Efficient Gene Transfer into Early Chicken Embryos by Electroporation of Stage X Blastoderms in Vivo, Applying Electric Pulses Vertically to the Blastoderm Layer

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In order to introduce exogenous DNA into early chicken embryos by transfecting the stage X (EG&K) blastoderm, in vivo electroporation was applied to it. A pair of novel electrodes was devised for applying electric pulses vertically to the blastoderm layer. Fertilised eggs at stage X were broken and the blastoderm was placed on top of the yolk. One of the electrodes was placed on the blastoderm layer through the yolk membrane, and the other electrode was placed on the bottom of the yolk. DNA (GFP gene) was injected into the blastoderm, and electric square pulses were applied 5 times at 5-100 V for loading periods of 50 msec per pulse at one-second intervals. The manipulated embryos were transferred into host eggshells and incubated for 4 days. The viability of the manipulated embryos was 67.0% (59/88) on day 3 of incubation. The expression pattern of GFP gene was mostly mosaic in the embryos, and the percentages of embryos that expressed the GFP gene were 97.7% (86/88), 93.2% (82/88) and 75.0% (66/88) on days 1, 2 and 3 of incubation. A high rate of GFP gene expression was observed in the embryonic and extra-embryonic tissues (54.2%, 32/59) and also in the extra-embryonic tissues only (27.1%, 16/59) on day 3 of incubation. The expression of the GFP gene throughout the embryonic tissues was also observed. These results suggest that the system for applying electric pulses vertically to the blastoderm layer is effective in introducing exogenous DNA into early chicken embryos.

Key words : blastoderm, chick embryo, electroporation, embryo culture, GFP gene

Introduction

The technique for gene transfer into chickens provides a powerful tool for studying gene functions, the production of pharmaceutical materials into eggs and the genetic
improvement of chickens. The introduction of exogenous DNA into chickens by non-viral method has been attempted by direct microinjection of DNA into fertilised ova (Sang and Perry, 1989; Perry et al., 1991; Naito et al., 1991, 1994; Naito, 1997), through chimaeric intermediates produced by the transfer of blastodermal cells (Etches et al., 1997) or primordial germ cells (Naito, 1998; Naito et al., 1998, 2000a; Hong et al., 1998), or by using spermatozoa as a vector (Squairs and Drake, 1997). So far, the only way of successfully transferring exogenous DNA into chicken chromosomes was achieved by direct microinjection of DNA into the fertilised ova (Love et al., 1994; Sherman et al., 1998). However, in this method, a limited number of ova can be manipulated because only one egg can be obtained from a hen, and a low integration frequency of exogenous DNA was obtained due to the cytoplasmic injection of DNA.

Freshly laid fertilised eggs (stage X, Eyal-Giladi and Kochav, 1976) can easily be obtained from chickens. So far, the introduction of exogenous DNA into chickens by manipulating the stage X blastoderm in vivo has been considered difficult because it is composed of about 60,000 cells (Eyal-Giladi and Kochav, 1976; Kochav et al., 1980). The recent development of an in vivo electroporation method, however, has made it possible to introduce exogenous DNA into early chicken embryos (Muramatsu et al., 1997, 1998; Momose et al., 1999). The in vivo electroporation method has some advantages over gene transfer such as simplicity, low embryonic damage and high transfection efficiency (Momose et al. 1999). In our previous study, the stage X blastoderm was transfected by in vivo electroporation (Naito et al., 2000b; Sano et al., 2002). Following the microinjection of DNA (GFP gene) into the blastoderm, parallel type electrodes were placed on both sides of the blastoderm and electric pulses were applied horizontally to the blastoderm layer. The exogenous DNA was efficiently expressed in the early embryos, but the transfection efficiency was varied in each embryo, and a low expression rate of the GFP gene in the embryonic tissues at stage 18 (Hamburger and Hamilton, 1951) was observed. This variation in transfection efficiency of the stage X blastoderm was thought to be caused by the electric pulses applied horizontally to the blastoderm layer. The structure of the stage X blastoderm is disc-like, about 4 mm in diameter, and the area pellucida of the blastoderm layer is about 30 μm thick (Eyal-Giladi and Kochav, 1976; Kochav et al., 1980). Thus, it seemed preferable to apply electric pulses vertically to this blastoderm layer structure rather than horizontally so that exogenous DNA could be introduced more efficiently into the blastoderm.

In this study, a novel technique of in vivo electroporation of stage X blastoderm was devised whereby electric pulses can be applied vertically to the blastoderm layer. By using this technique, exogenous DNA (GFP gene) was efficiently expressed in the embryonic tissues at stage 18, and also the embryos that expressed the GFP gene in the whole embryonic tissues were observed.

Materials and Methods

Fertilised eggs and animal care

Fertilised eggs of White Leghorn chickens were obtained by artificial insemination
from the genetic stocks maintained at the National Institute of Livestock and Grassland Science. All animals received humane care as outlined in the Guide for the Care and Use of Experimental Animals (National Institute of Agrobiological Sciences Animal Care Committee).

**Embryo manipulation and plasmid DNA**

Unincubated fertilised eggs at stage X were broken and the contents were put into glass vessels. The thick albumen capsule was removed from the yolk by tilting the glass vessel and, using rotation, blastoderm was placed on top of the yolk.

The plasmid DNA used in this study was pEGFP-N1 (6085-1, CLONTECH, CA). Circular form plasmid DNA was used for the transfection. DNA was diluted with HBS (20 mM Hepes 1 \(^{-1}\), 150 mM NaCl 1 \(^{-1}\), pH 7.4) at a concentration of 0.25 \(\mu\)g/\(\mu\)l.

**Transfection of stage X blastoderms by electroporation in vivo**

Micropipettes were made by pulling siliconised microcapillary tubes (1-000-0500, Drummond, PA) and bevelling the tips down (25 degrees) to an outside diameter of 30 \(\mu\)m. A micropipette, filled with DNA solution, was inserted into the blastoderm, and one \(\mu\)l of DNA solution was injected into each blastoderm.

Electroporation was carried out using an electroporator (CUY-21, TR Tech, Tokyo). The newly devised electrodes for electroporation are shown in Fig. 1. One lower electrode is disc-like in shape, 2 cm in diameter, and attached to the bottom of the vessel. The upper electrode is a stick type, 2 mm in diameter, and is exposed at its tip. The manipulated embryo (yolk) was placed in the vessel and the blastoderm was placed on top of the yolk by rotation. The stick type electrode was placed on the yolk membrane in the centre of the blastoderm. Electric square pulses were applied 5 times at 5-100 V for loading periods of 50 msec at one-second intervals.

**Embryo culture and detection of gene expression**

The manipulated embryos were transferred into host eggshells, which were then filled with thin albumen, sealed with cling film, and secured by plastic rings and elastic bands (Perry, 1988; Naito et al., 1990). The reconstituted eggs were incubated in a forced air incubator (P-008B, Showa Furanki, Saitama) for up to 4 days at 38°C and 50% relative humidity, with rocking through an angle of 90 degrees. Viability was expressed by the percentage of embryos to reach stage 18 (Hamburger and Hamilton,

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Fig. 1. *In vivo* electroporation of stage X blastoderm of chickens. Electric pulses were applied vertically to the blastoderm layer.
Expression of the GFP gene was detected under a fluorescent microscope (MZFL-III, Leica Microsystems, Tokyo) with the filters 480 nm excitation and 510 nm emission wavelengths through the aperture of the reconstituted egg. The GFP gene expression in the embryonic and extraembryonic tissues was observed at stage 18 (Hamburger and Hamilton, 1951) to adjust the stage to the determination of embryo viability. The degree of GFP gene expression was expressed in 5 grades depending on the number of GFP-expressing cells (−: 0, +: 1-10, ++: 11-50, +++: 51-100, ++++: 101 or more).

Results

Effects of electric pulses on embryo viability

The development of the embryos treated with electric pulses tended to lag for several hours compared with intact embryos. The viabilities of the embryos after applying electric pulses of 5-100V to the blastoderms are shown in Table 1. The viability of the intact embryos was 95%, and this tended to decrease with an increase in the voltage applied. When 5-20 V electric pulses were applied to the blastoderms, more than 70% of the embryos survived but at 25 V, viability decreased rapidly, and no embryo survived when 100 V pulses were applied.

GFP gene expression in developing embryos following electroporation

On day 1 of incubation, expression of the GFP gene was observed in most of the blastoderms manipulated (Table 2). The intensity of GFP gene expression after 24 hours of incubation was highest when 15 V electric pulses were applied and lowest at 10 V electric pulses. Although the site of GFP gene expression in the blastoderm on day 1 of incubation varied, the GFP gene tended to be expressed in the central part of the blastoderm when higher voltages were applied. A high rate of GFP gene expression in the manipulated embryos persisted from day 1 to day 3.

Table 1. Effect of electric pulses to stage X blastoderms on the development of chicken embryos

<table>
<thead>
<tr>
<th>Voltage</th>
<th>Number of blastoderms treated</th>
<th>Number (%) of embryos developed to stage 18 (H &amp; H)</th>
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<tbody>
<tr>
<td>0 V</td>
<td>40</td>
<td>38 (95.0)</td>
</tr>
<tr>
<td>5 V</td>
<td>31</td>
<td>29 (93.5)</td>
</tr>
<tr>
<td>10 V</td>
<td>32</td>
<td>26 (81.3)</td>
</tr>
<tr>
<td>15 V</td>
<td>32</td>
<td>25 (78.1)</td>
</tr>
<tr>
<td>20 V</td>
<td>32</td>
<td>25 (78.1)</td>
</tr>
<tr>
<td>25 V</td>
<td>32</td>
<td>18 (56.3)</td>
</tr>
<tr>
<td>30 V</td>
<td>31</td>
<td>14 (45.2)</td>
</tr>
<tr>
<td>40 V</td>
<td>30</td>
<td>7 (23.3)</td>
</tr>
<tr>
<td>50 V</td>
<td>30</td>
<td>6 (20.0)</td>
</tr>
<tr>
<td>100 V</td>
<td>10</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>
The viability of the manipulated embryos was high (more than 90%) when 10 V electric pulses were applied, but decreased to about 55% by applying 15–20 V pulses (Table 3). The viabilities of the manipulated embryos treated with 15–20 V electric pulses were lower compared with the cases in which only electric pulses were applied without DNA injection. High rates of GFP gene expression in the embryonic as well as extra-embryonic tissues at stage 18 were observed in whatever voltage was applied. Percentages of embryos expressing the GFP gene in both embryonic and extra-

Table 2. Degree of GFP gene expression in blastoderms after 24 hours of electroporation treatment

<table>
<thead>
<tr>
<th>Voltage</th>
<th>Number of blastoderms treated</th>
<th>Number (%) of blastoderms expressed GFP gene</th>
<th>Degree of GFP gene expression*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10 V</td>
<td>28</td>
<td>1</td>
<td>6</td>
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<tr>
<td></td>
<td></td>
<td>(3.6)</td>
<td>(21.4)</td>
</tr>
<tr>
<td>15 V</td>
<td>30</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3.3)</td>
<td>(10.0)</td>
</tr>
<tr>
<td>20 V</td>
<td>30</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.0)</td>
<td>(6.7)</td>
</tr>
</tbody>
</table>

* - : No expression
+ : 1–10 cells
++ : 11–50 cells
+++ : 51–100 cells
++++ : 101– cells

Table 3. Expression of GFP gene in early chicken embryos following transfection of stage X blastoderms in vivo by electroporation

<table>
<thead>
<tr>
<th>Voltage</th>
<th>Number of blastoderms treated</th>
<th>Number (%) of blastoderms or embryos expressing GFP gene</th>
<th>Number (%) of embryos developed to stage 18 (H &amp; H)</th>
<th>Expression of GFP gene in stage 18 embryos</th>
<th>No expression</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Embryonic and extra-embryonic tissues</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 V</td>
<td>28</td>
<td>27</td>
<td>25</td>
<td>26</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(96.4)</td>
<td>(89.3)</td>
<td>(67.9)</td>
<td>(92.9)</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>15 V</td>
<td>30</td>
<td>29</td>
<td>27</td>
<td>22</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(96.7)</td>
<td>(90.0)</td>
<td>(73.3)</td>
<td>(56.7)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 V</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>25</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(100.0)</td>
<td>(100.0)</td>
<td>(83.3)</td>
<td>(53.3)</td>
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</tbody>
</table>
Fig. 2. Expression of GFP gene in the developing chicken embryo transfected by electroporation of stage X blastoderm \textit{in vivo}. Intense GFP gene expression was observed in the whole blastoderm (a: day 1) and in all embryonic and extra-embryonic tissues (b: day 2; c: day 3). Scale bars indicate 1 mm in (a), (b) and (c).

embryonic tissues increased by increasing the voltage.

\textit{GFP gene expression in an embryo for three consecutive days}

Figure 2 shows the GFP gene expression in the blastoderm or embryo over three consecutive days treated with 15 V electric pulses following DNA microinjection. On
day 1 of incubation, intense GFP gene expression was observed in almost the whole area of the blastoderm. On day 2 of incubation, it was expressed intensely throughout the embryonic tissues and also in the extra-embryonic tissues. On day 3 of incubation, a strong expression persisted in both embryonic as well as extra-embryonic tissues. Many GFP-expressing cells were observed in the bloodstream (erythrocytes or primordial germ cells) of this embryo at stage 14.

Discussion

A chicken embryo at stage X contains a large amount of yolk surrounded by yolk membrane, an albumen capsule, eggshell membranes and eggshell. Advances in chicken embryo culture technique (Perry, 1988) made it possible to access the stage X blastoderm. Especially by removing the albumen capsule from the yolk, the stage X blastoderm can be manipulated very easily (Naito et al., 1990), and the manipulated embryos can be cultured through to hatching. By using this embryo culture technique, the electrodes for in vivo electroporation can directly touch the yolk membrane without interference from an albumen layer. The stage X blastoderm is composed of a single-layered area pellucida and a peripheral area opaca. Upon incubation, the area pellucida differentiates into two distinct layers, an upper epiblast and a lower hypoblast. The epiblast gives rise to the embryo proper, and the hypoblast gives rise to a portion of the extra-embryonic membrane (Petitte et al., 1999). Furthermore, primordial germ cells, which are the progenitor cells of ova and spermatozoa, may already be determined as early as stage X and distribute mostly in the central disc of the area pellucida and the ventral surface of the blastoderm layer (Ginsburg, 1994; Karagenc et al., 1996; Ginsburg and Eyal-Giladi, 1987; Naito et al., 2001). In order to introduce exogenous DNA into embryonic tissues and primordial germ cells by in vivo electroporation, it seems effective to apply electric pulses from bottom to top (to the central disc of the blastoderm) through the whole yolk following DNA microinjection into the blastoderm. This method enables us to apply electric pulses vertically to the blastoderm layer without injuring the yolk membrane.

The results show that electroporation treatment vertically to the central disc of the blastoderm as employed in this study was much more effective in introducing exogenous DNA into embryonic tissues compared with horizontal electroporation treatment (Naito et al., 2000). The viabilities of embryos treated with electric pulses vertically or horizontally were almost the same, suggesting that such pulses were applied effectively to the blastoderm layer in both methods irrespective of their direction or the distance between the two electrodes. When electric pulses were applied horizontally to the blastoderm layer, their effectiveness varied, and a low frequency of GFP gene expression in the embryonic tissues was observed. The exact structure of the stage X blastoderm is somewhat curved along the surface of the yolk and yolk membrane. When parallel type electrodes were placed on both sides of the blastoderm, the curved shape of the blastoderm layer becomes more pronounced. As a result, exogenous DNA was mainly introduced into the blastodermal cells in the area opaca of the blastoderm by applying electric pulses horizontally to the blastoderm layer (Naito et al., 2000). On
the other hand, when they were applied vertically to the blastoderm layer (as in this
study), exogenous DNA was successfully introduced into the blastodermal cells distri-
buted in the central disc of the area pellucida, and as a result exogenous DNA was
efficiently expressed in the embryonic tissues, and in some cases throughout the
embryonic tissues. Transfection efficiency was enhanced by increasing the voltage
applied, although the effect was pronounced in the extraembryonic membranes. Prob-
ably cells in the embryonic tissues proliferated rapidly compared with the extra-
embryonic tissues, so that the introduced DNA tended to disappear rapidly in the
embryonic tissues. Thus, vertical rather than horizontal application is more effective for
introducing exogenous DNA into early chicken embryos.

Introduction of exogenous DNA into developing embryos and chickens was earlier
achieved by transfecting the stage X blastoderm by lipofection (Rosenblum and Chen,
1995; Inada et al., 1997; Naito et al., 2000), although the resulting efficiency of
embryonic tissue expression of exogenous DNA was low. However by combining
lipofection and electroporation, transfection efficiency was enhanced when the stage X
blastoderm was manipulated (Naito et al., 2000). The DNA introduced into the stage
X blastoderm persisted in the developing embryos and was detected in various tissues
and organs including gonads (Sano et al., 2003). In chickens, DNA introduced into
embryos by lipofection, electroporation or some other method was usually present
episomally and was gradually lost during their development (Naito et al., 1994, 1998;
Muramatsu et al. 1997), although occasionally transmitted to the next generation (Li et
al., 1995). For the integration of exogenous DNA into host chromosomes, it is
necessary to culture cells for the long-term or to establish ES cells (Pain et al. 1996) or
EG cells (Park and Han, 2000) for selecting cells with transgenes into the chromo-
somes.

Dynamic morphogenetic movement and sexual differentiation in early chicken
embryos are induced by various gene effects (Schmid et al., 2000). Since the expression
of these genes is usually transient, it seems possible to study the function of various
genes by introducing and expressing exogenous DNA into the stage X blastoderm. By
coen- transfec- ting the gene of interest with the GFP gene, it should be possible to monitor
the distribution and movement of the gene introduced into the blastoderm and embryo
and to study the function of the gene of interest. The novel electroporation method
developed in this study could help to investigate the function of various genes in early
chicken embryos.

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