***In vivo* manipulation of foreign gene expression by steroid administration in the oviduct of laying hens**

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Abstract

The experiments described herein were conducted to examine whether or not steroid administration allows *in vivo* induction of foreign gene expression in the oviduct of laying hens. The chloramphenicol acetyltransferase reporter gene driven by several viral and cellular promoters with or without steroid response elements was transfected by *in vivo* electroporation. The results indicated that *in vivo*, as observed *in vitro*, steroid administration induced transcriptional activities of the promoters with steroid response

elements but it did not do so without steroid response elements. Our data implicate, therefore, that *in vivo* induction of foreign gene expression is possible in the oviduct of laying hens, and that the present *in vivo* gene transfer approach would serve as a useful tool to elucidate the mechanism of tissue-specific and steroid-induced transcription of chicken egg white genes.

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Introduction

In the magnum segment of the chicken oviduct, several egg white proteins, including ovalbumin (Ov) and conalbumin, are synthesized and secreted in large amounts under the control of steroid hormones. Therefore, the chicken oviduct is an attractive model to study the mechanism of transcriptional regulation of tissue-specific and steroid-induced gene expression in higher eukaryotes (Sanders & McKnight 1985).

In the past, most of the studies on the transcriptional mechanism of egg white protein genes such as the Ov gene have been carried out with cell lines (Muramatsu & Sanders 1995) or primary cultures of the oviduct cells from estrogen-stimulated immature chicks (Kato *et al.* 1992, Burbach *et al.* 1994, Muramatsu & Sanders 1995). However, no studies have been conducted to elucidate the role of such regulatory elements in oviduct cells of laying hens in which they originally function. There are at least two major reasons for this: (1) the cultured oviduct cells of laying hens were almost completely unresponsive to hormonal stimuli whereas those from immature chicks were responsive (Muramatsu *et al.* 1995), and (2) DNA transfection in the cultured oviduct cells of laying hens was not possible by popular means such as calcium phosphate precipitation or lipofection.

Recently, it has become possible to transfer DNA in tissues of living animals by various nonviral means such as microparticle bombardment (Yang *et al.* 1990, Jiao *et al.* 1993, Muramatsu *et al.* 1998a), lipofection (Thierry *et al.* 1995, Muramatsu *et al.* 1996a) and electroporation (Heller

et al. 1996, Muramatsu *et al.* 1996a,b, Nishi *et al.* 1996). Of these, *in vivo* gene electroporation has been more successful than any other nonviral means of transferring DNA into target tissues (Muramatsu *et al.* 1996a, 1997b). Ochiai *et al.* (1998) applied this *in vivo* electroporation technique to transfer the human erythropoietin gene into the oviduct cells of laying hens, and successfully expressed the corresponding recombinant protein albeit in a small amount.

Although nonviral *in vivo* gene transfer becomes feasible as stated above, it is not yet known whether or not the expression of foreign genes transfected *in vivo* can be controlled. The present study was conducted, therefore, to examine whether or not the expression of transfected foreign genes could be induced by the administration of steroid hormones when steroid responsive promoters were used.

Materials and Methods

All animal experiments described here were conducted in accordance with the Guidelines for the Care and Use of Experimental Animals, laid down by the Committee of Experimental Animals Care, Nagoya University, Nagoya, Japan.

Plasmid

The plasmid, pmiwLuc (Muramatsu *et al.* 1997b), was made by substituting the firefly luciferase (Luc) gene for

the bacterial lacZ gene of pmiwZ which was obtained from the Japanese Cancer Research Resources Bank (No. VE052), Osaka, Japan. The plasmid DNAs, pSVCAT and pSVLuc containing the SV40 early promoter (SV), were obtained commercially (Promega, Madison, WI, USA). The transfected reporter plasmid DNAs, pOvCAT100 and pOvCAT900 contained the Ov gene 5' upstream promoter of two different lengths, i.e. 100 bp (−87 to +9) and 900 bp (−900 to +9) respectively, fused upstream to the chloramphenicol acetyltransferase (CAT) reporter gene. These two plasmid DNAs were kindly provided by Dr M M Sanders (University of Minnesota, Minneapolis, USA). The CAT reporter plasmid DNAs driven by virus promoter pMTVCAT containing mouse mammary tumor virus-long terminal repeat (MMTV-LTR) was also kindly provided by Dr M M Sanders. The pRSVCAT containing the Rous sarcoma virus (RSV)-LTR was kindly provided by Dr H Kondou (Osaka University, Osaka, Japan). Both pOvgal100 and pOvLuc100 containing the Ov 100 bp (−87 to +9) promoter fused upstream to the bacterial β-galactosidase gene and firefly luciferase gene respectively, were constructed by substituting these reporter genes for the CAT gene of pOvCAT100.

In vivo gene electroporation

White Leghorn laying hens (Aichi line) at 18 months of age were used, and lightly anesthetized with diethyl ether. The abdominal cavity was opened surgically, and the magnum portion of the oviduct was exposed. An incision approximately 3 cm long was made longitudinally in the exposed magnum portion. Into one of the oviduct mucosal folds filled with glandular cells on the mucosa side, 50 µl DNA solution containing 20 µg pRSVCAT or equal moles of other plasmids were carefully injected together with pOvLuc100 at 5 µg to correct for transfection efficiency. After injection, electric square pulses were applied 8 times at 50 V/5 mm for the loading period of 50 ms/pulse with an electro-square porator T820 (BTX, San Diego, CA, USA). Pulses were administered to the tissue using a tweezers type electrode. Usually, 4–6 DNA transfers could be carried out in different mucosal folds per bird. After 24 h, the hens were killed and the oviduct portions were removed quickly.

Bioluminescence imaging

The tissue portion transfected with pSVLuc or pSVCAT was washed with phosphate buffered saline (137 mM NaCl, 2.68 mM KCl, 8.08 mM Na₂HPO₄, pH adjusted to 7.0), and transferred to a Petri dish to which 2 ml luciferase substrate buffer (Toyo Ink Co. Ltd, Tokyo, Japan) were added. Bioluminescence was monitored for 10 min.

Cell culture

Primary cultures of chicken oviduct cells were carried out as described previously (Sanders & McKnight 1985, 1988). The DNA transfection with calcium phosphate was carried out as described elsewhere (van der Eb & Graham 1980) immediately after dissociation since the timing of DNA transfection is of crucial importance. In general, the earlier the transfection, the better the steroid induction obtained (Muramatsu *et al.* 1997a). In combination with the CAT reporter plasmids, the pOvgal100 plasmid was co-transfected at 3.5 µg to correct for transfection efficiency. After the transfection, the cells were aliquoted into culture dishes containing the serum-free culture medium and 50 ng/ml insulin with or without steroid hormones (17β-estradiol at 2×10^{-7} M, and corticosterone at 1×10^{-6} M), and cultured at 39 °C for 30 h. The cells were then harvested.

Assays

The *in vitro* transfected and cultured oviduct cells were sonicated, and centrifuged at $1000 \times g$ for 10 min. The cell extract was used for measurements of β-galactosidase and CAT activities. CAT activity was determined as described previously (Sanders & McKnight 1985, 1988), and the values were expressed as relative CAT activity after the adjustment for β-galactosidase activity in cultured cells to take account of changes in transfection efficiency. The β-galactosidase activity was determined fluorometrically as described in the literature (Sambrook *et al.* 1989). The *in vivo* transfected oviduct samples were transferred to a 5 ml centrifuge tube to which 10 vol ice-cold buffer A (containing 15 mM Tris, 60 mM KCl, 15 mM NaCl, 12 mM EDTA, 1 mM dithiothreitol, 0.15 mM spermine and 0.4 mM phenylmethylsulfonyl fluoride, pH adjusted to 7.6) (Pothier *et al.* 1992) were added. After homogenization and sonication, the homogenate was centrifuged at $1000 \times g$ for 10 min. The supernatant was transferred to a new tube, and an aliquot of 20 µl was used for the luciferase assay. The remaining cell extract was heated at 70 °C for 3 h to inactivate endogenous acetylases, and centrifuged at $1000 \times g$ for 10 min. The resultant supernatant was used for the CAT assay as in the *in vitro* experiment. The values were expressed as relative CAT activity after the adjustment for luciferase activity to take account of changes in transfection efficiency. The luciferase activity was determined by using a Pica Gene kit (Toyo Ink Co. Ltd) according to the instructions given by the manufacturer.

Statistical analysis

Usually the same experiments were repeated at least twice, and analysis of variance was carried out on the pooled data (Snedecor & Cochran 1980). Where necessary,

Figure 1 Activity of firefly luciferase expressed *in vivo* in the oviduct of laying hens. *In vivo* DNA transfection was carried out by electroporation with 20 µg pmwLuc at different voltages. Luciferase activities were measured 24 h after the transfection. Results are means $\pm$ S.E.M. of 3 or 4 replicates. RLU, relative light units. ^{a,b}Means not sharing a common letter are significantly different at $P < 0.05$.

normalized CAT activities were transformed to their logarithmic values to stabilize error variance prior to the analysis. Significance of differences between means

was inspected by Duncan's multiple range test using a commercially available statistical package (SAS 1985).

Results

Voltage optimization of *in vivo* gene electroporation

In the first experiment, voltage optimization of *in vivo* gene electroporation was attempted. The results showed that the value for luciferase gene expression (relative light units/mg tissue) was significantly increased by raising voltages from 25 to 50 V, and decreased substantially from 50 to 75 V (Fig. 1). Therefore, the optimal voltage was considered to be 50 V under the present experimental conditions, and this voltage was used throughout the subsequent *in vivo* experiments.

Bioluminescence imaging

In the following experiment, 10 µg pSVLuc or pSVCAT were similarly transfected by *in vivo* gene electroporation in the oviduct of laying hens. Twenty-four hours after the transfection, bioluminescence imaging was carried out by using the single photon imaging system (Argus-50/2D, Hamamatsu Photonics, Hamamatsu, Japan). The result of photon imaging is shown in Fig. 2. Significant bioluminescence, the intensity of which is shown in the right column ranging from red (strong) to dark blue (weak), was

Figure 2 Bioluminescence imaging of the chicken oviduct. Either pSVLuc or pSVCAT was transfected *in vivo* in the chicken oviduct by electroporation at 10 µg. Intensity of bioluminescence detected by the single photon imaging system was indicated by the right column ranging from red (strong) to dark blue (weak).

observed only in the tissue portion transfected with the Luc reporter gene (pSVLuc), but not with CAT (pSVCAT) or the (Control) reporter gene.

Induction of transcriptional activity in the oviduct of laying hens in vivo by steroid treatment

To examine whether or not steroid administration induces transcription of foreign genes driven by viral and cellular promoters with or without steroid response elements, steroid hormones were injected subcutaneously around the neck for 3 consecutive days. The amounts of each steroid hormone were chosen to give equivalent (in total of 1.4×10^{-4} mol corticosterone; experiment 3), $\times 1$ (in total of 2.7×10^{-5} mol 17β -estradiol and 1.4×10^{-4} mol corticosterone; experiment 4) or $\times 10$ (10-fold) concentrations (experiment 4) of those added to the culture medium in the *in vitro* study, provided that the injected steroids were evenly distributed within the body of a 1.7-kg laying hen. Subsequently, the three viral promoters, i.e. SV, MMTV-LTR and RSV-LTR were transfected *in vivo* into the oviduct of laying hens (experiment 3), and the two viral promoters, i.e. SV and MMTV-LTR, and two cellular promoters, Ov100 and Ov900, were transfected *in vivo* likewise (experiment 4).

The values for relative CAT activity and fold induction by the corticosterone administration in experiment 3 are shown in Fig. 3 (panels A and B). In both the SV and RSV-LTR promoters, no *in vivo* induction in CAT values was found in the presence of corticosterone, whereas a substantial *in vivo* induction was detected in the MMTV-LTR promoter in the presence of this hormone.

The values for relative CAT activity in experiment 4 are given in Fig. 4 (panel A). The transcriptional activities of MMTV-LTR and Ov900 were significantly increased as reported in the *in vitro* experiment with steroid hormones (Ringold 1983, Otten *et al.* 1988, Haecker *et al.* 1995), although the extent of steroid induction in the MMTV-LTR and Ov900 appeared to be slightly weaker in experiment 4 than in experiment 3, approximately four to five times at the 10-fold concentration (Fig. 4, panel B). As was expected, no steroid induction was detected *in vivo* in the SV and Ov100 promoters.

Transcriptional activity in the in vitro oviduct cell culture system

Finally, to confirm the steroid induction of transcriptional activity of these viral and cellular promoters *in vitro*, the plasmid, pSVCAT, pMMTVCAT, pOvCAT100 or pOvCAT900 was transfected in primary cultures of oviduct cells from steroid-stimulated immature chicks by calcium phosphate precipitation. The results are shown in Fig. 5, panel A, indicating that the MMTV-LTR which contains a glucocorticoid receptor binding site (von der Ahe *et al.* 1985) showed an increased transcriptional

Figure 3 Activity of CAT driven by viral promoters together with the extent of steroid induction in the oviduct of laying hens injected with corticosterone at 1.4×10^{-4} mol. (A) *In vivo* DNA transfection was carried out by electroporation with pSVCAT (SV), pMMTVCAT (MMTV) or pRSVCAT (RSV) at a dose of 20 μ g (pRSVCAT) or equal moles of other plasmids. In all samples, 5 μ g pOvLuc100 were co-transfected as an internal control to normalize transfection efficiency. Relative CAT activity was measured 24 h after the transfection. Results are means $\pm$ S.E.M. of 4 replicates. ^{a,b,c}Any two means not sharing a common letter are significantly different at $P < 0.05$. (B) The extent of steroid induction was calculated from relative CAT values with steroid hormones divided by the corresponding ones without steroid hormones, and shown in terms of fold induction.

activity in the presence of corticosterone. The extent of induction is illustrated more clearly in Fig. 5, panel B. The Ov100 and Ov900 promoters had roughly the same levels of relative CAT activity with steroid hormones. However,

without steroid hormones, transcriptional activity of the Ov900 promoter was remarkably attenuated. Thus, as reported previously (Sanders & McKnight 1985, Haecker *et al.* 1995), steroid-induced gene expression was confirmed in the oviduct cells from steroid-stimulated immature chicks cultured *in vitro*, and the induction responses were analogous to those found *in vivo* in the oviduct of living layers.

Discussion

The experiments described herein suggest that steroid hormone administration induces foreign gene expression *in vivo* in the oviduct of laying hens when steroid responsive promoters are used (Figs 3 and 4). The extent of induction in the oviduct *in vivo* was found to be roughly the same as that in the oviduct cells cultured *in vitro*

(Fig. 5). For *in vivo* manipulation of gene expression in the present study, the MMTV-LTR and corticosterone were employed for the promoter and steroid hormone respectively in experiment 3, as the glucocorticoid-stimulated transcription through multiple glucocorticoid receptor binding sites in the MMTV-LTR has been well known (Ringold 1983, Otten *et al.* 1988). The Ov100 and Ov900 promoters were also employed in addition to the MMTV-LTR in experiment 4 because Ov900 contains negative regulatory element and steroid dependent response element in the 5'-flanking region which are known to confer responsiveness to steroidal stimuli (Haecker *et al.* 1995), but Ov100 does not have these coordinated steroid response elements.

The basal gene expression level conferred by the MMTV promoter in the absence of exogenous corticosterone was lower than those conferred by the SV40 and RSV promoters (Figs 3 and 4). Although this particular viral promoter retains some steroid inducible activity *in vivo* in the oviduct of laying hens, there may not be sufficient transcription factors to interact with the glucocorticoid response elements and other regulatory elements in oviduct glandular cells. In the absence of steroid hormones, the cellular promoters, i.e. Ov100 and especially Ov900, showed weaker transcriptional activity than did the SV40 promoter. However, this is not surprising since in an episomal form which would account for most transfected genes by the *in vivo* electroporation technique, gene transcriptional activity as high as that of endogenous levels is not always attained. Lack of some important factors associated with integrated genes such as higher order structure of DNA, or elements like locus control region and matrix attachment elements (Muramatsu & Sanders 1995) might be responsible for the poor foreign gene expression.

In vivo gene transfer by utilizing electroporation in the present study can be conducted relatively simply and

Figure 4 Activity of CAT driven by viral promoters together with the extent of steroid induction in the oviduct of laying hens injected with steroid hormones. Steroid hormones at two concentrations, i.e. $\times 1$ (in total of 2.7×10^{-5} mol 17β -estradiol and 1.4×10^{-4} mol corticosterone) and $\times 10$ (10 times the $\times 1$ concentration) were injected subcutaneously for 3 consecutive days. On the last day of steroid injection, CAT reporter plasmids were transfected *in vivo* in the oviduct. (A) *In vivo* DNA transfection was carried out by electroporation with pSVCAT (SV), pMMTVCAT (MMTV), pOvCAT100 (OV100) or pOvCAT900 (OV900) at a dose of 20 μ g pMMTVCAT or equal moles of other plasmids. In all transfection samples, 5 μ g pOvLuc100 were co-transfected as an internal control to normalize transfection efficiency. CAT activities were measured 24 h after the transfection. Results are means $\pm$ S.E.M. of 4 replicates. (B) To calculate the extent of steroid induction, CAT values with steroid hormones were divided by those without steroid hormones, and shown in terms of fold induction. * $P < 0.05$ compared with the corresponding no steroid control. # $P < 0.05$ compared with the corresponding $\times 1$ -treated group.

Figure 5 Activity of CAT together with the extent of steroid induction in primary cultures of oviduct cells from steroid-stimulated immature chicks. (A) *In vitro* transfection was carried out by calcium phosphate precipitation with pSVCAT (SV), pMMTVCAT (MMTV), pOvCAT100 (Ov100) or pOvCAT900 (Ov900) at a dose of 20 µg pMMTVCAT per dish or equal moles of other plasmids. The cells were cultured with (+) or without (–) steroid hormones, i.e. 17β-estradiol and corticosterone at 2×10^{-7} M and 1×10^{-6} M respectively for 30 h. In all transfection samples, 3.5 µg pOvgal100 were co-transfected as an internal control to normalize transfection efficiency. Results are means $\pm$ S.E.M. of 5 or 6 replicates. (B) To calculate the extent of steroid induction, CAT values with steroid hormones were divided by those without steroid hormones, and shown in terms of fold induction. ^{a,b,c,d}Any two means not sharing a common letter are significantly different at $P < 0.05$.

quickly. For one bird, the entire procedure is completed within an hour including operation. The efficiency of gene transfection in the oviduct *in vivo* is generally higher than that in the oviduct cell cultures *in vitro*. By utilizing a bacterial β-galactosidase expressing plasmid construct, the heterologous oviduct cell population expressing the transgene ranged from approximately 20 to 30% of the total cells mainly consisting of glandular cells in a transfected

area of mucosal folds (H-M Park & T Muramatsu, unpublished results). Good transfection efficiency in the *in vivo* gene electroporation system was also substantiated by the fact that the detectable amount of human erythropoietin protein was produced *in vivo* but not in the cultured oviduct cells *in vitro* (Ochiai *et al.* 1998). The data in the mouse testis further support a better gene transfer efficiency by gene electroporation in the *in vivo* system than in *in vitro* cultures (Muramatsu *et al.* 1996c).

In addition to controllable gene expression, tissue- or cell-specific foreign gene expression is attainable by *in vivo* gene electroporation as shown in the mouse testis (Muramatsu *et al.* 1997b). This would be of particular importance if one wishes to express foreign genes only in specific cells in a specific tissue. For example, by comparing the pattern of foreign gene expression in different cells and tissues including the oviduct, one might even be able to identify tissue-specific elements in the Ov gene promoter region. Such inter-tissue comparisons cannot be easily done in the *in vitro* primary culture system as the establishment of optimal culture conditions requires time-consuming work, whereas in the *in vivo* circumstances tissues are *a priori* in the best 'culture' condition. This is perhaps one of the most useful applications of *in vivo* gene electroporation in studying the Ov gene transcriptional regulation.

For *in vivo* gene electroporation, optimization is an essential step as previous studies suggested that the highest gene expression was obtained at different voltages, number of electric pulses, and loading periods depending on target tissues and animals of interest (Muramatsu *et al.* 1998b). In the present study, only the voltage was varied (Fig. 1) since it was empirically known that the longer period of 50 ms with 8 pulses would cause serious irreversible tissue damage.

In the *in vitro* cell culture system with steroid hormones in the present study and the work of others (Sanders & McKnight 1985), the concentrations of supplemented estradiol and corticosterone used were about 1000-fold higher than physiological levels (Freeman 1984, Wells & Gilbert 1984). The *in vivo* experiments showed that a pharmacological concentration equivalent to or 10 times higher than the *in vitro* culture levels was required to cause the induction of transcriptional activities of MMTV-LTR and Ov900 promoters. This suprapharmacological dose of exogenous steroid hormones would disrupt normal ovulation and oviposition cycles in laying hens, suggesting that the induced gene expression may not necessarily reflect endogenous regulation.

The reason for the apparently higher dose needed *in vivo* remains unknown. It may possibly be due to the delay of hormone delivery from the injection site to the oviduct *in vivo*. Alternatively, because of universal gene transfer into the entire oviduct cell population *in vivo*, the proportion of steroid responsive cells, i.e. glandular cells, into which foreign genes were transfected may be low. This

would, in turn, result in suprapharmacological doses being required for the induction of gene expression *in vivo*. Moreover, in the *in vivo* situation there are endogenous steroid hormones in plasma so that oviduct glandular cells are already in an induced state with respect to steroid responsive gene promoters. In order to induce gene transcription to a further substantial extent, such high levels may be needed.

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