandriani et al., 2010; Juranic Lisnic et al., 2013; Rossetto & Pari, 2014). Unlike these lncRNAs, the ERL lncRNA

**Fig. 6.** Overexpression of ADAR1 after SOCS1 si/miRNA silencing. (a, d) Relative expression of SOCS1 mRNA 48 h after lipofection with anti-SOCS1 (si-a/b/c) and negative control siRNAs or pCDNA-miR-21/155/-M4 constructs. mRNA was quantified by the RT-qPCR $2^{-\Delta\Delta C_{\text{t}}}$ method, with GAPDH mRNA as the reference. The histograms correspond to the mean of triplicates of three independent lipofections of DF-1 cells. SOCS1 mRNA levels were normalized with respect to si-neg (a) or pCDNA-miR-21 (d) lipofection results. SD are shown. *Student’s t-test, P-value <0.05 versus si-neg or pCDNA-miR-21. (b, e) Relative levels of SOCS1 protein (S) assessed by Western blot with GAPDH protein (G) as the reference in corresponding cells. SOCS1 protein amounts were normalized with respect to si-neg (b) or pCDNA-miR-21 (e) lipofection results. The Western blot shown is representative of three independent lipofections of DF-1 cells. m, Mean amounts of SOCS1 protein in each condition of the three experiments; SD, standard deviations of the three experiments; M, dual-colour protein ladder. (c, f) Relative expression of ADAR1 mRNA 48 h after lipofection with anti-SOCS1 (si-a/b/c) and negative control siRNAs or pCDNA-miR-21/155/-M4. Cells were cultured in medium with (P0.5) or without (P0) 0.5 µg ml⁻¹ poly(I:C)/LyoVec. ADAR1 mRNA was quantified by the RT-qPCR $2^{-\Delta\Delta C_{\text{t}}}$ method with GAPDH mRNA as the reference. The histograms show the mean of triplicates of three independent lipofections of DF-1 cells. ADAR1 mRNA levels were normalized relative to those after si-neg or pCDNA-miR-21 lipofections. SD are shown. *Student’s t-test, P-value <0.05 versus si-neg or pCDNA-miR-21.
displayed similar levels of transcription during primary cytolytic infection, lytic reactivation and latency (Fig. 2c). However, editing of the ERL lncRNA seems to be stronger during the cytolytic infection of CEF, with 26% of hyperedited transcripts (Table 1). By contrast, the hyperediting is not associated with lytic reactivation of the virus, since the treatment of GaHV-2 latently infected MSB-1 cells by sodium n-butyrate does not modify the level of hyperedited transcripts (1%). As no difference was found between native and treated MSB-1 cells, we can exclude the possibility that the 1% hyperediting in the native MSB-1 cell line corresponded to the 0.5% of cells in which the virus undergoes spontaneous reactivation (Strassheim et al., 2012). Moreover, as described for HHV-4 and HHV-8 (Cao et al., 2015; Gandy et al., 2007), editing rate is closely related to the level of ADAR1 expression (Fig. 4b), and the similarly low rates recorded in native and treated MSB-1 cells are consistent with the similar levels of ADAR1 present in these cells (data not shown). However, the low rate of editing during GaHV-2 reactivation reflects differences in the links between infection phases and hyperediting between GaHV-2 and other herpesviruses, as the HHV-8 K12 and HHV-4 oriP transcripts undergo site-specific editing and hyperediting, respectively, only during the lytic reactivation of the virus from latency in an infected cell line.

Both inter- and intramolecular dsRNAs can serve as a substrate for ADAR1 (Nishikura, 2010), although editing of the sense/anti-sense dsRNA duplex seems to be a minor event (Kawahara & Nishikura, 2006; Neeman et al., 2005). The lack of editing in the transcripts from the other strand in this and previous (Coupeau et al., 2012) studies suggests that ADAR1 recognizes the ERL lncRNA through internal dsRNA secondary structures, such as hairpins, and bulges and bubbles in long dsRNA regions (Fig. S2) (mfold; Zuker, 2003), a characteristic of ADAR1 substrates (Wahlstedt & Ohman, 2011). Finally, it should be noted that editing seems to be specific to the ERL lncRNA, as no A-to-G event was observed during our many transcriptomic studies, notably for the LAT lncRNA/ICP4 (sense/anti-sense) transcripts encoded by the Rb region (Strassheim et al., 2012).

The hyperediting of the ERL lncRNA is consistent with post-transcriptional regulation during the lytic phase of GaHV-2 infection. Such modifications greatly alter the secondary structure of RNAs, thereby also affecting their nuclear retention and stability and promoting Tudor-SN protein-mediated degradation (Scadden, 2005; Zhang & Carmichael, 2001). Furthermore, the extent of sequence modification clearly affects the potential functionality of this NAT, by disrupting hypothetical interactions with other-strand transcripts through changes in secondary structure or a loss of complementarity (Fig. 1). Hyperediting may also disrupt splicing, by eliminating or creating splice sites (Nishikura, 2010), as observed for the A4 and A5b A-SSs, or by interfering with the splicing of non-edited introns by modifying RNA structure and interactions with the spliceosome (Fig. 2b). These splicing modifications suggest that, as described for some cellular and viral RNAs (Wong & Lazinski, 2002; Zhang & Carmichael, 2001), the ERL lncRNA is edited at least partly in the nucleus, either before or during splicing. This conclusion is further supported by the description of associations of ADAR proteins in the supraspliceosome acting as key regulators of the processing of many pre-mRNAs (Shefer et al., 2014). Moreover, like the HHV-8 ALT lncRNA, which is anti-sense to eight of the 12 miRNAs of the HHV-8-mir-K12 cluster (Gandy et al., 2007), the ERL lncRNA presents a perfect match between exon 1 and the complete 75 nt pre-mdvl-mir-M1, which could be eliminated by editing the 22 adenosines of the complementary ERL lncRNA sequence. This loss of matching during lytic replication, but not during latency, may be responsible for the differential expression of mdv1-mir-M1-5p between lytic infection in CEFs and latent infection in MSB-1 cells, in which this miRNA accounts for a quarter and less than a twentieth of total GaHV-2 miRNAs, respectively (Burnside et al., 2006; Morgan et al., 2008).

The pattern recognition receptors TLR3 and MDA-5 have both been reported to be overexpressed during GaHV-2 infection (Feng et al., 2013; Heidari et al., 2010; Hu et al., 2016), but the mechanism of innate immune activation leading to the expression of ISGs like ADAR1 remains unclear. However, as TLR3-mediated signalling is essentially associated with endosomes (Kawai & Akira, 2006), the cytoplasmic MDA-5 is probably involved in GaHV-2 infection (Feng et al., 2013), as already shown for HHV-1 (Melchjorsen et al., 2010). Our data show that the MDA-5 pathway, which is specifically activated by poly(I:C)/LyoVec (Gitolin et al., 2006), is efficient in the chicken.

The natural induction of ADAR1 during the infection of CEFs with GaHV-2 (Fig. 4a) and the presence of two overlapping functional ISREs in its promoter (Fig. 4d), consistent with activation of the IFN-JAK/STAT pathway, extend the findings of previous transcriptomic studies showing the overexpression of IFN and of several ISGs during the infection of chickens and CEFs (Heidari et al., 2010; Morgan et al., 2001). Silencing of chSOCS1 demonstrated that this protein controlled the JAK/STAT pathway in chickens, as its homologue does in mammals (Fig. 6) (Fenner et al., 2006). Like hsa-miR-155-5p (Su et al., 2011), SOCS1 expression was repressed by gga-miR-155-5p and the viral analogue mdv1-mir-M4-5p (Fig. 6). It has been shown that gga-mir-155 is strongly downregulated during GaHV-2 infection (Yao et al., 2009), whereas mdv1-mir-M4-5p is expressed three times more strongly during lytic infection than in latency (Burnside et al., 2006; Morgan et al., 2008) and may mediate the viral control of SOCS1, partly accounting for the mRNA levels measured in lytic infections of CEFs, which are 80% lower than those in latently infected MSB-1 cells (data not shown).

In conclusion, we have characterized a new viral lncRNA, the ERL lncRNA, which is expressed during all phases of infection. We showed that hyperediting occurred specifically during the lytic phase and was correlated with ADAR1
expression under the control of an IFN-inducible promoter. Finally, as during the early lytic phase of GaHV-2 infection, previous published data showed (i) a strong expression of IFNs and ISGs (Heidari et al., 2010); (ii) the early expression of mdv1-miR-M4, the most expressed GaHV-2 miRNA (Zhao et al., 2015), and of the lytic-associated mdv1-miR-M1 (Burnside et al., 2008); and (iii) a transcription of the meq oncogene (Coupeau et al., 2012), but a low expression of the MEQ protein (Jie et al., 2013), we propose a preliminary scheme where the ERL lncRNA could be involved in the lytic phase of the viral cycle, as NAT RNA (Fig. 7). To be precise, we propose that, during oncogenesis, the non-edited ERL lncRNA could potentially repress the expression of both mdv1-miR-M4 and mdv1-miR-M1 via an excised stable intron with long half-life as previously described for HHV-1 (Thomas et al., 2002) and exon 1, respectively. The modifications of the sequence and of the splicing resulting from the IFN-induced hyperediting of the ERL lncRNA during the first step of GaHV-2 infection would prevent this NAT activity. This hypothesis is consistent with previous data showing that in vitro and in vivo poly(I:C) treatments decreased the level of Meq expression and reduced viral-induced oncogenesis (Hu et al., 2016; Parvizi et al., 2012). Finally, we propose a time regulation of viral and cellular factors according to the viral replication phases. During the cytolytic infection phase, we suggest that mdv1-miR-M4 through the downregulation of SOCS1 could promote a positive regulation loop in which the ADAR1 hyperediting of the ERL lncRNA could increase the rate of mdv1-miR-M4. This regulation loop may support the previously described role of IFNs in the induction of the viral latency (Volpini et al., 1996; Xing & Schat, 2000). Following lytic stage/latency switchover, the resulting amount of hyperedited forms of the ERL lncRNA would subsequently contribute to the described downregulation of IFNs expression, as the binding of inosine-containing RNAs by MDA-5 is known to inhibit its activity and to block the pathway of IFN expression (Li et al., 2012; Mannion et al., 2014; Vitali & Scadden, 2010).

**Fig. 7.** Putative functions of the ERL lncRNA in GaHV-2 biology. Main hypothesis of the non-edited ERL lncRNA functions during the latency/oncogenesis phases (broken lines) and during the early steps of the infection (solid lines). (a) Inhibition of the mdv1-miR-M1 by sequence complementarity, leading to a Meq increase during oncogenesis. (b) Inhibition of the mdv1-miR-M4 via stable exons during the latency/oncogenesis. (c) Blocking of the induction of IFN expression by MDA-5 after binding inosine-containing ERL lncRNA.
METHODS

Virus strain and cell cultures. The very virulent RB-1B strain of GaHV-2 used in this study was prepared from a stock of PBLs collected from B13/B13 chickens 42 days post-infection (dpi) that contained cell-associated GaHV-2-RB-1B virus (Stik et al., 2013). CEFs were prepared from 11-day-old specific-pathogen-free (SPF) White Leghorn B13/B13 embryos raised at INRA (PFIE) and were used as secondary monolayer cultures. CEFs were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Lonza) supplemented with 2.5% FCS, 1.25% chicken serum (CS), 1% penicillin/streptomycin and 1% amphotericin B (Fungizone) and 5% tryptose phosphate broth (TPB). We used GaHV-2-infected PBLs to infect secondary CEFs. After two successive rounds of infection of secondary CEF monolayer cultures, CEFs were infected with cell-associated GaHV-2 seed in a 3:1 ratio. The cells were then harvested. The GaHV-2-transformed lymphoblastoid cell line MSB-1 was maintained in RPMI 1640 medium (Lonza) supplemented with 1 mM sodium pyruvate, 10% FCS and 5% CS. The lymphoblastoid B-cell line DT40, obtained from a tumour induced by Rous-associated virus 1 infection, was cultured in a similar manner in DMEM supplemented with serum. The DF-1 chicken fibroblast cell line was cultured in DMEM supplemented with 10% FCS and 5% CS.

Construction of IR/ΔIR cDNA libraries. Total RNA was extracted from MSB-1 cells in Trizol reagent (Invitrogen). Residual DNA was removed with the RQI DNase (Promega), according to the manufacturer’s instructions. The mRNAs were reverse transcribed with a mixture of oligo(dT) and random primers (50 pmol each; Eurogentec) and SuperScript III reverse transcriptase (Invitrogen). The resulting cDNAs were amplified by PCR [30 cycles of denaturation (94°C, 15 s), annealing (57°C, 30 s) and extension (72°C, 45 s)] in a final volume of 50 μl containing 0.625 U GoTaq Polymerase (Promega), 2.5 mM MgCl2 and 0.1 mM of each primer (Table S1), in the reaction buffer provided by the manufacturer. All the PCR products were inserted into the pGEM-T Easy Vector (Promega). All the clones were sequenced by GATC Biotech, and the corresponding sequences were aligned with the GaHV-2-RB-1B genome.

5’ and 3’ RACE-PCR analysis. RACE-PCR was carried out on total RNA extracted from MSB-1 cells with the GeneRacer kit (Invitrogen) according to the manufacturer’s instructions. Each RACE-PCR was performed as nested-RACE-PCR with closely localized gene-specific and nested gene-specific primers: A647/A356 for ADAR1 mRNA, A969a/ A969b and A982/A983 for ERL IncRNA (Table S1). All products of both 5’ and 3’ RACE-PCR were inserted into pGEM-T Easy (Promega) and the corresponding sequences were determined by GATC Biotech.

Determination of editing rates. Amplification of the mdv1-miR-M9M4 transcript during the determination of ERL IncRNA editing rates was avoided by carrying out specific reverse transcription reactions on total RNA, extracted as previously described, with the specific A13 primers (Table S1) and SuperScript III reverse transcriptase (Invitrogen). The resulting cDNAs were amplified by PCR, as previously described, with the A342 and A13 primers, degenerate (A or G) for A and (T or C) T nucleotides, respectively, to improve the match with the potential editing sites. PCR products were inserted into the pGEM-T Easy Vector (Promega), and at least 96 random clones were systematically sequenced (GATC Biotech).

Induction of ADAR1 expression. We evaluated the natural induction of ADAR expression during infection, by infecting secondary CEFs with GaHV-2 and culturing them in DMEM for 72 h before harvesting for RNA extraction.

The artificial overexpression of ADAR1 was induced by culturing non-infected secondary CEFs for 64 h in DMEM before treatment for 8 h with 100 μM 5′-monophosphate (cyclic) (AbD-Serotec, PAP004) or 1 μg ml−1 poly(βC)/LyoVec (tlrl-picyl; Invivogen). Cells were then harvested for RNA extraction in parallel to untreated CEFs.

We assessed the impact of ADAR1 overexpression on hyperediting of the ERL IncRNA by also stimulating infected cells with poly(βC)/LyoVec as described above: GaHV-2-infected CEFs and MSB-1 cells, for lytic and latent infections, respectively.

Quantitative RT-PCR. We used the Power SYBR® Green Master system (Applied Biosystems) to quantify ADAR1 and SOCS1 mRNA levels by the 2−ΔΔCt method. We subjected 1 μg of extracted RNA to reverse transcription in a total volume of 20 μl, and 2 μl of a 1:10 dilution was used for qPCR, as indicated by the manufacturer, with specific primers (Table S1). GAPDH was used as the reference gene. All qPCRs were performed on the StepOnePlus™ Real-time PCR System (Applied Biosystems), under the conditions recommended by the manufacturer, and dissociation curves were generated post-run.

Luciferase reporter constructs. A 102 bp region of the SOCS1 mRNA containing the predicted target site was amplified by PCR from the cDNA of GaHV-2-infected CEFs, with primer pairs introducing XbaI and NotI restriction sites (Table S1). The desired mutation was generated by direct PCR with a mutated reverse primer. XbaI/NotI-digested PCR products were inserted into the 3′-UTR of the Renilla luciferase gene in the pRL-TK vector (Promega). Both plasmids were purified with a NucleoBond Xtra Midi kit (Macherey-Nagel), and inserts were fully sequenced (GATC Biotech).

Luciferase reporter assays. Luciferase reporter assays with miRNA expression vectors were performed in both DF-1 and DT40 cells. The miRNA expression vector, which had been used in a similar approach in a previous study (Muylkens et al., 2010), was used for the expression of gga-miR-155, mdv1-miR-M4 or the control miR gga-miR-21 in parallel to an empty pcDNA3.1 vector as a negative control. DF-1 chicken fibroblast cells were cotransfected with 400 ng of miRNA expression vector, 20 ng of luciferase reporter constructs containing wt or mutant putative target sites and 5 ng of luciferase control vector, in six-well plates (25×103 cells well−1), in the presence of Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocols. DT40 B cells were cotransfected by resuspending 2×106 cells in Nucleofector solution T (Amaza Biosystems, Cologne, Germany) and subjecting them to electroporation, with Nucleofector program B-023, in the presence of 4 μg of miRNA expression vector, 200 ng of luciferase reporter constructs containing wt or mutant putative miRNA response elements and 50 ng luciferase control vector. Luciferase activity was quantified with the Dual-Luciferase Reporter Assay system (Promega), according to the manufacturer’s protocol. For the standardization of inter- and intra-assay measurements of luciferase activity, we systematically used the control vector pcDNA-MLuc, encoding the firefly luciferase gene under the control of the CMV promoter. Firefly and Renilla luciferase activities were measured consecutively, 24 h after transfection, with a luminometer (Tristar® luminometer; Berthold Technologies). Three independent experiments were carried out in triplicate. Student’s t-test was used for statistical analysis; P<0.05 was considered significant.

We used the pGL3basic expression vector (Promega) to assess the strength of promoters inserted upstream from the firefly luciferase gene. The pRL-TK vector (Promega) carrying the Renilla luciferase gene was used under the control of the thymidine kinase promoter of HHV-1 was used for co-transfection, as a transfection efficiency control. Adherent DF-1 cells, plated in six-well dishes at a density of 20×104 cells well−1, were cotransfected in the presence of Lipofectamine 2000 (Invitrogen) with 100 ng of pGL3-prADAR1-wt or pGL3-prADAR1-mut in DMEM, cultured for 16 h in fresh medium and then treated for 8 h with or without 1 μg ml−1 of poly(βC)/LyoVec (tlrl-picyl; Invivogen). Luciferase activity was quantified as described above.
**SOCS1 silencing.** SOCS1 silencing was carried out in adherent DF-1 cells, which were plated in six-well dishes at a density of 5 × 10⁶ cells well⁻¹. DF-1 cells were transfected in the presence of Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocols, with 4 µg of DNA-ga-miR-155, pcDNA-mv1-miR-M4 or pcDNA-gga-miR-21, or with 200 pmol of SOCS1-siRNAs (si-a, CACGCACUUCCGAAACCUU-UTT; si-b, GGAGACGC AACGCAAGAAGAUU-UTT; si-c, GGAAAGACCUUACAGAAGAUU-UTT) or negative control sRNA (Eurogentec). We assessed the impact of SOCS1 silencing on ADAR1 expression, by culturing transfected cells for 40 h and then replacing the medium with fresh medium with (P0.5) or without (P0) 0.5 µg ml⁻¹ poly(C)/LysOve. Cells were harvested 48 h after transfection, for quantification of the SOCS1 and ADAR1 mRNAs, as previously described, and of the SOCS1 protein by Western blotting.

**Immunoblot analysis.** SOCS1 protein levels were estimated by Western blotting. Harvested cells were treated with Laemmli sample buffer and the proteins in the lysate were separated electrophoretically (SDS-PAGE) and blotted onto nitrocellulose membranes, as previously described (Stik et al., 2013). The membranes were blocked by incubation with blocking buffer (Odyssey) and probed with primary rabbit polyclonal anti-human SOCS1 (Abcam, ab83493) or mouse anti-GAPDH antibodies, followed by monoclonal goat anti-rabbit and anti-mouse infrared dye (IRD)-labelled secondary antibodies (Odyssey). The immunoreactions of interest were detected after excitation with light at wavelengths of 700 and 800 nm for the anti-mouse and anti-rabbit antibodies, respectively. SOCS1 signal intensities relative to the GAPDH loading control were calculated with Image Studio™ 3.1 software.

**Bioinformatics analysis.** Geneious software was used for sequence analysis (www.geneious.com). Genomatix software was used for the *in silico* prediction of promoter sequences (www.genomatix.de/cgi). The online miR target prediction algorithm TargetScan (release 6.2) was used to identify potential cellular target sites from the *Gaillus gallus* 3′-UTR database (http://www.targetscan.org/). GaHV-2 chicken miRNA sequences were downloaded from the miRBase sequence database (release 21; June 2014; http://www.mirbase.org/). The annotated genome sequence for GaHV-2-91 strain (GenBank reference: EF523590.1) and chicken miRNA sequences for SOCS1 (GenBank reference: NM_003745.1 and NM_001137648.1) and ADAR1 (GenBank reference: AM179858.1) were downloaded from NCBI (www.ncbi.nlm.nih.gov/nucleotide).

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