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Clonal analysis of early mammalian development

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[Plate 1]

Various extrinsic markers have been used to label single cells in the early mouse embryo. However, they are appropriate only for short-term experiments because of their susceptibility to dilution. Studies on cell lineage and commitment have therefore depended mainly on exploiting genes as markers by combining cells from embryos that differ in genotype at particular loci. Tissue recombination and transplantation experiments using such indelible intrinsic markers have enabled the fate of different cell populations in the blastocyst to be determined with reasonable precision. The trophectoderm and inner cell mass (i.c.m.) give rise to distinct complementary groups of tissues in the later conceptus, as do the primitive endodermal and primitive ectodermal components of the more mature i.c.m. When cloned by blastocyst injection, single i.c.m. cells colonize only those parts of host conceptuses that are derived from their tissue of origin. Thus, while clonal descendants of early i.c.m. cells can contribute to all tissues other than those of trophectodermal origin, primitive endodermal and primitive ectodermal clones are restricted, respectively, to the extraembryonic endoderm versus all i.c.m. derivatives except the extraembryonic endoderm. Interestingly, individual primitive ectoderm cells can include both germ cells and somatic cells among their mitotic descendants.

By using the genetically determined presence versus absence of cytoplasmic malic enzyme activity as a cell marker, the deployment of clones has been made visible *in situ* in whole-mount preparations of extraembryonic membranes. Very little mixing of donor and host cells was seen in either the endoderm of the visceral yolk sac or the mesodermal and ectodermal layers of the amnion. In contrast, mosaicism in the parietal endoderm was so fine grained that, in all except 1 of 15 fields from several specimens that were analysed, the arrangement of donor and host cells did not differ significantly from that expected on the basis of their random association.

Introduction

Very little can be learnt about cell lineage in early mammalian development by observation of the intact embryo in culture. Compaction leads to the loss of distinct boundaries between blastomeres by the late eight-cell stage (Lewis & Wright 1935) and morphogenesis of the blastocyst is disrupted if the embryo is maintained in a decompacted state much beyond this juncture (Johnson *et al.* 1979). Furthermore, although under certain conditions pre-implantation embryos can develop to an early somite stage *in vitro*, they do so unpredictably and, most interestingly, by a somewhat different route than *in utero* (Gonda & Hsu 1980; Wu *et al.* 1981). Hence, to follow the fate of cells during early embryogenesis in mammals, it is necessary to mark them in such a way that their clonal descendants can be distinguished unambiguously from all other cells in the embryo.

It is only quite recently that extrinsic markers such as horseradish peroxidase or certain

fluorochrome dyes have been used to label cells of the preimplantation embryo (Balakier & Pedersen 1982; Gearhart *et al.* 1982; Ziomek 1982; Surani & Handyside 1983; Surani & Barton 1984). Hitherto, [^{3}H]thymidine was the reagent of choice for this purpose (Mintz 1965; Hillman *et al.* 1972; Garner & McLaren 1974; Kelly 1979). In most cases use of extrinsic markers has entailed the recombination of cells from labelled embryos with those from unlabelled ones. However, intracellular injection of horseradish peroxidase has proved a satisfactory way of marking individual cells of the cleaving embryo and blastocyst *in situ* (Balakier & Pedersen 1982; Gearhart *et al.* 1982; Cruz & Pedersen 1984; Winkel & Pedersen 1984). Extrinsic cell labels have been particularly valuable in tackling the problem of allocation of blastomeres to the trophectoderm versus inner cell mass (i.c.m.). Their principal limitation is that, being susceptible to dilution through metabolic degradation or cell division, they can only be used in relatively short-term experiments.

The problem of dilution of label can obviously be circumvented by using genes as markers in longer-term lineage studies. However, other obstacles have to be overcome in harnessing such indelible intrinsic cell markers. First, few of the large number of polymorphic gene loci that have been documented in the mouse (Green 1981) exhibit the requisite characteristics. The properties of an ideal genetic marker have been discussed recently by West (1984), together with the merits and shortcomings of those that are currently available for the mouse. Needless to say, none of the existing markers measures up fully to the ideal, although several display most of the necessary attributes. The second difficulty is that of actually marking individual cells genetically. Evidence for the occurrence of mitotic recombination in mammals is still equivocal (Gruneberg 1966), and no other reliable way of changing the genotype of single cells *in situ* has yet been devised. Hence, at present, genes can only be exploited to study lineage in mammalian development by bringing together cells from embryos that differ in genetic constitution with respect to the marker locus. The strategy originally devised by Tarkowski (1961), and subsequently refined by Mintz (1962), was to aggregate pairs of approximately eight-cell embryos that had been divested of the zona pellucida. The resulting composite early embryos resemble those of *Drosophila* gynandromorphs (Janning 1978) in the sense that they consist of two genotypically different coherent clusters of cells with a presumably randomly oriented dividing line between them (Garner & McLaren 1974; Kelly 1979). Unfortunately, the extensive cell minging that occurs during later development of these aggregation chimeras (McLaren 1976) precludes their being used like *Drosophila* gynandromorphs to map the spatial relationships between tissue primordia in the early embryo.

For clonal analysis of early mammalian development it was obviously essential to refine procedures for chimera production so that a single cell from an embryo of one genotype could be introduced into that of another. In practice, this entails dissociating and reaggregating blastomeres (Hillman *et al.* 1972; Kelly 1975, 1977), usually between the 4- and 16-cell stage, or injecting cells into the blastocyst (Gardner 1968, 1978; Babinet 1980). It is important to bear in mind that cellular relationships are inevitably disturbed to a certain extent by using these cloning procedures. Some degree of perturbation of development may, indeed, be a price that has to be paid for using genes as clonal markers since tissue damage is also associated with the use of X-rays for changing the genotype of cells *in situ* by mitotic recombination in *Drosophila* (Haynie & Bryant 1977).

The following discussion is specifically concerned with studies on cell potency, lineage and deployment in the mouse embryo using genetic markers, and is divided into two sections. The

first summarizes what has been learnt about the developmental potential and lineage of cells of the early embryo with respect to the different tissues of the later conceptus and postnatal mouse. The second section deals with exploitation of the genetically determined presence versus absence of cytoplasmic malic enzyme activity as an *in situ* marker system for investigating cell deployment within certain tissues of the conceptus, and is essentially an interim report. Throughout, emphasis is placed on clonal studies since these give the greatest precision that is attainable in the types of investigation under consideration.

Lineage

Electrophoretically distinct allozymes of glucosephosphate isomerase (GPI) have been used as cell markers in most lineage studies because the enzyme appears to be ubiquitous in tissue distribution, and is cell-autonomous and cell-limited in expression. It is also synthesized from an early stage in development (Chapman *et al.* 1971; Brinster 1973; West & Green 1983). The principal limitation of GPI is that its allozymes can only be resolved following disruption of tissue architecture. Hence, this marker system can only be used to estimate the relative proportions of cells of different genotype in a given piece of tissue and provides no information about their spatial arrangement. This shortcoming poses particular problems with regard to elucidation of cell lineage relationships in structures like the chorioallantoic placenta whose constituent cell populations cannot readily be separated before analysis (Gardner 1985*a*; Rossant, this symposium). Nevertheless, GPI has enabled a basic fate map of the early embryo to be established and the time of restriction in developmental potential of certain cell populations to be defined (Gardner 1983).

In a series of elegant recombination experiments, Kelly (1975, 1977) was able to demonstrate that blastomeres of the eight-cell embryo retain totipotency. Some eight-cell blastomeres clearly normally contribute progeny to both the trophectoderm and i.c.m. although the number that do so is still a matter of debate (Graham & Lehtonen 1979; Handyside 1981; Johnson & Ziomek 1981; Gearhart *et al.* 1982; Pedersen *et al.* 1982; Gardner 1985*a*). More recently, Rossant & Vijh (1980) found that in one third of a series of chimeric conceptuses obtained following injection of individual one sixteenth blastomeres under the zona pellucida of 8- to 12-cell embryos, clonal descendants of the donor cell had contributed to derivatives of both the trophectoderm and i.c.m. This is the latest stage of development for which the presence of cells that are able to contribute progeny to both primary tissues of the blastocyst has been demonstrated unequivocally. Nevertheless, interconversion of presumptive trophectoderm and i.c.m. cells can still be achieved in the late morula and early blastocyst, respectively (Rossant & Vijh 1980; Handyside 1978; Hogan & Tilly 1978; Spindle 1978; Rossant & Tamura-Lis 1979; Nichols & Gardner 1984).

Three types of experiment using GPI allozymes as markers have enabled the fate of the various cell populations in the blastocyst to be determined with respect to all components of the conceptus except the placenta. The first entails transplantation of entire i.c.ms from expanded fourth-day blastocysts into vesicles of pure trophectoderm prepared from synchronous embryos of the opposite genotype (Gardner *et al.* 1973; Papaioannou 1982). The results demonstrated that the trophectoderm gives rise to the trophoblastic giant cells, ectoplacental cone, extraembryonic and chorionic ectoderm, and that all other tissues of the early postimplantation embryo are derived from the i.c.m. (see Snell & Stevens (1966) for a

description of early mouse development). No tissues were found to be of dual origin in these experiments. However, certain very recent findings have raised the possibility that the early i.c.m. makes a cellular contribution to the overlying polar trophectoderm during normal development (Cruz & Pedersen 1984; Winkel & Pedersen 1984; Rossant & Croy 1985). While there is compelling evidence that the early i.c.m. can form trophectoderm (Handyside 1978; Hogan & Tilly 1978; Spindle 1978; Rossant & Tamura-Lis 1979; Nichols & Gardner 1984), this has not been regarded hitherto as part of its normal development repertoire. If such an exodus of cells does indeed occur normally, it must presumably be restricted to the initial phase of blastocyst growth since there is general agreement that i.c.ms from expanded blastocysts do not form trophoblast either *in vivo* or *in vitro* (Gardner *et al.* 1973; Handyside 1978; Spindle 1978; Rossant & Tamura-Lis 1979; Papaioannou 1982). If confirmed, this exchange of cells between the two tissues of the early blastocyst will necessitate reappraisal of current ideas on the mechanism of differentiation of trophectoderm versus i.c.m. (Gardner 1985*a*).

The second type of experiment involves microsurgical dissection of the i.c.m. of mature fifth day blastocysts into its constituent primitive endoderm and primitive ectoderm followed by injection of the separated tissues into different blastocysts (Gardner & Papaioannou 1975; Gardner 1985*b*). The two tissues yield complementary patterns of chimerism in the resulting conceptuses. The donor cells are normally found in the parietal and visceral layers of the extraembryonic endoderm only in the primitive endoderm injections and in all i.c.m. derivatives except extraembryonic endoderm in corresponding ectoderm injections.

Injection of single cells into blastocysts constitutes the third and final type of experiment. So far, only i.c.m. cells have been cloned because of difficulty in obtaining pure populations of viable dissociated trophectoderm cells. Daughter cell pairs formed during short-term culture of dissociated tissue were used in early i.c.m. cell cloning experiments, as described elsewhere (Gardner 1984*a*), in an attempt to avoid transplanting those destined to die (Copp 1978). Thirty-nine of the 82 conceptuses obtained in these experiments were chimeric. The clones were confined to primitive ectoderm derivatives in 21 cases and to primitive endoderm derivatives in 11. A further six clones clearly spanned derivatives of both tissues. The remaining conceptus was chimeric in the trophoblast only, possibly reflecting exceptional contamination of donor i.c.m. tissue with trophectoderm cells (R. L. Gardner, unpublished observations).

Cloning of primitive endoderm versus primitive ectoderm cells from mature i.c.ms has yielded results that are very similar to those obtained in the corresponding tissue transplantation experiments (Gardner & Rossant 1979; Gardner 1982, and unpublished observations). The only notable difference was that primitive endoderm clones showed a marked preference for colonizing parietal endoderm only (see table 1) which was not apparent in either the tissue injections or in cases where daughter pairs of early i.c.m. cells had contributed to the extraembryonic endoderm. Nevertheless, clonal descendants of primitive endoderm cells were found in both parietal and visceral endoderm in a minority of cases. The majority of primitive ectodermal clones yielded as widespread chimerism as the entire undissociated tissue but, contrary to what might have been anticipated from earlier *in vitro* studies (Pedersen *et al.* 1977; Dziadek 1979), in no case did they encompass extraembryonic endoderm as well. There are, indeed, additional grounds for challenging the claim that such cells retain this option (Gardner 1985*b*).

More can be learnt about the developmental potential of primitive ectoderm clones by analysing postnatal chimeras in addition to conceptuses. By using combined pigmentation and

chromosomal markers, progeny of donor cells have been found in all somatic tissues of adult mice that have been examined so far (Gardner *et al.* 1985). A particularly interesting finding was that more than half the chimeras whose sex corresponded with that of the donor cell produced functional gametes of donor origin. Furthermore, at least two ectoderm cells from a single donor embryo can exhibit this behaviour (Gardner *et al.* 1985). This means that, despite having undergone some restriction in developmental potential, primitive ectoderm cells are antecedents of germ cells as well as somatic cells. Segregation of the germline must therefore occur after the embryo has implanted in the uterus.

Deployment of cells within tissues

Interspecific chimeras between mouse and rat were used initially to investigate cellular deployment during post-implantation embryogenesis because cells of the two species could readily be distinguished *in situ* by immunological techniques (Gardner & Johnson 1973, 1975). However, failure of the chimaeras to thrive during the latter part of gestation, possibly as a result of immunological assault on the rat component by the mouse uterine foster-mother, raised serious doubts about the validity of using such organisms to elucidate aspects of normal development. More recently, much greater success has been obtained by using embryos composed of *Mus musculus* and *Mus caroli* tissue in which the *musculus* cells can be identified *in situ* by using a species-specific cDNA probe, as discussed elsewhere (Rossant this symposium). However, these two species differ markedly in rate of development (Frels *et al.* 1980). While this difference clearly does not prevent them from cooperating in the formation of viable fertile chimeras (Rossant & Chapman 1983), it would be surprising if it had no effect on cell allocation and deployment. Unfortunately, too little is known at present about these processes in intraspecific *Mus musculus* chimeras to make the necessary comparisons. Hence, there is still a pressing need to develop intraspecific *in situ* genetic marker systems even if they are only applicable to some rather than all tissues of the conceptus. It is with the development and use of one such marker that the remainder of this presentation is concerned.

Extraembryonic membranes were used initially for testing potential *in situ* markers because they are one or, at most, a few cell layers thick. Thus, there was the prospect of treating them as whole-mount preparations, thereby avoiding the difficult and time-consuming task of reconstruction from serial sections. In addition, such membranes offer an opportunity of analysing patterns of chimerism in two dimensions as a prelude to tackling the largely three-dimensional organization within the foetus itself. Finally, extraembryonic tissues are of interest as rapidly expanding populations of functionally differentiated cells whose life span is relatively brief. This is particularly true for the parietal endoderm which originates in the late blastocyst on day 5 postcoitum (p.c.), exhibits increasingly conspicuous cell death from early in the second half of gestation, and has degenerated by the time that its secretory product, Reichert's membrane, ruptures a few days before birth (Clark *et al.* 1975; Dickson 1979; R. L. Gardner, unpublished observations).

Various candidate marker systems were tested in mid-gestation capsular parietal endoderm, visceral yolk sac and, more recently, amnion. The one that proved most satisfactory was based on a null mutation at the *Mod-1* locus on chromosome 9 which codes for the supernatant form of malic enzyme (Lee *et al.* 1980*a*, *b*). Homozygous mutant ($Mod\text{-}1^n/Mod\text{-}1^n$) mice lack both activity of the enzyme and material reactive with polyclonal antisera directed against it, but

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retain the mitochondrial form of enzyme encoded by the *Mod-2* locus on chromosome 7. Recently, an mRNA species that hybridizes with a specific *Mod-1*$^{+}$ cDNA probe has been detected in *Mod-1*^{n}/*Mod-1*^{n} tissue, suggesting that the mutant gene is transcribed. This mRNA is, however, larger than its wild-type counterpart (Glynias *et al.* 1984; Sul *et al.* 1984) and, intriguingly, can be translated into a correspondingly larger polypeptide in a rabbit reticulocyte system. Although the product of *in vitro* translation reacted with an antiserum against wild type enzyme (Sul *et al.* 1984), it was not tested for retention of activity.

Conditions were defined in which excellent differential staining of membranes from wild type versus mutant conceptuses was obtained following their brief fixation in dilute glutaraldehyde (Gardner 1984*a*, and unpublished data). The histochemical reaction was basically as described by Pearse (1972), using either nitroblue or tetranitroblue tetrazolium in conjunction with meldolablue or phenazine methosulphate (or, more recently, its 1-methoxy derivative (Hisada & Yagi 1977; Van Noorden & Tas 1982)) as intermediate electron acceptor. Providing the concentration of L-malate was reduced to approximately 10 mM, very little deposition of diformazan occurred in cells of mutant compared with wild-type specimens during incubation periods of 24–48 h at 37 °C. Indeed, under these conditions, approximately 0.2% or less cells in mutant parietal endoderms stained and the proportion of cells that failed to stain in corresponding wild-type preparations was under 2.0%. Similar results were obtained even if membranes of the two genotypes remained in intimate contact throughout incubation. Discrimination between cells in mutant and wild type specimens was even better in the case of visceral yolk sac endoderm and amnion (Gardner 1984*a*, and unpublished data). Furthermore, a very good correspondence between histochemical phenotype and genotype was found in mixed cultures of parietal endoderm in which mutant and wild-type cells could be distinguished unequivocally by an independent marker (Gardner 1984*a*). This did not constitute an ideal test of cell-autonomy because contact between cells was limited in such cultures as, indeed, it seems to be in this particular tissue *in vivo* (Jollie 1968). However, the conspicuous absence of phenotypically intermediate cells in stained specimens of other chimeric tissues, notably the endoderm of the visceral yolk sac in which cellular contact is very extensive, provides strong grounds for concluding that this histochemical marker is cell-autonomous.

Perhaps the most difficult question to answer is whether the MOD-1 marker can be regarded as a gratuitous one with respect to the aspects of embryogenesis that it was designed to elucidate. This is because there is persisting uncertainty about the normal role of cytoplasmic malic enzyme (see, for example, Chubb & Chasalow 1984). Preliminary breeding data suggested that the *Mod-1*^{n} allele may have an adverse effect on prenatal development (Lee *et al.* 1980*a*; Johnson *et al.* 1981), but this has not been confirmed (Gardner 1984*a*; J. Green, personal communication of unpublished data). The performance of mutant primitive endoderm cells certainly compares very favourably with that of their wild-type counterparts in terms of both cloning efficiency (table 1) and clone size in reciprocal blastocyst injection experiments (Gardner 1984*a*, and unpublished observations). Finally, it is perhaps relevant to note that in *Drosophila*, which has only one locus coding for malic enzyme, homozygous null flies are viable, fertile and lack any obvious abnormalities (Voelker *et al.* 1981). Hence, there is at present no reason to suppose that use of the MOD-1 marker system is likely to have affected the outcome in the cell cloning experiments discussed hereafter.

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Cloning experiments with the MOD-1 marker

As noted above, mutant embryos were used as donors in some cloning experiments to determine whether cells that lacked cytoplasmic malic enzyme activity were inferior in viability or growth potential to those which did not. Normally, however, wild-type cells were transplanted into null blastocysts because of the greater ease with which stained cells can be identified against an unstained background than *vice versa*. This is particularly so in the visceral yolk sac and amnion which both consist of more than one layer of cells. This practice also ensured that any wild-type cells that had lost enzyme activity through death or damage during handling of the membranes before fixation were misclassified as host rather than donor.

(*a*) *Chimerism in visceral and parietal endoderm*

Primitive endoderm cells from fifth day p.c. blastocysts were used in the first and most extensive series of single cell injection experiments undertaken so far using the MOD-1 marker. The results corrresponded closely with those obtained earlier using GPI as a marker (Gardner 1982) with regard to both the cloning efficiency of primitive endoderm cells and the gross distribution of the resulting clones (table 1).

Progeny of the transplanted cell were present in the capsular parietal endoderm in all 58 conceptuses that exhibited chimerism in their extraembryonic membranes. In the five cases in which the visceral yolk sac was also chimeric, examination of sectioned material confirmed that donor cells were confined to its endodermal layer. The pattern of mosaicism encountered in

Table 1. Summary of results of cloning single fifth day primitive endoderm cells in fourth-day blastocysts by using MOD-1 versus GPI markers

(Based on Gardner 1982, 1984*a*, unpublished data.)

					classification of chimeras according to the distribution of donor cells			
stage of analysis	genotype of donor blastocysts	genotype of host blastocysts	number of conceptuses analysed	number (percentage) of chimeric conceptuses	number of chimeric in parietal endoderm only	number of chimeric in both parietal and visceral endoderm	number of chimeric in visceral endoderm only	number of chimeric in additional or other tissues†
midgestation	*Mod-1*$^{+}$/*Mod-1*$^{+}$	*Mod-1*^{n}/*Mod*-1^{n}	59	36 (61)	33	3	0	0
	Mod-1^{n}/*Mod*-1^{n}	*Mod*-1^{+}/*Mod*-1^{+}	17	16 (94)	15	1‡	0	0
ninth day p.c.	*Mod*-1^{+}/*Mod*-1^{+}	*Mod*-1^{n}/*Mod*-1^{n}	10	6 (60)	5	1	0	0
total Mod-1 experiments			86	58 (67)	53	5	0	0
midgestation	*Gpi*-1^{b}/*Gpi*-1^{b}	*Gpi*-1^{a}/*Gpi*-1^{a}	117	63 (54)	48	8+2§	2	3¶

† The only other tissue examined in experiments with the MOD-1 marker were the visceral mesoderm, amniotic mesoderm, and amniotic ectoderm.

‡ In view of difficulty of identifying small unstained patches in the endoderm layer of visceral yolk sacs whose adjacent mesoderm is fully stained, modest contributions of null cells to the visceral endoderm might have been missed.

§ Both these conceptuses were unequivocally chimeric in the visceral endoderm, but had equivocal contributions of donor allozyme in their parietal endoderm.

¶ Donor allozyme was detected in the foetal and placental fractions only in one case and in the visceral yolk sac mesoderm only in the second. The third had a major donor contribution in the visceral yolk sac endoderm and a very weak one that may have been due to contamination with endoderm in its visceral mesoderm.

the parietal endoderm was, as discussed later, so diffuse that the task of systematically quantifying chimerism in this tissue has not been attempted. Nevertheless, it is clear from gross inspection of the whole-mount preparations that the proportion of donor cells varied considerably between specimens. At one extreme, a sparse smattering of donor cells was seen that might well have eluded detection using GPI as a marker; at the other extreme, donor cells were undoubtedly in the majority. The visceral endoderm proved more tractable for quantitative analysis because donor cells were almost entirely segregated from those of host origin in this tissue. However, in estimating visually that the donor contribution was less than 15% in each of the three chimeric yolk sacs mentioned in an earlier report (Gardner 1984*a*), no allowance was made for the presence of villi (Boe 1951). Such villi increase the surface of the proximal relative to the more distal region of the visceral endoderm to an extent that varies according to both stage of development and their position in the yolk sac. Nevertheless, morphometric analysis of sectioned material using McIntyre's method (see Aherne & Dunnill 1982) suggested that the surface area of the endoderm per unit area of membrane is normally between three and five times greater in villous than non-villous regions of mid-gestation yolk sacs. Corrected estimates of both the proportion and total number of donor cells are presented in table 2 for each of the four chimeric yolk sacs that were suitable for morphometry as whole-mount preparations. The fifth yolk sac, a previllous ninth day specimen, was too folded for analysis. It is evident from the table that the donor contribution varied by as much as fourfold, and was substantially larger in the specimen in which it was confined to the proximal region than in the remainder. This is perhaps not surprising in view of the elaboration of villi that takes place in this part of the yolk sac, although more cases of visceral endodermal chimerism are obviously needed to establish whether there are consistent regional differences in growth of the tissue.

TABLE 2. CONTRIBUTIONS OF FOUR PRIMITIVE ENDODERMAL CLONES TO THE VISCERAL YOLK SAC ENDODERM AT MIDGESTATION

clone code number	position along proximodistal axis of yolk sac	number of patches of donor cells	estimated percentage contribution by clone based on different correction factors for proximal villous region			estimated range in donor cell number†
			X3	X4	X5	
5RM6	proximal	2	19.9	22.5	24.5	81500–131400
5RM17	intermediate + distal	20+	9.9	8.6	7.7	16600–17400
5RM28	intermediate	1	5.2	4.6	4.2	22700–23800
5RM56	intermediate	1	5.9	6.1	6.2	33000–51600

† Based on an estimated mean surface area per cell of 84.7 μm².

Several points emerge from closer inspection of the spatial arrangement of donor cells within the endoderm of these five yolk sacs, plus others produced in multiple primitive endoderm and early i.c.m. cell injection experiments. First, as indicated earlier, donor cells occur almost exclusively in coherent patches (figure 1, plate 1). Second, the patches tend to be oriented with their longest axis perpendicular rather than parallel to the proximodistal axis of the yolk sac. Third, proximal contributions typically consist of one or, at most, a few largish patches, whereas more distal donor contributions are arrayed in more numerous smaller patches. Fourth,

regardless of location, donor cells show very little intermingling with host cells. Finally, the boundaries of patches are extremely irregular so that a substantial proportion of donor cells are in contact with one or more host cells. This would not be expected if lack of mingling was due to differential adhesiveness of the genetically different donor and host cells (Ransom & Matela 1984). Rather, it suggests that the visceral endoderm grows as a stable tensile sheet in which there is very little relative cell movement (Honda *et al.* 1984). If this is indeed the case, it is difficult to envisage how anisotropic growth can take place without mitoses being oriented so that daughter cells are more or less aligned in the appropriate direction. The shape of donor cell patches in visceral endoderm might therefore be expected to reflect the directional growth characteristics of the region in which they occur. This possibility is being examined more closely.

The distribution of primitive endodermal clones in the parietal endoderm differed from that in the visceral layer in two respects. First, although donor cells were often widely disseminated in the parietal endoderm, they were mainly located distally rather than proximally in the majority of specimens (table 3). Sometimes a more or less discrete focus or, more rarely, two foci were discernible in which these cells were clearly more abundant than elsewhere (figure 2). The frequency of donor cells tended to decrease gradually rather than abruptly with distance from the centre of a focus. Second, mosaicism was invariably very fine-grained throughout the parietal endoderm (figure 2), even in specimens that were recovered before midgestation.

TABLE 3. COLONIZATION OF DISTAL VERSUS PROXIMAL CAPSULAR PARIETAL ENDODERM BY FIFTH DAY PRIMITIVE ENDODERM CELL CLONES

	distal > proximal	distal ≈ proximal	proximal < distal
specimens scored by gross visual inspection	21 (4)†	5 (1)	2 (0)
specimens scored by proximal versus distal cell counts as described in Gardner (1984*a*)	15 (5)‡	1 (0)§	3 (2)¶

† Figures in parentheses show numbers of specimens in each category in which donor cells were null.
‡ Difference significant in 13/15 specimens ($p < 0.001$ in 11 cases, and $p < 0.01$ and $p < 0.02$, respectively, in remaining two.
§ Difference not significant.
¶ Difference significant in all three specimens ($p < 0.001$).
Overall bias towards preferential distal colonization is significant (χ^2, 7.155; $p < 0.01$).

The disposition of cells within the parietal endoderm is such that the great majority have one neighbour that is closer than others. Hence, the frequency with which nearest neighbours were of the same or opposite genotype was investigated in chimeric specimens at ×1000 by using a microscope fitted with a drawing tube. Fractional scoring had to be used for the small minority of cells that could not be assigned a single nearest neighbour, if both donor and host cells were included among their immediate neighbours. For example, a cell that was equidistant from one mutant and two wild-type cells was scored as having one-third mutant and two-thirds wild-type nearest neighbours. Ten fields from three midgestation specimens and a further five fields from four ninth day specimens have been analysed in this way. Each field contained a total of more than 100 cells and, being located both within and outside foci, had donor contributions ranging from 13 to 72%. Only in one field, in which the proportion of donor

cells was 18%, did the observed frequencies with which wild-type and mutant cells had nearest neighbours of the same or opposite genotype show a marginally significant departure ($p \approx 0.05$) from the frequencies expected on the basis of random mixing. The field in question was one of four analysed in a mid-gestation specimen.

A similar degree of intermingling of donor and host cells has been found in cases of parietal endoderm chimerism produced by injecting daughter pairs of early i.c.m. cells into blastocysts. However, preferential colonization of the distal region of this tissue was not observed by using these less advanced donor cells. It is conceivable that the fate of primitive endoderm cells *in situ* may be portrayed more accurately by the transplanted early i.c.m. cells that enter the endodermal lineage than by the primitive endodermal clones (Gardner 1982, 1984*a*).

Use of the MOD-1 marker has revealed consistent and striking differences in the amount of relative cell movement that takes place during the growth and differentiation of two tissues that share a common origin. This is demonstrated particularly clearly in the five cases in which clonal descendants of the same cell spanned both tissues. In seeking to explain the disparity in degree of cell mixing, it is relevant to consider the marked differences in organization of the two tissues. The visceral endoderm has both the ultrastructural and physiological characteristics of a tight epithelium (Freeman *et al.* 1981; Jollie 1984). In contrast, the capsular parietal endoderm consists of a discontinuous layer of cells throughout its existence (Jollie 1968; Enders *et al.* 1978). Hence, it is perhaps not surprising that its cells are more mobile than their visceral counterparts. It seems unlikely that parietal endoderm cells move *in vivo* by migrating between the two extraembryonic endodermal layers because multi-layering of these cells has not been recorded in either sectioned or whole-mount specimens of the tissue. Furthermore, there is no evidence to suggest that parietal cells can attach to the apical surface of cells in the adjacent visceral endoderm. Hence, the cells presumably accomplish their extensive peregrinations by insinuating themselves between neighbours while using Reichert's membrane as a substratum.

Recently, Hogan & Newman (1984) have suggested that cells may be recruited into the parietal endoderm layer from the visceral during the course of normal development via the junctional zone between them. This hypothesis might help to explain why there is such extensive cell mingling in the parietal endoderm. There is, indeed, convincing evidence that visceral endoderm cells of seventh day embryos can transform into parietal cells *in vitro* (Hogan & Tilly 1981), and are also able to colonize the parietal endoderm *in vivo* (Gardner 1982). However, it is difficult to account for the existence of foci of donor cells in the parietal endoderm

Description of plate 1

Figure 1. Part of a midgestation yolk sac showing most of the contribution made to the visceral endoderm by primitive endoderm clone 5RM6 (see table 2 for details). The stained wild-type donor cells are confined to the proximal villus-rich region of the organ, and are arranged in two irregularly shaped coherent patches separated by host tissue as indicated by the arrow. (Scale bar, 1 mm.)

Figure 2. Clonal descendants of a wild-type primitive endoderm cell in a ninth day specimen of parietal endoderm. Note the extremely fine-grained mosaicism around the nearly central distal focus of donor cells. Folding of the distal region during flattening of the specimen gives the impression that the arrangement of donor cells in the focus is more coherent than was actually the case. (Scale bar, 200 μm.)

Figure 3. A midgestation amnion which has been colonized by the clonal descendants of a wild-type primitive ectoderm cell. The strongly stained patches are in the mesoderm layer and the weakly stained patches in ectoderm. (Scale bar, 1 mm.)

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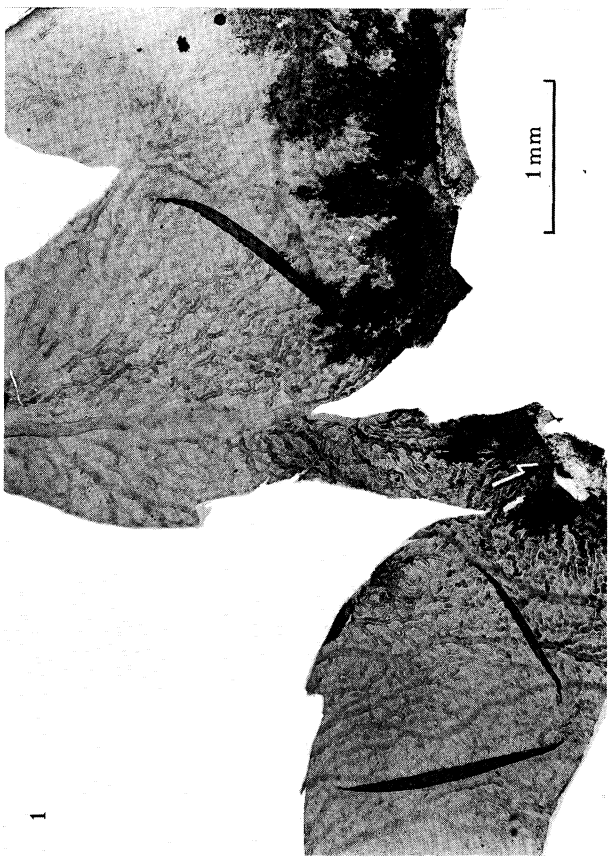

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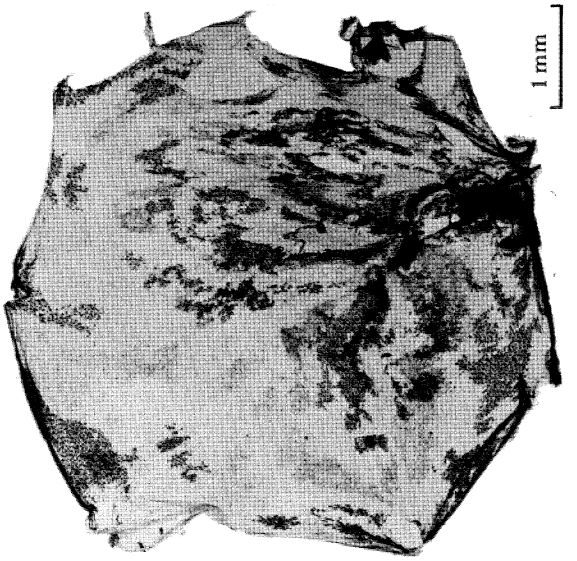

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FIGURES 1 – 3. For description see opposite.

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if its principal mode of growth is by recruitment. Such foci are more likely to represent the approximate sites to which donor progenitor cells migrated before undergoing clonal expansion and dispersal. Examination of the junctional zone in extraembryonic endodermal chimeras may yield further insight into the relationship between parietal and visceral endoderm in normal development. Satisfactory histochemical preparations of this zone can now be obtained routinely.

(*b*) *Chimerism in visceral mesoderm and amnion*

Most midgestation conceptuses obtained in a series of experiments in which daughter pairs of wild-type early i.c.m. cells had been injected into mutant blastocysts were found to be chimeric in one or more of their extraembryonic membranes (Gardner 1984*a*). The original aim of these experiments was to examine the parietal endoderm and visceral yolk sac only for chimerism. However, the amnion was also scored in those cases where part or all of it had remained attached to the visceral yolk sac during fixation and staining. Stained areas that looked very different from those encountered in the primitive endoderm injection experiments were seen in 9 of the 11 chimeric visceral yolk sacs. Staining in such areas appeared somewhat granular and diffuse compared with that produced by primitive endodermal clones and the remaining two early i.c.m. cell clones. Interestingly, three of the nine yolk sacs exhibiting this 'diffuse' staining contained coherent patches of donor cells as well. By examining histological sections from regions showing one or other type of staining, it was possible to equate the 'diffuse' pattern with mesodermal chimerism and to confirm that coherent staining was due to donor cells in the endoderm (Gardner 1984*a*). In all five cases in which there was chimerism in the visceral endoderm, the parietal endoderm was chimeric as well. A similar correlation was recorded for the visceral mesoderm and amnion whenever the latter was present. Hence, two of the early i.c.m. clones were evidently confined to primitive endoderm derivatives and six to primitive ectoderm derivatives, the remaining three showing no such restriction in distribution. Recently, an additional case of chimerism in both extraembryonic endoderm and mesoderm was obtained in a series of single i.c.m. cell injection experiments using donor blastocysts that were recovered late in the morning on the fifth day of gestation. So far, this is the latest stage of development from which such an unrestricted i.c.m. clone has been obtained.

The pattern of staining associated with chimerism in the visceral mesoderm was termed 'diffuse' simply because of its gross appearance compared with that seen in the visceral endoderm. In retrospect, choice of this term was unfortunate, because it has been interpreted as a summary description of the spatial arrangement of donor cells in the tissue (Schmidt *et al.* 1985). In fact, staining in the mesoderm is surprisingly coherent in sections of chimaeric specimens (for example, figure 13 in Gardner 1984*a*), suggesting that its appearance in whole-mount preparations is attributable more to local variation in the extent of layering and flattening of cells than to their mingling. Thick resin sections of boundary regions between mutant and wild-type areas of visceral mesoderm are currently being analysed as a way of determining the lineage relationships between its constituent mesenchymal, endothelial and mesothelial cells.

Amnia that had remained attached to the visceral yolk sacs in the foregoing early i.c.m. cell injection experiments were too fragmented and folded to warrant detailed examination. None the less, donor cells seemed to be largely segregated from host cells in those that were unquestionably chimeric. Much better histochemical preparations of the amnion were obtained subsequently by detaching the membrane from the visceral mesoderm immediately after fixing

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it *in situ*. Wild-type control specimens handled in this way exhibited strongly stained mesoderm cells and, where discontinuities in this layer enabled them to be discerned, weakly stained ectoderm cells. A very low level of background staining was observed throughout most corresponding mutant controls, although some contained up to 20 or so unequivocally stained cells. Curiously such exceptional stained cells usually occurred in tiny clusters rather than singly. Several satisfactory preparations of chimeric amnion have been obtained in recent cloning experiments that display fairly extensive donor contributions. Chimerism was invariably present in the mesoderm and was characterized by coherent patches of wild type donor cells that stood out very clearly against the background of mutant cells (figure 3). Only rarely were donor or host cells completely surrounded by those of the opposite genotype. In most midgestation specimens, coherent patches of stained cells were also discernible in the ectoderm (figure 3). Patches in the two layers bore no consistent spatial relationship to each other and, unexpectedly, the ectodermal ones did not seem in general to be larger than those in the mesoderm. Since cell density is less than twofold higher in the ectoderm than mesoderm, this implies that coherent growth is initiated at approximately the same stage of development in both layers. This is surprising because, while the ectoderm layer is evidently formed by extension of an existing epithelium, the mesoderm originates from cells that lose their epithelial arrangement before making their exit through the primitive streak during gastrulation (Snell & Stevens 1966; Spiegelman 1976; Batten & Haar 1979). Observations on interspecific chimeras suggest a lack of coherent growth in the primitive ectoderm that might account for this finding (see Rossant, this symposium). Another possibility is that mesoderm migration is accomplished with little cell mixing. Finally, the remarkably coherent pattern of growth of the amniotic mesoderm is perhaps also surprising in view of the fact that this tissue has, like the parietal endoderm, been described as a discontinuous layer of cells (Scott *et al.* 1982).

Conclusions

Knowledge of cell lineage and commitment in the early mouse embryo has been increased immeasurably by the introduction of techniques of clonal analysis. The recent development of a variety of genetic markers that enable donor and host cells to be distinguished *in situ* has further enhanced the value of this approach. Use of the MOD-1 marker, for example, has graphically demonstrated the precision with which most i.c.m. clones partition between extraembryonic tissues that originate from the primitive endoderm and primitive ectoderm. It has, in addition, confirmed the marked variation in both size and location of different clones in the same tissue. There are obvious parallels in the behaviour of these clones and those induced in *Drosophila* embryos by X-irradiation (see, for example, Garcia-Bellido, this symposium).

Application of *in situ* markers to other tissues of the conceptus is likely to pose greater problems if sectioning is required, because patterns of mosaicism in sections can be very misleading unless serial reconstruction is undertaken. Hence, there is a case for exploiting *in situ* markers that can be used on whole-mounts, whenever practicable. The recent work of Ponder and his colleagues on large intact sheets of chimeric intestinal mucosa is a particularly instructive example of the value of this strategy (Schmidt *et al.* 1984, 1985; Ponder *et al.* 1985).

Finally, it is worth noting that use of the blastocyst for cloning cells is only really appropriate for delineating the major limbs of the lineage tree. The task of tracing the smaller branches, especially those in the primitive ectodermal lineage from which the foetal soma and germ-line

originate, can be accomplished only by finding ways of making the early postimplantation embryo more accessible to experimental manipulation under conditions that permit its continued normal development (Gardner 1984*b*; Beddington 1985).

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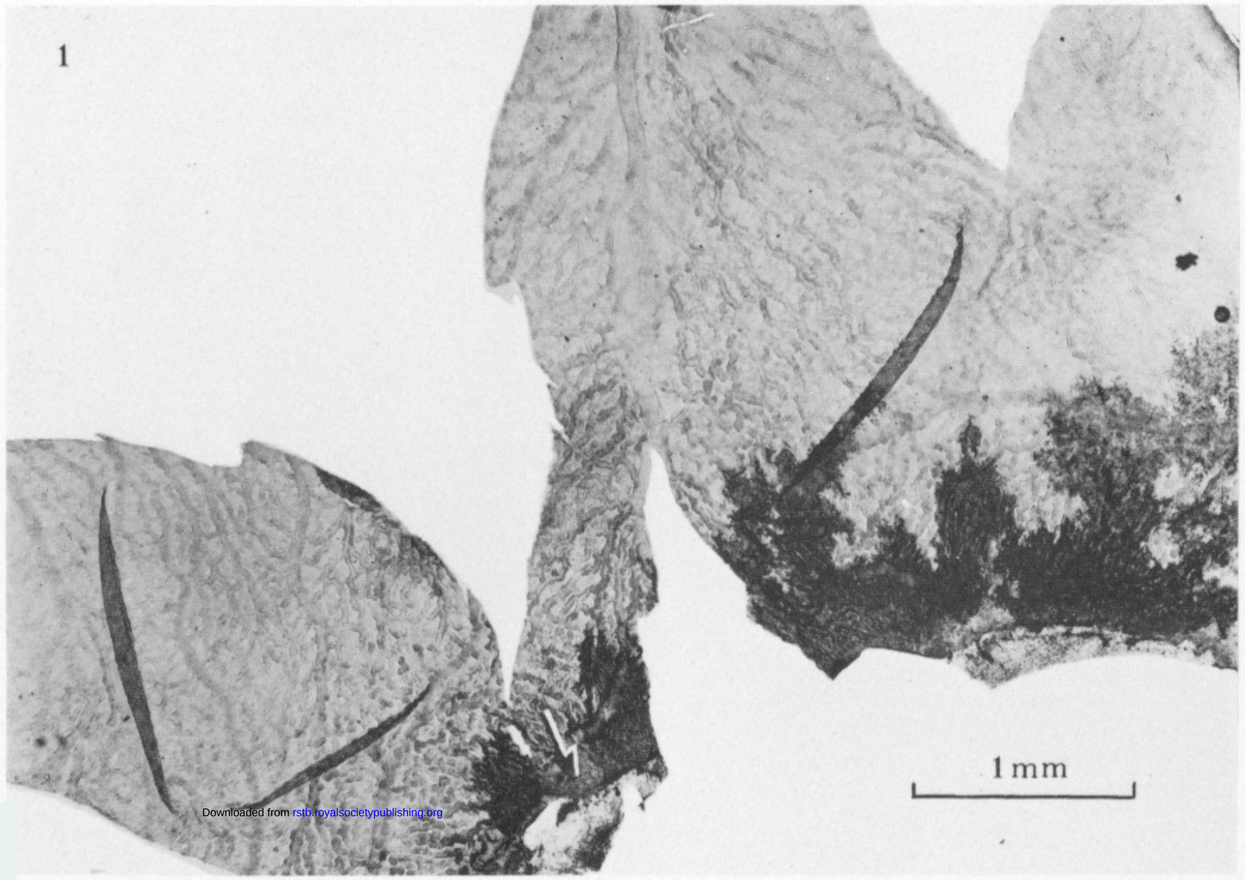

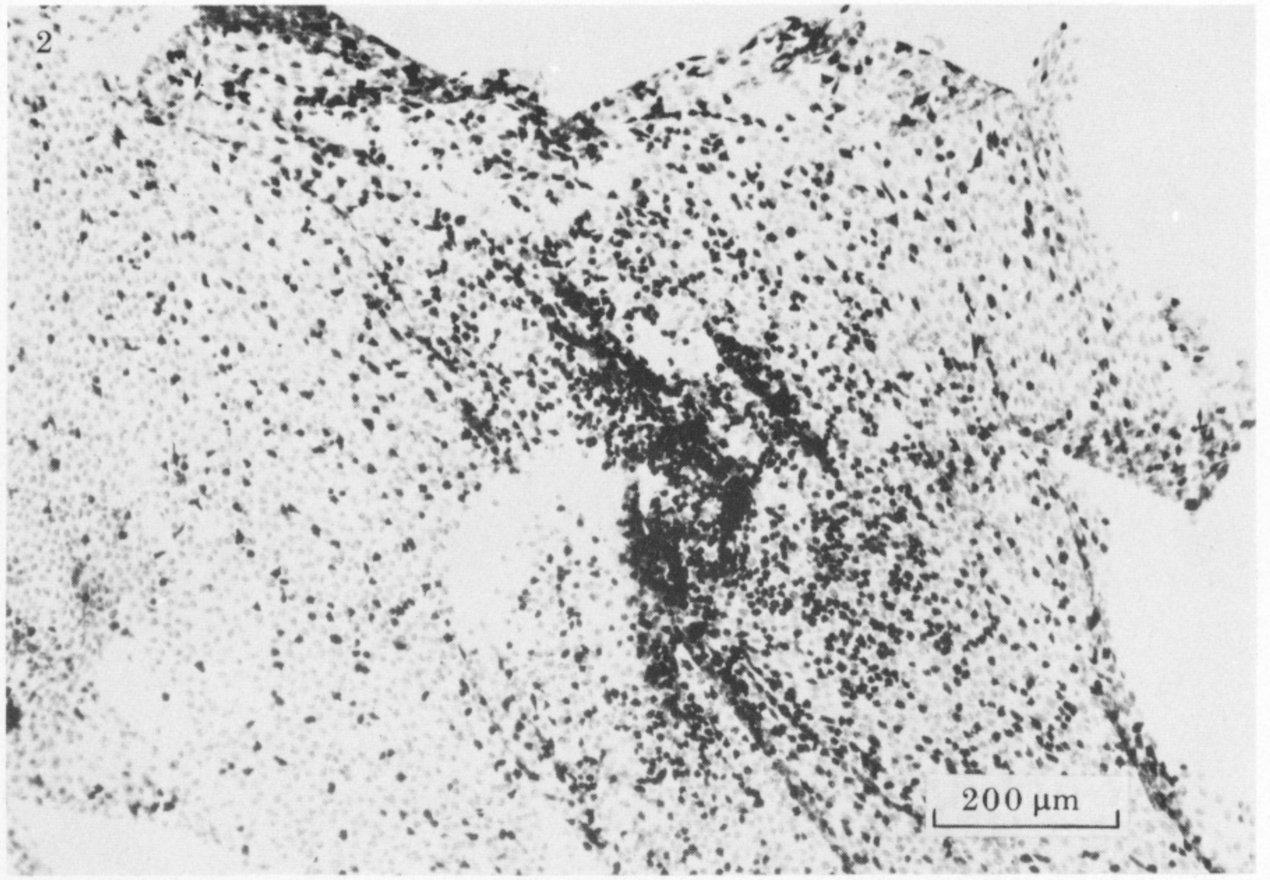

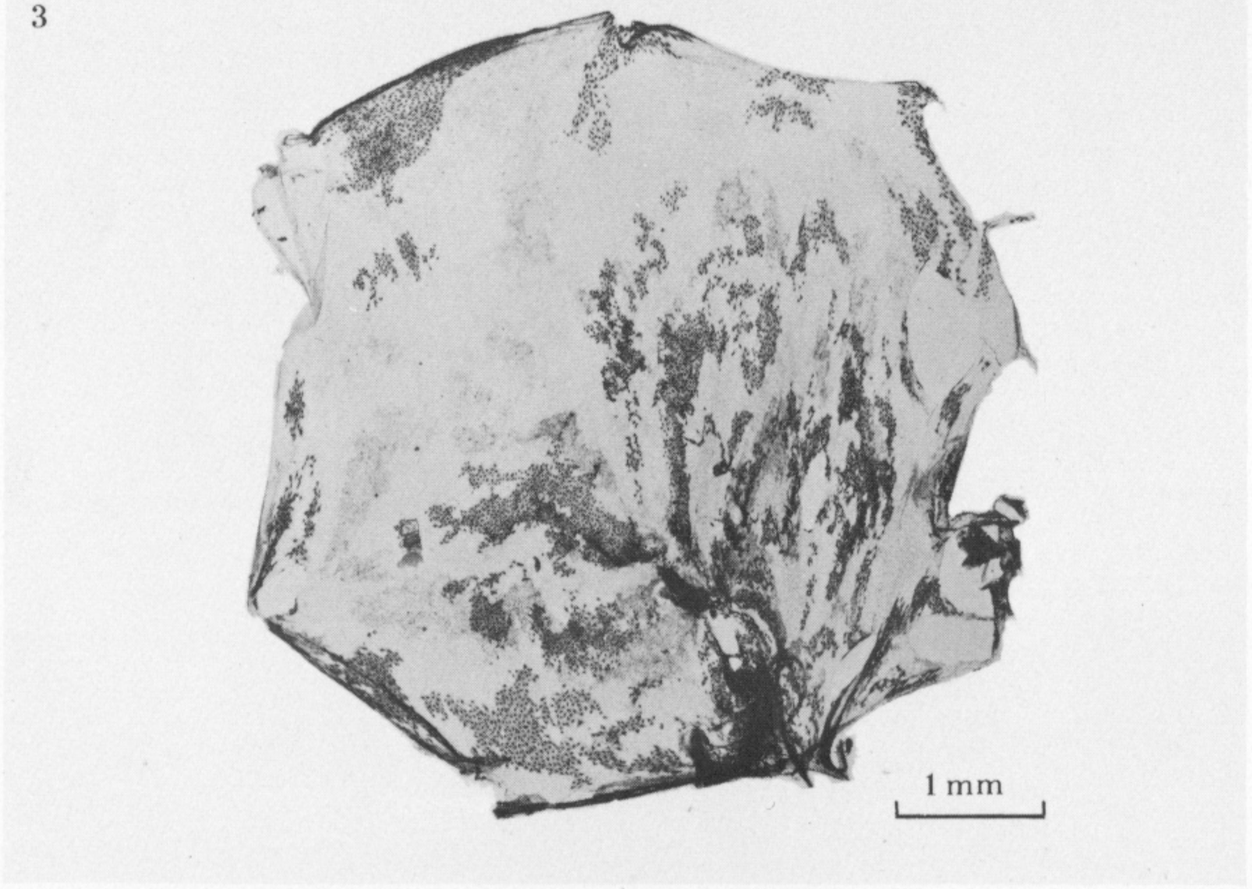

FIGURES 1 – 3. For description see opposite.

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