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An *in situ* assessment of the routes and extents of colonisation of the mouse embryo by embryonic stem cells and their descendants

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Summary

An embryonic stem (ES) cell line stably expressing *lacZ* under the control of an endogenous promoter has been isolated and used as a marker to follow the fate of ES cells injected into blastocysts and morulae, before midgestation. The results show a multisite pattern of blastocyst colonization by ES cells deposited into the blastocoel cavity and a low degree of mingling between ES cells and ICM cells. Furthermore, analysis of dispersal of ES cell descendants in postimplantation chimaeric embryos showed that colonization can be highly variable from one region of the embryo to another. In contrast, a high and reproducible degree of chimaerism was obtained when the ES cells were injected at the morula stage prior to ICM formation.

Key words: embryonic stem cells, chimaera, mouse embryo, β-galactosidase marker.

Introduction

Embryonic stem (ES) cells are established cell lines derived from the inner cell mass (ICM) of a mouse blastocyst (Evans and Kaufman, 1981). After microinjection into a host blastocyst, they can differentiate into any tissue of the conceptus, including the extraembryonic tissues (Beddington and Robertson, 1989; Suemori *et al.* 1990) and the germ line (Bradley *et al.* 1984; Gossler *et al.* 1986; Robertson *et al.* 1986). Furthermore they can be manipulated and cloned *in vitro* so as to provide genetically modified cells (Robertson *et al.* 1986). ES cells have been used to introduce mutations into the germ line of chimaeric mice, particularly targeted mutations obtained *via* homologous recombination in order to generate mutant mouse strains for desired genes (Koller *et al.* 1989; Schwartzberg *et al.* 1989; Thompson *et al.* 1989; Zijlstra *et al.* 1989).

To understand better the routes and extent of ES cell colonization of chimaeric mouse embryos, we have developed an intracellular genetic tracer. An ES cell line called N1 was created, which expressed to a high and stable degree the *lacZ* reporter gene coding for the β-galactosidase, an enzyme whose activity can be followed *in situ* directly on whole-mount embryos (Beddington *et al.* 1989).

We have used these marked cells to study their early fate in the preimplantation blastocyst and to assess the degree of ES cell descendant dispersal through the beginning of gastrulation. From 5.5 to 8.5 days *p.c.*, β-galactosidase activity was detected in every derivative of the embryonic ectoderm of chimaeric embryos but not in primitive endoderm or trophectoderm derivatives. Our results show that once ES cells are injected into the blastocoel cavity, (1) they often attach to the mural trophectoderm, (2) those that do attach to the ICM do not mingle extensively with ICM cells. A non-uniform pattern of chimaerism was also observed in the embryonic ectoderm of some postimplantation embryos. The results obtained by injection into blastocysts led us to test other methods of microinjection. We found that injection of ES cells in the morulae prior to ICM formation was an efficient way to increase the degree of chimaerism of the embryos.

Materials and methods

DNA

pGNA, the plasmid used in this work, is shown in Fig. 1. It is a derivative of a vector, pGN, used in gene-targeting experiments and described in detail elsewhere (Le Mouellic *et al.* 1990). It has a functional *E.coli lacZ* gene, with an eucaryotic translation initiation sequence immediately following a multiple cloning site. A neomycin-resistance transcription unit within pGNA was optimized for expression in ES cells so as to give the maximum number of G418-resistant clones.

In the present study, pGNA was linearized before transfection at the *Sac*II site, in the multiple cloning site. Since *lacZ* in pGNA does not have a promoter, *lacZ* gene expression in transfected ES cells was dependent upon the insertion of pGNA into the genome near a promoter functional in ES cells.

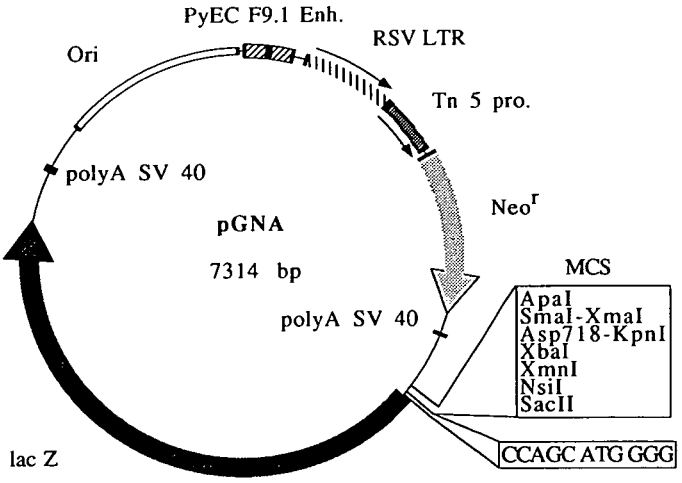

Fig. 1. pGNA plasmid vector. The neomycin resistance transcription unit begins with a tandem direct repeat of the enhancer unit B of the polyoma virus (a *Pvu*II–*Pvu*II fragment from the PyEC F9.1 strain (Herbomel *et al.* 1984)), in the late mRNAs orientation. An eukaryotic promoter, the RSV LTR, and a prokaryotic promoter, from Tn5, direct the transcription of the phosphotransferase gene Neo^r. The 5′ sequence flanking the Neo^r AUG initiation codon was modified to the vertebrate consensus sequence, CACCATG (Cavener, 1987), in order to increase the translation initiation efficiency in mammalian cells. *lacZ* represents the coding sequence of the *E.coli* β-galactosidase with a modified initiation codon fitting the vertebrate consensus sequence and is followed at the 3′ end by the SV40 early polyadenylation and transcription termination signals. A multiple cloning site has been inserted 5′ of the *lacZ* gene. Ori is the col E1 origin of replication. Before electroporation, pGNA was linearized at the *Sac*II unique site.

ES cell culture

The D3 ES cell line used in the following experiments was a gift from R. Kemler. It was originally derived from an XY blastocyst of the 129/Sv+/+ strain (Doetschman *et al.* 1985). Cells were cultured on a layer of primary embryonic fibroblasts as described by Robertson (1987) in Dulbecco's Modified Eagles Medium (DMEM) containing 15% (v/v) heat-inactivated fetal calf serum, in a 37°C, 5% CO_2 humidified incubator.

Electroporation and clone isolation

Electroporation was carried out in HeBS medium (20 mM Hepes pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose) (Chu *et al.* 1987) using a Gene Pulser^{TM} apparatus (Bio Rad) and the Special Gene Pulser^{TM} cuvettes (Bio Rad). 2 to 5×10^6 cells were submitted to an electric discharge (200 V; 960 μF) in the presence of 20 μg linearized pGNA plasmid. Selection was applied 24 h after the discharge at 250 μg ml^{-1} of G418. G418-resistant (neo^R) clones were visible after 12 days of selection.

After the pulse, the transfected cells were plated onto four 24-well tissue-culture dishes (Costar), so as to have no more than 4 to 5 clones per well. After 24 h selection, each well was subcultured into two wells and after three days X-gal staining was performed on one of the wells according to the following protocol: each plate was washed three times in phosphate-buffered saline (PBS), fixed in PBS containing 0.2% glutaraldehyde and 1% formaldehyde for five minutes, washed twice in PBS prior to staining in X-gal buffer (0.4 mg ml^{-1} X-gal, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6.3H_2O$, 4 mM $MgCl_2$: in PBS) overnight at 37°C.

For X-gal-staining-positive clones, cells in the original well were reseeded at low density for cloning purposes. Clones were picked with a fine pipette and seeded in duplicated wells for β-galactosidase assay. The cloning procedure was repeated twice. Isolated β-galactosidase producing ES clones were frozen and/or used directly for microinjection.

Embryoid bodies

Embryoid bodies were obtained as described elsewhere (Robertson, 1987), by culturing small aggregates of ES cells in DMEM plus 10% v/v fetal calf serum in bacterial culture dishes for a week. X-gal staining was performed following the same protocol as for ES cells.

Chimaeric embryos

Morulae and blastocysts were obtained from 4 week old, superovulated females mated with males of the same strain (F_1: Balb/c×SJL/J). The embryos were kept in DMEM microdrops covered with paraffin oil in a humidified incubator (37°C, 5% CO_2). Microinjections of ES cells into blastocysts were performed in DMEM microdrops as described by Bradley (1987). Injected blastocysts were reimplanted in the uterus of pseudopregnant recipient F_1 females (2.5 days *p.c.*) or cultured in DMEM. Microinjections of ES cells in morulae were performed in the same conditions except that, when necessary, morulae were kept in calcium-free PBS at 37°C to make them decompact. ES cells were injected through the zona pellucida and deposited between the blastomeres in the core of the embryo. Microinjected morulae were reimplanted in the oviduct of pseudopregnant recipient F_1 females (0.5 day *p.c.*).

X-gal staining of chimaeric embryos

Postimplantation embryos were recovered at the appropriate stages, considering the day of the plug of the pseudopregnant recipient mother as day 0.5 of development. Embryos were dissected in PBS and the protocol for β-galactosidase detection described above was applied as for cells but with a 20 min fixation and several 10 min washings.

Results

Construction of ES cell line N1

To study how ES cell descendants colonize the embryo between blastocyst and gastrula stages, we required an embryonic stem cell line that would not give any bias in its colonization of the various tissues and which would also express an *in situ* marker in the embryonic ectoderm cells and their descendants. To obtain such a cell line, we first put the *lacZ* gene under the control of endogenous promoters in transfected ES cells. Cell lines resulting from transfection and G418 selection were then screened for the capacity to express *lacZ* in the embryonic ectoderm and its derivatives in chimaeric embryos (Gossler *et al.* 1989).

The linearized pGNA was electroporated under conditions which mostly yield a single insertion in the genome (Boggs *et al.* 1986). In our construct, the *lacZ* gene lacked its own promoter. Its activity in ES cells was therefore dependent upon its insertion in the

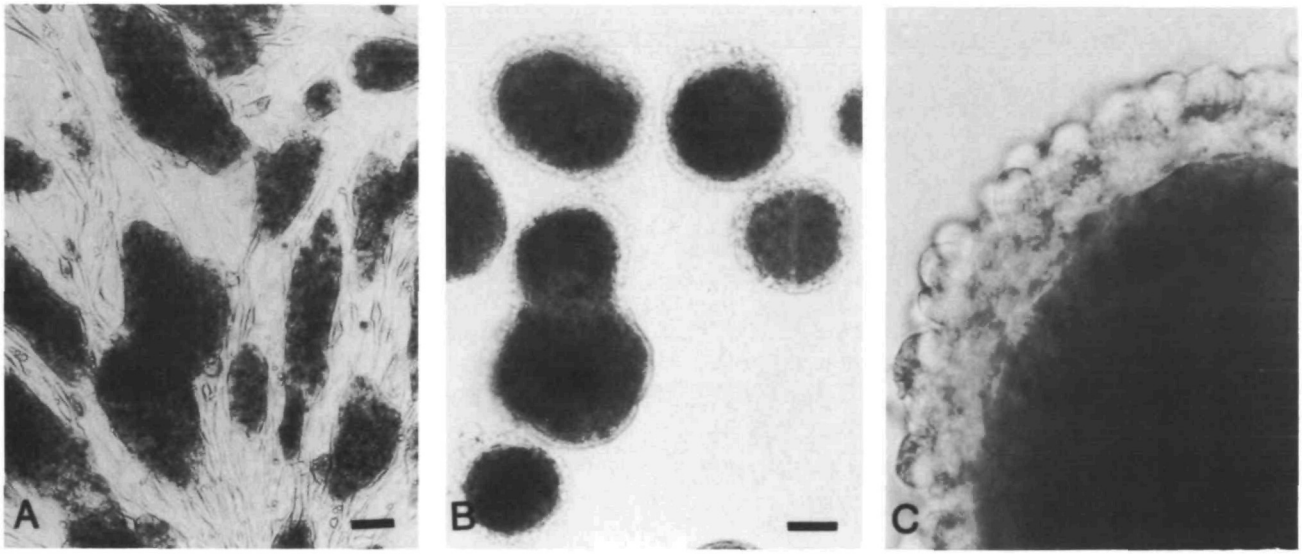

Fig. 2. N1 cells stained with X-Gal, in culture and after embryoid body formation. (A) N1 cells cultured on a primary embryonic fibroblast feeder-layer. All the ES cells exhibit β-galactosidase activity and appear blue after X-Gal staining, whereas embryonic fibroblasts are unstained. Bar, 100 μm. (B) Embryoid bodies obtained in bacterial dishes after one week. The layer of endodermal cells surrounding the core of β-galactosidase-expressing undifferentiated cells does not express *lacZ*. Bar, 100 μm. (C) High magnification of an embryoid body showing the demarcation of the endodermal cells and the inner core. Bar, 10 μm.

vicinity of an endogenous promoter active in ES cells. About 1% of the G418^R clones stained positively after X-gal treatment, indicating the production of β-galactosidase. Twelve clones were selected for subcloning, according to the level of expression of the enzyme and the percentage of β-galactosidase-expressing cells per clone. In one clone, named N1, 100% of the cells expressed *lacZ* when regularly passaged at low density (Fig. 2A).

Embryoid bodies were produced with N1 cells. In our culture conditions, a layer of mostly visceral endoderm appeared surrounding a core of undifferentiated ES cells (Fisher *et al.* 1989). Only the ES cells in the core of the embryoid body expressed *lacZ* whereas the endoderm cells displayed no β-galactosidase activity (Fig. 2B,C).

Further experiments, as described below, established that the pattern of *lacZ* expression in N1 cells allowed these cells to be used as an *in situ* marker in chimaeric embryos. The expression of *lacZ* in the other clones will not be discussed.

Colonization of preimplantation blastocysts by N1 cells

The early fate of N1 cells was first examined soon after injection into blastocysts. Several series of injections were performed with either a single N1 cell or multiple cells (15–20) deposited into the blastocoel cavity. Injections were done as precisely as possible by perforating the trophectoderm in front of the ICM to allow the later determination of this point, referred in the text as the injection point, after reexpansion of the blastocysts.

Injection of single cells

18 h after microinjection of single N1 cells, X-gal staining was performed. The results are summarized in Table 1a. The blastocysts could be distributed into three categories. (1) Those displaying no labelled cells in the blastocoel; the injected cell had either not colonized the embryo or was localized between the zona pellucida and the trophectoderm. (2) Blastocysts with a single N1 cell attached to the surface of the inner

Table 1. *Frequency of chimaerism among injected blastocysts and localization of the N1 ES cells*

Number of blastocysts microinjected*	Number of chimaeric blastocysts	N1 cells in ICM only	N1 cells in trophectoderm only	N1 cells in ICM and trophectoderm
(a) 79	14	6	8	0
(b) 53	39	11	5	23
(c) 16	14	2	1	11

* only well reexpanded blastocysts were taken in account.
(a) injection of a single cell and staining after 18 hours.
(b) injection of several cells (15–20) and staining after 18 hours.
(c) injection of several cells (15–20) and staining after 40 hours.

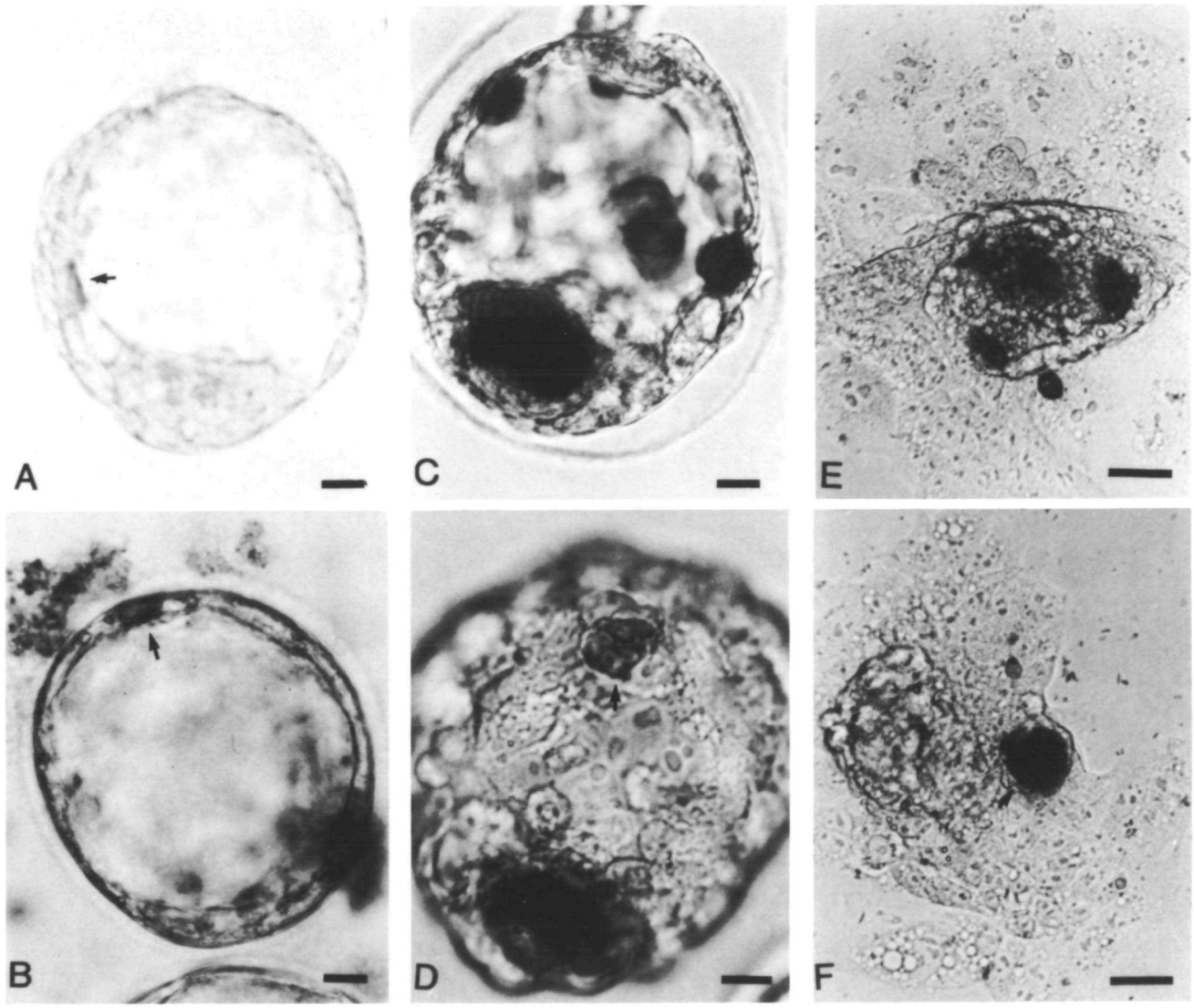

Fig. 3. Blastocysts stained with X-Gal after injection of N1 cells in the blastocoel through the mural trophectoderm. (A,B) Blastocysts after injection of a single N1 cell and 18 h of *in vitro* development. (C,D) Blastocysts after injection of 15–20 N1 cells and, respectively, 18 and 40 h of *in vitro* development. (E,F) Blastocysts after injection of 15–20 N1 cells and spreading of trophectoderm in Petri dishes. (A) A single N1 cell, indicated with a black arrow, is present at the border of the ICM. Bar, 10 μm. (B) A N1 cell, indicated with a black arrow, has been trapped in the trophectoderm, probably at the injection point (i.e: the hole made by the injecting pipette during microinjection). Bar, 10 μm. (C) A blastocyst displaying multicolonization by N1 cells. The ICM (lower part of the photograph) is highly colonized. N1 cells are also present in three other sites on the mural trophectoderm. Bar, 10 μm. (D) After 40 h of *in vitro* development, N1 cells, indicated with a black arrow, were still attached on the trophectoderm and displayed an undifferentiated aspect. The ICM (lower part of the photograph) is also colonized by N1 cells. Bar, 10 μm. (E) After spreading of the trophectoderm, a chimaeric blastocyst displayed few patches of β-galactosidase-expressing cells in its growing ICM. Bar, 20 μm. (F) A patch of N1 cell progenies, indicated with a black arrow, present apart from the ICM (left of the photograph) and not displaying any sign of differentiation. Bar, 20 μm.

cell mass, frequently at a border with the trophectoderm (Fig. 3A). No ES cell was ever found in the core of the inner cell mass. (3) Blastocysts with a blue cell on the surface of the trophectoderm. In one case, we found a single *lacZ*-expressing cell apparently trapped inside the trophectoderm. (Fig. 3B).

Injection of several (15–20) cells

Results similar to the previous ones were obtained after microinjection of 15 to 20 N1 cells and staining the day following microinjection. They are summarized in Table 1b. Many instances of ICM and trophectoderm colonization at the same time were observed. In 11 out of 39 colonized blastocysts, X-gal-positive cells were localized at the supposed injection point in front of the ICM. In other cases, blue cells were present on the trophectoderm, away from the injection point. In three blastocysts, more than one site of the trophectoderm

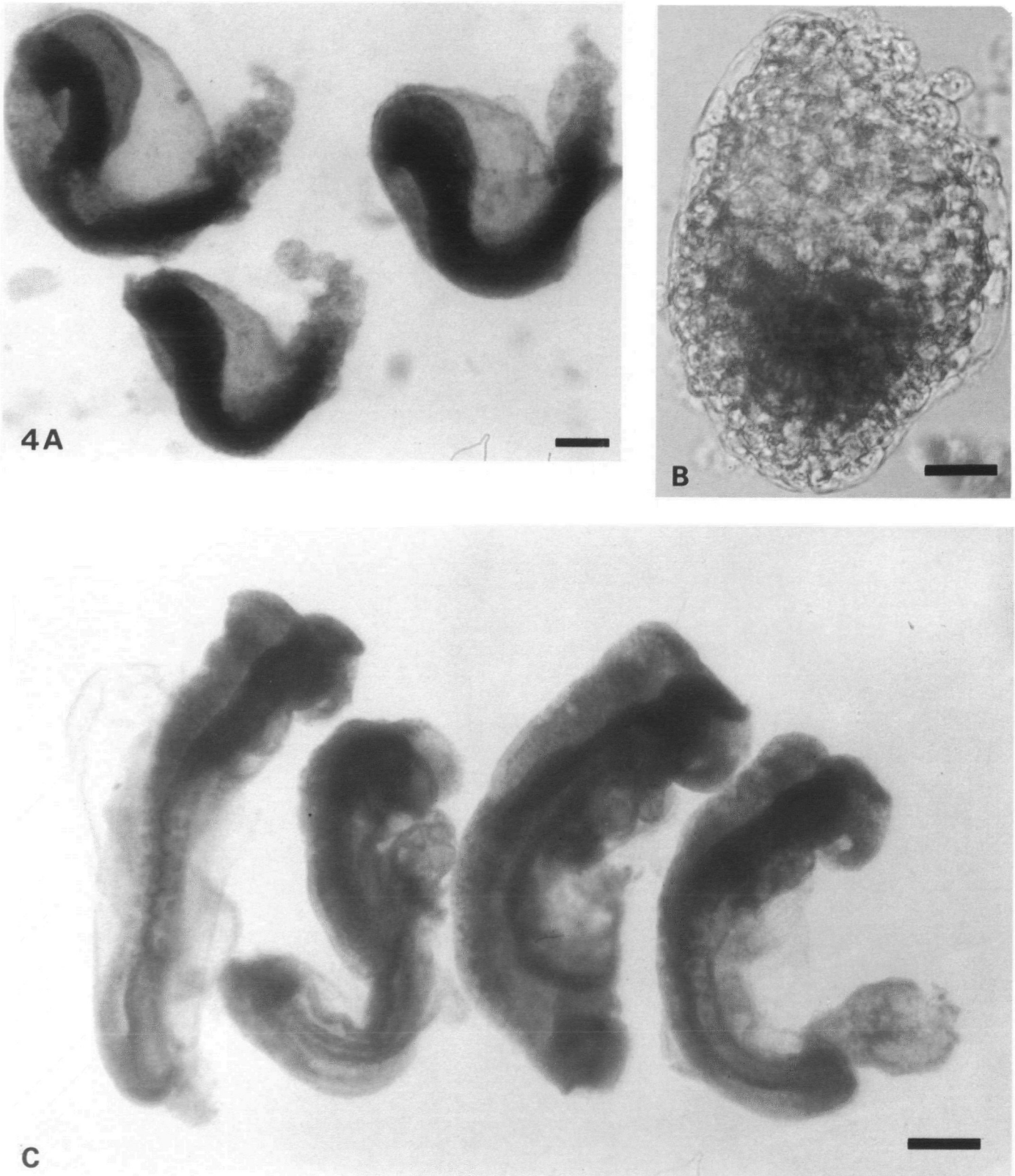

Fig. 4. Chimaeric embryos obtained by injection of about 10 N1 cells into Balb/c×SJL/J F_2 morulae (2.5 days *p.c.*) and stained with X-Gal. (A) Three 8.5 day old embryos of the same litter. Bar, 150 μm. (B) A 5.5 day old embryo. *lacZ* is highly and uniformly express in the embryonic ectoderm whereas no β-galactosidase activity can be detected either in the extraembryonic ectoderm or in the visceral endoderm. Bar, 20 μm. (C) Four 9.5 day old embryos from the same litter. Bar, 100 μm.

was colonized by the N1 cells (Fig. 3C), indicating that N1 ES cells could attach to the inner surface of the blastocyst outside the inner cell mass and outside the hole made by microinjection. Despite attachment to the trophectoderm, none of the injected cells displayed a trophectoderm cell morphology (large, flattened, polygonal). After a night in culture, all of these trophectoderm attached cells showed a morphology resembling that of ES cells (small cells with large nuclei, tightly bound together).

To ask if N1 ES cells attached onto the trophectoderm after microinjection would migrate to the inner cell mass, microinjected blastocysts were cultured in bacterial dishes for two days after microinjection. Results are summarized in Table 1c. After 48 h, N1 cells were still detected attached to the trophectoderm, sometimes in several sites, and still presenting the morphological aspect of ES cells (Fig. 3D).

As it was difficult to observe the ICM clearly in expanded blastocysts, a third experiment was done in which microinjected blastocysts were allowed to attach on the surface of cell culture Petri dishes. After two days of culture, trophectoderm spreads to form a layer of trophoblast giant cells surrounding the growing ICM (Hogan *et al.* 1986). It thus became easier to distinguish different cell types and to analyse N1 cells distribution among the different tissues of the spread blastocyst. Colonies of small blue cells resembling ES cells were detected in numerous cases (10/13), sometimes at a location distinct from the growing ICM (Fig. 3F). No blue trophoblast cells were ever observed after staining. Distinct patches of blue cells could also be distinguished within the ICM, showing that injected cells had clumped together within the ICM rather than mingling as individual cells with the ICM cells (Fig. 3E).

Chimaeric embryos obtained by injection of N1 cells into morulae

The preceding experiments showed that once injected through the mural trophectoderm, many ES cells do not colonize the ICM but instead attach to the trophectoderm, decreasing the probability of obtaining a high and reproducible level of chimaerism in subsequent chimaeric embryos. To enhance the degree of embryo chimaerism and to determine more rapidly the pattern of expression of the endogenous promoter driving *lacZ*, we studied another technique for producing chimaeric mice by injecting ES cells into morulae prior the formation of the ICM, assuming that this might give a developmental advantage to the ES cells.

Blastocyst stage

About 10 N1 cells were first injected in 2.5 day old decompacted morulae (8–16 blastomeres). After a night in culture most morulae had evolved to blastocysts, but they were not well expanded and the ICMs were difficult to visualise. This may have been due to the medium (DMEM), which is optimum for ES cell growth and blastocyst culture but not for growth of precompaction embryos (Bradley, 1987). Nevertheless, X-gal staining showed that 13 blastocysts among 21 were chimaeric. No patch of blue cells was detected on the trophectoderm.

Postimplantation stages

In a second experiment, ten similarly injected morulae were reimplanted into the oviduct of a recipient pseudopregnant mother. Embryos were recovered and stained at 8.5 days of gestation. Out of ten decidua visible in the uterus, seven were found to be empty, with the remaining three containing well-developed embryos. After X-gal staining these three embryos turned out to be highly colonized by N1 progeny (Fig. 4A) to a degree we had never obtained by blastocyst injection (see below). All the tissues derived from the embryonic ectoderm (embryo, allantois, amnion and mesodermal part of the yolk sac) were deeply and uniformly blue after X-gal staining. The tissues derived from the trophectoderm and the primitive endoderm did not stain blue.

At 9.5 days *p.c.*, two litters of four embryos each were recovered. The eight embryos were highly chimaeric (Fig. 4C), but apparently retarded in their development.

From 5.5 to 7.5 days *p.c.* (Fig. 4B), all the embryos recovered except one displayed a high degree of N1 cell colonization in the embryo proper but no β-galactosidase activity could be detected in the trophectoderm and extraembryonic endoderm derivatives. All the embryos recovered from 5.5 to 8.5 days *p.c.* displayed a normal morphology.

Postimplantation chimaeric embryos obtained by injection of N1 cells at the blastocyst stage

The results obtained in the previous experiments showed that the N1 cells could colonize all the embryonic ectoderm derivatives to a high degree without perturbing the development of the chimaeric embryos, at least until 8.5 days of gestation, and that the endogenous promoter was uniformly active in all these tissues. Thus the N1 clone appeared appropriate to study ES cell descendant dispersal during early embryogenesis. In order to assess cell mingling after the formation of the ICM, we injected ten N1 cells into blastocysts and recovered the embryos at different stages.

Primitive streak stage (8th day)

In this experiment, embryos were recovered at 7.5 days *p.c.* Chimaeric embryos displayed in Fig. 5A,B were obtained from the same microinjection experiment. In one case, the whole embryonic ectoderm had been colonized by ES cells. In the less-chimaeric embryo shown in Fig. 5B, the allantois was strongly colonized whereas the other tissues were virtually unstained. Other embryos displayed various degrees of colonization. No β-galactosidase staining was detected in the visceral endoderm even in the most chimaeric embryo (Fig. 5A).

Early embryonic ectoderm (6th day)

Seventeen embryos were recovered at this stage. In one

embryo, the degree of chimaerism was low, with N1 descendants uniformly dispersed throughout the embryo; the three other chimaeric embryos displayed patches of β-galactosidase-expressing cells. No dispersed N1 descendants could be detected in these three embryos.

Discussion

The N1 ES cell line: an in situ *cell marker in chimaeric embryos*

Several ES cell lines isolated from cultured blastocysts have been shown to colonize, upon blastocyst injection, the various embryonic tissues including the germ line, and to give chimaeric animals (Robertson *et al.* 1986; Gossler *et al.* 1986). However, the developmental status of ES cells is not yet quite established. They could be derived from some embryonic ectodermal cells (Evans and Kaufman, 1981) which, upon *in vitro* culture, revert to a more primitive cell type (Gardner and Beddington, 1988). Alternatively ES cultures could be a heterogeneous mixture of ectodermal and more primitive cells (discussed in Beddington and Robertson, 1989). In this case, upon injection into a blastocyst a bias toward colonization of the embryo might occur. Evidence was recently provided that ES cells contribute to derivatives of trophectoderm and primitive endoderm, although at a low frequency (Beddington and Robertson, 1989). It has been shown that early ICM cells display a similar behaviour after formation of chimaeras (Rossant and Tamura Lis, 1979), which suggests that ES cells most closely resemble early ICM cells (discussed in Beddington and Robertson, 1989).

Upon injection of N1 ES cells into blastocysts, we noticed that degrees and patterns of chimaerism varied among animals. But when N1 cells were injected prior to ICM formation, very strong and reproducible chimaerism was obtained, suggesting that chimaerism was dependent on the embryonal stage of development at which ES cells are introduced. When injected at the morula stage, N1 ES cells contributed heavily to the ICM. This supports the suggestion of Beddington and Robertson (1989) that ES cells resemble early embryonic cells (ICM cells) rather than multipotent cells of later stages (i.e. embryonic ectoderm cells of the 'egg cylinder' stage).

The intracellular marker chosen for this work is a reporter gene, *E. coli* β-galactosidase, whose activity can be visualized *in situ* directly on whole embryos. Such an *in situ* genetic marker was described in a transgenic mouse strain carrying several copies of *lacZ* under the control of the rat β-actin promoter (Beddington *et al.* 1989); β-galactosidase activity was detected in every epiblast derivative between 7.5 and 9.5 days *p.c.*, but no data were reported on earlier postimplantation stages. In contrast, we selected our N1 ES cells so that they could be a tool for cellular studies in early embryos before gastrulation. In those cells, *lacZ* expression was under the control of a functional endogenous promoter and, in our culture conditions, 100% of N1 cells expressed *lacZ* to a high level and in a stable manner. As seen also in Fig. 4A,B, a uniform X-gal staining was obtained in the embryonic ectoderm and its derivatives from 5.5 to 8.5 days *p.c.* without perturbing the embryos development. The promoter driving *lacZ* expression in the N1 clone was widely and uniformly active in all embryonic tissues during this period.

However, in embryos of various stages, no labeling was ever detected in primitive endoderm or trophectoderm derivatives. In the present study, we could not therefore evaluate *in situ* the contribution of N1 ES cells to the extraembryonic tissues.

Finally, as shown with the blastocysts injected with single N1 cells, the fate of single injected cells could be followed *in situ* and the distribution of their progenies in the embryos observed. N1 cells could therefore be used as an unbiased intracellular marker to study, eventually at the single cell level, the routes and extent of embryo colonization by ES cells descendants up to the ninth day of gestation.

Pattern of chimaerism obtained after ES cell injection into blastocysts and morulae

The blastocyst injection experiments, performed with single and multiple (up to 20) cells established that ES cells could bind to the ICM surface but did not mingle with the ICM cells, at least up to 18 h following microinjection. This was particularly obvious after single cell injections as shown in Fig. 3A, but also was evident when injected blastocysts were allowed to attach onto a substratum. Blue cells were clumped together and did not mingle as individual cells with ICM cells (Fig. 3E). Little dispersion of ES cells was also observed in some of the 5.5 day old chimaeric embryos; N1 descendants were not dispersed but remained as discrete patches of cells.

When X-gal staining of injected N1 ES descendants was performed on the gastrulating 7.5 day old embryos, we found that chimaerism varied not only from an embryo to another but could also, in a given embryo, vary from one region to another. This is illustrated in Fig. 5A,B. Although the embryonic ectoderm was colonized uniformly in some cases, we also found an example of strong colonization of mainly the allantois. These results suggest that a bias in the tissues that will become chimaeric can be introduced by the position injected ES cells assume relative to the ICM.

Highly variable results with blastocyst injections, together with the fact that many injected ES cells attached to the trophectoderm and did not colonize the ICM (Fig. 3C,D), led us to test another method of microinjection for its potential to enhance the degree of chimaerism. This method consisted of injecting ES cells into decompacted morulae before the formation of the ICM. A similar technique has already been described by aggregating morulae and clumps of ES or EC cells, but it requires the removal of the zona pellucida and culture of the morulae *in vitro* until blastocyst

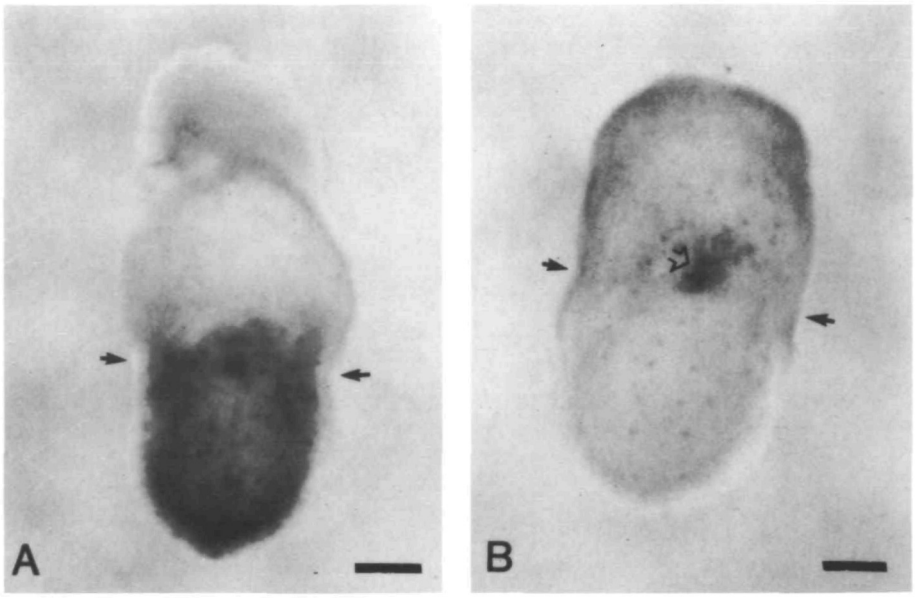

Fig. 5. Dorsal view of 7.5 day old chimaeric embryos obtained by injection, through the mural trophectoderm, of 10–15 cells into the blastocoel cavity of Balb/c×SJL/J F_2 blastocysts and stained with X-Gal. Embryos are placed with the extraembryonic part toward the top of the picture. Black arrows indicate the limit between the embryo proper and the extraembryonic cavity. (A) The embryonic ectoderm has been largely colonized by N1 cell progenies. Bar, 100 μm. (B) β-galactosidase-expressing cells are present mainly in the allantois (open arrow). Bar, 100 μm.

formation (Stewart, 1980; Stewart, 1982; Stewart *et al.* 1985). Our results suggest these delicate manipulations are unnecessary. By injecting ES cells into morulae through the zona pellucida, a high degree of chimaerism was obtained in nearly all the chimaeric embryos obtained. With the β-galactosidase marker, we could verify that all the embryonic tissues, from 5.5 to 9.5 days *p.c.*, were colonized at a high level and reproducibly by N1 progenies.

Through gene targeting in ES cells, strains of mice are being obtained with specific mutations in defined genes (Koller *et al.* 1989; Schwartzberg *et al.* 1989; Thompson *et al.* 1989; Zijlstra *et al.* 1989). A recent work has shown that the choice of the host strain is an important factor in obtaining a high degree of chimaerism and subsequently successful germ line transmission in chimaeras after blastocyst injection (Schwartzberg *et al.* 1989). According to the authors, the most efficient strain for host blastocysts is the C57Bl/6 strain. In this paper, we report that the degree of chimaerism is also influenced by the developmental stage of the host embryo.

These two factors are possibly correlated. We have noticed in our laboratory that C57Bl/6 3.5 day old blastocysts are retarded in their development as compared with those of other strains (unpublished observation) and hence are being injected at an earlier stage of blastocyst development. Our work indicates that strong chimaerism is obtained when ES cells are injected in decompacted morulae, before ICM formation. This suggests that the precise stage at which embryos are microinjected (morula, early cavitating blastocyst, well-expanded blastocyst) might influence the efficiency of ES cell colonisation and subsequently the probability of obtaining germ-line chimaeras. We are now optimizing the different parameters (number of cells injected; injection into morulae, early or late blastocysts etc...) of this technique for the germ line colonization of chimaeric mice by ES cells.

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