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Chicken oviduct-specific expression of transgene by a hybrid ovalbumin enhancer and the Tet expression system

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We generated genetically manipulated chickens and quail by infecting them with a retroviral vector expressing the human growth hormone under the control of chicken ovalbumin promoter/enhancer up to −3861 bp from the transcriptional start site. The growth hormone was expressed in an oviduct-specific manner and was found in egg white, although its level was low. The DNA sequence of the integrated form of the viral vector in the packaging cells was shown to be truncated and contained only the sequence spanning −3861 to −1569 bp. This represented only the DNase I hypersensitive site (DHS) III of the 4 DHSs and lacked the proximal promoter of the ovalbumin control region. We found several TATA-like and other promoter motifs of approximately −1800 bp and considered that these promoter motifs and DHS III may cause weak but oviduct-specific expression of the growth hormone. To prove this hypothesis and apply this system to oviduct-specific expression of the transgene, the truncated regulatory sequence was fused to an artificial transactivator–promoter system. In this system, initial weak but oviduct-specific expression of the Tet activator from the promoter element in the ovalbumin control sequence triggered a self-amplifying cycle of expression. DsRed was specifically expressed in oviduct cells of genetically manipulated chickens using this system. Furthermore, deletion of a short region possibly containing the promoter elements (−2112 to −1569 bp) completely abrogated oviduct-specific expression. Taken together, these results suggest that weak expression of this putative promoter causes oviduct-specific expression of the transgene.

[**Key words:** Hybrid promoter; Ovalbumin; Oviduct; Self amplificationt; Tet activator; Transgenic chicken]

Transgenic livestock such as goats and sheep have been generated for the production of recombinant proteins in milk (1–3). Chickens and quail have also attracted considerable attention as an alternative for establishing the so-called transgenic bioreactors because they have a relatively short breeding time for sexual maturation (4,5). We and other research groups have reported the production of recombinant proteins in eggs using genetically manipulated avian species (6–13). In most cases, recombinant proteins were accumulated in egg white because recovery of exogenous proteins from yolk has several obstacles including the fact that only a limited species of protein can be transported to the yolk by a receptor-mediated process (12,14).

Chicken ovalbumin (OVA) is a major protein in egg white and is produced specifically in oviduct cells. The gene has been used as a model system for hormonal and tissue-specific regulation of extremely high expression levels (15). Within 8.7 kb of the DNA region between ovalbumin and the upstream neighboring gene called the Y gene, there are 4 DNase I hypersensitive sites (DHSs) located at −0.15, −0.8, −3.2 and −6.0 kb upstream of the transcriptional start site (16). Two major regulatory elements have been identified in the chicken ovalbumin gene in relationship to these DHSs. These include a steroid-dependent regulatory element (SDRE) and a negative regulatory element (NRE) (15). DHS II corresponds to SDRE, spanning −892 to −780 bp, and is required for responsiveness to estrogen and glucocorticoid hormones. Estrogen inducibility is partly mediated by the estrogen-inducible transcription factor δEF-1 that binds to SDRE (17). DHS I corresponds to NRE spanning −88 to −308 bp and appears to have a dual role of repressing and activating transcription (18). For estrogen responsiveness, it has also been postulated that 4 half palindromic estrogen-responsive elements (EREs) that reside in DHS III act synergistically, at least *in vitro* (19). On the other hand, little is known about the mechanisms that control the tissue-specificity of OVA expression. The only documented evidence is that a silencer element that resides between −3.2 and −2.8 kb reduces the promoter activity in liver (20), although characterization of this silencer in other organs has yet not been performed.

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In this study, the upstream control region including DHS III of the chicken OVA gene was fused to an artificial promoter with a self-activating loop. This enabled oviduct-specific expression of the transgene in genetically manipulated chickens.

MATERIALS AND METHODS

Retroviral vector construction and virus production Genomic DNA containing the −3.9 to +3.6 kb region of the OVA gene was obtained from white leghorn genomic DNA as a phage clone using EMBL3 (Stratagene, Agilent Technologies, Tokyo, Japan). The OVA fragment (−3861 to +3576 bp) was isolated by *Sal*I and *Kpn*I digestion (Fig. 1A) and subcloned into pUC19. The 5′ terminus of the second exon was situated at +1637 bp, and therefore, the *Xho*I site was generated by PCR at +1641 bp (Figs. 1B, C), immediately upstream of the OVA initiation codon (+1654 to +1656 bp). The plasmid containing the artificial *Xho*I site was digested with *Xho*I and *Kpn*I and ligated with the GFP sequence tagged with the *Xho*I site at the N terminus and *Mlu*I, *Sph*I, and *Kpn*I sites at the C terminus. The resulting plasmid was designated pOVA-GFP.

Genomic DNA of human growth hormone (hGH) was isolated by PCR from human endothelial cells and cloned into the *Xho*I site of pZeoSV2 (Invitrogen, Carlsbad, CA,

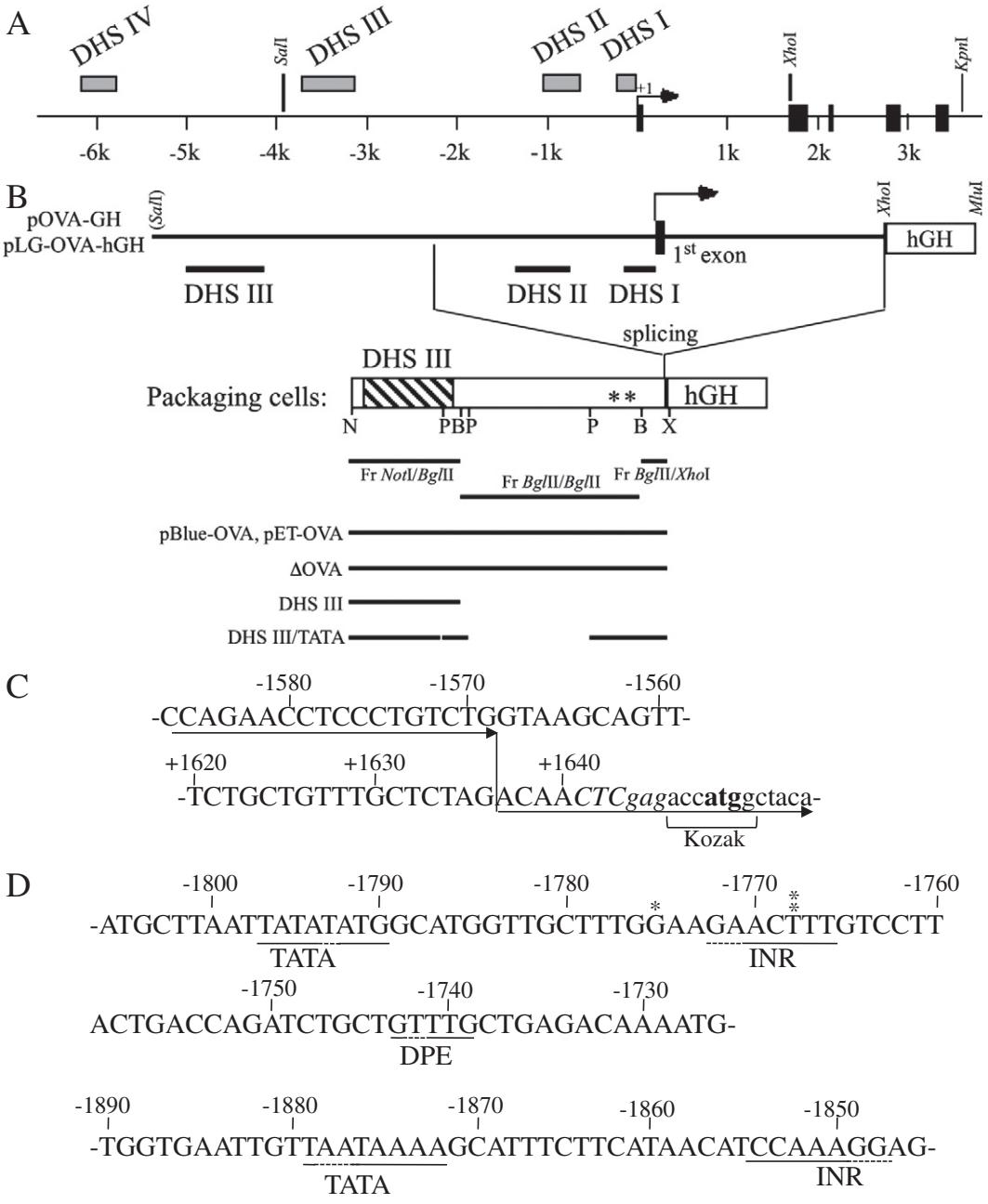

FIG. 1. (A) Structure of the ovalbumin promoter/enhancer region. The 4 DHS sites and 5 of the 8 exons are shown as gray and black boxes, respectively. Naturally occurring *Kpn*I site (3576 bp) and artificially introduced *Sal*I and *Xho*I sites used for plasmid construction are also shown. (B) Splicing of the retroviral vector in packaging cells. Top: The structure of the expression vector for hGH (pLG-OVA-hGH) and that in packaging cells in which retroviral vector sequences such as 5′ LTR, 3′ LTR, packaging signal, and the reporter gene (GFP) are omitted. The *Sal*I site was only present in pOVA-GH (see text). P, *Pvu*II (−3202, −3017, −2113 bp); B, *Bgl*II (−3055, −1752 bp); N, *Not*I; X, *Xho*I; *, TATA-like element. Bottom: Deletion mutants and DNA fragments used for plasmid construction. (C) The DNA sequence of the splicing junction. Arrows indicate the retroviral vector sequence in packaging cells after splicing. Lower case letters indicate the sequence other than the OVA region. The *Xho*I site used for cloning and first methionine of hGH are indicated by italic and bold letters, respectively. (D) Promoter motifs in the upstream region. There are putative INR and DPE but no BRE (TFIIB recognition element) or MTE (motif 10 element). Up: TATA-like element around −1800 bp. The solid and broken underlines indicate nucleotides that match and mismatch the motif sequences, respectively. Down: TATA-like element around −1880 bp. Motif sequence: INR, C/T-C/T-A-N-T/A-C/T-C/T (−2 to +5 bp in which +1 corresponds to the transcription start site); DPE, A/G-G-A/T-C/T-G/A/C (+28 to +32); TATA, T-A-T-A-A-A-A/T/G-A/G (−31 to −24 bp) (32). The numbers correspond to NCBI M29020 (−6658 to −1337 bp) and J00895 (−1342 to +7864 bp). Single asterisk, A in NCBI M29020; double asterisk, C in NCBI M29020.

USA). The plasmid was transfected into CHO cells, followed by isolation of mRNA. cDNA of hGH was isolated by reverse transcription-PCR (RT-PCR). Primers were designed to generate the *Xho*I site and Kozak sequence at the N terminus and consecutive *Mlu*I and *Xho*I sites after the C terminus. The cDNA was cloned into the *Xho*I site of pBluescript. A *Xho*I/*Mlu*I fragment of the hGH cDNA from the plasmid was ligated to *Xho*I/*Mlu*I-digested pOVA-GFP to construct pOVA-GH (Fig. 1B, top). In total, the region spanning −3861 to +1640 bp of OVA, followed by the artificial *Xho*I site, was linked immediately upstream of the coding sequence of hGH (Fig. 1C, lower line). Retroviral vector pLG, constructed by deleting the RSV promoter and the Neo^r gene from the retroviral vector pLGRN (21) and inserting the *Xho*I/*Mlu*I site, was digested with *Xho*I/*Mlu*I and ligated with a *Sal*I/*Mlu*I fragment from pOVA-GH containing hGH fused to the OVA upstream sequence. This resulted in construction of pLG-OVA-hGH (Fig. 1B, top). A packaging cell line was established using methods described previously (10,12,13,21). The viral titer was determined by counting GFP-positive cells after infecting NIH3T3 cells (21).

To obtain a truncated form of the OVA promoter, an approximate 220-bp fragment containing the junctional sequence of the artificial splice site of the transgene of the retroviral vector (−1791 to −1569 bp and +1637 to +1640 bp of OVA, followed by the *Xho*I site and 5′ terminal of hGH; Fig. 1C) was obtained by PCR using the genome of the packaging cells as the template. The following primers were used: direct, 5′-TGGCATGGTTGCTTTGAAAGAAC-3′ and reverse, 5′-CCTGTAGCCATGGTCTCGAGTTG-3′. A fragment obtained by digesting the PCR product with *Bgl*II (−1752 bp) and *Xho*I (designated as Fr *Bgl*II/*Xho*I; Fig. 1B) and a *Not*I (vector backbone)/*Bgl*II (−3055 bp) fragment of the pLG-OVA-hGH plasmid that contained the −3861 to −3055 bp region of the OVA control region (Fr *Not*I/*Bgl*II in Fig. 1B) were inserted into the *Not*I/*Xho*I site of pBluescript. This was followed by the recovery of the *Bgl*II/*Bgl*II fragment of the OVA promoter (−3055 to −1752 bp; Fr *Bgl*II/*Bgl*II in Fig. 1B), resulting in pBlue-OVA containing −3861 to −1569 bp and +1637 to +1643 bp as the promoter. A fragment obtained by digesting pBlue-OVA with *Not*I/*Xho*I was inserted into the same sites of pETBlue to generate pET-OVA in which the OVA promoter region was obtained as a *Sal*I/*Xho*I fragment. The sequence of tTA, the tetracycline-responsive transactivator (tetR/VP16 fusion protein) of pTET-OFF (Clontech, Takara Bio, Shiga, Japan), was tagged with the *Eco*RI/*Hin*dIII site and inserted into the *Eco*RI/*Hin*dIII site of a retroviral vector pMSCVneo (Clontech). This plasmid was designated pMSCV-tTA. A post-transcriptional regulation element derived from the Woodchuck hepatitis virus (WPRE) sequence that stabilizes mRNA (22) was inserted at the *Cla*I site, immediately downstream of the tTA gene of pMSCV-tTA (designated pMSCV-tTA-W). A *Kpn*I fragment, which contained the virus packaging signal, cytomegalovirus (CMV) promoter, expression unit for the Neo^r gene, and the deleted LTR of the moloney murine leukemia virus that lacked promoter activity, was removed from a Q vector pQCXIN (Clontech) and replaced by a *Kpn*I fragment of pMSCV-tTA-W. This fragment contained the virus packaging signal, tTA, WPRE, and LTR of the murine stem cell virus (MSCV) in this order to construct pQ-tTA-W. Using this replacement, a high titer of virus vector could be prepared by simple transfection without establishing a packaging cell (Q-vector system) (23). eGFP tagged with the *Eco*RI/*Xho*I site, derived originally from pIRES2-EGFP (Clontech), and the *Xho*I/*Eco*RI fragment of pTRE2 (Clontech) containing TRE were then inserted into the *Eco*RI site of pQ-tTA-W to generate pQ-eG-TRE-tTA-W. Finally, the 2.3-kb OVA upstream control region from pET-OVA (*Sal*I/*Xho*I fragment) was ligated to the *Xho*I site located immediately upstream of TRE in this plasmid. This plasmid was designated pQ-eG-OVA-TRE-tTA-W and is shown as ΔOVA in Figs. 1–4.

Deletion mutants (Fig. 1B, bottom) were constructed from this plasmid. For the DHS III plasmid, the OVA sequence downstream of −3050 bp was deleted by digestion with *Bgl*II (−3055 bp)/*Xho*I (immediately upstream of TRE), followed by Klenow fill-in and self-ligation. This resulted in deletion between DHSs II and III where the putative promoter sequences were present. For construction of the DHS III/TATA plasmid, pQ-eG-OVA-TRE-tTA-W was digested with *Pvu*II to remove *Pvu*II fragments (−3203 to −3017 bp and −3018 to −2113 bp) and ligated with a *Pvu*II fragment (−3203 to −3017 bp) again. In total, the downstream sequence of DHS III (−3016 to −2113 bp) was deleted while the putative promoter sequences remained.

For DsRed expression (24), the gene was amplified from pIRES2-DsRed-Express (Clontech) by PCR and cloned into pQ-eG-TRE-tTA-W (pQ-eG-TRE-DsRed-W). Fragments containing the OVA upstream control region (2.3 kb), internal ribosome entry site from EMCV (IRES), and tTA were inserted into this vector, designated pQ-eG-OVA-TRE-DsRed-IRES-tTA.

Pantropic retroviral vectors pseudotyped with vesicular stomatitis virus G protein (VSV-G) were produced as described previously (10,13,21). Briefly, vector plasmids were transfected into a retroviral packaging cell line, GP293 (Clontech), along with an expression vector for VSV-G (pVSV-G, Clontech) using a lipofection reagent (Lipofectamine 2000; Invitrogen). The culture medium containing viral particles was concentrated by ultracentrifugation.

Microinjection of viral vector into avian embryos and *in vitro* embryo culture A surrogated embryo culture was performed using the method of Perry (25) with some modifications (26). After the fertilized eggs (White Leghorn) were incubated with rocking for 50–55 h at 37.8°C to stages 14–15 according to Hamburger and Hamilton (27), the viral solution, except for pLG-OVA-hGH, was injected into the heart of the developing embryos (1.5–3.5 μl per embryo) as described previously

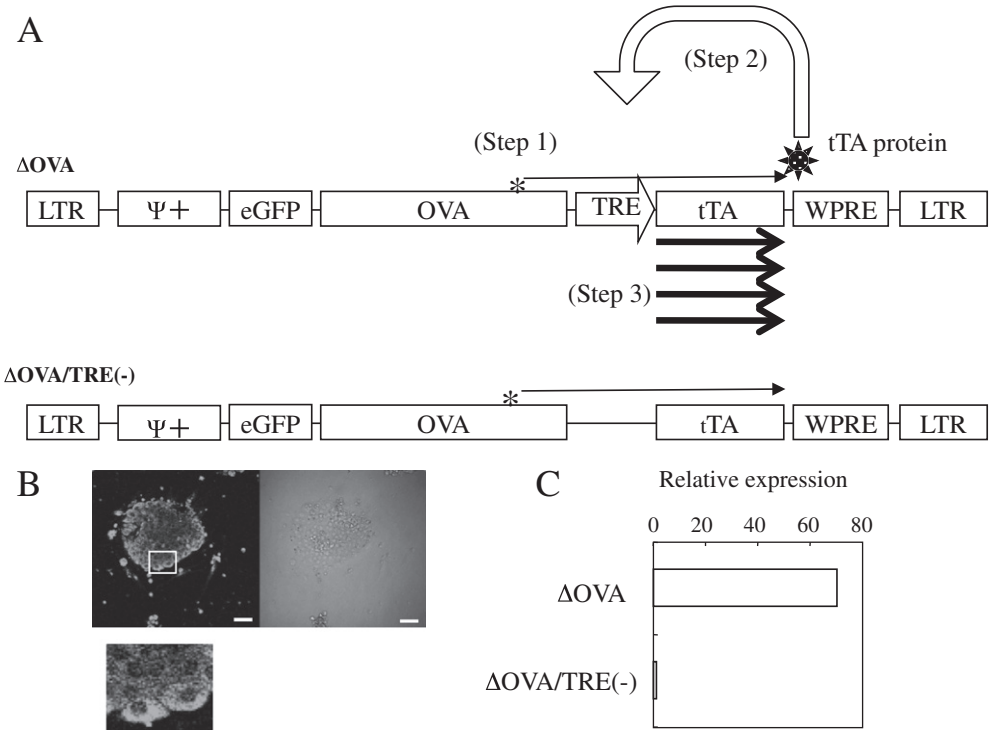

FIG. 2. Amplification of tTA expression in primary oviduct cells. (A) Structure of pQ-eG-OVA-TRE-tTA-W (ΔOVA; up) and its TRE-less derivative (ΔOVA/TRE(−); down) in the form of an integrated provirus and a possible mechanism for self-amplification of expression. Step 1: The OVA upstream region drives weak but oviduct-specific mRNA expression leading to the production of a small amount of the tTA protein. Step 2: tTA produced binds to TRE resulting in activation of transcription from TRE and the minimal CMV promoter. Step 3: The self-amplifying cycle of tTA expression supports further expression. Ψ+, virus packaging signal sequence. Asterisk, putative transcription start site. B: The primary cultures consisted mainly of OVA-expressing tubular gland cells. After 48 h of culture, the cells were stained with anti-OVA antibody. Left, fluorescent image (OVA); right, bright field image. Bottom: Magnified image of the boxed area. Bar: 50 μm. (C) Successful enhancement of tTA expression by the self-amplifying system in primary oviduct cells. The cells were infected with the vectors and tTA expression was analyzed by qRT-PCR.

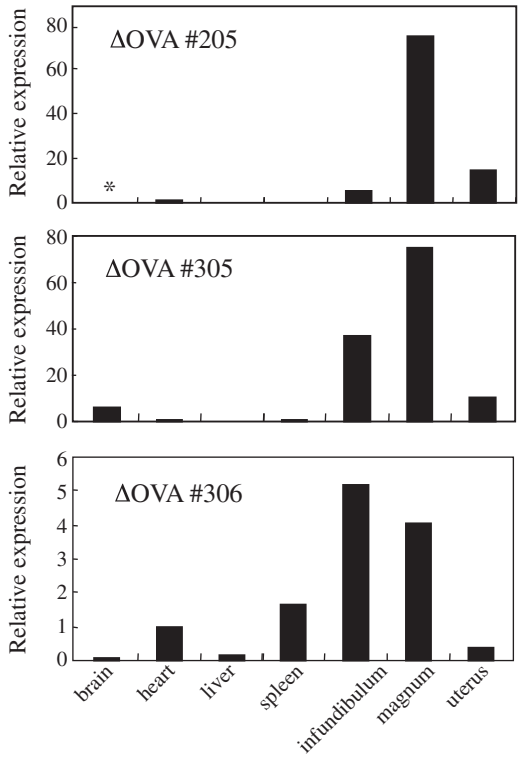

FIG. 3. Oviduct-specific tTA expression by the hybrid promoter. Estrogen-treated chicks (#205, #305) or laying hen (#306) was analyzed by qRT-PCR. Expression levels are expressed as relative values compared with those in the heart. * Not detected.

(6,10,12,13). Retroviral vector for pLG-OVA-hGH was injected into the embryos at the blastodermal stage as described previously (21) to effectively obtain transgenic progeny. All experiments were performed according to the ethical guidelines for animal experimentation of Nagoya University.

Preparation of primary oviduct cells from estrogen-induced immature hens Oviduct cells were isolated from the magnum of estrogen-treated female chicks essentially by the method of Sanders and McKnight (28) with some modifications (29). In brief, diethylstilbestrol dissolved in olive oil was intramuscularly injected into 2- to 3-week-old female chicks at a dose of 1 mg/day for 7–14 days. After 2 rounds of estrogen injections, the magnum of the developed oviduct, where most egg white proteins including OVA are produced, was minced. The cells were then dissociated in F12 medium containing penicillin (89 μg/ml), streptomycin (100 μg/ml), collagenase (250 U/ml), trypsin (20 μg/ml), and DNase I (100 μg/ml). Tubular grand cells were recovered from the magnum by 2 cycles of dissociation treatment. The isolated cells were combined and washed with F12 medium containing 0.1% bovine serum albumin (BSA). After removing cell clumps and undissociated materials by filtration, the cells were seeded in a collagen-coated dish. One hour after seeding, floating tubular gland cells were collected by pipetting to remove fibroblasts attached to the dish surface. Cells were then cultured in Dulbecco's modified Eagle medium (DMEM)/F12 containing penicillin, streptomycin, BSA (0.1%), β-estradiol (10^{-7} M), dexamethasone (10^{-7} M), and insulin (50 ng/ml).

A hepatocellular carcinoma cell line, LMH (30), was used as a control. LMH cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in DMEM/F12 supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 2 mM L-glutamine, penicillin and streptomycin on collagen-coated dishes.

Immunostaining Cells were cultured for 48 h and fixed with 1% formalin for 15 min, permeabilized by 0.5% Triton X100 for 5 min at room temperature, and then stained with rabbit anti-OVA antibody (Rockland, Gilbertsville, PA, USA; diluted 500 times with phosphate buffered saline (PBS) containing 3% BSA) and FITC-labeled anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 500 times with PBS containing 3% BSA). The cells were observed using a fluorescence microscope (BZ-8000, Keyence, Osaka, Japan).

Determination of mean copy number of integrated vector by quantitative PCR (qPCR) Genomic DNA was isolated from various organs, erythrocytes, and sperm using Mag Extractor-Genome (Toyobo, Osaka, Japan). A 20 ng aliquot of purified DNA was used for qPCR (LightCycler; Roche Diagnostics, Mannheim, Germany) using LightCycler FastStart DNA Master Hybprobe (Roche). Primers and probes were designed inside the packaging signal region of MSCV: forward, 5′-CAAGAAGAGACGTTGGGTTAC-3′; reverse, 5′-CTTCCCAGGTCACGATGT-3′; 5′-LCRed, 5′-GGTGATGGAGGTCTCGGTTAAAGGTGCC-3′; 3′-FITC, 5′-GGCCAGGTGAAAAGACCTTGATCTTAACCT-3′. The genome of erythrocytes from a G2 transgenic chicken producing single chain Fv fused to Fc (10) (1 copy) and non-transgenic chickens (zero copy) were used as a copy number standard. The copy number was determined using LightCycler Software version 3.5 (Roche Diagnostics).

Quantitative RT-PCR (qRT-PCR) Total RNA was extracted using a QuickPrep total RNA extraction kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and converted to cDNA with reverse transcriptase (ReverTraAce; Toyobo) using oligo-dT as a primer. cDNA was then subjected to qPCR to estimate the amount of mRNA of tTA using the following primers: forward, 5′-TAGAGCAGCCTACATTGTAT-3′; reverse, 5′-CGATGACTTAGTAAAGCACA-3′; 5′-LCRed, 5′-AGAAGGGGAAAGCTGGCAAGATTTTT-TACG-3′; 3′-FITC, 5′-TGTTAGATAGGCACCATACTCACTTTTGCCCTT-3′. The amount of each gene was first determined on the basis of a standard curve of the corresponding plasmid, and then normalized by calculating the ratio of mRNA to the copy number of the integrated transgene.

Frozen sections Oviduct of estrogen-treated female chicks were embedded in Tissue-Tek OCT compound (Miles Scientific, Naperville, IL, USA). Cryostat serial sections (20 μm) were cut at −21°C, mounted on glass slides, and observed using a fluorescence microscope (BZ-8000).

RESULTS

A large deletion in the OVA promoter still supports oviduct-specific expression of the transgene We constructed a retroviral vector to express hGH under the control of the OVA promoter/enhancer. The OVA control region spanning −3.9 to +1.5 kb including DHSs III, II, and I, first exon and intron, and 7 nucleotides of the second exon located immediately upstream of the initiation codon was used to express hGH (Fig. 1B). By injecting the high-titer virus vector into embryos, G_0 chicken and quail were generated. G_1 transgenic quail were generated by successive mating. G_1 quail and G_0 chicken produced hGH in egg white, although the levels were low at approximately 1–5 ng/ml (Fig. S1A). To determine the possible reasons for this low productivity, Southern blots were used to analyze the transgene in the packaging cells, established by infecting GP293 cells with a low-titer virus preparation. This showed that the major band of the transgene was short, with the full-size transgene being detected only rarely (data not shown). Sequence analysis showed that deletion occurred between nucleotides −1568 bp in the OVA control region and +1636 bp that corresponded to the 3′ end of intron 1 of the OVA gene. The latter was a naturally occurring splicing acceptor for OVA (Figs. 1B, C). The flanking sequence around −1568 bp matched the consensus splicing donor site sequence. We therefore considered that this deletion was caused by splicing of the genomic RNA of the retroviral vector. We transfected the transgene and VSV-G gene into GP293 cells to produce the low-titer virus preparation and then infected GP293 cells with this preparation to establish packaging cells producing high-titer-virus. Because a shorter transgene has an advantage in viral particle formation, we assumed that packaging cells with a truncated transgene were dominant during screening for cells producing a high titer of the viral vector. The structure of the transgene is shown in Fig. 1B. In total, the large deletion caused a lack of the major part of the known OVA regulatory sequences, including DHSs II, I, and TATA box, which may have resulted in very low production of hGH. It is also possible that hGH expression may be silenced, which was observed for the transgene infected to very early stages of embryo (21). However, to our surprise, hGH was expressed in an oviduct-specific manner (Fig. S1B), suggesting that the sequence −3861 to −1569 bp containing DHS III and its downstream sequence may be responsible for tissue-specific expression of the hGH gene. To determine the mechanism of this tissue-specific expression, putative binding sites for transcription factors were analyzed by transfac (http://mbs.cbrc.jp/research/db/TFSEARCH.html). In addition to the previously reported 4 half sites of ERE within DHS III, we predicted that a TATA-like element existed close to the 3′ end of the spliced form of the OVA control region (−1797 bp; Fig. 1D). There also appeared to be other putative core promoter elements such as initiator (INR), which contains a transcriptional start site, at a location that permitted INR to work together with the TATA element (31) and downstream core promoter element (DPE). Similar to typical DPE, this DPE was located

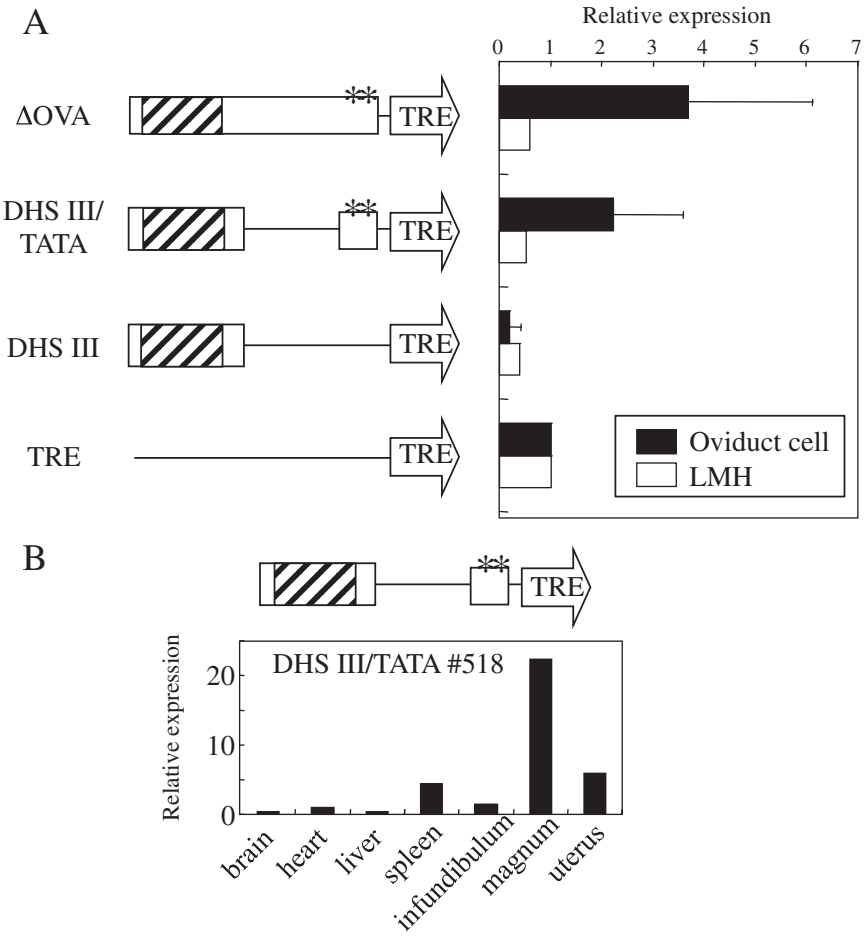

FIG. 4. Oviduct-specific expression by the hybrid promoter requires the region containing TATA-like elements. (A) Promoter activity in primary oviduct cells. Primary oviduct cells were infected *in vitro* with the vectors, and tTA expression was assessed by qRT-PCR. LMH cells were transfected using Lipofectamine 2000. (B) tTA expression in the magnum of an estrogen-treated chick infected with a DHS III/TATA construct. Hatched box, DHS III; asterisk, TATA-like element.

approximately 30 bp downstream of the putative INR, despite it usually being found in the TATA-less promoter (32). The TATA–INR structure was also found near −1880 bp.

Enhanced expression of tTA by self-amplifying system in oviduct cells An *in silico* study as well as tissue-specific hGH expression in transgenic birds suggested that weak but oviduct-specific hGH expression may rely on DHS III and core promoter elements located around −1.8 kb in the absence of authentic OVA promoter sequences. Expression from the upstream core promoter appeared weak, and therefore, a strategy was adopted to use this specific expression system in transgenic chickens in which the products were deposited in egg white. Fig. 2A shows the structure of the viral vectors used. To induce oviduct-specific tTA expression, the cassette of the tTA coding sequence flanked with TRE and minimal CMV promoter sequences was introduced downstream of the OVA control region (−3861 to −1569 bp containing DHS III and putative core promoter sequences). tTA is a chimeric protein containing the DNA binding domain of the Tet repressor that recognizes TRE and the transcription activation domain of VP16 (33). With this system, weak and oviduct-specific expression of tTA from the upstream OVA promoter (step 1 in Fig. 2A) may trigger self-amplification cycle of transcription from TRE plus minimal CMV promoter (steps 2–3).

To confirm that amplification was effective in chicken oviduct cells, we used primary cultures of oviduct tubular grand cells. Female chicks were treated with estrogen to prematurely develop the oviduct, followed by the recovery of tubular gland cells, the purity of which was confirmed to be 80−90% by immunostaining with anti-OVA antibody (Fig. 2B). In the first trial, we transfected DNA into the cells using a conventional lipofection reagent; however, the efficiency of this method was very low. In contrast, infection of the retroviral vector at a high multiplicity of infection enabled reliable gene manipulation. We therefore used this method to examine tTA expression driven by the hybrid promoter. qRT-PCR revealed that the tTA expression level was 70 times higher in ΔOVA-infected cells than in the ΔOVA/TRE(−) construct without a TRE sequence (Fig. 2C). This result indicated that the self-amplifying system efficiently enhanced tTA expression in chicken oviduct cells.

Truncated OVA promoter supports oviduct-specific expression We then introduced ΔOVA vector into the embryonic hearts and obtained G_0 chickens. In non-treated female G_0 chicks aged approximately 1 month, tTA was not expressed in any organs (data not shown). This confirmed the lack of basal activity of the construct. After estrogen treatment to develop a premature oviduct, tTA was produced in oviduct cells but not in any other organs (Fig. 3). In these experiments, a high level of expression was observed in the infundibulum as well as the magnum in some chickens. Because the magnum and infundibulum are anatomically distinct and OVA expression is restricted to the magnum, this result suggested that the tissue specificity of the artificial promoter is slightly different from that of the original OVA promoter. These results also demonstrated that the self-amplifying hybrid promoter can be used for the chicken oviduct-specific expression system. After sexual maturation, tTA was expressed in the magnum and infundibulum of laying hen that had not been treated with hormone (#306), although moderate

expression was observed in the spleen and heart but not in any other organs. Because large amounts of the viral vector were injected into the hearts, a background level of expression may occur in this organ. The reason for moderate expression in the spleen remains unclear. Overall, it is suggested that oviduct-specific OVA expression may be mediated, at least in part, by the far upstream region spanning −3.9 to −1.6 kb that contains DHS III and putative TATA-like elements.

Sequence requirement for oviduct-specific expression To investigate the role of TATA-like elements for oviduct-specific expression, several deletion constructs were introduced into primary oviduct cultures (Fig. 4A). Deletion of the intervening region between DHS III and TATA-like elements (DHS III/TATA, lacking −3016 to −2113 bp) caused a slight reduction in expression. Further deletion including the TATA-like elements completely abrogated the expression (DHS III). On the other hand, LMH, a hepatocellular carcinoma line of chicken origin, expressed tTA very weakly with ΔOVA. Taken together, these deletion analyses support the association between this weak promoter and oviduct-specific regulatory mechanisms that rely on DHS III of OVA. Consistent with these results, tTA expression was observed in the magnum of a genetically manipulated chicken infected with the DHS III/TATA vector (Fig. 4B).

Hybrid promoter can be used for expression of exogenous proteins DsRed was inserted downstream of the promoter to confirm that a target protein other than tTA can be expressed in an oviduct-specific manner by the hybrid promoter (Fig. 5A). DsRed and tTA were linked with IRES, which enabled translation of both genes from a bicistronic mRNA. We injected the viral vector and obtained 3 G_0 chickens. tTA expression was higher in the magnum than in other organs, except for the heart of estrogen-treated chicks (Fig. 5B). Injecting the viral vector into embryonic hearts may cause relatively high expression in this organ. Microscopic analysis of frozen sections of the oviduct clearly demonstrated DsRed fluorescence (Fig. 5C). Consistent with our previous observations that the LTR promoter of MSCV did not function in transgenic chickens (10), we did not detect

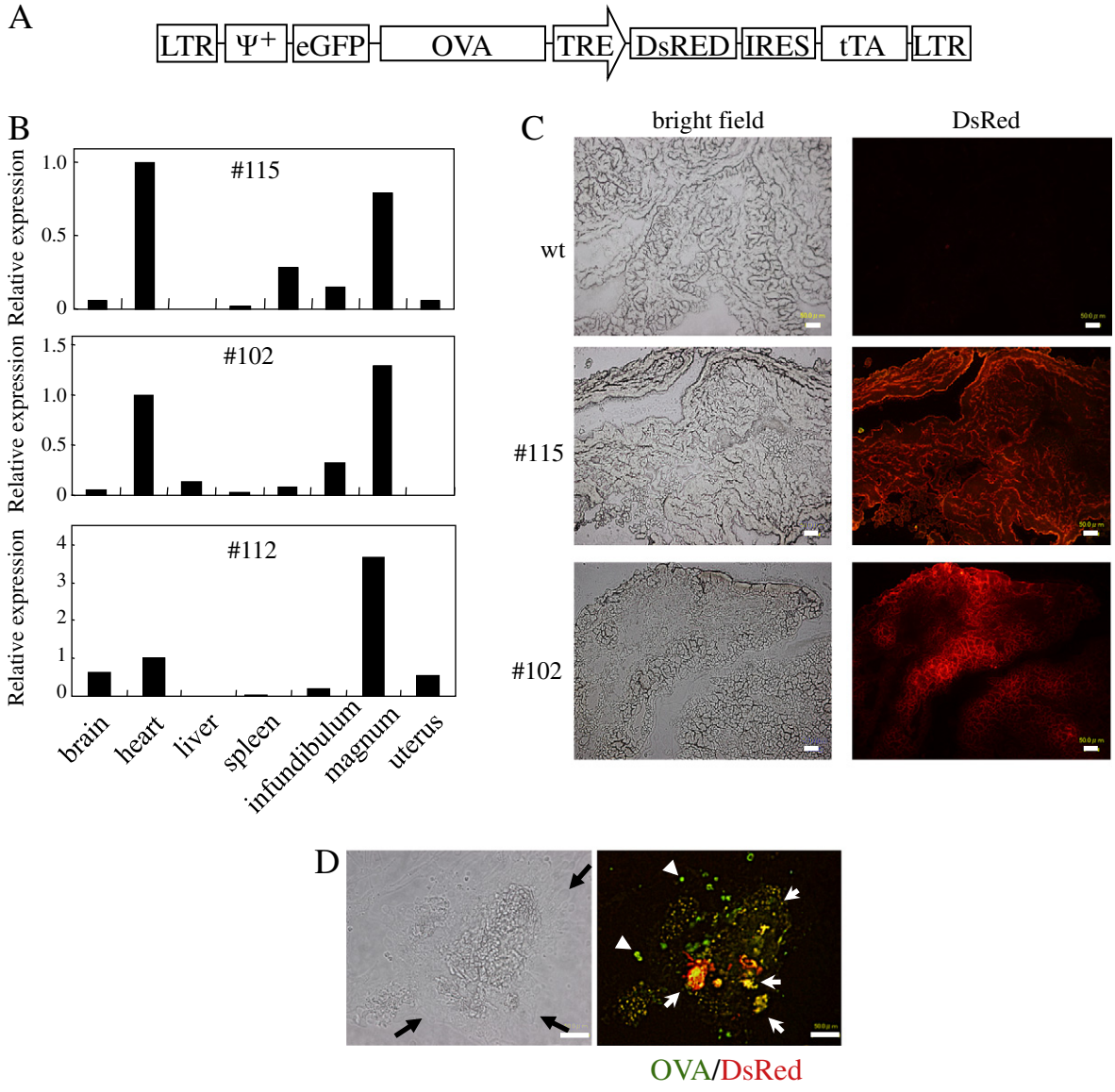

FIG. 5. The hybrid promoter can be used for expression of DsRed as a model target gene. (A) Schematic representation of the infected retrovirus vector pQ-eG-OVA-TRE-DsRed-IRES-tTA. (B) Tissue specificity of expression in estrogen-treated chicks was evaluated as in Fig. 3. (C) DsRed expression in the magnum. Frozen sections (20 μm) were analyzed by microscopy. Note that the mosaicism of the transgene in the G_0 chickens. wt, wild type. (D) Oviduct tubular gland cells expressed DsRed. The induced oviduct in #112 was dissociated and cultured for 48 h. OVA was stained with anti-OVA antibody. Left, bright field; the arrows indicate fibroblasts. Right, fluorescent image [merge of OVA (green) and DsRed (red)]; the arrows indicate DsRed-expressing tubular gland cells (yellow); the triangles indicate tubular gland cells without DsRed expression (green). Bar: 50 μm.

eGFP expression under the control of this promoter (data not shown). Oviduct cells of the genetically manipulated chicken were dissociated and stained with anti-OVA antibody after 48 h of culture (Fig. 5D). Fibroblasts did not express DsRed or OVA, while tubular gland cells, which were considerably smaller than the fibroblasts and loosely attached to these fibroblast cells as round shapes or aggregates, were stained with anti-OVA antibody. A portion of OVA-expressing cells were shown to express DsRed. Because the cells were a mixture of non-infected and successfully infected cells in the G_0 generation, it is likely that the non-expressing tubular gland cells were not transduced by the retroviral vector. These results demonstrated that our hybrid expression cassette in which the OVA upstream region was fused with the TRE–tTA system worked very efficiently.

DISCUSSION

It has been reported that the 1-kb region proximal to the transcription start site, which contains both DHSs II and I and TATA sequences, is not sufficient for tissue-specific regulation of OVA (20). The results of the present study suggested that the region far upstream of OVA (−3.9 to −1.6 kb) may act as a switch that determines tissue specificity. Our results also demonstrated that the specificity of the far upstream region alone is not sufficient for the strict specificity of OVA. OVA is expressed at considerably higher levels in the oviduct than in other tissues, the levels being more than 1000-fold higher (15 and data not shown), whereas expression of our hybrid promoter in the oviduct was not as strong as that of OVA (Figs. 3–5). In addition, expression of the hybrid promoter in the infundibulum, tissue adjacent to the magnum, was detected in several chickens (Fig. 3), while OVA expression was barely detected (data not shown). In this regard, it is noteworthy that putative binding sites for other tissue-specific regulators have been suggested to be within the promoter proximal region (15,18). These studies indicated the co-operative effects of far upstream and promoter proximal regions conferring oviduct-specific OVA expression. However, the detailed mechanisms, including the hierarchy of each regulatory region, have yet to be elucidated.

Our study suggests that oviduct specificity may be determined by the region spanning −2.1 to −1.6 kb (containing TATA-like elements), in association with the region spanning −3.9 to −3.0 kb (containing DHS III).

Analysis of the truncated OVA promoter indicated that the region around −1800 bp that contained 2 TATA-like sequences was essential for oviduct-specific expression of the transgene (Fig. 4). We therefore assumed weak transcription around −1800 bp may cause oviduct-specific expression. However, we have been unable to detect mRNA transcribed from the putative promoter around −1800 bp in normal hens because of its low abundance. Furthermore, magnum-specific mRNA that covered the OVA transcriptional initiation site and had the opposite orientation could be detected by RT-PCR and Northern blotting (Okino et al., unpublished results). This result suggested that several RNA species may be generated from the OVA control and coding regions. Precise analyses of the mRNA species and their transcriptional start site are now being undertaken.

The importance of DHS III in regulation of OVA expression has been suggested by several studies. First, DHSs III and II are controlled hormonally and closely linked to OVA expression itself. After premature development of the oviduct achieved by injecting estrogen, OVA expression ceases within 3 days in the absence of estrogen (16,19). DHSs III and II disappeared after short-term estrogen withdrawal, while DHS I remained unchanged. Furthermore, under this condition, OVA expression could be induced again by a very short exposure to estrogen (1 day). This is in marked contrast to the first induction that took 2 weeks. The second induction was also associated with the reappearance of DHSs III and II. Second, CpG methylation within DHS III was decreased by OVA expression. Although demethylation was complete in DHSs I and II, demethylation was partial in DHS III (29). Third, estrogen responsiveness (19) or silencer activity in the liver (20) has been reported for DHS III or the overlapping region. Therefore, DHS III may have a possible role in oviduct-specific OVA expression.

Several roosters were grown to maturity, and the copy number of the transgene in semen was analyzed by qPCR. However, the copy number was found to be quite low (data not shown), and we could not obtain transgenic offspring. Therefore, all analyses were performed in the G_0 generation. In these chickens, mean copy number is variable among organs, but this can be overcome by normalizing mRNA expression with an integrated copy number. Furthermore, analyses using G_0 chickens have an advantage that deviation due to the chromosome environment of the integration sites, which has profound effects on mRNA expression, can be minimized in G_0 chickens. The data obtained correspond to the mean value of the various integration sites. In fact, the specificity of expression was generally uniform for each construct between individuals, with the exception of some organs.

Oviduct-specific expression is especially useful for the production of pharmaceuticals in eggs of transgenic chickens. Several trials have been reported (9,34), although the expression levels need to be improved. Preliminarily, it was suggested that erythropoietin productivity by our hybrid promoter was approximately one third compared with that by actin promoter (Kodama et al., unpublished result). Further refinement will greatly facilitate the development of transgenic chickens that produce highly bioactive pharmaceuticals in egg white, which may even be toxic to the host if expressed ubiquitously.

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