Development 124, 1133-1137 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV7558

Following cell fate in the living mouse embryo

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SUMMARY

It has been difficult to follow many of the dramatic changes in cell fate and cell migration during mouse development. This is because there has been no enduring marker that would allow cells to be recognised in the living embryo. We believe that we have overcome this problem by developing a novel form of green fluorescent protein, named MmGFP, that proves to be easily visible and non toxic to mouse cells and does not perturb embryogenesis. We show that synthetic mRNA encoding MmGFP can be injected into blastomeres to follow the fate of their progeny during preimplantation development. We have made a stable embryonic stem cell line that expresses MmGFP and introduced these fluorescent cells into mouse embryos. For the first time, we have been able to follow the fate of embryonic stem cells in living embryos and to observe directly the contribution of these cells to distinct lineages of the postimplantation embryo. This approach should lead to a more complete description of the dynamics of cell fate in the mouse.

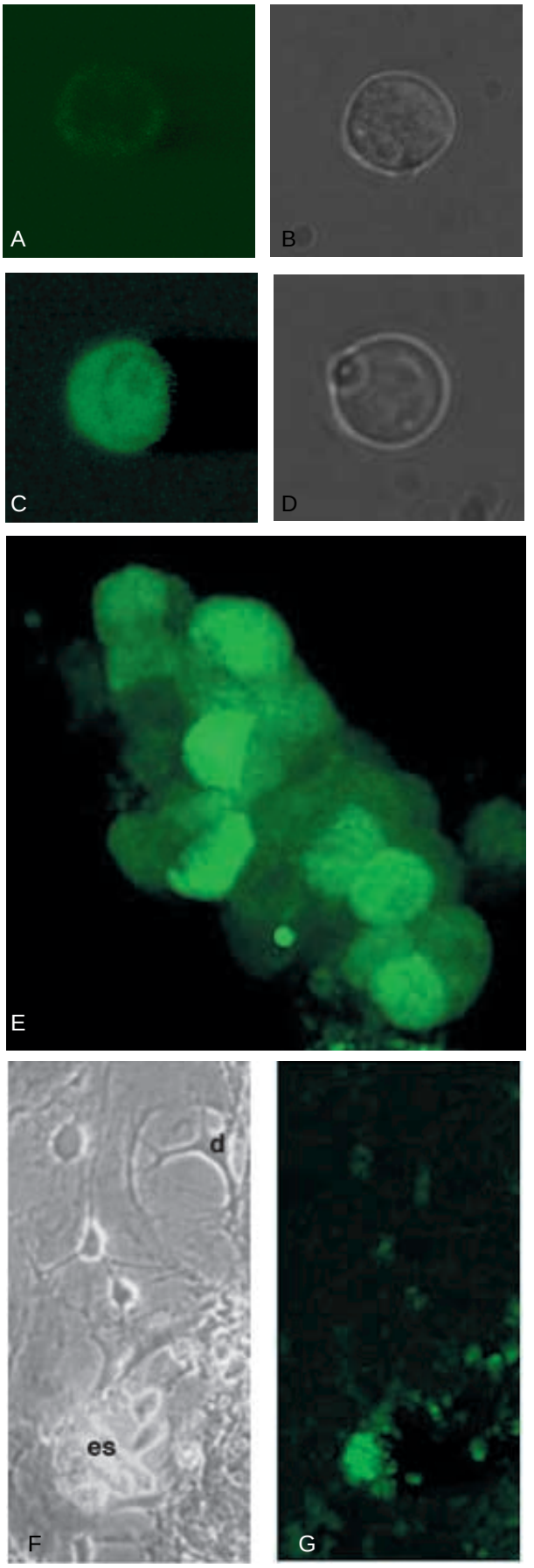
Key words: green fluorescent protein, GFP, cell fate, mouse development

INTRODUCTION

In order to trace cell lineages during development, a cell marker is needed. Ideally this would cause no interference with normal development and be visualised in the living tissue. Exogenously applied vital dyes such as DiI are very useful, but a controllable endogenously expressed marker would be preferable in many instances. Moreover, such a marker might be usable as a reporter gene to visualise cell-specific control of gene expression. Recently, proteins derived from cnidaria, which have a property of being spontaneously fluorescent, have been described and one, in particular, the green fluorescent protein (GFP) from *Aequorea victoria* has been cloned (Prasher, 1995) and is able to function as a vital cell marker in many organisms, including nematodes, insects, fish and frogs (Amsterdam et al., 1995; Chalfie et al., 1994; Kerrebrock et al., 1995; Peters et al., 1995; Prasher, 1995; Wang and Hazelrigg, 1994; Zernicka-Goetz et al., 1996). In the mouse, a favourable method for tracing cells would be to introduce a vital marker into embryonic stem (ES) cells (Evans and Kaufman, 1981). However, no ES cell lines expressing GFP have been reported until now. We have found that neither the wild-type protein, nor many of the mutants described so far (Ehrig et al., 1995; Heim et al., 1994, 1995), are visible in stable mouse ES cell lines. Therefore, we have developed a novel form of the protein, named MmGFP, that can be used in the mouse, either when expressed as a transgene, or when microinjected as mRNA. MmGFP allowed us to follow the fate of early blastomeres throughout preimplantation development and to visualise the progeny of ES cells during both preimplantation and postimplantation development. Our results demonstrate the potential of MmGFP to be used as a cell fate marker for the mouse.

MATERIALS AND METHODS

Mutations were introduced into *mGFP5* (Siemering et al., 1996) by PCR mutagenesis as previously described (Zernicka-Goetz et al., 1996). Constructs were sequenced using an ABI automatic sequencer. Embryos were collected from F_1 (CBA/H×C57BL/10) mice induced to superovulation by injections of 5 IU of pregnant mare's serum gonadotrophin (PMSG, Intervet) and 5 IU of human chorionic gonadotrophin (hCG, Intervet) 48-52 hours apart and mated to F_1 males. Synthetic MmGFP RNA was transcribed with T3 polymerase from the GFP.RN3 vector (Zernicka-Goetz et al., 1996), and ~12 pl at 80 μg/ml injected into one blastomere of a late 2-cell-stage embryo (40-44 hours after hCG). Both injection and holding pipettes were straight and held in micromanipulators (Nikon). The holding pipette was drawn from hard glass capillary tubes and flame polished to a flat, blunt end (id: ~10 μm). The injection pipette was made from capillaries with an internal filament (Clark Biomedical Supplies, UK) pulled on an automatic micropipette puller (Sutter, USA). RNA was loaded into the back of the capillary and the tip broken against the holding pipette just before injection. Microinjections were performed using air pressure from a gas-tight syringe. All injections were made into embryos suspended in drops of M2 medium supplemented with 4 mg/ml BSA (M2+BSA) (Whittingham, 1971) surrounded by



paraffin oil in a depression slide. After injection embryos were cultured in Whitten's medium (Whitten, 1971) under paraffin oil at 37˚C until the blastocyst stage. For visualisation under the confocal microscope embryos were transfered to flattened capillary tubes filled with M2+BSA medium and sealed at the ends.

CCB embryonal stem cells were electroporated with a 1:10 ratio of pCdc2-MmGFP and a DNA construct carrying a neomycin-resistant (neo^R) gene under the PGK promoter (Colledge et al., 1994). Cells were selected in 200 μg/ml G418 for 14 days. 43 clones resistant to G418 were identified of which 21 visibly expressed MmGFP under epifluorescence microscopy with a FITC filter set (BP 450-490 nm, DM 512 nm, BF 515 nm). To induce ES cells to differentiate, cells were aggregated in culture in order to form embryonal bodies (Martin and Evans, 1975). These were allowed to attach to dishes after 10 days. Chimaeras were formed by aggregation of 8- to 16-cell-stage embryos and CCB cells transformed with pCdc2-MmGFP. Embryos were pretreated (3 minutes) with a 1:20 dilution of phytohemaglutinin (Gibco) before aggregation with CCB cells in M2+BSA under paraffin oil at 37˚C and cultured in vitro for 24 hours before transfer to foster mothers. The expression of MmGFP was examined during embryogenesis between 6.5 and 9.5 days p.c. Cells and embryos were imaged on a Nikon Optiphot-2 using the 488 nm line of a KrAr laser for MmGFP, and the 568 nm laser line for autofluorescence, on a Bio-Rad 1024 confocal microscope with a triple dichroic filter block.

RESULTS

We based MmGFP on *mGFP5* (Siemering et al., 1996), which has two mutations, V163A and S175G, that improve the folding of the apoprotein and, therefore, its solubility, at higher temperatures. There are also codon usage changes that remove a cryptic splice site recognised in plants (Haseloff and Amos, 1995). Two further mutations, F64L and S65T, were introduced by PCR mutagenesis. The S65T mutation enhances the blue light absorption of GFP (Heim et al., 1995) and the F64L mutation (Cormack et al., 1996) enhances its solubility in bacterial cells, and therefore may also improve apoprotein folding in mammalian cells. (MmGFP also contains two apparently silent mutations, H25Y and G191D, that do not measurably affect fluorescence intensity.) MmGFP matures well at 37˚C and is easily detectable in living mammalian cells using a standard FITC filter set.

To be able to follow the lineage of proliferating cells during development, we placed the *Mm GFP* cDNA under the control of the human cdc2 promoter, which is repressed in quiescent cells (Dalton, 1992). We used an 850 bp region of the cdc2 promoter and inserted MmGFP 60 bp downstream of the major transcriptional start site and upstream of the SV40 small t intron and poly adenylation signal. This construct was introduced into ES cells by electroporation with a plasmid encoding the *neo* resistance gene under the PGK promoter. MmGFP was detected in neomycin-resistant cells by epifluorescence microscopy, and was present in the nucleus and the cytoplasm

Fig. 1. MmGFP is visible in ES cells in culture but not in non-proliferating, differentiated cells. Confocal images (A,C,E,G) and transmitted light images (B,D,F). (A,B) Control ES cell. (C,D) ES cell expressing MmGFP under the cdc2 promoter. The diameter of an ES cell is approximately 10 μm. (E) Colony of MmGFP-expressing ES cells. (F,G) Areas of non-proliferating, differentiated cells (d) derived from embryonal bodies and proliferating ES cells (es) expressing MmGFP are shown.

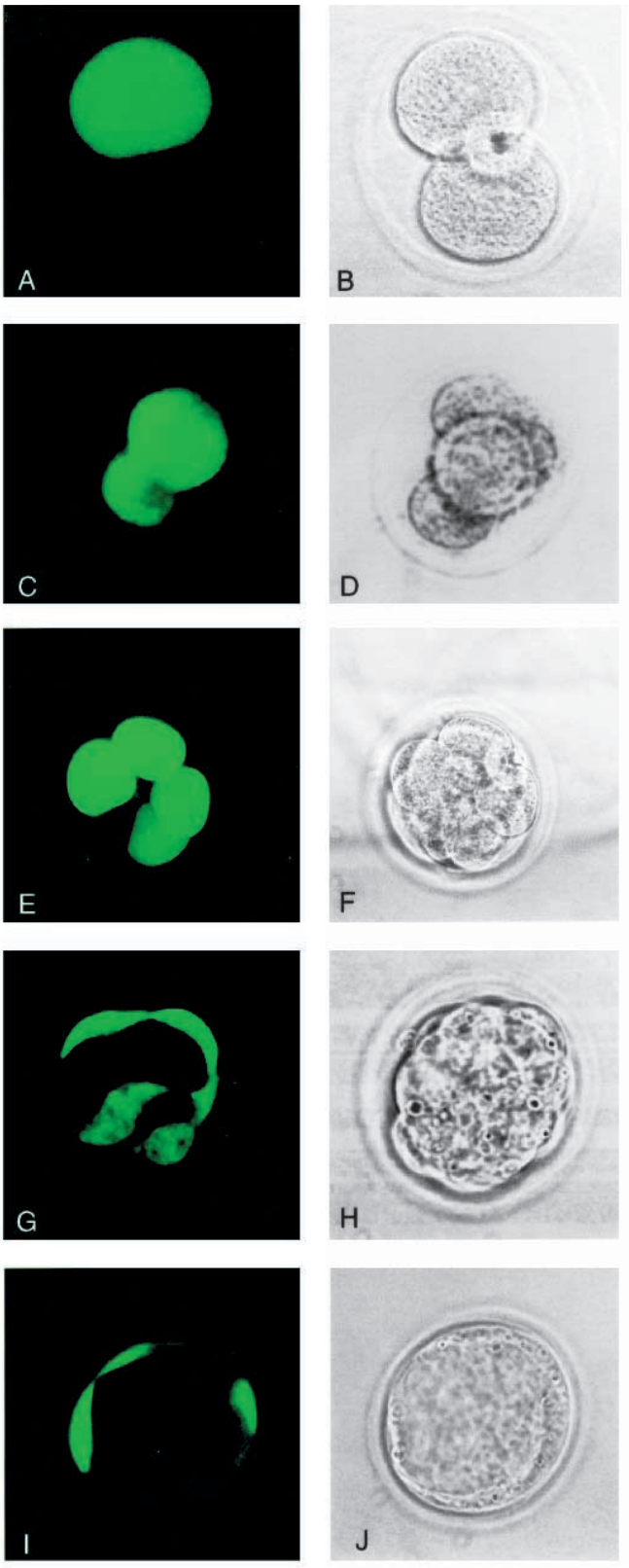


Fig. 2. Blastomeres injected with MmGFP mRNA can be followed through preimplantation development. Fluorescence (A,C,E,G,I) and transmitted light (B,D,F,H,J) images of a developing mouse embryo. (A,B) 2-cell stage; (C,D) 4-cell stage; (E,F) 8-cell stage; (G,H) morula; (I,J) blastocyst. G-I show single optical sections of the imaged embryo. The diameter of the embryo is ~85 μm.

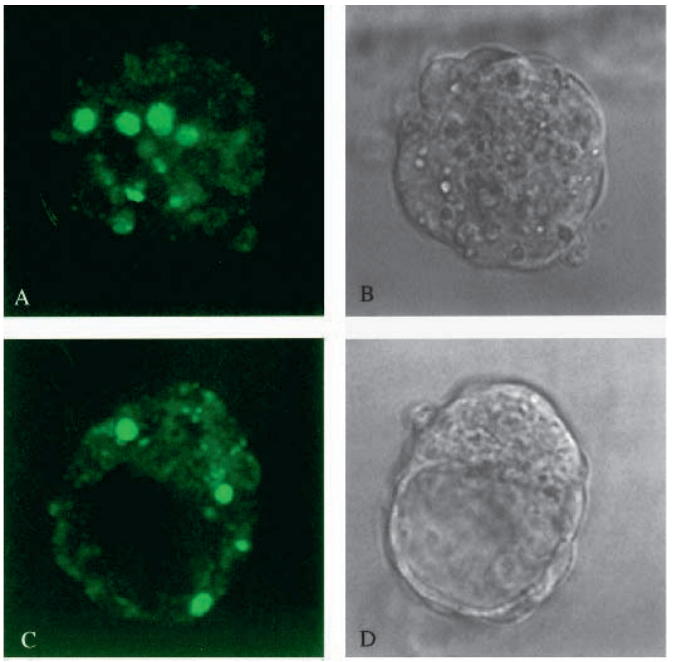


Fig. 3. Colonisation of preimplantation embryos by MmGFP-expressing ES cells. Fluorescence (A,C) and transmitted light images (B,D) of morula stage chimaeric embryo (A,B) and chimaeric blastocyst (C,D). The diameter of the embryo is ~85 μm.

(Fig. 1A,C,E). We confirmed that the fluorescence was due to MmGFP by immunoblotting cell lysates with a specific anti-GFP antibody. These stable ES cell lines have continued to express MmGFP under the cdc2 promoter after several months in culture. To demonstrate that MmGFP expressed from the cdc2 promoter can act as a marker for proliferating cells, we induced MmGFP-expressing ES cells to exit the cell cycle and differentiate in culture. Indeed, we were able to detect differentiated, non-dividing cells that did not show MmGFP fluorescence (Fig. 1F,G).

We then asked whether MmGFP could provide an enduring marker when its synthetic mRNA was injected into early blastomeres of the mouse embryo. We injected MmGFP mRNA into one blastomere at the late 2-cell-stage embryo and showed that the progeny of this cell can be followed until the blastocyst stage (Fig. 2). These experiments also showed that frequent viewing by confocal microscopy did not perturb embryonic development.

We wished to determine whether ES cells expressing MmGFP could be identified during embryogenesis and whether they could contribute to normal mouse development. We produced chimaeras by aggregating ES cells with 8- to 16-cell-stage embryos and followed marked cells during preimplantation development in vitro (Fig. 3). We found MmGFP-expressing cells in the morula (Fig. 3A,B), and in blastocysts our ES cells appear to contribute both to inner cell mass (ICM) and trophectoderm (Fig. 3C,D). These chimaeric blastocysts, and blastocysts into which marked ES cells were directly injected, were transplanted to the uteri of foster mothers to allow their implantation and further development. We examined the expression of MmGFP during embryogenesis between 6.5 and 9.5 days of development. Gastrulation in the

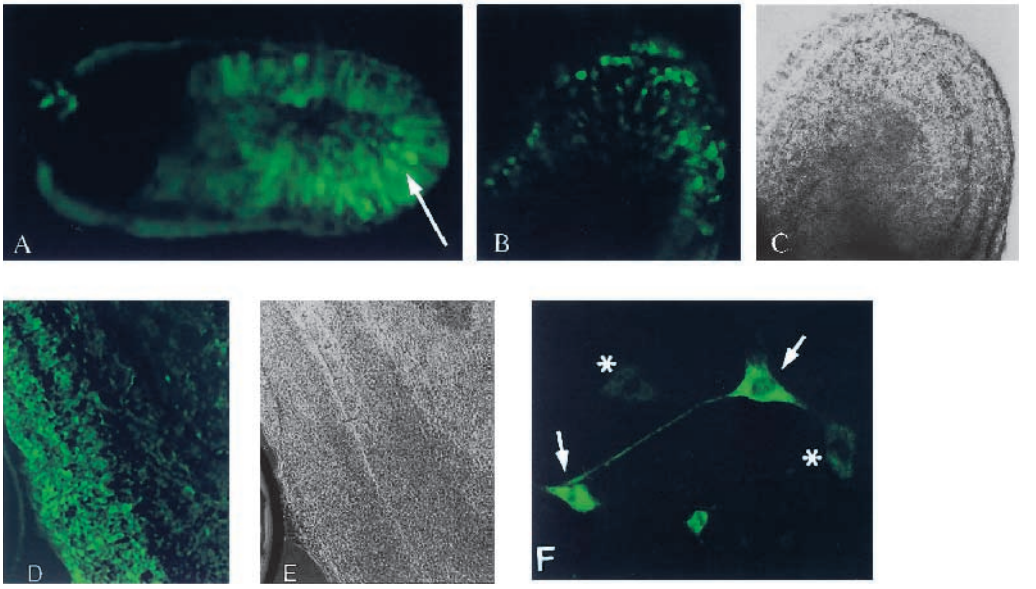


Fig. 4. Developmental potential of MmGFP-expressing ES cells in postimplantation embryos. (A) Fluorescence image of an optical section of a 6.5 day p.c. chimaeric embryo. MmGFP-expressing cells are seen in a large proportion of the embryonic ectoderm (indicated by the arrow). The length of an embryo at this stage is approximatly 1 mm. (B,C) Fluorescence (B) and transmitted light (C) images of a 7 day p.c. chimaeric embryo showing MmGFP expression in individual cells of the embryonic ectoderm. (D,E) Fluorescence (D) and transmitted light (E) image of an optical section of tissues from the mid trunk region of a 9.5 day p.c. chimaeric embryo with MmGFP-expressing cells in the surface ectoderm and neuroepithelium. Somites are largely derived from host embryonic cells. (F) Fluorescence image of cells from a primary culture of fibroblasts established from the adult skin with pigmented hair (i. e. of ES cell orgin) of chimaeric mice. Two MmGFP-expressing cells (arrowed) can be distinguished by their bright green fluorescence from two non-GFP-expressing cells (marked with a *). (The bright dot on the bottom of the figure is a refractive dust particle).

mouse begins at about day 6.5 p.c. and is associated with rapid cell proliferation. Analysis of the cell number and mitotic index has shown that the cells of the primitive ectoderm divide extremely rapidly between 5.5 and 7.5 days of development (Snow, 1977). Thus, as could be predicted, cells in the embryonic ectoderm showed strong MmGFP expression from the cdc2 promoter (Fig. 4A,B). We found MmGFP in a variety of different proliferating cell types in apparently normal tissues at 9.5 day p.c. (Fig. 4D,E) and normal development was confirmed when chimeric embryos developed to term and into healthy adult mice. MmGFP expression could be detected in primary fibroblast cultures established from the skin of adult chimaeric mice (Fig. 4F).

DISCUSSION

In this paper, we have described MmGFP, a novel, autonomous marker for living cells in the mouse. MmGFP contains coding sequence mutations that increase its ability to fold up correctly at higher temperatures, and others (S65T, I167T) that alter the spectral properties of the chromophore. Together these mutations generated a form of GFP that, by flow cytometry analysis, is at least 50-fold brighter than the wild-type protein at 37˚C (M. Jackman and J. P., unpublished observations), and should be of substantial value in studies of mammalian development. MmGFP allows marked cells and their progeny to be visualised in living mouse embryos and it has enabled us to follow the fate of ES cells in chimaeric embryos. There are still several unanswered questions about the developmental potential and deployment of ES cells introduced into early embryos, many of which can only be addressed by determining the distribution of individual ES cells, and by observing their proliferation and migration in living tissues (reviewed in Gardner and Brook, 1997). Previously, although it was known that ES cells can colonise a preimplantation embryo, it has only been possible to distinguish them from carrier embryo cells by using ES cells expressing *lacZ* and staining fixed embryos, or by assaying for isoenzymes. We can now tackle these issues using MmGFP.

Our modified GFP provides the means to follow the developmental potential and distribution of cells at successive stages in the development of the same, living, embryo. We have shown that it is possible to use MmGFP either as a stable cell marker when the cDNA is integrated, or as an enduring marker when the mRNA is injected into cells. Our studies have shown that visualisation of MmGFP-expressing embryos under the confocal microscope did not perturb development, and that the protein was not toxic. MmGFP allowed us to follow the fate of cells throughout the whole of preimplantation development, which can take place in vitro. It should also be possible to trace cell fate in 6.5- to 8.5-day-old embryos, since embryos at these stages can be recovered from the mother and cultured in vitro for a further 2 days, a period extending from just before gastrulation to early organogenesis. Although Ikawa et al. (1995) reported mice expressing GFP after injection of a transgene into the male pronucleus, their study used wild-type GFP, which, in our hands, is invisible in ES cells. Also, all other published studies using GFP in mammalian cells have needed to use a modified form to achieve a visible signal, even when overexpressed in transiently transfected cells (Hampton et al., 1996; Heim et al., 1994; Marshall et al., 1995; Olson et al., 1995; Pines, 1995; Rizzuto et al., 1995).

The results that we describe should have a major impact on future studies of mammalian embryogenesis. The novel ability to visualise marked cells in living embryos has implications for both cell migration and cell lineage analyses. When expressed under tissue-specific promoters, MmGFP should be a useful

reporter for mapping the lineages of specific cell types. Such analyses could be extended using site-specific recombination at later developmental stages to delineate the fates of subpopulations of cells. Furthermore, MmGFP driven by the cdc2 promoter promises to allow the domains of cell proliferation to be traced throughout embryogenesis and postnatal development.

We thank Brendan Cormack for communicating results prior to publication, Hartmund Land for the cdc2 promoter and numerous colleagues for helpful discussions. M. Z.-G. thanks the EMBO for a long-term postdoctoral fellowship. This work was supported by funds from the Wellcome Trust to M. J. E., the MRC to J. H. and the Cancer Research Campaign to J. P.

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(Accepted 20 January 1997)