

# Development and in vitro evaluation of recombinant chicken promoters to efficiently drive transgene expression in chicken oviduct cells

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**ABSTRACT** Virus injection into EGK-X embryos is a well-defined approach in avian transgenesis. This system uses a chicken ovalbumin gene promoter to induce transgene expression in the chicken oviduct. Although a reconstructed chicken ovalbumin promoter that links an ovalbumin promoter and estrogen-responsive enhancer element (**ERE**) is useful, a large viral vector containing the ovalbumin promoter and a target gene restricts viral packaging capacity and produces low-titer virus particles. We newly developed recombinant chicken promoters by linking regulatory regions of ovalbumin and other oviduct-specific genes. Putative enhancer fragments of the genes, such as *ovotransferrin* (**TF**), *ovomucin alpha subunit* (**OVOA**), and *ovalbumin-related protein X* (**OVALX**), were placed at the 5'-flanking

region of the 2.8-kb ovalbumin promoter. Basal promoter fragments of the genes, namely, *pTF*, *lysozyme* (*pLYZ*), and *ovomuroid* (*pOVM*), were placed at the 3'-flanking region of the 1.6-kb ovalbumin ERE. The recombinant promoters cloned into each reporter vector were evaluated using a dual luciferase assay in human and chicken somatic cells, and LMH/2A cells treated with 0-1,000 nM estrogen, and cultured primary chicken oviduct cells. The recombinant promoters with linking ovalbumin and *TF*, *OVOA*, *pOVM*, and *pLYZ* regulatory regions had 2.1- to 19.5-fold ( $P < 0.05$ ) higher luciferase activity than the reconstructed ovalbumin promoter in chicken oviduct cells. Therefore, recombinant promoters may be used to efficiently drive transgene expression in transgenic chickens.

**Key words:** chicken, ovalbumin promoter, recombinant promoter, reporter analysis

2021 Poultry Science 100:101365

<https://doi.org/10.1016/j.psj.2021.101365>

## INTRODUCTION

Recombinant proteins are widely produced in diverse platforms, such as microorganisms, animal cell lines, plants, and animals (Zhu, 2012). Avian species have several advantages over other platforms as bioreactors to produce recombinant proteins because of the benefits of glycosylation and posttranslational modification (Lillico et al., 2005; Kojima et al., 2014). The low cost, efficient scale-up, and relatively simple purification of the proteins are feasible in avian bioreactor systems (Ivarie, 2006; Lillico et al., 2007). Therefore, many transgenic chickens that produce recombinant proteins were generated. These chickens recently exhibited meaningful performances and received approval from the Food and Drug Administration (**FDA**) (Sheridan, 2016).

Unique approaches for producing transgenic avian species were used because of their species-specific reproductive mechanism, and virus injection into the subgerminal cavity of Eyal-giladi and Kochav Stage-X (**EGK-X**) chicken embryos is a well-defined method (McGrew et al., 2004). This system uses an ovalbumin gene promoter to induce transgene expression in transgenic chickens due to its oviduct-specific regulation and productivity. Four DNase I-hypersensitive sites (**DHSS**) are located between the ovalbumin locus and its upstream neighboring gene (Kaye et al., 1986), and the estrogen-responsive enhancer element (**ERE**) DHS III is responsible for estrogen inducibility and regulation of oviduct-specific expression (Kato et al., 1992; Kodama et al., 2012). The estrogen hormone and receptor complex activates transcription of the target gene via binding to the ERE in the promoter of the gene (Giguere, 1999). Steroid-dependent regulatory elements (**SDREs**) and negative-dependent regulatory elements (**NREs**) in the 5'-flanking region of the ovalbumin transcription start site functionally collaborate for

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Received February 25, 2021.

Accepted June 24, 2021.

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oviduct-specific gene expression and regulation (Dougherty and Sanders, 2005).

Several types of ovalbumin promoters were used to construct transgene expression cassettes in viral vectors, and a higher level of recombinant protein expression, such as human functional cytokines and lysozyme, was observed when the ovalbumin ERE was placed upstream of the ovalbumin promoter (Lillico et al., 2007; Cao et al., 2015; Herron et al., 2018). Although a reconstructed ovalbumin promoter that links an ovalbumin promoter and the ERE is useful, a large viral vector containing the ovalbumin promoter and a target gene restricts viral packaging capacity and produces low-titer virus particles. Lentivirus titers are significantly decreased for large transfer vectors (Kumar et al., 2001; Yacoub et al., 2007). Therefore, the production of chimeric chickens with germline transmission of a transgene may have low efficiency following injection of a low-titer virus into EGK-X embryos.

The present study newly developed recombinant chicken promoters by linking regulatory regions of ovalbumin and other oviduct-specific genes. The recombinant promoters were designed to be more compact than the reconstructed ovalbumin promoter, and these constructs were evaluated in several human and chicken somatic cells and cultured primary chicken oviduct cells.

## MATERIALS AND METHODS

### Experimental Animals and Animal Care

The experimental protocol used in this study was performed under an approved animal-use document and according to the animal care and use guidelines of the committee of the National Institute of Animal Science, Republic of Korea (approval no: 2017-219).

### Cell Preparation and Culture

Human cervix epithelial (HeLa and CCL-2; ATCC), human ovarian epithelial (MES-SA and CRL-1976; ATCC), and chicken liver epithelial (Leghorn male hepatoma [LMH]/2A and CRL-2118; ATCC) cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured, and stocked according to the manufacturer's instructions. Cells were cultured with Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA) and 1% antibiotic/antimycotic (Gibco, Carlsbad, CA). Chicken embryonic fibroblast cells (cEFs) were trypsinized and collected via centrifugation (Schmid et al., 1983), and the DF-1 chicken embryonic fibroblast cell line (CRL-12203; ATCC) was cultured in DMEM supplemented with 10% FBS, 1% antibiotic/antimycotic, and 2% chicken serum (Gibco, Carlsbad, CA). Chicken oviduct epithelial cells (cOECs) were isolated and cultured according to a previous study (Kasperczyk et al., 2012; Yang et al., 2021). Briefly, the magnum from a 35-wk egg-laying hen (White Leghorn) oviduct was

surgically separated, and the tissue was horizontally torn off to expose the inner surface. The inner surface was scraped, and the scraped tissue fragments were minced, digested with 10 mL (1 mg/mL) of collagenase P (Roche, Basel, Switzerland) supplemented with 10 mM HEPES (Sigma-Aldrich, Saint Louis, MO), and placed in an incubator with 5% CO<sub>2</sub> at 37°C for 30 min with vortexing every 5 min. The digested tissue masses were centrifuged and cultured on collagen-treated dishes (Corning, New York, NY) with keratinocyte complete medium (K-SFM; Gibco, Carlsbad, CA) supplemented with 5% chicken serum and 1% antibiotic/antimycotic.

### Vector Construction

As a control, a reconstructed ovalbumin promoter (*Mut-4.4-kb-pOV*) linking the 2.8-kb ovalbumin promoter and the 1.6-kb ovalbumin ERE was synthesized (Herron et al., 2018). Two restriction enzyme sites (*Nhe I* and *Xho I*) were inserted at the end of the 5'- and 3'-flanking regions via PCR amplification, and the promoter was cloned into the pGL4.11/luc2p firefly DNA vector (Promega, Madison, WA). Recombinant promoters linking putative enhancer elements of chicken oviduct-expressing genes, namely *ovotransferrin (TF)*, *ovomucin alpha subunit (OVOA)*, and *ovalbumin-related protein X (OVALX)*, to the 5'-flanking region of the 2.8-kb ovalbumin promoter were synthesized using 2 restriction enzyme sites (*Nhe I* and *Xho I*). Recombinant promoters linking basal promoters of the genes, namely *pTF*, *lysozyme (pLYZ)*, and *ovomuroid (pOVM)*, to the 3'-flanking region of the 1.6-kb ovalbumin ERE were synthesized using 2 restriction enzyme sites (*Xho I* and *EcoR V*). These 6 recombinant promoters were cloned into the pGL4.11 reporter vectors.

### Transfection

Each pGL4.11 reporter vector cloned with *Mut-4.4-kb\_pOV* and the 6 recombinant promoters were used together with the pGL4.74/hRluc Renilla DNA vector (Promega, Madison, WA). Renilla is driven by the herpes simplex virus thymidine kinase promoter. pGL4.11 and pGL4.74 were cotransfected into HeLa cells, MES-SA cells, cEFs, LMH/2A cells, and cOECs using a Lipofectamine 3,000 kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, cells were seeded into a 12-well culture plate, and transfection was performed at 70% cell density, considering the molecular ratio of each vector. To perform transfection, 50  $\mu$ L of Opti-MEM (Gibco, Carlsbad, CA) containing 1  $\mu$ g of pGL4.11, 0.01  $\mu$ g of pGL4.74, and 2  $\mu$ L of P3,000 per well and another 50  $\mu$ L of Opti-MEM containing 1.5  $\mu$ L of Lipofectamine reagent per well were mixed for 20 min at room temperature. A volume of 100  $\mu$ L of the mixture per well was added to 12-well culture plates, and the culture plate was placed in an incubator with 5% CO<sub>2</sub> at 37°C for 24 h.

## Luciferase Assay

To analyze the promoter activity, a dual luciferase assay was performed. The cell culture medium was removed, and 250  $\mu$ L of passive lysis buffer was added to each well of the 12-well culture plate. The plate was stirred for 20 to 30 min to lyse the cells. The lysed cells were transferred into 1.5-mL E-tubes, which were centrifuged at 12,000 rpm for 10 min, and 20  $\mu$ L of each lysate was transferred to a 96-well white microplate (Nunc, Rochester, NY). To analyze the estrogen response reaction in LMH/2A cells, the cells were additionally cultured for 24 h with medium containing 0-1,000 nM 17 $\beta$ -estradiol (Sigma-Aldrich, Saint Louis, MO) at 24 h post transfection. These cells were prepared as described above. Each luciferase activity was measured using a Centro LB 960 luminometer (Berthold, Bad Wildbad, Germany). The normalized luciferase value was calculated as the firefly luminescence/Renilla luminescence ratio. The relative luciferase ratio for promoter activity was recombinant promoter luciferase ratio/*Mut-4.4-kb-pOV* luciferase ratio. The relative luciferase ratio for the estrogen response was estrogen treatment luciferase ratio/non-estrogen treatment luciferase ratio.

## Immunofluorescence

cOECs at passage 1 were seeded into a 4-well culture plate, fixed with 4% paraformaldehyde (Molecular Probes, Eugene, OR), and permeabilized with 0.5% Triton-X (Gibco, Carlsbad, CA) for 15 min at room temperature. Cells were blocked with 5% bovine serum albumin (BSA; Molecular Probes, Eugene, OR) for 1 h at room temperature and incubated with a primary rabbit polyclonal anti-ovalbumin antibody (1:250; 1 mg/mL; Abcam, Cambridge, UK) overnight at 4°C. Cells were incubated with a secondary mouse anti-rabbit FITC conjugated antibody (1:500; 2 mg/mL; Abcam) for 1 h and washed with PBS (Gibco, Carlsbad, CA). Cells were counterstained for DNA using Hoechst 33258 (1:4,000; 10 mg/mL; Life Technologies, Carlsbad, CA). Immunofluorescence was analyzed under a fluorescence microscope.

## Western Blot Analysis

Total protein was extracted from the chicken oviduct as a positive control and from chicken leg tissue and embryonic fibroblast cells (DF-1) as negative controls. Total protein was also extracted from the cOECs. RIPA lysis buffer (Thermo Scientific, Waltham, MA) containing a protease and phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA) was used for protein extraction. The protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA), and 15  $\mu$ g of protein extract was electrophoresed using an SDS-PAGE 4 to 12% gel system (Invitrogen, Carlsbad, CA). Separated proteins were transferred to a polyvinylidene fluoride membrane (PVDF; Invitrogen, Carlsbad, CA). The membrane was blocked with 5% skim

milk in PBS (Thermo Scientific, Waltham, MA) for 1 h and incubated with a rabbit polyclonal anti-ovalbumin antibody (1:1,000; 1 mg/mL; Abcam, Cambridge, UK) and rabbit monoclonal anti-vinculin antibody (1:1,000; 0.054 mg/mL; Abcam, Cambridge, UK) overnight at 4°C. The membrane was washed and incubated with a mouse anti-rabbit HRP-conjugated antibody (1:2,000; 0.4 mg/mL; Santa Cruz, Dallas, TX) for 30 min at room temperature. Amersham ECL prime (GE Healthcare, Buckinghamshire, UK) substrate was used to visualize the target bands, and the bands were analyzed using EZ-Capture II (Atto, Tokyo, Japan).

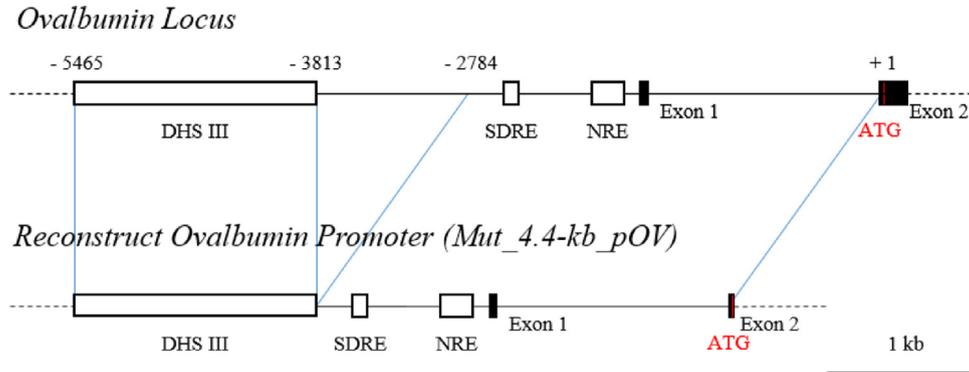
## Statistical Analysis

Statistical analysis was performed using GraphPad Prism statistical software (GraphPad Prism 5.03 software, San Diego, CA). One-way ANOVA was used to compare the relative luciferase ratio, and a *P*-value less than 0.05 was considered statistically significant. The reporter analysis data are expressed as the mean  $\pm$  standard error of the mean from three independent experiments.

## RESULTS

### Construction of the Recombinant Chicken Promoters

To develop chicken recombinant promoters to efficiently drive transgene expression, a reconstructed ovalbumin promoter (*Mut\_4.4-kb\_pOV*) was used as a control in reporter analysis, and 6 recombinant promoters (*TF\_pOV*, *OVOA\_pOV*, *OVALX\_pOV*, *ERE\_pTF*, *ERE\_pLYZ*, and *ERE\_pOVM*) were synthesized and cloned into the pGL4.11 reporter vector. The *Mut\_4.4-kb\_pOV* structure is shown in [Figure 1](#), and the structures of the 6 recombinant promoters are shown in [Figure 2](#). Notably, 958 bp of *TF*, 977 bp of *OVOA*, and 1,468 bp of *OVALX* were linked upstream of the 2.8-kb ovalbumin promoter ([Figure 2A](#)), then 1,126 bp of *pTF*, 857 bp of *pLYZ*, and 1,350 bp of *pOVM* were linked downstream of the 1.6-kb ovalbumin ERE ([Figure 2B](#)). These recombinant sequences, except *pLYZ*, had several putative estrogen receptor (ER) alpha binding sites as follows: 5 (1.6-kb ovalbumin ERE), 3 (2.8-kb ovalbumin promoter), 2 (*TF*), 2 (*OVOA*), 3 (*OVALX*), 1 (*pTF*), and 1 (*pOVM*) in the direction of 5' to 3' ([Table 1](#)). As a result, the lengths of the recombinant promoters were 3,742 (*TF\_pOV*), 3,761 (*OVOA\_pOV*), 4,252 (*OVALX\_pOV*), 2,778 (*ERE\_pTF*), 2,509 (*ERE\_pLYZ*), and 3,002 bp (*ERE\_pOVM*). Therefore, the recombinant promoters were more compact than *Mut\_4.4-kb\_pOV* (4,436 bp) and may have oviduct-specific regulation and expression via the ovalbumin regulatory regions, 2.8-kb ovalbumin promoter or 1.6-kb ovalbumin ERE.

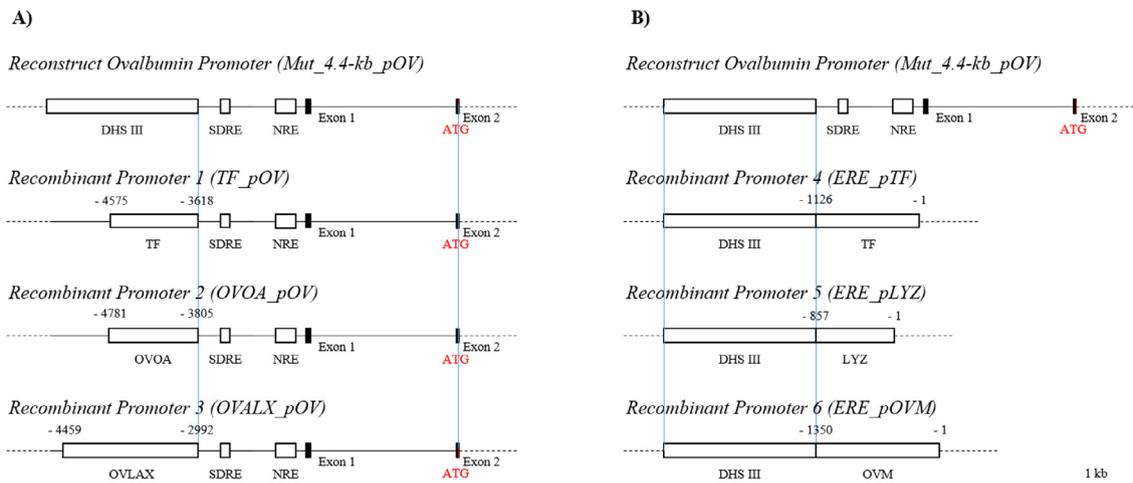


**Figure 1.** Structure of the reconstructed chicken ovalbumin promoters. *Mut\_4.4-kb\_pOV* with a 1-kb deletion between the 1.6-kb ovalbumin ERE and the 2.8-kb ovalbumin promoter in the ovalbumin locus was constructed via gene synthesis for use as a control in the luciferase assay. Nucleotide sequences are denoted based on the translation start site (ATG) as a +1. Abbreviations: DHS III, DNase hypersensitive site III; NRE, negative-dependent regulatory element; SDRE, steroid-dependent regulatory element.

## Activity and Estrogen Response of the Recombinant Chicken Promoters in Somatic Cells

To validate the characteristics of the recombinant promoters, such as promoter activity, cell-specificity, and estrogen responsiveness, each reporter vector cloned with *Mut\_4.4-kb\_pOV* and the 6 recombinant promoters were transfected into HeLa cells, MES-SA cells, cEFs and LMH/2A cells. A dual luciferase assay was performed to evaluate the relative promoter activity in these cells (Figure 3). Although the promoter activities of *TF\_pOV* and *OVOA\_pOV* showed no large differences from each other in HeLa cells (Figure 3A), *OVOA\_pOV* had 2.0-fold ( $P < 0.01$ ) higher activity in MES-SA cells compared to the control (Figure 3B). There were no significant differences between all groups in chicken embryonic fibroblast cells (Figure 3C). *TF\_pOV* and *OVOA\_pOV* showed 2.1- ( $P < 0.05$ ) and 1.7-fold ( $P > 0.05$ ), respectively, higher relative luciferase expression in LMH/2A cells compared to the control (Figure 3D).

However, *ERE\_pLYZ* and *ERE\_pOVM* showed no significant differences compared with the control, but *ERE\_pTF* had significantly higher luciferase activity in all cells, especially in chicken embryonic fibroblast cells (Figure 3E–3H). The seven reporter vectors mentioned above were transfected into LMH/2A cells followed by treatments with 0 to 1,000 nM estrogen 24 h post-transfection. A dual luciferase assay was performed to investigate the estrogen response at 24 h posttreatment (Figure 4). The luciferase activity was significantly increased 2.5-fold ( $P < 0.01$ ) after treatment with 1,000 nM estrogen in the control (Figure 4A). *TF\_pOV* and *OVOA\_pOV* showed 56.4- ( $P < 0.01$ ) and 15.9-fold ( $P < 0.01$ ) increases, respectively, with 1,000 nM estrogen treatments (Figure 4B, 4C), but there were no increases following estrogen treatments in *OVALX\_pOV* or *ERE\_pTF* (Figure 4D, 4E), and only 2.0- ( $P < 0.05$ ) and 1.4-fold ( $P < 0.001$ ) increases were confirmed in *ERE\_pLYZ* and *ERE\_pOVM*, respectively (Figure 4F, 4G). Taken together, *TF\_pOV*, *OVOA\_pOV*, *ERE\_pLYZ*, and *ERE\_pOVM* were considered



**Figure 2.** Structures of the six recombinant promoters constructed by linking regulatory regions of ovalbumin and other oviduct-specific genes. (A) Chicken oviduct expression genes (958 bp of *TF*, 977 bp of *OVOA*, and 1,468 bp of *OVALX*) were linked upstream of the 2.8-kb ovalbumin promoter. (B) In addition, 1,126 bp of *pTF*, 857 bp of *pLYZ*, and 1,350 bp of *pOVM* were linked downstream of the 1.6-kb ovalbumin ERE. Nucleotide sequences are denoted based on the translation start site (ATG) as a +1. Abbreviations: LYZ, lysozyme; OVOA, ovomucin alpha subunit; OVALX, ovalbumin-related protein X; OVM, ovomucoid; TF, ovotransferrin.

**Table 1.** Prediction of putative estrogen receptor alpha-binding sites.

Name	Sequences (5' to 3')	Start position	End position	Dissimilarity
1.6-kb ERE	TTGACCTGATA	-5,395	-5,385	0.000000
	GTGACCTGAGC	-5,268	-5,258	0.722887
	TTGACCTGTGA	-5,068	-5,058	0.000000
	CTGACCTTTTC	-4,916	-4,906	2.007569
	CTGACCTCAGT	-3,928	-3,918	4.617205
2.8-kb pOV	ACAGAGGTCAG	-2,568	-2,558	4.617205
	TAATAGGTCAC	-2,138	-2,128	3.734241
	TCAAAGGTCAA	-1,735	-1,725	2.007569
TF	TTGACCTATTG	-4,505	-4,495	4.617205
	GTGACCTGACC	-4,494	-4,484	2.328738
OVOA	GTGACCTGGCA	-4,711	-4,701	3.934590
OVALX	CAATAGGTCAA	-4,156	-4,146	4.617205
	CTGACCTCTTT	-4,389	-4,379	4.617205
pTF	CTGACCTGTAG	-3,763	-3,753	3.211703
	ATGACCTATGA	-3,107	-3,097	4.457128
pLYZ	CGTTAGGTCAT	-150	-140	7.668831
pOVM	-	-	-	-
	CTGGAGGTCAG	-186	-176	7.828908

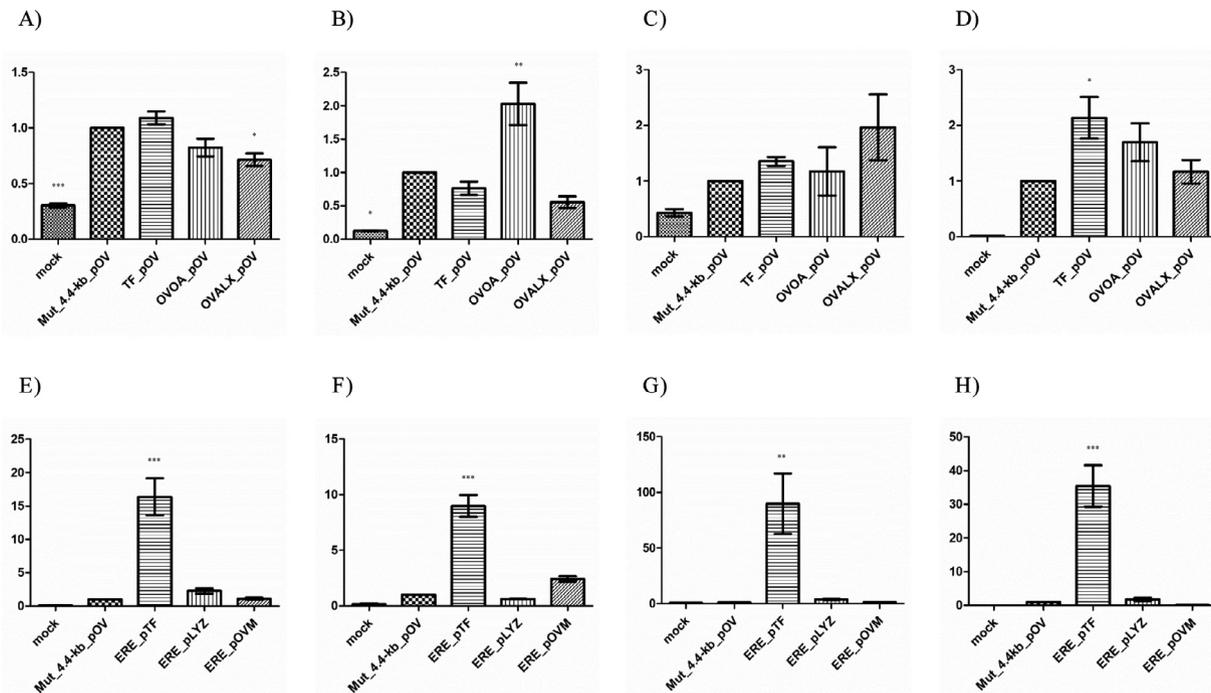
Nucleotide sequences are denoted based on translation start site (ATG) as a +1.

to have the potential to efficiently drive transgene expression, and these constructs were selected for additional evaluation of promoter activity using the dual luciferase assay in cultured primary cOECs.

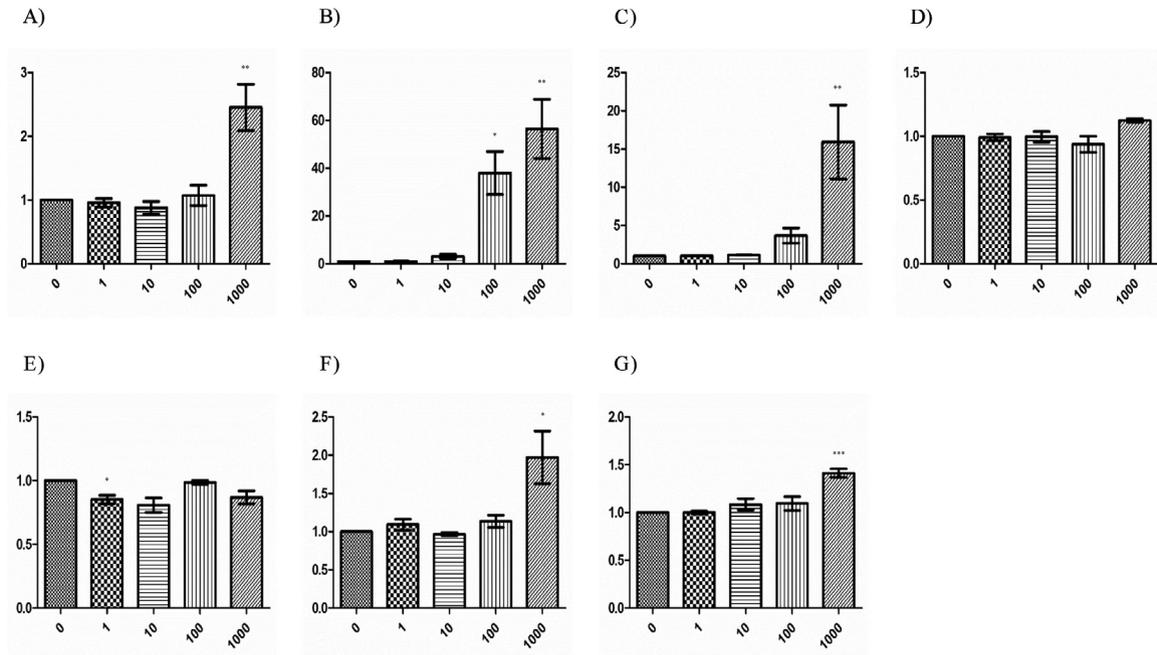
### Activity of the Recombinant Chicken Promoters in Chicken Oviduct Cells

To additionally evaluate the relative promoter activity of the recombinant promoters, *TF\_pOV*, *OVOA\_pOV*, *ERE\_pLYZ*, and *ERE\_pOVM*, we isolated and

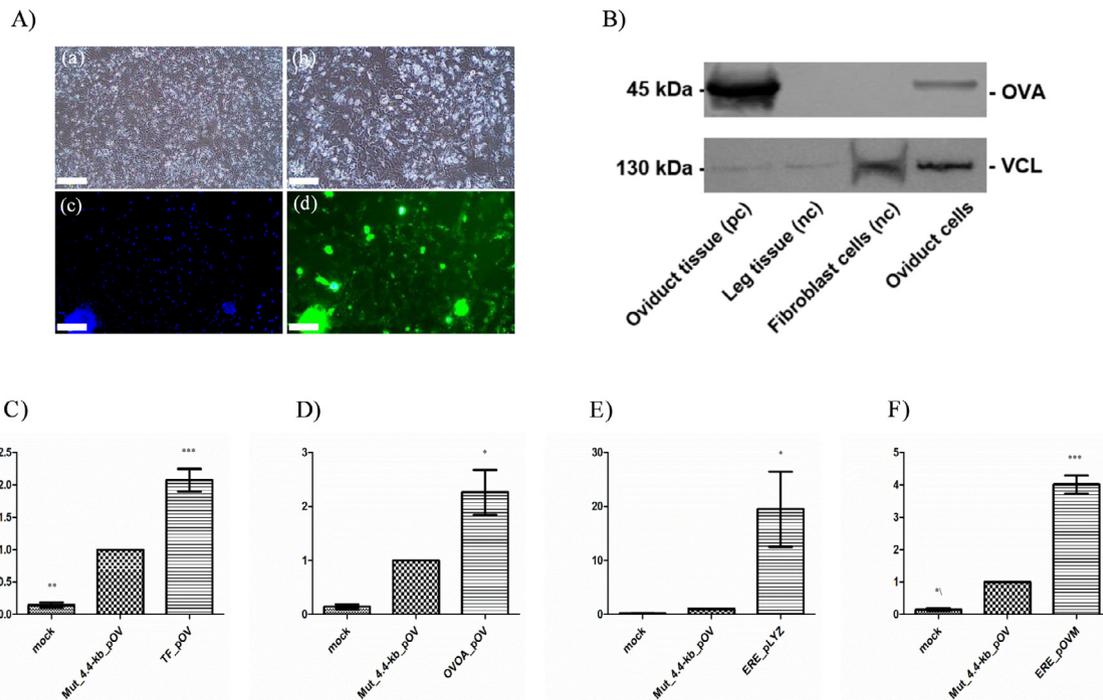
cultured primary chicken oviduct cells from a 35-wk egg-laying hens, and the cells were confirmed to express ovalbumin protein using immunofluorescence (Figure 5A). Western blot analysis indicated that chicken ovalbumin protein was clearly detected in the cOECs and oviduct tissue but not in chicken leg tissue or chicken embryonic fibroblast cells (Figure 5B). Each reporter vector was cloned with *Mut\_4.4-kb\_pOV* and four recombinant promoters (*TF\_pOV*, *OVOA\_pOV*, *ERE\_pLYZ*, and *ERE\_pOVM*) were transfected into the cOECs. A dual luciferase assay was performed to compare relative promoter activities. Comparative luciferase analysis showed



**Figure 3.** Relative luciferase ratio of recombinant promoters in human and chicken somatic cells. Each reporter vector cloned with a control and the six recombinant promoters was transiently transfected into HeLa cells (A, E), MES-SA cells (B, F), cEFs (C, G), and LMH/2A cells (D, H). Luciferase assays were performed 24 h post-transfection. Data represent relative values obtained with *Mut\_4.4-kb\_pOV*. The normalized luciferase value was calculated as firefly luminescence/Renilla luminescence ratio, and the relative luciferase ratio was calculated as recombinant promoter luciferase ratio/*Mut\_4.4-kb\_pOV* luciferase ratio. Error bars indicate the mean  $\pm$  SEM (n = 3). \*  $P < 0.05$ , \*\*  $P < 0.01$ , and \*\*\*  $P < 0.001$  compared to *Mut\_4.4-kb\_pOV*.



**Figure 4.** Estrogen responsiveness of recombinant promoters following estrogen treatments in LMH/2A cells. Each reporter vector cloned with a control and the six recombinant promoters was transiently transfected into LMH/2A cells followed by treatment with 0, 1, 10, 100, and 1,000 nM estrogen 24 h post-transfection. Luciferase assays were performed 24 h after estrogen treatments. Data represent the relative values obtained with 0 nM (non-E2) estrogen treatment in *Mut\_4.4-kb\_pOV* (A), *TF\_pOV* (B), *OVOA\_pOV* (C), *OVALX\_pOV* (D), *ERE\_pTF* (E), *ERE\_pLYZ* (F), and *ERE\_pOVM* (G). The normalized luciferase value was calculated as firefly luminescence/Renilla luminescence ratio, and the relative luciferase ratio was calculated as estrogen treatment luciferase ratio/non-estrogen treatment luciferase ratio. Error bars indicate the mean ± SEM (n = 3). \*  $P < 0.05$ , \*\*  $P < 0.01$ , and \*\*\*  $P < 0.001$  compared to 0 nM estrogen treatment.



**Figure 5.** Characterization of chicken oviduct cells and relative luciferase ratio of the recombinant promoters in cells. (A) Primary chicken oviduct epithelial cells were cultured. Cell morphology at passage 1 (a, b) and immunofluorescence images for nuclei (c) and ovalbumin (d) at passage 2 are presented. Scale bars: 100  $\mu\text{m}$ . (B) Western blot analysis of oviduct epithelial cells. Chicken oviduct magnum (pc, positive control), chicken leg muscle tissue and DF-1 (nc, negative control) are shown. Ovalbumin (OVA) and vinculin (VCL) proteins were used as oviduct-specific markers and loading controls. (C–F) Relative luciferase ratios of the *TF\_pOV* (C), *OVOA\_pOV* (D), *ERE\_pLYZ* (E), and *ERE\_pOVM* (F) recombinant promoters in oviduct cells are shown. Luciferase assays were performed at 24 h post transfection. Data represent the relative values obtained with *Mut\_4.4-kb\_pOV*. The normalized luciferase value was calculated as firefly luminescence/Renilla luminescence ratio, and the relative luciferase ratio was calculated as recombinant promoter luciferase ratio/*Mut\_4.4-kb\_pOV* luciferase ratio. Error bars indicate the mean ± SEM (n = 3). \*  $P < 0.05$ , \*\*  $P < 0.01$ , and \*\*\*  $P < 0.001$  compared to *Mut\_4.4-kb\_pOV*.

that *TF\_pOV*, *OVOA\_pOV*, *ERE\_pLYZ*, and *ERE\_pOVM* had 2.1- ( $P < 0.001$ ), 2.3- ( $P < 0.05$ ), 19.5- ( $P < 0.05$ ), and 4.0-fold ( $P < 0.001$ ) higher activity than the control in the cOECs, respectively (Figure 5C–5F). Consequently, recombinant promoters linking ovalbumin and *TF*, *OVOA*, *pOVM*, and *pLYZ* regulatory regions showed higher or similar levels of promoter activity in somatic cells and higher levels of estrogen response in LMH/2A cells. These constructs also showed significantly higher promoter activity in chicken oviduct cells. These results confirmed that reconstitution with regulatory regions of ovalbumin and other oviduct-specific genes contributed to an efficient driving of transgene expression in chicken oviduct cells.

## DISCUSSION

The present study newly developed recombinant chicken promoters, and these recombinant promoters were evaluated in human and chicken somatic cells and cultured primary chicken oviduct cells with a dual luciferase assay. Virus injection into EGK-X chickens and quail embryos and the transfer of primordial germ cells (PGCs) into blood vessels are widely used in avian transgenesis (van de Lavoie et al., 2006; Lee et al., 2019). Although PGC-mediated transgenesis requires establishment via isolation, the in vitro culture, characterization, modification, selection and transfer of PGCs, virus injection into blastoderm cells is a relatively simple process.

The recombinant viral particles used for injection to generate transgenic chickens that produce recombinant proteins are constructed using packaging and transfer plasmids, including a transgene and promoter. The packaged viral particles are injected into EGK-X embryos. Several types of ubiquitous promoters, such as cytomegalovirus,  $\beta$ -actin, rous sarcoma virus, and human phosphoglycerate promoter, were reported in viral vector constructs (Woodfint et al., 2018). Although the constitutive promoter CMV induced a high concentration of human erythropoietin (hEPO) in chicken blood, pathophysiological abnormalities such as reddish appearance, vasodilation, and death at an early stage, occur (Koo et al., 2017). However, these unfavorable phenotypes were improved by driving hEPO under the control of the chicken ovalbumin promoter (Kwon et al., 2018). Accordingly, oviduct-specific promoters for chicken bioreactors may be a solution to uncontrolled and unexpected side effects.

SDREs, NREs, and EREs are located in the 5' -flanking regions of the ovalbumin transcription start site. The chicken ovalbumin promoter has a translation start site (ATG) at the beginning of exon 2, and the 2.8-kb ovalbumin promoter consists of SDRE, NRE, exon 1, intron 1, and the beginning of exon 2. The use of this 2.8-kb ovalbumin promoter with a 0.7- to 1.6-kb ERE induced higher expression levels of transgenes (Lillico et al., 2007; Cao et al., 2015; Herron et al., 2018) compared to our previous study, in which green

fluorescence protein or extracellular human superoxide dismutase was driven by ovalbumin promoters without intron 1 (Byun et al., 2011; Byun et al., 2013). The 6 recombinant promoters in the present study were constructed based on these regulatory elements and had a more compact size (184- to 1,928-bp) than *Mut\_4.4-kb\_pOV*, which may enable a wider selection of a target gene and efficient processing for lentivirus packaging.

The relative luciferase activity ratio under the control of the 6 recombinant promoters was analyzed in HeLa cells, MES-SA cells, cEFs, LMH/2A cells, and cOECs compared to *Mut\_4.4-kb\_pOV*. HeLa and MES-SA cells are somatic epithelial cells from the human cervix and ovarian cancer, respectively, and these cells were used to perform a comparative analysis of promoter activity between recombinant promoters and the control in somatic epithelial cells. Chicken embryonic fibroblast cells (cEFs), were used to analyze cell-specificity. LMH/2A was used to perform the comparative analysis of promoter activity in somatic epithelial cells and investigate estrogen responsiveness under the control of all promoters following estrogen treatments. The recombinant promoters shared one promoter organization, such as the 2.8-kb ovalbumin promoter or 1.6-kb ovalbumin ERE. Therefore, their ability to induce luciferase activity was expected to be similar in somatic cells. However, there were significant differences according to the promoter type and somatic cells type.

*TF\_pOV* and *OVOA\_pOV* had significantly higher expression levels in LMH/2A cells, and moderate expression levels in HeLa cells in the present study. *OVOA\_pOV* showed significantly higher expression levels in MES-SA cells. The results showed that these regulatory element of 958 bp of *TF* and 1,002 bp of *OVOA* effectively induced luciferase gene expression in somatic epithelial cells compared to DHS III in the control. However, *OVALX\_pOV* was a less effective organization than *TF\_pOV* and *OVOA\_pOV*, due to its weak performance. *ERE\_pLYZ* and *ERE\_pOVM* also showed no large differences, but *ERE\_pTF* showed significantly higher activity in the cells, especially 89.8-fold in cEFs compared to the control. Because *ERE\_pTF* was thought to have lost tissue-specific regulation, it was excluded from subsequent validation in oviduct cells. As a result, significant differences in luciferase expression levels may be caused by interactions between cell-specific transcription factors and nucleotide sequences in regulatory regions.

LMH/2A cells are a chicken liver epithelial cell line that originated from the parental LMH cell line established by the integration of the chicken ER alpha expression vector (Binder et al., 1990), and ovalbumin promoters function in chicken embryonic hepatocytes (Dierich et al., 1987). Therefore, the LMH/2A cell line was additionally used to validate the estrogen response for all recombinant promoters. The recombinant promoters in this study were designed to have at least one ER alpha binding site, as predicted with PROMO 3.0 (Messegueur et al., 2002). The reconstructed ovalbumin promoter (*Mut\_4.4-kb\_pOV*) clearly showed an

## REFERENCES

increase after estrogen treatment. *TF\_pOV*, *OVOA\_pOV* and *OVALX\_pOV*, which consist of a 2.8-kb ovalbumin promoter, showed different estrogen responsiveness, which indicates that regulatory regions, such as *ERE*, *TF*, *OVOA*, or *OVALX*, may determine the estrogen effect. However, *ERE\_pTF* did not show a sufficient increase with estrogen treatment, despite small increases in *ERE\_pLYZ* and *ERE\_pOVM*. To the best of our knowledge, this discordance of results may be affected by a misguided prediction of ER alpha binding sites in regulatory regions or accidental inhibitory effects of the reconstitution of nucleotide sequences from different genes.

Chicken ovalbumin genes are specifically expressed and regulated in chicken oviduct tissue. Therefore, the newly developed ovalbumin promoters should be validated in chicken oviduct cells. The isolation, in vitro culture, and characterization of cOECs were reported recently (Jung et al., 2011; Kasperczyk et al., 2012; Stadnicka et al., 2018). We also previously established the isolation and in vitro culture of these cells, and the cells were maintained at least until passage 5 (Yang et al., 2021). The *TF\_pOV*, *OVOA\_pOV*, *ERE\_pLYZ*, and *ERE\_pOVM* recombinant promoters showed 2.1- to 19.5-fold higher luciferase activity than the control in the present study, but increases in luciferase activity following estrogen treatments were not confirmed (data not shown). The long-term in vitro culture of the cells remains challenging. Therefore, stably established chicken oviduct cell lines are required to deeply investigate oviduct cell biology and its applications.

Synthetic recombinant promoters linking regulatory regions of ovalbumin and other oviduct-specific genes, such as *TF*, *OVOA*, *LYZ*, and *ovomucoid*, efficiently drove higher transgene expression in chicken oviduct cells. In conclusion, *TF\_pOV* and *ERE\_pLYZ* were expected to be used to generate lentivirus particles, and application in further avian transgenic studies. These findings provide an understanding of the potential to reconstitute chicken oviduct-expressing genes and produce recombinant proteins, such as pharmaceuticals, in transgenic chickens.

## ACKNOWLEDGMENTS

This work was supported by the National Institute of Animal Science (Grant No. PJ01260301), Rural Development Administration (RDA), Republic of Korea.

## DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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