Biochemical and Biophysical Research Communications **292,** 88–93 (2002)
doi:10.1006/bbrc.2002.6604, available online at http://www.idealibrary.com on IDEAL®

Human Alkaline Phosphatase Expression and Secretion into Chicken Eggs after *in Vivo* Gene Electroporation in the Oviduct of Laying Hens

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Received February 4, 2002

***In vivo* gene electroporation was used to examine whether or not a recombinant protein is synthesized in the chicken oviduct and subsequently secreted into eggs. A plasmid DNA containing a secretion form of the human alkaline phosphatase gene was injected into mucosa of the chicken magnum. Immediately, *in vivo* gene electroporation was conducted. The human alkaline phosphatase activity in the oviduct mucosa increased and reached its peak at 2 days posttransfection, followed by a sharp decrease to a negligible level at 4 days posttransfection. In the egg white, the alkaline phosphatase activity showed a similar change to that in the magnum mucosa except for a delay of 4 days. The present results imply that *in vivo* gene electroporation method in the oviduct may serve as a rapid production system of recombinant proteins into chicken eggs.**

***Key Words:* human alkaline phosphatase; *in vivo* gene electroporation; oviduct; chicken egg.**

A number of researchers have long been attracted to produce chicken egg white that contains pharmaceutical proteins such as recombinant human interleukins, interferons and hormones. As these proteins are never synthesized naturally, foreign genes should be introduced and expressed in the chicken oviduct where egg white proteins are synthesized, and secreted into the oviduct lumen to form egg albumen. So far, however, no such attempt has been reported to use chickens as a pharmaceutical protein producer.

In mammalian species, extensive studies on the synthesis and secretion of pharmaceutical proteins into milk, saliva, blood and urine have been conducted by generating a variety of transgenic animal species (1). The pioneering and yet the most successful way for gene transformation in mammals is microinjection of transgenes into pronuclei of fertilized oocytes. In avian species, however, transgenic bird production has scantily been reported. Retroviral vectors succeeded for the first time in generating transgenic birds (2). Several years later, a microinjection method was used for gene transformation of this animal species (3). In spite of these efforts, the production efficiency of transgenic birds is still substantially poorer than that of mammalian species, although the use of retroposon in transgene constructs improved to a certain extent the production efficiency (4).

Apart from the above-established approaches for gene transformation, an alternative gene transfer and expression system has been proposed: somatic cells and tissues are directly subjected to *in vivo* foreign gene transfer. The gene expression by this system is usually confined to a localized area. Such localized *in vivo* gene transfer can be achieved by various viral and nonviral methods. Of these, *in vivo* gene electroporation (EP) is by far the most efficient and convenient means as far as nonviral localized *in vivo* gene transfer methods are concerned (5). Intensive gene expression by *in vivo* EP has been demonstrated in a broad spectrum of target tissues including mouse testis (6), mouse muscle (7, 8), mouse liver (9, 10), and pig muscle (11, 12).

Although *in vivo* gene EP technology has seldom been employed in avian species, its promising potential is beginning to emerge as shown in chicken embryos (13, 14), and testes of Japanese quail and chickens (15, 16). Application of *in vivo* EP to the oviduct of laying hens might serve as a new tool for the analysis of transcriptional mechanism and pharmaceutical protein production. In the oviduct of live laying hens, for example, Ochiai *et al.* (17) found that by *in vivo* gene EP, recombinant human erythropoietin was produced albeit at a small amount. Using *in vivo* gene EP, Park and Muramatsu (18) studied the nature of foreign gene

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0006-291X/02 $35.00

expression in the oviduct, and found that exogenous steroid hormone administration induced reporter gene expression *in vivo* when driven by promoters containing steroid response elements.

At present, gene expression levels attained by the oviduct gene EP are far from satisfactory for commercial protein production. A key to efficient recombinant protein production is how to enhance foreign gene expression. First, characterization of *in vivo* EP conditions is of crucial importance. In addition, some improvement in DNA constructs should be made for induced gene expression. In cultured cells, several methods are known to induce foreign gene expression. Kitajewski *et al.* (19) found, for example, that cotransfecting with a gene encoding adenovirus associated RNA induced the expression of foreign genes of interest. Transcriptional modulation of foreign gene expression has also been attempted with various nonnatural chemical compounds including tetracycline, rapamycine, and modified steroids that function as transactivators (20). Applications of some of these induction systems to *in vivo* conditions might help enhance the synthesis and production of recombinant proteins in the oviduct, and thereby in eggs laid.

In the present study, *in vivo* gene EP was used in the oviduct of laying hens to examine whether or not a recombinant protein is synthesized and subsequently secreted into eggs.

MATERIALS AND METHODS

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

Plasmid DNA. The plasmid, pmiwluc (6), was made by substituting the firefly luciferase gene for the bacterial lacZ gene of pmiwZ that was obtained from Japanese Cancer Research Resources Bank (No. VE052), Osaka, Japan. The cotransfection plasmid vector, pAdV having a gene encoding adenovirus associated RNA, and the reporter plasmid vector, pSVluc, were obtained commercially (pAdVAntage and pGL2 control respectively, Promega, Madison, WI). The construction of plasmid containing a secretion form of the firefly luciferase gene, pSVsecluc, was given elsewhere (21). The secretion form of human alkaline phosphatase (SEAP) gene was obtained commercially (pSEAP control, Clontech Laboratories, Palo Alto, CA).

In vivo EP procedures. A brief schematic presentation of *in vivo* gene EP in the oviduct is given in Fig. 1A. White Leghorn laying hens (Aichi Line) at 17 months of age were used, and anesthetized with diethyl ether. The abdominal cavity was opened surgically, and the magnum portion of the oviduct was exposed. An incision of approximately 3-cm long was made longitudinally in the exposed magnum portion. Into one of the oviduct mucosal folds filled with glandular cells, 50 μl DNA solution containing 30 μg pmiwZ (Experiment 1) and 10 μg pmiwluc (Experiments 2 and 3) was carefully injected. In Experiment 4, pmiwluc at 10 μg was injected with or without pAdV at 5 μg. For luciferase imaging, either pmiwluc at 150 μg, pSVsecluc at 30 μg, pSVluc at 30 μg, or pSVGal at 30 μg was injected together with pAdV at 10 μg (Experiment 5). In the secretion experiment (Experiment 6) in which 6 laying hens were used, pSEAP control was injected at 150 μg together with pAdV at 10 μg. Immediately after injection, electric square pulses of direct current were applied 6 times at 75 V unless otherwise stated. Square pulses with the loading period at 100 ms per pulse were applied with an electro-square porator CUY21 (NEPA Gene, Ichikawa, Japan) in combination with pincette-type electrodes (CUY641, Tokiwa Science, Tsukushino, Japan). The electrodes had one circular gold-plated steel of 10 mm in diameter on each end of forceps with separate electrical connection to the above electro-square porator. Three pulses were administered in row to the tissue, and another three pulses were applied by changing polarity of the electrodes through a switching device connected between CUY21 and the pincette-type electrodes.

Chemical analysis. At designated time points after *in vivo* gene EP, the hens were euthanized and the oviduct portions were removed quickly. The mucosa samples were transferred to a 5-ml centrifuge tube to which 10 volumes of 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) (22) were added. After homogenization and sonication, the homogenate was centrifuged at 8000*g* for 10 min. The supernatant was transferred to a new tube, and an aliquot of 20 μl was used for firefly luciferase assay. The luciferase and SEAP activities were determined by using a Pica Gene kit (Toyo Ink Co. Ltd., Tokyo Japan), and Great EscAPe SEAP Genetic Reporter System (Clontech Laboratories, Palo Alto, CA) according to the instruction given by the manufacturer. In Experiment 6, eggs were collected for 8 days post-transfection. For the first 4 days after EP, no eggs were laid, and for the following 4 days, 3 to 5 eggs were collected daily. Egg white was separated from egg shell and yolk, and homogenized with 10 volumes of ice-cold buffer A. The SEAP activity in the egg white was determined similarly as done in the mucosa.

For bioluminescence imaging, the tissue portions transfected with either pmiwluc, pSVluc, pSVsecluc or pSVGal were washed with phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 8.08 mM Na_2HPO_4, pH adjusted to 7.0). Then, they were transferred to a petri dish to which 2 ml luciferase substrate buffer (Toyo Ink Co. Ltd., Tokyo, Japan) was added. Bioluminescence was monitored with Argus 50 (Hamamatsu Photonics, Hamamatsu, Japan) for 10 min.

Statistical analysis. The data were treated statistically by a one-way analysis of variance. Where necessary, logarithmic transformation was done prior to analysis to stabilize error variance. Significance of differences was tested by Duncan's multiple range test by using a commercially available statistical package (SAS Institute, Cary, NC).

RESULTS

LacZ Expression

Figure 1B represents bacterial β-galactosidase gene expression in the target site of the oviduct. The site that showed specific blue color by X-gal staining was confined to a small area of approximately 100 μm wide and 200 μm long. Although gene expression in the tela submucosa or muscle layer was not clearly demonstrated, some mucosal cells lining toward the oviduct lumen appeared to express the lacZ gene driven by the miw promoter. The miw promoter, i.e., hybrid RSV LTR and chicken β-actin tandem promoter, was used since in the chicken oviduct, it was found to have stronger transcriptional activity than any other promoter tested (17).

Voltage Optimization

The results of voltage optimization of *in vivo* gene EP are shown in Fig. 2A. The value for luciferase gene

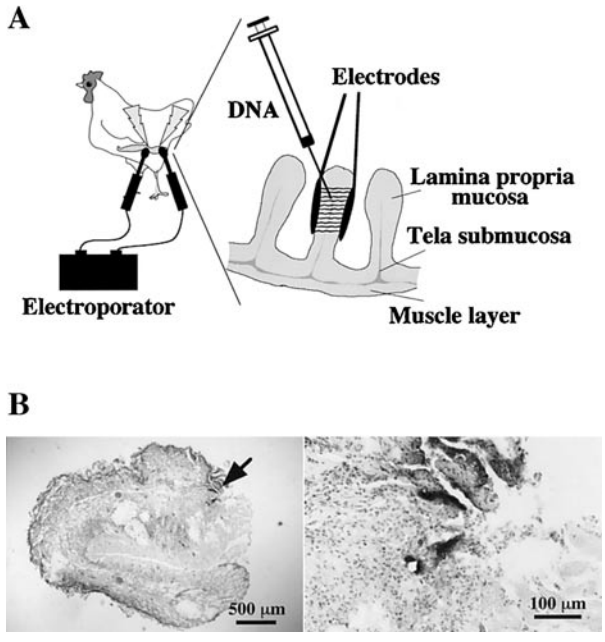

FIG. 1. Schematic presentation of *in vivo* gene electroporation into the oviduct of laying hens (A), and the results of Xgal staining of the magnum where a lacZ gene was transferred *in vivo* (B). The plasmid DNA containing the bacterial lacZ gene, pmiwZ, was transferred into oviduct by electroporation. At 24 h after electroporation, the transferred site was removed, fixed with 1% glutaraldehyde, and stained with X-gal for 1 h. Magnification is indicated by horizontal bars.

expression was significantly increased, almost 20-fold, by raising voltages from 25 to 75. Therefore, the optimal voltage was considered to be 75 V under the present experimental conditions, and this voltage was used throughout the subsequent *in vivo* experiments.

Time course of firefly luciferase activity is given in Fig. 2B. During the posttransfection period of 12 days, the luciferase activity rapidly declined for the first 4 days followed by a continuous, slow decrease until 12 days, and significant reduction was detected from 1 vs 4, and 4 vs 8 or 12 days posttransfection ($P < 0.05$). At 8 days of posttransfection or later, the foreign gene expression was negligible and virtually disappeared, suggesting that the gene expression in this tissue would not last long as reported in skeletal muscle (7, 8) or testis (6) of mice.

Effect of Adenovirus Associated RNA on Reporter Gene Expression

The effect of cotransfection of pAdV vector is shown in Fig. 2C. Luciferase activity at 2 days posttransfection was measured in the presence or absence of the pAdV vector. As shown clearly, the cotransfection with the pAdV vector significantly fortified luciferase activity by 2-fold when compared with the luciferase expression vector alone ($P < 0.05$). The result suggests that the adenovirus associated RNA induces reporter gene expression *in vivo* in the oviduct of laying hens as was expected from results with cultured cells (19).

Photon Imaging

The results of photon imaging analyses are given in Figs. 3A and 3B. In Fig. 3A, the firefly luciferase gene was transferred and expressed in an area of the mag-

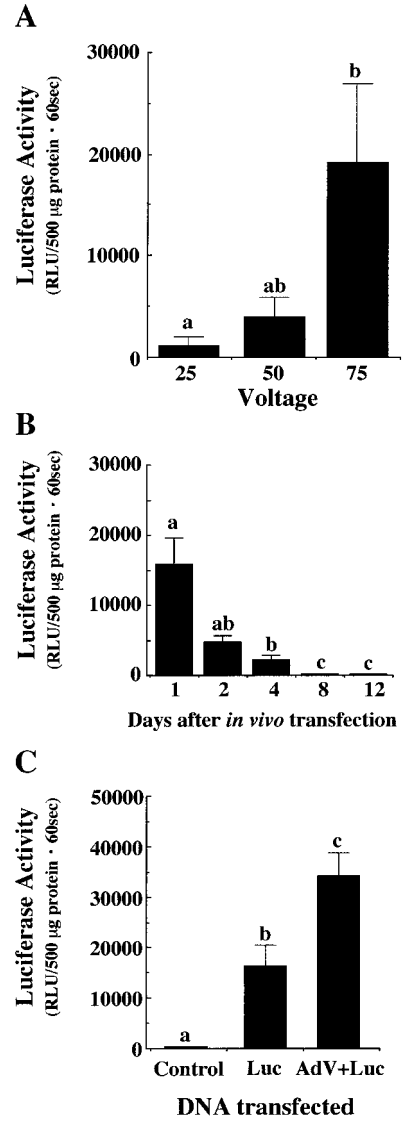

FIG. 2. Firefly luciferase activity in the oviduct of laying hens. (A) With varying voltages, *in vivo* gene electroporation was done. At 24 h after electroporation, firefly luciferase activity was measured. (a, b) Significantly different at $P < 0.05$. (B) Time course of firefly luciferase activity was measured after *in vivo* gene electroporation as in A except for a fixed voltage at 75 V. (a, b, c) Significantly different at $P < 0.05$. (C) Effect of cotransfer of pAdVantage (AdV) in combination with pmiwluc (Luc) on firefly luciferase activity in the oviduct of laying hens. At 24 h after electroporation, firefly luciferase activity was measured. Values are means ± SEM of 4 or 5 replicates. (a, b, c) Significantly different at $P < 0.05$.

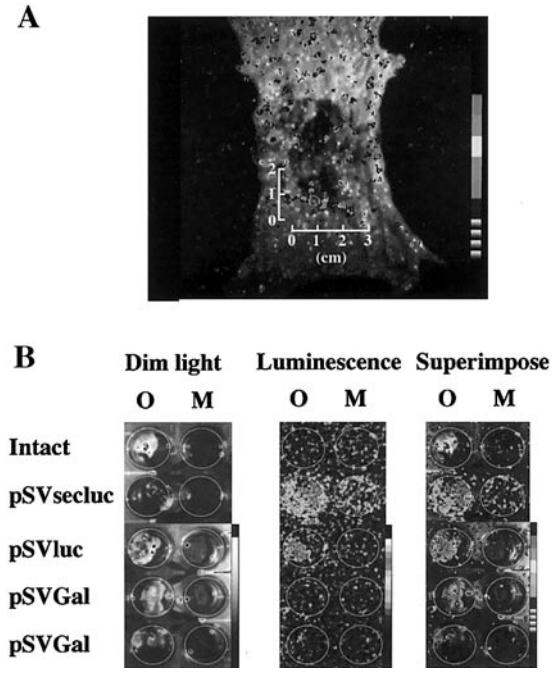

FIG. 3. Bioluminescence imaging analysis of the oviduct segment of laying hens. (A) Photon imaging was done on a large piece of the magnum. The plasmid DNA, pmiwluc, was transfected *in vivo* by electroporation. At 24 h after electroporation, the magnum segment including the transferred site was removed for bioluminescence imaging analysis. (B) Photon imaging was done on a small magnum segment and medium to examine luciferase secretion into the incubation medium. Three plasmid vectors, pSVsecluc, pSVluc, and pSVGal were transfected *in vivo* by electroporation. The transfected segment was removed at 24 h after electroporation, kept in a culture medium for 1 h, and then placed to a new petri dish. The incubation medium was filtered, and poured into a new dish adjacent to the magnum tissue. Abbreviations: O, transfected oviduct segment; M, incubated medium.

num segment, approximately 25 mm wide and 10 mm long. However, the expression was scattered and unevenly distributed, and its intensity was not consistent according to the color of intensity bar shown on the right. In Fig. 3B, photon imaging was done on both magnum segment and the medium in which the segment was placed for 1 h prior to direct imaging of the tissue segment. Strong bioluminescent signals were detected in the magnum segments from pSVluc- and pSVsecluc-transfected birds, while no signals were found in those from birds of any other experimental groups. In the medium, in contrast, clear, though weak, signals were detected only from the pSVsecluc-transfected hens. These results imply that this particular firefly luciferase was secreted from the glandular cells of the oviduct into the medium. However, since the luminescence intensity in the medium of the pSVsecluc was not strong, the secretion signal originated from bovine α-lactalbumin in the pSVsecluc gene (21) was considered to function poorly in the oviduct. Accordingly, instead of pSVsecluc, the commercially available secretion-type reporter gene, SEAP, was used in the last experiment.

SEAP Expression and Secretion into Egg White

Time course of SEAP activity in the magnum segment and egg white is shown in Fig. 4. In the magnum, a rapid and significant increase in SEAP activity was observed at 2 days posttransfection ($P < 0.05$) with a sharp decrease reaching almost the initial level at 4 days posttransfection. The values for egg white SEAP activity were expressed as means ± SEM of 3 eggs that showed positive chemiluminescence signals out of maximally 5 eggs collected. In egg white, essentially the same pattern was observed as that of the magnum segment, but there was a delay of 4 days: SEAP activity elevated rapidly at 6 days after *in vivo* electroporation ($P < 0.05$), and then decreased sharply afterwards within 2 days. During the 4-day delay period, no egg production was observed.

DISCUSSION

In the present study, we demonstrated not only the reporter protein synthesis in the oviduct of laying hens (Figs. 1B and 2), but also the luciferase protein secretion from the magnum segment (Fig. 3B). Moreover, the SEAP reporter protein appeared to be secreted and transported into egg white (Fig. 4). Calculation from the peak SEAP activity suggested that the maximum amount of SEAP protein detected in the egg white was approximately at 500 μg per egg on average. For the amount of SEAP protein detected in egg white, dose dependency on transferred DNA was suspected, at least at the peak secretion time point, but this was not

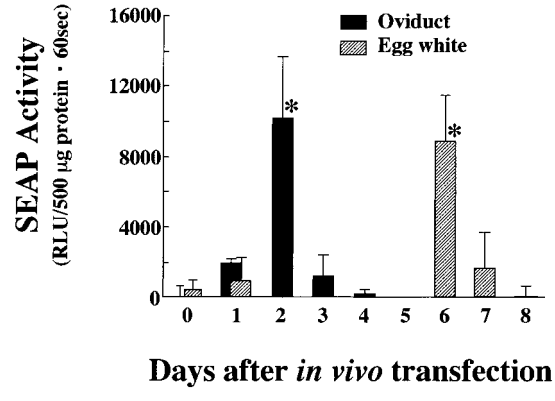

FIG. 4. Time course of human alkaline phosphatase (SEAP) activity in the oviduct of laying hens and egg white in eggs laid. The expression plasmid, pSEAP control, was transfected *in vivo* into the oviduct of laying hens by electroporation. At designated time points after electroporation, the transferred magnum segment was removed, and eggs laid were collected for measurement of SEAP activity. Values are mean ± SEM of 3 replicates. *Significantly different from the Day 0 value of the magnum segment at $P < 0.05$.

examined due to the limitation in the number of birds available.

To our knowledge, this is the first report in which a recombinant protein was synthesized and secreted into egg white after *in vivo* gene EP in the magnum of laying hens. Although successful synthesis of foreign proteins in the oviduct has been reported previously (17), no recombinant protein was detected in the egg white. Thus, the present results imply that *in vivo* gene EP method in the oviduct may serve as a rapid production system of recombinant proteins into chicken eggs.

The *in vivo* gene EP into the magnum of laying hens has a large potential to produce pharmaceutical proteins into chicken eggs, a sterilized package of proteins. The recombinant proteins are rapidly obtained within a matter of days. In this respect, the *in vivo* gene EP is advantageous over the transgenic bird method, which requires dozens of months. Such superiority of the *in vivo* gene EP implies that it provides a rapid and inexpensive means of quality analyses of recombinant proteins to be produced in avian egg white. However, our results also indicated that reporter proteins both in the oviduct and egg white diminished rapidly, having a sharp peak at 1 or 2 days in the former, and at 6 days in the latter, after *in vivo* gene EP. Since in mouse skeletal muscle, the firefly luciferase expression lasted for more than several months and human erythropoietin for 15 months (8), there might be high activities of DNase and protease in the oviduct, which in turn accounts for the rapid decrease in the luciferase activity.

If the *in vivo* gene EP is to be used for screening purposes of pharmaceutical protein quality, this short period of gene expression would have to be improved. To do so, two approaches may be available. First, extra chromosomal replication of plasmids helps prolong foreign gene expression. In primate cells, for example, the presence of the Epstein–Barr virus self-replicating region consisting of replicating origin and nuclear antigen would result in episomal amplification of transfected plasmids in cultured cells from primate origin (23). Indeed, in the mouse testis, this was substantiated (24). In avian species, however, the same Epstein–Barr virus self-replication region did not function properly in the testes of chickens and Japanese quail (15, 16), and therefore a similar, but functional self-replicating region in avian species should be identified. Second, promotion of foreign gene integration should result in longer gene expression. In the mouse testis, cotransfer of a reporter gene and bovine virus integrase gene prolonged foreign gene expression probably due to an enhanced integration rate (25). The combined gene transfer of a reporter gene and RSV integrase gene also resulted in prolonged gene expression in chicken embryos *in ovo* (Muramatsu *et al.*, unpublished).

Besides the duration of gene expression, intensity of recombinant protein gene expression should be fortified. For this purpose, a gene encoding adenovirus associated RNA was cotransfected with a reporter gene since Kitajewski *et al.* (19) reported that the cotransfection induced reporter gene expression. As was expected, gene expression increased by 2-fold, but the improvement was far from satisfactory. Further enhancement should be needed such as the use of a tetracycline-on or -off system (26). In addition, replacement of poly(A) signal sequences may enhance foreign gene expression (27, 28). These modifications on plasmid constructs remain to be examined in the future.

In the end of a series of experiments, a serious problem became evident. The present *in vivo* gene EP imposed an interruption of egg production for several days following the EP treatment. This was probably due to a stress from EP stimuli or more likely from operation procedures. It would appear that the voltage at 75 V employed in the present study per se may not have a serious effect on egg production rate, because EP even at 200 V with different electrodes and simple operation procedures resulted in milder disturbance (Muramatsu *et al.*, unpublished). Already at 2 days after EP, egg production rate returned completely to a pre-experimental level of 75%. The exposure of magnum portion might have been the primary cause, although the entire operation and electroporation procedures for one bird was completed within an hour. In any case, such a delay might facilitate degradation of recombinant proteins in egg white, and therefore result in lowered recovery of recombinant proteins. The pause of egg production should be overcome by changing methods of operation, types of electrodes and routes of DNA delivery. Further study is in progress.

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