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Rainbow trout chimeras produced by injection of blastomeres into recipient blastulae

(fish/*Oncorhynchus mykiss*/salmonid)

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ABSTRACT **In mammals, the ability to successfully introduce isolated cells into a recipient embryo and to document their development has been an important experimental advance in determining the developmental potential of cells. These techniques are also useful in reestablishing the genome of embryonic cells into a germ line. The objective of the present study was to determine whether blastomeres isolated from rainbow trout (*Oncorhynchus mykiss*) will incorporate and continue to develop when injected into recipient embryos. In the first experiment, donor cells, previously labeled by injecting fluorescein isothiocyanate-dextran into the zygote, were isolated from blastulae; ≈1000 of these cells were microinjected into each unlabeled recipient embryo of the same developmental stage. Following subsequent development through gastrulation, microscopic examination revealed that 19 of 114 injected embryos (17%) contained fluorescent cells. These labeled cells were present at numerous sites within embryos, and the pattern of distribution of these cells varied among embryos. In experiment two, blastomeres from normal diploid embryos were injected into triploid blastulae. The injected embryos were incubated until hatching and then sacrificed; cells from these embryos were dispersed and treated with 4′,6-diamidino-2-phenylindole. The proportion of diploid cells, as determined by flow cytometry, varied from 2.0% to 12%. From these results we conclude that blastomeres isolated from rainbow trout blastulae will incorporate and continue to develop following injection into recipient embryos.**

Chimeras are organisms composed of cells from different parental lineages. The pluripotential nature of mammalian blastomeres has been demonstrated in numerous studies by the experimental production of chimeras. In the initial studies, Tarkowski (1) and Mintz (2) demonstrated unequivocally that mammalian chimeras could be produced by combining the blastomeres of early mouse embryos. In these studies it was demonstrated that early mammalian embryos could aggregate together and then develop into a functional embryo. Subsequently, it was demonstrated that cells isolated from the inner cell mass and injected into the blastocoel of a recipient mammalian blastula would form a functional chimera (3, 4), as would injection of cultured cells derived from teratocarcinomas (5) and embryonic stem cells (6). In the chimeras produced by any of these methods, it has been clearly shown that the germ line can be made up of cells derived from both embryonic sources (7).

The successful experimental formation of chimeric animals has also been useful in reestablishing isolated embryonic cells into a germ line. Embryonic stem cells, pluripotent cells that remain undifferentiated under proper culture conditions, have been established from embryos of the mouse (8, 9) and the hamster (10). Because embryonic stem cells contribute to the germ-cell population when injected into early embryos, these cells have been utilized for gene transfer in the mouse (11, 12).

Early development of the teleost fish embryo differs from that of mammals in that the fish embryo forms as a blastoderm on a large mass of yolk, similar to early development in the bird. For example, following 72 hr of incubation at 11.5°C, the rainbow trout (*Oncorhynchus mykiss*) blastula is composed of a single external layer of tightly joined cells enveloping a loose ball of deep, ameboid cells. Both of these cell types overlie the periblast, a syncytial layer that separates the yolk from the developing embryo. No true blastocoel is evident during teleost development; instead a space forms between the cellular blastoderm and the underlying periblast called a subgerminal cavity (13).

Results of transplantation and grafting studies by Oppenheimer (14) first suggested that cells of the blastula stage were pluripotent and that cell determination for fish occurs at the time of gastrulation. This conclusion is supported by more recent investigations of zebrafish cell lineage. Studies of the clonal progeny of specific blastomeres injected with a tracer dye show that the developmental fate of the descendants of specific cells varies between individual fish. This indicates that these blastomeres have a degree of pluripotency (15).

Because the introduction of blastomeres into the early embryo can be used to investigate cell determination and to transfer new genes into the germ line, the objective of this investigation is to produce chimeric rainbow trout. Our hypothesis is that blastomeres isolated from rainbow trout embryos will be incorporated and continue to develop when injected into the subgerminal cavity of recipient embryos.

MATERIALS AND METHODS

Gamete Handling. Rainbow trout (*Oncorhynchus mykiss*) gametes were obtained either from the Clear Springs Trout Company (Buhl, ID) or from Mt. Lassen Trout Farms (Red Bluff, CA) and stored at 2°C under 100% oxygen.

Eggs were fertilized by adding 50 μl of sperm to ≈200 eggs followed by the addition of 6 ml of an activating solution (94.4 mM NaCl/30.1 mM Tris/32.2 mM glycine). After 1 min, the eggs were flooded with dechlorinated tap water. Unless otherwise indicated, fertilized eggs were incubated in a Heath incubator (Heath Techna, Kent, WA) at 11–11.5°C.

Manufacture of Injection Micropipettes. Microhematocrit capillary tubes (VWR Scientific; no. 15401-650) were pulled with a vertical pipette puller (Kopf Instruments, Tujunga, CA). The pulled micropipette ends were cut with a DeFonbrune microforge (A. S. Aloe, St. Louis) and the tip was beveled to a 45° angle using a variable-speed diamond wheel pipette grinder.

Production of Fluorescent Blastomeres. To monitor the incorporation and subsequent development of isolated blas-

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Abbreviation: FITC, fluorescein isothiocyanate.

tomeres, donor embryos were labeled with fluorescein isothiocyanate (FITC)-dextran. Rainbow trout eggs were fertilized and incubated at 11.5°C for 12 hr. The blastodisc was well formed at this time and was injected with 0.02 μl of 12% FITC-dextran (Sigma, no. FD-4) in 0.1 M KCl (16), using a glass microinjection pipette held in a Leitz micromanipulator as described by Chourrout *et al.* (17) with the modification that a microsurgery probe (Micromanipulator Microscope, Carson City, NV) was used to incise the outer chorion layer.

Production of Fluorescent Chimeric Embryos. After ≈3 days of incubation, the embryos labeled at late stage 6 or early stage 7 (13) were manually dissected free of their chorion, and the blastoderm was separated from the yolk sac and incubated in Ca^{2+}-, Mg^{2+}-free Nui Twitty's solution (18). The resultant isolated blastomeres were aspirated into a microinjection pipette with a tip measuring 40–60 μm (inside diameter). Approximately 1000 FITC-dextran-labeled blastomeres were injected into the subgerminal cavity of nonfluorescent recipient embryos of the same developmental stage.

In all replicates noninjected fertilized trout eggs served as controls, and only replicates in which >80% of the controls developed normally were used.

Determination of Fluorescent Blastomere Incorporation. Two to 5 days after blastomere injection, recipient embryos were examined for the presence of fluorescein-labeled blastomeres. For each injected embryo, the chorion was manually removed, and the blastoderm was dissected free of its yolk sac and examined using an inverted microscope (Nikon Diaphot) equipped with epifluorescence. The presence of incorporated fluorescent cells was considered evidence of embryonic chimerism.

Production of Triploid Recipient Embryos. Eggs were fertilized as described above. At 10 min postfertilization, the eggs were immersed in a 28°C water bath for 10 min (19). These conditions inhibited the extrusion of the second polar body, resulting in triploid zygotes.

Injection of Diploid Blastomeres into Triploid Recipient Embryos. After ≈3 days of incubation, diploid donor embryos were dissected free of their chorion, and the blastoderm was separated from the yolk sac and incubated in Ca^{2+}-, Mg^{2+}-free Nui Twitty's solution (18). The resultant isolated blastomeres from the diploid embryos were aspirated into a microinjection pipette, and ≈1000 donor blastomeres were injected into the subgerminal cavity of triploid recipient embryos of the same developmental stage.

Determination of Diploid/Triploid Chimerism. Sac fry from diploid and triploid control groups and from the injected treatment group were sacrificed from 1 week prior to the time of expected hatch to 2 weeks after hatching. In all cases the fry were dissected free of their yolk sac and finely minced in a solution of 10 mM Trisma base/0.8% NaCl/1 mM $CaCl_2$/1 mM $MgCl_2$/0.05% bovine serum albumin/0.1% Nonidet P-40 (Sigma no. N3516)/10 μg of 4′,6-diamidino-2-phenylindole per ml (Accurate Chemicals, Westbury, NY, no. 18860). The minced material was filtered through a stainless steel screen, and samples were frozen in 10% dimethyl sulfoxide at −70°C. The samples were shipped on dry ice to the University of Washington for flow cytometric analysis of nuclear fluorescence to determine the proportion of diploid and triploid cells in each sample (20).

In addition to these flow cytometric studies, the number of chromosomes per cell in additional treated embryos was determined as described by Kligerman and Bloom (21).

RESULTS

Fluorescent Blastomere Incorporation. The embryos from uninjected control and treatment groups were incubated through gastrulation. All injected embryos that contained fluorescent and nonfluorescent cells appeared to have developed normally and were at the same developmental stage as uninjected controls. At the time at which the embryos were observed, the optic and otic vesicles were present, and the somites were well defined; these embryos were determined to be stage 13 of development (13).

Microscopic examination of the resultant embryos that survived and completed gastrulation revealed that fluorescent cells were present in 19 (17%) of the original 114 recipient embryos injected with donor blastomeres. The proportion of embryos that survived injection and the incidence of chimerism increased with successive replicates (Table 1).

Fluorescent cells were distributed throughout the injected embryos (Fig. 1) and appeared to be smaller than the FITC-labeled blastomeres initially injected into recipient embryos. The distribution of these fluorescent cells was not consistent within the injected embryos and varied among embryos. For example, fluorescent cells were present predominantly in the anterior or posterior region of some embryos or evenly scattered throughout others. Labeled cells were not associated with any single embryonic structure but were present in various tissues. No fluorescent cells were observed in embryos of the uninjected control group (data not shown).

Diploid/Triploid Chimerism. To evaluate whether the injected blastomeres were maintained throughout embryogenesis, it was necessary to find a longer-lasting cell marker. Since triploid rainbow trout are viable and since ploidy is a simple and reliable marker, triploid embryos were used as recipients, and injected blastomeres were derived from diploid embryos. Of six recipient embryos that survived to hatching and had their cells sampled, flow cytometry showed that five embryos contained diploid and triploid cells (Fig. 2*C*). The proportion of diploid cells in these embryos ranged from 2.0% to 12.0%, with a mean ± SEM of 3.7% ± 4.2%. No diploid cells were detected in the six triploid control embryos examined.

In an additional trial, five embryos underwent karyotyping. From the chromosome spreads, diploid and triploid cells were identified in three of five additional injected recipient embryos.

DISCUSSION

The results of these experiments demonstrate that isolated blastomeres from trout embryos have the potential to incorporate and continue development following injection into the subgerminal cavity of recipient embryos.

The wide distribution of labeled cells observed in these chimeric embryos suggests that injected blastomeres are contributing to the development of different types of embryonic tissues. These results support the hypothesis that blastomeres isolated from rainbow trout embryos at stage 6c to stage 7b (13) are pluripotential. This conclusion is supported by evidence in fundulus (14) and zebrafish (15) that pregastrulation stage embryonic cells are pluripotential.

It was noted that the donor-derived cells observed in chimeric embryos were qualitatively smaller than the FITC-labeled blastomeres initially injected into recipient embryos. Also, the hatched fry of the diploid/triploid chimera exper-

Table 1. Percentage of chimeric embryos formed by injecting FITC-dextran-labeled blastomeres into recipient embryos

Replicate	Embryos injected	% survival*	% chimerism†
1	40	17.5	7.5
2	45	28.9	6.7
3	29	55.2	44.8

*Proportion of embryos injected that were alive at the time of assay for chimerism.

†Proportion of embryos injected that contained FITC-dextran-labeled cells when assayed: (no. FITC-positive − no. injected) × 100.

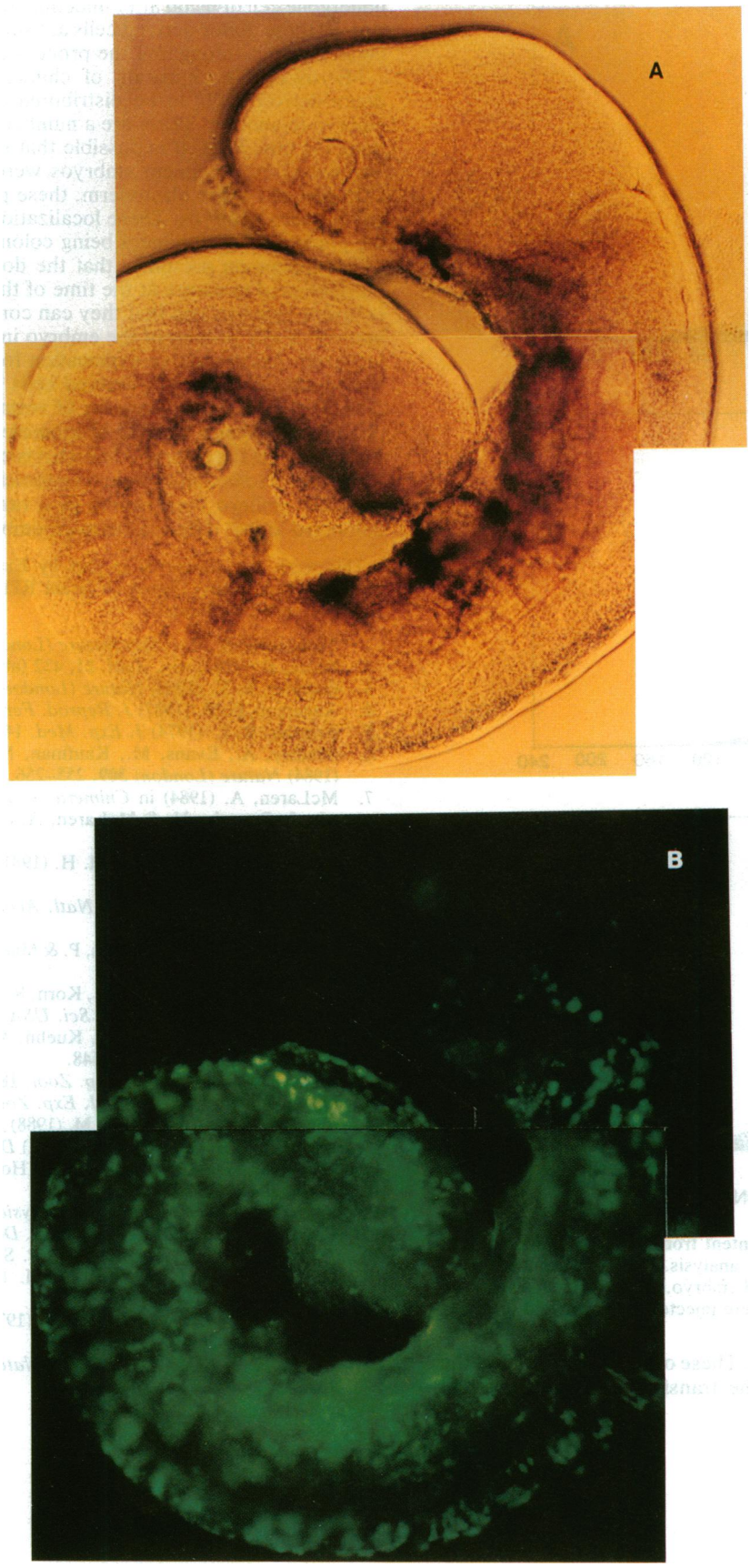

FIG. 1. Chimeric fish embryo produced by injecting fluorescent blastomeres into unlabeled recipient blastulae. (*A*) Embryo as viewed with normal tungsten illumination. (*B*) The same embryo as viewed with epifluorescence. Green foci are cells derived from FITC-dextran-labeled blastomeres that have become incorporated into the developing recipient embryo. (×115.)

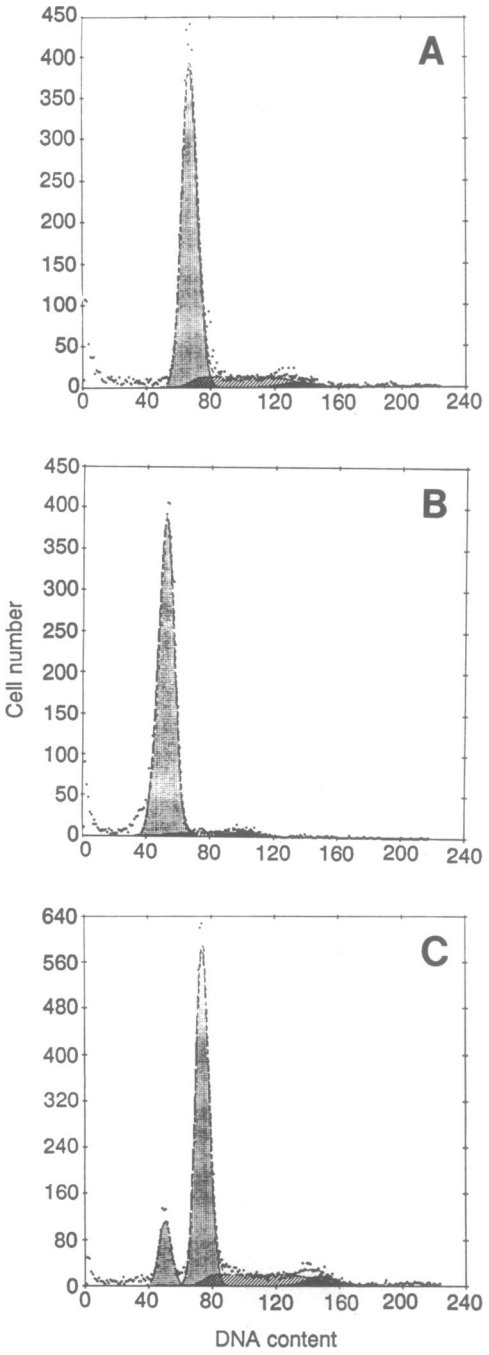

FIG. 2. Profiles of DNA content from dispersed rainbow trout embryos using flow cytometric analysis. (*A*) Cells from a triploid embryo. (*B*) Cells from a diploid embryo. (*C*) Cells from an embryo in which diploid blastomeres were injected into a triploid blastula.

iment contained diploid cells. These observations support the following conclusions: (*i*) the transferred blastomeres had undergone cell division after injection into recipient embryos and (*ii*) these donor-derived cells are stably maintained in the chimeric trout throughout the process of embryogenesis.

Microscopic examination of chimeric embryos showed donor-derived cells to be distributed differently from one embryo to another. There are a number of possible explanations for this. First, it is possible that the donor cells being introduced into recipient embryos were localized into specific portions of the blastoderm, these portions being different between embryos. These localizations resulted in differing portions of the chimera being colonized by donor cells. This explanation presumes that the donor blastomeres are pluripotential in nature at the time of their introduction into recipient embryos and that they can contribute to the development of any portion of the embryo in which they become localized. An alternative explanation for the differing distribution patterns of donor-derived cells between embryos is that the donor blastomeres are of restricted developmental potential, but each injected blastomere will migrate (22) to that portion of the embryo of which it is capable of colonizing. The random selection of donor blastomeres injected into each recipient embryo then results in a random distribution of colonized areas, differing between embryos.

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