Identification of embryonic cell lineages in histological sections of *M. musculus* ↔ *M. caroli* chimaeras

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SUMMARY

An *in situ* cell marker system has been developed which allows identification of *Mus caroli* and *Mus musculus* cells in interspecific chimaeras. A radioactively labelled, cloned DNA probe to *M. musculus* satellite DNA was hybridized *in situ* to sections of *M. musculus* and *M. caroli* adult tissues. Autoradiography revealed high levels of hybridization to the nuclei of *M. musculus* cells, but little or no label bound to *M. caroli* cells. The DNA probe could also distinguish *M. musculus* and *M. caroli* cells in the same tissue section. Patches of labelled and unlabelled cells were clearly identified in sections of adult chimaeric tissues and also in the embryonic ectoderm of 6-5-day embryonic chimaeras.

The ability to recognize *M. musculus* and *M. caroli* cells in sections of chimaeras should provide a powerful new tool in analyses of cell lineages in both embryonic and adult mouse chimaeras. The marker system has several advantages over other marker systems so far developed, the most important of which is its ubiquity. Since it is a nuclear marker, only cells without nuclei should be unsuited to its use. The potential of the marker system has been shown by its use in demonstrating directly for the first time the postimplantation derivatives of inner cell mass and trophectoderm in blastocysts 'reconstituted' with *M. musculus* trophectoderm and *M. caroli* inner cell mass.

INTRODUCTION

The use of mammalian chimaeras for detailed cell lineage analysis in development has been limited by the lack of a suitable ubiquitous *in situ* cell marker system (McLaren, 1976; Gearhart & Oster-Granite, 1978). In the preceding paper (Siracusa *et al.*, 1982), it has been shown that a marker system exists which may allow identification of the two component cell types in interspecific chimaeras between *Mus musculus* and *Mus caroli*. A cloned DNA probe to *M.
musculus satellite DNA was hybridized in situ to M. musculus and M. caroli cell spreads and hybridization was detected by autoradiography. This particular satellite DNA sequence showed little cross hybridization with M. caroli repetitive DNA sequences so that M. caroli cells appeared virtually unlabelled after in situ hybridization. The probe was able to distinguish M. caroli and M. musculus cells in bone marrow preparations from interspecific chimaeras. However, to be truly useful as a marker system, it must be able to distinguish M. caroli and M. musculus cells in histological sections of chimaeric tissue. In this study we describe extension of the technique of in situ DNA–DNA hybridization to sectioned material and demonstrate that the cloned probe to M. musculus satellite DNA can be used to distinguish M. musculus and M. caroli cells in sections of adult and embryonic chimaeric tissue. The marker system was also used experimentally to demonstrate directly for the first time the postimplantation derivatives of the inner cell mass (ICM) and trophectoderm in blastocysts ‘reconstituted’ with M. musculus trophectoderm and M. caroli ICM.

MATERIALS AND METHODS

Embryo collection and manipulation

Mus musculus blastocysts were obtained by flushing the uteri of Ha/ICR female mice on the afternoon of the fourth day after natural mating. Mus caroli blastocysts were obtained following hormonal induction of ovulation and natural mating as described previously (Rossant & Frels, 1980). Embryos were placed in PBI medium (Whittingham & Wales, 1969) plus 10% foetal calf serum for manipulation and transfer. M. caroli blastocysts were subjected to immunosurgery (Solter & Knowles, 1975) and the resulting ICMs were injected microsurgically into either M. musculus blastocysts or M. musculus trophectoderm vesicles. Trophectoderm vesicles were obtained by slitting the zona pellucida overlying the ICM and allowing the ICM plus its overlying polar trophectoderm to extrude during a period of culture at 37°C (Papaioannou, 1981). When the host ICM was clearly outside the zona, a M. caroli ICM was injected into the trophectoderm vesicle still enclosed by the zona. The last stage of the procedure, which involved cutting off the extruded ICM, was performed by hand using glass microneedles under the dissecting microscope and not by micromanipulation as described previously (Papaioannou, 1981). Both ‘reconstituted’ blastocysts and injected blastocysts were allowed to recover for an hour before transfer to pseudopregnant recipients.

Embryo transfer and development

M. caroli blastocysts, M. musculus blastocysts injected with M. caroli ICMs, and blastocysts reconstituted with M. musculus trophectoderm and M. caroli ICM were transferred to the uteri of Ha/ICR females on the third day of
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pseudopregnancy. Some females carrying injected blastocysts were allowed to go through to term. Live interspecific chimaeric offspring were recognized by coat colour and glucose phosphate isomerase (GPI) mosaicism (Rossant & Frels, 1980). Other females carrying all three types of embryo were killed at 6-5 days of pregnancy. Uteri containing decidua were removed and fixed in glacial acetic acid: absolute ethanol (1: 3) at 4°C overnight.

Processing of tissues for hybridization

Various small pieces of tissue from adult M. musculus, M. caroli and chimaeric animals were fixed as described for embryonic tissue. All tissues were then rinsed in two 30 min changes of absolute ethanol at 4°C, transferred to a 50: 50 mixture of ethanol and ester wax (BDH 1960) in a 50°C water bath for 1 h, infiltrated with two 1 h changes of ester wax and embedded in the wax at 50°C. Blocks were cooled and sectioned at 7μm. A few serial sections were mounted on chromic-acid-cleaned glass slides. Sections were heated to 40°C for the minimum time required for section spreading and then allowed to dry for a few hours at room temperature. Care was taken throughout to ensure that slides remained clean and free of dust.

In situ DNA–DNA hybridization

Conditions for in situ DNA–DNA hybridization on sectioned material were essentially similar to those described for cell spreads (Siracusa et al., 1982), except that only heat denaturation was used as alkali denaturation usually removed the sections from the slides. Slides were dewaxed in xylene, washed in ethanol and air dried briefly before heat denaturation and hybridization overnight. The slides were then washed in 2×SSC to remove non-specifically bound DNA, dehydrated, air dried and dipped in Kodak NTB-3 emulsion diluted 1:1 with water. Autoradiographs were exposed for five days and then developed for 1-5 min in Kodak D-19 developer, rinsed in distilled water and fixed for 5 min in Kodak fixer. After washing with distilled water, slides were stained with haematoxylin and eosin.

RESULTS

In situ hybridization of M. musculus satellite DNA sequence to histological sections

In situ hybridization of the cloned M. musculus satellite sequence to histological sections of M. musculus adult tissues was successfully achieved. The procedure outlined resulted in specific binding of the probe to the nuclei of cells from a variety of adult tissues, including kidney, liver, uterus, and brain (Fig. 1). There was always some background labelling in the cytoplasm, which varied between experiments. However, most label was clearly localized to the nucleus
and often showed patches of intense labelling as observed previously in interphase nuclei hybridized with satellite DNA in cell spreads (Pardue & Gall, 1970; Singh, Purdom & Jones, 1977; Siracusa et al., 1982). The amount of label bound was visually similar to the level observed when the same probe was hybridized to cell spreads (Siracusa et al., 1982), but there was more variation between nuclei because of the varying amounts of DNA exposed in the plane of the section. When the probe was hybridized to adult *M. caroli* tissue, only background labelling was observed; no nuclear localization of label was apparent (Fig. 1B).

Fig. 1. Autoradiographs of sections of adult tissues from *M. musculus* and *M. caroli* after hybridization with pMR196. (A) *M. musculus* uterus, showing nuclear localization of label; (B) *M. caroli* kidney, showing only background labelling. Grid bar on this and succeeding figures = 50 μm.
The low level of binding to *M. caroli* cells suggested that misidentification of *M. caroli* as *M. musculus* in chimaeric material would be unlikely. However, occasional classification of a *M. musculus* cell as unlabelled and hence *M. caroli* might occur. Several counts of labelled versus unlabelled nuclei were made from photographs of *M. musculus* tissues after *in situ* hybridization. Nuclei were

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**Fig. 2.** Autoradiographs of sections of adult chimaeric tissues after *in situ* hybridization. Patches of labelled and unlabelled cells clearly visible. Diagrams illustrate patchiness by scoring all labelled nuclei with a solid dot (●) and all unlabelled nuclei with open dot (○). (A) chimaeric liver; (B) chimaeric brain.
scored as unlabelled if there were fewer than five grains over the nucleus. This represents a very conservative estimate since *M. caroli* tissues never showed this high a level of labelling. An average of 8% unlabelled cells (range 3–14%) was observed in all tissues analysed. In most cases, nuclei scored as unlabelled were clearly sectioned tangentially and might have appeared labelled in succeeding sections.

**Identification of M. musculus and M. caroli cells in adult chimaeric tissue**

Tissues from adult interspecific chimaeras that showed fairly balanced GPI mosaicism were used for in situ hybridization. A mixture of labelled and unlabelled nuclei was evident in sections from liver and cerebral hemispheres (Fig. 2). In both tissues, the patch sizes of the two species types were small. The distinction between labelled and unlabelled cells was particularly evident in the brain tissue, where nuclei were widely spaced (Fig. 2B). Counts of labelled versus unlabelled cells in random samples of the tissues gave *M. caroli : M. musculus* ratios similar to those calculated from quantitative GPI analysis of other samples of the same tissues from the same chimaera (Table 1).

**In situ hybridization on embryonic cells**

An initial experiment revealed that the *M. musculus* cloned sequence could be used to distinguish *M. musculus* and *M. caroli* blastomeres in cell spreads from aggregation chimaeras showing that, as expected, hybridization was equally effective on embryonic and adult tissue. Hybridization of the probe to sectioned

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<th>Table 1. Comparison of mosaicism estimated by GPI analysis or by in situ DNA–DNA hybridization in adult interspecific chimaeric tissues</th>
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*M. musculus* embryos at 6-5 days was also successful (Fig. 3A). In fact, the large size of the nuclei and the distinctness of their boundaries, as well as the epithelial arrangement of many cell types, resulted in clearer localization of label than in many adult tissues. The mean percentage of cells that would be scored as unlabelled was 7% (range 5–9%). Again, sections of *M. caroli* embryos at similar stages showed no nuclear localization of label, even when enclosed in the *M. musculus* uterus. In such cases, *M. musculus* decidual cells were clearly labelled but all *M. caroli* embryonic cells were unlabelled (Fig. 3B).
Identification of M. musculus and M. caroli cells in embryonic chimaeras

Three 6-5-day potential interspecific chimaeras were sectioned and subjected to in situ DNA–DNA hybridization. In one embryo, no patches of unlabelled cells could be detected but the remaining two showed patches of unlabelled, presumably M. caroli, cells in the embryonic region. Two successive sections of one of these chimaeras are shown in Fig. 4. All cells of the extraembryonic ectoderm, trophoblast and endoderm were labelled, but patches of unlabelled M. caroli cells were apparent in the embryonic ectoderm. Complete serial reconstruction of the embryo was not possible since some sections were lost, but limited analysis suggested that the M. caroli cells formed a fairly contiguous group of cells showing little mixing with M. musculus cells.

Analysis of reconstituted blastocysts

Five out of eleven reconstituted blastocysts had formed decidua when analysed at 6-5 days p.c. and three implants contained embryos when sectioned. Two of the three embryos were analysed using in situ hybridization with the M.

Fig. 3. (A) Autoradiograph of section of ectoplacental cone and extraembryonic ectoderm from 6-5 day M. musculus embryo, showing virtually all nuclei labelled. (B) Autoradiograph of M. caroli egg cylinder in M. musculus uterus, showing labelling of nuclei of uterine tissue and only background labelling (fairly high!) over M. caroli embryo. Note unlabelled trophoblast giant cells (arrows) around periphery of M. caroli embryo, surrounded by labelled M. musculus nuclei.
Fig. 4. Autoradiographs of sections of 6-5-day *M. musculus* ↔ *M. caroli* chimaera. (A) Micrograph and diagram of embryonic region, revealing group of unlabelled *M. caroli* cells in embryonic ectoderm. Hatched areas represent unlabelled cells. (B) Micrograph and diagram of succeeding section showing continuity of unlabelled area through the embryo.

*musculus* probe. This analysis revealed that all cells of the ectoplacental cone, extraembryonic ectoderm and trophoblast giant cell layer were labelled, whereas the embryonic ectoderm, and visceral and parietal endoderm were unlabelled in both cases (Fig. 5). There was no evidence for cross contamination of tissue types. Boundaries between extraembryonic ectoderm and embryonic ectoderm
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Fig. 5. Autoradiograph of section of 6-5 day reconstituted M. caroli/M. musculus embryo, showing labelled (M. musculus) extraembryonic ectoderm (arrow) and unlabelled (M. caroli) proximal endoderm.

were clear and the hybridization patterns corresponded exactly to the tissue boundaries.

DISCUSSION

A cloned probe to M. musculus satellite DNA has been used to distinguish M. musculus and M. caroli cells in histological sections. Heavy labelling, localized to the cell nucleus, was observed when the probe was hybridized to M. musculus tissues but no such labelling was observed in M. caroli cells. Patches of labelled and unlabelled cells were observed in all three adult chimaeric tissues examined and the proportions of unlabelled to labelled cells were similar to the proportions of M. caroli and M. musculus cells assessed by GPI analysis. This suggested that the patches were not an artifact but truly represented differential binding of the probe to M. musculus and M. caroli cells. The small size of the patches was also consistent with the pattern of mosaicism predicted by previous reports of fine-grained mosaicism in adult chimaeric tissues, using other in situ markers (Dewey, Gervais & Mintz, 1976; West, 1976; Oster-Granite & Gearhart, 1981). Confirmation of the ability of the system to distinguish M. musculus and M. caroli cells in the same section was provided by sections in which unlabelled M. caroli embryo cells were clearly distinguishable from surrounding labelled M. musculus uterine cells. The marker system also revealed patches of unlabelled M. caroli
cells in the embryonic regions of 6-5-day interspecific chimaeras. The distribution of the *M. caroli* cells suggested that growth of the injected *M. caroli* ICM cells was fairly coherent and that little cell mixing had yet occurred. Similar results have been reported previously in rat ↔ mouse chimaeras (Gardner & Johnson, 1973, 1975).

The most powerful test of the marker system was its use in following the fate of *M. caroli* ICM and *M. musculus* trophectoderm in reconstituted blastocysts. There have been many previous experimental studies aimed at delineating the fate of these two cell types (Gardner, Papaioannou & Barton, 1973; Rossant & Lis, 1979; Papaioannou, 1982) and it has generally been agreed that the ICM gives rise to the embryonic ectoderm and endoderm, while the trophectoderm produces the extraembryonic ectoderm, ectoplacental cone and giant cells (reviewed by Rossant & Papaioannou, 1977). However, most studies have relied on electrophoretic analysis of GPI isozymes in tissue homogenates of later embryos, where cross contamination of tissues is possible and a minor contribution to a given tissue may go unnoticed. Direct analysis of the development of reconstituted blastocysts using the marker system has confirmed all previous indirect studies of ICM and trophectoderm cell fate and simultaneously provided powerful confirmation that in situ labelling with the recombinant DNA probe was specific to *M. musculus* cells.

The genetic marker system described here has several advantages over previous marker systems used for chimaera analysis. First, the marker can be detected in histological sections and does not require destruction of the spatial integrity of the chimaeric tissue being analysed. The wax embedding method used in association with the in situ DNA–DNA hybridization should allow serial reconstruction of the clonal distribution of *M. musculus* and *Mus caroli* cells in chimaeras. Second, the marker is cell autonomous; no invasive method of cell marking is required. Exogenous marker systems, such as [3H]thymidine (Kelly & Rossant, 1976) or horse-radish peroxidase (Balakier & Pedersen, 1982), may alter cell behaviour and make interpretation of cell relationships difficult. Third, the marker is confined to the cell nucleus, since the probe hybridizes to a non-transcribed satellite DNA sequence (Flamm, Walker & McCallum, 1969; Pietras, 1981). This nuclear localization was clearly preserved in histological sections. Some other marker systems, such as β-glucuronidase activity variants, suffer from problems of enzyme transfer between cells (Feder, 1976).

The most important advantage of this marker system over any other in situ marker system used experimentally in mammals is its ubiquitous nature. The marker system has already been used to distinguish *M. caroli* and *M. musculus* cells in a variety of chimaeric tissues from embryo to adult and should be applicable to any nucleated tissue, since satellite DNA is present in all nuclei. A variety of histologically detectable marker systems has been developed, including activity variants of β-glucuronidase (Mullen & Herrup, 1979) and β-galactosidase (Dewey et al., 1976), the ichthyosis nuclear marker (Goldowitz & Mullen, 1982).
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and GPI immunocytochemistry (Gearhart & Oster-Granite, 1978; Oster-Granite & Gearhart, 1981), but none has so far proved applicable to more than a limited selection of chimaeric tissues.

This in situ cell marker system thus possesses many of the properties of an ideal cell marker (McLaren, 1976) and, although it utilizes interspecific rather than intraspecific chimaeras, we have no evidence to suggest that this should impose any serious limitations on its use. This question is considered in more depth in the succeeding paper (Rossant & Chapman, 1982). The in situ DNA–DNA hybridization marker system does, however, have some minor technical limitations, which it should be possible to eliminate with further refinement of the system. The extent of hybridization and of background labelling was somewhat variable. However, it was always easy to distinguish evenly distributed background labelling over M. caroli cells from high-density nuclear-localized labelling over M. musculus cells. Improvements in hybridization conditions will continue to be sought in order to reduce this background labelling. The marker also cannot unequivocally identify every single cell in a chimaeric section as M. musculus or M. caroli. M. musculus cells will occasionally be scored as unlabelled and therefore M. caroli, because of the varying depth of sectioning of the nuclei. A single diploid M. caroli cell in the middle of a mass of M. musculus cells would, therefore, be unlikely to be recognized. However, this is common to most cell marker systems and is unlikely to be a major problem since contiguous growth of clones of cells is more likely to be observed in embryos than migration of isolated cells. Patches of M. musculus and M. caroli were readily identified in adult chimaeric tissue and the resolution in earlier embryonic stages was even greater. Another limitation is that the marker scores M. caroli cells as unlabelled and M. musculus cells as labelled. For technical reasons, interspecific chimaeras are usually made by injecting M. caroli cells in M. musculus blastocysts and transferring to the M. musculus uterus. This means that the injected M. caroli cells will usually be the minority population, especially if single cell injections are performed. Clearly it would be preferable, although not essential, to arrange the marker system so that M. caroli cells are recognized as labelled and M. musculus cells as unlabelled. Preliminary observations using M. caroli repetitive DNA as a probe for in situ hybridization suggest that this should be possible (Siracusa et al., 1982). We are currently attempting to clone sequences from M. caroli repetitive DNA in order to obtain the reverse marker system.

CONCLUSIONS

The use of in situ DNA–DNA hybridization of a M. musculus satellite DNA probe to interspecific M. musculus ↔ M. caroli chimaeras should provide a powerful new tool in analyses of cell lineages in both embryonic and adult mouse chimaeras. The system has several advantages over other marker systems so far developed, the most important of which is its ubiquity. We have not yet tested
it on all tissues, but, in theory, only cells without nuclei, like erythrocytes, should be unsuited to its use. Its utility has already been demonstrated by analysis of reconstituted *M. caroli/M. musculus* blastocysts.

REFERENCES


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