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Abstract

Covalently closed circular DNA (cccDNA) forms a template for the replication of hepatitis B virus (HBV) and duck HBV (DHBV). Recent studies suggest that activation-induced cytidine deaminase (AID) functions in innate immunity, although its molecular mechanism of action remains unclear, particularly regarding HBV restriction. Here we demonstrated that overexpression of chicken AID caused hypermutation and reduction of DHBV cccDNA levels. Inhibition of uracil-DNA glycosylase (UNG) by UNG inhibitor protein (UGI) abolished AID-induced cccDNA reduction, suggesting that the AID/UNG pathway triggers the degradation of cccDNA via cytosine deamination and uracil excision.

Highlights
• AID reduced DHBV NC-DNA and cccDNA in chicken hepatoma cells.
• AID induced G-to-A and C-to-T hypermutations in cccDNA.
• UNG inhibition abolished AID-induced reduction of cccDNA but not NC-DNA.
• The AID/UNG pathway can be a trigger for cleavage of DHBV cccDNA.

Keywords
AID, APOBEC, base excision repair, hepatitis B virus, cccDNA

Abbreviations
AID, activation-induced cytidine deaminase; APOBEC, apolipoprotein B mRNA-editing enzyme catalytic subunit; UNG, uracil-DNA glycosylase; DHBV, duck hepatitis B virus; cccDNA, covalently closed circular DNA; 3D-PCR, differential denaturation DNA PCR.

1. Introduction

The covalently closed circular DNA (cccDNA) of the hepatitis B virus (HBV) plays an essential role in chronic hepatitis. cccDNA exists as an episome in the nucleus of infected hepatocytes and transcribes all viral RNAs, including pregenomic (pg) RNA. pgRNA is assembled into the nucleocapsid (NC) in the cytoplasm and is then converted into a relaxed circular DNA (rcDNA) by viral polymerase activity. Mature NCs are secreted after combining with envelope proteins or translocated to the nucleus. In the nucleus, the rcDNA is again converted into cccDNA for the next replication cycle. Only one or a few copies of cccDNA per infected cell are stably maintained by an unknown mechanism [1].

Despite the crucial role of cccDNA in viral persistence, little is known about the host factors that target this viral nuclear intermediate owing to the absence of an efficient experimental system that can produce HBV cccDNA at a level sufficient for analysis. In view of the limitation of HBV *in vitro* systems, the duck HBV (DHBV) model has been commonly used to study HBV replication [2]. Recently, we reported that an anti-viral factor APOBEC3G (A3G) induces hypermutation on DHBV cccDNA [3]. A3G belongs to the APOBEC (apolipoprotein B mRNA-editing catalytic polypeptide) family of proteins. In humans, this family comprises at least 11 members including AID and APOBEC-1, -2, -3A, -3B, -3C, -3DE, -3F, -3G, -3H and -4. These AID/APOBEC proteins have enzymatic activity that can deaminate a cytosine base in DNA and/or RNA, thereby generating a uracil base. A3G is known to restrict the replication of retroviruses, including human immunodeficiency virus type 1 (HIV-1), HBV and retrotransposable elements [4-6]. AID was initially identified as an essential factor for class-switch recombination (CSR) and somatic hypermutation (SHM) of immunoglobulin genes in B cells [7,8]. It was also found that AID is expressed in response to cytokine stimulation in human hepatocytes [9] and HBV patients [10], induces HBV hypermutation in NC-DNA [10] and pgRNA [11] and restricts viral replication [12]. It remains to be established whether AID targets hepadnavirus cccDNA.

UNG is a host nuclear factor that removes uracil bases from uracilated DNA and initiates base excision repair (BER), resulting in DNA repair or cleavage [13]. The involvement of UNG activity in A3G-induced viral restriction has been vigorously studied in the HIV field, but remains a subject of debate [14-17]. We previously demonstrated that UNG repairs DHBV cccDNA hypermutation induced by A3G [3]. The involvement of UNG in the anti-HBV activity of AID is of interest because AID is well known to initiate CSR and SHM by coupling with downstream UNG-directed BER pathways [13,18].

Here we investigated the effects of chicken AID on DHBV replication using an *in vitro* DHBV replication system with a chicken hepatoma cell line LMH, which we utilized to analyse cccDNA formation and function [2,19,20]. We found that the expression of chicken AID in LMH cells resulted in the reduction of DHBV NC-DNA and cccDNA. Furthermore, UNG inhibition abolished AID-induced reduction of cccDNA but not NC-DNA, suggesting that viral DNA degradation is induced by the AID/UNG pathway in the nucleus.

2. Materials and Methods

2.1. Plasmids

The DHBV replicon plasmid pCSD3.5ΔS [21] and UGI expression vector pIRES-UGI [3] were described previously. The pCSD3.5ΔS plasmid produces a DHBV surface-deficient mutant that accumulates cccDNAs more efficiently than wild-type DHBV [20,21]. Other expression vectors are described in the Supporting Methods.

2.2. Cell culture and transfection

LMH cells were maintained in Dulbecco's modified Eagle medium (DMEM; Sigma, St. Louis, MO, USA) containing 10% foetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Plasmid transfection was performed using Fugene 6 (Roche, Basel, Switzerland), according to the manufacturer's instructions. The total DNA amount (6 μg for a 60-mm dish) for each transfection was kept constant by adding mock vector as needed. The ER-fusion protein that was expressed as an inactive form was activated by tamoxifen (OHT, Wako Pure Chemical Industries, Osaka, Japan) treatment [22]. In the subcellular localization experiments of GFP fusion proteins, cells were treated with 10 ng/mL leptomycin B (LMB, Sigma) for 4 h.

2.3. Purification of viral DNAs

Cells were lysed in 0.5% NP40 lysis buffer, and nuclei were collected by centrifugation to separate the cytoplasmic and nuclear fractions. Cytoplasmic supernatants were treated with DNase I and RNase A and digested with proteinase K and sodium dodecyl sulphate (SDS) to extract viral NC-DNA. A modified Hirt extraction procedure was used to extract cccDNA from the nuclear fraction [3]. The nuclear pellet was lysed in 50 mM Tris–HCl (pH 7.5), 10 mM EDTA and 2% SDS. After a 20-min incubation at room temperature, 0.5 M KCl was added to the lysate and incubated at 4°C overnight. All purified DNA solutions were treated with DpnI restriction enzyme to digest any transfected plasmid DNA.

2.4. Mutation analysis

Hypermutation was detected using differential DNA denaturation PCR (3D-PCR) with DHBV cccDNA-selective primers. The specific amplification of cccDNA with cccDNA-selective primers was verified previously [3]. 3D-PCR was performed as follows: initial denaturation for 5 min at 94–83°C, followed by 35 cycles of 30 s at 94–83°C, 30 s at 55°C and 2 min at 72°C, with a final elongation for 7 min at 72°C. To determine the hypermutation frequency, PCR fragments were cloned into T vectors (Promega, Madison, WI, USA). Successfully recombined clones were randomly selected and sequenced using ABI PRISM 3130 (Applied Biosystems, Foster City, CA, USA).

2.5. Southern blotting

Purified DHBV DNAs were analysed by Southern blotting using double-stranded DHBV DNA probes spanning the entire viral genome. Probe labelling and signal development were performed using the AlkPhos direct labelling system (GE Healthcare, Piscataway, NJ, USA), and signals were detected using the LAS1000 imager system (GE Healthcare).

2.6. Rolling circle amplification (RCA)

Specific amplification of DHBV cccDNA by RCA method was described previously [3,23]. The RCA concatemerized product was converted to the monomeric full-length DHBV genome by digestion with EcoRI, which cuts the DHBV cccDNA once. After electrophoresis, the DNA fragments were analysed by Southern blotting.

2.7. Western blotting

Transgene expression was analysed by Western blotting as described previously [3]. The following antibodies were used in this study: rabbit anti-GAPDH (G9545, Sigma), mouse anti-FLAG (M2, Sigma), rabbit anti-ER (HC-20, Santa Cruz Biotechnology, CA, USA) and anti-rabbit Igs-horseradish peroxidase (HRP) (ALI3404, eBioscience, San Diego, CA, USA).

3. Results

3.1. Chicken AID induced cccDNA reduction via a UNG-dependent pathway

A DHBV replication system in a chicken hepatoma cell line LMH was utilized to analyse cccDNA. With this *in vitro* system, we could detect cccDNA by Southern blotting of nuclear Hirt extracts (Fig. 1A). The three DNA forms (rc, dl and cccDNA) in the nuclear Hirt extract were all converted into dlDNA position as 3.0-kb bands after EcoRI digestion (Fig. 1A). To investigate the anti-DHBV activity of AID, a chicken AID expression vector and DHBV replicon plasmid pCSD3.5 were transfected into LMH cells. Concurrently, the effect of UNG inhibition was also tested by co-transfection of a UGI expression vector. UGI is an irreversible inhibitory protein that forms a stable complex with UNG. We previously demonstrated a 91% reduction of UNG activity under the conditions shown in Fig. 1B [3]. Six days after transfection, cytoplasmic nucleocapsid (NC)-DNAs and nuclear Hirt-extracted DNAs were purified. Southern blotting showed that AID overexpression reduced DHBV NC-DNA and cccDNA (Fig. 1B). UNG inhibition by UGI abolished AID-induced reduction of cccDNA but not NC-DNA. We confirmed that the protein level of chicken AID was not affected by UGI overexpression (Fig. 1C).

To determine cccDNA level at an earlier time point, we used RCA, a highly sensitive method widely used to measure the level of closed circular DNA of various viral species [24]. Three days after transfection, cccDNA from nuclear Hirt-extracted DNAs was amplified by RCA and digested with EcoRI to cleave the concatemer into monomers of full-length viral genomes (3.0 kb). Amplified cccDNA was detected by Southern blotting. Consistent with data obtained from day 6 (Fig. 1B), AID overexpression reproducibly suppressed both NC-DNA and cccDNA levels and UGI abolished the reduction of only cccDNA at day 3 (Fig. 2A and B).

3.2. Chicken AID induced cccDNA hypermutation

Next, we investigated the hypermutation activity of AID for DHBV cccDNA by 3D-PCR using cccDNA-specific primers (Fig. 3A). 3D-PCR can detect hypermutated DNA by gradually decreasing the denaturation temperature [25]. Our results showed that only AID–UGI co-expression resulted in lower temperature amplification (83.9°C and 83.0°C, indicated by triangles in Fig. 3B). Sequence analysis of DNA amplified at the lower denaturation temperature revealed G-to-A and C-to-T hypermutations (Fig. 3C and D and Fig. S1). Dinucleotide context analysis of the hypermutated cccDNA showed a preference for GpC and ApC dinucleotides as a mutation target (Fig. 3E), which is consistent with a previous report of human AID-induced HBV hypermutation [10].

3.3. Chicken AID is a protein that shuttles between the cytoplasm and nucleus

Chicken AID has a nuclear localization signal (NLS) in the N-terminus and a nuclear export signal (NES) in the C-terminus [26]. To determine the subcellular localization of chicken AID in chicken hepatocytes, an AID–GFP vector was transfected into LMH cells. Similar to human AID [27], chicken AID–GFP protein localized predominantly in the cytoplasm (Fig. 4A). Cells were treated with leptomycin B (LMB) to inhibit exportin 1-dependent nuclear export [27,28]. LMB treatment for 4 h changed the localization of AID from the cytoplasm to both cytoplasm and nuclear. Consistent with this observation, deletion of C-terminal residues containing NES (AIDΔNES in Fig. 4B,

corresponding to JP8Bdel human mutant) resulted in the nuclear localization of AID with minor cytoplasmic retention (Fig. 4A). These observations indicate that chicken AID shuttles between the cytoplasm and nucleus in LMH cells and that the subcellular localization pattern of AID is conserved in mammals and chicken.

3.4. Nuclear retention of AID enhanced cccDNA-reduction activity

Because the majority of AIDΔNES–GFP protein was localized mainly to the nucleus, we examined the cccDNA-reduction activity of this mutant. Wild-type AID or AIDΔNES was co-transfected with a DHBV plasmid. Southern blotting following RCA showed that AIDΔNES had a stronger DHBV cccDNA-reduction activity than wild-type AID, whereas reduction of cytoplasmic NC-DNA levels by AIDΔNES was slightly weaker than that by wild-type AID (Fig. 5A). Protein levels of AIDΔNES were equivalent to that of wild-type AID (Fig. 5B). These data suggest that nuclear-localized AID is responsible for cccDNA reduction.

4. Discussion

In this study, we overexpressed chicken AID and inhibited UNG activity in LMH cells to evaluate anti-viral roles of AID and UNG against DHBV. We found that AID reduced both DHBV NC-DNA and cccDNA levels, and UNG inhibition abolished the reduction of cccDNA but not NC-DNA (Fig. 1 and 2). The UNG inhibition also revealed the AID-induced G-to-A and C-to-T hypermutation in cccDNA (Fig. 3). Furthermore, a nuclear-localized AID mutant more strongly reduced cccDNA levels than wild-type AID (Fig. 5). These results suggest that AID introduces C-to-U conversion in both strands of cccDNA and that UNG-mediated BER may degrade uracilated cccDNA (Fig. 6).

This study suggests that the molecular mechanism of AID-induced NC-DNA reduction could be different from that for cccDNA because it was found to be UNG-independent. We previously showed a physical association between human AID and HBV core proteins [11], suggesting the encapsidation of AID proteins in NC as reported in the case of A3G. Unlike HIV, UNG incorporation was not detected in HBV NC [3], implying no or little contribution of BER-mediated DNA cleavage in NC-DNA restriction. Similar to A3G [29,30], AID may suppress NC-DNA formation through deaminase-independent activity such as inhibition of reverse transcription. We also obtained the result, suggesting that human P19-AID mutant carrying a mutation in the deaminase motif [31] has the ability to reduce HBV NC-DNA as efficiently as wild-type AID (unpublished). In contrast, when AID deaminates cccDNA in nucleus, UNG-mediated BER can efficiently excise uracils from cccDNA and cleave DNA, similar to the case of the immunoglobulin gene undergoing CSR [13,18]. This can be a reason why cccDNA reduction was abolished by UNG inhibition.

Our previous study revealed that A3G reduces both DHBV cccDNA and NC-DNA [3]. In contrast to AID, cccDNA reduction by A3G was not abolished when UNG was inhibited. It is likely that AID has a different activity from A3G to suppress cccDNA. cccDNA reduction by A3G may simply be reflected by the reduction of NC-DNA, whereas *de novo* cccDNA degradation could be triggered by nuclear-localized AID followed by UNG activity (Fig. 6). Enhancement of cccDNA-reduction activity in the AIDΔNES mutant (Fig. 5) supports the idea that AID is directly targeted to nuclear cccDNA. Furthermore, mutation analysis showed AID-induced hypermutation in both strands of cccDNA (Fig. 3), which is different from the A3G-induced hypermutation exclusively occurring in the minus strand DNA [3]. cccDNA reduction mediated by the AID/UNG pathway can be explained by double-strand breaks initiated by deamination of both strands. AID is proposed to recruit UNG to the immunoglobulin gene to cause CSR [32]. We speculate that such a property may also underlie the different outcomes in cccDNA reduction observed between AID and A3G.

Recently, Vartanian et al. clearly demonstrated that APOBEC3 proteins are major deaminases that cause HBV NC-DNA hypermutation *in vivo* using dinucleotide context analysis of hypermutated NC-DNA obtained from patients [10]. However, the contribution of AID to cccDNA reduction and deaminase-independent NC-DNA reduction was still unknown *in vivo*. Our data suggests cccDNA as a novel target of the anti-HBV activity of AID for the first time. Further studies are required to reveal the mechanism of AID/UNG-mediated HBV cccDNA degradation *in vitro* and *in vivo*.

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Figure Legends

Fig. 1. Chicken AID induced DHBV cccDNA reduction depending on UNG activity.
(A) Southern blotting for DHBV DNA. The DHBV replicon plasmid pCSD3.5ΔS was transfected into LMH cells. Six days later, cells were harvested and cytoplasmic NC-DNA and nuclear Hirt-extracted DNA were purified. Nuclear sample without EcoRI treatment showed two bands at the expected positions of relaxed circular (rc) DNA and cccDNA, respectively. Treatment with EcoRI (a single cutter for the DHBV genome) resulted in conversion of both bands into one band at the position of double-stranded linear (dl) DNA. (B) Expression vectors for chicken AID-ER, UGI-ER and Mock-ER were used to transfect LMH cells with pCSD3.5ΔS. The ER-fusion protein was expressed as an inactive form that could be activated by tamoxifen (OHT) treatment [22]. OHT was added 3 days after transfection and cells were harvested at day 6. The cytoplasmic NC-DNA and nuclear Hirt-extracted DNA were purified from the indicated transfectants and subjected to Southern blotting with a DHBV probe. The percent density of each band is indicated (top, rcDNA in NC; bottom, cccDNA in nucleus). The density of the negative control (Mock-GFP) was defined as 100%. (C) Exogenous protein expression of chicken AID- and Mock-ER was detected by Western blotting with anti-ER antibody. pIRES-UGI was used to test whether UGI affects AID expression levels. Expression of GAPDH is also shown to demonstrate equivalent protein loading.

Fig. 2. Rolling circle amplification (RCA) analysis of cccDNA. Three days after transfection (OHT stimulation for 2 days), cytoplasmic NC-DNA and nuclear Hirt-extracted DNA were purified. (A) Southern blotting for the cytoplasmic NC-DNA. (B) Semi-quantification by cccDNA-specific amplification. The Hirt-extracted DNAs were serially diluted to 1:4 and 1:16 as shown in (B) and subjected to RCA with phi29 DNA polymerase. Concatemeric viral DNA were digested with EcoRI and analysed by Southern blotting.

Fig. 3. Chicken AID induced DHBV cccDNA hypermutation. (A) A schematic representation of cccDNA-selective primers. Left, cccDNA; right, rcDNA with gaps in both strands (− and +), which leads to inefficient amplification of rcDNA with the cccDNA-selective primers [3]. (B) 3D-PCR analysis of DHBV cccDNA. The Hirt-extracted DNA from each transfectant was analysed by 3D-PCR with a denaturation temperature (Td) gradient of 94.0°C, 85.3°C, 83.9°C and 83.0°C. PCR fragments (83.9°C and 83°C) indicated by triangles were sequenced, and results from 4 independent clones are shown in C-E. (C) Mutation matrix of cccDNA from AID–UGI transfectants. (D) Proportion of G-to-A and C-to-T mutations per clone. (E) Dinucleotide context analysis of C-to-T and G-to-A hypermutations. Underlined characters indicate mutated residues. The expected and observed frequencies are shown in white and black bars, respectively.

Fig. 4. Chicken AID is a protein that shuttles between the cytoplasm and nucleus.
(A) Chicken AID-GFP or AIDΔNES-GFP was transfected into LMH cells. The nucleus was visualized by co-transfection of the DsRed-NLS vector. LMB was treated for 4 h to block nuclear export. LMB treatment caused accumulation of AID–GFP in the nucleus.

(B) Domain structure of chicken AID. Numbers indicate amino acid residue positions. NLS, nuclear localization signal; NES, nuclear export signal.

Fig. 5. Deletion of NES domain enhanced the cccDNA-reduction activity of chicken AID. (A) Southern blotting for cytoplasmic NC-DNA and RCA-amplified cccDNA. Three days after transfection (OHT stimulation for 2 days), DHBV DNAs were analysed. (B) Exogenous protein expression of wild-type AID- and AIDΔNES-ER was detected by Western blotting with anti-ER antibody. Expression of GAPDH is also shown to demonstrate equivalent protein loading.

Fig. 6. A proposed model for AID-induced DHBV cccDNA reduction. Chicken AID shuttles between the cytoplasm and nucleus. Cytoplasmic AID reduces NC-DNA levels, presumably by an UNG-independent mechanism. Nuclear AID deaminates cccDNA, and then uracilated cccDNA can be cleaved by the UNG-mediated BER pathway. Previous HBV studies demonstrated that human AID hypermutates both pgRNA and NC-DNA [10,11].

Figure 1

A

EcoRI — EcoRI
- +   - +   (kb)

rcDNA
dlDNA
cccDNA

4.2
3.4
2.7
1.8

nuclear
Hirt

cytoplasmic
NC

B

GFP   UGI
Mock AID Mock AID

cytoplasmic
NC

◄ rcDNA

100   63   98   59   rcDNA density (%)

nuclear
Hirt

◄ rcDNA
◄ cccDNA

100   36   118   100   cccDNA density (%)

C

GFP   UGI
Mock AID Mock AID   (kDa)

68
50
36

anti-ER

anti-GAPDH

Figure 2

A

Mock   UGI
Mock AID Mock AID

rcDNA

cytoplasmic
NC

B

Mock UGI   Mock UGI   Mock UGI
Mock AID Mock AID Mock AID Mock AID Mock AID Mock AID

cccDNA
(3.0 kb)

1   1/4   1/16

Figure 3

A

cccDNA   rcDNA

B

Td (°C)
94.0 85.3 83.9 83.0

Mock {
Mock
AID
}

UGI {
Mock
AID
}

C

	A	G	C	T
A		3		
G	**217**			
C				**32**
T		1	3	

total 5,612 nt

D

# of G-to-A/C-to-T mutation

■ G-to-A
□ C-to-T

clone #

E

Dinucleotide context %

C-to-T

TpC CpC GpC ApC

% G-to-A

□ expected
■ observed

GpA GpG GpC GpT

Figure 4

A

	GFP	DsRed	merge	LMB
AID-GFP DsRed-NLS				−
AID-GFP DsRed-NLS				+
AIDΔNES-GFP DsRed-NLS				−

B

cytidine
deaminase
domain

NLS   NES

AID

198

AIDΔNES

1 9 26   55   94   183

Figure 5

A

	Mock	AID	AIDΔNES	
				cytoplasmic NC-DNA
				nuclear cccDNA

B

	AID	AIDΔNES	
			anti-ER
			anti-GAPDH

Figure 6

Supplementary Data

Supporting Figure Legend

Fig. S1. Alignment of hypermutated DHBV sequences. PCR fragments from the 83.9°C denaturation temperature reaction in Fig. 3B were excised from the agarose gel and cloned into T vectors. Subsequently, four randomly selected clones were sequenced. The reference sequence from the replicon plasmid is shown above. Dots in the alignment represent identity with the reference sequence.

Figure S1

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DHBV3  ATACACCCCTCTCTCGAAAGCAATATATATTCCACATAGGCTATGTGGAACTTAAGAATTACACCCCTCTCCTTCGGAGCTGCTTGCCAAGGTATCTTTA
#1     ...................................................................A.........................A..A.......AA.......
#2     .................................................................T...........................T...........
#3     ............................................................................................A........
#4     ..................................................................................................

DHBV3  CGTCTACATTGCTGTTGTCGTGTGTGACTGTACCTTTGGTATGTACCATTGTTTATGATTCTTGCTTATATATGGATATCAATGCTTCTAGAGCCTTAGC
#1     ...........................A.A........................A...................AT..................AT........
#2     .............................T.............................................A.........................
#3     ...................A.A........A.....................................................................A.
#4     ..............................................T..................................................

DHBV3  CAATGTGTATGATCTACCAGATGATTTCTTTCCAAAAATAGATGATCTTGTTAGAGATGCAAAAGATGCTTTAGAACCTTATTGGAGATCAGATTCAATA
#1     ...................A....................................T......A.........T...........................
#2     ...........................................................................................C.....
#3     ....A.A...A.........A..........G.............A..A....A.......A.............A...............
#4     .............T.....................................................................C.....

DHBV3  AAGAAACATGTTTTAATTGCAACTCACTTTGTGGATCTTATTGAAGACTTCTGGCAGACTACACAGGGTATGCATGAAATAGCCGAAGCATTAAGAGCAG
#1     ..................................................................................................
#2     .......................................................T.........T...............T..
#3     ........A.........A...........A.AA.................AA.............AA...A...A.....A..A..A.........A..A
#4     .........................................A........................................................

DHBV3  TTATACCACCTACTACTACTCCTGTACCCCAAGGATATCTCATTCAGCACGACGAGGCAGAAGAGATTCCTTTGGGAGATTTATTTAAACATCAGGAAGA
#1     .....T...T..T..........................A.............................................................
#2     ..................................................................................................
#3     ...............A.................................................A...............A..A.
#4     .........................A....................................................................

DHBV3  AAGGATAGTTAGTTTCCAACCTGATTATCCAATTACGGCTAGAATTCATGCTCATTTGAAAGCTTATGCTAAGATCAACGAGGAATCCCTGGATAGGGCT
#1     ..............................A..........A....A..A.......A..........A...........A.....A..
#2     ..................................T.C.............................................................
#3     ...A..A.....................A...A.......A........A..A.............AA.................
#4     ..........................................T.........T....T...........................A..

DHBV3  AGGAGATTGCTTTGGTGGCATTACAACTGTTTACTGTGGGGAGAAGCTAATGTAACTAACTATATTTCTCGATTGCGTACTTGGTTATCAACTCCTGAGA
#1     ...A...A......A.....................A....A.................A.A...AA.............
#2     ........................G....................................................................
#3     .........A...........A.........A.......................A.............A.
#4     .......A.......A.........A.......A......A..A.....A................A...........A.

DHBV3  GATATAGAGGTAGAGATGCCCCAACCATTGAAGCAATCACTAGACCAATCCAAGTGGCTCAGGGAGGCCGAAAAACAACTTCGGGTACTAGAAAACCTCG
#1     A.......A....A.................A...............A.........A...............................
#2     ....................T.............................................................................
#3     A...........A..............AA.....A...............AAA.............A
#4     ........A...........A.......A.......A..A...A..A.................A...........

DHBV3  TGGACTCGAACCTAGAAGAAGAAAAGTTAAAACCACAGTTGTCTATGGGAGAAGACGTTCAAAGTCCAGGGAAAGGAGAGCCCCTACACCCCAACGTGCG
#1     .....................................................................A...................A.A.A
#2     ................T...................................................T..T....T...T.
#3     ...........A....A...........A..A...........A............................A.A..
#4     ............A..A....A.........A.....A.A.A...A.........A.....A................A.A..

DHBV3  GGCTCCCCTCTCCCACGTAGTTCGAGCAGTCACCACAGATCTCCCTCGCCTAGGAAATAAATTACCTGCTAGGCATCACCTAGGAAAATTGTCAGGTCTC
#1     .A..............A..A...A.A...................................A...............A.................
#2     ...........................T.............T...T.......T.T...................
#3     .................A.....................A.................A..............A.........
#4     .A.............A..A.....A..A........AA..........A....A...................

DHBV3  TATCAAATGAAGGGCTGCACATTTAACCCAGAATGGAAAGTACCAGATATTTCGGATACTCATTTTGATTTAGAAGTAATAAATGAGTGCCCTTCCCGAA
#1     ..........A..A.........A..........A......A..........A.........A.....A......
#2     ..............................................................................T..
#3     ..........A.A.A...........A...............AA.........A..........A.........A.A.
#4     ...........A..A.........A.......A...............A.....A.

DHBV3  ATTGGAAATATTTGACTCCAGCCAAATTCTGGCCCAAGAGCATTTCCTACTTTCCTGTCCAGGCAGGGGTTAAACCAAAATATCCTGACAATGTGATGCA
#1     ...A...............................................................A.................
#2     .....................A...............A.A.............T.......A...................
#3     ...AA................A.........A.....A........A......A..............
#4     ...A...............A.A...............A....AA...................A..

DHBV3  ACACGAATCAATAGTAGGTAAATATTTAACCAGGCTCTATGAAGCAGGAATCCTTTATAAGCGGATATCTAAACATTTGGTCACATTTAAAGGTCAGCCT
#1     ................................A...........A..................
#2     ...................................................A...........................
#3     ............A..AA...................A........A..A..............G....
#4     ..............AA...............A........A...A.................A......

DHBV3  TATAATTGGGAACAGCAACACCTTGTCAATCAACATCAAATTCCTGATGGGGCAACATCCAGCAAAATCAATGGACGTCAGGAGAATAGAAGGAGGAGAA
#1     ...........A..................................................................
#2     ............G..................................................................
#3     ..........................................................................
#4     .......A...............................A................A.................

DHBV3  CTCCTA
#1     ......
#2     .....
#3     .....
#4     ......
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Supporting Methods

Vector construction

A DNA fragment encoding the oestrogen receptor was amplified from pFERp [3] using the primers AAAGGATCCAGATTACAAGGA and TTTTTGCGGCCGCTCATCAAGCTGTGGCA, digested with BamHI and NotI and inserted into the BamHI/NotI site of pEGFP-N1 (Clontech, Mountain View, CA, USA) to prepare pCMVfER. A multiple cloning site in pCMVfER was modified by inserting synthetic double-stranded DNAs (5′-GAATTCCGGAACTAGTCGACGGATCC-3′ and 5′-GAATTCTAGCACCATGGCTAAGGATCC-3′) into the EcoRI/BamHI site and the constructed vectors were designated pCMVfER2 and pCMVmockER, respectively. pCMVmockER was used to express the Mock-ER protein. A chicken AID open reading frame (NM_001243222) [26] was amplified with the primers 5′-AAGAATTCACCATGGACAGCCTCTTGATGA-3′ and 5′-TTTTGTCGACGTAAGTCCCAGAGTTTTAAAGGCA-3′, trimmed with EcoRI/SalI and inserted into the EcoRI/SalI site of pCMVfER2 or pEGFP-N1 to prepare chicken AID–ER or AID–GFP expression vectors, respectively. An open reading frame of chicken AIDΔNES was amplified with the primers 5′-AAGAATTCACCATGGACAGCCTCTTGATGA-3′ and 5′-AAAAGGATCCGGCAGAAGTATCCGTCGGAGTTTCCTGGA-3′, trimmed with EcoRI/BamHI and inserted into the EcoRI/BamHI site of pCMVfER or pEGFP-N1 to prepare chicken AIDΔNES–ER or AIDΔNES–GFP expression vectors. A DNA fragment of UGI was generated by PCR with the primers 5′-AAAGAATTCATGACAAATTTATCTGAC-3′ and 5′-AAAGGATCCGCTAACATTTTAATTTTA-3′, trimmed with EcoRI/BamHI and inserted into the EcoRI/BamHI site of pCMVfER to prepare UGI–ER expression vector. All plasmids derived from PCR products and their cloning junctions were verified by DNA sequencing.