APOBEC1-mediated editing and attenuation of HSV-1 DNA implicates an antiviral role in neurons during encephalitis

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Abstract

APOBEC1 (A1) is a cytidine deaminase involved in the regulation of lipids in the small intestine. HSV-1 is a ubiquitous pathogen that is capable of infecting neurons in the brain, causing encephalitis. Here, we show that A1 is induced during encephalitis in neurons of rats infected with HSV-1. In A1-stably expressing cells, HSV-1 infection resulted in significantly reduced virus replication when compared with infection in control cells. Infectivity could be restored to levels comparable to those observed in control cells if A1 expression was silenced by specific A1 shRNA. Moreover, cytidine deaminase activity appeared to be essential for this inhibition and lead to an impaired accumulation of viral mRNA transcripts and DNA copy number. The sequencing of the viral gene UL54 DNA, extracted from infected A1-expressing cells, revealed G to A and C to T transitions, indicating that A1 associates with HSV-1 DNA. Taken together, our results demonstrate a model in which A1 induction during encephalitis in neurons may aid to thwart HSV-1 infection.

Running Title: APOBEC1 inhibits HSV-1 infection

INTRODUCTION:

The human apolipoprotein b editing catalytic polypeptide (ABOBEC) family is a group of zinc-dependent DNA and RNA cytidine deaminases and consists of AID, APOBEC1 (A1), APOBEC2 (A2), seven APOBEC3s (A3A-H), and APOBEC4 (A4). A1, the first APOBEC to be discovered, is known to introduce a premature stop codon into host apolipoprotein B mRNA in the gastrointestinal tract, an event critical for lipid metabolism (17, 39, 60). The editing by A1 is highly precise and specifically converts C to U at position 6666 of the apolipoprotein B mRNA substrate (45). Along with apobec 1 complementation factor ACF, these two proteins constitute the minimal required components necessary for the editing of apolipoprotein B mRNA *in vitro* (36).

Cytidine deaminases as antiviral factors first came into the limelight after A3G was identified as a cellular restriction factor capable of inhibiting HIV-1 dissemination in the absence of HIV-1 virus infectivity factor (vif) (55). This molecule was later shown to inhibit retrovirus infection by inducing massive hypermutation of the murine leukemia virus (MLV) genome (22). Further detailed studies revealed that APOBEC molecules are packaged into HIV-1 virions in virus producer cells via a specific interaction with gag and viral RNA and then exert their deaminase activity in subsequent target cells on a single ssDNA intermediate synthesized by the reverse transcriptase (3, 27, 54). Editing can lead to non-synonymous mutations, such as premature stop codons, in critical proteins (e.g. reverse transcriptase) necessary for virus replication and infectivity, severely impairing the next round of infection (53, 63). Extensive studies to assess the antiviral nature of these APOBEC enzymes have been performed across a broad range of retroviruses and the hepatitis B virus (HBV) (7, 21, 34, 35, 41, 42, 49, 55, 57).

Herpes simplex virus (HSV) is an enveloped, dsDNA virus and a member of the genus alphaherpesviridae. One in every 250,000 to 500,000 individuals infected with HSV type 1 (HSV-1) or type 2 (HSV-2) experiences a devastating disease known as HSV encephalitis (HSE), characterized by acute inflammation and/or hemorrhaging in the central nervous system (CNS) (61). HSV-1 is the predominant causative agent of sporadic encephalitis in western countries and it is estimated to be responsible for over 90 % of HSE cases (61).

In this study, we show a novel finding that A1 expression is induced during HSV-1 infection in neurons of infant rat brains, more than 30 % of which were able to recover from HSE. Moreover, an investigation into the potential antiviral role of A1 *in vitro* revealed that A1 inhibited virus replication directly by mainly targeting viral DNA in a deaminase dependent manner, resulting in a stall of virus replication. To the best of our knowledge, this is the first report implicating the potential antiviral function of A1 against herpes viruses in the context of HSE.

Materials and methods

Cells, plasmids, viruses and infection. Vero and RS cells were maintained in DMEM and supplemented with FCS and antibiotics as previously described (4). Rat A1 cDNA was obtained by RT PCR of mRNA derived from HSV-1-infected rat brain tissue and inserted into pcDNA3.1/Zeo(+) (Invitrogen). An A1 mutant, E63A, was generated by site-directed mutagenesis using a QuickChange® II Site-Directed Mutagenesis Kit (Stratagene). HA-tagged empty vector, A1-WT, or E63-mutant DNA were transfected into RSC and then cultured for 10 days in the presence of Zeocin. Colonies were then screened and confirmed for protein expression by western blotting with an anti-HA Mab. GFP-expressing replication-competent HSV-1, YK333 (59), was used as previously described (4). YK333 HSV-1 was created by the intergenic insertion of a GFP expressing cassette between UL3 and UL4 of the virus genome. Green fluorescence can be detected after HSV infects host cells and starts viral gene expression (59); however, since this event occurs before viral DNA replication, the GFP signal detected in these cells is not indicative of HSV-1 progeny production. For inhibition of HSV-1, we added 25 μg/ml of acyclovir (Sigma-Aldrich, St. Louis, MO, USA) in culture medium.

Generation of shRNA and shRNA-expressing cells. shRNA plasmids were generated by cloning A1 specific or luciferase control ORF targeted sense and antisense sequences into a pBAsi-hU6 Puromycin vector (TaKaRa), via *Bam*H1 and *Xba*I restriction sites. The targeted A1 and luciferase sequences are as follows: A1-736, 5'-GATCCCCGACGCTCCGTTACCCGGTTACGTGTGCTG TCCGTAACCAGGTAATGGAGCATCTTTTTGGAAAT-3'; A1-1119, 5'-GATCCCCGTTC TTCAAGGCTGCCGTTACGTGTGCTGTCCGTAATGGCAGCTTTGAAGAGCTTTTTGG

AAAT-3'; Luciferase, 5'-GATCCCCGTGCGTTGTTGGTGTTAATACGTGTGCTGTCCGTATTGGCACCAGCAGCGCACTTTTTGGAAAT-3'. A1-expressing RSCs were transfected with the shRNA plasmids, respectively, using lipofectamine 2000, selected by puromycin for individual clones and finally analyzed by western blotting for the suppression of A1.

Animal models and tissue collection. Fourteen-day-old Wistar Hannover GALAS rats (CLEA Japan, Inc.) were anaesthetized and then intracranially inoculated with 1.0×10^6 $TCID_{50}$ HSV-1 (YK333), or phosphate-buffered saline (PBS) as a negative control. Rats exhibiting a range of symptoms were classified into four groups as described in Table 1. For histological examinations, tissue samples were fixed by immersion in 4% PFA and processed as described before (4). All animal experiments were carried out according to the guidelines for animal experimentation at Kyoto University.

Transcriptome analysis. RNA was prepared from rat brains using an RNeasy extraction kit (QIAGEN) and transcriptome analysis was performed using a GeneChip Rat Genome 230 2.0 Array (Affymetrix) according to the manufacturer's instructions. Microarray data was analyzed using Gene Spring software (Agilent).

Real-time PCR. Nucleic acids were extracted as previously described (4). For the analyses of HSV-1 mRNA products, 1 μg of RNA was reverse transcribed using a QuantiTect Reverse Transcription Kit (QIAGEN), according to the manufacturer's instructions. The cDNA (50 ng) was used as a template to amplify UL54 (immediate early), UL30 (early), and UL27 (late) genes by real-time PCR using a Power SYBRGreen PCR Master Mix (Applied Biosystems). The threshold was set to a value

of 1.00, and the number of cycles required to reach the threshold cycle, Ct, was determined and then normalized with the Ct of the housekeeping gene, GAPDH. The HSV-1 DNA copy number was measured as previously described (4). Thermocycler conditions for the real-time PCR reaction are as follows: 95°C for 10 min, (95°C for 15s, 60°C for 1 min) x 40 cycles. The primers used for real-time PCR are as follows: A1 Forward, 5'-ACCACGCAGATCCTCGAAAT-3'; A1 Reverse, 5'-TCTTGCTCCGTCATGATCTGG-3'; HSV-1 UL54 Forward, 5'-CCGCGACGACCTGGAATCGG-3'; HSV-1 UL54 Reverse: 5'-GGCGAGCGGCGTCGAGTATC-3'; HSV-1 UL30 Forward, 5'-AGAGGGACATCCAGGACTTTGT-3'; HSV-1 UL30 Reverse, 5'-CAGGCGCTTGTTGGTGTAC-3'; HSV-1 UL27 Forward, 5'-TCGCCTTTCGCTACGTCAT-3'; HSV-1 UL27 Reverse, 5'-GGTTCTTGAGCTCCTTGGTGG-3'; GAPDH Forward, 5' -ACTAAAGGGCATCCTGGGCTA-3'; GAPDH Reverse: 5' -TGGAAGAATGGGAGTTGCTGT- 3'.

Immunohistochemistry and antibodies. Brain sections and culture cells were treated as described before (4, 30). The following primary and secondary antibodies were used: goat anti-apobec1 polyclonal antibody (Pab) (Santa Cruz Biotechnology), rabbit anti-HSV-1 Pab (DakoCytomation), mouse anti-MAP2 monoclonal antibody (Mab) (Upstate), anti-HA Mab (Roche), Alexa Fluor 594-conjugated anti-goat IgG (Invitrogen), Cy5-conjugated anti-mouse IgG or Alexa Fluor 647-conjugated streptavidin (Invitrogen). Nuclei were stained using Hoechst33342 (Invitrogen). Each sample was examined

under a confocal laser microscope (TCS SP2 AOBS, Leica Microsystems) using 405, 543, 633 nm excitations with 10x, 20x and 40x objectives.

Sequencing analysis of UL54. Nested PCR of UL54 was carried out with Pfu Ultra II Fusion DNA polymerase (Stratagene). In the first round of PCR, 100 ng of template cDNA or DNA, derived from either HSV-1 infected A1-expressing or control cells, was used with the appropriate primers in a total volume of 25 µl under the following thermocycler conditions: 1 min at 98°C, (20 s at 98°C, 20 s at 57°C, and 30 s at 72°C) x 35, and 3 min at 72°C. For the second round of PCR, 2.5 µl of the first PCR reaction was used as a template in a total volume of 25 ul under the following thermocycler conditions: 1 min at 93.4°C, (20 s 93.4°C, 20 s at 57°C, and 30 s at 72°C) x 35, and 3 min at 72°C. The UL54 amplicon was cloned into a pUC19 vector and the DNA was extracted from transformed bacteria. A BigDye® Terminator Cycle Sequencing Kit (v3.1) was next used and samples were read on an ABI® Prism 3130 sequencer. Sequences were analyzed using Sequencher 4.9 software. The primers used for the nested PCR are listed as follows: First PCR UL54 forward, 5'-AGCTTTGGCCGCAGCGCACA-3'; First PCR UL54Reverse, 5'-GAGTTGCAATAAAAATATTTGCCGTGCAC-3'; Second PCR UL54 Forward, 5'-GGTCTAGAAGCTTTGGCCGCAGCGCACA-5'; Second PCR UL54 Reverse, 5'-ATCAAGCTTCCTCGCGCCTTCAGGTAGCA-3'.

Statistical analysis. Statistical significance was determined using the student's t test and χ^2 analysis. A p value of less than 0.05 was considered statistically significant.

Results

Survival rat model from encephalitis with intracranial HSV-1 inoculation

In the past, rats have been reported to be useful models for examining HSE induced by both HSV-1 and HSV-2 and, depending on the age of the animal, there appears to be an age-dependent resistance to infection for survival (5, 15). In line with these reports, our preliminary studies revealed that infant GALAS rats before the age of 14 days old suffered from 100% mortality when infected by an intracranial injection of GFP-expressing HSV-1 (YK333) (Supplementary Data 1). On the other hand, rats 18 days or older displayed 100% survival after HSV-1 inoculation. Interestingly, the infection of 14 day-old-rats resulted in half of the animals surviving (Supplementary Data 1). These results indicated that possible intrinsic factors expressed in rats from this age may help to protect against HSV.

In order to the elucidate factors contributing to the survival of the HSV-1-infected 14-day-old rats, we focused on the observed phenotype in this age group. We proceeded to inoculate 63 animals with HSV-1 for a more in-depth assessment of HSE. We found that 17 HSV-1-injected rats (27%, indicated as severe in Table 1 and Figure 1A) died after showing severe signs of encephalitis such as weight loss and quadriplegia within 5 days after inoculation. Fourteen injected rats (22%, indicated as mild in Table 1 and Figure 1A) died after showing a milder degree of weight loss and quadriplegia between 5 and 7 days. Interestingly, 24 injected rats (38.1%, indicated as survived in Table 1 and Figure 1A) recovered from HSE after showing temporary paralysis; HSV-1 infection in these mice was confirmed by GFP^+ and $HSV\text{-}1^+$ regions. The final group of 8 injected rats did not exhibit symptoms of encephalitis and did not possess GFP^+ and $HSV\text{-}1^+$

183 regions (indicated as healthy in Table 1). The range of symptoms displayed by the rats
184 indicated that some were able to cope with HSV-1 infection more than others, which we
185 speculated to be due to the containment of virus infection.

186 In the brain tissue of the severe and mild rats, taken at the time of death from HSE, we
187 found vast hemorrhagic and necrotic damage (Figure 1A). Furthermore, disseminated
188 GFP^+ regions, which were confirmed as $HSV\text{-}1^+$ cells by immunostaining with an anti-
189 HSV antibody, indicated massive spreading and a multifocal distributed infection in the
190 brain, typical of encephalitis in human infants (Table 1 and Figure 1A) (61). Severe and
191 mild rats were also associated with extensive leukocyte infiltration by $CD3^+$ T cells and
192 $CD68^+$ macrophages (Figure 1B, Red), however, brain samples from survived rats
193 taken 36 h after the disappearance of paralysis showed less infiltration in parenchyma
194 and fewer GFP^+ cells (Table 1 and Figure 1B, 3rd row of panels). Meanwhile we could
195 not detect pathological changes, GFP^+ or HSV^+ cells in the brains of mock-infected rats
196 (in the bottom panels Figure 1A), confirming that the observed results were indeed
197 dependent on HSV-1 infection. These data also indicated that limited but clear HSV-1
198 infection occurred in the brain of the survived rats.

199 To investigate whether our initial observation of reduced HSV-1 infection in survived
200 rats was due to the control of HSV-1 dissemination over the course of infection or
201 simply due to an inadequate initial infection, brain samples of survived rats were taken
202 12, 24, and 36 h after the disappearance of paralysis, and the extent of GFP^+ regions
203 was determined. Results showed that the HSV-1-infected regions diminished over a
204 span of 36 h, suggesting that the virus was being contained and cleared (Figure 1C).
205 These data also suggest the existence of an anti-HSV factor(s) induced upon HSV-1

infection, which appears to be expressed in CNS parenchymal cells, including neurons or glia, since the number of invading lymphocytes and macrophages did not seem to correlate with clearance of the virus.

Apolipoprotein B editing catalytic subunit 1 is induced during HSV-1 infection in brain tissue

In order to identify potential candidate molecules which are induced in the brain upon HSV-1 infection, we isolated RNA from the GFP^+ and neighboring regions of two survived rat brains at 4 and 6 days post infection (corresponding to 12 and 36 h after the disappearance of paralysis, respectively) and performed microarray analysis of mRNA from both survived or mock-infected rat brains. In comparing the level of mRNA from these rats, 47 genes showed augmented expression (Supplementary Data 2). Among them, the level of A1 mRNA was clearly increased by 4.59 and 41.6 fold at 4 and 6 days post infection, respectively.

Since A1 and related A3 family proteins are known to be strong inhibitors of HIV-1 infection, we examined the possibility of an association between A1 and HSV-1 infection. From rat brain tissue, we were able to detect significantly elevated levels of A1 cDNA exclusively in HSV-1-infected rats by real-time PCR (Figure 2A). As expected, we confirmed high levels of A1 cDNA expression in the small intestine of mock and HSV-1-infected rats, where A1 is constitutively expressed (Figure 2E). Immunofluorescence staining of brain tissue with an A1-specific antibody demonstrated $A1^+$ staining only after HSV-1 infection, but not after mock infection (Figure 2B), corroborating PCR findings.

To further investigate the cell type specific expression of A1 after HSV-1 infection, we performed co-immunostaining with an anti-MAP2 (neuron-specific) and an anti-A1 antibody. $A1^+$ staining was predominantly observed in $MAP2^+$ cells which were HSV-1 infected but not in $MAP2^-$ cells, indicating that the A1 expression was induced in neurons within the HSV-1 infected brain (Figure 2B-D). On the other hand, the expression levels of A1 in the small intestine were high regardless of HSV-1 infection (Figure 2F). These data suggested that A1 may serve as an intrinsic neuronal tissue factor for the control of HSV-1 infection in the CNS.

A1 inhibits HSV-1 infection in a deaminase-dependent manner

To examine the potential anti-HSV-1 activity of A1, we generated N-terminal HA-tagged wild type rat A1 ($A1_{WT}$) stably-expressing rabbit skin cells (RSC). A1 is a zinc-dependent deaminase with 3 residues, (His^{61}, Cys^{93}, Cys^{96}) needed for coordinating zinc binding and a glutamate residue at position 63 in the catalytic domain essential for cytidine deaminase activity (38). Hence, we also generated N-terminal HA-tagged A1 deaminase-deficient mutant ($A1_{E63A}$) stably-expressing RSC. Control A1 ($A1_{Ctrl}$) RSC were generated using an empty HA vector. Western blotting analysis using an anti-HA antibody confirmed that the expression of $A1_{WT}$ and $A1_{E63A}$ was equivalent in these cell lines (Figure 3A). Immunofluorescence staining of $A1_{WT}$ and $A1_{E63A}$ showed similar localization which was comparable to that in $A1^+$ neuronal cells of HSV-1-infected brain tissue (Figure 2B and 3B, respectively).

We next proceeded to assess the potential HSV-1 antiviral activity of A1 by infecting $A1_{WT}$ or $A1_{E63A}$ RSC with GFP-expressing HSV-1 at a MOI of 0.01. As shown in Figure

3C, the level of GFP expression was significantly attenuated in $A1_{WT}$ RSC in comparison to $A1_{E63A}$ and $A1_{Ctrl}$ RSC 48 h post infection. Interestingly, $A1_{E63A}$ cells also had a modest inhibitory effect on HSV-1, although this activity did not appear to be as potent as in wild type expressing RSC, suggesting a somewhat deaminase-independent inhibition mechanism (Figure 3C). When two different shRNA were used to specifically target $A1_{WT}$, the infectivity of HSV-1 was restored and comparable to that observed in $A1_{Ctrl}$ (Figure 3D). Overall, the low and high level of A1 protein expression resulting from specific shRNA (A1/736 and A1/1119) against A1 and control luciferase, respectively, correlated inversely with HSV-1 infectivity (Figure 3D) and suggested that A1 expression is essential for the inhibition of HSV-1 infection.

In order to analyze the level of attenuation in HSV-1 replication, we looked at the virus titer of supernatants taken from $A1_{ctrl}$, $A1_{WT}$ and A_{E63A} cells infected at higher MOI. The virus titer of supernatant from HSV-1-infected $A1_{WT}$ cells was significantly attenuated when compared with that from empty vector or $A1_{E63A}$ stably expressing cells in a dose dependent manner (Figure 3E). Additionally, the $A1_{E63A}$ mutant also modestly attenuated virus titers, although not to the same extent as wild type A1. These data indicated again that cytidine deaminase activity was also crucial for potent inhibition to take place. Taken together, even at a high MOI, HSV-1 replication was being significantly inhibited in $A1_{WT}$ expressing cells when compared with $A1_{Ctrl}$ RSC in a deaminase-dependent manner.

A1 inhibits HSV-1 gene transcription and DNA replication

HSV-1 gene expression is highly ordered and occurs in a cascade-dependent manner which is divided into three stages with the transcription products subdivided into 3 broad groups termed α, β, and γ, also known as immediate early (IE), early (E), and late (L), respectively (26). The α genes, produced soon after infection, promote β gene transcription and increase viral protein synthesis. Thereafter, β genes are transcribed and enhance DNA replication which, in turn, signals γ gene transcription for the production of viral proteins necessary for virion assembly (12).

To better understand the mechanism by which A1 inhibits HSV-1 replication, we first examined the expression level of representative genes from α, β, and γ groups. UL54, an α gene that plays a role in the shut off of host protein synthesis and also enhances viral gene expression, UL30, a β gene and the DNA polymerase responsible for DNA replication, and UL27, a γ gene and envelope glycoprotein, were analyzed by real-time PCR and normalized to GAPDH. As can be seen from the expression level of HSV-1 cDNA extracted from $A1_{Ctrl}$ and $A1_{WT}$ stably expressing cells at 8 h post infection at a MOI of 0.01, all three genes analyzed were significantly lower in $A1_{WT}$ cells compared to $A1_{Ctrl}$ cells (Figure 4A).

Given that α gene synthesis precedes DNA replication, we expected that the observed low level of the immediate early genes would affect HSV-1 DNA replication and, thus, the viral DNA copy number. Indeed, at 8 h post infection, the HSV-1 DNA copy number was nearly 4 times lower in the $A1_{WT}$ cells when compared with $A1_{Ctrl}$ cells (Figure 4B). As an additional control, infection of control RSC with a UV-inactivated virus did not result in viral DNA replication (Figure 4B). Altogether, our results indicated that A1 affected viral gene expression and also DNA replication.

A1 induces mutations in HSV-1 DNA

A1 homologues are putative inducers of mutations in retrovirus DNA and also in DNA of A1-expressing bacteria (8, 23, 28, 43, 48). Thus, to investigate the mechanism of A1-dependent inhibition of HSV-1 gene expression, we looked at the potential editing of HSV-1 viral DNA. The UL54 DNA extracted from HSV-1-infected $A1_{WT}$ RSC showed evidence of both G→A and C→T transitions, indicating that plus and minus strands were being edited (Figure 5B). The majority of the 51 clones analyzed from $A1_{WT}$ RSC contained 1 mutation within the sequenced region; however, a few clones harbored 2 or 3 mutations (Figure 5E). On the other hand, $A1_{Ctrl}$ derived clones showed no signs of mutations (Figure 5A and 5D), suggesting that the observed nucleotide changes were induced by A1.

Given that rat A1 is also known to edit retroviral RNA, we looked at cDNA extracted from $A1_{WT}$ RSC 8 hpi at a MOI of 0.01 for evidence of additional deaminase activity (8). Similar to DNA sequencing results, G→A and C→T transitions in viral cDNA were observed, with the majority of clones containing 1 mutation, while clones with 2 or 3 mutations were also observed (Figure. 5C and 5F). Interestingly, 1 clone contained 10 G→A mutations, suggesting that a second round of editing may be occurring on mRNA transcribed from the mutated DNA (Figure 5F); yet, the main editing appeared to be occurring on the viral DNA which was mirrored by the mutations observed in viral cDNA (Figure 5B and 5C). In looking at the type of mutations arising from 39 commonly mutated positions in UL54 cDNA and 30 commonly mutated positions in UL54 DNA, approximately 70% of mutations were found to lead to non-synonymous substitutions in the analyzed gene (Figure 5G).

APOBECs, such as A3G, are known to have an editing preference for 5'CpC and 5'TpC and we attempted to assess the dinucleotide preference of rat A1 (58). As can be seen in Figure 5H, we found the preferred context to be 5'GpC on both viral DNA and RNA, although previous studies have indicated a tendency of rodent A1 to prefer 5'TpC (Figure 5H) (11, 28, 48). Altogether, these results indicate that rat A1 directly acts on DNA for editing.

A1 inhibits HSV-1 transcription independently of viral mRNA editing

To further elucidate the mechanism by which A1 inhibits HSV-1 infection, we focused on the initial transcription of viral genes in the presence of the nucleoside analogue inhibitor, acyclovir, which effectively inhibits nascent DNA synthesis. If A1 mainly acts on ssDNA substrates for mutagenesis then we expected to see reduced deamination of HSV-1 cDNA during transcription and few clones harboring signature A1-mediated mutations. At a MOI of 1, the sequencing of UL54 mRNA extracted 2 hpi revealed a low frequency of C → U and G → A transitions in samples taken from $A1_{WT}$ expressing cells similar to nucleic acids obtained from $A1_{Ctrl}$, further indicating that A1 is acting on DNA and not mRNA (Figure 6A and 6B). In addition, only 1 clone containing these mutations was observed from each $A1_{Ctrl}$ and $A1_{WT}$ RSC (Figure 6C and 6D). However, when we looked at the levels of viral gene transcripts at this time point, α, β, and γ gene expression was still significantly lower in $A1_{WT}$ than $A1_{Ctrl}$ RSC (Figure 6E). Altogether, these data suggest that although A1 did not induce mutations in viral RNA, its association with ssDNA may serve to block and inhibit transcription.

Discussion:

From our findings, we propose a novel role for A1 as an inhibitor of HSV-1 infection in the context of HSE in rats. Although the induction mechanism remains to be elucidated, we posit that HSV-1 infection triggers the expression of A1 in neuronal cells which inhibits the early stages of the virus life cycle. The molecule may edit viral DNA in the nucleus, and/or serve as a physical blockade by binding to DNA during transcription, resulting in reduced expression and dysfunction of the edited viral genes. Stalling of the HSV-1 gene cascade may prevent the virus from ramping up to full scale production and allow for a controlled immune response.

We found that both viral DNA and RNA harbored G → A transitions, however, DNA was the main target of A1 during HSV-1 infection (Figure 5B and 5C). Furthermore, when DNA synthesis was blocked, cDNA isolated from virus-infected cells displayed few mutations, further reinforcing the assumption that A1 is acting on a DNA substrate (Figure 6A and 6B). HSV-1 is known to replicate within the nucleus and while A1 has been reported to shuttle between the cytosol and nucleus in the presence of apobec 1 complementation factor (ACF) for a nuclear distribution, if ACF is mutated or absent, then A1 will primarily have a cytoplasmic localization (9, 24). Here, we show similar distribution of A1 in the cytosol *in vivo* in HSV-1-infected neurons and *in vitro* in A1 expressing RSC (Figure 2B and 3B). Yet, because we observed mutations in HSV-1 DNA *in vitro* (Figure 5B), this suggests that a small pool of A1 also exists in the nucleus and is involved in the direct interaction with and mutation of genomic viral DNA.

Although we obtained evidence of cytidine deaminase dependent mutations in HSV-1 nucleic acids, it is possible that our observed results could be an underestimate of the

361 actual degree of mutagenesis which is taking place inside of A1-expressing cells. HSV
362 encodes a uracil DNA glycosylase which is able to remove uracil bases from DNA
363 allowing for subsequent repair of the damaged DNA (31, 37, 62). This activity may be
364 masking the true extent of deamination taking place by A1.

365 It is worth noting that the editing of HSV-1 DNA alone may not completely account for
366 the inhibition of HSV-1. We found 33% of mutated positions in the sequenced region of
367 UL54 DNA and 28% of UL54 RNA to be synonymous mutations, meaning that their
368 function would remain intact (Figure 5G). Furthermore, mutations alone do not entirely
369 account for the observed inhibition of α gene transcription (Figure 4A) since
370 transcription of these genes are highly dependent on incoming tegument protein VP16
371 which acts as a transactivator (1, 2). Therefore, it is possible that A1 may also inhibit
372 gene transcription through an undefined mechanism similar to the A3 deaminase-
373 independent inhibition of HIV-1 reverse transcription products, perhaps through a
374 physical block of transcription or DNA replication (6, 18, 19, 40, 56). Supporting this
375 assumption is data from our deaminase-deficient mutant A_{E63A} that showed partial
376 inhibition of HSV-1 infection, although not to the same extent as the $A1_{WT}$ protein
377 (Figure 3C and 3E). Moreover, diminished HSV-1 transcript levels were observed in
378 $A1_{WT}$ RSC when DNA synthesis was inhibited, even in the absence of RNA cytidine
379 deamination (Figure 6E). Yet, because of the presence of repair mechanisms encoded
380 by HSV-1, such as the uracil DNA glycosylase described above, we were not able to
381 rule out the possibility that viral ssDNA is mutated during transcription.

382 If mutated viral transcripts are synthesized and make their way to the cytoplasm for
383 translation, we suspect the functionality of the synthesized viral protein may be affected.

As mentioned above, the gene expression of HSV-1 is tightly regulated and defects in certain genes can have detrimental effects on the virus life cycle. For instance, studies on HSV-1 temperature mutants have revealed that defects in the IE genes ICP4, and UL54 render the virus replication deficient (16, 46, 50). Furthermore, IE genes have pleiotropic functions as transcriptional activators of viral genes and suppressors of host protein synthesis. In the case of UL54, other functions include the inhibition of mRNA splicing, an increase in viral mRNA levels, and the export of viral mRNA from the nucleus (10, 20, 29, 51, 52). Given that HSV-1 proteins have essential regulatory roles, it is conceivable that disruptions of these genes can cause a stall in the virus replication cycle. Our results also support this possibility as evidenced by the reduced HSV-1 mRNA and DNA levels, GFP expression, and virus titer in infected A1 expressing RSC (Figure 3C, 3E, 4A, and 4B, respectively). This effect was dependent on A1 expression as silencing of A1 by specific shRNA restored HSV-1 infection (Figure 3D).

Our *in vitro* results raise the issue of whether the expression of A1 in rat brains induced during HSE aids to inhibit virus infection or not. Rat A1 is ubiquitously expressed in virtually all tissues such as the spleen and liver and while A1 functions within an editing complex in the small intestine for the deamination of apolipoprotein B mRNA, it is also expressed in tissues where apolipoprotein B mRNA is not present, bringing into question whether there is an additional role for this enzyme (25). Indications that A1 also has antiviral activity on top of its already known physiological function have been shown using mouse models infected with either MLV or HBV. Splenocytes taken from MLV infected mice or hepatocytes taken from HBV infected transgenic mice both displayed signs of A1 specific editing of viral genomes, implicating a direct role in virus

inhibition (44, 48). From our *in vivo* findings, survived and mild rats showed an increase in A1 mRNA expression which was not observed in mock infected rats (Figure 2A and 2B). Moreover, rat brain tissue infected by HSV-1 revealed that the induction of A1 occurred predominantly in HSV-1 infected neuronal cells and suggests a possible antiviral function for this molecule in the brain.

The consequence of increased cytidine deamination of the HSV-1 genome on virus infectivity has been exemplified by a previous report showing that HSV-1 mutants containing a defective uracil DNA glycosylase were 100,000 times less neuroinvasive in mice intracranially inoculated with HSV-1 than their wild type counterparts (47). On the other hand, uracil DNA glycosylase deficient-HSV-1 did not have issues replicating *in vitro*, suggesting that this enzyme is necessary for virus infectivity in rodent neurons because HSV-1 is more prone to cytidine deamination in these cells (47). It is tempting to speculate that A1 in rats may also be playing a role in mutating the HSV-1 DNA *in vivo* to inhibit HSV-1 infection in the brain in our model.

A1 may also help to reduce HSE *in vivo* by slowing down virus dissemination long enough to allow for an appropriate immune response to be mounted, thus eliminating the virus without causing excessive damage from infiltrating macrophages during encephalitis or lytic damage resulting from massive viral infection. In fact, it has been speculated that an overzealous immune response may be causing more harm than good in the CNS (13). For instance, the depletion of macrophages during HSV-1 infection in mice has been demonstrated to lead to a higher survival rate (33). On the other hand, uncontrolled virus dissemination can also lead to a fatal outcome in mice missing type I interferon receptor (14, 32). Needless to say, a balance must be struck

between the host immune response and the extent of virus infection. It is plausible that A1 helps to achieve both of these feats by inhibiting virus infection, resulting in a toned down immune response, as was shown *in vitro* (Figure 3C and 3E). Here, we observed diminished leukocyte infiltration in survived rats compared to mild and severe rats which died within 5-6 days after infection in our *in vivo* model (Figure 1A). Moreover, HSV-1 infected areas, diminished over time in survived rats, suggesting the containment of virus dissemination (Figure 1C).

In conclusion, we identified rat A1 as a novel anti-HSV-1 molecule induced during virus infection in rat brains. The mechanism by which A1 inhibits virus replication appears to be through the interference of the HSV-1 gene cascade by a predominantly deaminase dependent manner involving the editing of viral DNA. In order to assess the extent of A1 inhibition on HSV-1 infection during encephalitis, future studies using knockout animals may provide insights into the contribution of A1 in controlling HSE.

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Footnotes

Author contributions: Y.K., Y.A., H.K., and P.G. designed research; Y.A., H.K., S.P.Y., Y.K., H.E., and P.G. performed research; Y.K. contributed new reagents/analytic tools; Y.K., Y.A., H.K., and P.G. analyzed data; and Y.K. and P.G. wrote the paper.

The authors declare no conflict of interest.

References

1. **Ace, C. I., M. A. Dalrymple, F. H. Ramsay, V. G. Preston, and C. M. Preston.** 1988. Mutational analysis of the herpes simplex virus type 1 trans-inducing factor Vmw65. J Gen Virol **69 (Pt 10):**2595-605.
2. **Ace, C. I., T. A. McKee, J. M. Ryan, J. M. Cameron, and C. M. Preston.** 1989. Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. J Virol **63:**2260-9.
3. **Alce, T. M., and W. Popik.** 2004. APOBEC3G is incorporated into virus-like particles by a direct interaction with HIV-1 Gag nucleocapsid protein. J Biol Chem **279:**34083-6.
4. **Ando, Y., H. Kitayama, Y. Kawaguchi, and Y. Koyanagi.** 2008. Primary target cells of herpes simplex virus type 1 in the hippocampus. Microbes Infect **10:**1514-23.
5. **Bergstrom, T., B. Svennerholm, N. Conradi, P. Horal, and A. Vahlne.** 1991. Discrimination of herpes simplex virus types 1 and 2 cerebral infections in a rat model. Acta Neuropathol **82:**395-401.
6. **Bishop, K. N., R. K. Holmes, and M. H. Malim.** 2006. Antiviral potency of APOBEC proteins does not correlate with cytidine deamination. J Virol **80:**8450-8.
7. **Bishop, K. N., R. K. Holmes, A. M. Sheehy, N. O. Davidson, S. J. Cho, and M. H. Malim.** 2004. Cytidine deamination of retroviral DNA by diverse APOBEC proteins. Curr Biol **14:**1392-6.
8. **Bishop, K. N., R. K. Holmes, A. M. Sheehy, and M. H. Malim.** 2004. APOBEC-mediated editing of viral RNA. Science **305:**645.
9. **Blanc, V., J. O. Henderson, S. Kennedy, and N. O. Davidson.** 2001. Mutagenesis of apobec-1 complementation factor reveals distinct domains that modulate RNA binding, protein-protein interaction with apobec-1, and complementation of C to U RNA-editing activity. J Biol Chem **276:**46386-93.
10. **Chen, I. H., K. S. Sciabica, and R. M. Sandri-Goldin.** 2002. ICP27 interacts with the RNA export factor Aly/REF to direct herpes simplex virus type 1 intronless mRNAs to the TAP export pathway. J Virol **76:**12877-89.
11. **Chen, Z., T. L. Eggerman, A. V. Bocharov, I. N. Baranova, T. G. Vishnyakova, G. Csako, and A. P. Patterson.** 2010. Hypermutation induced by APOBEC-1 overexpression can be eliminated. RNA **16:**1040-52.
12. **Conley, A. J., D. M. Knipe, P. C. Jones, and B. Roizman.** 1981. Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of gamma polypeptides. J Virol **37:**191-206.
13. **Conrady, C. D., D. A. Drevets, and D. J. Carr.** 2010. Herpes simplex type I (HSV-1) infection of the nervous system: is an immune response a good thing? J Neuroimmunol **220:**1-9.
14. **Conrady, C. D., M. Thapa, T. Wuest, and D. J. Carr.** 2009. Loss of mandibular lymph node integrity is associated with an increase in sensitivity to HSV-1 infection in CD118-deficient mice. J Immunol **182:**3678-87.
15. **Crawford, J. P., D. H. Percy, and L. A. Hatch.** 1979. Experimental encephalitis in the newborn rat due to herpes simplex virus type 2. Exp Mol Pathol **31:**44-55.

16. **DeLuca, N. A., M. A. Courtney, and P. A. Schaffer.** 1984. Temperature-sensitive mutants in herpes simplex virus type 1 ICP4 permissive for early gene expression. J Virol **52:**767-76.
17. **Driscoll, D. M., and Q. Zhang.** 1994. Expression and characterization of p27, the catalytic subunit of the apolipoprotein B mRNA editing enzyme. J Biol Chem **269:**19843-7.
18. **Gaddis, N. C., E. Chertova, A. M. Sheehy, L. E. Henderson, and M. H. Malim.** 2003. Comprehensive investigation of the molecular defect in vif-deficient human immunodeficiency virus type 1 virions. J Virol **77:**5810-20.
19. **Guo, F., S. Cen, M. Niu, J. Saadatmand, and L. Kleiman.** 2006. Inhibition of formula-primed reverse transcription by human APOBEC3G during human immunodeficiency virus type 1 replication. J Virol **80:**11710-22.
20. **Hardwicke, M. A., and R. M. Sandri-Goldin.** 1994. The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection. J Virol **68:**4797-810.
21. **Hardy, W. R., and R. M. Sandri-Goldin.** 1994. Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. J Virol **68:**7790-9.
22. **Harris, R. S., K. N. Bishop, A. M. Sheehy, H. M. Craig, S. K. Petersen-Mahrt, I. N. Watt, M. S. Neuberger, and M. H. Malim.** 2003. DNA deamination mediates innate immunity to retroviral infection. Cell **113:**803-9.
23. **Harris, R. S., S. K. Petersen-Mahrt, and M. S. Neuberger.** 2002. RNA editing enzyme APOBEC1 and some of its homologs can act as DNA mutators. Mol Cell **10:**1247-53.
24. **Henderson, J. O., V. Blanc, and N. O. Davidson.** 2001. Isolation, characterization and developmental regulation of the human apobec-1 complementation factor (ACF) gene. Biochim Biophys Acta **1522:**22-30.
25. **Hirano, K., J. Min, T. Funahashi, and N. O. Davidson.** 1997. Cloning and characterization of the rat apobec-1 gene: a comparative analysis of gene structure and promoter usage in rat and mouse. J Lipid Res **38:**1103-19.
26. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J Virol **14:**8-19.
27. **Huthoff, H., and M. H. Malim.** 2007. Identification of amino acid residues in APOBEC3G required for regulation by human immunodeficiency virus type 1 Vif and Virion encapsidation. J Virol **81:**3807-15.
28. **Ikeda, T., T. Ohsugi, T. Kimura, S. Matsushita, Y. Maeda, S. Harada, and A. Koito.** 2008. The antiretroviral potency of APOBEC1 deaminase from small animal species. Nucleic Acids Res **36:**6859-71.
29. **Jean, S., K. M. LeVan, B. Song, M. Levine, and D. M. Knipe.** 2001. Herpes simplex virus 1 ICP27 is required for transcription of two viral late (gamma 2) genes in infected cells. Virology **283:**273-84.
30. **Kitayama, H., Y. Miura, Y. Ando, and Y. Koyanagi.** 2008. Human immunodeficiency virus type-1 vulnerates nascent neuronal cells. Microbiol Immunol **52:**78-88.

31. **Lindahl, T.** 1979. DNA glycosylases, endonucleases for apurinic/apyrimidinic sites, and base excision-repair. Prog Nucleic Acid Res Mol Biol **22:**135-92.
32. **Luker, G. D., J. L. Prior, J. Song, C. M. Pica, and D. A. Leib.** 2003. Bioluminescence imaging reveals systemic dissemination of herpes simplex virus type 1 in the absence of interferon receptors. J Virol **77:**11082-93.
33. **Lundberg, P., C. Ramakrishna, J. Brown, J. M. Tyszka, M. Hamamura, D. R. Hinton, S. Kovats, O. Nalcioglu, K. Weinberg, H. Openshaw, and E. M. Cantin.** 2008. The immune response to herpes simplex virus type 1 infection in susceptible mice is a major cause of central nervous system pathology resulting in fatal encephalitis. J Virol **82:**7078-88.
34. **Mahieux, R., R. Suspene, F. Delebecque, M. Henry, O. Schwartz, S. Wain-Hobson, and J. P. Vartanian.** 2005. Extensive editing of a small fraction of human T-cell leukemia virus type 1 genomes by four APOBEC3 cytidine deaminases. J Gen Virol **86:**2489-94.
35. **Mangeat, B., P. Turelli, G. Caron, M. Friedli, L. Perrin, and D. Trono.** 2003. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. Nature **424:**99-103.
36. **Mehta, A., and D. M. Driscoll.** 1998. A sequence-specific RNA-binding protein complements apobec-1 To edit apolipoprotein B mRNA. Mol Cell Biol **18:**4426-32.
37. **Mullaney, J., H. W. Moss, and D. J. McGeoch.** 1989. Gene UL2 of herpes simplex virus type 1 encodes a uracil-DNA glycosylase. J Gen Virol **70 (Pt 2):**449-54.
38. **Navaratnam, N., S. Bhattacharya, T. Fujino, D. Patel, A. L. Jarmuz, and J. Scott.** 1995. Evolutionary origins of apoB mRNA editing: catalysis by a cytidine deaminase that has acquired a novel RNA-binding motif at its active site. Cell **81:**187-95.
39. **Navaratnam, N., J. R. Morrison, S. Bhattacharya, D. Patel, T. Funahashi, F. Giannoni, B. B. Teng, N. O. Davidson, and J. Scott.** 1993. The p27 catalytic subunit of the apolipoprotein B mRNA editing enzyme is a cytidine deaminase. J Biol Chem **268:**20709-12.
40. **Newman, E. N., R. K. Holmes, H. M. Craig, K. C. Klein, J. R. Lingappa, M. H. Malim, and A. M. Sheehy.** 2005. Antiviral function of APOBEC3G can be dissociated from cytidine deaminase activity. Curr Biol **15:**166-70.
41. **Noguchi, C., H. Ishino, M. Tsuge, Y. Fujimoto, M. Imamura, S. Takahashi, and K. Chayama.** 2005. G to A hypermutation of hepatitis B virus. Hepatology **41:**626-33.
42. **Okeoma, C. M., N. Lovsin, B. M. Peterlin, and S. R. Ross.** 2007. APOBEC3 inhibits mouse mammary tumour virus replication in vivo. Nature **445:**927-30.
43. **Petersen-Mahrt, S. K., and M. S. Neuberger.** 2003. In vitro deamination of cytosine to uracil in single-stranded DNA by apolipoprotein B editing complex catalytic subunit 1 (APOBEC1). J Biol Chem **278:**19583-6.
44. **Petit, V., D. Guetard, M. Renard, A. Keriel, M. Sitbon, S. Wain-Hobson, and J. P. Vartanian.** 2009. Murine APOBEC1 is a powerful mutator of retroviral and cellular RNA in vitro and in vivo. J Mol Biol **385:**65-78.

45. **Powell, L. M., S. C. Wallis, R. J. Pease, Y. H. Edwards, T. J. Knott, and J. Scott.** 1987. A novel form of tissue-specific RNA processing produces apolipoprotein-B48 in intestine. Cell **50:**831-40.
46. **Preston, C. M.** 1979. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant tsK. J Virol **29:**275-84.
47. **Pyles, R. B., and R. L. Thompson.** 1994. Evidence that the herpes simplex virus type 1 uracil DNA glycosylase is required for efficient viral replication and latency in the murine nervous system. J Virol **68:**4963-72.
48. **Renard, M., M. Henry, D. Guetard, J. P. Vartanian, and S. Wain-Hobson.** 2010. APOBEC1 and APOBEC3 cytidine deaminases as restriction factors for hepadnaviral genomes in non-humans in vivo. J Mol Biol **400:**323-34.
49. **Rosler, C., J. Kock, M. Kann, M. H. Malim, H. E. Blum, T. F. Baumert, and F. von Weizsacker.** 2005. APOBEC-mediated interference with hepadnavirus production. Hepatology **42:**301-9.
50. **Sacks, W. R., C. C. Greene, D. P. Aschman, and P. A. Schaffer.** 1985. Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J Virol **55:**796-805.
51. **Sandri-Goldin, R. M.** 1998. ICP27 mediates HSV RNA export by shuttling through a leucine-rich nuclear export signal and binding viral intronless RNAs through an RGG motif. Genes Dev **12:**868-79.
52. **Sandri-Goldin, R. M.** 1994. Properties of an HSV-1 regulatory protein that appears to impair host cell splicing. Infect Agents Dis **3:**59-67.
53. **Sato, K., T. Izumi, N. Misawa, T. Kobayashi, Y. Yamashita, M. Ohmichi, M. Ito, A. Takaori-Kondo, and Y. Koyanagi.** 2010. Remarkable lethal G-to-A mutations in vif-proficient HIV-1 provirus by individual APOBEC3 proteins in humanized mice. J Virol **84:**9546-56.
54. **Schafer, A., H. P. Bogerd, and B. R. Cullen.** 2004. Specific packaging of APOBEC3G into HIV-1 virions is mediated by the nucleocapsid domain of the gag polyprotein precursor. Virology **328:**163-8.
55. **Sheehy, A. M., N. C. Gaddis, J. D. Choi, and M. H. Malim.** 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature **418:**646-50.
56. **Shindo, K., A. Takaori-Kondo, M. Kobayashi, A. Abudu, K. Fukunaga, and T. Uchiyama.** 2003. The enzymatic activity of CEM15/Apobec-3G is essential for the regulation of the infectivity of HIV-1 virion but not a sole determinant of its antiviral activity. J Biol Chem **278:**44412-6.
57. **Suspene, R., D. Guetard, M. Henry, P. Sommer, S. Wain-Hobson, and J. P. Vartanian.** 2005. Extensive editing of both hepatitis B virus DNA strands by APOBEC3 cytidine deaminases in vitro and in vivo. Proc Natl Acad Sci U S A **102:**8321-6.
58. **Suspene, R., P. Sommer, M. Henry, S. Ferris, D. Guetard, S. Pochet, A. Chester, N. Navaratnam, S. Wain-Hobson, and J. P. Vartanian.** 2004. APOBEC3G is a single-stranded DNA cytidine deaminase and functions independently of HIV reverse transcriptase. Nucleic Acids Res **32:**2421-9.

59. **Tanaka, M., H. Kodaira, Y. Nishiyama, T. Sata, and Y. Kawaguchi.** 2004. Construction of recombinant herpes simplex virus type I expressing green fluorescent protein without loss of any viral genes. Microbes Infect **6:**485-93.
60. **Teng, B., C. F. Burant, and N. O. Davidson.** 1993. Molecular cloning of an apolipoprotein B messenger RNA editing protein. Science **260:**1816-9.
61. **Whitley, R. J.** 2006. Herpes simplex encephalitis: adolescents and adults. Antiviral Res **71:**141-8.
62. **Worrad, D. M., and S. Caradonna.** 1988. Identification of the coding sequence for herpes simplex virus uracil-DNA glycosylase. J Virol **62:**4774-7.
63. **Yu, Q., R. Konig, S. Pillai, K. Chiles, M. Kearney, S. Palmer, D. Richman, J. M. Coffin, and N. R. Landau.** 2004. Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome. Nat Struct Mol Biol **11:**435-42.

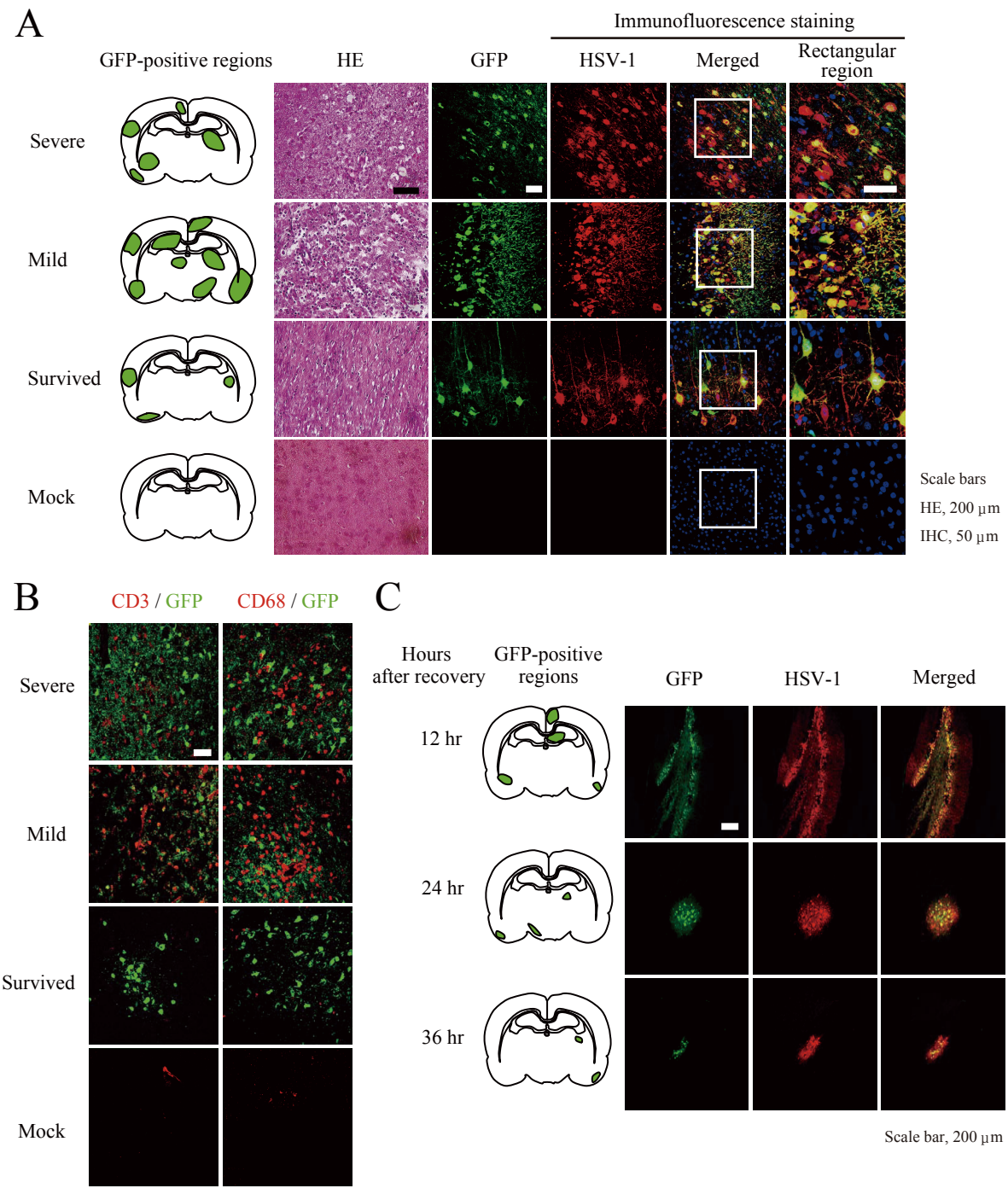


Figure 1. HSE model induced by GFP-HSV-1 inoculation of infant rats. Fourteen day-old infant rats were intracranially injected with GFP-expressing HSV-1 (YK333), and brain tissues were collected from rats exhibiting severe or mild encephalitis as described in Table 1 or from survived rats 36 h after the disappearance of paralysis. PBS (Mock)-inoculated rats were used as negative controls and brain tissues were collected at the end of the experiment. (A) GFP+ (green) regions in the coronal plane, HE, GFP+ (green) and HSV antigen+ (red) brain cells in the tissue section. Nuclei were also stained with Hoechst33342 (blue) and shown in the merged image. A zoom in view of the rectangular region in the merged image is shown to the right. (B) GFP+ (green), CD3+ (red) or CD68+ (red) cells in the tissue section. (C) GFP+ regions in the coronal plane (right panels) and GFP+ and HSV antigen+ cells in the tissue section (left panels) are shown in survived rats at indicated h after the disappearance of paralysis. Representative results are shown. Scale bars are 200 μm in HE section and 50 μm in the other sections.

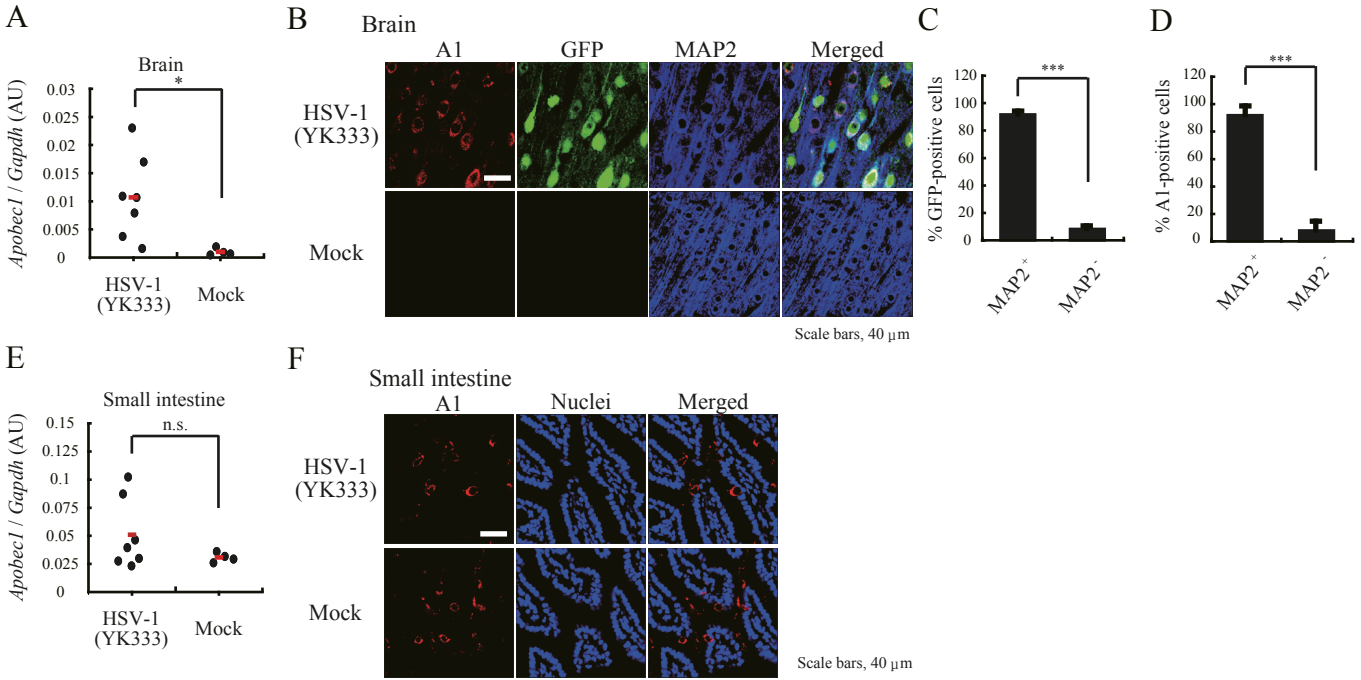


Figure 2. Expression of A1 in HSV-1-infected rat brains. (A) The level of A1 cDNA expression quantified by real-time PCR in the brain and small intestine of HSV-1-inoculated rats, which consisted of 4 mild rats and 2 recovery rats, and of 4 PBS-inoculated rats was assessed. (B) A1+ (red), GFP+ (green), and MAP2+ (blue) neuronal cells of an HSV-1 and mock-infected rat brain (upper panels) and A1+ cells in the small intestine of HSV-1 and mock-infected rats (bottom panels). Representative results are shown. Scale bars are 200 μm. Quantification data were presented as a percentage of MAP2 antigen-positive cells in GFP+ (C) or A1+ (D). *, $P<0.05$ compared to mock, ***$P<0.005$ compared to MAP2 antigen-negative cells.

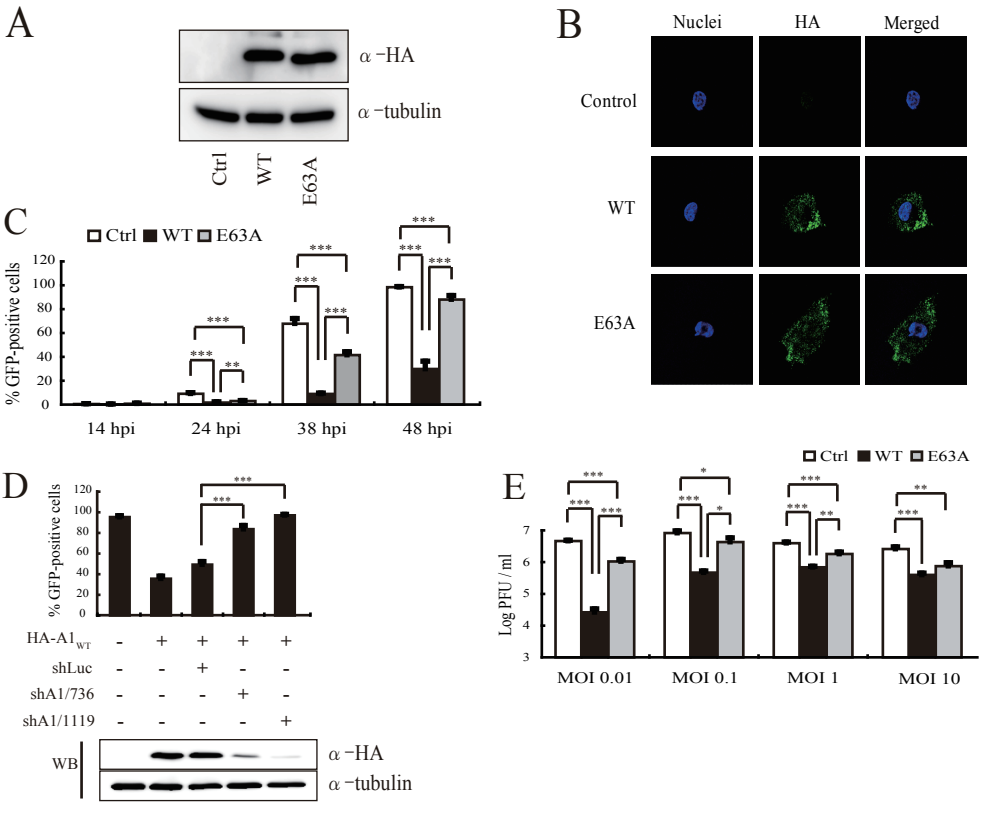


Figure 3. A1 inhibits HSV-1 infection. (A) Western blotting of HA-tagged WT, E63A, and Ctrl stably transfected cells probed with an anti-HA for A1 expression or an anti-tubulin antibody an an internal standard. (B) Immunofluorescence staining of HA-tagged A1WT, A1E63A or A1Ctrl RSC with an anti-HA antibody (green). The nucleus was stained with Hoechst33342 (blue). (C) Longitudinal analysis of GFP-expressing HSV-1 infection in A1WT, A1E63A mutant, or Control stably transfected cells. **, P<0.05 and ***P<0.005. (D) Level of GFP in HA-tagged A1WT RSC stably expressing A1 targeted shRNAs or luciferase (Luc) 48 h after YK333 infection. ***P<0.005 compared to shLuc-treated cells. Western blot probed with an anti-HA antibody for the expression of A1 and anti-tubulin as an internal standard. (E) Level of PFU in the supernatant of Ctrl, WT, and E63A RSC 48 h after the infection by YK333 at varying MOI. *, P<0.05, **, P<0.005, and ***P<0.0005.

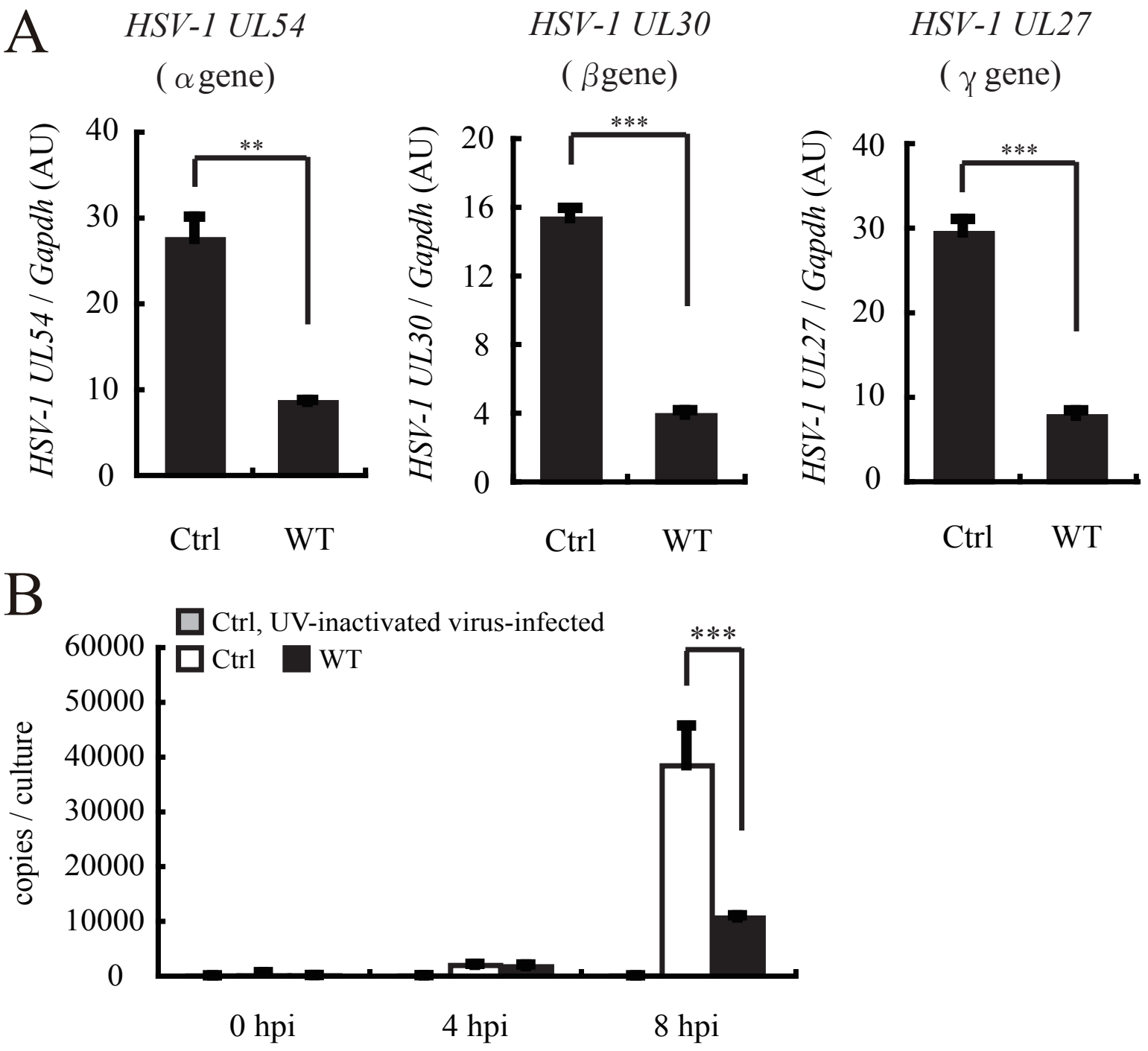


Figure 4. Inhibition of HSV-1 mRNA and DNA. (A) HSV-1 gene expression for UL54, UL30, and UL27 were measured by real-time PCR and normalized to GAPDH. Nucleic acids were extracted from HSV-1 infected WT and Ctrl RSC 8 hpi. (B) DNA copy number was measured by real-time PCR in A1 and control RSC 4 and 8 h after infection. As a control, UV-inactivated virus was also used to infect control RSC. **, $P<0.05$, and ***$P<0.005$

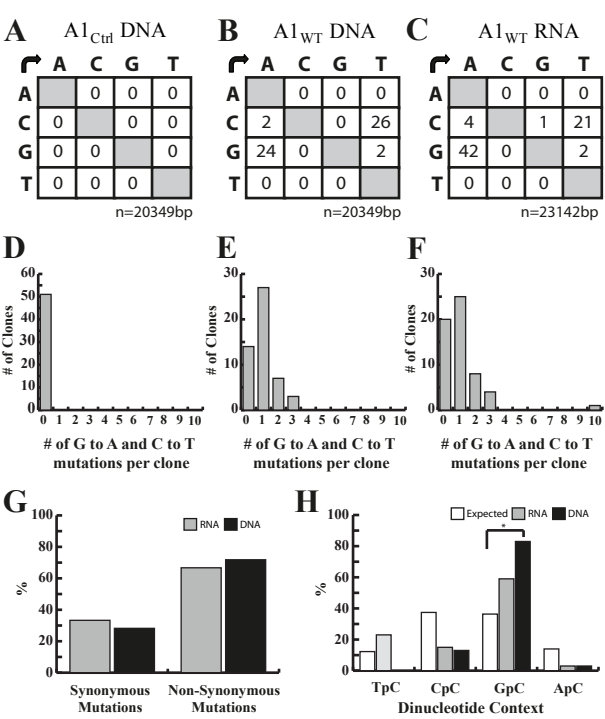
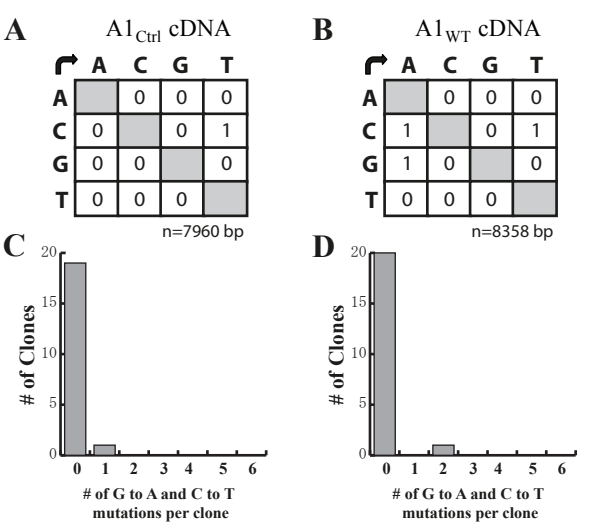


Figure 5. Sequencing analysis of UL54 DNA and cDNA from A1WT and A1 Ctrl HSV-1 infected RSC. A 399 bp region of UL54 DNA or cDNA, extracted 8 hpi, was amplified by nested PCR and cloned for sequencing. Mutation matrices are depicted for (A) DNA extracted from A1Ctrl RSC, 51 clones; (B) DNA extracted from A1WT RSC, 51 clones; and (C) cDNA extracted from A1WT RSC, 58 clones. The number of clones harboring G to A and C to T transitions was assessed and represented in (D) DNA extracted from A1 Ctrl RSC; (E) DNA extracted from A1WT RSC; and (F) cDNA extracted from A1WT RSC. (G) Mutations from UL54 cDNA and DNA were narrowed down to 39 and 30 commonly mutated positions, respectively. The % of synonymous or non-synonymous mutations is depicted in the bar graph. (H) Rat A1 had a tendency in dinucleotide context for 5'GpC in viral UL54 DNA and cDNA and was statistically significant in the DNA. A $\chi 2$ analysis showed that when compared to expected values, 5'GpC was significantly favored on UL54 DNA as indicated by the asterisk ($p < 0.001$).



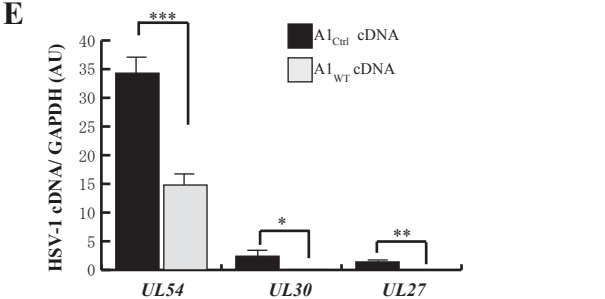


Figure 6. A1 inhibition of HSV-1 RNA transcripts is independent of RNA mutagenesis. A1WT and A1Ctrl RSC were infected with YK333 at a MOI of 1 for 2 h in the presence of 25 μg/mL of acyclovir. Mutation matrices for UL54 cDNA extracted from (A) A1Ctrl RSC and (B) A1WT RSC are shown. The number of clones harboring G to A and C to T transitions from (C) cDNA extracted from A1Ctrl RSC, 20 colonies and (D) cDNA extracted from A1WT RSC, 21 colonies, was assessed and represented in the bar graphs. (E) The level of HSV-1 mRNA for UL54, UL30 and UL27 in A1Ctrl and A1WT RSC were measured by real time PCR and normalized to GAPDH levels. *, $p< 0.05$; **, $p<0.0005$; ***, $p<0.00005$.

Table I Classification of rats exhibiting varying degrees of HSE

Classification[a]	Number of rats (%)	Number of rats showing the following neurological symptoms[b]			Pathological Change			GFP-positive regions[f]	HSV-1-positive regions[f]
		Monoplegia	Quadriplegia	Seizure	Tissue damage[c]	CD3-positive cells[d]	CD68-positive cells[e]		
Severe	17 (27)	1	15	11	+	++	++	++	++
Mild	14 (22.2)	7	7	4	++	+++	+++	+++	+++
Survived	24 (38.1)	22	0	0	-	+	+	+	+
Healthy	8 (12.7)	0	0	0	-	-	-	-	-
Total	63								

a HSV-1-infected rats were classified as follows: Severe, which died within 5 days after showing symptoms of severe encephalitis; Mild, which died between 5-7 days after showing symptoms of mild encephalitis; Survived, which recovered after transient encephalitis; Healthy, which did not display HSE symptoms.

b The number of rats exhibiting neurological symptoms such as monoplegia, quadriplegia, and seizures.

c The percentage of damaged areas including cell loss, degeneration of cells, and/or hemorrhaging in the coronal section was presented as follows: -, <5%; +, 5-10%; ++, > 15%

d The number of CD3-positive cells in one infected area of the coronal section was quantified and presented as follows: -, not detected; +, <5 cells; ++, 5-20 cells; +++, >20 cells.

e The number of CD68-positive cells in one infected area of the coronal section was quantified and presented as follows: -, not detected; +, <5 cells; ++, 5-50 cells; +++, >50 cells.

f The percentage of GFP- or HSV-1-positive areas in the coronal section was presented as follows: -, not detected; +, <5%; ++, 5-20%; +++, >20%.