INTRODUCTION

Eggs and spermatozoa are derived from a specialized lineage of cells that is separated at an early stage of embryonic development from cells that form somatic tissues (Nieuwkoop and Satasurya, 1979). In the chicken, fertilization occurs within 1 hour after ovulation and embryonic development is initiated 3 hours later as the male and female pronuclei fuse (Perry, 1987). The first cleavage furrow develops within 2 hours as the ovum enters the shell gland. The shell is deposited during the next 18-20 hours and, when the egg is laid, the embryo is composed of 40 000-80 000 morphologically undifferentiated cells designated as stage-X by Eyal-Giladi and Kochav (1976). The precise timing of differentiation of the germline during embryonic development has yet to be established but it is thought to occur at approximately stage-X (Ginsburg and Eyal-Giladi, 1987). Access to the population of cells in the newly laid, but unincubated egg, that differentiates into the lineage giving rise to the gametes of the adult chicken, would provide a powerful tool to probe the complex interactions that regulate sexual differentiation. In addition, cells in the germline lineage are attractive candidates for genetic modification because manipulation of their DNA, which could include specific gene targeting, would ensure transmission of the genetic alteration to the next generation.

Although we have previously demonstrated that germline chimeras can occasionally be produced by injecting dispersed blastodermal cells from stage-X embryos into recipients at the same stage of development (Petitte et al., 1990, 1993), manipulations of the cells in the germline have been thwarted because techniques to direct the developmental destiny of isolated chicken blastodermal cells into functional gametes in chimeric chickens have been unavailable. By combining isolated blastodermal cells that give rise to functional gametes in the adult chicken with recipient embryos whose development has been compromised by exposure to $\gamma$ irradiation, we have now developed a proce-
Materials and Methods

Donor embryos were obtained from inter se matings of Barred Plymouth Rocks that are homozygous recessive at the \( I \) locus. Recipient embryos were obtained from inter se matings of White Leghorns that are homozygous dominant at the \( I \) locus. Donor cells were obtained by collecting stage-X blastodermis from freshly laid, unincubated eggs using filter paper rings (Petitte et al., 1990). The appropriate exposure of recipients to irradiation, prior to injection, was determined that yields germline chimeras consistently and repeatably. The predictable incorporation of donor cells into the germline of chimera makes these birds ideal intermediates through which genetic alterations in dispersed blastodermal cells made in vitro might be transferred to the germline in vivo.

Donor embryos were obtained from inter se matings of Barred Plymouth Rocks that are homozygous recessive at the I locus. Recipient embryos were obtained from inter se matings of White Leghorns that are homozygous dominant at the I locus. Donor cells were obtained by collecting stage-X blastodermis from freshly laid, unincubated eggs using filter paper rings (Petitte et al., 1990). The area pellucida from each embryo was dissected while submerged in Dulbecco’s phosphate-buffered saline (PBS) containing 5.6 mM glucose (PBS-G; Gibco BRL, Burlington, Ontario). Isolated area pellucidae were transferred into fresh PBS-G, rinsed once in calcium- and magnesium-free PBS containing 2% (v/v) chick serum (2% CS-PBS-CMF) and dissociated with 0.25% trypsin (Sigma Chemical Company, St. Louis, Missouri) in 2% CS-PBS-CMF containing 0.04% (w/v) EDTA at 4°C. Dissociation of the cells was halted by suspending the cells in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% (v/v) Fetal Bovine Serum (DMEM-FBS) prior to injection into recipients.

Recipients were prepared by exposing freshly laid, unincubated eggs to approximately 500-700 rads of \( \gamma \) irradiation from a \( ^{60}\text{Co} \) source. The embryo was accessed subsequently through a window cut into the long axis of the egg at its widest sector. The shell was removed to expose an 8 mm \( \times \) 8 mm area of the shell membrane and then a 4 mm \( \times \) 4 mm window was cut into the membrane to expose the embryo. Approximately 100 or 200-400 cells in 2-5 \( \mu \)l of DMEM-FBS were injected into the subgerminal cavity using a finely drawn micropipet. The window was closed by aligning a piece of freshly isolated shell membrane to overlap the exposed shell membrane of the recipient egg without contacting the shell. The membranes were allowed to adhere by exposure to air and a second piece of membrane was placed over the hole in the shell. When the outer covering of shell membrane was dry, it was glued to the shell with cement for plastic models and, after approximately 1 hour, was covered with Opsite™ surgical membrane (Smith & Nephew, Montreal). The eggs were placed into a conventional forced air incubator maintained at 37.5°C and 50% relative humidity and turned through 90°/hour for 19 days after sealing and the eggs were incubated until hatch. Somatic chimeras were identified at hatch by the presence of black pigmented down. At sexual maturity, they were mated to Barred Plymouth Rocks and the distribution of black and yellow offspring was recorded to assess the contribution of the donor and recipient lineages to the germline, respectively (Fig. 1). The frequency of germline chimerism when approximately 100 or 200-400 donor cells were injected into irradiated recipients was compared to the frequency of germline chimerism when approximately 100 donor cells were injected into a non-irradiated recipient. Fisher’s Exact Test was used to identify statistically significant differences between these frequencies.

Fig. 1. The formation and analysis of chimeras. Donor embryos were obtained from inter se matings of Barred Plymouth Rocks that are homozygous recessive at the \( I \) locus. Recipient embryos were obtained from inter se matings of White Leghorns that are homozygous dominant at the \( I \) locus. Donor cells were obtained by isolating the area pellucida from stage-X blastodermis collected from freshly laid, unincubated eggs. These area pellucidae were dispersed into a single cell suspension prior to injection into recipients. Recipients were prepared by exposing freshly laid, unincubated eggs to 490-680 rads of \( \gamma \) irradiation from a \( ^{60}\text{Co} \) source. The embryo was accessed subsequently through a window cut into the long axis of the egg at its widest sector. Approximately 100 or 200-400 cells were injected into the subgerminal cavity of the recipient. The window in the egg was sealed and the eggs were incubated until hatch. Somatic chimeras were identified at hatch by the presence of black down. At sexual maturity, they were mated to Barred Plymouth Rocks and the distribution of black and yellow offspring was recorded to assess the contribution of the donor and recipient lineages to the germline, respectively.

The appropriate exposure of recipients to irradiation, prior to injection of donor cells, was determined by exposing freshly laid, unincubated, White Leghorn eggs to 0, 552, 691, or 898 rads of \( \gamma \) irradiation from a \( ^{60}\text{Co} \) source (Atomic Energy of Canada). Embryos were subsequently incubated at 37.5°C and 50% relative humidity for 48, 72 and 96 hours or allowed to complete development and hatch. Development of irradiated embryos was compared to development of embryos that were not irradiated but incubated for the same length of time. An estimate of embryonic development in hours of incubation under standard incubation conditions and according to the classification of Hamburger and Hamilton (1951) was assigned to irradiated and non-irradiated
embryos. Mortality and hatching rates were also compared between irradiated and non-irradiated embryos.

**RESULTS**

The development of embryos exposed to 552-898 rads was delayed relative to the development of embryos not exposed to irradiation and the extent of the delay was proportional to the exposure to $\gamma$ irradiation (Fig. 2A-D). This delay in development was maintained throughout incubation and irradiated embryos emerged from the shell approximately one day later than non-irradiated embryos (data not shown). Of the embryos exposed to 552, 691 and 898 rads, 28.9%, 36.1% and 59.1%, respectively, did not hatch. By comparison 13% of the embryos that were not exposed to irradiation failed to hatch (Fig. 2E). These observations indicated that exposure of embryos to approximately 500-700 rads of $\gamma$ irradiation prior to incubation would compromise development without reducing hatchability to impracticable levels. Chicks that developed from White Leghorn embryos exposed to 500 rads, but otherwise not manipulated, grew normally, possessed unimpaired fertility and produced only yellow chicks typical of the White Leghorn breed (data not shown).

Of 809 White Leghorn embryos exposed to 500-700 rads of $\gamma$ irradiation and injected with approximately 100 donor cells, 192 developed past day 14 of incubation or hatched. Among the 192 embryos and chicks, somatic chimerism was evident on 118 (58%); on average 48% of the feather follicles of these somatic chimeras had been colonized by donor-derived melanocytes. In 2 hatched chicks, 75-90% of
The frequency of germline chimerism following injection of approximately 100 donor cells was significantly ($P<0.001$) increased from 2/106 to 3/24 by using an irradiated rather than a non-irradiated recipient. Whereas increasing the number of cells injected had no effect on the frequency of chimerism when non-irradiated recipients were used (Petitte et al., 1993), the frequency of germline chimerism was significantly ($P<0.01$) increased to 8/14 when 200-400 cells were injected into irradiated recipients. Using the ratio of germline to somatic chimeras as the basis for comparison, the frequency of germline chimerism increased significantly ($P<0.01$) from 2/21 when non-irradiated recipients were used, to 3/14 when approximately 100 cells were injected into recipients. Injecting 200-400 cells into irradiated recipients significantly ($P<0.01$) increased the ratio even further to 8/12.

When White Leghorn embryos were injected with approximately 100 cells from Barred Rock donors, 9 somatically chimeric males and 5 somatically chimeric females were produced and mated to Barred Plymouth Rocks. Two of the males have sired black chicks (Table 1) providing evidence that derivatives of the donor cells colonized the germline in these chimeras. Among the 5 somatically chimeric females one has produced a Barred Plymouth Rock chick (Table 1). Four putatively chimeric males and 6 putatively chimeric hens have produced only yellow chicks (Table 1) indicating that the donor-derived cells did not enter the germline in any of these birds.

Six somatically chimeric males, 6 somatically chimeric females and 2 putatively chimeric females produced by injecting 200-400 cells into irradiated embryos have been mated to Barred Plymouth Rocks (Table 1). Donor-derived cells were incorporated into the testes of 5 of the 6 male somatic chimeras (Table 1) and, in one case (042-195 in Fig. 3 and Table 1), all of the spermatozoa that sired chicks were descendants of donor cells. Three of the 6 somatically chimeric females contained donor-derived oocytes in their ovary (Table 1) whereas neither of the putatively chimeric females produced black offspring to date.

**DISCUSSION**

We have previously demonstrated that it is possible to make somatic and germline chimeras by injecting dispersed cells from a stage-X chick embryo into non-irradiated recipient embryos at the same stage of development (Petitte et al., 1990) and interspecific chimeras have been reported by Naito et al. (1991) and Watanabe et al. (1992). Subsequent experience with non-compromised recipients, however, has revealed that somatic chimeras are produced infrequently, donor-derived cells have colonized the gonads in only 2 of 106 chimeras and the transmission rate of donor-derived gametes from germline chimeras was less than 0.4% (Petitte et al., 1993).

By contrast, extensive colonization of the recipient by donor cells was promoted by exposing the recipient White Leghorn eggs to 500-700 rads from a $^{60}$Co source within one hour after oviposition. Whereas only 32% of the embryos and chicks produced by the injection of approximately 100 cells into a non-irradiated recipient exhibited

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**Fig. 3.** Chimera number 042-195 and 11 of his offspring. This chimera was made by injecting approximately 400 dispersed blastodermal cells from stage-X Barred Plymouth Rock embryos into a White Leghorn recipient at the same stage of development. The recipient was exposed to 550 rads from a $^{60}$Co source prior to the introduction of the Barred Plymouth Rock blastodermal cells. The body conformation and earlobes of this bird are typical of the White Leghorn breed but his plumage pigmentation, with the exception of a few white feathers, is indistinguishable from that of a Barred Plymouth Rock. The entire germline of this chimera is presumed to be donor-derived because he has produced only Barred Plymouth Rock offspring when mated to Barred Plymouth Rock hens (Table 1).
black pigmentation in their down, 58% and 64% of the chimeras injected with approximately 100 or 200-400 cells, respectively, into irradiated recipients exhibited donor-derived down pigmentation. The proportion of feather follicles colonized by donor-derived melanocytes was increased from 29% when about 100 cells were injected into non-irradiated recipients to 48% and 66% when approximately 100 cells and 200-400 cells, respectively, were injected into irradiated recipients.

The frequency of somatic and germline chimeras was unaffected by the number of cells that were injected into non-irradiated recipients (Petitte et al., 1992). By contrast, both the frequency and extent of incorporation of donor cells into somatic and germline lineages was increased when the number of cells injected into irradiated recipients was increased (Table 1). From these data we have concluded that irradiation compromises the ability of cells within the recipient embryo to divide and that the ratio of donor/recipient cells is increased as embryonic development proceeds during the first few hours or days of development. We assume that the relative rate of replication of cells in the chimera can favour colonization of any tissue

### Table 1. The number of donor- and recipient-derived offspring from matings to Barred Plymouth Rocks of somatic and putative chimeras made using compromised White Leghorn recipients

<table>
<thead>
<tr>
<th>Description of the chimera</th>
<th>Approximate number of donor cells injected</th>
<th>Percentage of black pigmentation</th>
<th>Number of donor-derived offspring</th>
<th>Number of recipient-derived offspring</th>
<th>Percentage of donor-derived offspring</th>
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<tr>
<td>Somatic ♂</td>
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<td>50</td>
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<td>119-1592</td>
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<td>30</td>
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<td>830</td>
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<td>101-102</td>
<td>100</td>
<td>30</td>
<td>0</td>
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<td>103-104</td>
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<td>85</td>
<td>0</td>
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<tr>
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<td>809</td>
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<td>9</td>
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<td>1001</td>
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<td>175-176</td>
<td>400</td>
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by the more rapidly dividing donor cells although we have
direct evidence to support this assumption only for
germinals and germ cells.

From the data presented in Table 1, it is evident that
germinal chimeric chickens can be made frequently and
reliably using a recipient that has been compromised by
exposure to irradiation. The production of chimeras from
compromised recipients significantly increased the number of
germinal chimeras when an irradiated rather than a non-
irradiated recipient was used, and increasing the number of
donor cells from approximately 100 to 200-400 significa-
cantly improved the frequency of germine chimeras to
more than 50%. In addition to improving the number of

germinal chimeras, the use of irradiated recipients also
increased the frequency of transmission of gametes of
donor-cell origin from the gonads of the chimeras. The 2

germinal chimeras made using non-irradiated recipients
yielded donor-derived offspring at a rate of less than 0.4%,
whereas the minimum rate of transmission using compro-
mised recipients was 1.3%, the median rate was 5.8% and
the maximum rate was 100%.

Our results to date indicate that male chimeras are more
likely to accommodate donor cells in the germine than
female chimeras but this sex difference is not yet under-
stood. In a previous study, male chimeras were also
observed more frequently than females and female cells
were usually excluded from adult male chimeras (Shaw et
al., 1992). Further analysis of male germine chimeras made
with female donor cells and compromised recipients are
required to resolve this enigma.

All of the germinal chimeras listed in Table 1 are also
somatic chimeras but there is little correlation between the
extent of feather pigmentation in somatic chimeras and the
likelihood that donor cells will have colonized the germine.
For example, hen 028-029 possessed only a small patch of
black feathers on her neck, but approximately 1 in 5 oocytes
was donor-derived (Table 1). The melanocyte population of
males 048-049 and 042-195 was almost exclusively donor-
derived (Table 1 and Fig. 3) but the proportion of func-
tional, donor-derived spermatozoa in their semen was
approximately 3% and 100%, respectively (Table 1). These
data support the interpretation that entry of cells into the
germine and into the ectoderm, from which the
melanocytes are derived, occurs separately and independ-
ently in chimeras made with compromised recipients. In
addition, the rates of proliferation of donor and recipient
cells in the ectoderm and the germine may not be equal.

By our definition, putative chimeras show no evidence of
donor-derived feather pigmentation and to date, no puta-
tive chimaera produced in the currently reported experiments
has proved to be germine. The lack of success in produc-
ing extensive somatic or germine chimerism in every
embryo may be due to donor cells being injected into an
inappropriate location in the irradiated egg and we are
addressing this issue. Nevertheless, the ease with which
large numbers of fertile eggs can be irradiated and injected
with donor cells, and with which somatic chimeras can be
identified, makes the production of germine chimeric chick-
ens a routine and practicable procedure.

The simplicity and practicability of obtaining germine
chimeras using compromised recipients creates new oppor-
tunities for manipulation of the avian genome. Blastoder-
mal cells can be frozen and retain their ability to form
chimeras after they are thawed (Petitte et al., 1993; Naito
et al., 1992); therefore, there is a unique opportunity to cry-
opreserve the genome of chickens using these readily avail-
able cells. Blastodermal cells can be transfected and have
been shown previously to express their modified genotype
in the non-compromised recipient (Brazolot et al., 1991).

More recently, we have shown extensive incorporation of
transgenic cells in compromised recipients (Fraser, Car-
sience, Clark, Etches and Verrinder Gibbins, unpublished
results) indicating the possibility of using the compromised
recipient as a vehicle to move genetically modified blasto-
dermal cells from in vitro systems into the germine. We
have also shown that blastodermal cells retain their ability
to form germine chimeras when introduced into compro-
mised recipients after 48 hours in culture (Etches, Toner,
Clark, Viekkind and Verrinder Gibbins, unpublished results)
allowing time for selection of some types of genetically
modified donor cells. Genetically modified stocks can be
dispersed quickly and inexpensively since founder chimeras

produce more than 1500 offspring in six months (e.g.
1587-120 in Table 1). Taken together, these data indicate
that the compromised recipient has the potential to provide
experimental biologists with a powerful new tool that will
facilitate molecular and cellular manipulations of the de-
veloping chick embryo. To date, such manipulations have only
been possible using the mouse as the vertebrate model. The
application of these techniques to the chick embryo via
transgenic germine chimeras will allow the amalgamation
of classical information regarding development, gleaned
from anatomical studies of the chick embryo, with the
nascent understanding of the molecular control of cellular
function during both embryonic and adult life.

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Germline chimeric chickens


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