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Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos

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We show that a gene introduced into cells of mouse embryos by a retrovirus can serve as a heritable marker for the study of cell lineage *in vivo*. We constructed a defective recombinant retrovirus in which the *Escherichia coli* β-galactosidase (*lacZ*) gene is inserted in the genome of a Muloney murine leukemia virus (M-MuLV). Expression of lacZ was detected with a histochemical stain that can be applied to cultured cells and embryonic tissue. Infection of cultured cells showed that lacZ has no detectable deleterious effects on cell viability or growth, that the enzyme is stably expressed in the progeny of infected cells for many generations in the absence of selective pressure, and that the virus can induce lacZ in a variety of cell types. Following injection of the virus into mid-gestation mouse embryos, clones of lacZ-positive cells were detected in skin, skull, meninges, brain, visceral yolk sac, and amnion. We identified the cell types comprising a series of lacZ-positive clones in the visceral yolk sac and skin to learn the lineage relationships of the labelled cells. In each tissue, we obtained evidence that several cell types have a pluripotential ancestor and that cell fate is progressively restricted as development proceeds.

Key words: cell lineage/lacZ/retrovirus/skin/visceral yolk sac

Introduction

Because cell lineage may play a crucial role in determining cell phenotype, knowledge about the genealogical relationships of cells is a prerequisite to understanding the mechanisms that underlie their developmental choices. During the past decade, considerable progress has been made in unravelling lineage patterns in several invertebrates and lower vertebrates (reviewed in Gardner and Lawrence, 1985). In mammals, lineage studies have relied largely on the use of chimeric animals, which are produced by combining cells of two genotypically different embryos (reviewed in Le Douarin and McLaren, 1984). Recently, this work has been complemented by the use of dyes that can be injected into the cytoplasm of individual embryonic cells and remain detectable through many cell divisions (Balakier and Pedersen, 1982; see also Jacobson, 1985; Kimmel and Warga, 1986). In both of these methods, however, cells are marked at pregastrula stages of development, and it is therefore early lineages that are most amenable to study. Later aspects of mammalian cell lineage, such as the determination of familial relationships among cells within individual tissues, have been difficult to study *in vivo*.

A novel approach to this problem is suggested by the recent development of recombinant retroviral vectors in which exogenous genes replace portions of a retroviral genome. These recombinant viruses, like the wild type, can integrate into the host genome and be inherited by daughter cells (reviewed in Weiss *et al.*, 1985). Such retroviruses have been used to introduce a number of genes into cultured cells and post-implantation murine embryos (Stuhlman *et al.*, 1984 and references therein). Because most recombinant retroviruses are incapable of producing new virions without a helper virus, their genomes are passed to only the progeny of the infected cell. If the exogenous gene encodes a protein that can be detected within single cells, the individual clonal descendants of the infected cell can be histochemically or immunohistochemically labelled. By infecting embryos and analyzing labelled clones at appropriate stages, it should be possible to derive the lineage relationships of the labelled cells. Here, we have tested the feasibility of this approach, using a defective recombinant retroviral vector containing the *Escherichia coli lacZ* gene. Using this virus, we have obtained novel data on cell lineage in the visceral yolk sac and skin.

Results

A recombinant retrovirus containing the lacZ gene

We constructed a recombinant retrovirus which has the following characteristics: (i) it contains *cis*-acting portions of the Moloney murine leukemia virus (M-MuLV) genome necessary for viral transcription, packaging, and integration. (ii) It bears the *lacZ* gene, whose product, a β-galactosidase that we designate lacZ, can be revealed histochemically. (iii) The *lacZ* gene is under the transcriptional control of the SV40 early promoter; expression from the retroviral promoter is suppressed in early embryonic cells (Jaenisch *et al.*, 1981; Peries *et al.*, 1977), but this suppression can be partially overcome by insertion of an internal promoter (Rubenstein *et al.*, 1984: Wagner *et al.*, 1985). (iv) The virus is defective in replication, so that once an infection has occurred, viral propagation is blocked and the provirus is restricted to the progeny of the initially infected cells.

To produce virions, we transfected the plasmid encoding the retrovirus (pM-MuLV-SV-*lacZ*; Figure 1a) into ψ2 cells (Mann *et al.*, 1983) as described in Materials and methods; the virus produced is named LZ1. Restriction analysis (Figure 1b) showed that the structure of LZ1 provirus did not differ detectably from that of the plasmid from which it was derived. For injection into embryos, LZ1 was concentrated 100-fold by centrifugation (Figure 1c). Using a saturating concentration of polybrene (Figure 1d), NIH 3T3 cells as recipient, and the histochemical assay described below, we detected $\sim 10^6$ infectious particles per ml of viral concentrate.

A histochemical stain for lacZ

We used a histochemical stain for β-galactosidase (Lojda, 1970) to detect expression of lacZ in virus-infected cells. Under the conditions detailed in Materials and methods, essentially all (>98%) cells of several LZ1-infected NIH 3T3 clones were

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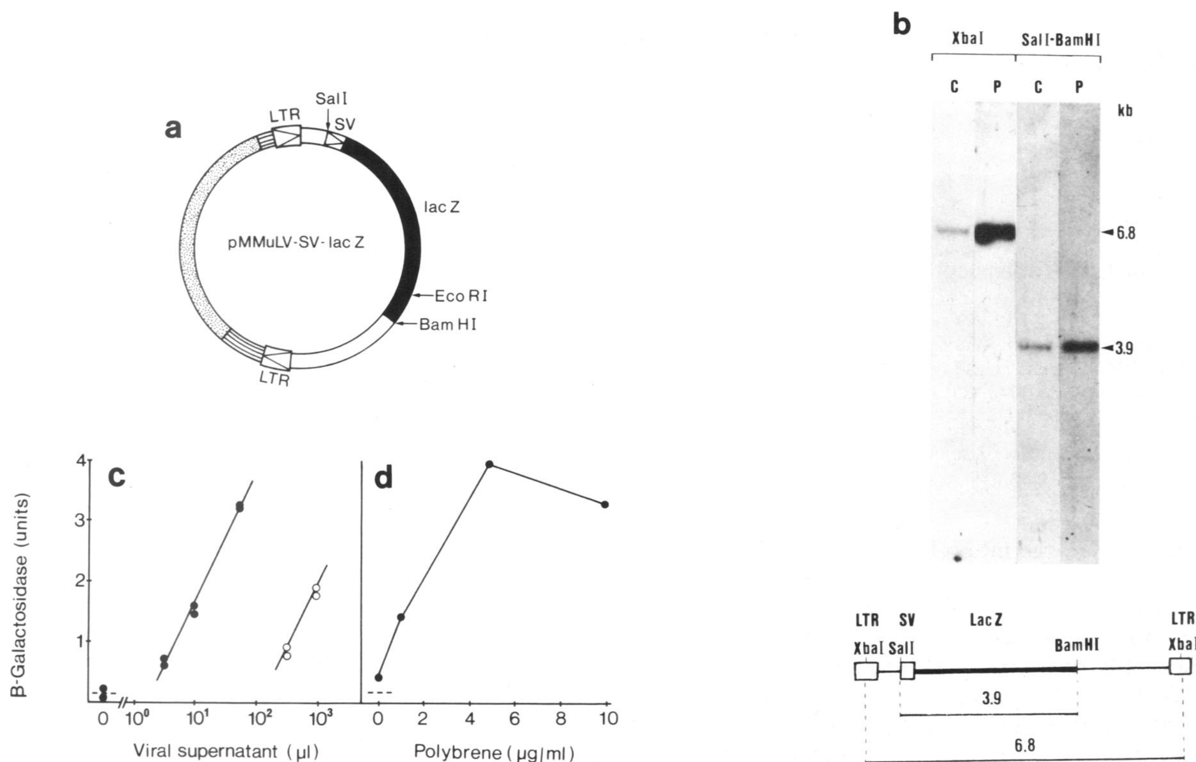


Fig. 1. Production of the recombinant retrovirus LZ1. (**a**) Diagram of the plasmid encoding the retroviral vector pM-MuLV-SV-*lacZ*. Variations in shading are used to distinguish sequences derived from M-MuLV (□), SV40 (⧅), *E. coli* (■), mouse (⊟) and pBR322 (▨). *Eco*RI, *Sal*I and *Bam*HI sites used in the construction are indicated, as are positions of the viral long terminal repeats (LTR) and the SV40 promoter (SV). (**b**) Analysis of the structure of pM-MuLV-SV-*lacZ* and its provirus. Plasmid DNA (lanes labelled P) or DNA from NIH 3T3 cells infected with LZ1 (lanes labelled C) were digested either with *Xba*I or with a combination of *Sal*I and *Bam*HI. A purified SV-*lacZ* fragment was used to probe the Southern blot. The diagram below the blot shows the predicted sizes of the *Xba*I and *Sal*I−*Bam*HI restriction fragments. (**c**) β-galactosidase activity of NIH 3T3 cells infected with viral supernatant either before (○) or after (●) concentration. The concentrated virus was resuspended in 1% of its original volume; the titration shows that nearly complete recovery was achieved. Units of activity are as defined by Miller (1972), per mg cellular protein. (**d**) Dependence of apparent viral titer on polybrene. Plates of cells were infected with 50 µl of concentrated virus in the presence of the indicated concentration of polybrene. While some productive infection occurs in the absence of polybrene, the apparent titer is agumented ≫10-fold by the use of 5−10 µg/ml polybrene.

β-galactosidase-positive after 4 h incubation, while no uninfected NIH 3T3 cells were detectably stained after 48 h (Figure 2). As expected for lacZ, staining was inhibited by *D*-galactolactone or lactose (4-β-*D*-galacto-*D*-glucose) but not by cellobiose (4-β-*D*-glucose-*D*-glucose; all at 30 mM). In most cell and tissues, background staining was negligible, presumably because the predominant mammalian β-galactoside hydrolyzing activity is a lyzosomal, acidic enzyme that is inactive at the neutral pH optimal for lacZ.

In partially infected populations of several cell types (Table I), stained cells were frequently observed directly adjacent to unstained cells, suggesting that enzyme is not transferred among cells in contact. The reaction product can apparently diffuse extracellularly before precipitating (e.g. Figure 4), but once formed, the precipitate is sufficiently stable to survive embedding in plastic. The ability to locate stained cells in whole mounts and then to characterize them in sections greatly facilitates the study of small groups of lacZ-positive cells within lacZ-negative tissues.

Infection of cultured cells with the lacZ retrovirus

To use LZ1 for studies of cell lineage, it is important that the virus be able to induce selectively neutral and stable expression of lacZ in many cell types. We used cultured cells to ask whether LZ1 possessed these abilities.

To seek effects of lacZ on cell growth, NIH 3T3 cells were infected and replated at low density the next day. At 1-day intervals thereafter, plates were stained for lacZ and the number of cells per colony counted. Cells in lacZ-positive and -negative colonies divided at the same rate, and this rate was indistinguishable from that of uninfected cells (Figure 3a). In addition, the percentage of lacZ-positive cells in a partially infected population did not change significantly for at least 30 generations (5 weeks; Figure 3b). Thus, we detected no effect of lacZ on viability or growth.

The constant proportion of lacZ-positive cells in a partially infected population suggests that the progeny of LZ1-infected cells stably express lacZ. To obtain direct evidence on this point, we isolated single cells from an infected population by limiting dilution, and studied lacZ expression in the clones to which they gave rise. In each of 6 clones, nearly all (>97, >98, >98, >98, >99 and >99%) of the cells were lacZ-positive, and expression was stable for at least 50 generations (Figure 3b). We also found one clone in which >50% of the cells were lacZ-negative; studies of this clone will be reported elsewhere (J.R.S., J.L.R.R. and J.F.N., in preparation). In the present context, however, it is important to note that such instability is rare: in >90% (48/50) of colonies derived from single infected NIH 3T3 cells, >95% of cells were lacZ-positive.

To ask whether the LZ1 virus could infect and promote lacZ expression in cells other than fibroblasts, we used mouse and rat clonal cell lines. Table I shows that lacZ expression was detectable following infection of a variety of cell types. LZ1 was

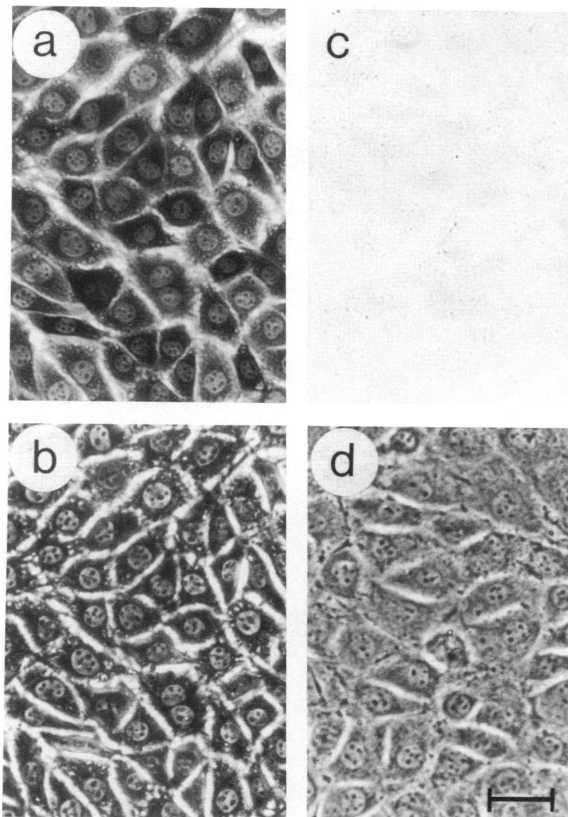


Fig. 2. Histochemical staining of LZ1 virus-infected cells for lacZ. LZ1-infected (**a,b**) and uninfected NIH 3T3 cells (**c,d**) fixed and stained for lacZ as described in Materials and methods. **a** and **c** are brightfield micrographs; **b** and **d** are phase contrast micrographs of the same fields. Bar is 50 μM.

Table I. Cultured cell lines that express lacZ following infection with LZ1 virus

Designation	Cell type	Reference
C2	muscle	Yaffe and Saxel, 1977
C6	glioma	Benda *et al.*, 1968
NIE-115	neuroblastoma (adrenergic)	Amano *et al.*, 1972
NS20Y	neuroblastoma (cholinergic)	Amano *et al.*, 1972
3T3	fibroblast	Todaro and Green, 1963
Fao	hepatoma	Deschatrette and Weiss, 1974
BWIJ	hepatoma	Cassio and Weiss, 1979
LT	embryonal carcinoma	Jakob and Nicolas, 1986
PCC3	embryonal carcinoma	Nicolas *et al.*, 1976
PCC7S	embryonal carcinoma	Pfeiffer *et al.*, 1981

Cells were infected with 1–5 μl LZ1 viral concentrate; polybrene was added at 5 μg/ml. Three to 6 days after infection, cultures were fixed and stained for lacZ.

also able to induce expression of lacZ in primary cultures of E8-E18 mouse embryos and extra-embryonic membranes. Together, these results demonstrated that LZ1 can be used to stably mark embryonic cells of several types, and motivated us to inject virus directly into embryos.

Injection of the lacZ retrovirus into mouse embryos

Concentrated LZ1 virus (0.2–0.5 μl, or a few hundred virions) was injected through the uterine wall into E7–11 embryos. Two

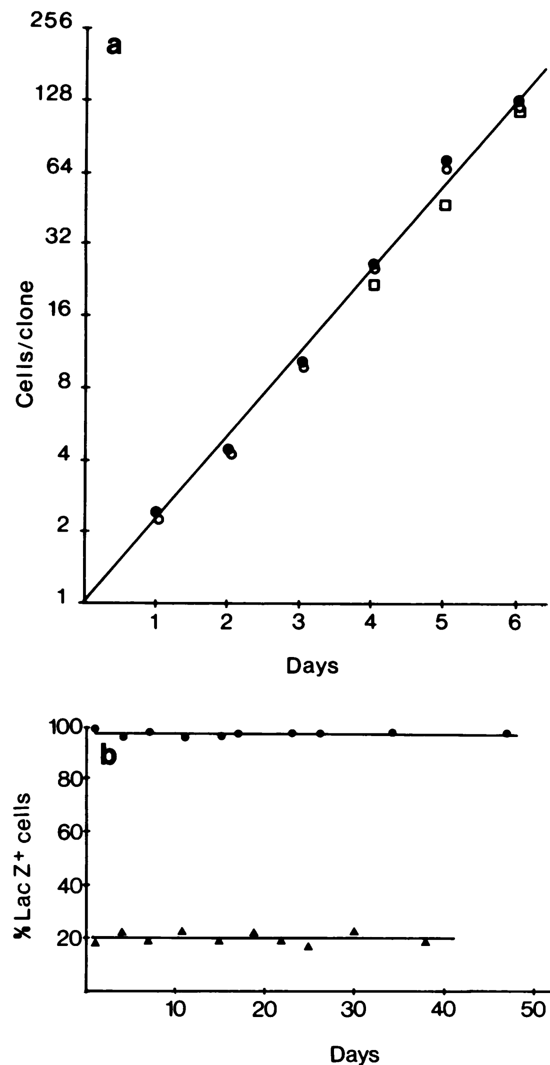


Fig. 3. Growth of LZ1-infected cells and stability of lacZ expression. (**A**) An LZ1-infected population of NIH 3T3 cells was replated at 5 cells/cm², and dishes were fixed and stained for lacZ at daily intervals. Average number of cells per colony was determined for lacZ-positive colonies (●), lacZ-negative colonies in the same dishes (○), and colonies in separate, uninfected dishes (□). (**b**) An LZ1 infected population (▲) and a lacZ-positive clone isolated from the population (●) were passaged at 3–4 day intervals; at each passage a duplicate plate was stained and the percentage of lacZ-positive cells determined.

Table II. Efficiency of obtaining lacZ-positive cells in the skin, amnion or yolk sac following microinjection of LZ1 virus

Age at injection	Number of embryos (number of litters)					
	Injected	Survived	Yolk sac positive	Skin positive	Amnion positive	Total positive
E7	68(9)	60(8)	1(1)	1(1)	1(1)	2(2)
E8	44(5)	25(5)	2(2)	0	0	2(2)
E9	137(17)	78(16)	30(14)	17(9)	1(1)	38(14)
E10	36(5)	11(5)	0	3(2)	1(1)	3(2)
E11	76(9)	39(7)	0	5(3)	7(3)	10(4)
Total	361(45)	214(41)	33(17)	26(15)	10(6)	55(24)

Embryos were injected with LZ1 virus concentrate at the indicated gestational ages, then fixed and stained for lacZ 2–8 days later.

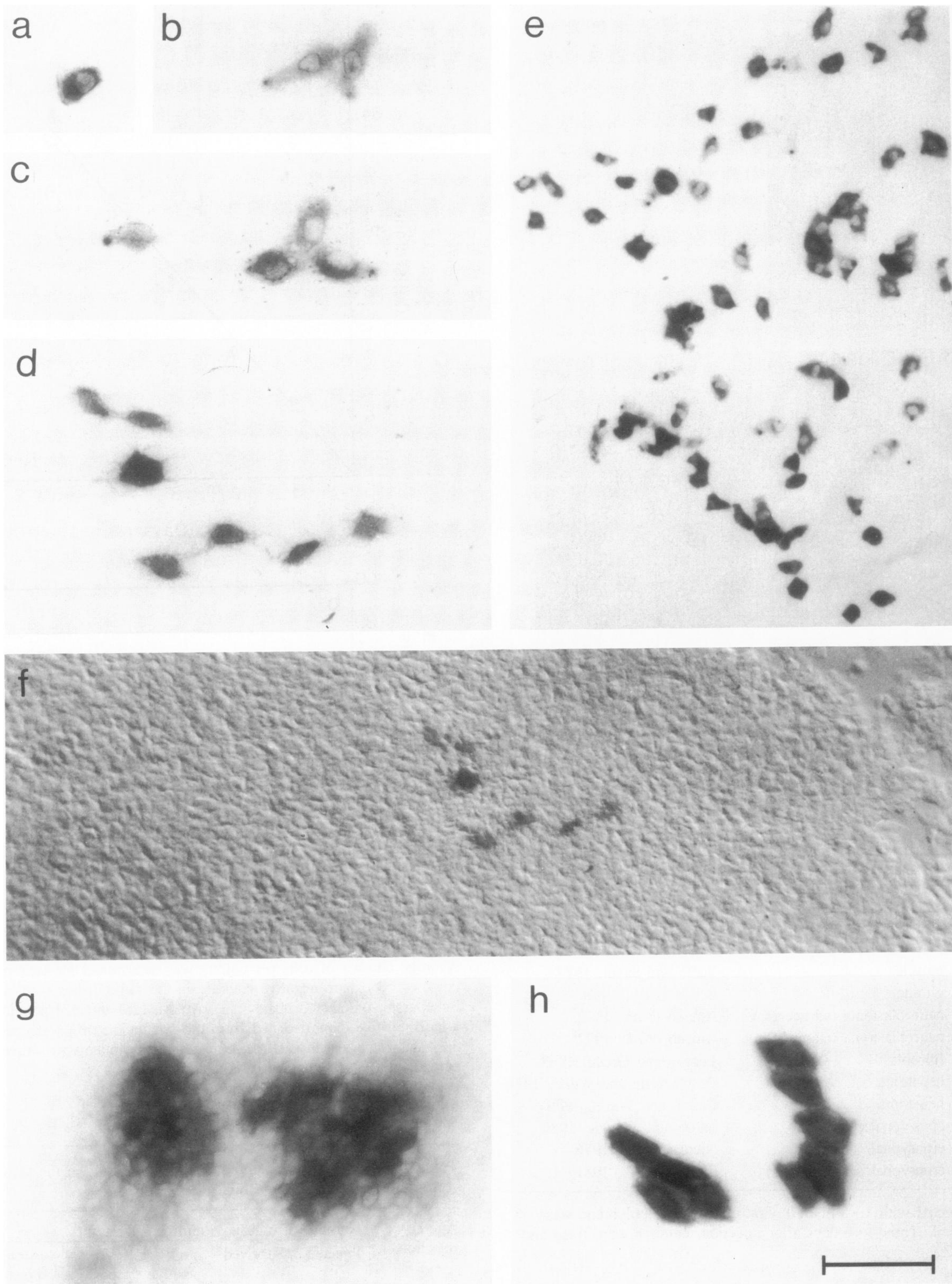


Fig. 4. LacZ-positive cells in the visceral yolk sac (**a–f**) and skin (**g–h**) of embryos injected with LZ1 virus. Embryos were injected on E9 (**a–d,g,h**) or E7 (e), fixed and stained for lacZ 2 (**a,b**), 3 (**c,d**) or 5 (**e,g,h**) days later, then viewed as whole mounts. Unstained cells are nearly invisible with the brightfield optics used for these micrographs. Granular precipitates near stained cells may represent reaction product that diffused before precipitating. (**f**) Shows the same cells as (d) at lower power and with Nomarski optics, to show that clusters of stained cells are surrounded by large areas of unstained tissue. Bar in **h** represents 50 μm in a–d, g, h; 80 μm in e, f.

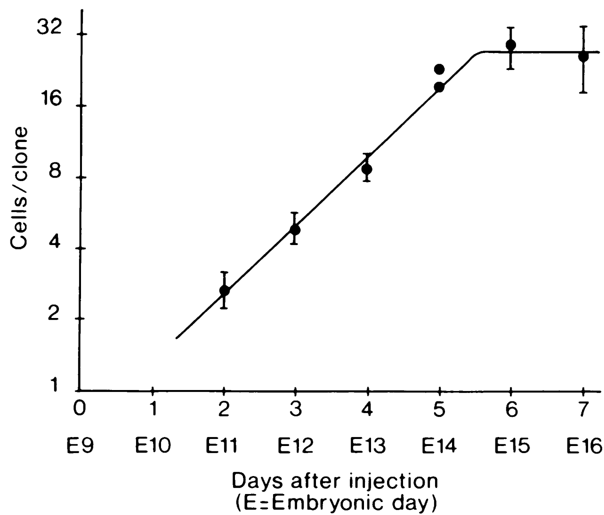


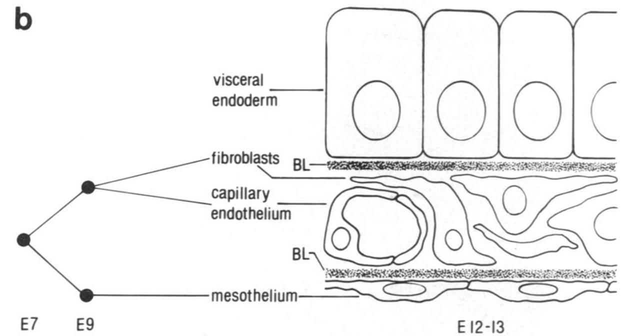
Fig. 5. Growth of lacZ-positive clones in the visceral yolk sac. Embryos injected with LZ1 virus on E9 were sacrificed at daily intervals starting at E11, then fixed and stained for lacZ. The number of lacZ-positive cells per clone was counted in whole mounts such as those shown in Figure 4. Mean number of cells per clone ± SE is shown. The number of clones analyzed for the 2−7 day points is **13, 19, 20, 2, 7** and **5**, respectively.

to eight days later, the embryos and extra-embryonic membranes were dissected, fixed, and stained for lacZ. We began by seeking infected cells in the skin, visceral yolk sac, and amnion because these tissues are thin, flat and easy to examine as whole mounts. Table II shows that 60% of the embryos survived injection, and that lacZ-positive cells were detectable in a quarter of the survivors. Infections were successful at all embryonic ages tested, but injection at E9 was consistently most productive: about half of all embryos surviving injection at E9 had lacZ-positive cells. No positive cells were observed in tissues from 30 uninjected embryos (or in 159 of the injected embryos), demonstrating that staining was not attributable to endogenous galactosidase-like activities. Thus, the LZ1 virus can infect cells following injection into embryos.

In each tissue examined, lacZ-positive cells occurred in small clusters (Figure 4). Cells within a cluster were closely spaced, although often not in contact with each other, while clusters were separated from each other by relatively long distances (generally > 10 cluster diameters). Positive animals bore 1−10 labelled clusters each. In both yolk sac and skin, the average number of lacZ-positive cells per cluster increased with the interval between injection and sacrifice. In the yolk sac, cluster size increased for about 5 days following injection of virus into E9 embryos (Figure 5); the lack of growth after E14−15 is consistent with a report

a *lacZ*-positive clones in the yolk sac

Clone	Day of injection	*lacZ*-positive cells: Visceral endoderm	Capillary endothelium	Fibroblasts	Mesothelium
1	7	−	+	+	+
2	7	−	+	+	+
3	9	−	−	−	+
4	9	−	+	+	−
5	9	−	+	+	−
6	9	−	+	+	−
7	9	−	+	+	−
8	9	−	+	+	−
9	9	−	+	+	−
10	9	−	−	+	−



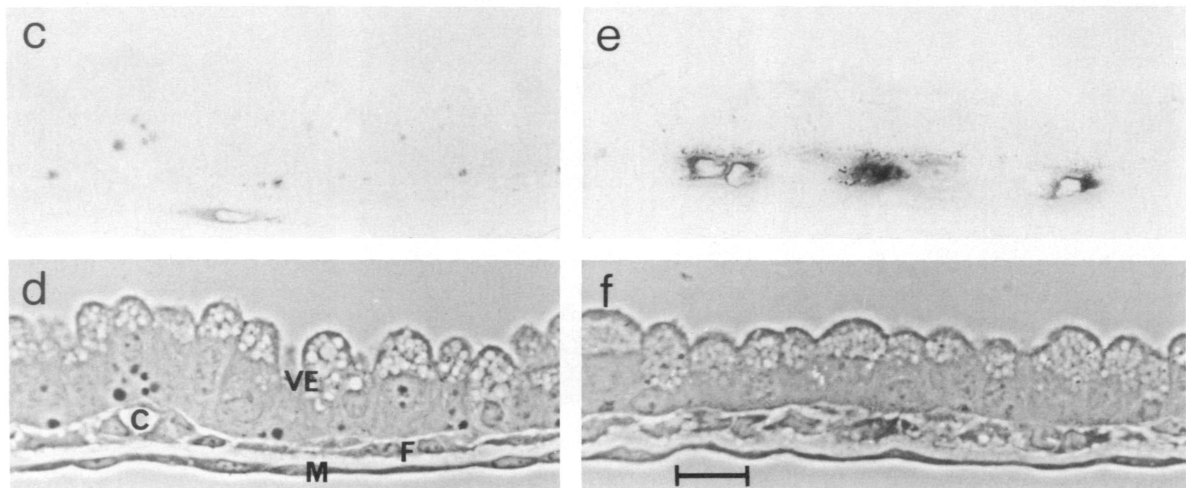
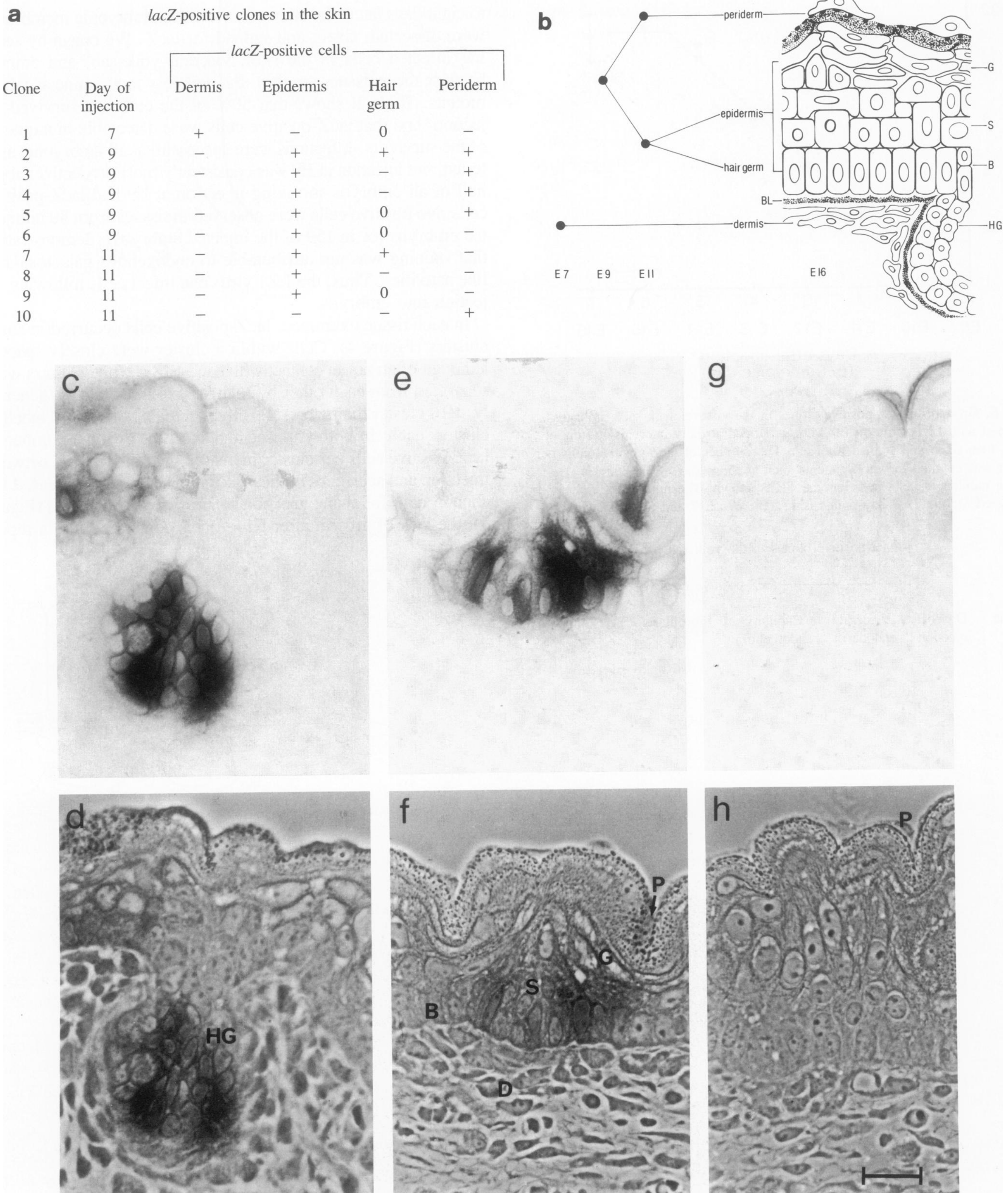


Fig. 6. Clonal analysis in the visceral yolk sac. (**a**) LacZ-positive cell types identified in a series of 10 clones that were embedded in plastic and semi-serially sectioned. (**b**) Lineage diagram constructed from the results in a. (**c** to **f**) Sections from clone #3 (**c,d**) and clone #4 (**e,f**), photographed with brightfield (**c,e**) and phase (**d,f**) optics. **VE**, visceral endoderm; **C**, capillary; **F**, fibroblast; **M**, mesothelium; **BL**, basal lamina. Bar is 20 μm.



a *lacZ*-positive clones in the skin

Clone	Day of injection	*lacZ*-positive cells: Dermis	Epidermis	Hair germ	Periderm
1	7	+	−	0	−
2	9	−	+	+	+
3	9	−	+	−	+
4	9	−	+	−	+
5	9	−	+	0	+
6	9	−	+	0	−
7	11	−	+	+	−
8	11	−	+	−	−
9	11	−	+	0	−
10	11	−	−	−	+

Fig. 7. Clonal analysis in the skin. **(a)** Lac-Z-positive cell types in 10 clones that were embedded in plastic and semi-serially cross sectioned. **(b)** Lineage diagram constructed from the results in (a). '0' indicates that no hair germ was present in the tissue fragment. **(c–h)** Sections from clones #2 **(c,d)**, 3 **(e,f)** and 10 **(g,h)**, photographed with brightfield **(c,e,g)** and phase **(d,f,h)** optics. **B**, stratum basale; **S**, stratum spinosum; **G**, stratum granulosum; **P**, periderm; **HG**, hair germ; **D**, dermis; **BL**, basal lamina. Bar is 20 μm.

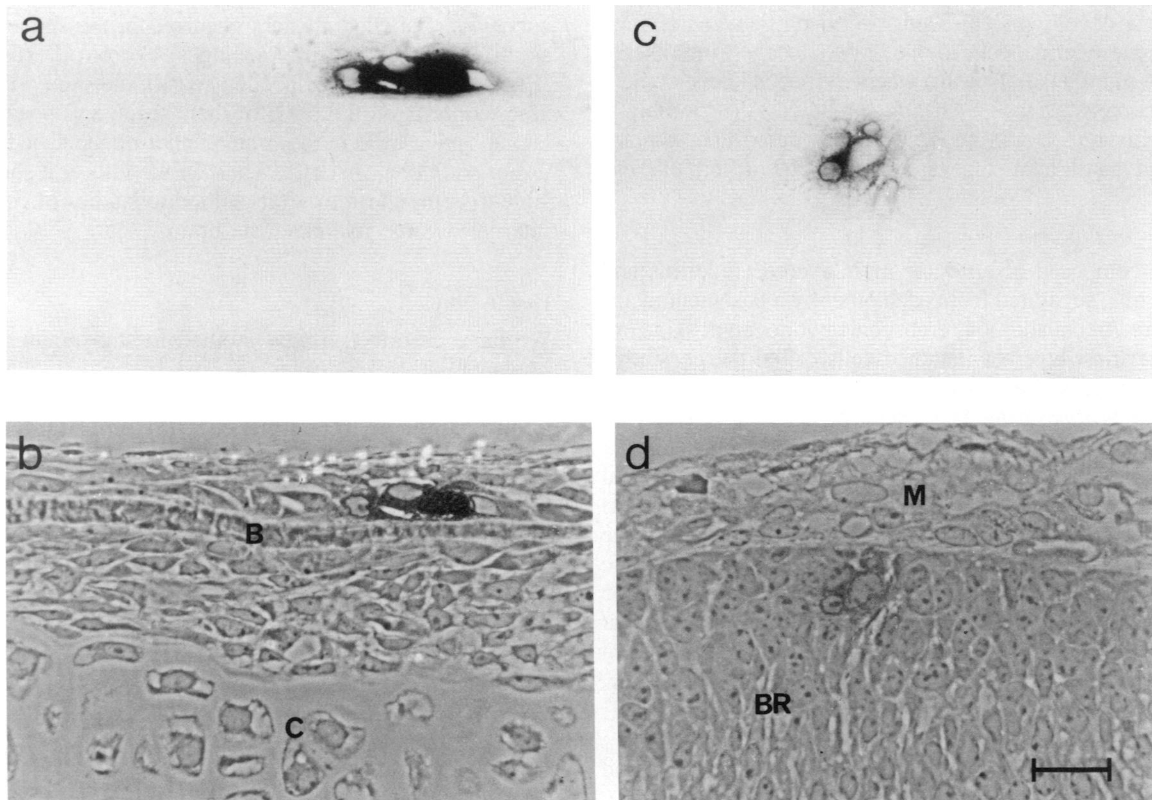


Fig. 8. LacZ-positive cells in the cranium. LacZ-positive cells in the skull (**a,b**) and brain (**c,d**) of embryos injected with virus on E13 and killed on E17. Stained areas were identified in whole mounts, embedded in plastic, sectioned at 2 μm, and photographed with brightfield (**a,c**) or phase (**b,d**) optics. The section of the skull is from near the base of the orbit, an area where membranous and cartilaginous bone lie in close proximity. **C**, cartilage; **B**, brain; **M**, meninges. Bar is 20 μm.

that little DNA synthesis occurs in extraembryonic membranes after this stage (Saccoman *et al.*, 1965). In the skin, average size increased continuously with the interval from injection to staining; a cluster of several hundred cells was observed in a neonate following injection at E9. Our interpretation of these patterns is that each cluster comprises the progeny of a single LZ1-infected cell and is therefore clonal in origin.

Cell lineage in the visceral yolk sac

From E8 to term, the visceral yolk sac consists of three cellular layers separated from each other by basal laminae: a monolayer of visceral endoderm faces outward, a thin mesothelium faces the embryos, and a layer of fibroblasts and capillaries is interposed between the two (Figure 6b). The central and inner layers both develop from a sheet of mesodermal cells that underlies the endoderm by E7. Proliferation in the mesoderm results in the formation of cell 'masses' that give rise to capillary endothelial cells and erythroblasts; cells within or between the masses differentiate to form the fibroblasts and mesothelium (Wislocki and Padykula, 1953; Haar and Ackerman, 1971).

To study cell lineage in the visceral yolk sac, we embedded and semi-serially sectioned 10 fragments of tissue containing lacZ-positive clones and identified the cell types that had been stained; the clones were from embryos injected on E7 and fixed on E12 or injected on E9 and fixed on E13 (Figure 6a). Both clones marked on E7 contained three mesodermal derivatives: mesothelial, fibroblastic, and capillary endothelial cells. In contrast, clones marked on E9 contained fewer cell types. LacZ-positive cells were confined to the mesothelium in one such clone (Figure 6c and d) and to the central layer in the others (Figure 6e and f). In the latter seven cases, some cells were identifiable as fibroblastic cells, endothelial cells of the capillary wall were also stained in six of these clones. In no clones were cells of the visceral endoderm labelled.

Our interpretation of these results is presented in the form of a lineage diagram in Figure 6b. The result that clones injected on E7 contain mesothelial, fibroblast, and capillary endothelial cells demonstrates the existence of an ancestor that can give rise to these three cell types. We believe that the ancestor is present on E7 because studies *in vitro* indicate that retroviral infection is rapid (Weiss *et al.*, 1982) and that free virions break down rapidly ($t_{1/2}$ *of* ~5 h at 37°C; data not shown). Similarly, we postulate ancestors present on E9 that give rise to mesothelial or fibroblast–endothelial progeny. In constructing the lineage diagram, we hypothesize that ancestors of the type infected on E7 gave rise to the cells infected on E9. The lack of lacZ-positive visceral endoderm cells in these clones does not permit us to conclude that the visceral endoderm arises from separate ancestors,

because we do not know whether the *lacZ* gene in LZ1 provirus can be expressed in this cell type. Taken together, our results suggest that a developmental event occurring between E7 and E9 allocates mesodermal cells to one of two lineages: mesothelial or fibroblast-endothelial. To learn whether a second developmental step occurring after E9 further subdivides the fibroblast-endothelial lineage, it will be necessary to determine whether cells infected at still later stages give rise to one or both of these cell types.

Cell lineage in the skin

The skin is composed of an outer multilayered epidermis and an inner dermis, separated from each other by a basement membrane (Figure 7b). In addition, embryonic but not adult skin contains an outermost layer of flattened cells called the periderm. The epidermis and periderm arise from an ectodermal monolayer, while the dermis arises from the mesoderm. As development proceeds, the layers that comprise the adult epidermis form. These are, from inside out: the stratum basale, a single layer of cuboidal cells lying in direct contact with the basal lamina; the stratum spinosum, consisting of several layers of polyhedral cells; the stratum granulosum, containing flattened cells with pycnotic nuclei and granular cytoplasm; and the stratum corneum, made up of anucleate, keratin-rich scales. Mitoses occur largely (but not exclusively) in s. basale, and basal cells differentiate to form the suprabasal layers. The periderm is shed during the last few days of embryogenesis and disappears by birth (Hanson, 1947; Stern *et al.*, 1971; Hayward and Kent, 1982).

We sectioned nine lacZ-positive clones from embryos that had been injected with LZ1 virus on E9 or E11 and killed on E16 (Figure 7a). At E16, the s. basale, s. spinosum and s. granulosum are present, the s. corneum has not yet formed, and the periderm is being shed. Labelled cells were confined to the epidermis and periderm in all nine of these clones (Figure 7c–h). Both peridermal and epidermal cells were labelled in four of the five clones marked on E9, while either periderm or epidermis was labelled in all four clones initiated on E11. Thus, between E9 and E11, a class of initially bipotential ancestor cells are apparently allocated to one of two lineages: epidermal or peridermal. Cells of all epidermal layers (s. basale, spinosum and granulosum) were lacZ-positive in seven of the eight clones that contained epidermal cells; this result provides direct evidence that these cells arise from a single ancestor. In one epidermal clone, labelled cells were confined to the s. basale, suggesting that proliferation in this layer is not necessarily followed by prompt differentiation into suprabasal cells. Cells of a hair germ, the epidermal invagination that gives rise to a hair, were labelled in two clones that also contained lacZ-positive epidermal cells. Thus, progeny of a single cell can give rise to both hair and ordinary epidermal cells. Finally, we sectioned one lacZ-positive clone of $>10^2$ cells from an embryo that had been injected on E7 and killed on E13; labelled cells were confined to the dermis in this clone. A lineage diagram summarizing these results was constructed as described above for the yolk sac and is shown in Figure 7b.

LacZ-positive cells within the cranium

In further studies of cell lineage using the LZ1 virus, it will be essential to detect labelled cells in internal organs and useful to target virus to particular parts of the body. To test the feasibility of achieving these goals, we injected LZ1 into the head in a series of E12 and E13 embryos; E12 was the earliest stage that the head could be clearly seen through the uterine wall. Four days later, the heads were fixed, bisected in the mid-sagittal plane, and stained for lacZ. Of 45 embryos injected, 29 survived, and intracranial clusters of lacZ-positive cells were detected in 10 (34%) of the survivors. Labelled clusters occurred in the subdermal fascia, skull (Figure 8a and b), meninges, choroid plexus, and brain (Figure 8c and d). The labelled cells in the skull are identifiable as osteoblasts on the basis of their shape and position, but the lacZ-positive cells in the brain cannot be identified without the use of additional markers. Thus, in the future, it should be possible to apply the retroviral method to studies of cell lineage in internal organs, including the brain.

Discussion

We have described a new method to study mammalian post-implantation cell lineages *in vivo* by infecting cells with a replication-defective recombinant retrovirus and detecting the virally-introduced gene product in the progeny of the infected cells. In developing the method, we: (i) produced a recombinant retrovirus, LZ1, containing the *lacZ* gene; (ii) modified existing histochemical methods to permit detection of *lacZ*-encoded β-galactosidase in whole mounts of tissue and in sections; (iii) used cultured cells to show that expression of lacZ is stable and selectively neutral in the progeny of infected fibroblasts, and that LZ1 can direct lacZ expression in a variety of cell types; (iv) detected lacZ-positive cells in several tissues (visceral yolk sac, amnion, skin, skull, meninges, choroid plexus, fascia and brain) following injection of viral concentrate into mid-gestation embryos; and (v) tested the method by applying it to an analysis of cell lineage in the visceral yolk sac and skin. This discussion focuses on the final of these steps.

In all tissues where lacZ-positive cells were detected, they were grouped in clusters. The clusters occurred either singly in a given tissue or separated from each other by large distances. The number of cells per cluster increased with the interval from injection to analysis. Stained cells were not seen in uninjected or sham-injected embryos, demonstrating that they were not attributable to endogenous β-galactosidase-like activities. Furthermore, the cells were readily identifiable in sections as belonging to types endogenous to the tissue; thus, they were not extrinsic cells (e.g. macrophages) that had migrated to injection sites. We have little doubt, athough we have not proved directly, that most clusters are composed of the progeny of a single cell and are therefore clonal in origin. If some members of the clone have migrated away or contain undetectable levels of lacZ in their cytoplasm, the cluster will comprise only part of a clone. Conversely, it is possible that, in rare cases, separate viruses could infect two closely spaced cells, the progeny of which would be mistakenly identified as a single clone. However, to the extent that our assumption of the clonal origin of lacZ-positive cell clusters is valid, analysis of the composition of the cluster should provide information about cell lineage relationships in the tissue in question.

To apply the method to the visceral yolk sac and skin, we semi-serially sectioned a series of lacZ-positive clones, identified the labelled cells, and constructed lineage diagrams based on this analysis. Because we analyzed a small number of clones (10) in each tissue, and took these clones from embryos injected and killed at few different ages, our results constitute only the beginning of a definitive study of cell lineage. None the less, the results are consistent with and extend previous developmental studies in several respects.

First, patterns of clonal growth reflected patterns of tissue growth. In the skin, the average number of lacZ-positive cells

per clone increased with the interval between virus injection and sacrifice, as expected for a continuously growing tissue. In the yolk sac, on the other hand, clones increased in size only until E15, consistent with a report that DNA synthesis in extra-embryonic membranes has nearly ceased at this time (Saccoman *et al.*, 1965).

Second, in each clone, lacZ-positive cells were restricted to derivatives of a single primary germ layer. In the yolk sac, mesodermally derived cells were labelled while the overlying endoderm was unlabelled; in the skin, either ectodermal (epidermal and peridermal) or mesodermal (dermal) cells, but not both, were labelled in any single clone.

Third, many clones contained cells of more than a single type, demonstrating the existence of multipotential precursors within each tissue. In the yolk sac, clones comprising mesothelial, fibroblast and capillary endothelial cells provide the first evidence for a common precursor to these three cell types. (Primitive erythrocytes also arise from the mesoderm, but have migrated from the yolk sac at the time of our observations; Haar and Ackerman, 1971). In the skin, the labelling of cells in several epidermal strata within the same clone confirms the common origin of these cells from a single ancestor, presumably in the stratum basale. The presence of both epidermal and peridermal cells within single clones initiated on E9 provides direct evidence that these two cell types originate from a common precursor (cf. Hanson, 1947) and clearly disproves the hypothesis (Bonneville, 1968) that the periderm arises from invading amnionic cells. The existence of a clone containing both hair germ and ordinary epithelial cells demonstrates that hair-forming precursors have not been singled out for specialization as late as E11.

Finally, clones marked at early times contain more cell types than clones marked at late times, providing evidence for a progressive restriction of cell fate. In the yolk sac, injection on E7 labelled cells of both the mesothelial and central layers within a single clone, while injection on E9 labelled either the mesothelial or the central layer. In the skin, clones marked on E9 contained both epidermal and peridermal cells, while clones marked on E11 contained either epidermal or peridermal cells. Thus, in each tissue, our method has provided new information on the time at which a developmental event occurs that allocates cells to one developmental pathway or another. Interestingly, these restrictions of cell fate occur at times at which lamination occurs in both yolk sac (separation of mesothelium from central layer on E8; Haar and Ackerman, 1971) and skin (formation of separate epidermal and peridermal layers ~E10; Hanson, 1947).

The agreement of our results with those obtained by traditional histological methods reinforces the reliability of the retroviral approach for genealogical studies. However, in undertaking new experiments, it may be necessary to modify the method in a number of ways: (i) some tissues may require improved histochemical or surgical techniques, e.g. because of interfering endogenous β-galactosidase-like activities or because of difficulties in delivering the virus to the appropriate site. (ii) Even though LZ1 virus induces lacZ expression in a variety of cell types, some cells may be immune to infection (e.g., they may lack appropriate virus receptors) or may not express lacZ even if they are infected. In this case, it may be necessary to construct new retroviruses, with different envelope types or internal promoters. (iii) Because it is impossible to distinguish the progeny of one infected cell from those of another, we have studied tissues in which the outlines of entire individual clusters can be distinguished in whole mounts. Thus, we define clonal origin morphologically. Recently, Keller *et al.* (1985) and Soriano and Jaenisch (1986) have used the site of integration (determined by Southern blotting) as a clonal marker in studies of the lineage of retrovirally infected cells; however, this method is currently inapplicable to individual cells. Therefore, new criteria will be required to evaluate the clonal origin of cells in clones that span multiple tissues, or include migratory cells. (iv) Because only the offspring of an infected cell are detected, identification of ancestors that give rise to lineages of interest will require infections and analyses at multiple, overlapping intervals.

Finally, it is important to note that the retroviral method we have used provides information about the actual fates of cells but not about their range of potentials. For example, while we found that cell fate becomes progressively restricted in both yolk sac and skin, we do not know whether developmental potential is similarly restricted; it is equally likely that initially equipotent cells express different phenotypes as a consequence of exposure to different environments. Issues of developmental potential and of the mechanisms that underly developmental choices have been profitably studied by analyzing the fates of cells that can be induced to differentiate under controlled conditions in culture (see Raff *et al.*, 1984; Doupe, Patterson and Landis, 1985; Muller-Sieburg *et al.*, 1986, for recent examples). Correlative studies *in vivo* and *in vitro* may be reasonably expected to provide a more complete understanding of cell lineage than either alone.

In conclusion, the application of a new retroviral approach to the analysis of cell lineage in the yolk sac and skin has yielded new results that are consistent with and readily interpretable in light of previous work. The fact that few other methods are currently available to study post-implantation cell lineages in mammalian embryos should motivate efforts to improve the retroviral approach and thus increase the range of developmental issues to which it can be applied. At the same time, the protocols and reagents currently in hand may prove useful in studying the genealogy of many cell types.

Materials and methods

Plasmid construction

The plasmid pM-MuLV-SV-*lacZ* (Figure 1a) was constructed by methods described in Maniatis *et al.* (1982) using enzymes obtained from Boehringer-Mannheim. Three fragments were used. The first, containing bacterial and retroviral sequences, was derived from pB6 (Yermanian, 1984), a plasmid that contains a M-MuLV proviral genome (Harbers *et al.*, 1981). pB6 was digested with *Sal*I and *Bam*HI and the 8.2-kb fragment was isolated. The second fragment, containing the SV40 promoter and part of the *E. coli lacZ* gene, was derived from pCH110-3-SVE (a gift of P.Herbomel, Institut Pasteur), a modified version of pCH110 (Hall *et al.*, 1983) in which a *Sal*I site has been inserted at a *Pvu*II site just 5′ to the SV40 promoter. This plasmid was digested with *Sal*I and *Eco*RI, and the 3.6-kb fragment was purified. The third fragment, containing the 3′ end of the *lacZ* gene, derived from a modified form of pMBL524 (Parsot *et al.*, 1982) in which we changed the *Ava*I site to a *Bam*HI site. The 320-bp fragment was isolated from a *Bam*HI – *Eco*RI digest of this plasmid. These three fragments were then ligated.

Production of retrovirus

A calcium phosphate precipitate formed from 10 μg each of pM-MuLV-SV-*lacZ* and pSVtkneoβ (Nicolas and Berg, 1983) was used to transfect ψ2 cells (Mann *et al.*, 1983) by the method of Graham and Van der Eb (1973). ψ2 is a 'helper' cell line that contains a M-MuLV provirus genome with a deleted packaging signal; it produces no wild-type virus but provides viral proteins in trans to complement replication-defective retroviruses. The pSVtkneoβ plasmid encodes the gene for resistance to the aminoglycoside G418; its inclusion allowed us to select stable co-transformants by growth in G418 (Colbere-Garapin *et al.*, 1981; Southern and Berg, 1982). Neo transformants were tested for β-galactosidase activity by the method in Miller (1972). Clones with the highest activity were tested for virus production by exposing NIH 3T3 cells to their supernatants in the presence of 10 μg/ml polybrene and assaying the NIH 3T3 cells for β-galactosidase 2 days later. The clone producing the highest level of lacZ transducing activity was recloned by limiting dilution and called LZ1. We have not yet determined whether the *lacZ* gene is transcribed from the viral LTR or from the internal SV promoter.

Using the S+ L− test described in Weiss *et al.* (1982), we found that the LZ1 producing line produced no wild-type virus (<1 per 4 ml supernatant).

The virus was concentrated from culture medium by a modification of a method provided by Constance Cepko (Harvard Medical School). Medium was centrifuged for 10 min at 4°C in a clinical centrifuge. The supernatant was filtered through a 0.22 μm Millipore filter into alcohol-sterilized polyallomer tubes and then centrifuged for 14−16 h at 14 000 r.p.m. at 2°C in an ultracentrifuge, using a SW27 rotor. The supernatant was poured off and the pellet resuspended in 0.2−2% of its original volume of culture medium: calf serum (1:1). Nearly complete recovery of viral activity was generally achieved (Figure 1c) and the resuspended virus was stable for 1 week at 4°C and at least 2 months in liquid nitrogen.

Injection of virus into embryos

Male mice of the 129/SV strain were mated with females that were the F1 progeny of a cross between C57BL/6 males and CBA-J females. None of these strains have been reported to harbor infective M-MuLV (Weiss *et al.*, 1985). Pregnancy was timed by checking for vaginal plugs, and the day the plug was detected was counted as E0. Pregnant mice were anesthetized by interperitoneal injection of tribromoethanol (0.5 ml of a 150 μg/ml solution). Vertical incisions were made in the skin and body wall musculature at the abdominal ventral midline, and the uterine horns were withdrawn. Viral concentrate was then injected into individual embryos through a micropipette. Pipettes were either pulled by hand from 5 μl disposable capillaries (Corning) or with a horizontal electrode puller from electrode glass (GC 100-10; Clark Instruments). 0.2−0.5 μl liquid was injected per embryo. Because infection of cultured cells is enhanced by polybrene (Figure 1d), the viral concentrate was mixed wtih polybrene to a final concentration of 1 mg/ml in most experiments; tests on NIH 3T3 cells showed that this procedure was not deleterious to the virus. Positive results were, however, also obtained when polybrene was omitted or replaced by 1 mg/ml DEAE-dextran (Duc-Nguyen, 1968). For E7 and E8 embryos, injections were made from the ventral midline (just opposite the myometrium) into the decidual swelling; for E9−E11 embryos, injections were aimed at the extra-embryonic cavity; for E12 and E13 embryos, we visualized and attempted to inject into the head. Following injection the body wall muscle was closed with sutures and the skin with surgical staples. Two or more days later, embryos or neonates were retrieved and stained for lacZ.

β-Galactosidase histochemistry

Cultured cells were rinsed with 150 mM NaCl, 15 mM Na phosphate, pH 7.3 (PBS) and then fixed for 5 min at 4°C in 2% formaldehyde plus 0.2% gluteraldehyde in PBS. The cells were then washed with PBS and overlaid with a histochemical reaction mixture containing 1 mg/ml 4-Cl-5-Br-3-indolyl-β-galactosidase (X-Gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS. The X-Gal was dissolved in dimethoxysulfoxide (DMSO) at 40 mg/ml, and then diluted into the reaction mixture. In some experiments, 1% agar was added to the incubation mixture to minimize detachment of cells from their substrate. Incubation was for 14−18 h at 37°C.

To reveal β-galactosidase activity in embryonic tissues, this procedure was modified in the following ways: (i) Fixation was prolonged for 40−60 min. (ii) Agar was omitted from the reaction mixture. (iii) The detergents NP-40 and sodium deoxycholate were added to final concentrations of 0.02% and 0.01%, respectively, to enhance permeability of the tissue. (iv) Incubation was at 30°C instead of 37°C to reduce background staining and minimize tissue damage. (v) Tissues were rinsed with 3% DMSO in PBS and then stored in PBS at 4°C following reaction.

β-Galactosidase-positive areas in embryos were identified at 50× magnification under the dissecting microscope, cut out, and analyzed either as whole mounts or in sections. For whole mounts, tissue was thinned by dissection, refixed briefly in ethanol:glycerol (2:1) and mounted in glycerol:PBS (9:1). Pieces to be sectioned were rinsed in 0.1 M cacodylate buffer, refixed in 1% OsO_4 in cacodylate for 40 min, rinsed again, dehydrated in alcohol, rinsed in propylene oxide, and infiltrated in Araldite. It was important to minimize the time of exposure to propylene oxide (<10 min), as the X-Gal reaction product is slightly soluble in this solvent. Following infiltration, β-galactosidase-positive areas were reidentified under the dissecting microscope, trimmed, and embedded. Sections were cut at 2 μm and examined without counterstaining.

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