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*Original Article*

# Efficient incorporation of transfected blastodermal cells into chimeric chicken embryos

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**ABSTRACT** **The formation of transgenic chimeric chickens for use in developmental studies and as intermediates in the production of transgenic chickens requires the incorporation of stably transfected blastodermal cells into a chimera. To obtain blastodermal cells, area pellucidae of stage X (Eyal-Giladi and Kochav, *Dev. Biol. 49:* 321-337, 1976: E.-G.&K.) embryos were collected from unincubated, freshly oviposited Barred Plymouth Rock eggs and dissociated in 0.25% trypsin/0.04% EDTA (w/v) and 2% (v/v) chicken serum in phosphate-buffered saline ($Ca^{2+}$ and $Mg^{2+}$ free) at 4°C for 10 min. The blastodermal cells were suspended in Dulbecco's Modified Eagle's Medium (DMEM) and transfected by lipofection with superhelical pmiwZ, a plasmid containing a hybrid *lacZ* gene encoding bacterial ß-galactosidase (ß-gal) under the control of a chicken ß-actin/Rous sarcoma virus promoter. A mixture of 2.5 µg Lipofectin™ and 1.56 µg pmiwZ in 250 µl DMEM was incubated for 30 min at 37°C and added to 500 µl of 20-40,000 cells in suspension. Cells incubated with the transfection reagents in the presence or absence of pmiwZ were either plated and cultured for 48 h at 37°C in 5% $CO_2$/95% air, or injected through a shell window into the subgerminal cavity of White Leghorn stage X (E.-G.&K.) embryos previously exposed to 500-600 rads from a $^{60}Co$ source, after which the window was sealed and the egg incubated at 38±1°C for 72 h. Specific expression of the bacterial *lacZ* gene was determined by staining with X-gal at pH 7.4 and detected in 30% of the cells surviving exposure to Lipofectin™ and pmiwZ; no stain was detected in cells lipofected in the absence of pmiwZ. Embryos that were injected with blastodermal cells transfected with pmiwZ demonstrated bacterial ß-gal activity in cells in both extra- and intra-embryonic tissues. Cells expressing the bacterial *lacZ* gene were observed in tissues derived from the mesoderm, endoderm and ectoderm. None of the embryos injected with cells lipofected without pmiwZ revealed a blue stain when incubated with the X-gal substrate at pH 7.4. Chimeras bearing genetically modified cells could be used to study cell lineages by observing the developmental fate of transfected blastodermal cells bearing reporter genes and could serve as intermediates in the production of transgenic chickens if stably transfected cells enter the germline.**

KEY WORDS: *chimera, chicken, transfection, blastoderm, transgenic*

## Introduction

The production and study of various transgenic animals such as the mouse and *Drosophila* has benefitted both fundamental and applied biological research. In contrast, although transgenic birds would provide an excellent model for developmental studies, could be an asset to the poultry breeding industry and could constitute a production system for pharmaceutically important proteins (Verrinder-Gibbins, 1993), few have been available for study (Shuman, 1991) because of the difficulty experienced in accessing the avian genome. One method proposed for the production of transgenic birds involves the development of transgenic chimeric intermediates (Petitte *et al.*, 1990). This strategy is similar to that used for the generation of transgenic mice through chimeric intermediates (Bradley *et al.*, 1984; Gossler *et al.*, 1986; Robertson *et al.*, 1986) and has the added, optional benefit of allowing the introduction of specific genetic modifications achieved by homologous recombination (for review see Cappechi, 1989a,b).

As a first step in developing a system to make specific genetic modifications in birds, Petitte *et al.* (1990) demonstrated that dispersed blastodermal cells from stage X (Eyal-Giladi and Kochav, 1976; E.-G.&K.) embryos of Barred Plymouth Rock chickens could

*Abbreviations used in this paper:* DMEM, Dulbecco's modified Eagle's Medium; ß-gal, ß-galactosidase; E.-G.&K., Eyal-Giladi and Kochav; H.& H., Hamburgerand Hamilton; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PBS-G, PBS-glucose; PBS-CMF, calcium- and magnesium-free PBS; CS-PBS-CMF, chick serum-PBS-CMF.

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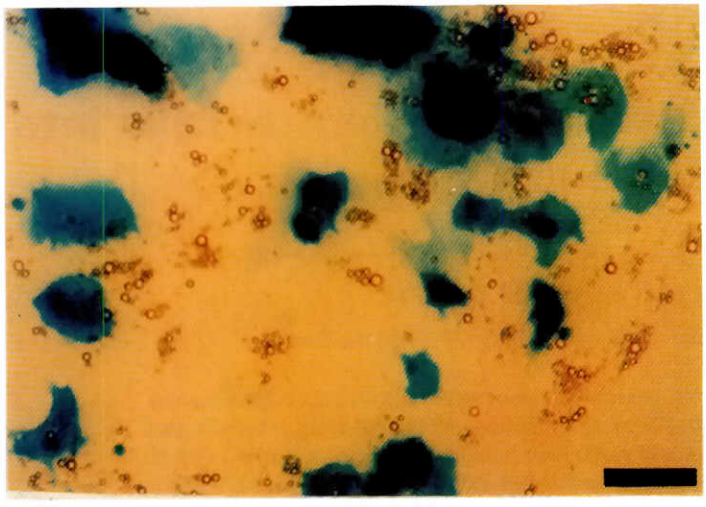

**Fig. 1. Chicken blastodermal cells stained for bacterial *lacZ* gene expression following lipofection treatments with 2.5 μg Lipofectin™ incubated with 1.56 μg superhelical pmiwZ DNA.** *Cells were cultured for 48 h at 37° C in 5% $CO_2$/95% air in four wells of a 96-well plate. Cultured cells were fixed and tested for exogenous ß-gal activity using X-gal as a substrate as described in Materials and Methods. The intense blue coloration indicates the presence of exogenous ß-gal activity. Bar, 80 μm.*

be injected into the subgerminal cavity of exposed stage X (E.-G.&K.) embryos from White Leghorn chickens and could enter the germline, the hematopoietic tissues and at least one derivative of neural crest cells, the melanocytes. In addition, it was demonstrated that the blastodermal cells could be transfected and that these transfected cells could be incorporated into chimeric chicken embryos (Brazolot *et al.*, 1991); descendants of dispersed blastodermal cells transfected with a bacterial *lacZ* gene were incorporated in the prosencephalon, head ectoderm and ventricle of the heart in an embryo that had developed to stage 11 (Hamburger and Hamilton, 1951; H.&H.). Although these observations demonstrated the feasibility of producing genetically modified chickens via a chimeric intermediate, the production of germline chimeras using this technique was rare, as was the incorporation of transfected cells into chimeric embryos.

To overcome these obstacles, the proportion of donor-derived cells that is incorporated into chimeras has been increased markedly by exposing the recipient embryo to γ-irradiation prior to the injection of dispersed stage X (E.-G.&K.) blastodermal cells (Carsience *et al.*, 1993). Under optimal conditions, somatic chimerism was observed in 64% of chimeric embryos and hatched chicks, and 84% of surviving male chimeras transmitted the donor genotype through the germline. To assess the likelihood of incorporation of genetically modified donor cells into chimeras made with irradiated recipients, donor blastodermal cells transfected with *lacZ* were injected into stage X embryos previously exposed to 500-600 rads of γ-irradiation.

## Results

### *Transfected cells in culture*

Approximately 30% of plated cells that were cultured for 48 h following lipofection with a preincubated mixture of 2.5 μg Lipofectin™ and 1.56 μg superhelical pmiwZ DNA consistently showed intense exogenous ß-gal activity throughout the cell body similar to that shown in Fig. 1. These cells formed predominantly confluent single layers. Most cells were packed with large yolk-containing vacuoles and appeared broad, flat and firmly attached. Some cells had long projections and appeared to be neuroblast-like. There was no ß-gal activity detected at pH 7.4 in any of the cells lipofected in the absence of pmiwZ DNA (data not shown).

### *Transfected cells within the chimeric embryo*

Stage 11 and 15 (H.&H.) chimeric embryos produced by injecting 92 γ-irradiated White Leghorn blastoderms with dispersed Barred Plymouth Rock blastodermal cells exposed to a preincubation mixture of 2.5 μg Lipofectin™ and 1.56 μg superhelical pmiwZ DNA were recovered after 72 h of incubation. All of the 45 recovered embryos possessed extra-embryonic cells expressing the bacterial *lacZ* gene in some or all of the following tissues: the cells surrounding and contained in the vitelline arteries (Fig. 2A), blood islands (Fig. 2B) and red blood cells within the vitelline veins and arteries (Fig. 2C); the somatopleure surrounding the anterior and/or posterior regions of the embryo; vasculature within the yolk sac; and cells contained within the yolk sac epithelium (data not shown). The following tissues contained cells with exogenous ß-gal activity

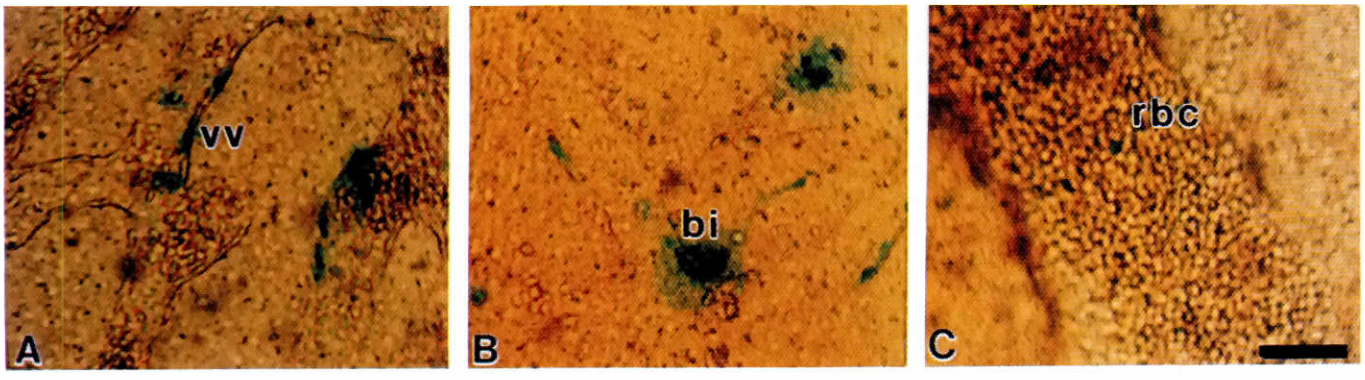


**Fig. 2. Extra-embryonic exogenous ß-gal activity localized in (A) vitelline vessels (vv), (B) blood island (bi) cells and (C) red blood cells (rbc) within the vitelline artery of a stage 15 (H.&H.) chimeric chick embryo.** *Chimeric embryo production and staining for bacterial lacZ gene expression is explained in Materials and Methods. The intense blue coloration indicates the presence of exogenous ß-gal activity. Bar, 80 μm.*

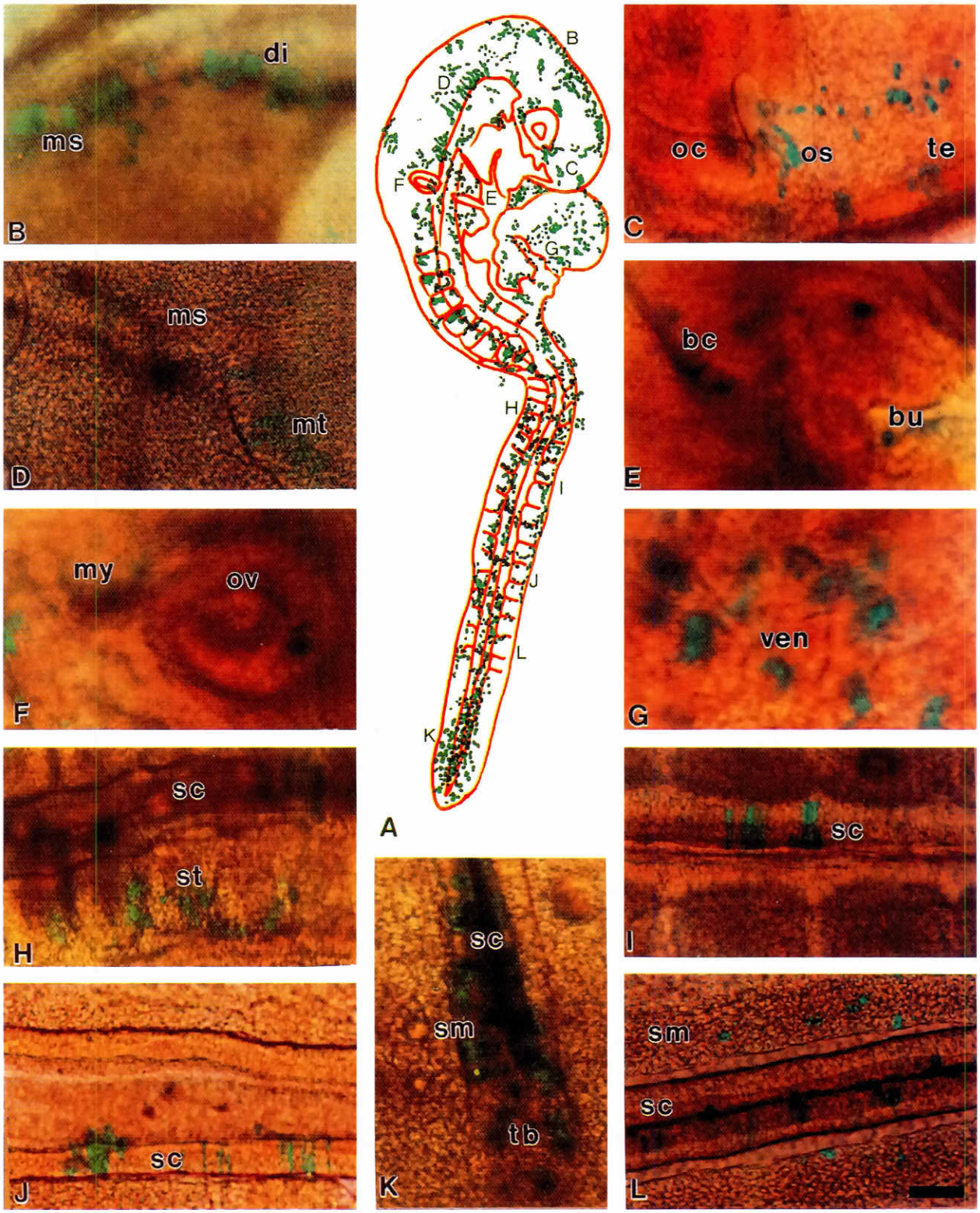


**Fig. 3. Exogenous ß-gal activity in chimeric chicken embryos incubated for 72 h before analysis. (A)** *Compilation of cells with exogenous ß-gal activity (indicated in blue) within a representative stage 15 (H.&H.) embryo (in red). Examples are shown of extensive exogenous ß-gal activity (blue stained cells) taken from various chimeric embryos; these regions are indicated by the corresponding letter in A as follows:* **(B)** *mesencephalon (ms), diencephalon (di);* **(C)** *optic cup (oc), optic stalk (os) and telencephalon (te);* **(D)** *metencephalon (mt) and mesencephalon (ms);* **(E)** *branchial cleft (bc) and bulbis cordis (bu);* **(F)** *myelencephalon (my) and otic vesicle (ov);* **(G)** *ventricle (ven);* **(H)** *spinal cord (sc) and somites (st);* **(I and J)** *spinal cord (sc);* **(K)** *tail bud (tb), spinal cord (sc) and segmental mesoderm (sm);* **(L)** *segmental mesoderm and spinal cord (sc). Bar, 80 μm*

TABLE 1

**THE GERM LAYER ORIGIN OF STRUCTURES CONTAINING CELLS EXHIBITING ß-GAL ACTIVITY IN CHIMERIC CHICKEN EMBRYOS FORMED FROM INJECTING *lacZ*-TRANSFECTED BLASTODERMAL DONOR CELLS INTO γ-IRRADIATED STAGE X (E.-G.&K.) EMBRYOS**

| INTRA-EMBRYONIC | | EXTRA-EMBRYONIC |
|---|---|---|
| **Ectoderm** | **Mesoderm** | **Ectoderm and Somatomesoderm** |
| telencephalon | mandibular arch | somatopleure |
| diencephalon | bulbis cordis | |
| mesencephalon | atrium | **Endoderm and Splanchnic Mesoderm** |
| metencephalon | ventricle | yolk sac |
| myelencephalon | sinus venosus | |
| optic cup | somites | **Mesoderm** |
| optic stalk | segmental mesoderm | vitelline and yolk sac vasculature |
| lens vesicle | notochord | hematopoietic cells |
| otic vesicle | embryonic coelom | red blood cells |
| neural folds | body wall | |
| spinal cord | tail bud | |
| | **Endoderm** | |
| | pharynx | |
| | tail fold | |
| | primitive streak | |

intra-embryonically in 3 of the 9 stage 11 (H.&H.) (data not shown) and 12 of the 36 stage 15 (H.&H.) embryos recovered (Fig. 3): telencephalon (Fig. 3C), diencephalon (Fig. 3B), mesencephalon (Fig. 3B and 3D), metencephalon (Fig. 3D), myelencephalon (Fig. 3F), optic cup (Fig. 3C), optic stalk (Fig. 3C), lens vesicle, otic vesicle (Fig. 3F), mandibular arch, bulbus cordis (Fig. 3E), atrium, ventricle (Fig. 3G), pharynx, sinus venosus, somites (Fig. 3H), neural folds, notochord, spinal cord (Fig. 3H-L), segmental mesoderm (Fig. 3L), embryonic coelom, body wall, primitive streak, tail bud (Fig. 3K) and tail fold. There was no ß-gal activity at pH 7.4 in any of the embryos produced by injecting White Leghorn blastoderms with dispersed Barred Plymouth Rock blastodermal cells incubated with 2.5 µg Lipofectin™ in the absence of pmiwZ DNA (data not shown).

## Discussion

Compromising the development of the recipient chick embryo by exposure to γ-irradiation increases the incorporation of transfected donor blastodermal cells into the intra-embryonic tissues of chimeric embryos from 1 out of 60 recovered embryos (Brazolot *et al.*, 1991) to approximately 1 out of 3 recovered embryos. These data confirm and extend previous results (Brazolot *et al.*, 1991), which demonstrated that dispersed stage X (E.-G.&K.) blastodermal chicken cells, transfected with a bacterial *lacZ* reporter gene, could be incorporated into a chimeric chick embryo. The increase in the number of cells that express the foreign reporter gene in chimeras made with compromised recipients may be related to the effects of irradiation on embryonic development. The recovered chimeric embryos lagged in their development by approximately 24 h; after 72 h of incubation the chimeric embryos had progressed to approximately the equivalent stages expected after 45 to 55 h of development. The retarding effect of γ-irradiation on the development of the recipient embryo has been demonstrated previously (Carsience *et al.*, 1993). Although the mechanisms involved are not understood, the delay in development of the recipient embryo caused by γ-irradiation apparently allows more donor cells to become established into the tissues of the developing chimera.

In the current series of experiments, approximately 30% of cells in culture expressed the bacterial *lacZ* gene following exposure of blastodermal cells in suspension to a preincubated mixture of 2.5 µg Lipofectin™ and 1.56 µg superhelical pmiwZ DNA (Fig. 1). If the ratio of transfected:non-transfected cells were further enriched, prior to the injection of donor cells into the subgerminal cavity, the resultant chimeric embryo should contain a greater number of genetically altered cells. In mice, the development of established lines of embryonic stem cells provided a means whereby clones of transfected cells can be selected to provide a genetically uniform population that is introduced into the recipient embryo (Bradley *et al.*, 1984; Gossler *et al.*, 1986; Robertson *et al.*, 1986). Immortalized lines of chicken blastodermal stem cells are not presently available, but alternative methods of enriching the donor cell population in stably transfected cells are being developed in our laboratory.

Recent electron microscopic studies of the stage X (E.-G.&K.) blastoderm indicate that the cells at this stage are uniform in size and shape (Watt *et al.*, 1993) indicating that stage X (E.-G.&K.) cells may be pluripotent. In support of this possibility, dispersed stage X (E.-G.&K.) blastodermal cells transfected with pmiwZ DNA were identified in most tissues of 72 h chimeric embryos (Fig. 3A) and differentiated into the appropriate cell type within these tissues (Fig. 3B-L). For instance, exogenous ß-gal activity was detected in extra-embryonic cells located in the somatopleure (composed of ectoderm and somatomesoderm), cells constituting the yolk sac (composed of endoderm and splanchnic mesoderm; data not shown), the vitelline arteries and the vasculature within the yolk sac (derived partially from the mesoderm; Fig. 2A) blood island cells (derived from mesoderm; Fig. 2B) and red blood cells arising from hematopoietic cells (Fig. 2C). Cells expressing the bacterial *lacZ* gene within the embryo were found in a variety of ectodermally-, mesodermally- and endodermally-derived tissues (Fig. 3, Table 1). Stage X (E.-G.&K.) blastodermal cells, therefore, appear to be suitable vehicles for the incorporation of cells bearing foreign genes into a wide range of embryonic tissues. By using donor cells transfected with hybrid genes containing tissue specific promoters controlling the expression of a reporter gene, it should be possible to analyze the ontogeny of developing tissues.

The dramatic increase in incorporation and the widespread distribution of transfected donor blastodermal cells in chicken chimeras constructed with irradiated recipient embryos (Carsience *et al.*, 1993) supports the possibility of employing transgenic chimeras as intermediates in the production of transgenic chickens. To facilitate this approach, substantial numbers of genetically modified donor cells are required, and it would be advantageous if all cells within the population were transfected. Populations of stably transformed cells might be isolated from freshly dispersed, transfected blastodermal cells, or lines of chicken embryonic stem cells might be developed to enable the selection of uniformly modified donor cells. The production of transgenic chickens from chimeric intermediates constructed with genetically modified blastodermal cells could then become routine.

## Materials and Methods

### *Transfection of stage X (E.-G.&K.) chicken blastodermal cells*

Stage X (E.-G.&K.) blastoderms were collected from freshly oviposited Barred Plymouth Rock eggs, using filter paper rings (Petitte *et al.*, 1990). The area pellucida was dissected from each embryo while submerged in Dulbecco's phosphate buffered saline (PBS) containing 5.6 mM glucose

(PBS-G; Gibco BRL, Burlington, Ontario). Two isolated blastoderms were transferred to 1 ml of PBS-G, rinsed once with calcium and magnesium free PBS (PBS-CMF) containing 2% (v/v) chick serum (2% CS-PBS-CMF) and the cells were dissociated in 0.05% (w/v) trypsin (Sigma Chemical Company, St. Louis, Missouri)/ 0.04% (w/v) EDTA in 2% CS-PBS-CMF at 4°C for 10 min. Trypsin digestion was halted by replacement of the dissociation solution with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) Fetal Bovine Serum (FBS). The cells were dispersed by subjecting them to gentle vortexing. To remove the FBS, the cells were centrifuged (300xg for 4 min) and resuspended in DMEM three times. The concentrations of cells in the DMEM preparations and their viability, estimated by exclusion of trypan blue, were determined prior to dispensing 500 µl volumes containing 20-40,000 cells each into round-bottomed, polystyrene culture tubes. The pmiwZ plasmid, kindly provided by H. Kondoh, Nagoya University, Japan, contains the bacterial *lacZ* gene under the control of Rous sarcoma virus and chicken ß-actin tandem promoters, and is believed to be constitutively expressed in the chicken; the encoded ß-gal has an optimum pH of 7.4, distinguishing it from the endogenous chicken ß-gal which has an optimum pH of 4.3 (Brazolot *et al.*, 1991). A modification of the procedure reported by Brazolot *et al.* (1991) was employed in the transfection of blastodermal cells with pmiwZ DNA using a cationic liposome preparation, Lipofectin™ (Gibco BRL, Burlington, Ontario): 2.5 µg Lipofectin™ and 1.56 µg superhelical pmiwZ in 250 µl DMEM was incubated for 30 min at 37°C prior to combining it with the 500 µl cell suspension. For comparison, cells were also lipofected similarly in the absence of pmiwZ. After the suspensions were incubated for an additional 3.5 h at 37°C in 5% $CO_2$/95% air, FBS was added to a final concentration of 10% (v/v) to inhibit further transfection. The cell suspension was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 350xg for 2 min. The cells were then resuspended in DMEM containing 10% FBS to give either a droplet of 40 µl final volume for immediate injection into recipient blastoderms or a 1 ml final volume divided between four wells of a 96 well culture plate for 48 h culture at 37°C in 5% $CO_2$/95% air.

***Injection of transfected cells into the stage X (E.-G.&K.) blastoderm***

Freshly oviposited Shaver White Leghorn (WL) eggs were exposed to 500-600 rads of γ-radiation from a $^{60}Co$ source (Atomic Energy of Canada) (Carsience *et al.*, 1993). The eggs were swabbed with 70% (v/v) ethanol and a 0.8 $cm^2$ window was ground out of the equatorial plane of the eggshell directly over the blastoderm (Petitte *et al.*, 1990), without damaging the membrane. A 0.4 $cm^2$ window was cut from the shell membrane to access the stage X (E.-G.&K.) embryo. Approximately 700-1000 transfected cells in 2 µl of DMEM and 10% FBS, drawn up from a concentrated region of the 40 µl droplet, were injected into the center of the subgerminal cavity using a finely drawn micropipet. Both the shell and shell membrane windows were covered with White Leghorn egg shell membrane, air dried and the outer layer sealed with «cement for plastic models» (Testor Canada, Weston, Ontario) and covered with a permeable adhesive film (Opsite™, Smith and Nephew, Quebec). The injected eggs were slowly rotated through 360° in an incubator at 38±1°C and 50% relative humidity for 72 h. Cells that were not injected were incubated for 48 h in 200 µl of DMEM and 10% FBS in a 96-well plate at 37°C in 5% $CO_2$/95% air, and stained for bacterial ß-gal activity to assess the transfection reactions.

***Assessment of exogenous ß-gal activity***

After 72 h of incubation, embryos were removed from eggs and fixed as previously reported (Brazolot *et al.*, 1991), except that the embryos were washed with PBS instead of avian saline, the washed embryos were fixed for 30 min instead of 20 min and the fixative contained 0.8% (v/v) instead of 2% (v/v) formaldehyde. The embryos were then incubated for 16 h at 22°C in 2 ml of X-gal staining solution which contained 0.5 mg X-gal/ml of 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide, 0.05% (v/v) Triton X-100 in 2mM $MgCl_2$ and 50mM phosphate buffer, pH 7.4. The X-gal was added fresh from a stock solution of 20 mg/ml dimethyl-formamide. All fixative, washing and staining solutions were maintained at pH 7.4 to maximize exogenous ß-gal activity (Brazolot *et al.*, 1991). Cultured blastodermal cells were similarly fixed and washed for 5 min each and stained for 1 h at 37°C. The expression of the bacterial *lacZ* gene was indicated by the presence of blue coloration within the body of the cell.

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