

An autoradiographic analysis of the potency of embryonic ectoderm in the 8th day postimplantation mouse embryo

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SUMMARY

The potency of 8th day mouse embryonic ectoderm cells has been studied by injecting them into synchronous embryos which were subsequently cultured for 36 h. The development of injected embryos *in vitro* was comparable to that of embryos maintained *in vivo*. Tritiated thymidine was used to label the donor cells so that chimaerism could be analysed histologically. The results demonstrate the pluripotency of embryonic ectoderm *in situ* in the late primitive-streak-stage embryo. In addition, the patterns of donor cell colonization vary according to the site of origin and injection of the donor tissue.

INTRODUCTION

Analysis of cell lineages in the mammalian embryo at the time of gastrulation has been severely hampered by the inaccessibility of the embryo *in utero*. One solution to this problem is to study embryos grown in culture. Rat embryos can develop successfully *in vitro* from the pre-primitive streak stage until the 30- to 40- somite stage, during which time gastrulation is completed and organ differentiation well advanced (Buckley, Steele & New, 1978). However, no comparable success has been achieved with mouse embryos over a similar period, although they can develop normally in culture from a later stage, the early somite stage, for the first 48 h of organogenesis (Sadler, 1979). Despite these recent advances in mammalian embryo culture, these systems have not been exploited for the purpose of studying cell fate in postimplantation embryos. Only indirect approaches have been adopted to analyse cell lineages in these later embryos. Culture and ectopic transfer of various tissues and of defined areas from the postimplantation embryo (Švajger & Levak-Švajger, 1974; Škreb & Švajger, 1975; Diwan & Stevens, 1976; Rossant & Ofer, 1977; Škreb & Crnek, 1977) have provided some insight into the developmental potential of particular cell types prior to organogenesis. Similarly, injection of post-implantation extraembryonic ectoderm and visceral endoderm into the pre-

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implantation mouse blastocyst (Rossant, Gardner & Alexandre, 1978) has suggested probable fates for these cells types in the later embryo.

It would seem important, if any understanding is to be gained of the mechanisms underlying tissue specification at the time of gastrulation, that lineage analyses should be made on the intact embryo. This paper describes a method for culturing 8th day mouse embryos from the late primitive streak stage, when gastrulation is still progressing, up until the early somite stage. Although this is a relatively short period (36 h) it is a time of intensive cell division and differentiation and is also marked by substantial morphogenetic activity. The developmental potential of embryonic ectoderm cells, from the late primitive streak stage, has been studied following the injection of these cells into 8th day embryos, which were subsequently grown in culture. As the culture period is short, tritiated thymidine (^3H thymidine) could be used to label the injected cells, thus allowing analysis of *in vivo* fates to be made at the histological level. A similar strategy has been used to study cell fate in the 9th day embryo (Copp & Beddington, in preparation).

MATERIALS AND METHODS

General strategy of experiments

Embryos of the late primitive streak stage were injected with ^3H thymidine-labelled donor cells and subsequently cultured for 36 h before being analysed autoradiographically for colonization by donor tissue. The validity of such an approach for studying cell fate in the postimplantation embryo is subject to several conditions. Figure 1 illustrates some of the different categories of embryos which served as controls to ensure that such conditions were met.

(1) The development of embryos which have been grown in culture must be comparable to that of embryos of an identical age which have been maintained *in vivo* (*in vivo* controls). Certain characteristics of all embryos grown in culture (*unlabelled controls*, *labelled controls* and *injected embryos*) were recorded and compared with those of *in vivo* controls.

(2) The ^3H thymidine-labelling conditions should be such that not only will all donor cells be labelled but also that sufficient ^3H thymidine will be incorporated to ensure the detection of donor cells, despite dilution of the label, after 36 h in culture. Furthermore, the incorporation of such a quantity of ^3H thymidine should not produce any adverse effects during subsequent development. Embryos fixed immediately after labelling (*uptake controls*) provide a standard for the initial labelling density as well as measure of the percentage of cells labelled. Embryos grown in culture for 36 h following labelling (*labelled controls*) provide a control for the dilution of label over the culture period and were also used in assessing the compatibility of radioisotope incorporation with normal development.

(3) Donor cells labelled with ^3H thymidine must be able to survive and proliferate in host embryos. A comparison of the grain density over donor

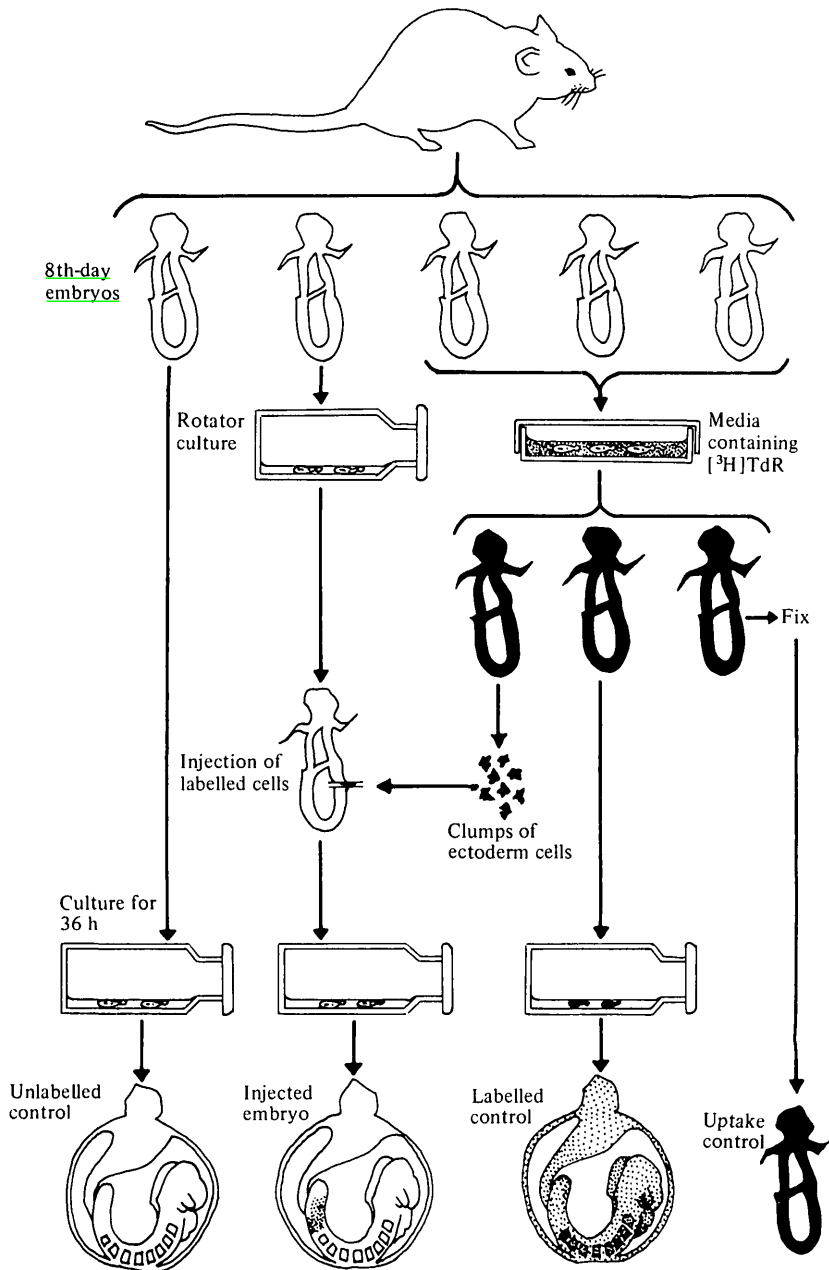


Fig. 1. A diagram of the general strategy of the experiments.

tissue in chimaeras with the density of labelling seen in both *uptake controls* and *labelled controls* provides an estimate of the extent of donor cell proliferation in *injected embryos*. The viability of [³H]thymidine-labelled cells after injection was tested directly by injecting labelled cells which also carried a genotypic isoenzyme marker. After culture, such *injected embryos* were analysed by starch gel electrophoresis thereby introducing an independent test for chimaerism and the viability of donor cells dependent on the expression of a ubiquitous enzyme.

Recovery and culture of embryos

All embryos were recovered on the morning of the 8th day of gestation, except for *in vivo controls* which were obtained 36 h later. Donor embryos were recovered either from random-bred CFLP mice or from CBA/H-T6 mice, which are homozygous for the *Gpi-1^b* allele of the enzyme glucose phosphate isomerase (GPI). All other embryos, including host embryos, were from CFLP mice and were *Gpi-1^a/Gpi-1^a*. The deciduae were removed from the uterus and the embryos dissected out into PBI medium (Whittingham & Wales, 1969) containing 10% foetal calf serum (FCS) instead of bovine serum albumen. Reichert's membrane with its attached trophoblast was removed using watch-makers' forceps. *In vivo controls* were obtained from CFLP mice and were recovered just before embryos were removed from culture thus ensuring that cultured embryos and *in vivo controls* were of the same age.

Embryos were cultured in 30 ml siliconized (Repelcote; Hopkins & Williams) glass bottles with ground glass stoppers (Scientific Supplies), on a roller system similar to that described by New, Coppola & Terry (1973). Each bottle contained two or three embryos, in 2.5 ml of medium, and was gassed with a mixture of 5% CO₂/5% O₂/90% N₂ (New, Coppola & Cockcroft, 1976a). For the first 6–10 h, embryos were grown in 50% rat serum in Waymouth's MB 751/2 medium (Flow Laboratories) supplemented with glutamine (0.35 mg/ml) and glucose (2.5 mg/ml). For the second part of the culture period 100% rat serum was used, again supplemented with glutamine and glucose. The rat serum was prepared by immediately centrifuging freshly drawn blood and was heat-inactivated at 56 °C for 30 min (Steele, 1972; New, Coppola & Cockcroft, 1976b). All the media were filtered before use (Sartorius; pore size 0.45 μm). In all cultures containing embryos either labelled with [³H]thymidine or injected with [³H]thymidine-labelled cells the medium contained an additional 5 μM/ml of [³H]thymidine. The cultures were gassed at approximately 12 h intervals and terminated after 36 h.

Labelling of embryos

Following the removal of Reichert's membrane embryos were transferred to plastic bacteriological dishes (Sterilin) containing the α modification of Eagle's medium supplemented with 30 μM adenosine, guanosine, cytidine and

uridine, and 10 μM of thymidine (Flow Laboratories), and were cultured at 37 °C in an atmosphere of 5% CO₂ in air. The medium also contained 10 $\mu\text{Ci}/\text{ml}$ of [³H]thymidine (Radiochemicals, Amersham) made up to a specific activity of 10.5 $\mu\text{Ci}/\text{mm}$. After 2 h the embryos were removed from the labelling medium and washed for 15 min in three changes of PBI+10% FCS containing an additional 10 μM of thymidine. A few embryos were fixed in Carnoy's fixative (Carnoy, 1887) immediately after washing and prepared for autoradiography. These embryos served as *uptake* controls (Fig. 1). At least two labelled embryos in each experiment were placed in roller bottles and cultured for 36 h. These embryos were used as *labelled controls* (Fig. 1). The remaining embryos served as a source of donor cells.

Preparation and injection of donor cells

After washing, the extraembryonic region of the [³H]thymidine-labelled embryos was removed and the required segment of the remaining embryonic portion dissected out using glass needles. The endoderm and any mesoderm was removed from these segments by incubating them in a mixture of 0.5% trypsin and 2.5% pancreatin (Difco) in calcium-magnesium-free Tyrode saline at pH 7.4 for 10 min at 4 °C (Levak-Švajger, Švajger & Škreb, 1969), followed by gentle pipetting. A few of the resulting lumps of ectoderm were prepared for routine histology and later scanned in serial sections for contamination with endoderm and/or mesoderm cells. No obvious contamination was detected and so it was assumed that donor cells represented a pure population of embryonic ectoderm. The remaining ectoderm lumps were further disaggregated into small clumps of a suitable size for injection, using a fine hand-drawn Pasteur pipette. Donor cells homozygous for the *Gpi-1^b* allele, with or without [³H]thymidine label, were prepared in an identical fashion except that the final clumps were larger. Some representative clumps, of both sizes were used for cell counts following dissociation in TVP (0.025% trypsin, 1 mM-Na₂EDTA, 1% (v/v) chick serum in phosphate-buffered saline lacking calcium and magnesium) (Bernstine, Hooper, Grandchamp & Ephrussi, 1973). Donor clumps labelled with [³H]thymidine comprised approximately 15–30 cells (mean = 21.6 ± 4.2 ; $n = 11$) whereas those carrying the GPI-B enzyme contained approximately 85–115 cells (mean = 97.3 ± 9.2 ; $n = 11$).

The clumps of ectoderm cells were transferred to a drop of PBI+10% FCS containing 10 $\mu\text{M}/\text{ml}$ of thymidine in the lid of a plastic tissue culture dish (Sterilin). The drop was entirely covered by liquid paraffin (Boots, U.K. Ltd) and placed on a micromanipulation assembly (Leitz). The host embryos were also transferred to the drop and held by suction on the end of a thin-walled pipette (internal diameter 150 μm). Injections were made using a thin-walled pipette (internal diameter 5 μm). Both pipettes were made from capillary tubing pulled on an electro puller and broken off to the required diameter using a De Fonbrune microforge (Gardner, 1976). The clump of donor cells

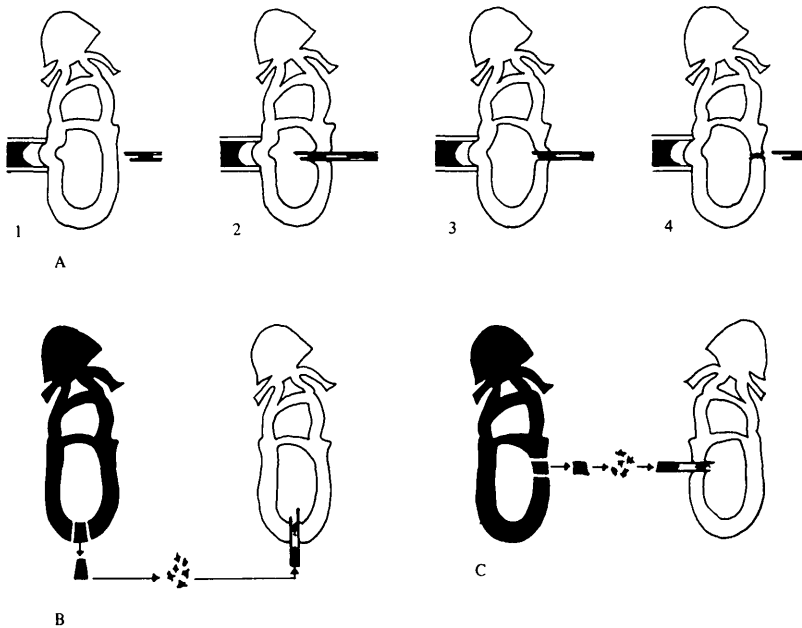


Fig. 2. (A) A diagram of the injection technique. (1) The egg cylinder is held by suction with a holding pipette opposite the intended site of injection. (2) The injection pipette, containing the donor tissue, is pushed through the egg cylinder wall into the amniotic cavity. (3) The injection pipette is withdrawn slowly and the donor tissue gently expelled into the ectoderm layer of the egg cylinder. (4) The injection pipette is removed and the embryo released from the holding pipette. (B) Injection of distal ectoderm into the distal tip of the egg cylinder. (C) Injection of anterior ectoderm into the anterior region of the egg cylinder.

was placed in the ectoderm layer of the host egg cylinder and the host embryos were held in such a way that injections could be made into a similar site to that from which the donor ectoderm had originated (Fig. 2). *Injected embryos* were replaced in rotator bottles and cultured for 36 h.

Evaluation of cultured embryos

At the end of the culture period embryos were placed in warm phosphate-buffered saline (PBS) and the presence or absence of heartbeat at yolk-sac circulation was recorded for each embryo. The number of somites was counted, either after removal of the yolk sac or after embryos had been cleared in toluene and features such as the extent of axial rotation and fusion of the neural folds were also noted.

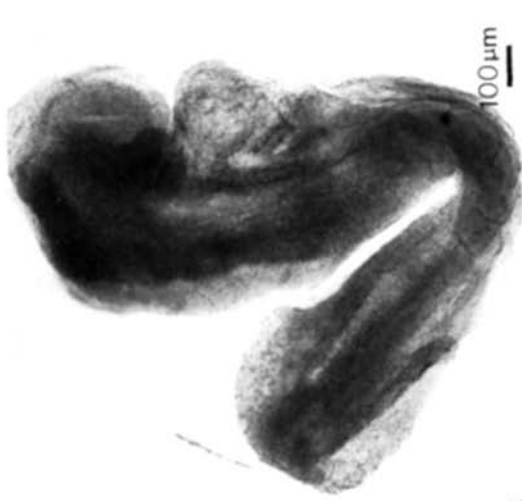
Several embryos from each control and experimental category were dissolved in 0.1 M-sodium hydroxide and analysed for total protein content (Lowry, Rosebrough, Farr & Randall, 1951) to provide a gross estimate of embryonic growth. No selection was exercised in the choice of embryos for this analysis except that any grossly abnormal embryos were excluded. All other embryos

were either prepared for starch gel electrophoresis or fixed in Carnoy's, dehydrated, cleared and embedded in paraffin wax (m.p. 56 °C) and subsequently serially sectioned at 5 μm . The slides were stained, either immediately or following autoradiography, with haemalum and eosin or haemalum alone. Mitotic indices and dead cell indices were calculated in three tissues, the neural tube, the gut and somitic mesoderm, in sections of embryos from experimental and control categories. The numbers of nuclei in each of the three tissues was counted in three adjacent sections and the numbers of mitoses and dead cells were also recorded for each tissue, dead cells being defined by the criteria adopted by Copp (1978). The counts were made at the level of the last somite (i.e. the most recently formed somite rather than a numerically defined somite) except in those embryos where gut closure did not coincide with this region in which case the gut was scored just posterior to the point of hindgut closure. Mitotic and dead cell indices were used for comparison as the variability in orientation of sections from different embryos made it invalid to compare total cell numbers. All the recorded characteristics of embryos taken from culture were compared with those of *in vivo* controls.

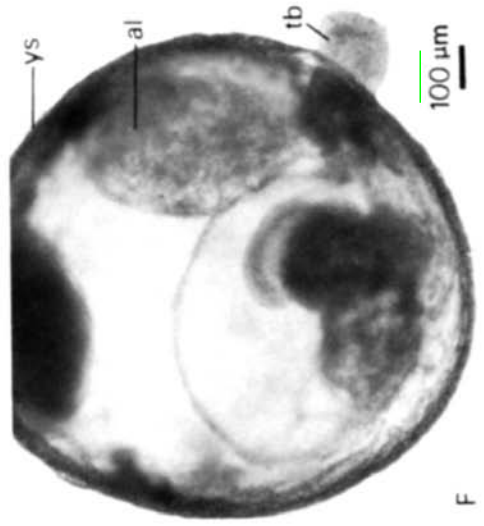
Autoradiography

Sections of *labelled control* embryos and *injected embryos* were hydrated and immediately incubated in a 5% solution of trichloroacetic acid (BDH Chemicals Ltd) at 4 °C for 30 min. remove any unincorporated [³H] thymidine. After washing in running water the slides were placed in distilled water and, in a dark room, covered with AR 10 fine-grain autoradiographic stripping film (Kodak Ltd). The slides were left to expose at 4 °C for 3 weeks, developed using D-19 developer (Kodak Ltd) and fixed in a Kodafix solution (Kodak Ltd). When dry, the slides were stained with haemalum and scanned in a light microscope.

Injected embryos were only considered to be chimaeric if they contained at least three labelled cells showing equivalent grain density to that found over the same tissue in *labelled controls*. Grains had to be present over an individual nucleus in two adjacent sections in order for that cell to qualify as a labelled cell. Nuclei showing extremely dense labelling, similar to that seen in *uptake controls*, were not considered to be viable colonizing cells as it was unlikely that they had undergone cell division following injection. In addition, lumps of labelled cells clearly not incorporated into embryonic tissues were not considered to be colonizing cells. In those embryos, judged to be chimaeric by these criteria, the distribution of labelled cells was subdivided into head, trunk and posterior structures. The head region comprised that part of the embryo anterior to the first somite, the trunk region was the part of the embryo which included all the somites, and the posterior region consisted of structures posterior to the last somite. The number of colonizing cells in each chimaera was estimated by counting every labelled cell in alternate sections. As the



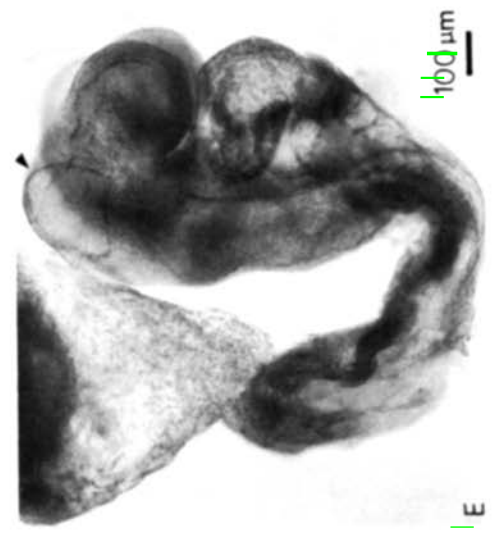
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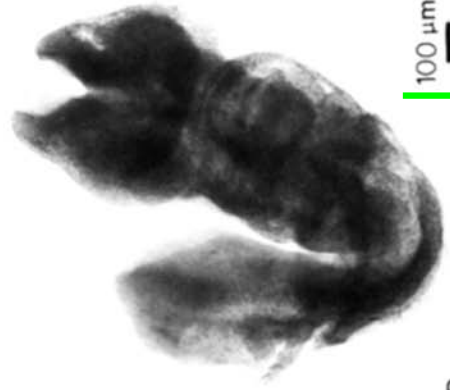
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D

sections were $5\ \mu\text{m}$ in width it was considered unlikely that a single labelled nucleus would span more than two sections.

Electrophoresis

After removal from culture, embryos injected with *Gpi-1^b/Gpi-1^b* donor cells were washed thoroughly in PBS. They were dissected into four portions: yolk sac and ectoplacental cone; anterior region of the embryo (from the first somite); trunk region of the embryo (segment containing all the somites); posterior region and allantois. The four samples were analysed electrophoretically for GPI using the method described by Chapman, White & Ruddle (1971).

RESULTS

Evaluation of cultured embryos

At the start of the culture period embryos were at the late primitive streak stage and either had formed an amnion or had very advanced amniotic folds (Fig. 3A) After 36 h *in vitro* these embryos had developed beating hearts, the visceral yolk sac had expanded and a yolk-sac circulation had been established. The neural folds were well developed and had fused along most of the trunk region. Somites had formed along both sides of the neural axis and some of the embryos had begun, and in a few cases completed, axial rotation (Fig. 3C, D). The yolk sac of embryos developed *in vitro* tended to be more expanded than that seen in *in vivo* controls and, perhaps as a corollary of this, the degree of curvature of the embryos before turning was less pronounced in those from culture. In addition, embryos grown in culture appeared more translucent than those recovered from *in vivo*. These discrepancies were not considered to be gross abnormalities. A few embryos, both from *in vitro* and *in vivo*, did show gross abnormalities such as an obvious lack of growth or organization of the embryo, or particular deformities such as a lack of somites or vesiculation of the surface ectoderm (Figs. 3E, F).

Table 1 shows a comparison of the macroscopic features of the three cate-

Fig. 3 (A) 8th day mouse embryo at the time of manipulation. Reichert's membrane and trophoblast have been reflected and the amnion is clearly visible.

(B) *In vivo* control embryo after removal from the yolk sac. This embryo is unturned. Somites are visible in the trunk region and the heart is well developed.

(C) Unlabelled control embryo which is beginning to turn and somites are distinguishable in the trunk region.

(D) Labelled control embryo showing distinct axial rotation.

(E) Abnormal injected embryo with vesiculated surface ectoderm (arrow).

(F) A grossly abnormal labelled control embryo with intact yolk sac (ys). The allantois has not fused with the chorion and the tail bud (tb) is growing outside the yolk sac. (magnification $\times 56$, bar $100\ \mu\text{m}$).

Table 1. *Comparison of development between embryos grown in vivo and the three classes of embryo grown in vitro*

Type of embryo	<i>In vivo</i> controls	Unlabelled controls	Labelled controls	Injected
Total no. embryos	41	23	32	98
No. abnormal	3 (7.3%)	2 (8.7%)	3 (9.4%)	16 (16.3%)
No. normal	38 (92.7%)	21 (91.3%)	29 (90.6%)	82 (83.7%)
Normal embryos showing				
Heartbeat	37 (97.4%)*	21 (100%)	29 (100%)	82 (100%)
Yolk sac circulation	34 (89.5%)*	20 (95.2%)	29 (100%)	77 (93.9%)
Turning	26 (68.4%)	13 (61.9%)	26 (89.7%)	53 (64.6%)
Trunk neural tube fusion	36 (94.7%)	21 (100%)	25 (96.6%)	79 (96.3%)
Cranial neural tube fusion	5 (13.2%)	2 (9.5%)	5 (13.8%)	4 (4.9%)
Somite number†	9.5 ± 2.7	9.3 ± 1.5	10.5 ± 2.1	9.5 ± 2.0
Total protein content‡	81.3 ± 31.7 (n = 14)	81.8 ± 12.8 (n = 6)	63.7 ± 15.6 (n = 8)	76.2 ± 20.1 (n = 8)

* The reduced number of *in vivo* controls showing a heartbeat and yolk-sac circulation is probably an artifact. During the time taken to dissect out a whole litter the heartbeat and/or yolk-sac circulation may cease.

† Somite numbers of embryos developed *in vitro* are not significantly different from those developed *in vivo* (*t*-test; $P \geq 0.05$).

‡ The total protein contents of embryos developed *in vitro* are not significantly different from those of embryos developed *in vivo* (*t*-test; $P \geq 0.05$).

gories of cultured embryos with *in vivo* controls. Only injected embryos showed an obvious increase in the incidence of abnormalities but, nonetheless, over 80% of them showed development comparable to that found in *in vivo* controls. All cultured embryos which were classified as normal, had beating hearts and of these over 90% also showed signs of a yolk sac circulation. The incidence of axial rotation was close to that seen *in vivo* for *unlabelled controls* and *injected embryos*, but was rather higher in *labelled controls*. There was no reduction in the frequency of trunk neural tube fusion among cultured embryos. Cranial neural tube fusion was somewhat reduced in *unlabelled controls* and *injected embryos* but this was quite a rare feature even in *in vivo* controls. When the

Fig. 4. (A) Sagittal section through part of an *uptake control* showing dense labelling over all the nuclei. (Magnification $\times 450$; Bar $20 \mu\text{m}$.)

ECT, embryonic ectoderm; MES, mesoderm, END, visceral endoderm.

(B) Transverse section through a *labelled control* embryo showing silver grains over most nuclei.

(C) Transverse section through chimaera no. 4 (from the distal series of injections). Donor cells are present in the posterior loose mesoderm.

(D) Transverse section through chimaera no. 3 (from the distal series of injections). Donor cells are present in the notochord and gut.

Donor tissue in Figs. 4C and 4D are marked by arrows. NT, neural tube; N, notochord; G, gut; NP, neural plate; M, mesoderm; CA, caudal artery. (Magnification $\times 450$; bar = $20 \mu\text{m}$.)

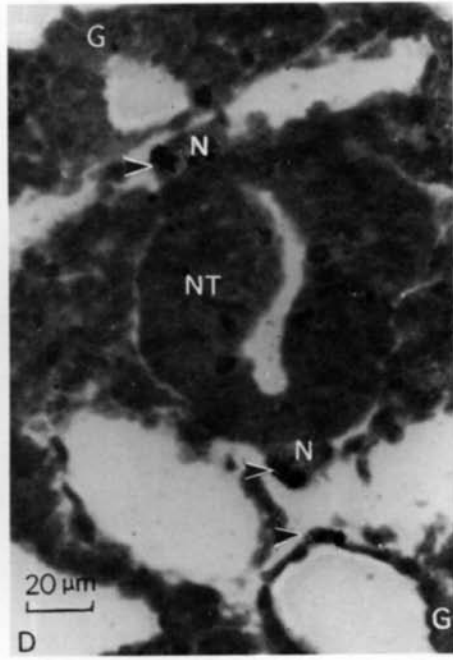
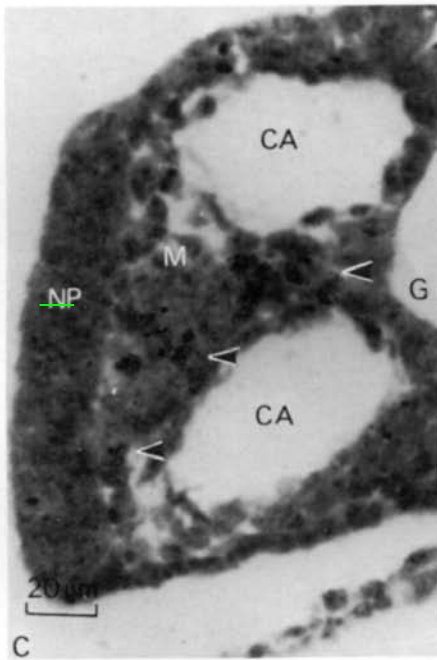
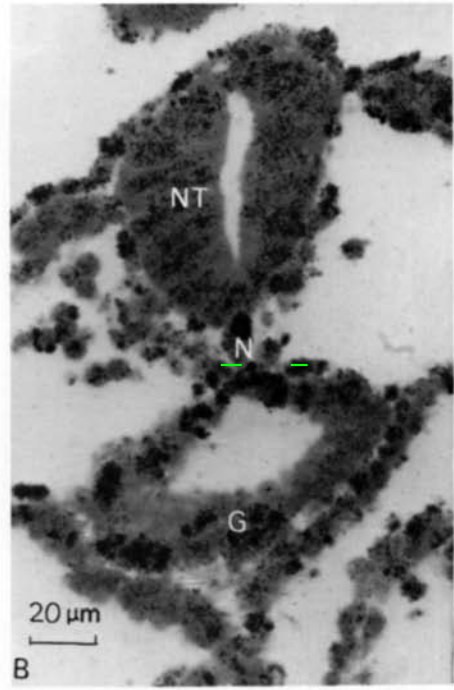
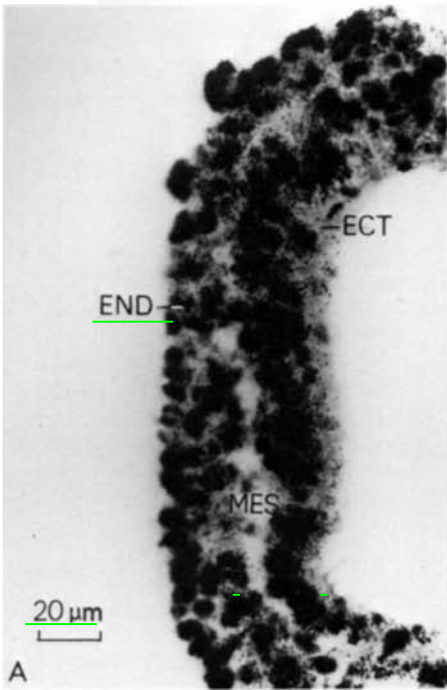


Table 2. Analysis of chimaerism by Gpi gel electrophoresis: Rates of chimaerism in embryos injected with embryonic ectoderm carrying either the isoenzyme marker + [³H]thymidine or the isoenzyme alone

<u>Donor cell</u>	<u>No. of embryos</u>	<u>No. of chimaeras</u>	<u>% of chimaeras</u>
<u>Gpi-1^b/Gpi-1^b</u>	<u>17</u>	<u>3*</u>	<u>17.7</u>
<u>Gpi-1^b/Gpi-1^b + ³H TdR</u>	<u>25</u>	<u>5†</u>	<u>20.0</u>

* 11 embryos injected in the primitive-streak region generated one embryo which was chimaeric in the posterior region. Six embryos injected in the anterior region generated one embryo chimaeric in the head and one embryo chimaeric in the yolk sac (this embryo had a distinct lump in the yolk sac).

† 25 embryos injected in the primitive streak region generated four embryos chimaeric in the posterior region and one embryo chimaeric in the head (this embryo had a lump sticking to its cranial folds).

total somite number of normal cultured embryos from each category were compared separately with those of embryos maintained *in vivo* no significant differences were found (*t*-test; $P \geq 0.05$). Total protein contents were compared in the same way and, although for embryos were included in the analysis, no statistically significant differences were found between the different experimental categories of embryos and *in vivo* controls. However, as the samples are small the low value obtained for *labelled controls* may reflect a real, although not great, reduction in protein content. The mitotic indices and cell death indices calculated for a small segment of the neural tube, the gut and one somite in sections of three *labelled controls* and three *injected embryos* did not differ significantly, for either category of embryo, from those found in the same tissues of three *in vivo controls* (χ^2 -test; $P \geq 0.05$).

Detection and viability of [³H]thymidine-labelled cells

Autoradiographs of *uptake controls* showed that all the nuclei in the embryo were heavily labelled with silver grains. The labelling over most individual nuclei was so heavy that it was not possible to count individual grains (Fig. 4A). *Labelled controls* also showed the presence of grains over the vast majority of nuclei. The density of grains varied over cells of different embryonic tissue: gut endoderm and notochord consistently showed the highest density of labelling while neurectoderm showed the lowest (Fig. 4B). All nuclei were covered by a sufficient number of grains to allow the label to be seen easily at a low magnification in the light microscope.

The comparison of *labelled controls* with *in vivo* controls (Table 1) strongly suggest that embryonic cells containing [³H]thymidine incorporated into their DNA are capable of participating in normal development for the duration of the culture period. The viability of donor cells, both labelled and unlabelled, in *injected embryos* was tested directly using GPI gel electrophoresis. The results

Table 3. The distribution of ³H-thymidine-labelled cells in chimaeras

Chimaera number	Distal ectoderm injected into distal part of egg cylinder											Anterior ectoderm injected into anterior part of egg cylinder										
	1	2	3	4	5	6	7	8	9	10	11	1	2	3	4	5	6	7	8	9	10	
Dead cells																						
Unincorporated lumps																						
No. of incorporated cells	10	44	133	61	83	46	61	5	6	4	8	49	20	39	65	53	75	57	57	18	85	
Head surface ectoderm																						
Head neuroectoderm																						
Head loose mesoderm																						
Heart; blood vessels																						
Foregut																						
Trunk surface ectoderm																						
Trunk neuroectoderm																						
Somite																						
Trunk loose mesoderm																						
Midgut																						
Notochord																						
Posterior surface ectoderm																						
Posterior neuroectoderm																						
Posterior loose mesoderm																						
Primitive streak																						
Hindgut																						
Notochord																						

Although an estimate of the number of incorporated labelled cells is provided this does not reflect the extent of donor cell proliferation as there is no control for the loss of cells following injection. That some injected embryos do not form chimaeras, and show no sign of dead cells or unincorporated lumps, indicates that some grafts must be extruded from the embryo following injection. It is therefore possible that in some cases only part of a graft may be retained within the embryo and an unknown number of cells may be lost into the culture medium.

of the electrophoretic analysis are shown in Table 2. The GPI assay used is unable to detect less than a 2% contribution of either isoenzyme (Gardner, Papaioannou & Barton, 1973), and therefore although larger clumps of donor cells were used in these experiments the assay is probably at the limits of its sensitivity. Despite this, a donor contribution could be detected in some of the *injected embryos* and these results establish that injected cells can survive in host embryos even if they are labelled with [³H]thymidine.

Distribution of [³H]thymidine-labelled cells in chimaeras

Twenty embryos were injected with [³H]thymidine-labelled ectoderm dissected from the distal tip of the egg cylinder, just anterior to the primitive streak (Fig. 2B). The injections were made into the ectoderm layer, also at the tip, of the host egg cylinder. Analysis of the *injected embryos* after culture by autoradiography revealed that 11 out of the 20 embryos had been colonized by donor cells. The tissue distribution of labelled cells in these chimaeric embryos is shown in Table 3. The number of incorporated or colonizing cells (see Materials and Methods) shows considerable variation between different chimaeras. However, in all those chimaeras showing relatively slight colonization, 'dead' cells or unincorporated lumps were evident. In all but two of the chimaeras (nos. 4 and 6) there were densely labelled cells adhering to the amnion or trapped inside the neural tube, but not incorporated into the embryos. Since the degree of labelling over these cells did not differ from that seen in *uptake controls* it was assumed that these cells were dead, or at least, had not divided. In addition, two of these chimaeras containing 'dead' cells (nos. 2 and 9) also had lumps of labelled cells floating free in the amniotic cavity. These lumps showed reduced labelling compared with *uptake controls* suggesting that some cell division had occurred despite a failure to incorporate into the embryo. Four embryos containing only lumps of unincorporated labelled cells were not classified as chimaeras and therefore are not represented in Table 3.

A further 18 embryos were injected in the anterior region of the egg cylinder with ectoderm removed from the anterior part of donor embryos (Fig. 2C). Ten of these injected embryos were chimaeric and a further two contained unincorporated lumps of labelled cells in the amniotic cavity. The distribution of colonizing cells in these chimaeras is also shown in Table 3. Three out of the ten colonized embryos contained 'dead' cells and one of the colonized embryos had an unincorporated lump attached to the amnion.

DISCUSSION

Embryos have been grown successfully *in vitro* from the late primitive streak stage until the early somite stage. Over 90% of both *unlabelled controls* and *labelled controls* develop normally and do not differ significantly from *in vivo controls* in any of the features which were recorded. (Table 1) Similarly, almost

85% of injected embryos appear to grow and develop in culture at a rate comparable to that occurring *in vivo*. The frequency of grossly abnormal embryos is somewhat higher among injected embryos than among embryos which have not been injected. This discrepancy is almost certainly due to the trauma of manipulation. Nonetheless, the majority of embryos grown *in vitro* although showing certain differences from those maintained *in vivo* (such as the size of the yolk sac) appear to develop sufficiently normally to validate cell lineage studies. In addition, the incorporation of [³H]thymidine, in the amounts used in these experiments, into the DNA of cultured embryos does not seem to disrupt their development and therefore it is justifiable to use this radioisotope as a cell marker. This makes it possible to study cell fate *in situ* in the developing postimplantation embryo.

The presence of [³H]thymidine-labelled cells in histological sections of injected embryos strongly suggests that donor cells can incorporate into the embryo and participate in subsequent development. It is obviously important to establish that these cells are (1) alive and of donor origin, and (2) that they are participating in the normal development of the host. The density of grains over donor cells found incorporated into the host embryos was considerably less than seen in uptake controls, implying that the cells had undergone cell division. In addition, the number of grains found over donor cells in chimaeras was similar to the number of grains over cells of the same tissue in labelled controls. Therefore, the dilution of label in donor cells was equivalent to that occurring in the cells of labelled controls and hence the two must be dividing at a comparable rate. The presence of excess cold thymidine in the culture medium (Weston, 1963) would make it extremely unlikely that those cells qualifying as colonizing donor cells (see Materials and Methods) could in fact have been host cells. Any free [³H]thymidine that might be released from dead donor cells would be considerably diluted by the cold thymidine and therefore even if host cells did take it up the extent of their labelling would be well below that seen in labelled controls. The results from the electrophoretic analysis of embryos injected either with *Gpi-1^b/Gpi-1^b* plus [³H]thymidine-labelled tissue or with cells carrying only the isozyme marker and further evidence that injected cells remain viable and continue to divide in the host embryo although due to the limited sensitivity of GPI assay, it was necessary to inject larger lumps of donor cells in these experiments and it could be argued that labelled cells have a higher survival rate under these circumstances. The electrophoretic results do however indicate that injected [³H]thymidine-labelled cells are not at any selective disadvantage compared with their counterparts which have not been labelled with radiotope.

The distribution of labelled cells in the chimaeras is consistent with true tissue colonization rather than aberrant development. Injected embryos did not show anomalous structures, either labelled or unlabelled, which were part of the embryo itself. The labelled cells tended to be clearly segregated into specific

embryonic organs, such as gut, notochord, or somite, and did not appear as contiguous patches overlapping a variety of tissue types within a single area. However, the distribution of labelled cells in chimaeras indicated that no extensive cell mixing occurred between host and donor cells following injection. Labelled cells tended to be found in groups and, with the exception of chimaeras showing bilateral colonization, the distance between two groups of labelled cells was seldom more than 50 μm .

The pattern of colonization in those embryos injected at their distal extremity with labelled ectoderm dissected from the same region in donor embryos show several interesting features (Table 3). First, it appears that 8th day ectoderm can give rise to definitive gut endoderm in the embryo (embryos 1–3). This supports results from previous work, notably histological description, ectopic transfers, and injection of cells into the blastocyst, which indicated that definitive endoderm or gut endoderm, is derived not from visceral endoderm but from embryonic ectoderm (Jolly & Ferester-Tadie, 1936; Levak-Švajger & Švajger, 1974; Diwan & Stevens, 1976; Gardner & Papaioannou, 1975; Gardner & Rossant, 1979). Secondly, ectoderm in this region contributes only rarely to definitive ectoderm derivatives (surface ectoderm and neurectoderm). The origin of the injected cells and their site of injection correspond closely to the location of the proliferative zone described by Snow (1977). The dearth of definitive ectoderm derivatives colonized in the chimaeras does not support his suggestion that this zone is responsible for generating all the ectoderm of the head-fold-stage embryo (Snow, 1977). The results are more consistent with the fate map in the chick at the time of gastrulation, analysed by a similar autoradiographic technique, which indicates that the definitive endoderm, notochord and some mesoderm originate from the epiblast at the anterior extreme of the primitive streak (Nicolet, 1970). Thirdly, the spatial distribution of the colonized tissues gives the impression that the overall cell movement from the tip of the egg cylinder may be in a caudal direction. It is difficult to substantiate such an impression since there is no reference point in the *injected embryos* at the time of analysis for the initial injection site. Therefore, what may appear to be caudal cell movement may in fact be a reflection of embryonic growth occurring predominantly in the anterior half of the embryo. Similarly, it is not possible to determine whether the labelled mesoderm cells have emerged following invagination through the primitive streak or whether they have arisen directly from the ectoderm in the vicinity of the injection site. If the latter were the case it would imply that the primitive streak is not necessarily the only route for mesoderm formation at this stage.

Embryos injected anteriorly with anterior ectoderm show a rather different spectrum of colonization. Here the predominant tissues containing labelled cells are surface ectoderm and neurectoderm. Only two embryos show colonization of the mesoderm, and in the embryo in which the gut is also labelled (embryo 10), the donor cells are found at the anterior extreme of the foregut.

It is possible that this region represents the ectodermal component of the foregut, the stomodeum, rather than definitive endoderm. Without the use of tissue specific markers it is not possible to determine which of these two cell types the donor cells have formed. Nonetheless, the preponderance of ectodermal tissues colonized in these chimaeras contrasts strongly with the results from orthotopic distal injections, where only one chimaera (embryo 11) showed very slight colonization of the trunk neurectoderm.

The difference in the pattern of colonization between the two series of injections suggests that there may be some regionalization in the embryonic ectoderm in the 8th day embryo. This regionalization could reflect the acquisition of a mosaic prepattern or it may reflect a gradient or gradients within the egg cylinder which are responsible for specifying particular tissues in different regions. It is hoped that heterotopic injections of embryonic ectoderm may give some insight into this question.

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